Characterization of the AhR−hsp90−XAP2 Core Complex and the Role of the Immunophilin-Related Protein XAP2 in AhR Stabilization†

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ABSTRACT: The unliganded aryl hydrocarbon receptor (AhR) exists in the cytoplasm in a tetrameric 9S core complex, consisting of the AhR ligand-binding subunit, a dimer of hsp90, and the hepatitis B virus X-associated protein 2 (XAP2), an immunophilin-related protein sharing homologous regions with FKBP12 and FKBP52. Interactions between the recently identified XAP2 subunit and other members of the unliganded AhR complex and its precise role in the AhR signal transduction pathway are presently unknown. Mapping studies indicate that XAP2 requires the PAS, hsp90, and ligand binding domain(s) of the AhR for binding, and that both proteins directly interact in the absence of hsp90. XAP2 is also able to interact with hsp90 complexes in the absence of the AhR, and C-terminal sequences of XAP2 are required for this interaction. XAP2 binds to the C-terminal end of hsp90, which contains a tetratricopeptide repeat domain acceptor site, whereas the AhR binds to a domain in the middle of hsp90. XAP2 was not found to be associated with the AhR−Arnt heterocomplex either in vitro or in nuclear extracts isolated from Hepa 1 cells treated with TCDD. Transient expression of XAP2 in COS-1 cells resulted in enhanced cytosolic AhR levels, suggesting a role for XAP2 in regulating the rate of AhR turnover.

The aryl hydrocarbon receptor (AhR)[1] is a member of the bHLH-PAS (basic helix−loop−helix Per-Arnt-Sim) protein superfamily and mediates the response to halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and planar polycyclic aromatic hydrocarbon compounds (*1*−*3*). This superfamily includes the AhR dimerization partner, the aryl hydrocarbon nuclear translocator (Arnt), the hypoxia inducible factor 1a (HIF-1α), and others, which have a bHLH motif and a PAS domain (*4*, *5*). The N-terminal half of the AhR contains the bHLH motif; the HLH acts as a dimerization interface with Arnt, and the basic region mediates the ability of the AhR−Arnt heterodimer to bind to dioxin responsive elements (DREs) (*6*−*8*). The PAS region is composed of highly conserved hydrophobic A and B repeats. Both of these highly conserved regions are homologous to *Drosophila* proteins Per and Sim, which are involved in circadian rhythm regulation and midline development, respectively (*9*, *10*). This region has been demonstrated to be a dimerization interface of the AhR−Arnt heterodimer and is also required for ligand binding and hsp90 interaction (*7*, *8*, *11*). The C-terminus of the AhR contains a complex transactivation domain that mediates transcriptional activation (*12*−*14*).

The unliganded AhR resides in the cytoplasm in a tetrameric 9S core complex consisting of the AhR ligand binding subunit, a dimer of hsp90, and the hepatitis B virus X-associated protein 2 (XAP2) (*15*−*18*). XAP2 was originally observed to interact and suppress the hepatitis B virus X protein, a promiscuous transactivator (*19*). XAP2 is related to the FK506 binding class of immunophilins, and shares regions of homology with FKBP12 and FKBP52. However, unlike these immunophilins, XAP2 is unable to bind to FK506 (*20*). XAP2 is also termed AIP and ARA9 by laboratories that have independently cloned this protein (*17*, *18*). Following activation by ligand, the AhR translocates to the nucleus and heterodimerizes with Arnt with the concurrent loss of hsp90 (*21*). This heterodimer then binds to dioxin responsive elements (DREs) in the promoters of genes such as *CYP1A1* and *CYP1B1*, which in turn results in the production of xenobiotic metabolizing enzymes. This signal transduction pathway has been linked to teratogenesis, tumor promotion, and slow wasting syndrome in animals (*2*).

Like the AhR, several other ligand-inducible receptors are complexed with an hsp90 dimer and an immunophilin component. Immunophilins belong to a family of intracellular receptors that are able to bind to immunosuppressive drugs such as FK506 and cyclosporin A. Several immunophilins contain tetratricopeptide repeat (TPR) domains, which have

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[1] Abbreviations: AhR, aryl hydrocarbon receptor; XAP2, hepatitis B virus X-associated protein 2; AIP, AhR-interacting protein; ARA9, AhR-activated 9; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *CYP1A1* and *CYP1A2*, structural genes for cytochromes P4501A1 and P4501A2, respectively; hsp90, 90 kDa heat shock protein; Arnt, Ah receptor nuclear translocator protein; TPR, tetratricopeptide repeat; Hepa 1 or Hepa 1c1c7, mouse hepatoma cell line 1c1c7; GAM, goat anti-mouse; SAM, sheep anti-mouse; DAR, donkey anti-rabbit; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinyl difluoride); mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid.

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been demonstrated to mediate protein−protein interactions (*22*). FKBP52 (FKBP59, hsp56, HBI, p56), an FK506 binding immunophilin has been demonstrated to form complexes with the glucocorticoid (GR), progesterone (PR), estrogen (ER), and androgen receptors (AR) with an hsp90 dimer (*23*, and references therein). The FK506 binding domain of FKBP52 is located in its N-terminal portion, whereas three TPR domains reside in its C-terminus (*24*). FK506 binding to the FK506 binding domain of FKBP52 does not appear to affect the GR signal transduction pathway (*25*). The TPR domains, however, have been demonstrated to mediate its ability to bind to hsp90 (*26*). The specific role of FKBP52 in these complexes is yet to be determined, although it has been suggested that it mediates nuclear translocation of the GR (*27*). The immunophilin CyP-40, a cyclophilin, has also been isolated in complexes with hsp90 and ligand-activated transcription factors such as the GR, PR, and ER (*23*, *28*). CyP-40 contains an N-terminal domain that binds to the cyclosporin class of immunosuppressive drugs, and contains three TPR domains in its C-terminal region, which have been demonstrated to mediate its binding to hsp90 (*29*, *30*). Recently, CyP-40 has been demonstrated to inhibit c-Myb DNA binding activity, and this inhibition is blocked by cyclosporin A (*31*). Thus, an important role for immunophilins in soluble receptor and transcription factor activity is emerging.

Recently, XAP2 was identified as a protein that was capable of stably interacting with the AhR (*17, 18*), and identified as a member of the unliganded tetrameric AhR core complex (*16*). Although its precise role in the AhR signal transduction pathway is unknown, XAP2 transient expression above endogenous levels is capable of enhancing AhR-mediated transcriptional activity (*16*, *17*). In this report, XAP2 was demonstrated to interact directly with the AhR in the absence of hsp90, bind at or near the PAS domain of the AhR, bind to the C-terminal end of hsp90 in the absence of the AhR, and enhance the level of the AhR when transiently expressed in COS-1 cells, suggesting a role for XAP2 in regulating AhR turnover.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Culture. Escherichia coli DH5α (Gibco BRL) was used for all plasmid expression. COS-1 and Hepa 1c1c7 (Hepa 1) cells were grown in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 0.1 μg/mL streptomycin at 37 °C in 94% air/6% CO_2.

Construction of Plasmids. Forward oligonucleotide primers containing a restriction endonuclease cleavage site, Kozak consensus sequence (*32*), and an ATG start codon were used in a standard PCR protocol with reverse oligonucleotide primers that encode a FLAG amino acid epitope (DYKDDDDK) and restriction endonuclease cleavage site to generate XAP2-FLAG and mAhR-FLAG deletion mutants. PCR was performed using pCI/XAP2 and pcDNA3/bmAhR as the templates to generate XAP2-FLAG and mAhR-FLAG deletion mutants, respectively. Oligonucleotide primer sequences and PCR conditions will be made available on request. Full-length XAP2-FLAG and XAP2-FLAG deletion mutants were cloned into pCI (Promega). The designation 1−330 denotes full-length XAP2-FLAG (amino acids 1−330), and the same format was used to describe XAP2-FLAG deletion mutants. The FLAG epitope was added to the mAhR deletion mutant pcDNA3/N315/AhR (*6*) and pcDNA3/C386/βmAhR using the procedure described above. The nucleotide sequences of all cloned PCR products were confirmed by DNA sequencing. pcDNA3/bmAhR, pcDNA3/βmAhR-FLAG, and pcDNA1/Arnt-FLAG have been previously described (*33*, *34*).

