

# Carboxyl Terminus of hsc70-Interacting Protein (CHIP) Can Remodel Mature Aryl Hydrocarbon Receptor (AhR) Complexes and Mediate Ubiquitination of Both the AhR and the 90 kDa Heat-Shock Protein (hsp90) *in Vitro*<sup>†</sup>

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**ABSTRACT:** The regulation of the aryl hydrocarbon receptor (AhR) protein levels has been an area of keen interest, given its important role in mediating the cellular adaptation and toxic response to several environmental pollutants. The carboxyl terminus of hsc70-interacting protein (CHIP) ubiquitin ligase was previously associated with the regulation of the aryl hydrocarbon receptor, although the mechanisms were not directly demonstrated. In this study, we established that CHIP could associate with the AhR at cellular levels of these two proteins, suggesting a potential role for CHIP in the regulation of the AhR complex. The analysis of the sucrose-gradient-fractionated *in vitro* translated AhR complexes revealed that CHIP can mediate hsp90 ubiquitination while cooperating with unidentified factors to promote the ubiquitination of mature unliganded AhR complexes. In addition, the immunophilin-like protein XAP2 was able to partially protect the AhR from CHIP-mediated ubiquitination *in vitro*. This protection required the direct interaction of the XAP2 with the AhR complex. Surprisingly, CHIP silencing in Hepa-1c1c7 cells by siRNA methods did not reveal the function of CHIP in the AhR complex, because it did not affect well-characterized activities of the AhR nor affect its steady-state protein levels. However, the presence of potential compensatory mechanisms may be confounding this particular observation. Our results suggest a model where the E3 ubiquitin ligase CHIP cooperates with other ubiquitination factors to remodel native AhR–hsp90 complexes and where co-chaperones such as the XAP2 may affect the ability of CHIP to target AhR complexes for ubiquitination.

The aryl hydrocarbon receptor (AhR)<sup>1</sup> is a member of the basic helix–loop–helix PER–ARNT–SIM family and a cytosolic ligand-activated transcription factor (*1*). It plays a central role in mediating the adaptive responses to exogenous

compounds such as halogenated polycyclic hydrocarbons, including the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*2*). Some papers have also indicated that endogenous and dietary substances can directly activate the AhR (*3, 4*), although a putative endogenous ligand for the AhR remains unidentified. The mature unliganded AhR complex is primarily composed of a homodimer of the 90 kDa heat-shock protein (hsp90) and at least one molecule of the immunophilin-like protein XAP2 and/or p23 (*5–8*). In the presence of a ligand, the receptor rapidly accumulates in the nucleus, where it heterodimerizes with the AhR nuclear translocator (ARNT) and activates a myriad of genes primarily encoding phase I and II metabolism enzymes (*3*).

The chronic activation of the AhR has been associated with tumor promotion, as well as a number of birth and reproductive defects, such as cleft palate and reduced numbers of primordial follicles, respectively (*9, 10*). As a result, the regulation of the AhR protein levels has been the subject of several important studies, because this may have a profound impact in the activation or repression of genes directly regulated by the AhR. The AhR protein levels can be rapidly depleted following its activation by high-affinity ligands or after treatment with the hsp90 inhibitor geldanamycin (*11–13*). Several findings have implicated the 26 S proteasome in the ligand and geldanamycin-mediated turn-

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<sup>1</sup> Abbreviations: AhR, aryl hydrocarbon receptor; hAhR, human AhR; mAhR, murine AhR<sup>b-1</sup>; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XAP2, hepatitis B virus X-associated protein 2, also known as ARA9; ARNT, AhR nuclear translocator; hsp90, 90 kDa heat-shock protein; TPR, tetratricopeptide repeat motif; TSDS–PAGE, tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHIP, carboxyl terminus of hsc70-interacting protein; CHIPΔU-BOX, CHIP deletion construct lacking its C-terminus U-box domain, also known as E4-like domain; CHIPΔTPR, CHIP deletion construct lacking its N-terminal 3-tandem TPR motifs; hsc70, 70 kDa heat-shock cognate protein; hsp70, inducible 72 kDa heat-shock protein; HOP, hsp90/hsp70 organizing protein; Hsp40, 40 kDa heat-shock protein; CHIP-*myc*, CHIP that is tagged with the *myc* epitope at its C terminus; p23, 23 kDa heat-shock protein; PBS, phosphate-buffered saline; RRL, rabbit reticulocyte lysate; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E2-DM, dominant negative mutant of E2; E3, ubiquitin ligase enzyme; UbcH5a, human ubiquitin-conjugating enzyme family 5 isoform a.

over of the AhR, while no direct evidence of AhR ubiquitination has been published. A possible role for the E3 ubiquitin ligase carboxyl terminus of hsc70-interacting protein (CHIP) in the regulation of AhR protein levels has been debated in recent studies (14, 15). Interestingly, the calcium-dependent protease calpain has recently been suggested to be the true protease responsible for the ligand-mediated activation and degradation of the AhR (16). However, this paper did not reconcile observations previously made in ts20 cells expressing a temperature-sensitive mutant of the ubiquitin-activating enzyme (12). Therefore, it appears that the AhR can be targeted by both proteolytic pathways.

The hsp90 is a highly abundant protein, accounting for approximately 1% of the total cellular protein pool. Its high abundance reflects its rather essential role in the chaperone-assisted protein-folding machinery that also prevents the aggregation of denatured proteins (17, 18). Briefly, *de novo* synthesized hsp90 client proteins are rapidly engaged by the hsc70 and co-chaperone hsp40 in an ATP-dependent fashion (19). As the client continues to fold, the hsc70–client complex interacts with the hsp90 by the assistance of the hsp70–hsp90 organizing protein (HOP). Through an uncharacterized mechanism, the client protein achieves its mature conformation and the hsc70 and HOP dissociate from the Hsp90–client complex. Finally, the co-chaperone p23 joins the mature Hsp90–client complex, and it is thought to modulate the ligand-binding properties of some receptors (20). In the case of the AhR, the XAP2 protein forms part of the mature AhR complex and it is thought to modulate the stability of the AhR by preventing its ubiquitination and degradation through the proteasome (21). However, the time at which XAP2 joins the AhR complex and its true role remains under investigation (22–24). Interestingly, the hsp90 has also been associated with the degradation of some client proteins such as the apoprotein B (25).

