The Aryl Hydrocarbon (Ah) Receptor Transcriptional Regulator

**Hepatitis B Virus X-associated Protein 2 Antagonizes p23 Binding to Ah Receptor-Hsp90 Complexes and Is Dispensable for Receptor Function**

Received for publication, July 12, 2004, and in revised form, August 17, 2004
Published, JBC Papers in Press, August 20, 2004, DOI 10.1074/jbc.M407840200

**Brett D. Hollingshead§, John R. Petrulis‡, and Gary H. Perdew¶¶**

*From the §Center for Molecular Toxicology and Carcinogenesis and the Department of Veterinary Science and the ¶¶Graduate Program in Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802*

To further understand the role that the hepatitis B virus X-associated protein 2 (XAP2) plays in regulating aryl hydrocarbon receptor (AhR) function, a point mutation was introduced at tyrosine 408 of the AhR, changing the residue to an alanine or lysine. These mutations resulted in the loss of AhR binding to endogenous XAP2 in COS-1 cells and reduced binding of exogenously expressed XAP2. Cellular localization of the mutant AhR-yellow fluorescent protein fusion proteins remained nuclear when XAP2 was co-expressed, while the non-mutant receptor was redistributed to the cytoplasm. XAP2 expression caused an overall repression of constitutive and ligand-induced AhR transcriptional activity. However, increased expression of XAP2 had no effect on the AhRY408A mutant transcriptional activity. Additionally the XAP2 binding-deficient AhR mutants showed overall higher transcriptional activity when compared with the non-mutant receptor. Interestingly reduced incorporation of the Hsp90 associated co-chaperone p23 in the unliganded AhR complex was observed with increasing XAP2 expression. The displacement of p23 from Hsp90 did not occur when increasing levels of XAP2 were introduced in COS-1 cells in the absence of the AhR; thus this displacement event occurs specifically within an AhR complex. Finally XAP2 itself was capable of existing in multimeric complexes, and these complexes did not require Hsp90 or AhR to form. However, it is not yet clear whether XAP2 can exist within the AhR complex in more than one copy.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor in the basic helix-loop-helix Per/ARNT/Sim family of proteins. The activity of many basic helix-loop-helix Per/ARNT/Sim proteins is regulated by an external stimulus such as light/dark cycle or oxygen stress (for reviews, see Refs. 1 and 2). Analogously the AhR can be activated by a wide variety of structurally diverse exogenous ligands as well as some endogenous compounds (for a review, see Ref. 3). The prototypic high affinity AhR ligand TCDD mediates the incidence of a myriad of toxic and developmental effects through AhR activation (4, 5). AhR transcriptional activity, however, is not simply regulated by the binding and release of ligand. A variety of chaperone and co-chaperone proteins are found in a complex with the AhR at different stages of AhR maturation and transcriptional activation. Understanding how these auxiliary complex proteins act in concert to differentially regulate AhR activity is important in further elucidating the molecular mechanism by which the AhR functions.

The unliganded cytoplasmic AhR exists in a complex with a dimer of Hsp90 (6, 7) and at least one molecule each of the co-chaperones XAP2 (8–10) and p23 (11, 12). Ligand binding to the AhR initiates the formation of the nuclear transcriptionally active AhR/ARNT heterodimer through an ill defined mechanism referred to as transformation. The binding of the AhR/ARNT heterodimer to its specific DNA enhancer element results in the increased transcription of a battery of metabolically important enzymes (13).

XAP2, also known as AhR-associated protein 9 (8) or AhR interacting protein (9), was initially discovered by its ability to interact with and repress transactivation by the hepatitis B virus X protein (14). It was subsequently identified as an AhR-interacting protein by a yeast two-hybrid screen and was also found as part of the AhR complex within mammalian cells (10). It displays primary sequence homology with the FKBP family of immunophilins (8) but is not characterized as an immunophilin because it does not appear to possess the ability to bind immunosuppressant drugs such as FK506 (15). Like some immunophilins, XAP2 can interact with the carboxyl-terminal half of Hsp90 through its TPR domain. In addition to interacting with Hsp90, XAP2 can interact directly with the AhR in the absence of Hsp90 (16). Expression of XAP2 in Saccharomyces cerevisiae using ectopically introduced AhR results in increased receptor activity compared with when the AhR is expressed alone (15, 17). Moreover expression of XAP2 in mammalian cells can result in increased AhR activity (9). This elevated AhR activity observed with high XAP2 expression is likely due to increased AhR stability caused by XAP2 (17). Stabilization of the AhR by XAP2 in mammalian cells has been attributed to reduced levels of AhR ubiquitination when XAP2 is co-expressed (18). Along those lines, XAP2 reduces the ability of the E3 ligase carboxyl-terminal Hsc70-interacting protein to initiate proteasome-mediated degradation of AhR (19). Con-
versely XAP2 has been shown to repress constitutive and ligand-induced mouse AhR activity in the presence of low ligand concentrations (20). Additionally repression of human AhR activity by XAP2 in cell culture has been reported (21). The discrepancies observed in the modulation of AhR activity by XAP2 may be related to the interplay between AhR stabilization caused by overexpression of XAP2 and other potential repressive properties such as cytoplasmic retention of the AhR.