In Vitro Protein−Protein Interaction Analysis. In vitro protein−protein interaction assays were performed by generating proteins using a coupled transcription/translation system in rabbit reticulocyte lysate (RL) (Promega). In several experiments, proteins were labeled with [^{35}S]methionine (1000 Ci/mmol) (Amersham). Immunoadsorption of FLAG epitope-tagged constructs was performed with a 25 μL packed bed volume of M2 anti-FLAG affinity gel (Sigma). All immunoadsorptions were performed for 2 h at 4 °C with rocking in IP buffer [MENG [25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, and 10% glycerol (pH 7.5)], 2 μg/mL ovalbumin, 2 μg/mL bovine serum albumin, 50 mM NaCl, 0.5% Triton X-100, and 10 mM sodium molybdate]. Following immunoadsorption, the M2 affinity gel was washed three times in IP buffer, followed by two washes in MENG buffer. The immunoadsorbed complexes were eluted with FLAG peptide (Sigma), followed by addition of an equal volume of 2× tricine sample buffer (2× TSB), resolved on 8% tricine SDS−PAGE, followed by fluorography and autoradiography.

Protein−Protein Interaction Analysis in COS-1 Cells. For protein−protein interactions, COS-1 cells were grown in 10 cm^2 tissue culture dishes (Falcon) and transfected with 9 μg of pCI/XAP2-FLAG and pCI/XAP2-FLAG deletion mutants using Lipofectamine according to the manufacturer's instructions (Gibco BRL). Following the transfection procedure, cells were rinsed once in PBS, trypsinized, washed once in PBS, and lysed in MENG buffer containing 10 mM sodium molybdate, 1% NP-40, and 1× protease inhibitor cocktail (Sigma) for 15 min at 4 °C. Lysates were centrifuged at 100000*g* for 1 h at 4 °C. Lysate was incubated with 25 mL of packed M2 affinity gel in IP buffer for 2 h at 4 °C with rocking, washed in IP buffer three times, washed in MENG buffer two times, eluted with FLAG peptide (Sigma), followed by addition of an equal volume of 2× TSB, resolved on 8% tricine SDS−PAGE, and transferred to a PVDF membrane for immunoblot analysis.

Antibodies Used in Immunoadsorption and Immunoblot Analysis. RPT1, an anti-AhR mouse monoclonal antibody, was used to detect AhR in immunoblot analysis (*35*). RPT9, an anti-AhR mouse monoclonal antibody, was used in immunoadsorption analysis (*35*). C1p50, an anti-p50^{cdc37} mouse monoclonal antibody, was used for detection of p50 in immunoblot analysis (*36*). 2B10, an anti-Arnt mouse monoclonal antibody (*37*), and hsp84, hsp86, and anti-hsp84/86 rabbit polyclonal antibody were used for detection of hsp90 by immunoblot analysis (*38*). M2, an anti-FLAG mouse monoclonal antibody, was used in immunoblot analysis to detect FLAG-tagged proteins (Sigma). Anti-FKBP52 and anti-FKBP52 rabbit polyclonal antibody were used to detect FKBP52 (*39*). Rabbit polyclonal anti-XAP2 antibodies were provided by E. Crose (Berlex). For immunoblot analysis, primary antibodies were visualized with 5 μCi of [^{125}I]SAM IgG or 5 μCi of [^{125}I]DAR IgG (Amersham) followed by visualization by autoradiography. In some

experiments, primary antibodies were visualized with GAM−HRP or DAR−HRP (Jackson ImmunoResearch).

GST−hsp90 Fusion Protein Interaction Assays. GST−hsp90 fusion proteins were generated in *E. coli* and isolated as previously described (*40*). Equivalent amounts of each protein were incubated with glutathione−agarose (Sigma) in PBS for 2 h and washed three times in PBS and two times in binding buffer (MENG, 1% w/v Chaps, 100 mM NaCl, and 20 mM sodium molybdate). [^{35}S]Methionine-labeled proteins were then incubated in 0.5 mL of binding buffer overnight, washed three times in binding buffer and two times in MENG buffer, resuspended in an equal volume of 2× TSB, resolved by SDS−PAGE, and Coomassie Blue stained to visualize GST−hsp90 fusion proteins. The [^{35}S]-methionine-labeled proteins were visualized by autoradiography.

Hepa 1 Nuclear Extract Isolation. Hepa 1 cells were grown to confluency in 175 cm^2 tissue culture flasks (Falcon). Following induction with 20 nM TCDD or carrier solvent for 1 h, cells were washed once in PBS, trypsinized, washed once in PBS, and homogenized in a dounce homogenizer in 0.5 mL of MENG buffer containing 20 mM sodium molybdate and 1× protease inhibitor cocktail at 4 °C. The cytosolic fraction and nuclear extracts were prepared exactly as previously described (*41*). Immunoadsorption of the AhR−Arnt heterocomplex from nuclear extracts was performed with 5 μg of the anti-Arnt monoclonal antibody 2B10 or mouse IgG as a control absorbed to 50 μL of Protein G−Sepharose (Sigma). Immunoadsorbed heterocomplexes were washed three times in MENG and 500 mM NaCl, followed by one wash in MENG, incubated in an equal volume of 2× TSB, resolved by SDS−PAGE, and transferred to a PVDF membrane for immunoblot analysis.

Stabilization of AhR by XAP2. COS-1 cells were grown in 10 cm^2 tissue culture dishes (Falcon) and transfected with 3 μg of pcDNA3/βmAhR and 6 μg of pCI/XAP2 or pCI as a control using Lipofectamine according to the manufacturer's instructions (Gibco BRL). Following the transfection procedure, cells were rinsed once in PBS, trypsinized, washed once in PBS, and lysed in MENG buffer containing 10 mM sodium molybdate, 1% NP-40, and 1× protease inhibitor cocktail (Sigma) for 15 min at 4 °C. Lysates were centrifuged at 100000*g* for 1 h at 4 °C. The total cell lysate (150 μg) from each transfection was incubated in an equal volume of 2× TSB, resolved on 8% tricine SDS−PAGE, and transferred to a PVDF membrane for immunoblot analysis.

RESULTS AND DISCUSSION

Several laboratories have demonstrated that XAP2 is part of the unliganded cytosolic AhR complex (*15*−*18*). Previous studies have shown that XAP2 is not required for the AhR to bind to hsp90 (*16*). In addition, transient expression of XAP2 leads to enhanced AhR-mediated transactivation activity (*16*, *17*). The goal of this study was to further characterize the properties of XAP2 in the AhR unliganded tetrameric complex, explore possible mechanism(s) of its role in enhancing AhR activity, and test whether it is capable of interacting with the AhR−Arnt heterocomplex.

XAP2 Requires the PAS, hsp90, and Ligand Binding Domain(s) of the AhR for Binding. It is unknown if XAP2 directly interacts with the AhR, hsp90, or both proteins in the unliganded AhR tetrameric complex. The XAP2 binding site on the AhR was mapped using a coupled in vitro transcription/translation system in rabbit reticulocyte lysate (RL). A series of FLAG epitope-tagged AhR cDNA constructs were generated, and C-terminal AhR deletion mutants (*6*) were used for AhR−XAP2 interaction studies (Figure 1A). First, FLAG-tagged AhR constructs were radiolabeled with [^{35}S]methionine, mixed with [^{35}S]methionine-labeled XAP2, immunoadsorbed with anti-FLAG M2 resin, resolved by SDS−PAGE, and transferred to a PVDF membrane. The FLAG-tagged AhR constructs and XAP2 were visualized by autoradiography, and hsp90 was visualized by immunoblot analysis with anti-hsp90 antibodies (Figure 1B). These data indicated that the carboxyl-terminal AhR mutant C386/AhR/FLAG, which lacks the 386 carboxyl-terminal amino acids of the AhR, but includes the bHLH, PAS A, PAS B, and ligand/hsp90 binding domains of the AhR, is able to bind to XAP2 as does full-length AhR (Figure 1B, lanes 3 and 5). Next, the N315/GAL4/AhR-FLAG mutant, which lacks the first 315 amino-terminal amino acids of the AhR, including the bHLH, PAS A, and a portion of the PAS B domain, was used in XAP2 binding studies (Figure 1A). In this mutant, the bHLH, PAS A, and a portion of the PAS B domain has been replaced with a Gal4 DBD (*6*). This Gal4 domain does not bind to XAP2 using the yeast two-hybrid system (*17*). This deletion resulted in the almost total loss of XAP2 specific binding, suggesting that the region spanning the N-terminal end of the AhR is necessary. However, AhR constructs with bHLH and PAS A domain deletions were also tested and were found to interact with XAP2 (unpublished data). These results suggested that the PAS B domain, or including a region between PAS A and B, was important for XAP2 binding. A significant amount of hsp90 still complexed with the N315/GAL4/AhR mutant (Figure 1B, lane 2), as has been previously reported (*6*, *11*), demonstrating that hsp90 binding to the AhR was not sufficient for efficient recruitment of XAP2. Arnt was utilized as a control because it is unable to interact with hsp90 or XAP2 (Figure 1B, lane 4) (*17*, *18*). Similar mapping experiments using AhR deletion mutants and XAP2 have also been performed, and it was demonstrated in these studies that an N-terminal mutant lacking the first 130 amino acids of the AhR, including the bHLH region, but containing the PAS A and PAS B domains, was able to bind to XAP2 (ARA9) (*20*). Taken together, these results suggest that a region between the PAS A and PAS B domains is required for efficient binding of XAP2.