The CHIP is an E3/E4 ubiquitin ligase and interacting partner of the hsp90 and hsp70 proteins. As such, the CHIP can modulate chaperone ATPase activity and promote the degradation of client proteins in a ubiquitin- and proteasome-dependent manner (26). Some known CHIP protein targets are the GR (27) and mutant p53 (28), in addition to the hsp70 and the hsp90 chaperone proteins themselves, under stressful conditions (29, 30). CHIP contains a U-box domain homologous to the yeast Ufd2 protein that is thought to participate in the assembly of polyubiquitin chains (31). The U-box domain is also required for the recruitment of the UBC5 family of ubiquitin-conjugating enzymes in the ubiquitination of substrates (30). The N-terminal three-tandem TRP domains of CHIP mediate its interaction with the chaperones hsp90 and hsp70. The conjugation of ubiquitin to a substrate is a precise mechanism requiring various enzymes and has been extensively studied (32, 33). Briefly, an ubiquitin-activating enzyme E1 forms a thiol–ester intermediate with ubiquitin, in the presence of ATP. In the second step, ubiquitin is transferred to an ubiquitin-conjugating enzyme termed E2, and finally, in the presence of an ubiquitin ligase termed E3, the ubiquitin moiety is transferred to a lysyl in the target protein in the form of an isopeptide bond. The assembly of polyubiquitin chains is thought to be mediated by a distinct enzymatic activity termed E4.

In a previous paper, the ubiquitin ligase CHIP was associated with the turnover of hsp70 and hsp90 protein

levels (29). We now demonstrate through the use of sucrose-gradient fractionation methods and *in vitro* experiments with purified components that CHIP can directly mediate both multisite and polyubiquitination of the hsp90 and the AhR, while ubiquitination of the AhR required yet unidentified factors. A model is proposed by which CHIP works in concert with other ubiquitination factors in the remodeling of the mature unliganded AhR complex.

## EXPERIMENTAL PROCEDURES

**Source of Mammalian Expression Constructs.** The pcDNA3/ $\beta$ mAhR and pCI/hAhR-FLAG were generated previously (7). The pcDNA3/CHIP, pcDNA3/CHIP-myc, pcDNA3/CHIP $\Delta$ E4 (CHIP $\Delta$ U-box), and pcDNA3/CHIP $\Delta$ TPR were kindly provided by Cam Patterson (University of North Carolina, Chapel Hill, NC) (30, 34, 35). The pCI/XAP2 and pCI/G272D-XAP2 were previously generated (7, 23).

**Source of Recombinant Proteins.** Recombinant human E1, UbCH5a, UbCH5a-DM (dominant negative), ubiquitin, ubiquitin aldehyde, Ub-K0, Ub-K11, Ub-K29, Ub-K48, Ub-K63, and Ub-K48R were all purchased from Boston Biochem (Cambridge, MA). The plasmids pGST/hCHIP (1–303), pGST/hCHIP (1–197), and pGST/hCHIP (143–303) were kindly provided by C. Patterson (University of North Carolina, Chapel Hill, NC) and were used to produce recombinant human CHIP, CHIP $\Delta$ U-box, and CHIP $\Delta$ TPR in *Escherichia coli*, respectively. The human proteins hsp70 and hsp90- $\alpha$  used for *in vitro* ubiquitination assays were purchased from Stressgen Bioreagents (British Columbia, Canada).

**Cell Culture Conditions.** Cells were routinely grown at 37 °C and 5% CO<sub>2</sub> in modified Eagle's  $\alpha$ -minimum essential medium (Sigma, St. Louis, MO), supplemented with 1000 units/mL penicillin, 100  $\mu$ g/mL streptomycin (Sigma, St. Louis, MO), and 8% fetal bovine serum (HyClone, Logan, UT).

**Western Blotting.** Proteins were transferred from 6 to 8% polyacrylamide gels to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) using standard procedures, unless stated otherwise. The primary antibodies used in Western blots included rabbit anti-AhR polyclonal (Biomol, Plymouth Meeting, PA); the F5 mouse MAb anti-HOP was kindly provided by David Toft (Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN); the anti-hsp86 (hsp90- $\alpha$ ) polyclonal rabbit antibodies were generated previously (36); the anti-hsp70 MAb and affinity-purified rabbit anti-CHIP polyclonal antibodies were purchased from Affinity Bioreagents (Golden, CO); and anti-CHIP rabbit polyclonal used in Figures 1B and 3 were generated previously and a generous gift from C. Patterson (University of North Carolina, Chapel Hill, NC) (27). Proteins were visualized by autoradiography using radioactive <sup>125</sup>I-goat anti-rabbit or anti-mouse antibodies, the combination of biotin-conjugated secondary antibodies with radioactive streptavidin, or enhanced chemiluminescence using horseradish-peroxidase-conjugated secondary antibodies, as indicated in the figure captions. The quantification of proteins was performed by phosphor-image analysis of radioactive blots.

