Studies using transient expression systems have implicated the XAP2 protein in the control of aryl hydrocarbon receptor (AHR) stability and subcellular location. Thus, studies were performed in cell lines that expressed endogenous rat or mouse Ahb-1 (C57BL/6) or Ahb-2 (C3H) AHRs with similar levels of endogenous XAP2. Unliganded rat and mouse Ahb-2 receptor complexes associated with reduced levels of XAP2 and exhibited dynamic nucleocytoplasmic shuttling in comparison with Ahb-1 receptors. Rat and mouse Ahb-2 receptors also exhibited a greater magnitude of ligand-induced degradation than Ahb-1 receptors. Small interfering RNA reduction of endogenous XAP2 by >80% had minimal impact on the level of Ahb-2 receptors but resulted in a 25–30% reduction of Ahb-1 receptor. XAP2 reduction resulted in increased susceptibility of the Ahb-1 receptor to ligand-induced degradation yet produced higher levels of endogenous CYP1A1 induction. Stable expression of the Ahb-2 receptor in the C57BL/6 background resulted in a protein instability and localization or expression level of the human AHR in transient expression systems utilizing the Ahb-1 receptor complex but appears to have a minimal impact on endogenous rat or Ahb-2 receptors. This implies that the analysis of the AHR-mediated signaling via rat and mouse Ahb-2 receptors may better represent the physiology of this signal transduction pathway.

The hepatitis B virus X-associated protein, XAP2 (also termed ARA9 and AIP) was identified in 1996 by its interaction and inhibition of the hepatitis B virus X protein (1). Structurally, XAP2 shares sequence identity with the FKBPs class of immunophilins that contain a tetrastricopeptide repeat motif typical of proteins that interact with hsp90 (reviewed in Ref. 2). The function of the immunophilins is still an evolving process, but FKBPs have been shown to participate in the subcellular trafficking of steroid hormone receptors and also influence their stability and transactivation potential (reviewed in Ref. 3). However, XAP2 is not a true immunophilin, since it does not bind to compounds such as FK506 and does not share full identity with other immunophilins in the consensus tetrastricopeptide repeat motif domain residues (2, 4). Inter-

The abbreviations used are: AHR, Ah receptor; CHIP, C-terminal hsp70-interacting protein; GAR-HRP, goat anti-rabbit horseradish peroxidase; GAM-HRP, goat anti-mouse horseradish peroxidase; GAR-rhodamine, goat anti-rabbit IgG conjugated to rhodamine; siRNA, small interfering RNA; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; LA-I, Hepa-1 variant cell line with reduced AHR; AHWT, stable LA-I Hepa-1 cells expressing the Ahb-1 receptor; Hepa-1, Hepa-1 c17; Ahb-1, stable LA-I Hepa-1 cells expressing the Ahb-1 receptor; GA, geldanamycin; LMB, leptomycin B; E3, ubiquitin-protein isopeptide ligase; ARNT, aryl hydrocarbon nuclear translocator.

EXPERIMENTAL PROCEDURES

Materials—TCDD (98% stated chemical purity) was obtained from Radian Corp. (Austin, TX) and was solubilized in Me2SO. Geldanamycin (GA) and leptomycin B (LMB) were purchased from Sigma.

Antibodies—Specific antibodies against either the AHR (A-1 and A-1A) or aryl hydrocarbon nuclear translocator (ARNT) protein (R-1)
Role of XAP2 and CHIP in Ah Receptor Stability

Western Blot Analysis and Quantification of Protein—Equal amounts of protein were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose. Immunoblotting was carried out with varying concentrations of primary antibody (see figure legends) in BLOTTO buffer supplemented with DL-histidine (20 mM) for 1–2 h at 22 °C. Blots were washed with three changes of TTBS+ for a total of 45 min. The blot was incubated in BLOTTO buffer containing a 1:10,000 dilution of appropriate peroxidase-conjugated secondary antibody for 1 h at 22 °C and washed in three changes of TTBS+ as above. Prior to detection, the blots were rinsed in phosphate-buffered saline. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (GE/Healthcare, 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 (23–25).