To determine if additional sequences proximal to the PAS region were required for XAP2 binding, a series of progressively truncated AhR constructs (Figure 1A) deleted at the C-terminal end (*6*) were radiolabeled with [^{35}S]methionine, mixed with [^{35}S]methionine-radiolabeled XAP2, immunoadsorbed with the anti-AhR monoclonal antibody RPT9 coupled to Protein G−Sepharose, resolved by SDS−PAGE, and visualized by autoradiography (Figure 1C). XAP2 interacted with AhR deletion mutant C313, which contains both the PAS A and PAS B domains and 157 amino acids C-terminal to the PAS B domain. XAP2 was unable to bind to mutant C425, however, which contains both the PAS A and PAS B domains and 44 amino acids C-terminal to the PAS B domain. These results indicate that XAP2 binding also requires sequences in the hsp90/ligand binding domain

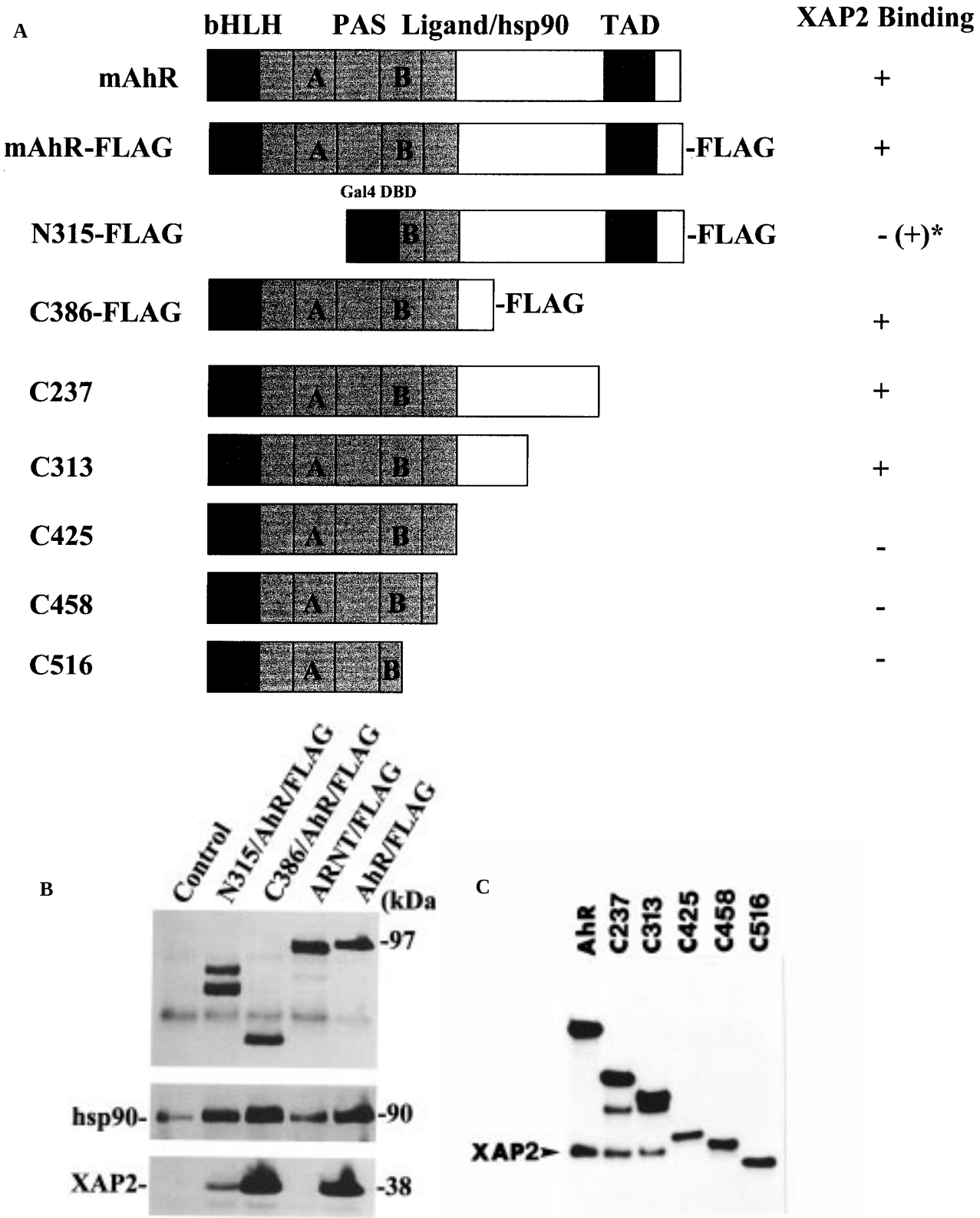

FIGURE 1: XAP2 requires the PAS, hsp90, and ligand binding domain(s) of the AhR for binding. (A) Schematic representation of mAhR deletion mutants used in immunoadsorption experiments and for binding to XAP2; the asterisk denotes partial binding to XAP2. (B) mAhR-FLAG, mAhR FLAG deletion mutants, and ARNT-FLAG were labeled with [^{35}S]methionine in RL, mixed with [^{35}S]methionine-labeled XAP2, immunoadsorbed with M2 resin, resolved by SDS−PAGE, and transferred to a PVDF membrane. (Top) AhR deletion mutants visualized by autoradiography. (Middle) Immunoblot analysis of top panel to detect hsp90 using anti-hsp90 antibodies (hsp84/86). (Bottom) [^{35}S]Methionine-labeled XAP2 co-immunoadsorbed with AhR and visualized by autoradiography. (C) mAhR and mAhR C-terminal deletion mutants were labeled with [^{35}S]methionine in RL, mixed with [^{35}S]methionine-labeled XAP2, immunoadsorbed with RPT9−Protein G−Sepharose, resolved by SDS−PAGE, and visualized by autoradiography.

of the AhR in addition to the PAS A and B domains. During the preparation of this paper, mapping studies were performed using these AhR mutants, with the results supporting the observations presented here that XAP2 binding requires

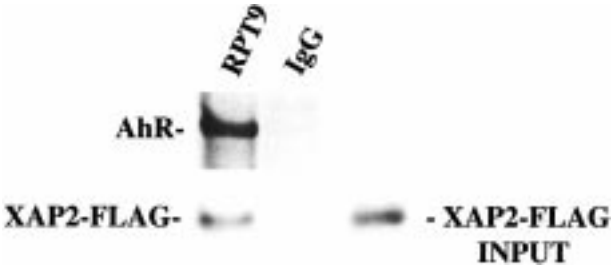

FIGURE 2: XAP2 binds to the AhR in the absence of hsp90 in a cell-free system. mAhR and XAP2-FLAG were labeled with [^{35}S]-methionine in RL independently. mAhR was immunoadsorbed with RPT9−Protein G−Sepharose, and XAP2-FLAG was immunoadsorbed with M2 resin and eluted with FLAG peptide. Each immunoadsorption mixture was washed in PBS to remove hsp90: lane 1, immobilized mAhR mixed with 40% of the XAP2-FLAG eluted from M2 resin; lane 2, immobilized control IgG mixed with 40% of the XAP2-FLAG; and lane 3, 20% of purified XAP2-FLAG resolved by SDS−PAGE, followed by immunoblot analysis using the M2 antibody.

sequences in the PAS, ligand, and hsp90 binding domains of the AhR for binding (*20*). If it is considered that XAP2 requires sequences in this region for binding, it is possible that it modulates the ability of the ligand to bind to the AhR. For example, a steric change imparted by XAP2 on the AhR could influence its ability to bind to specific classes of ligands.