**Immunoprecipitation of Cytosolic AhR.** Endogenous AhR from Hepa-1c1c7 was immunoprecipitated with affinity-

purified rabbit anti-AhR polyclonal antibody (Biomol, Plymouth Meeting, PA) or control rabbit IgG prebound to 40  $\mu$ L of protein-A resin (Pierce, Rockford, IL; 80  $\mu$ L of a 1:1 slurry) and in the presence of 3% bovine serum albumin. Hepa-1c1c7 (Hepa-1) cells were grown to near 95% confluence on 100 mm plates. Protein extracts were prepared by scraping cells into a 500  $\mu$ L/100 mm plate of MENG buffer [16.2 mM 3-(*N*-morpholino)-propanesulfonic acid sodium salt, 10 mM free acid 3-(*N*-morpholino)-propanesulfonic acid, 0.02% sodium azide, 10% glycerol, and 4 mM ethylenediaminetetraacetic acid (EDTA)] containing 1% NP-40 and a protease inhibitor cocktail P8340 (Sigma, St. Louis, MO). Cells from up to 2  $\times$  100 mm dishes were collected, pooled, and manually homogenized in lysis buffer using 40 strokes with a Dounce homogenizer. The cell lysates were centrifuged at 100000g for 45 min at 4 °C. The supernatant was collected, and a total of 200  $\mu$ g of protein was diluted in MENG buffer with protease inhibitors and used for immunoprecipitations. Protein extracts were then mixed with the protein-A/anti-AhR or protein-A/rabbit-IgG resin and incubated at 4 °C for 1 h on a platform rocker. The resin was then centrifuged at 1000g and washed 4 times with MENG buffer containing 150 mM NaCl, followed by washing once with MENG buffer alone. The immunoprecipitated proteins were then heated in 2 $\times$  sodium dodecyl sulfate (SDS) sample buffer at 95 °C for 5 min and separated via 8% tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (TSDS–PAGE) (37). Proteins were visualized by autoradiography using biotin-conjugated secondary antibodies and <sup>125</sup>I-streptavidin.

**Immunoprecipitation of CHIP-myc from COS-1 Cells.** COS-1 cells propagated on 100 mm dishes were transfected with 6  $\mu$ g of pCI/hAhR-FLAG and 3  $\mu$ g of pcDNA3 (Invitrogen, Carlsbad, CA) or 6  $\mu$ g of pCI/hAhR-FLAG and 3  $\mu$ g of pcDNA3/CHIP-myc using Lipofectamine and Plus reagents (Invitrogen), according to the instructions of the manufacturer. The cells were harvested 24 h post-transfection by trypsinization. Protein extracts were prepared in MENG buffer (MENG plus 20 mM molybdate) plus protease inhibitors and 40 strokes in a stainless-steel Dounce homogenizer. A total of 500  $\mu$ g of cell lysate obtained from cells expressing hAhR-FLAG alone or CHIP-myc and hAhR-FLAG was independently combined with 80  $\mu$ L of a 1:1 slurry anti-myc tag, clone 4A6, agarose conjugate (Millipore, Billerica, MA). The mixture was incubated at 4 °C for 1 h and washed 6 times in MENG buffer with 50 mM NaCl and twice with MENG buffer. The immunoprecipitates were combined with 2 $\times$  SDS sample buffer and resolved in an 8% polyacrylamide gel. Protein bands were visualized by enhanced chemiluminescence (ECL) with horseradish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies.

**Silencing of CHIP Expression in Hepa-1c1c7 Cells by Small Interfering RNA.** Cells growing on 6-well plates were transfected with Dharmacon's standard On-Target siGenome duplex against mouse CHIP (D-063143-04; siCHIP) or nontargeting siRNA number 1 (D-001210-01; siCONTROL) using the DharmaFECT 4 transfection reagent according to the instructions of the manufacturer. Mock transfections included just the transfection reagent. Cells were harvested 48 h post-transfection. Whole cell lysates were prepared in RIPA buffer supplemented with protease inhibitors. RIPA buffer is 10 mM Tris at pH 8.0, 1 mM EDTA, 0.5 mM

ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1% Triton-X, 0.1% deoxycholate, and 140 mM NaCl. Proteins were resolved by 8% TSDS–PAGE and transferred to PVDF membranes. The protein was detected by autoradiography using biotin-conjugate secondary antibodies and <sup>125</sup>I-streptavidin.

**Real-Time Quantitative Polymerase Chain Reaction (qPCR).** Total mRNA was isolated using the TRIzol reagent method (Invitrogen, Carlsbad, CA) and amplified using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA), according to the instructions of the manufacturer. The level of CYP1B1 mRNA induction by TCDD after CHIP knockdown (48 h post-siCHIP-transfection; 4 h treatment with TCDD or vehicle) was assessed by real-time qPCR using the MyIQ single-color PCR detection system (BioRad, Hercules, CA) and the iQ SYBR Green supermix (BioRad). CYP1B1 mRNA was detected using the forward primer, 5'-TTC CTA GAG CTG CTC AGC CAC AAT-3', and reverse primer, 5'-GAA CGA AGT TGC TGA AGT TGC GGT-3'. GAPDH mRNA was assessed to correct for CYP1B1 values with the forward primer, 5'-TCA ACA GCA ACT CCC ACT CTT CGA-3', and reverse primer, 5'-ACC CTG TTG CTG TAG CCG TAT TCA-3'.

**Transient CHIP and AhR Expression Experiments.** COS-1 cells at 90% confluence in 60 mm dishes were transfected with 0.25, 0.5, or 1.0  $\mu$ g of pcDNA3/CHIP along with 1  $\mu$ g of pcDNA3/ $\beta$ mAhR using the Lipofectamine and Plus transfection reagents and under the instructions of the manufacturer (Invitrogen, Carlsbad, CA). For the pcDNA3/CHIP $\Delta$ U-box and pcDNA3/CHIP $\Delta$ TPR constructs, a total of 1.0  $\mu$ g was transfected. The total amount of DNA transfected was normalized to 3  $\mu$ g using pcDNA3, where necessary. The cells were washed 18–20 h post-transfection twice with phosphate-buffered saline (PBS) at 25 °C, harvested by trypsinization, collected into 1.5 mL tubes, and centrifuged at 100g for 3 min. Cell pellets were each resuspended in 100  $\mu$ L of RIPA buffer supplemented with the protease inhibitor cocktail P8340 (Sigma, St. Louis, MO). Cells in lysis buffer were kept on ice with periodic single-pulse vortexing every 5 min. Whole cell lysates were centrifuged at 14000g for 15 min, and the supernatants were transferred to a fresh tube. Protein extracts were quantified, and a total of 80  $\mu$ g of protein per sample was combined with 2 $\times$  gel-loading buffer and analyzed by 8% TSDS–PAGE. Proteins were detected by autoradiography using biotin-conjugate secondary antibodies and <sup>125</sup>I-streptavidin.