The Hsp90-associated co-chaperone p23 has been shown to modulate the function of a number of steroid hormone receptors (22). For example, the glucocorticoid receptor shows increased stability and higher capacity to bind hormone when complexes are associated with p23 (23). Like many members of the steroid hormone receptors that bind Hsp90, p23 has been shown to be associated with p23 in the unliganded AhR complex (12). Additionally ligand-induced formation of the DNA-bound AhR/ARNT heterodimer is enhanced by p23 (12). Additionally ligand-induced formation of the DNA-bound AhR/ARNT heterodimer is enhanced by p23 in vitro (26).

This report examines the properties of AhR complexes that fail to bind XAP2. By introducing a single point mutation in the XAP2 binding region of the AhR we determined how the loss of functional XAP2 association with the AhR affects its transcriptional activity, its cellular localization, and the ability of p23 to exist in the unliganded AhR complex. These results suggest that XAP2 incorporation in the AhR complex is not required for the formation of a functional AhR but that it acts as a modulator of differentially regulate AhR activity possibly by regulating the levels of p23 incorporation in the AhR complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Expression Constructs—COS-1 cells were grown at 37 °C in 5% CO₂ in α modified minimal essential medium (Sigma) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1000 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). The plasmids pcDNA3/mAhR (27), pEYFP/mAhR (28), pcDNA3/mAhR-FLAG and pcXAP2 (29), pcDNA3/mAhRY408A-FLAG (29), and pGudLuc 6.1 (30) were generated previously. AhR point mutants were created by PCR amplifying the XAP2 cDNA and introducing the appropriate mutations. The Hsp90-associated co-chaperone p23 has been shown to associate with AhR (24).

Transient Transfections and Luciferase Assays—All transfections in COS-1 cells were performed using LipofectAMINE PLUS (Invitrogen) as specified by the manufacturer. Reporters assays were carried out in 6-well dishes using 70–80% confluent cells at the time of transfection. Luciferase gene expression in each well was as follows: 0–100 ng of AhR, 0 or 200 ng of pXAP2, 30 or 100 ng of the AhR-driven luciferase reporter plasmid pGUDLUC 6.1, and empty expression vector to total 1.5 μg of DNA/well. Cells were dosed for 8 h with mouse AhR carrier solvents (Sigma), 1 μl TCD2 (a gift from Dr. Steven Safe), or 10 μM benzo[a]pyrene (Aldrich) 18 h after starting the transfection. Cells were lysed with 1× Passive Lysis Buffer (Promega, Madison, WI). Luciferase assays were carried out using a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA), and values were normalized to protein content (Pierce) determined using a Spectracount™ spectrophotometer (PerkinElmer Life Sciences).

Fluorescent Microscopy—COS-1 cells were transfected in 30-mm glass bottom dishes (MatTek Corp., Ashland, MA) with 0.75 μg of pEYFP/AhR, pEYFP/AhRY408A, or pEYFP/AhRY408K plus 0.75 μg of pcXAP2 or pcDNA3 empty vector to a total of 1.5 μg of total DNA/dish. Approximately 12 h after transfection cells were visualized using a Nikon 60×/1.40 objective with a Nikon TE300 inverted microscope (Nikon Corp., Tokyo, Japan) and photographed using a SPOT™ RT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

In Vitro Interaction of XAP2 with AhR Mutants—pcDNA3/mAhR-FLAG, pcDNA3/mAhRY408A-FLAG, and pcXAP2 were separately in vitro translated using a Tnt® T7 coupled reticulocyte translation/translation system (Promega) in the presence of [35S]methionine. AhR and XAP2 were translated in 50- and 100-μl reactions, respectively. 10-μl aliquots of each translation were mixed and incubated on ice for 1 h. The incubated mixture was then diluted with 700 μl of binding buffer (MENG (25 mM MOPS, 2 mM EDTA, 0.02% NaN₃, and 10% glycerol (pH 7.5)), 30 mM Na₂MoO₄, and 2 mg/ml BSA), applied to 40 μl of anti-FLAG M2-agarose affinity gel (Sigma), and mixed on a platform rocker for 2 h at 4 °C. Immunoprecipitations were washed four times with binding buffer minus BSA plus 50 mM NaCl, and samples were resolved by SDS-PAGE and transfered to PVDF membrane (Millipore, Bedford, MA), and visualized by autoradiography using BioMax MS film (Dupont-Nenmark Co.).

FLAG Immunoprecipitations—COS-1 cells were transfected in 10-cm² dishes with pcDNA3/mAhR-FLAG, pcDNA3/mAhRY408A-FLAG, or pcDNA3/mAhRY408K-FLAG plus pcXAP2 or empty vector to equal 8–10 μg of total DNA/transfection. After 18 h transfected cells were trypsinized, washed with phosphate-buffered saline, and lysed in 500 μl of MENG, 20 mM Na₂MoO₄, 1% Nonidet P-40, 2 mg/ml BSA, and 1× protease inhibitors. The resulting mixture was then diluted to 0.5% Nonidet P-40, 20 mM Na₂MoO₄, and 1× protease inhibitors. Cytosol was obtained by centrifugation of lysates for 30 min at 21,000 × g. Cytosol was applied to 40 μl of anti-FLAG M2-agarose affinity gel. Samples were mixed on a platform rocker for 1.5 h at 4 °C and then washed three times with MENG, 0.5% Nonidet P-40, 20 mM Na₂MoO₄, 100 mM NaCl. Samples were resolved by SDS-PAGE and transferred to PVDF membrane.