XAP2 Binds to the AhR in the Absence of hsp90 in a Cell-Free System. Studies using the yeast two-hybrid system demonstrated that XAP2 (AIP−ARA9) interacted with the AhR (*17*, *18*). These results suggested that XAP2 could interact with the AhR. It was unknown, however, if hsp90 was a member of, or participated in, the two-hybrid interaction complex. Thus, a cell-free system was used to determine if there was a direct interaction between the AhR and XAP2 in the absence of hsp90. To address this issue, plasmids pcDNA3/βmAhR and pCI/XAP2-FLAG encoding the AhR and XAP2−FLAG, respectively, were transcribed and translated independently in RL. The AhR was immunoadsorbed with the anti-AhR monoclonal antibody RPT9 coupled to Protein G−Sepharose, and XAP2-FLAG was immunoadsorbed with the anti-FLAG M2 affinity gel. The immunoadsorbed AhR was washed sufficiently in PBS to remove hsp90 which forms a complex with the AhR following its translation (*16*). The immunoadsorbed XAP2-FLAG was also washed in PBS, although it has been previously demonstrated that XAP2 does not form a complex with hsp90 directly in RL (*16*). The presence of hsp90 was examined using immunoblot analysis in each purified sample, and no hsp90 was detected (data not shown). XAP2-FLAG was then eluted from the M2 affinity gel with the FLAG peptide for use in binding studies. Eluted XAP2-FLAG was incubated with immunopurified AhR immobilized to the RPT9−Protein G−Sepharose, and an equal amount was incubated with mouse IgG−Protein G−Sepharose in MENG and 150 mM NaCl for 1 h, washed three times in the same buffer, resolved by SDS−PAGE, and transferred to a PVDF membrane. Immunoblot analysis using the anti-AhR monoclonal antibody RPT1 and anti-FLAG M2 antibodies indicated that a portion of XAP2-FLAG bound directly to the AhR in the absence of hsp90 (Figure 2, lane 1), whereas no specific binding was observed in the control immunoadsorption (Figure 2, lane 2). Twenty percent of the total XAP2-FLAG elution was resolved by SDS−PAGE followed by immunoblot analysis to assess the efficiency of binding to the AhR and control IgG (Figure 2, lane 3). The amount of XAP2 that bound to the AhR was significant, when compared to the input of XAP2-FLAG (Figure 2). These results indicate that XAP2 can stably bind to the AhR in the absence of hsp90 in a cell-free system.

It has also been established by chemical cross-linking studies that the AhR can bind directly to hsp90 (*15*). In addition, XAP2 is not required for AhR−hsp90 assembly in rabbit RL (*16*). Thus, the AhR is capable of interacting with XAP2 and hsp90 independently. Cross-linking studies performed with other 9S complexes, including the GR, PR, and ER complexes, have revealed that they also exist as heterotetrameric complexes, similar to results obtained with the AhR 9S core complex (*42*−*44*). However, no evidence, to our knowledge, exists that FKBP52, an FK506-binding immunophilin, which has been isolated in the 9S GR complex, directly interacts with the GR in the absence of hsp90 (*45*). Thus, the AhR−XAP2 interaction is the first demonstration of a ligand-activated receptor that directly and stably interacts with an immunophilin-related protein within the context of a heterotetrameric hsp90 complex.

The C-Terminal End of XAP2 Is Required for Binding to hsp90 in COS-1 Cells. Previously, it has been observed that XAP2 is unable to interact with hsp90 in rabbit RL, yet is a member of hsp90 complexes in cells (*17*). To determine the region of XAP2 that mediates its interaction with hsp90, a series of XAP2 N- and C-terminal sequence deletion mutants were generated (Figure 3A). Each mutant was epitope-tagged at the C-terminal end with a FLAG epitope for immunoadsorption studies using the anti-FLAG M2 affinity gel. To determine if each mutant could be recognized by the M2 affinity gel, each mutant was labeled with [^{35}S]methionine in RL, immunoadsorbed by the M2 gel, resolved by SDS−PAGE, and visualized by autoradiography (Figure 3B). These results demonstrated that each mutant migrated at its calculated molecular weight and was recognized by the affinity gel. Next, plasmids encoding each construct were transiently expressed in COS-1 cells for hsp90 binding studies. COS-1 cells were selected because they essentially lack AhR expression; thus, XAP2−hsp90 interactions could be studied in the absence of AhR in cells. Following transient transfection of XAP2 full-length and deletion constructs, XAP2-FLAG−hsp90 complexes were immunoadsorbed with the anti-FLAG M2 affinity gel, resolved by SDS−PAGE, and analyzed by immunoblot analysis with anti-hsp90 antibodies and M2 anti-FLAG antibodies to detect hsp90 and XAP2-FLAG proteins, respectively. These results indicated that hsp90 was able to bind to N-terminal XAP2-FLAG mutant/17−330 as did full-length XAP2, indicating that the N-terminal 17-amino acid sequence of XAP2 is not required for binding to hsp90 (Figure 3C). It is important to note that full-length XAP2 was expressed at a higher level than the XAP2 mutant/17−330, yet both mutants were able to bind to equivalent amounts of hsp90 (Figure 3C). The lower level of expression of the XAP2 mutant/17−330 may be due to higher turnover of this N-terminal mutant in cells when compared to full-length XAP2-FLAG. XAP2-FLAG mutants lacking C-terminal sequences, however, were unable to bind to hsp90 (Figure 3C). Thus, C-terminal sequences of XAP2 are required for binding to hsp90. The XAP2 mutant/1−

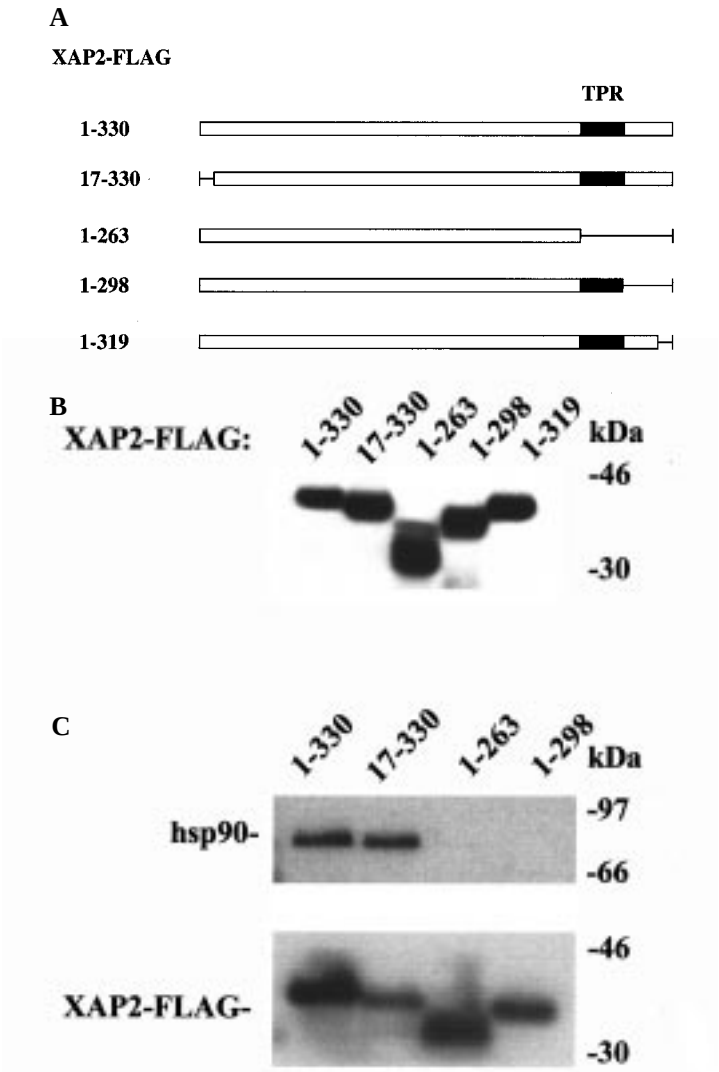

FIGURE 3: C-Terminal end of XAP2 required for binding to hsp90 in COS-1 cells. *(*A) Schematic representation of full-length XAP2-FLAG and XAP2-FLAG deletion mutants. (B) Constructs labeled with [^{35}S]methionine in RL, immunoadsorbed with M2 resin in IP buffer, resolved by SDS−PAGE, and visualized by autoradiography. (C) COS-1 cells were transfected with pCI/XAP2-FLAG and pCI/XAP2-FLAG deletion mutant constructs, cytosol isolated, immunoadsorbed with M2 resin in IP buffer, resolved by SDS−PAGE, followed by immunoblot analysis using anti-hsp90 antibodies (hsp86/84) to detect hsp90 and M2 anti-FLAG antibodies to detect XAP2-FLAG, and visualized using [^{125}I]DAR IgG or [^{125}I]SAM IgG, respectively, followed by visualization by autoradiography.