Immunofluorescence Staining and Microscopy—All immunocytochemical procedures (cell plating, fixation, and staining) were carried out as previously described (22–24). Cells were observed on an Olympus IX70 microscope. On average, 15–20 fields (5–20 cells each) were evaluated on each coverslip, and 3–4 fields were photographed with a digital camera at the same exposure time to generate the raw data. Nuclear fluorescence intensities of 25–50 cells in three distinct fields of view, were obtained using MicroSuite image analysis software (Olympus America Inc.).

Generation of Stable Cell Lines—cDNA to the Ah−2 receptor was a generous gift from Alan Pollard (NIOSH). The Ah−2 cDNA was ligated into the pLNCX2 retroviral expression vector (Clontech, Mountain View, CA), and virus particles used to infect the LA-I Hepa-1 variant cell line as described previously (26). For all experiments described in this report, three independent stable cell lines were evaluated. Parental LA-I cells and stable cell lines expressing murine C57Bl/6 Ah (Ah<sub>WT</sub>) have been described previously (26). All cells were maintained in selective medium during propagation, but G418 was not present in the medium when cells were plated for experiments.

RNA Interference—Annealed small interfering RNA (siRNA) complexes containing 21-bp regions of identity to regions of the murine XAP2 or an annealed 21-bp RNA to sequence not present in the mouse genome (siRNA control) were purchased from Ambion (Austin, TX). siRNA (final concentration of 50–100 nM) was transfected into 35-mm dishes containing 1–2 × 10⁶ cells using Lipofectamine<sup>TM</sup> reagent (Invitrogen). 36–48 h after transfection, cells were treated as detailed in the figure legends, and total cell lysates were harvested for Western blotting. The efficiency of siRNA gene knockdown of XAP2 was determined by Western blotting. Transfection efficiency was monitored by microscopy using fluorescein isothiocyanate-labeled RNA (Clontech). In general, transfection efficiency in all experiments was >85%.

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

RESULTS

The Endogenous Rat and Mouse Ah<sup>−2</sup> Receptors Are Not Associated with High Levels of Endogenous XAP2—It has been proposed that XAP2 functions in AHR-mediated signal transduction by influencing the stability and shuttling behavior of the AHR (5–7, 9–19). However, these conclusions have been based primarily on the analysis of AHR and XAP2 in transient transfection systems. There is minimal information on the function of endogenous XAP2 especially as it relates to interac-
Role of XAP2 and CHIP in Ah Receptor Stability

FIGURE 1. Analysis of AHR and XAP2 expression and association in Hepa-1, A7, and C2C12 cells. A, equal amounts of total cell lysates from the indicated cells were resolved by SDS-PAGE, blotted, and stained with A1-A rabbit IgG (1.0 µg/ml), β-actin rabbit IgG (1:1000), hsp90 rabbit IgG (1:500), XAP2 mouse IgG (1:750), or p23 mouse IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP or GAM-HRP IgG (1:10,000). B, cytosol was prepared from Hepa-1, A7, or C2C12 cells as detailed under “Experimental Procedures.” 800 µg of cytosol was precipitated with either affinity-pure A1-A IgG (5 µg) or affinity-pure preimmune rabbit IgG (5 µg) along with Protein A/G-agarose (25 µl) for 2.5 h at 4 °C with rocking. Pellets were washed three times for 5 min each with TTBS supplemented with sodium molybdate (20 mM) and then boiled in 30 µl of SDS sample buffer. 15 µg of cytosol (input) or 15 µl of the eluted protein were resolved by SDS-PAGE, blotted, and stained with A1-A IgG; P, precipitated with preimmune IgG. The numbers under the XAP2 blot represent the percentage of XAP in relation to the level in Hepa-1 (100%).

AHR suggests that the endogenous rat and mouse Ahb-2 receptors are actively shuttling between the cytoplasm and nucleus with a periodicity of 90–180 min. LMB-induced nuclear localization of the endogenous Ahb-2 receptor was observed in the MCF7 human breast cancer cell line and has been shown for the endogenous zebrafish Ahb2R in the zebrafish cell line (34).3 These results indicate that there exist strain and species differences in the nucleocytoplasmic shuttling of the endogenous AHR and that increased shuttling correlates to a reduced level of XAP2 associated with the AHR complex.