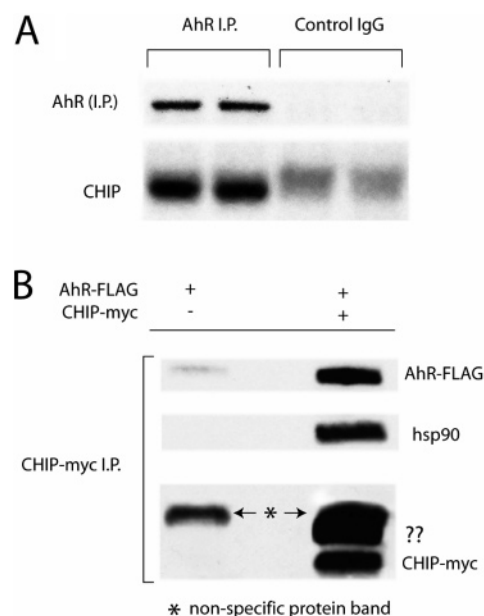
**Sucrose-Density Fractionation of *In Vitro* Translated Proteins.** *In vitro* translations were performed using the TNT Quick Coupled Transcription/Translation System following the instructions of the manufacturer (Promega, Madison, WI). Briefly, 200  $\mu$ L of *in vitro* translated AhR in the presence of <sup>35</sup>S-methionine or methionine were diluted to 300  $\mu$ L with M/N buffer (MENG buffer without glycerol and EDTA) and subjected to a M/N buffer 10–30% sucrose-density-gradient analysis, as previously described (38). A total of 25  $\times$  200  $\mu$ L fractions were collected, and 10  $\mu$ L from each fraction was combined with 2 $\times$  SDS sample buffer, heated at 95 °C for 5 min, and resolved by 6% TSDS–PAGE. Proteins were transferred to the PVDF membrane. Finally, the membranes were dried and exposed to Biomax film (Kodak) overnight at –80 °C to assess the distribution of the AhR protein. AhR protein bands were quantified by phosphor-image analysis.

**In Vitro Ubiquitination Assays.** *In vitro* translated AhR or XAP2 proteins in the presence or absence of  $^{35}\text{S}$ -methionine were treated with 50  $\mu\text{g}/\text{mL}$  cycloheximide, 100  $\mu\text{M}$  MG-132, and 3  $\mu\text{M}$  ubiquitin aldehyde before their use in reactions. A total of 1–2  $\mu\text{L}$  of *in vitro* translated protein or 5–9  $\mu\text{L}$  of sucrose-gradient-fractionated AhR were used for individual ubiquitination assays. The final concentrations in 20  $\mu\text{L}$  reactions for the CHIP ubiquitination cocktail included 100 nM E1, 1  $\mu\text{M}$  UbcH5a or UbcH5a-DM, 350 nM CHIP, CHIP $\Delta\text{TPR}$ , or CHIP $\Delta\text{U-box}$ , 1 $\times$  energy-regenerating system (ERS) containing 50 mM magnesium, 50 mM ATP, and a proprietary recipe of ATP-regenerating enzymes (Boston Biochem, Boston, MA), and 600  $\mu\text{M}$  ubiquitin or its mutant variations Ub-K0, Ub-K11, Ub-K29, Ub-K48, and Ub-K63. It should be noted that the addition of ERS is not necessary for the ubiquitination of *in vitro* translated AhR or rabbit hsp90, reflecting a high abundance of ATP in rabbit reticulocyte lysate (RRL). However, the ERS was added to reactions for consistency and to rule out ATP deficiency in the interpretation of results. For purified hsp90 (Stressgen Bioreagents, Ann Arbor, MI) ubiquitination experiments, hsp70 and hsp90 were added at a final concentration of 350 nM, although hsp70 was not required for hsp90 ubiquitination. All ubiquitination reactions were always performed at 30  $^{\circ}\text{C}$  for 1 h. The reaction buffer [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 8.0] was used to normalize reaction volumes and to dilute commercial proteins, where applicable. For reactions carried out in the presence of XAP2 or G272D-XAP2, whole *in vitro* translated AhR and XAP2 were combined in the ratio of 1:3 (3:9  $\mu\text{L}$ ) and preincubated on ice for 10 min before performing the ubiquitination reactions. Reactions were quenched with an equal volume of 2 $\times$  SDS sample buffer and heated at 95  $^{\circ}\text{C}$  for 5 min. Proteins were analyzed by 6% TSDS–PAGE and ran overnight. The gels were pre-equilibrated in transfer buffer for 30 min, followed by protein transfer to PVDF membranes.  $^{35}\text{S}$ -Methionine-labeled proteins were visualized by autoradiography and quantified by phosphor-image analysis.

**Photoaffinity Ligand-Binding Assay.** The sucrose-gradient fraction containing the 9 S AhR (nonradioactive) (fraction 14) was used for ubiquitination reactions as described above, and the reaction products were used in ligand-binding assays. Briefly, ubiquitination reaction products were diluted to 150  $\mu\text{L}$  with MENGEM and transferred to borosilicate glass tubes. A total of 500 000 cpm 2-azido-3-[ $^{125}\text{I}$ ]iodo-7,8-dibromodibenzo-*p*-dioxin was added to the diluted samples and incubated at room temperature for 30 min, followed by a brief 5 min incubation on ice. Samples were then treated with dextran-coated charcoal at a final concentration of 1/0.1% and incubated on ice for 20 min. Samples were centrifuged for 10 min at 3000g and 4  $^{\circ}\text{C}$  and exposed to 15 W UV lamps (>302 nm) at 8 cm for 4 min. Supernatants were then combined with 2 $\times$  SDS sample buffer and heated at 95  $^{\circ}\text{C}$  for 5 min for TSDS–PAGE analysis. The gels were dried and exposed to X-ray film. The bands were quantified by phosphor-image analysis.