319 was not expressed in COS-1 cells; thus, the ability of the 11 C-terminal amino acids of XAP2 to bind to hsp90 could not be addressed. This may be due to turnover of this mutant in cells, as it efficiently translates in RL (Figure 3B). The requirement of XAP2 C-terminal sequences for binding to hsp90 is consistent with the requirement of FKBP52 C-terminal sequences for hsp90 binding. It has been demonstrated that C-terminal residues in the TPR of FKBP52 are required for binding to hsp90 (*26*). Given that the third TPR of FKBP52 and the conserved TPR in XAP2 are highly homologous, this TPR and C-terminal sequences may mediate the XAP2−hsp90 interaction as has been previously described for immunophilins in hsp90 complexes. Thus, the TPR domain alone is not sufficient for binding of XAP2 or FKBP52 to hsp90, but requires proximal C-terminal sequences of the TPR domains which may participate in protein folding (*26*). These results are consistent with the observation that C-terminal sequences of the TPR domain in the immunophilin FKBP51 are also required for binding to hsp90 (*46*). Recent results using truncated forms of XAP2 have demonstrated that the C-terminal end of XAP2 (ARA9) are required for complexing with the AhR and hsp90, whereas deletion of 150 amino acids from the N-terminal end of XAP2 (ARA9) did not affect its ability to bind to AhR−hsp90 complexes (*20*). Collectively, these results indicate that the C-terminal sequences of XAP2 are required for complexation with hsp90 and in AhR−hsp90 complexes (*20*). Future experiments will focus on understanding the role of the C-terminal sequences of XAP2 in its ability to interact with hsp90.

XAP2 Binds to a C-Terminal Domain of hsp90, and the AhR Binds to a Middle Domain of hsp90. Studies presented here and from other laboratories indicate that XAP2 binds to hsp90 complexes in cells in the absence of the AhR (*17*). The hsp90 domains involved in the XAP2 and AhR complex formation are unknown. To address this question, GST−hsp90 fragment fusion proteins were used in interaction studies with XAP2. Recently, GST−hsp90 fragment fusion proteins were used to demonstrate that the C-terminal domain of hsp90 is the binding domain of p60, FKBP52, and CyP-40 (*40*). Thus, similar experiments were performed to identify the binding site of XAP2 on hsp90. Previously described GST−hsp90 fusion proteins corresponding to the N-terminal (residues 9−236), middle (residues 272−617), and C-terminal (residues 629−732) ends of hsp90 were generated in *E. coli* and bound to glutathione−agarose (*40*). Each glutathione−agarose−GST−hsp90 fusion was then incubated with [^{35}S]methionine-labeled XAP2, washed, and resolved by SDS−PAGE. As a control, [^{35}S]methionine-labeled FKBP52 and the TPR domains of the serine/threonine phosphatase PP5 were also immunoadsorbed with each GST fusion protein. The GST fusion proteins were visualized with Coomassie Blue staining to demonstrate that an equal amount of fusion protein was bound to glutathione−agarose (Figure 4A). The gel was visualized by autoradiography to detect radiolabeled XAP2, FKBP52, and PP5 TPR. These results indicated that XAP2, like FKBP52, binds to the C-terminal fragment of hsp90 (Figure 4B). Additionally, the TPRs of PP5 also bound to the C90 fragment, which has been demonstrated to compete for binding of full-length PP5 to hsp90 (Figure 4B) (*47*). Collectively, these results demonstrate that XAP2, like other TPR-containing hsp90-associating proteins, binds to the C-terminal end of hsp90, which has been hypothesized to act as a TPR domain recognition site. Recent studies have also demonstrated that a conserved MEEVD motif in the C-terminal end of hsp90 is required for binding of the TPR-containing proteins Hop and CyP-40 (*48*, *49*). It is not known if XAP2 requires the same motif for binding to the C-terminus of hsp90.

In a similar experiment, [^{35}S]methionine-labeled AhR was incubated with each of the GST−hsp90 fusion proteins. As a control, the binding site of the estrogen receptor on hsp90 was also examined. These results demonstrated that the binding sites of the AhR and steroid receptors are on the M90 fragment of hsp90 (Figure 4B). This is the first report examining the binding sites of the AhR and XAP2 on hsp90. Collectively, these results suggest that XAP2, like immunophilins found in hsp90 complexes, binds to the C-terminal

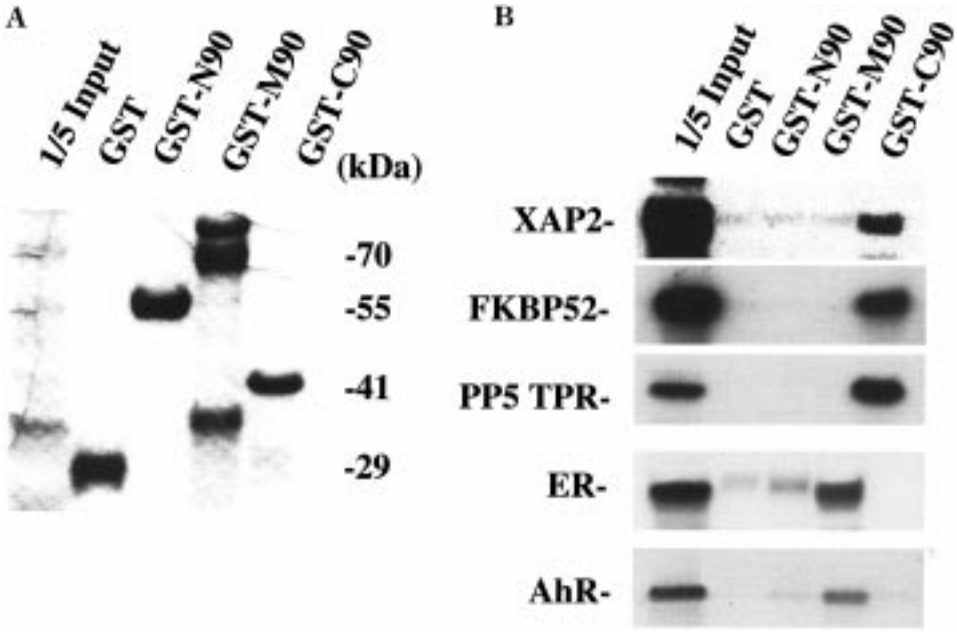

FIGURE 4: XAP2 binds to a C-terminal domain of hsp90, and the AhR binds to a middle domain of hsp90. GST−hsp90 fragment fusion proteins were bound to glutathione−agarose in PBS and then mixed with an individual [^{35}S]methionie-labeled protein, XAP2, FKBP52, PP5 TPR, ER, and AhR. Bound complexes were resolved by SDS−PAGE followed by (A) Coomassie Blue staining after a representative experiment and (B) autoradiography of each experiment to identify hsp90 binding sites.

fragment of hsp90, whereas the chaperoned protein, either the AhR or a steroid receptor, binds to the M90 fragment of hsp90.

The AhR−Arnt Heterocomplex Excludes XAP2 in Vitro. XAP2 has been previously demonstrated to be a stable subunit of the unliganded 9S AhR core complex both in Hepa 1 and in COS-1 cells (*16*, *17*). Following ligand binding, the AhR translocates to the nucleus where it binds to Arnt and hsp90 dissociates from the AhR in a process called transformation. It is not known if XAP2 dissociates along with hsp90 in the nucleus or remains bound to the AhR during heterodimerization with Arnt. Previous studies and those presented here have established that XAP2 does not directly bind to Arnt (*17*, *18*). However, it is not known if XAP2 is able to bind to the AhR in the presence of Arnt. Thus, to determine if XAP2 is a member of the AhR−Arnt heterocomplex, an in vitro analysis was performed using an assembled AhR−hsp90−XAP2 heterotetrameric complex mixed with Arnt in the absence or presence of ligand. First, [^{35}S]methionine-labeled mAhR-FLAG and XAP2 were generated in RL, and then mixed to form the heterotetrameric complex, which has previously been demonstrated to form a 9S complex (*16*). The complexes were treated with 20 nM TCDD or carrier solvent, immunoadsorbed with an M2 affinity gel, and resolved by SDS−PAGE, followed by autoradiography (Figure 5A, lanes 1 and 2). These results demonstrated the relative amount of XAP2 that co-immunoadsorbs with the AhR−hsp90 complex, and also indicated, as previously described, that XAP2 can bind to the AhR in the presence of ligand (*16*). To determine if XAP2 was able to bind to the AhR−Arnt heterocomplex, [^{35}S]methionine-labeled mAhR and XAP2 were generated in RL, and then mixed to form the heterotetrameric complex. Next, pcDNA1/Arnt-FLAG was generated in RL and added to the heterotetrameric complex followed by the addition of 20 nM TCDD or carrier solvent. AhR−Arnt heterocomplexes were immunoadsorbed with the M2 affinity gel, washed, and resolved by SDS−PAGE, followed by autoradiography. Immunoblot analysis was then performed with the anti-FLAG M2 antibody to detect immunoadsorbed Arnt. This assay allowed for the detection of the heterocomplex in that the immuoadsorption was specific for Arnt due to the presence of the FLAG epitope, as demonstrated by the control (Figure 5A, lane 5). Immunoadsorbed AhR−Arnt complexes were then compared to the relative amounts of XAP2 that were immunoadsorbed with the 9S complex generated in vitro (Figure 5A, lanes 3 and 4). These results indicated that XAP2 is not a component of the AhR−Arnt heterocomplex. These results are consistent with the AhR mapping data, suggesting that XAP2 and Arnt may occupy, at least in part, the same or adjacent binding site on the AhR. It is possible that following ligand activation, Arnt may act to displace XAP2 from the 9S complex in the nucleus. It is also possible that ligand binding coupled with hsp90 dissociation from the AhR may be the determining factors in the destabilization of XAP2 rather than displacement by Arnt. AhR−Arnt heterodimer formation was also observed in these studies in the absence of ligand. The formation of such AhR−Arnt heterodimers in RL may be due to an endogenous ligand in RL, as it has been previously observed by gel shift analysis that AhR−Arnt heterodimers form in rabbit RL in the absence of ligand (*6*).