3 R. S. Pollenz, unpublished results.
The Endogenous Rat and Mouse Ahb-2 Receptors Show Distinct Profiles of Ligand-induced Degradation in Comparison with the Ahb-1 Receptors—The next series of studies were designed to evaluate the stability of the endogenous AHR following ligand exposure. Hepa-1, C2C12, and A7 cells were treated with TCDD for 0–120 min, and the level of AHR was evaluated in total cell lysates by quantitative Western blotting. A representative experiment is shown in Fig. 3. Treatment of C2C12 or A7 cells with TCDD results in a dramatic reduction of cellular AHR protein by the 120-min time point. In C2C12 cells, AHR protein was not detectable at the 90-min time point, whereas the level of AHR in A7 cells decreased by 88% of control levels by the 120-min time point. Consistent with previous reports, the AHR was also reduced by greater than 90% following 120 min of TCDD exposure in the 10T cell line (22, 30). In contrast, TCDD-induced degradation of the Ahb-1 receptor exhibited a shallow degradation profile and was only reduced by 30–40% after 120 min. These results indicate that the endogenous Ahb-2 and rat receptors are degraded to near completion within 2 h of ligand exposure and highlight another striking difference in the response of these AHRs to the Ahb-1 receptor. Since previous studies suggest that 15–25% of the endogenous Ahb-1 receptors in Hepa-1 cells are not degraded even after 4–16 h of TCDD exposure (24, 26, 27, 35–37), it was of interest to determine whether this population was still associated with XAP2. Cytosols prepared from Hepa-1 cells were treated with TCDD or vehicle for 4 h, and the AHR was immunoprecipitated as described under “Experimental Procedures.” The results are shown in Fig. 3C. Although the endogenous Ahb-1 receptors were reduced by >80% following TCDD exposure, there were no changes in the level of endogenous XAP2, and the precipitated receptors were clearly associated with XAP2. It is noteworthy that the Ahb-1 receptors that are not degraded are still associated with XAP2.

Reduction of Endogenous XAP2 with siRNA Results in Reduced Levels of Endogenous Ahb-1 but Not Ahb-2 Receptors—To directly investigate the impact of XAP2 on the stability of the endogenous AHR, siRNAs were used to reduce the endogenous level of XAP2 in 10T, C2C12, and Hepa-1 cells. For these studies, siRNAs specific to mouse XAP2 were transfected into cells, and the level of endogenous AHR and XAP2 was evaluated after 36–48 h. To control for the cellular effects of siRNA transfection, siRNAs with no sequence identity to the mouse genome were included in all studies. A representative set of experiments is shown in Fig. 4. In all studies, the level of endogenous XAP2 expression...
was reduced by 80–90% as determined by quantitative Western blotting. Importantly, the level of endogenous Ahb<sup>-1</sup> receptor was decreased by 26 ± 5% in Hepa-1 cells with reduced XAP2 (Fig. 4D). These findings were consistent and reproducible over four experiments. In contrast, the reduction of XAP2 in 10T or C2C12 cells had no measurable impact on the endogenous concentration of the Ahb<sup>-2</sup> receptor (Fig. 4). The impact of XAP2 reduction in A7 cells could not be evaluated as transcription efficiency was not sufficient to reduce XAP2 levels below 50%. Collectively, these results support a role of endogenous XAP2 in the stability of a portion of the unliganded Ahb<sup>-1</sup> receptor and illustrate another distinction between the Ahb<sup>-1</sup> and Ahb<sup>-2</sup> receptors.