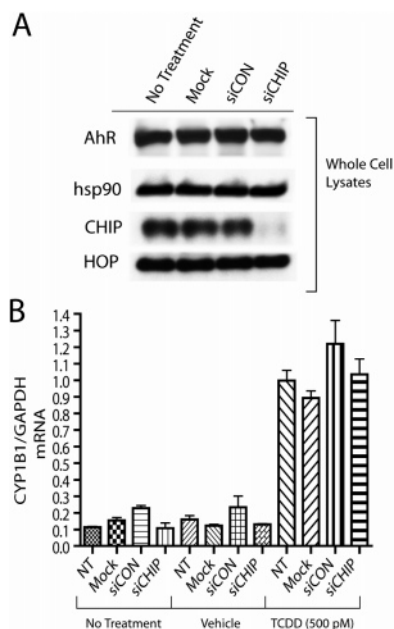
## RESULTS

**CHIP Can Interact with the AhR at Endogenous Levels.** The observation that the AhR protein is highly sensitive to hsp90 disruption (13, 39), in addition to the relationship



**FIGURE 1:** Interaction of endogenous Hepa-1c1c7 AhR with CHIP. (A) Hepa-1c1c7 cell protein extracts were prepared as described in the Experimental Procedures. For each immunoprecipitation, a total of 200  $\mu\text{g}$  of protein was incubated with rabbit anti-AhR or control IgG prebound to protein A (80  $\mu\text{L}$  of a 1:1 slurry) for 1 h on ice. Proteins were resolved via TSDS–PAGE analysis and transferred to the PVDF membrane. The AhR and CHIP protein bands were visualized with biotin-conjugated secondary antibodies and  $^{125}\text{I}$ -streptavidin via autoradiography. (B) COS-1 cells were transfected with 6  $\mu\text{g}$  of pCI/hAhR-FLAG alone or with the addition of 3  $\mu\text{g}$  of pcDNA3/CHIP-myc or empty vector. The cells were harvested 24 h post-transfection, and protein extracts were prepared. A total of 500  $\mu\text{g}$  of cell lysate from each sample was incubated with anti-myc resin at 4  $^{\circ}\text{C}$  for 1 h and washed 6 times in MENGEM with 50 mM NaCl and twice with MENGEM. The AhR and CHIP were resolved as in A but visualized via ECL. The hsp90 was also visualized via ECL. A nonspecific protein band (\*) was typically detected in Western blots. An unidentified protein band possibly representing a post-translationally modified form of CHIP is denoted as “??”.

between CHIP and the turnover of numerous hsp90 client proteins, prompted us to investigate the potential role of the CHIP on the AhR protein stability. Before determining if CHIP could modulate AhR function or stability, it was logical to first examine if CHIP could interact with the AhR complex, especially at normal cellular levels. Immunoprecipitations of the AhR from Hepa-1c1c7 cells suggested that the CHIP could associate with the unliganded AhR (Figure 1A). A modest level of background binding was observed with our control antibody against CHIP. Nevertheless, the level of CHIP immunoprecipitated with the AhR was notably above the background. We also verified if the immunoprecipitation of CHIP could pull down the AhR. However, because of the lack of a suitable antibody, the epitope-tagged CHIP-myc was transiently expressed in COS-1 cells along with the hAhR-FLAG. The immunoprecipitation of CHIP-myc via its myc tag revealed that the hAhR could associate with CHIP (Figure 1B) when transiently expressed in COS-1 cells, similar to previous observations (15). Intriguingly, the expression of CHIP-myc in COS-1 cells resulted in the appearance of a slower migrating protein band above CHIP-myc. While the nature of this protein band remains unknown, it is unlikely that it represents mono-ubiquitinated CHIP as previously hypothesized (15). This is due to its lower than

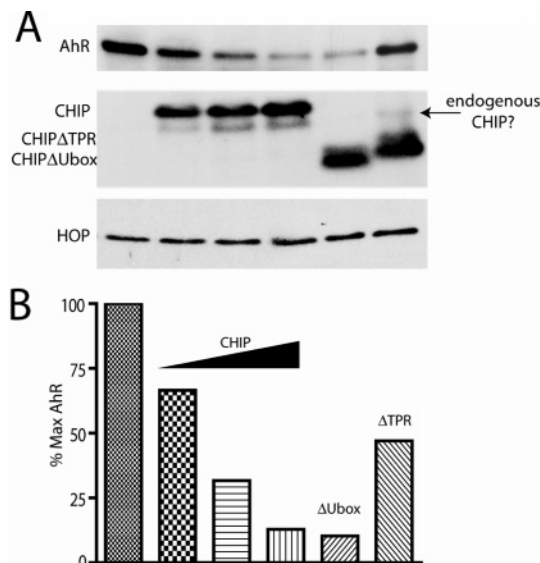


**FIGURE 2:** Silencing of CHIP expression in Hepa-1c1c7 and evaluation of AhR transcriptional activity. (A) Exponentially growing Hepa-1c1c7 cells were transfected, according to the instructions of the manufacturer (Dharmacon), in 6-well plates with a nontargeting or targeting siRNA depicted as siCON or siCHIP, respectively. A mock transfection with Dharmafect-4 alone was used to assess for nonspecific effects. Whole cell lysates were prepared in RIPA buffer supplemented with protease inhibitors. A total of 40  $\mu$ g of protein per sample was resolved by TSDS-PAGE analysis and visualized as in Figure 1A. These results are representative of three independent experiments. (B) Hepa-1c1c7 cells growing on 6-well plates were transfected with a control nontargeting siRNA (siCON) or siCHIP using the instructions of the manufacturer. After 48 h, cells were treated with vehicle control (DMSO) or 500 pM TCDD for 4 h. The no-treatment control did not receive DMSO or TCDD treatment. Total mRNA was extracted, and cDNA was synthesized. Specific mRNA levels were assessed using real-time quantitative PCR. All experiments were performed in triplicates, and the error bars indicate the standard deviation. These results are representative of three independent experiments.

expected molecular-weight shift and the lack of reactivity to anti-ubiquitin antibodies (data not shown).