XAP2 Immunoprecipitations—COS-1 cells were transfected in 10-cm² dishes with pcDNA3 empty vector, pcDNA3/mAhR, or pcDNA3/mAhRY408A plasmids and brought up to 8 μg of total DNA transfection with additional pcDNA3. After 18 h transfected cells were trypsinized, washed with phosphate-buffered saline, and lysed in 1 ml of MENG, 20 mM Na₂MoO₄, 0.5% Nonidet P-40, and 1× protease inhibitor mixture for 4 °C. Cytosol was isolated by centrifugation at 21,000 × g for 30 min. 400 μl of lysate plus 400 μl of binding buffer (MENG, 20 mM Na₂MoO₄, 0.5% Nonidet P-40, and 2 mg/ml BSA) was applied to 50 μl of Protein A-agarose (Pierce) prebound to either control rabbit IgG or polyclonal XAP2 antibody (31). Samples were mixed at 4 °C for 2 h and washed three times with MENG, 0.5% Nonidet P-40, 20 mM Na₂MoO₄, 50 mM NaCl. Samples were resolved by SDS-PAGE and transferred to PVDF membrane.

Geldanamycin Treatment of Transfected Cells—Duplicate 10-cm² dishes of COS-1 cells were transfected with empty vector, pcDNA3/mAhR, or pcDNA3/mAhRY408A plasmids and brought up to 8 μg of total DNA/dish. After 18 h transfected cells were treated with 500 nM geldanamycin or Me2SO carrier solvent for 2 h. Following geldanamycin treatment p23 immunoprecipitations were performed in the same manner as described above.

Assessment of XAP2 Binding in More than One Copy—COS-1 cells were transfected in 10-cm² dishes with pcXAP2/FLAG, pcXAP2/HA, and pcDNA3/mAhR in the combinations indicated. For each transfection pcDNA3 empty vector was used to a total of 8 μg of DNA/dish. Cytosol preparation and FLAG immunoprecipitations were performed as described above.

Antibodies and Immunoblotting—TSDS-PAGE-resolved proteins were visualized by immunoblot using the following antibodies: for AhR, RPT-1 or RPT-9 (32); for Hsp90, anti-Hsp84 and anti-Hsp96 (33); for XAP2, ARA-9 monoclonal (Novus Biologicals) or anti-XAP2 rabbit polyclonal (Affinity BioReagents); for p23, JJ3; for FLAG, anti-FLAG (Affinity BioReagents); and for HA, anti-HA (Santa Cruz Biotechnology). Chemiluminescence visualization was performed using horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) combined with the SuperSignal®
detergent (0.05–0.4% deoxycholate) efficiently stripped XAP2 from the immunoprecipitated AhR. This result suggests that XAP2 association in the AhR complex is mediated by nonpolar or hydrophobic amino acids.

**Tyrosine 408 Is Essential for XAP2 Interaction with the AhR**—Past experiments using AhR deletion constructs have determined the AhR region required for interaction with XAP2 to be contained by amino acid residues 380–419 (15, 16). In an attempt to create a mutant AhR that does not bind XAP2 but is otherwise unaltered we focused on mutating amino acids of the AhR that were not shared between XAP2, Hsp90, and ligand binding but were exclusive to mediating only XAP2 interaction with the AhR. It was determined by single site alanine scanning mutagenesis that tyrosine 408 (Tyr-408) is vital in maintaining stable XAP2 interaction with the AhR. As illustrated in Fig. 2A, Tyr-408 was found to be outside of the Hsp90 and ligand binding domains of the receptor.

The mutation of Tyr-408 to either an alanine (AhRY408A) or lysine (AhRY408K) was created in the AhR and AhR-FLAG plasmids, and the ability of XAP2 to interact with these mutant receptors was analyzed in COS-1 cells (Fig. 2, B, C, and E) or by using *in vitro* translated products (Fig. 2D). COS-1 cells were transfected to express AhR-FLAG, AhRY408A-FLAG, or AhRY408K-FLAG in the presence or absence of co-expressed XAP2. After 18 h, cytosol from transfected cells was prepared, and FLAG immunoprecipitations were performed. As seen in Fig. 2B, AhRY408A-FLAG and AhRY408K-FLAG lacked the ability to bind endogenous XAP2, and each mutant showed reduced binding to XAP2 when it was co-expressed with the receptor. *In vitro* translated AhRY408A-FLAG and AhRY408K-FLAG were also deficient in binding to translated XAP2 when the constituents were separately translated and incubated together on ice for 1 h (Fig. 2D). Furthermore immunoprecipitation of endogenous XAP2 from COS-1 cells expressing non-FLAG-tagged AhR or AhRY408A again showed a lack of XAP2 interaction with the mutant AhR (Fig. 2E).