Reduction of Endogenous XAP2 with siRNA Results in Increased Levels of Ligand-induced Degradation of the AHR and Enhanced Levels of CYP1A1 Induction in Hepa-1 Cells—The reduction of endogenous Ahb<sup>-1</sup> receptor by 25–30% is intriguing, since it has been hypothesized that there are distinct pools of AHR-hsp90 complexes in Hepa-1 cells (12), and previous studies have consistently shown that ~20–25% of the endogenous AHR pool remains in Hepa-1 cells after ligand exposure (Fig. 3) (24, 26, 27). Therefore, it was of interest to evaluate the ligand-induced degradation of the AHR in Hepa-1 cells with reduced levels of XAP2. Hepa-1 cells were transfected with control or XAP2 siRNA as described and allowed to recover for 48 h. Cells were then treated with TCDD for 0–6 h, total cell lysates were produced, and the level of XAP2, AHR, and CYP1A1 was evaluated by quantitative Western blotting. A representative study is shown in Figs. 5 and 6. As observed in Fig. 4, XAP2 was significantly reduced (85%), and there was a corresponding reduction in the level of endogenous AHR (Fig. 5). Importantly, in cells with reduced levels of XAP2, the endogenous Ahb<sup>-1</sup> receptor was more susceptible to TCDD-induced degradation (Fig. 5). Indeed, the Ah<sup>Δh-l</sup>AHR was reduced by 52% at the 2-h time point compared with 30% in cells transfected with control siRNA. The overall level of AHR degradation at the 6-h time point was also higher in cells with reduced XAP2. Thus, these results suggest that the association of XAP2 with the Ahb<sup>-1</sup> receptor complex impacts the time course and magnitude of ligand-induced degradation.

To assess the impact of reduced XAP2 on induction of the endogenous CYP1A1 gene, the identical samples shown in Fig. 5, were evaluated for the expression of CYP1A1 protein (Fig. 6). Importantly, reduction in XAP2 resulted in an increase in the endogenous CYP1A1 protein detected after 2, 4, and 6 h of TCDD exposure. At each time point, the level of CYP1A1 protein was increased by ~26% compared with the levels in cells that did not have reduced XAP2. These findings support the hypothesis that endogenous XAP2 acts to repress a population of AHRs from the induction of the endogenous CYP1A1 gene. This observation would be expected if the same populations of AHR that are resistant to degradation were also incapable of mediating gene regulation.

Ahb<sup>-2</sup> Receptors Expressed in the Hepa-1 Background Are Associated with Reduced Levels of XAP2 and Exhibit Higher Levels of Ligand-in
Reduced Degradation—To further assess the differences in stability and shuttling observed between the Ahb-1 and Ahb-2 receptors, retroviral infection was used to generate stable cell lines expressing each AHR in the same genetic background. The Hepa-1 LA-I variant line was used for these studies, since the cells express low levels of endogenous AHR (38). This cell line has also been used to generate stable cell lines expressing physiological levels of mouse AHR (26). Fig. 7A shows the expression of AHR, hsp90, XAP2, and actin in the parental LA-I variant, wild type Hepa-1 (He), and the stable cell lines designated AHWT (expressing the Ahb-1 receptor) (see also Ref. 26) and AHb2 (expressing the Ahb-2 receptor). Importantly, the level of AHR expressed in each of the stable cell lines approximates that of the endogenous AHR in wild type Hepa-1 cells, and the levels of hsp90 and XAP2 are also similar. To assess the composition of the unliganded AHR complex, cytosol was generated from two independent AHWT cell lines as well as the AHb2 cell line and immunoprecipitated with antibodies specific to the AHR. As shown for the endogenous Ahb-1 receptor in Hepa-1 cells, immunoprecipitation of the AHR from the AHWT cells co-precipitated a high level of XAP2 (Fig. 7B). In contrast, as shown for the endogenous rat and Ahb-2 receptors, immunoprecipitation of the AHR from both of the AHb2 cell lines pulled down significantly less amounts of endogenous XAP2 (Fig. 7B). Over several experiments, the average amount of XAP2 co-precipitated from the AHWT cells was ~21 ± 8% of the level co-precipitated from AHWT. Thus, the species of receptor and not the cellular context of its expression determine the association with XAP2.

Because the AHRs expressed in the A7 and C2C12 cells exhibited increased susceptibility to TCDD-induced degradation, it was pertinent to assess the degradation of the AHR in the AHb2 cell line. A representative Western blot experiment is shown in Fig. 7C, and the quantified results are presented in Fig. 7D. TCDD-induced degradation in the AHb2 line is more rapid and proceeds to a greater magnitude than that in the AHWT. Indeed, the Ahb-2 receptor is reduced by 52% after 2 h and >85% after 6 h of TCDD exposure compared with 28 and 74% for the Ahb-1 receptor. Overall, the profile of AHR degradation in the AHb2 line is more representative of that observed in the A7, C2C12, and Hepa-1 line with reduced XAP2 than the WT Hepa-1 line (compare Figs. 3B, 5C, and 7D).