**Evaluation of AhR Protein Levels and Activity in Hepa-1c1c7 Cells Following the Silencing of CHIP by Small Interfering RNA.** After we established that the AhR and CHIP could interact at endogenous cellular levels, it was critical to determine whether the CHIP protein could influence AhR protein levels in cells. Surprisingly, the use of siRNA technology to silence CHIP protein expression did not appear to have any measurable effects on the steady-state levels of the AhR protein in Hepa-1c1c7 cells (Figure 2A). In addition, the hsp90 protein levels remained unaffected by CHIP downregulation, in agreement with previously published results (14).

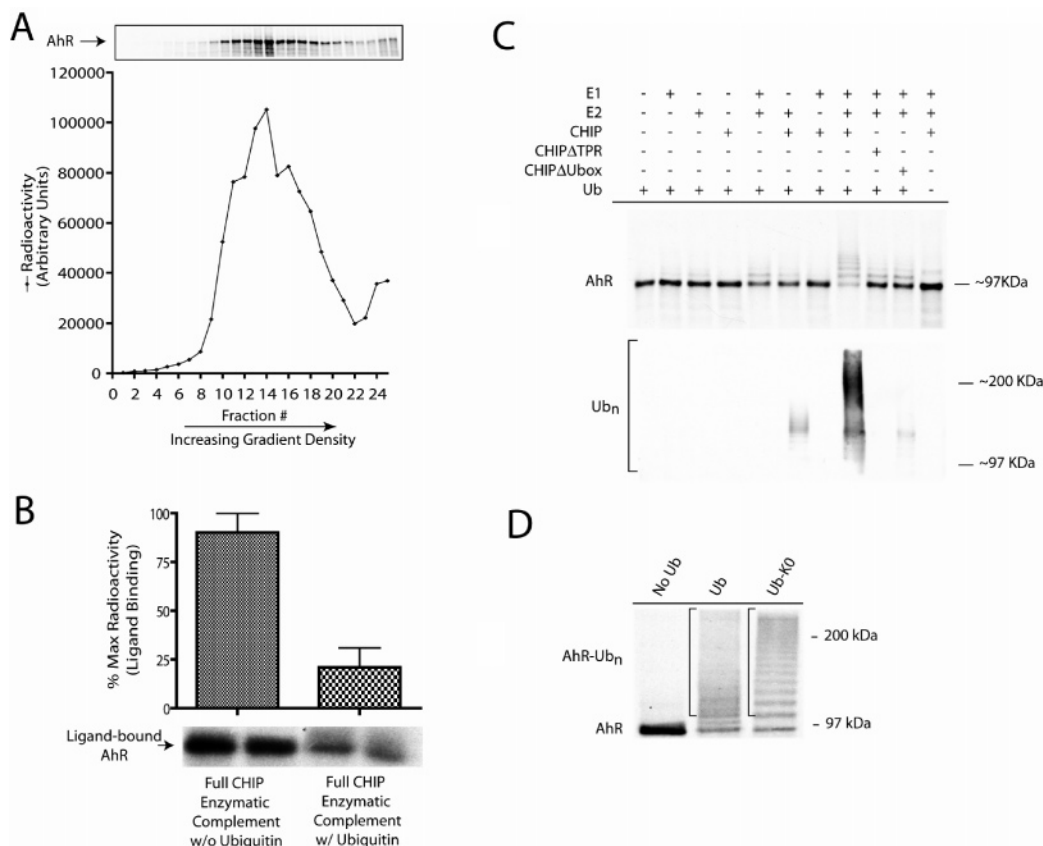
We also examined whether the silencing of CHIP expression could influence the transcriptional activity of the AhR. The TCDD-mediated induction of CYP1A1 is known to be unaffected by the loss of CHIP expression (14). To rule out any potential promoter-specific transcriptional effects, we evaluated whether another gene regulated by the AhR could possibly be affected by a reduction in CHIP expression. Therefore, the TCDD-mediated induction of the CYP1B1 gene by the AhR was assessed through real-time quantitative PCR analysis, following the silencing of CHIP in Hepa-1c1c7



**FIGURE 3:** CHIP and CHIP $\Delta$ U-box overexpression in COS-1 promotes AhR turnover. COS-1 cells propagated on 60 mm dishes were transfected with 1  $\mu$ g of pcDNA3/ $\beta$ mAhR and increasing amounts of pcDNA3/CHIP (0.25, 0.50, and 1.0  $\mu$ g) and pcDNA3/CHIP $\Delta$ U-box (1  $\mu$ g) or pcDNA3/CHIP $\Delta$ TPR (1  $\mu$ g). Whole cell lysates were prepared 24 h post-transfection in RIPA buffer, and proteins were resolved and analyzed as in Figure 1A. The AhR protein bands were quantified by phosphor-image analysis.

cells. The silencing of CHIP had no impact in the transcriptional activity of the AhR (Figure 2B). An apparent increase in the basal expression of CYP1B1 was observed in the presence of our control siRNA. However, the knockdown of CHIP had no impact in the induction of CYP1B1 by TCDD. This result, in addition to an earlier paper (14), further supports the notion that CHIP is not required for the transcriptional activity of the AhR, at least under the conditions utilized. Thus, the knockdown of CHIP mRNA and protein levels in cell culture did not yield insights into the role of CHIP in the AhR complex.