**AhR-YFP Point Mutant Cellular Localization Is Unaltered by Expression of XAP2**—Previous reports have demonstrated that XAP2 can sequester the AhR in the cytoplasm (17, 28). Therefore, it was expected that the XAP2 binding-deficient AhR mutants would display altered cellular localization compared with the non-mutant receptor. As shown in Fig. 3, expression of AhR-YFP in COS-1 cells resulted in a mostly nuclear localization of fluorescent receptor. Additionally the co-expression of XAP2 along with AhR-YFP resulted in a redistribution of fluorescence exclusively to the cytoplasm. The expression of AhRY408A-YFP or AhRY408K-YFP alone showed a similar cellular distribution of receptor compared with AhR-YFP. Unlike AhR-YFP, however, XAP2 expression had no effect on AhRY408A-YFP or AhRY408K-YFP localization confirming that direct interaction of XAP2 with the AhR is required for the cytoplasmic retention of the AhR upon co-expression of XAP2 in COS-1 cells. In a previous study using COS-1 cells (28) it was established that AhR-YFP exhibits a mostly nuclear localization when ~25% of AhR complexes are bound by endogenous XAP2. In this study we used slightly larger amounts (0.75 versus 0.5 μg/10-cm² culture dish area) of receptor plasmid in each transfection. Under these conditions we would expect the non-mutant AhR-YFP to be expressed at high enough levels that less than 25% of the receptor is bound by endogenous

---

**RESULTS**

**XAP2 Interaction with the AhR Complex Is Mediated through Nonpolar Amino Acid Residues**—To analyze AhR function in the absence of XAP2 we desired to create a mutant form of the AhR unable to bind XAP2. We first sought to determine whether the presence of XAP2 in the AhR complex was mediated through electrostatic or nonpolar forces. To accomplish this, AhR-FLAG and XAP2 were separately *in vitro* translated and then incubated together followed by FLAG immunoprecipitation of the AhR. As seen in Fig. 1, washing the immunoadsorbed complexes with buffer (0.05–1 M NaCl) of increasing ionic strength had little effect on the ability of XAP2 to co-precipitate with the AhR. Conversely washing the complexes with buffer containing increasing concentrations of non-ionic detergent (0.05–0.4% deoxycholate) efficiently stripped XAP2 from the immunoprecipitated AhR. This result suggests that XAP2 association in the AhR complex is mediated by nonpolar or hydrophobic amino acids.

---

**FIG. 1.** The interaction of AhR and XAP2 is mediated through nonpolar residues. pcDNA3/AhR-FLAG and pCMV/XAP2 were separately *in vitro* translated in the presence of [35S]methionine and subsequently mixed together and incubated on ice for 1 h. Complexes were FLAG-immunoprecipitated and washed with MENG buffer supplemented with increasing concentrations of ionic (NaCl) or nonionic (deoxycholate) components. Samples were resolved by SDS-PAGE and transferred to PVDF membrane, and proteins were visualized by autoradiogram. AhR and XAP2 band intensities were quantified by filmless autoradiographic analysis, and the relative percentage of XAP2 bound to the AhR was calculated and graphed.

West Pico chemiluminescent substrate kit (Pierce) and visualized with X-Omat Blue film (Kodak). For autoradiography one of two methods was used: either 125I-secondary IgG antibodies or 125I-streptavidin (Amersham Biosciences) were used with biotin-conjugated secondary IgG antibodies (Jackson Immunoresearch Laboratories, Inc.). Autoradiograms were visualized using Biomax MS film (Kodak).
XAP2, thus explaining a perceived inability of endogenous XAP2 to sequester the receptor in the cytoplasm.

The Absence of XAP2 in the AhR Complex Results in Elevated Basal and Ligand-induced Transcriptional Activity—Contrary to reports by other laboratories (9, 15, 17), we have demonstrated that basal and ligand-induced AhR activity can be globally repressed upon the increased expression of XAP2 in COS-1 cells (21, 34). Based on the XAP2-mediated effects we have observed, it was anticipated that AhR complexes lacking the functional binding of XAP2 would display altered transcriptional activity. AhR-FLAG or AhRY408A-FLAG was expressed in COS-1 cells in the presence or absence of co-expressed XAP2. Transfected cells were treated for 8 h with normal medium, Me2SO vehicle, or 1 nM TCDD, and transcriptional activity was assessed by luciferase production. As we have previously reported, basal AhR-FLAG activity was repressed by XAP2 co-expression. However, the co-expression of XAP2 with AhRY408A-FLAG had no effect on the basal transcriptional activity (Fig. 4A). Additionally ligand-induced activity of AhR-FLAG was reduced by the increased expression of XAP2, while the XAP2 binding-deficient mutant displayed the same activity in the presence or absence of exogenously expressed XAP2.

Results from Fig. 4A suggest that the AhRY408A-FLAG protein displays an overall increase in transcriptional activity. To verify that the increase in activity seen with the XAP2 binding-deficient AhR mutant was not merely due to the increased expression of that protein, the experiment was repeated, and cell lysates were prepared from a subset of untreated cells following an 8-h ligand treatment with 1 nM TCDD or 10 μM benzo[a]pyrene.