The AhR−Arnt Heterocomplex Excludes XAP2 in Hepa 1 Nuclear Extracts. To determine if XAP2 was also excluded from the AhR−Arnt heterocomplex in cells, Hepa 1 cells were treated with 10 nM TCDD for 1 h, the nuclear fraction was isolated, and the transformed AhR−Arnt heterocomplex was isolated by immunoadsorption using anti-Arnt 2B10−Protein G−Sepharose or IgG−Protein G−Sepharose as a control. AhR was detected in the Arnt immunoadsorption, whereas it was not in the control (Figure 5B). Immunoblot analysis using anti-XAP2 antibodies failed to detect XAP2 in immunoadsorbed AhR−Arnt heterocomplexes (Figure 5B). These results support the in vitro data which demon-

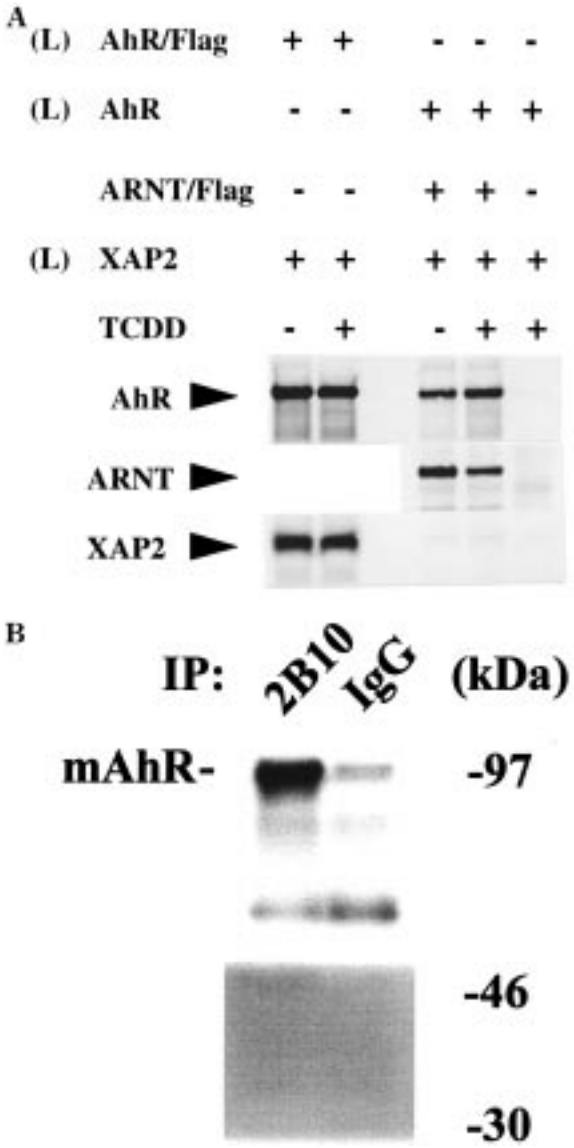

FIGURE 5: XAP2 is excluded from the AhR−Arnt heterocomplex in vitro and in Hepa 1 nuclear extracts. (A) mAhR, mAhR-FLAG, and XAP2 were labeled with [^{35}S]methionine in RL in independent reactions. In the first two lanes, mAhR-FLAG and XAP2 were mixed, treated with carrier solvent or 20 nM TCDD, immunoadsorbed with M2 resin in IP buffer, resolved by SDS−PAGE, and visualized by autoradiography. In the last three lanes, AhR and XAP2 were mixed to form the 9S heterotetramer. Arnt-FLAG (unlabeled) was mixed with heterotetrameric complexes, treated with carrier solvent or 20 nMTCDD, immunoadsorbed with M2 resin, resolved by SDS−PAGE, transferred to PVDF membrane, and visualized by autoradiography to detect AhR and XAP2. Immunoblot analysis was used to detect Arnt using M2 antibody. In the last lane, AhR and XAP2 were mixed in the absence of Arnt-FLAG. (B) Hepa 1 cells were treated with 10 nM TCDD for 1 h, and nuclear extracts were isolated, immunoadsorbed with 2B10−Protein G−Sepharose or control IgG−Protein G−Sepharose, resolved by SDS−PAGE, followed by immunoblot analysis using mAb RPT1 to detect AhR and anti-XAP2 polyclonal antibodies to detect XAP2, and visualized using [^{125}I]SAM IgG or [^{125}I]DAR IgG, respectively, followed by visualization by autoradiography.

strate that XAP2 is excluded in the AhR−Arnt heterocomplex. However, the possibility cannot be excluded that the incubation and washing procedure used for isolation and immunoadsorption of the AhR−Arnt heterocomplex caused XAP2 dissociation.

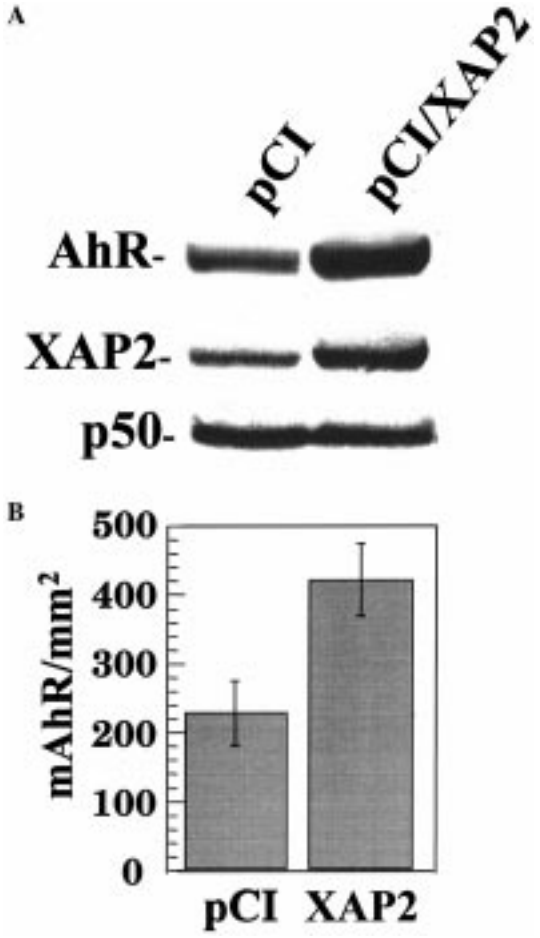

FIGURE 6: XAP2 acts to enhance cytosolic AhR levels in COS-1 cells. COS-1 cells were transfected with pcDNA3/bmAhR and pCI or pCI/XAP2 in triplicate. The cytosol was isolated; 150 μg from each transfection was resolved by SDS−PAGE, followed by immunoblot analysis using RPT1, anti-p50 antibody C1p50, and anti-XAP2 antibodies, and visualized with GAM−HRP or DAR−HRP. (A) Representation of a single experiment. (B) Quantitation of an experiment whose results are depicted in panel A in triplicate. PCI vs pCI/XAP2, control, $p < 0.01$.