**CHIP and CHIP $\Delta$ U-box Can Equally Mediate AhR Turnover when Transiently Expressed in COS-1 Cells.** To gain some insights into the function of CHIP in the AhR complex, both proteins were transiently expressed in COS-1 cells. The expression of full-length CHIP promoted the turnover of AhR protein levels in a dose-dependent manner, consistent with an earlier study (Figure 3) (15). Unexpectedly, the CHIP $\Delta$ U-box, which is unable to interact with its cognate ubiquitin-conjugating enzyme UBC5 (a, b, and c isoforms) (30) and therefore unable to mediate ubiquitination of its direct substrates, was equally capable of mediating AhR turnover when expressed at similar levels in COS-1 cells. This result is in direct contrast to previous observations where the CHIP $\Delta$ U-box was unable to mediate turnover of the AhR (15) or the dominant-negative effect observed on the cystic fibrosis transmembrane-conductance regulator (40). The blot was also probed for HOP to show that the CHIP-mediated effect was not global in nature. Last, the CHIP $\Delta$ TPR mutant was modestly capable of inducing AhR degradation. However, this increased degradation of the AhR may be due to the induction of an unknown protein band in the presence of CHIP $\Delta$ TPR. This band migrates at the expected molecular weight for CHIP. It is likely that CHIP $\Delta$ TPR is leading to the induction of endogenous CHIP in COS-1 cells by an uncharacterized mechanism. It is important to indicate that



**FIGURE 4:** CHIP can mediate ubiquitination of the AhR *in vitro*. (A) AhR *in vitro* translated in the presence of  $^{35}\text{S}$ -methionine was subjected to a 10–30% sucrose-density-gradient analysis as described in the Experimental Procedures. A total of  $25 \times 200 \mu\text{L}$  fractions were collected. A total of  $10 \mu\text{L}$  from each fraction was combined with  $2\times$  SDS–PAGE sample buffer and resolved by 6% TSDS–PAGE to assess the AhR distribution in the fractions. Proteins were transferred to a PVDF membrane, dried, and exposed to X-ray film. AhR protein bands were quantified by phosphor-image analysis. (B) Photoaffinity labeling of the 9 S AhR with 2-azido-3-[ $^{125}\text{I}$ ]iodo-7,8-dibromodibenzo-*p*-dioxin (see the Experimental Procedures for details) was performed after *in vitro* CHIP-mediated ubiquitination of the AhR in the presence or absence of ubiquitin at  $30^\circ\text{C}$  for 1 h. Error bars denote the standard deviation. (C) As in B, *in vitro* ubiquitination reactions were carried out with the AhR from fraction 14, containing the 9 S AhR unliganded receptor (38). Reactions were incubated for 1 h at  $30^\circ\text{C}$  and quenched by storing samples on ice and diluting 50-fold with ice-cold MENG buffer. Each diluted ubiquitination reaction was then incubated on ice for 1 h with  $50 \mu\text{L}$  of protein-A resin with prebound rabbit anti-AhR antibody. Immunoprecipitated complexes were then washed 4 times with MENG buffer (MENG, 20 mM molybdate, and 150 mM NaCl) and finally combined with  $2\times$  SDS–PAGE sample buffer. Proteins were resolved and analyzed as in A, except that the ubiquitin blot was detected by the enhanced chemiluminescence method (see the Experimental Procedures). (D) *In vitro* ubiquitination reactions were performed in the presence of wild-type ubiquitin or the Ub-K0 mutant and in the absence of ubiquitin. All reactions contained the full complement of enzymes and were carried out as in B. However, the whole reaction products were loaded on a gel and analyzed as in A.

CHIP expression in COS-1 cells does not appear to affect the steady-state levels of hsp90 (data not shown and ref 34). At this point, it was difficult to assess whether CHIP is capable of directly ubiquitinating the AhR or whether it may simply promote AhR degradation by influencing hsp90 function, such as influencing the ATP-binding state (27). Because of the complexity of the ubiquitin and proteasome pathways, it was necessary to differentiate between the above two possibilities using an *in vitro* approach.

**CHIP Can Promote AhR Ubiquitination *in Vitro*.** The turnover of the AhR by CHIP in transient expression experiments was previously associated with the inability of transiently expressed AhR to properly fold in cells (14). Therefore, we wanted to evaluate whether CHIP could promote the ubiquitination of the native unliganded AhR. To accomplish this task, it was necessary to use a system that allows proper folding of the AhR into a ligand- and hsp90-binding state. Hence, these experiments were performed initially with whole *in vitro* translated AhR. However, a modest level of background ubiquitinating activity could be triggered by the simple addition of the UbcH5a enzyme

(data not shown). Consequently, these experiments were performed with sucrose-gradient-fractionated *in vitro* translated AhR in the presence or absence of  $^{35}\text{S}$ -methionine, as indicated. This approach allowed us to isolate fractions containing the 9 S AhR, also known as the mature unliganded complex, which also lacks XAP2 (38). A representative sucrose gradient was analyzed by TSDS–PAGE to assess the distribution of the AhR in the fractions collected (Figure 4A). To demonstrate that the AhR complex is in a competent ligand-binding state, it was necessary to employ the use of the AhR photoaffinity ligand 2-azido-3-[ $^{125}\text{I}$ ]iodo-7,8-dibromodibenzo-*p*-dioxin. Sucrose-gradient fractionation of *in vitro* translated AhR in the absence of  $^{35}\text{S}$ -methionine was performed as in Figure 4A, and fraction 14 containing the 9 S AhR was selected for the assay. Notably, following a prior 1 h incubation in the presence of the full CHIP ubiquitination cascade components (see Figure 4C for details), the ability of the AhR to bind the ligand was severely impaired (Figure 4B). This indicated that CHIP could modulate the ability of the correctly folded AhR protein to bind the radioligand and likely by mediating its ubiquitination. Therefore, subsequent