To assess any differences in the ability of the Ahb-1 or Ahb-2 receptors to induce CYP1A1, AHb2 and AHWT cells were treated with TCDD for 0–6 h, and the level of CYP1A1 protein was evaluated by Western blotting (Fig. 8). Fig. 8A shows that CYP1A1 was detectable within 2 h of TCDD exposure in both cell lines and increased proportionally over time. To determine whether there were any quantitative differences in the induction of CYP1A1 protein in the two cell lines, cells were treated with TCDD for 16 h, and identical levels of total cell lysate were evaluated by quantitative Western blotting. To show that the induction of CYP1A1 protein was due to the expression of the integrated AHRs, the parental LA-I cell line was also evaluated over the same time frame. Surprisingly, the level of CYP1A1 protein induced after 16 h was not statistically different between the AHWT and AHb2 cell lines although the Ahb-2 receptor was associated with less XAP2 and degraded with a more rapid profile (Fig. 8, B and C). Thus, in these model systems, a reduction in the level of XAP2 in the core AHR complex did not appear to impact induction of the endogenous CYP1A1 gene.

Ahb-2 AHR Expressed in the Hepa-1 Background Exhibits Nucleocytoplasmic Shuttling—The endogenous Ahb-2 receptor expressed in C2C12 and 10T cells shows clear nucleocytoplasmic shuttling in comparison with the Ahb-1 receptor expressed in the Hepa-1 cell line (Fig. 2). Since each of these cells represents a different genetic background, the lack of shuttling in the Hepa-1 could be related to this background or be specific to the AHR expressed in the cell. To evaluate this question, AHWT and AHb2 cells were propagated on glass coverslips, exposed to TCDD or LMB, and then fixed and stained for AHR protein. As expected, the location of the Ahb-1 receptor in the AHWT cells was predominantly cytoplasmic and became nuclear following 1 h of TCDD treatment (Fig. 9). In addition, as observed in the wild type Hepa-1 lines, 4 h of LMB exposure did not influence the location of the Ahb-1 receptor. In contrast, the unliganded Ahb-2 receptor exhibited a nuclear and cytoplasmic location that became strongly nuclear in the presence of TCDD. Importantly, exposure of the AHb2 cells to LMB resulted in an...
cells were treated with Me₂SO (0.1%) for 6 h or TCDD (2 nM) for the indicated times. Total cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE, blotted, and stained with A-1A IgG (1:1000) or control siRNA (siCON) as detailed under “Experimental Procedures.” 48 h later, AHR protein bands were quantified in identical exposures and normalized as detailed (23–25). Samples are shown from cells transfected with control siRNA (siCON) or siRNA specific to XAP2 (siXAP2). B, the levels of CYP1A1 protein in the control siRNA and XAP2-specific siRNA samples were divided by the corresponding level of actin, and the average ± S.E. of the three independent samples was plotted as normalized densitometry units. *, statistically different from the control siRNA; P < 0.001. The level of XAP2 reduction in these samples is shown in Fig. 5, A and B.

Figs. 7 and 8, these findings show that the Ahb⁻² receptor expressed in the C57BL/6 background exhibits distinct susceptibility to ligand exposure and nucleocytoplasmic shuttling when compared with the Ahb⁻¹ receptor. If the association of the AHR with XAP2 in indeed involved in inhibiting nucleocytoplasmic shuttling (13, 16–18), these findings further support the hypothesis that endogenous levels of XAP2 have minimal impact on Ahb⁻² receptors.

Ligand-dependent and -independent Degradation of the Endogenous Ahb⁻¹ Receptor Is Not Mediated by CHIP—It was next of interest to investigate the mechanism responsible for the reduction in the endogenous Ahb⁻¹ receptor when XAP2 was reduced by siRNA. CHIP is an E3 ubiquitin ligase that has been implicated in the degradation of several target proteins that are associated with hsp90 (reviewed in Ref. 39).

Recently, CHIP was shown to degrade a constitutively nuclear form of the Ahb⁻¹ receptor (14). In addition, these same studies showed that CHIP mediated degradation of the AHR was inhibited by the overexpression of XAP2. However, these studies were performed on a constitutively nuclear AHR that does not exhibit ligand-induced degradation and has a 2-h half-life (15). Therefore, it was pertinent to investigate whether there was a connection between the reduction in XAP2, CHIP, and the degradation of the endogenous Ahb⁻¹ receptor.