Western blot analysis confirmed that when AhR-FLAG, AhRY408A-FLAG, and AhRY408K-FLAG were expressed at equal levels a considerable increase in basal and ligand-induced activity was observed (Fig. 4B). These results suggest that the endogenous XAP2 found in COS-1 cells acts to appreciably maintain the AhR in a constitutively inactive state and attenuate the level of basal and ligand-induced AhR activity. It should be noted that the transfected amounts of receptor plasmid used in each of these experiments was at least 7.5 times less (per cm² cell culture area) than those used for the microscopy experiment in Fig. 3, and therefore it is

 FIG. 2. Tyrosine 408 of the AhR is necessary for the stable interaction with XAP2. The location of the mutated tyrosine relative to other known domains of the AhR is illustrated (A). COS-1 cells were transfected in 10-cm² dishes with 4 μg of pcDNA3/AhR-FLAG, pcDNA3/AhRY408A-FLAG, or pcDNA3/AhRY408K-FLAG plus 4 μg of pcCI/XAP2 or pcCI. AhR-FLAG proteins were immunoprecipitated with anti-FLAG resin, and proteins were visualized by Western blot using 125I-secondary antibodies (B) or chemiluminescence (C). pcDNA3/AhR-FLAG, pcDNA3/AhRY408A-FLAG, pcDNA3/AhRY408A-FLAG, and pcCI/XAP2 were separately in vitro translated in the presence of [35S]methionine. Equal volumes of AhR and XAP2 translations were mixed together and incubated on ice for 1 h. FLAG immunoprecipitations were performed, and proteins were visualized by autoradiogram (D). COS-1 cells were transfected with 4 μg of pcDNA3/AhR or pcDNA3/AhRY408A. Endogenous XAP2 was immunoprecipitated with an XAP2-specific antibody, and proteins were visualized by Western blot using biotin-conjugated secondary antibodies and 125I-streptavidin (E). IP, immunoprecipitation; bHLH, basic helix-loop-helix; PAS, Per/ARNT/Sim; A, acidic; Q, glutamine rich; PST, proline/serine/threonine-rich.
XAP2 Antagonizes p23 within the AhR Complex

Expected that endogenous XAP2 has a more pronounced effect on influencing the cytoplasmic retention of the non-mutant AhR due to the decreased levels of expressed receptor present.

Increased Expression of XAP2 Results in the Displacement of p23 from the Unliganded AhR Complex—Recent publications have attempted to assess the role of the Hsp90-associated co-chaperone protein p23 in regulating AhR function. The absence of p23 allows AhR/ARNT heterodimer formation in the absence of ligand (12). p23 also mediates an increased abundance of ligand-induced, DNA-bound AhR/ARNT heterodimer (26). Because of the emerging functional role of p23 in regulating AhR activity we wanted to determine whether p23 presence in the receptor complex was altered by the presence or absence of XAP2.

AhR-FLAG or AhRY408A-FLAG was expressed in COS-1 cells in the presence of increasing amounts of XAP2. Cell lysates were prepared, and the AhR was immunoprecipitated with p23-bound Hsp90 but had little or no effect on the amount of Hsp90 associated with p23. It should be pointed out that based on the input levels (Fig. 6B) it appears as though virtually all of the p23 in the cell lysates was immunoadsorbed. Therefore, it is unlikely that different p23/Hsp90 populations were preferentially selected leading to misinterpreted results. Taken together, these data suggest that the dissociation of p23 from the AhR complex by XAP2 is specific to the AhR complex and not a general effect seen with Hsp90 alone.

XAP2 Is Capable of Existing in Multimeric Complexes That Are Not Dependent on Hsp90—XAP2 can exclusively bind to the AhR in the absence of Hsp90 (16). It also can bind directly to Hsp90 in cell culture when expressed in high amounts (10) and interacts weakly in vitro (35). Because of this, there may be more than one potential binding site for XAP2 within the unliganded AhR-Hsp90 complex. To see whether XAP2 can exist in a complex in more than one copy COS-1 cells were

**FIG. 3.** XAP2 is unable to redistribute AhRY408A-YFP mutants into the cytoplasm. COS-1 cells were transfected in 30-mm glass bottom dishes with 0.75 μg of pEYFP/mAhR, pEYFP/mAhRY408A, or pEYFP/mAhRY408K plus 0.75 μg of pClXAP2 or pCl. The cellular localization of expressed AhR proteins was analyzed by fluorescent microscopy ~12 h after transfection.

binding to the AhR results in the decreased ability of the AhR to recognize the nuclear import protein β-importin (20), p23 may act to enhance nuclear import (25). Therefore, we had reason to believe that the ligand-bound AhR may undergo a conformational change in the cytoplasm resulting in the loss of XAP2 and recruitment of p23 to enhance this nuclear uptake of the AhR. To test this hypothesis a constitutively cytoplasmic receptor, AhRK13A-FLAG, that contains a single point mutation in the NLS of the AhR was expressed in COS-1 cells. After 18 h transfected cells were treated for varying times (0, 1, 2, or 3 h) with 10 nM TCDD. Cell lysates were prepared, and AhRK13A-FLAG was immunoabsorbed using anti-FLAG resin. As shown in Fig. 5C, XAP2 and p23 binding to the cytoplasmic AhR did not appear to be significantly altered after exposure to a saturating amount of TCDD. Filmless autoradiographic analysis indicated no more than a 10% change in XAP2 and p23 recruitment relative to the amount of AhR adsorbed (data not shown). Since the AhRK13A-FLAG protein has been shown to efficiently bind ligand (29) it is possible that the cytoplasmic ligand-bound AhR does not preferentially recruit either XAP2 or p23 to the AhR complex.