XAP2 Acts To Enhance Cytosolic AhR Levels in COS-1 Cells. Previously, we and others have demonstrated that overexpression of XAP2 resulted in enhanced AhR-mediated transactivation (*16*, *17*). To determine if XAP2 had an effect on AhR levels in the cell, COS-1 cells were used as a model system to address this question, as this cell line has been used previously to study the AhR signal transduction pathway (*16*). COS-1 cells were transfected with pcDNA3/bmAhR and pCI or pCI/XAP2. Following transient transfection, cells were lysed, and the cytosolic fraction was isolated and analyzed by immunoblot analysis using antibodies against the AhR, XAP2, and p50. A representative immunoblot of a single experiment and quantitation of the mAhR bands from three independent experiments are depicted in Figure 6. These results demonstrated that there was a statistically significant 1.9-fold increase ($p < 0.01$) in AhR levels in the presence of XAP2 when compared to AhR levels in the presence of control vector alone (Figure 6B). Thus, in the unliganded form, XAP2 was able to stabilize AhR levels. Transfection of pCI/FKBP52 or pCI/XAP2-FLAG(1−263), a nonbinding AhR XAP2 mutant, did not result in enhancing the level of AhR, and thus further demonstrates the specificity

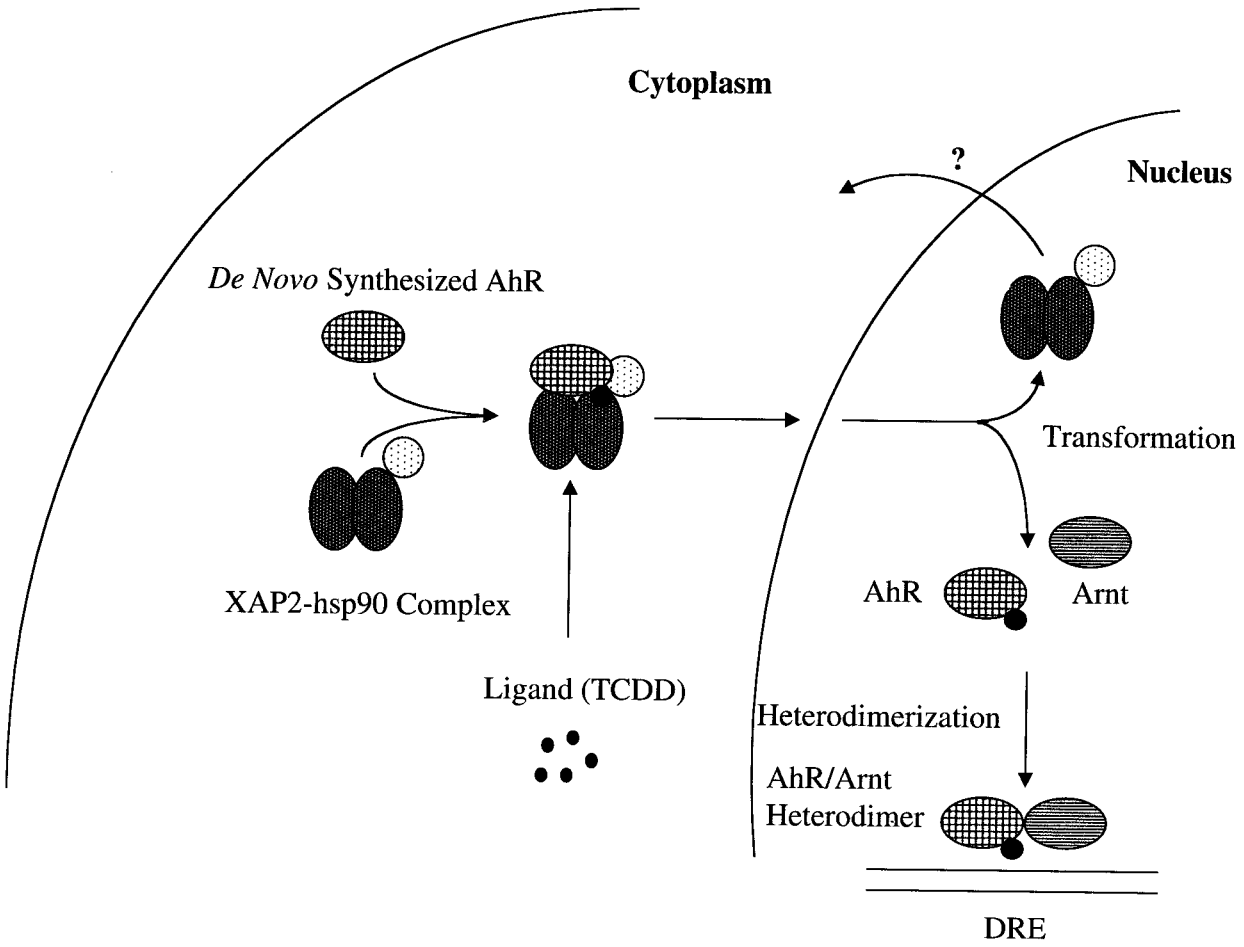

FIGURE 7: Model of the AhR signal transduction pathway. This model suggests that de novo synthesized AhR binds to preassembled XAP2−hsp90 complexes in the cytoplasm. Following ligand activation, the AhR core complex translocates to the nucleus and undergoes transformation with its heterodimeric partner Arnt. The AhR−Arnt heterodimer then binds to DREs in the promoters of genes such as *CYP1A1*.

of the XAP2-mediated effect (data not shown). Another hsp90 binding protein, p50, was used as a loading control. The mechanism by which XAP2 enhances cytosolic AhR levels is unknown. One possibility is that XAP2 expression results in the assembly of more 9S unliganded core AhR complexes. Another possibility is that XAP2 blocks the proteolytic turnover of the AhR.

Hsp90 exists in distinct complexes with several different proteins in the cell. For example, hsp90 has been isolated in complexes containing p50, a mammalian homologue of the *Saccharomyces cerevisiae* cell cycle control protein Cdc37 and the protein kinase $pp60^{v-src}$ or v-Raf (*36*, *50*). Hsp90 has also been isolated in complexes consisting of a steroid receptor, such as the GR, PR, or ER, and a TPR-containing protein, such as FKBP52 or CyP-40, two members of the immunophilin family (*23*, and references therein). Other proteins with TPR motifs, such as the serine/threonine phosphatase PP5, have also been isolated in hsp90 complexes with the glucocorticoid receptor (*51*). The TPR domains of FKBP52, CyP-40, and PP5 have been demonstrated to mediate binding to hsp90 in these complexes. For example, the CyP-40 TPR domain is able to compete for the binding site of FKBP52 and CyP-40 on hsp90 (*52*). Thus, these data and other studies suggest that only one TPR-containing protein can associate with a given hsp90 heterocomplex. Another example of hsp90 binding specificity is that hsp90 complexes with TPR-containing proteins such as PP5 do not contain $p50^{cdc37}$, and it is thought that steric interference of the TPR-containing proteins prevents the binding of $p50^{cdc37}$ (*53*).

XAP2 is a candidate protein that may bind to the TPR binding site of hsp90, much like FKBP52, CyP-40, and PP5. Previous sucrose density gradient analysis revealed that most, if not all, of the cytosolic XAP2 sedimented as a complex near hsp90 in COS-1 extracts (*16*). This, coupled with the fact that hsp90 complexes coprecipitate with XAP2 (Figure 3), would support the hypothesis that de novo synthesized AhR binds to preassembled XAP2−hsp90 complexes. It is important to note that we have not been able to detect other proteins in AhR complexes by silver stain analysis of immunoprecipitations. Additionally, neither FKBP52 nor CyP-40 has been detected in the unliganded AhR core complex (*15*, *54*). Thus, it is possible that XAP2−hsp90 complexes are specific for de novo synthesized AhR. A second less likely hypothesis is that the AhR binds to hsp90 followed by the association of XAP2 with the AhR−hsp90 complex. A model of the process by which de novo synthesized AhR binds to preassembled XAP2−hsp90 complexes is depicted in Figure 7. Following the assembly of the 9S heterotetrameric complexes in the cytosol, ligands such as TCDD bind to these complexes followed by translocation to the nucleus. Once in the nucleus, Arnt may

compete for the XAP2−hsp90 binding site of the AhR, resulting in the loss of the XAP2−hsp90 complex and heterodimerization of the liganded AhR with Arnt. The AhR−Arnt heterodimer, which lacks XAP2, then binds to the promoters of genes such as *CYP1A1* (Figure 7). Specific aspects of this model that are currently unknown include the following: (1) the possibility that XAP2 exists in preassembled complexes with hsp90, (2) the possibility that Arnt participates in the dissociation of XAP2−hsp90 complexes, and (3) the fate of XAP2−hsp90 complexes following dissociation of the AhR.