Initial studies were performed on Hepa-1 and C2C12 cell lines using siRNA to CHIP. Although the endogenous CHIP protein could be reduced by 70% in each of the cell lines, there was no significant difference in the level of AHR expression or its degradation profile in the presence of TCDD or geldanamycin (16). Therefore, there may have been sufficient CHIP enzyme remaining in the cells to mediate AHR degradation. In order to circumvent this problem, we utilized transformed...
lung fibroblasts derived from CHIP knockout mice (40). The initial characterization of these cells is shown in Fig. 10. Importantly, both CHIP −/− and CHIP +/+ cells express endogenous levels of AHR and exhibit no differences in the level of ARNT, XAP2, or hsp90. CHIP −/− cells appear homozygous for the Ahb-1 receptor, whereas CHIP +/+ cells express the Ahb-1 and the Ahd receptor (from the DBA-derived embryonic stem cells). Quantification of the AHR expression in both cell lines showed that the total level of AHR protein was equal. To assess whether CHIP affected ligand-induced degradation, cells were treated with TCDD for 4 h, and the level of AHR in each line was evaluated by Western blotting (Fig. 10B). The level of ligand-induced degradation of the AHR was identical in both CHIP −/− and CHIP +/+ cells. Thus, CHIP is not an enzyme used in the ligand-induced degradation of either the Ahb-1 or Ahd receptor.

The AHR is also degraded in a ligand-independent manner following exposure to geldanamycin (26, 35, 41). GA binds to hsp90 and is thought to cause a conformational change in the AHR complex that predisposes it to degradation (41). GA exposure also results in displacement of XAP2 from AHR-hsp90 complex in vitro, although overexpression of XAP2 does not appear to protect the AHR from GA-induced degradation in transfected cells (13). To assess the role of CHIP in GA-induced degradation, cells were treated with GA for 2 h, and the level of AHR in each line was evaluated by Western blotting (Fig. 10C). The level of GA-induced degradation of the AHR was identical in both CHIP −/− and CHIP +/+ cells. Thus, CHIP does not participate in the GA-induced degradation of either the Ahb-1 or Ahd receptor.

Finally, it was of interest to determine whether CHIP was involved in the loss of AHR that is observed following the reduction of XAP2 by siRNA (Figs. 4 and 5). For these studies, CHIP −/− cells were transfected with XAP2 siRNA and allowed to recover for 48 h. Cells were then exposed to TCDD for 4 h, and the levels of endogenous AHR, XAP2, and actin were evaluated by Western blotting (Fig. 11). Consistent with the results in the Hepa-1 cell line, the endogenous Ahb-1 receptor was decreased by 26% when XAP2 was reduced by siRNA. Collectively, these studies show that the CHIP E3 ubiquitin ligase is not involved in the degradation of the endogenous Ahb-1 receptor whether it is induced by TCDD, GA, or a reduced level of XAP2. In addition, the studies with the CHIP KO cells validate the CHIP siRNA work in the Hepa-1 and C2C12 cells and suggest that CHIP does not impact the level of the Ahb-2 receptor.

DISCUSSION

The role of XAP2 in the stability and nucleocytoplasmic shuttling behavior of the Ahb-1 receptor complex is based on overexpression studies that detected a higher level of cytoplasmic AHR protein and increased levels of AHR-mediated gene induction in the presence of exogenously expressed XAP2 in yeast or mammalian cells (5–7, 9–11). A general assumption has been that the functions ascribed to XAP2 on...
the Ahb-1 receptor complex are universal for all other AHRs. The key findings of the current report challenge this view by showing that (i) very low levels of endogenous XAP2 associate with the endogenous rat and mouse Ahb-2 receptors, (ii) rat and mouse Ahb-2 receptors show dynamic nucleocytoplasmic shuttling in the presence of endogenous XAP2, (iii) endogenous XAP2 stabilizes only a small fraction of the Ahb-1 receptor pool, (iv) endogenous XAP2 has a negative impact on gene regulation mediated by the Ahb-1 receptor, and (v) endogenous XAP2 is not competing with the CHIP E3 ubiquitin ligase. Collectively, these results imply that XAP2 may only impact AHR-mediated signaling in the context of the Ahb-1 receptor and suggest that the analysis of the AHR-mediated signaling via rat and mouse Ahb-2 receptors may better represent the physiology of this signal transduction pathway.:

So what is the role of XAP2 in AHR-mediated signaling? For the Ahb-1 receptor, the results support a role of XAP2 in the stabilization of a fraction of the Ahb-1 receptor pool, since there was only a modest reduction (25–30%) in endogenous AHR following XAP2 knockdown in Hepa-1 or lung fibroblast cells. This modest reduction of AHR is consistent with the finding that there may be multiple pools of endogenous AHR/hsp90 complexes in Hepa-1 cells and that less than 40% of these complexes are associated with XAP2 in vivo (12). Unlike the studies that utilized exogenous expression of AHR and XAP2 to assess the function of these proteins, the analysis of the endogenous proteins support a hypothesis that the populations of Ahb-1 receptor complexes that are associated with XAP2 are more resistant to ligand-induced degradation and to the formation of AHR/ARNT dimers capable of regulating genes. This hypothesis is supported by the finding that depletion of endogenous XAP2 by 85% results in increased susceptibility of the endogenous Ahb-1 receptor to ligand-induced degradation and a corresponding increase in the induction of CYP1A1 protein over control-treated cells. In addition, the 15–25% of Ahb-1 receptors that are resistant to ligand-induced degradation appear to be associated with XAP2. These results imply that the stabilization of the Ahb-1 receptor by XAP2 actually has a negative impact on AHR-mediated signaling. Important, the ability of XAP2 to exert a negative function on gene regulation has recently been demonstrated in transient studies of the human AHR (20) and by analysis of a mutant Ahb-1 receptor that does not associate with XAP2 (21). In addition, it is important to note

FIGURE 8. Western blot analysis of CYP1A1 protein in stable cell lines expressing Ahb-1 or Ahb-2 receptors following exposure to TCDD. A, AhWT and Ahb2 cells were treated with Me2SO (0.1%) for 6 h or TCDD (2 μM) for the indicated times. Total cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE, blotted, and stained with rat CYP1A1 IgG (1.0 μg/ml) and actin IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000), B, LA-I, AhWT, and Ahb2 cells were treated with Me2SO (0.1%) or TCDD (2 μM) for 16 h. Total cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE, blotted, and stained with rat CYP1A1 IgG (1.0 μg/ml) and actin IgG (1:1000). Reactivity was visualized by ECL with GAR-HRP (1:10,000), and protein bands were quantified and normalized as detailed (23–25). C, the levels of CYP1A1 protein in the LA-I, AhWT, and Ahb2 samples were divided by the corresponding level of actin, and the average ± S.E. of the three independent samples was plotted as normalized densitometry units. Con, average density of Me2SO-treated cells.

FIGURE 9. Subcellular localization of AHR AHWT and Ahb2 cells exposed to TCDD or LMB. Cells were grown on glass coverslips exposed to the compounds detailed below and then fixed as detailed previously (22–24). Coverslips were incubated with A-1 IgG (1.0 μg/ml) and visualized with GAR-rhodamine IgG (1:400). A, B, D, and E, AHWT cells exposed to Me2SO (0.1%) for 1 h, TCDD (2 μM) for 1 h, methanol (0.5%) for 4 h, or LMB (20 μM) for 4 h (F). A–E, AHb2 cells exposed to Me2SO (0.1%) for 1 h (F), TCDD (2 μM) for 1 h (G), methanol (0.5%) for 4 h (H), or LMB (20 μM) for 4 h (I). C, the parental LA-I cell line stained and photographed under the identical conditions to the AHWT and Ahb2 cells. All panels were exposed for identical times. Bar (A), 10 μm.
that the initial function of XAP2 was as a negative transcriptional regulator of the hepatitis B virus X protein (1).