The Displacement of p23 from the Unliganded AhR Complex by XAP2 Is Not a General Hsp90-mediated Effect—Previous work using in vitro components has shown that p23 presence in the AhR complex is mediated through association with Hsp90 (25). Although unlikely, we also wanted to be sure that p23 does not directly associate with co-expressed XAP2. To do this, duplicate plates of COS-1 cells were transfected with pC/ DNA3/AhR in the presence or absence of co-expressed pCI/XAP2. After 12 h, one plate of transfected cells was treated with 500 nM geldanamycin for 2 h to dissociate p23 and AhR from Hsp90, while the other plate was treated with Me2SO carrier solvent. Cell lysates were prepared, and p23 immunoprecipitations were performed. In the absence of geldanamycin treatment (Fig. 6A) the same results were observed as in Fig. 5B. Immunoprecipitations of p23 in geldanamycin-treated samples resulted in the loss of AhR, Hsp90, and XAP2 co-adsorption with p23 (Fig. 6A). This confirms that p23 is associated with the AhR and XAP2 indirectly through the interaction with Hsp90.

Since p23 and XAP2 both bind directly to Hsp90 in the absence of the AhR we wanted to determine whether XAP2-induced displacement of p23 from Hsp90 is a general effect that can be observed in complexes lacking the AhR. To this end, cells were transfected with increasing amounts of XAP2 without co-expressed AhR. Cell lysates were prepared, p23 immunoprecipitations were performed, and samples were visualized by Western blot. As seen in Fig. 6B, increased expression of XAP2 resulted in increasing levels of XAP2 associated with p23-bound Hsp90 but had little or no effect on the amount of Hsp90 associated with p23. It should be pointed out that based on the input levels (Fig. 6B) it appears as though virtually all of the p23 in the cell lysates was immunoadsorbed. Therefore, it is unlikely that different p23/Hsp90 populations were preferentially selected leading to misinterpreted results. Taken together, these data suggest that the dissociation of p23 from the AhR complex by XAP2 is specific to the AhR complex and not a general effect seen with Hsp90 alone.

AhR-FLAG or AhRY408A-FLAG was expressed in COS-1 cells in the presence of increasing amounts of XAP2. Cell lysates were prepared, and the AhR was immunoprecipitated using anti-FLAG resin. As shown in Fig. 2, XAP2 binding to AhRY408A-FLAG was absent from the endogenous XAP2 pool and markedly reduced with co-expressed XAP2 (Fig. 5A). Surprisingly p23 presence in the immunoadsorbed AhR-FLAG complex was reduced or eliminated by the increased presence of XAP2 but virtually unaltered by increasing XAP2 in the AhR-FLAG complex (Fig. 5A). Similarly immunoprecipitation of p23 from COS-1 cells expressing AhR-FLAG or AhRY408A-FLAG in the presence or absence of co-expressed XAP2 demonstrated the same effect. AhR-FLAG and AhRY408A-FLAG alone were able to be immunoadsorbed with p23, but when XAP2 was co-expressed only AhRY408A-FLAG was still in a complex with p23 (Fig. 5B). Additionally it was observed that, in the presence of co-expressed AhR, exogenously expressed XAP2 was found in a complex with p23, presumably through indirect interaction with Hsp90, but no endogenous XAP2 binding was seen (Fig. 5B).
transfected with FLAG- and HA-tagged forms of XAP2 in the presence and absence of AhR. FLAG immunoprecipitations were then performed to adsorb XAP2-FLAG and additional proteins found associated with it. As expected XAP2-FLAG was able to co-precipitate Hsp90 and the AhR (Fig. 7A). Surprisingly XAP2-HA was found to co-purify with adsorbed XAP2-FLAG. This association occurred in the presence and absence of expressed AhR. Therefore, it is clear that XAP2 can be found assembled in a complex composed of at least two molecules of itself.

Next we wished to determine whether Hsp90 and the AhR are required to mediate the association of more than one molecule of XAP2 in a complex. To do this we used the point mutant XAP2G272D-FLAG that contains a single glycine to aspartic acid substitution in the TPR domain of XAP2. This mutant is unable to directly interact with Hsp90 or the AhR (36). As previously noted, immunoprecipitated XAP2-FLAG associated in complex with XAP2-HA as well as Hsp90 and AhR (Fig. 7B). Substituting XAP2G272D-FLAG for the non-mutant FLAG-tagged protein resulted in the loss of Hsp90 and AhR binding to XAP2. Surprisingly, even without the association of the XAP2 point mutant with Hsp90 or the AhR, co-precipitation of XAP2-HA with XAP2G272D-FLAG still occurred (Fig. 7B). Therefore it is evident that XAP2 can exist in multimeric complexes that do not require the presence of Hsp90 or the AhR and that XAP2 most likely utilizes a distinct domain to facilitate this interaction. Whether multiple copies of XAP2 can exist in a complex with Hsp90 or the AhR remains to be determined.