This report established that (1) XAP2 requires the PAS, hsp90, and ligand binding domain(s) of the AhR for efficient binding, (2) XAP2 can directly interact with the AhR in the absence of hsp90 in a cell-free system, (3) the C-terminal end of XAP2 is required for binding to hsp90 complexes, (4) XAP2 binds to the C-terminal end of hsp90 whereas the AhR binds to the middle of hsp90, (5) XAP2 is excluded from the AhR−Arnt heterodimer in vitro and in Hepa 1 cells, and (6) XAP2 acts to enhance AhR levels in COS-1 cells. This latter conclusion may suggest that the relative XAP2 concentration in a given cell type is an important determinate of the steady-state level of the AhR.

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REFERENCES

1. Rowlands, J. C., and Gustafsson, J. A. (1997) *Crit. Rev. Toxicol. 27*, 109−134.
2. Schmidt, J. V., and Bradfield, C. A. (1996) *Annu. Rev. Cell. Dev. Biol. 12*, 55−89.
3. Hankinson, O. (1995) *Annu. Rev. Pharmacol. Toxicol. 35*, 307−340.
4. Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. (1991) *Science 252*, 954−958.
5. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5510−5514.
6. Dolwick, K. M., Swanson, H. I., and Bradfield, C. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8566−8570.
7. Dong, L., Ma, Q., and Whitlock, J. P., Jr. (1996) *J. Biol. Chem. 271*, 7942−7948.
8. Fukunaga, B. N., Probst, M. R., Reisz-Porsazasz, S., and Hankinson, O. (1995) *J. Biol. Chem. 270*, 29270−29278.
9. Huang, Z. J., Edery, I., and Rosbash, M. (1993) *Nature 364*, 259−262.
10. Nambu, J. R., Lewis, J. O., Wharton, K. A., Jr., and Crews, S. T. (1991) *Cell 67*, 1157−1167.
11. Perdew, G. H., and Bradfield, C. A. (1996) *Biochem. Mol. Biol. Int. 39*, 589−593.
12. Jain, S., Dolwick, K. M., Schmidt, J. V., and Bradfield, C. A. (1994) *J. Biol. Chem. 269*, 31518−31524.
13. Ko, H. P., Okino, S. T., Ma, Q., and Whitlock, J. P., Jr. (1996) *Mol. Cell. Biol. 16*, 430−436.
14. Rowlands, J. C., McEwan, I. J., and Gustafsson, J. A. (1996) *Mol. Pharmacol. 50*, 538−548.
15. Chen, H. S., and Perdew, G. H. (1994) *J. Biol. Chem. 26*, 27554−27558.
16. Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P., and Perdew, G. H. (1998) *Mol. Cell. Biol. 18*, 978−988.
17. Ma, Q., and Whitlock, J. P., Jr. (1997) *J. Biol. Chem. 272*, 8878−8884.
18. Carver, L. A., and Bradfield, C. A. (1997) *J. Biol. Chem. 272*, 11452−11456.
19. Kuzhandaivelu, N., Cong, Y. S., Inouye, C., Yang, W. M., and Seto, E. (1996) *Nucleic Acids Res. 24*, 4741−4750.
20. Carver, L. A., LaPres, J. J., Jain, S., Dunham, E. E., and Bradfield, C. A. (1998) *J. Biol. Chem. 273*, 33580−33587.
21. McGuire, J., Whitelaw, M. J., Pongratz, I., Gustafsson, J. A., and Poellinger, L. (1994) *Mol. Cell. Biol. 14*, 2438−2446.
22. Lamb, J. R., Tugendreich, S., and Heiter, P. (1995) *Trends Biochem. Sci. 20*, 257−259.
23. Pratt, W. B., and Toft, D. O. (1997) *Endocr. Rev. 18*, 306−360.
24. Callebaut, I., Renoir, J. M., Lebeau, M. C., Massol, N., Burny, A., Baulieu, E. E., and Mornon, J. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6270−6274.
25. Hutchison, K. A., Scherrer, L. C., Czar, M. J., Ning, Y., Sanchez, E. R., Leach, K. L., Deibel, M. R., Jr., and Pratt, W. B. (1993) *Biochemistry 32*, 3953−3957.
26. Radanyi, C., Chambraud, B., and Baulieu, E. E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11197−11201.
27. Czar, M. J., Lyons, R. H., Welsh, M. J., Renoir, J. M., and Pratt, W. B. (1995) *Mol. Endocrinol. 9*, 1549−1560.
28. Ratajczak, T., Carrello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L., and House, A. K. (1993) *J. Biol. Chem. 268*, 13187−13192.
29. Kieffer, L. J., Seng, T. W., Li, W., Osterman, D. G., Handschumacher, R. E., and Bayney, R. M. (1993) *J. Biol. Chem. 268*, 12303−12310.
30. Hoffman, K., and Handschumacher, R. E. (1995) *Biochem. J. 307*, 5−8.
31. Leverson, J. D., and Ness, S. A. (1998) *Mol. Cell 1*, 203−211.
32. Kozak, M. (1987) *Nucleic Acids Res. 15*, 8125−8132.
33. Fukunaga, B. N., and Hankinson, O. (1996) *J. Biol. Chem. 271*, 3743−3749.
34. Tsai, J. C., and Perdew, G. H. (1997) *Biochemistry 36*, 9066−9072.
35. Perdew, G. H., Abbott, B., and Stanker, L. H. (1995) *Hybridoma 14*, 279−283.
36. Perdew, G. H., Wiegand, H., Vanden Heuvel, J. P., Mitchell, C., and Sing, S. S. (1997) *Biochemistry 36*, 3600−3607.
37. Hord, N. G., and Perdew, G. H. (1994) *Mol. Pharmacol. 46*, 618−626.
38. Perdew, G. H., Hord, N., Hollenback, C. E., and Welsh, M. J. (1993) *Exp. Cell Res. 209*, 350−356.
39. Nair, S. C., Rimerman, R. A., Toran, E. J., Chen, S., Prapapanich, V., Butts, R. N., and Smith, D. F. (1997) *Mol. Cell. Biol. 17*, 594−603.
40. Young, C. A., Obermann, W. M. J., and Hartl, F. U. (1998) *J. Biol. Chem. 273*, 18007−18010.
41. Long, W. P., Pray-Grant, M., Tsai, J. C., and Perdew, G. H. (1998) *Mol. Pharmacol. 53*, 691−700.
42. Rexin, M., Busch, W., and Gehring, U. (1991) *J. Biol. Chem. 266*, 24601−24605.
43. Rehberger, P., Rexin, M., and Gehring, U. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8001−8005.
44. Segnitz, B., and Gehring, U. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2179−2183.
45. Czar, M. J., Owens-Grillo, J. K., Dittmar, K. D., Hutchison, K. A., Zacharek, A. M., Leach, K. L., Deibel, M. R., Jr., and Pratt, W. B. (1994) *J. Biol. Chem. 269*, 11155−11161.
46. Barent, R. L., Nair, S. C., Carr, D. C., Ruan, Y., Rimerman, R. A., Fulton, J., Zhang, Y., and Smith, D. F. (1998) *Mol. Endocrinol. 12*, 342−354.
47. Silverstein, A. M., Galigniana, M. D., Chen, M. S., Owens-Grillo, J. K., Chinkers, M., and Prat, W. B. (1997) *J. Biol. Chem. 272*, 16224−16230.
48. Chen, S., Sullivan, W. P., Toft, D. O., and Smith, D. F. (1998) *Cell Stress Chaperones 3*, 118−129.
49. Carrello, A., Ingley, E., Minchin, R. F., Tsai, S., and Ratajczak, T. (1999) *J. Biol. Chem. 274*, 2682−2689.

50. Stepanova, L., Leng, X., Parker, S. B., and Harper, J. W. (1996) *Genes Dev. 10*, 1491−1502.
51. Chen, M. S., Silverstein, A. M., Pratt, W. B., and Chinkers, M. (1996) *J. Biol. Chem. 271*, 32315−32320.
52. Owens-Grillo, J. K., Czar, M. J., Hutchison, K. A., Hoffman, K., Perdew, G. H., and Pratt, W. B. (1996) *J. Biol. Chem. 271*, 13468−13475.
53. Silverstein, A. M., Grammatikakis, N., Cochran, B. H., Chinkers, M., and Pratt, W. B. (1998) *J. Biol. Chem. 273*, 20090−20095.
54. Prokipcak, R. D., Faber, L. E., and Okey, A. B. (1989) *Arch. Biochem. Biophys. 274*, 648−658.

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