The results presented in the current report also call into question the proposed mechanism whereby XAP2 stabilizes the endogenous Ahb−1 receptor complex through competition with the CHIP E3 ubiquitin ligase (14). Our results utilizing a cell line devoid of CHIP protein clearly establish that CHIP is not involved in the degradation of the endogenous Ahb−1 receptor whether it is induced by TCDD, GA, or a reduced level of XAP2. In addition, siRNA knockdown of CHIP in the C2C12 cell line did not impact the TCDD or GA induced degradation of the Ahb−2 receptor. Therefore, these results indicate that the hypothesized competition between CHIP and XAP2 for binding to hsp90 is unlikely to be occurring in a physiological setting. The difference in results of this report from those of Lees et al. (14) are probably related to our analysis of an endogenous AhR that is functioning under physiological conditions, compared with the analysis of a tagged version of the AhR that is turning over rapidly. Since CHIP does not appear to impact AhR stability, the precise mechanism(s) that control ligand-induced or ligand-independent degradation is still not resolved. It is intriguing to consider that several studies have shown that the degradation of some nuclear receptors is correlated to transactivation, DNA binding, and the recruitment of co-activators to the promoter/enhancer region of responsive genes (42–46). The strong association of XAP2 with the Ahb−1 receptor complex (as compared with rat and Ahb−2 receptors), may slow down the transformation process and prevent a population of AHRs from interacting with ARNT, binding DNA, and being degraded. Interestingly, recent studies suggest that the Ahb−1 receptor remains associated at the CYP1A1 promoter during ligand stimulation, whereas the human AHR shows periods of association and dissociation at the CYP1A1 promoter and is suggestive of turnover (47, 48). Future studies will be aimed at further analyzing gene regulation and degradation in the AhWT and AHRα2 cell lines to see if there are differences that result from the reduced level of XAP2 association with Ahb−2 receptors.

Does endogenous XAP2 impact AHR-mediated signaling in species other than C57BL/6? In general, the results regarding XAP2 function on endogenous rat and Ahb−2 receptors are in strong contrast to those of the Ahb−1 receptor. When evaluated in the physiological stoichiometry of a cell, endogenous rat and mouse Ahb−2 receptors are associated with greatly reduced levels of endogenous XAP2. In addition, the cellular concentration of endogenous Ahb−2 receptors does not show a decrease when XAP2 is reduced by siRNA. Finally, endogenous rat and mouse Ahb−2 as well as stably expressed Ahb−2 receptor show dynamic nucleocytoplasmic shuttling in the presence of endogenous XAP2. The finding that the Ahb−2 and rat receptors are functioning in a distinct manner compared with the Ahb−1 receptor is not unprecedented. Indeed, when compared with human, rat and mouse AHRs from other strains, the Ahb−1 receptor expressed in the C57BL/6 mouse exhibits differences in biophysical stability (27, 28, 31), ligand-induced degradation (24, 30), ligand binding affinity (32, 49) and cycling at the CYP1A1 promoter (47, 48). All of these characteristics are consistent with an AHR protein that possesses a level of stability not found in the receptors from other mouse strains, rats, and humans. The dramatic differences between the mouse Ahb−1 and Ahb−2 receptors are particularly striking, since the two receptors exhibit 99.1% amino acid sequence identity and differ only at amino acids 408 (Asn), and 758 (Ala/Thr). In addition, the Ahb−2, rat, and human receptors all have a conserved tyrosine at amino acid 408 that has been implicated in the interaction of the Ahb−1 receptor with XAP2 (21). Thus, it is unlikely that the functional differences between the mouse Ahb−1 and Ahb−2 receptors are related to changes in the amino acid sequence over the first 805 amino acids. However, it is pertinent to note that the Ahb−1 receptor contains a point mutation that prematurely truncates the receptor at amino acid 805, whereas the mouse Ahb−1, rat, and human AHRs all contain an additional 42–45 amino acids at their COOH terminus that have regions of 70% identity (8, 29, 50, 51). This difference clearly distinguishes the Ahb−1 receptor from all other mammalian AHRs. Therefore, the analysis of AHR-mediated signaling via rat, human, and mouse Ahb−2 receptors may better represent the physiology of the AHR signal transduction pathway, since the mouse Ahb−2 receptor appears to be the outlier and is actually missing amino acid sequence that could be functionally significant.
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Redefining the Role of the Endogenous XAP2 and C-terminal hsp70-interacting Protein on the Endogenous Ah Receptors Expressed in Mouse and Rat Cell Lines

Richard S. Pollenz and Edward J. Dougherty

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