**DISCUSSION**

Presently almost all mammalian in-cell studies attempting to ascertain the role that XAP2 plays in regulating AhR function have looked at effects resulting from transiently expressed XAP2. Many common cell lines, however, contain substantial levels of endogenous XAP2 (36) that are presumably already

---

**Fig. 4.** Transcriptional activity of AhRY408-FLAG mutants is elevated by the absence of XAP2 binding. COS-1 cells were transfected in 6-well dishes with 100 ng of pcDNA3/mAhR-FLAG or pcDNA3/mAhRY408A-FLAG, 0 or 200 ng of pCI/XAP2, and 100 ng of the AhR-driven luciferase reporter vector/well. After 18 h, transfected cells were treated with Me2SO (DMSO) vehicle or 1 nM TCDD for 8 h. Transcriptional activity was assessed by luciferase production and corrected to total protein content (A). To obtain equal expression of AhR, 0–60 ng of receptor plasmid/well was transfected into 6-well dishes, and reporter assays were performed in the same manner as in A except 30 ng of reporter plasmid was used, and in addition to 1 nM TCDD cells were treated with 10 μM benzo[a]pyrene. Western blot using 125I-secondary antibodies was performed with 1% Nonidet P-40 cell lysates from non-treated cells to analyze the level of AhR expression between transfections. Samples that showed equal expression of AhR were used to compare activity between the mutant and non-mutant receptors (B). Data presented are the mean values of triplicate samples (relative activity per μg of protein (A) or per 0.1 mg of protein (B)). Error bars are representative of the standard deviation for those values.
regulating AhR function. Additionally many studies have utilized chimeric AhR constructs, and some of these are constitutively nuclear. Since XAP2 mediates a spectrum of functional changes in the AhR (i.e. cellular localization, NLS recognition, and AhR stability) leading to altered transcriptional activity we desired to look at the properties of full-length AhR in the absence of any effects contributed by endogenous XAP2.

Antisense RNA methods have been used in an attempt to reduce levels of endogenous and transiently expressed XAP2 with moderate success (19, 37) but have been unable to completely deplete cells of endogenous XAP2. The in vitro stripping experiment (Fig. 1) suggests that XAP2 interaction with the AhR is probably not mediated through highly charged amino acids. Using that knowledge we set out to create a targeted point mutation in the AhR that would abrogate the interaction of XAP2 with the AhR. Tyrosine 408 is located within the XAP2 binding domain of the AhR but outside of the Hsp90 and ligand binding regions. The mutation of this amino acid to alanine or lysine resulted in the loss of endogenous XAP2 binding to the AhR (Fig. 2). When XAP2 was co-expressed with the AhR, an increased level of XAP2 binding was observed, but the AhRY408 point mutants had reduced binding affinity by ~50% as quantified by filmless autoradiographic analysis (data not shown). Hsp90 does not appear to bind to endogenous XAP2 (10), but increasing exogenous expression of XAP2, as shown in Fig. 6B, resulted in binding of XAP2 to Hsp90 in a dose-dependent manner. Thus the binding of expressed XAP2 to the AhRY408 mutants is likely attributed to an interaction between Hsp90 and XAP2 in the AhR complex rather than a direct interaction with the AhR. This incorporation of XAP2 into the mutant AhR complex appears to exert few if any functional effects on the AhR as will be discussed below.

The ability of XAP2 to cause AhR cytoplasmic retention is well established (17, 28, 38). We wanted to exploit this effect to determine whether the binding of XAP2 to the mutant AhR complexes observed in immunoprecipitation experiments (Fig. 2) was able to regulate AhR cellular localization to the same degree as with the non-mutant receptor. Although XAP2 was incorporated into the unliganded AhRY408 mutant complex (Fig. 2) when it was co-expressed, it was unable to redistribute AhRY408A-YFP or AhRY408K-YFP from the nuclear compartment to the cytoplasm (Fig. 3). The presence of XAP2 in the AhR complex reduces the efficiency of the bipartite NLS of the AhR to recognize the nuclear import protein β-importin (20).
XAP2 is speculated to function through direct interaction with the AhR. The inability of XAP2 to alter AhRY408-YFP point mutant cellular localization suggests that the mutant complexes are not capable of XAP2-mediated conformational change in the AhR. As mentioned above, exogenously expressed XAP2's presence in the AhRY408 mutant complex is most likely mediated via XAP2 interaction with Hsp90. Direct interaction of XAP2 with the AhR is essential for cytoplasmic sequestration of the AhR as opposed to XAP2 influencing Hsp90 conformationally, which would in turn affect the AhR.

Since the binding of expressed XAP2 to the AhRY408 mutants was not functional in the context of regulating AhR cellular localization, it is plausible that it also would not have a marked effect on modulating transcriptional activity of the receptor. As postulated, co-expressed XAP2 had no effect on the transcriptional output of AhRY408A-FLAG, while the activity of the non-mutant receptor was repressed constitutively and upon ligand induction (Fig. 4A). When AhR-FLAG, AhRY408A-FLAG, and AhRY408K-FLAG were expressed in equal amounts the point mutants displayed elevated constitutive and ligand-induced activity (Fig. 4B). This result supports the idea that the endogenous pool of XAP2 is present in high enough levels to significantly influence AhR function specifically to maintain it in an overall repressed state.

In addition to the recognition of 5-importin, the unliganded AhR complex that is associated with p23 can interact with the nuclear import receptor protein pendulin (25). Therefore, it is possible that p23 presence in the AhR complex is reflective of a conformational status that favors nuclear import. Along those lines, it was found that the co-expression of XAP2 along with AhR caused the displacement of p23 from the AhR complex (Fig. 5, A and B). The AhRY408A mutant proteins, however, showed no substantial change in p23 association with the increased binding of XAP2. The lack of p23 loss from the AhRY408A complex furthers lends support to the previous suggestion that XAP2 does not functionally bind to the AhRY408 mutant receptors. Thus, the loss of p23 from the AhR complex is concomitant with the cytoplasmic retention and transcriptional repression observed upon increased XAP2 levels. Interestingly, p23 was not displaced from Hsp90 by XAP2 in the absence of the AhR (Fig. 6A) and did not bind directly to XAP2 (Fig. 6B). Consequently, it appears as though p23 displacement from Hsp90 may only occur when Hsp90 is bound to a client protein, in this case the AhR. It is important to note that only the combination of XAP2 and the AhR antagonized p23 association with Hsp90. Thus, these proteins together appear to induce a unique conformational change in Hsp90 that was not observed when either protein was bound to Hsp90 individually. ATP-bound conformation of Hsp90 is necessary for p23 binding (39). Likewise, XAP2 interaction in the AhR complex is favored by the presence of ATP and non-hydrolyzable ATP analogs, although it does require ATP for interaction in the AhR complex (35). Therefore, the XAP2-induced displacement of p23 from the AhR complex is probably not due to altered nucleotide-bound status of Hsp90 in the unliganded receptor complex. It remains to be determined whether XAP2 also can cause this p23 displacement effect in the peroxisome proliferator-activated receptor, which also in-
teracts with XAP2 directly and with p23 through Hsp90 (40, 41).

XAP2 is an immunophilin-like protein due to the homology it shares with the FKBP family of proteins. Like XAP2, FKBP51 and FKBP52 interaction with Hsp90 is mediated through their TPR domains and regions carboxyl-terminal to the TPR domain (42). The immunophilins cyclophilin 40, FKBP51, and FKBP52 have been reported to bind Hsp90 at a stoichiometry of two immunophilins per Hsp90 dimer (43). However, other reports conclude that only one molecule of FKBP52 exists for each Hsp90 dimer (44). Simplistically the unliganded AhR complex contains three potential binding sites with which XAP2 could interact. Each molecule of the Hsp90 dimer has a carboxyl-terminal TPR acceptor site, and the AhR itself contains one additional site. As observed in Fig. 7, XAP2 can exist in a complex of at least two molecules. Utilizing the XAP2G272D-FLAG TPR point mutant, which does not bind Hsp90 or the AhR, it was concluded that the presence of neither Hsp90 nor the AhR is necessary to mediate the multimeric binding properties of XAP2. As a result, it is possible that XAP2 is capable of binding to itself, requiring no auxiliary proteins. It is also possible that proteins other than Hsp90 or the AhR could mediate multiple XAP2 binding, such as Hsc70, which has been shown to interact with XAP2 (45). It still remains to be determined whether XAP2 can exist within the AhR complex in greater than one copy, although previous quantitative (28) and cross-linking studies (46) would suggest that it does not. Additionally if XAP2 cannot exist within the AhR complex in more than one copy, then the multimeric complexes of XAP2 may serve to regulate the amount of available monomeric XAP2 that can incorporate into AhR complexes and regulate receptor function.

As mentioned previously, XAP2 has been reported to enhance AhR signaling through the stabilization of and overall increase in AhR levels. Additionally we have observed that XAP2 can repress AhR activity. There is evidence to support the idea that XAP2 could repress AhR function. First, as mentioned above, XAP2 acts as a cytoplasmic retention factor, preventing AhR nucleocytoplasmic shuttling (20, 21). Second, upon ligand exposure AhR complexes exhibit a delayed rate of nuclear translocation when expressed with XAP2 (18). Third, as revealed in this report the expression of XAP2 in cells resulted in the displacement of p23 from the AhR complex. Although limited, the existing AhR research supports that p23 acts as a stimulatory factor in regulating AhR activity. It has also been suggested that p23 may act in an ATP-dependent manner to enhance client protein release from Hsp90 (47) and if applied to AhR could expedite ARNT binding leading to increased transcriptional activity. These three factors taken together would provide a mechanistic explanation for the repression in AhR activity observed in our studies.

Undoubtedly some of these seemingly contradictory results found in the literature examining XAP2 function may be experimental artifacts caused by the overexpression of these protein constituents. We attempted to circumvent these issues by creating an AhR mutant incapable of binding endogenous XAP2 or functionally interacting with co-expressed XAP2. Ultimately these questions will have to be addressed further using gene-silencing technology combined with the appropriate animal models. In conclusion, this report provides a plausible mechanistic explanation for the observed XAP2-mediated repression of AhR transcriptional activity in COS-1 cells by showing that XAP2 can induce a conformational change in the AhR.
complex that excludes p23 and is concomitant with the cytoplasmic retention of the AhR.

Acknowledgments—Many thanks to Dr. Steven Safe, Dr. David Toft, and Dr. Graeme Bolger for supplying TCDD, JJ3 antibody, and XAP2 antibody, respectively.

REFERENCES