ubiquitination reactions were carried out with the 9 S  $^{35}\text{S}$ -methionine-labeled AhR. This method allowed us to easily monitor any post-translational modifications of the AhR that could otherwise be missed by the inability of our antibodies to bind modified forms of the AhR. Interestingly, the addition of E1 and E2 (UbcH5a) alone triggered a minor level of ubiquitination of the 9 S AhR, suggesting the modest presence of AhR-ubiquitinating components in this fraction (Figure 4C). The combination of E1 and E2 or E2 and CHIP also resulted in higher but relatively minor levels of AhR ubiquitination. As predicted, the combination of all enzymes was required for efficient AhR ubiquitination *in vitro*. The CHIP $\Delta$ U-box still caused a minor level of AhR polyubiquitination as seen in the anti-ubiquitin blot that may explain its promotion of the AhR degradation in COS-1 (Figure 3). However, we expected a level of ubiquitination comparable to full-length CHIP.

After we established that CHIP could mediate ubiquitination of the AhR *in vitro*, whether directly or indirectly, a parallel experiment was performed with the same AhR fraction (number 14) in the presence of the ubiquitin mutant Ub-K0, to estimate the number of lysyl residues on the AhR being targeted for ubiquitination. The Ub-K0 mutant is quite useful because all lysine residues have been mutated to arginine, allowing this mutant to be effectively used only in the first ubiquitination step of a protein by an E3 ligase. The murine AhR protein has a total of 33 lysyl residues. As anticipated, a very clear stepwise banding pattern could be observed in the presence of Ub-K0, consistent with multi-lysine ubiquitination of the AhR. However, the high number of lysyl residues targeted for ubiquitination in the AhR was surprising. A total of 20 or more ubiquitination sites could be estimated (Figure 4D), given the typical 6–8 kDa shift in molecular weight per ubiquitin molecule attached. It should be noted, however, that the conjugation of several ubiquitin molecules to a protein decreases the linearity of the molecule and this may affect its migration properties in gel. Therefore, we can only estimate the number of potential ubiquitination sites on the AhR by this method. In addition, because the 9 S AhR (fraction 14) was evaluated, it is unlikely that the high number of ubiquitination sites is simply due to misfolded AhR. In other words, all proteins required to achieve a stable and mature unliganded AhR conformation are present in this fraction (38).

*The Immunophilin-like Protein XAP2 Is Capable of Protecting a Subset of the AhR Protein from CHIP-Mediated Ubiquitination.* Recent observations indicated that the XAP2 could antagonize the effects of CHIP on the AhR when expressed in 293T cells (15). Therefore, we wanted to determine if this process could be recapitulated in our *in vitro* ubiquitination assay of the AhR. Unlike most of the experiments presented above, this experiment was performed with nonfractionated *in vitro* translated AhR and XAP2. The reason for this approach was due to the interaction of XAP2 with the AhR, which may affect the sedimentation properties of the complex, making the direct comparisons between XAP2-plus and XAP2-minus samples rather difficult. Therefore, *in vitro* translated AhR and XAP2 were mixed at the ratio of 1:3 (v:v) and stored for 10 min on ice before carrying out the reactions. In this assay, we exploited the use of the previously described G272D-XAP2 and Y408A-AhR mutants as controls (23). In brief, the G272D-XAP2 TPR mutant

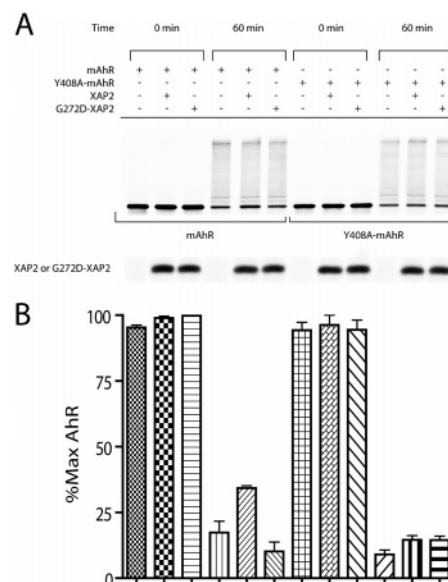


FIGURE 5: XAP2 can partially protect the AhR protein from CHIP–UbcH5a-mediated ubiquitination. (A) Whole *in vitro* translated AhR or Y408A-AhR in the presence of  $^{35}\text{S}$ -methionine was used for CHIP–UbcH5a-dependent ubiquitination reactions in the presence or absence of radiolabeled XAP2 or G272D-XAP2. Reactions were carried out at 30 °C for 1 h. As a control, reactions in the absence of XAP2 received equal amounts of reticulocyte lysate that had a pcDNA3 empty vector used during translation. Reactions were quenched in 2× SDS–PAGE sample buffer and analyzed as in Figure 4C. (B) Non-ubiquitinated AhR values were determined by phosphor-image analysis. Mean non-ubiquitinated AhR values in the presence of XAP2 were significantly higher than those in the absence (*t* test,  $p = 0.0093$ ;  $n = 3$ ) or presence (*t* test,  $p = 0.0014$ ;  $n = 3$ ) of G272D-XAP2. Error bars denote standard deviation.

is unable to interact with the hsp90 and therefore unable to bind AhR complexes, while the Y408A-AhR mutant does not bind XAP2. These controls were critical to establish whether XAP2 was capable of protecting the AhR from CHIP-mediated ubiquitination through its direct interaction with the complex or through a downstream event. Consistent with this notion and the proposed ability of the XAP2 protein to partially protect the AhR from ubiquitination (21), the XAP2 was capable of protecting a subset of the AhR from CHIP-mediated ubiquitination *in vitro* (Figure 5). From three independent experimental replicates, the mean non-ubiquitinated AhR values were significantly higher in the presence of XAP2 than reactions carried out in the absence of XAP2 or presence of the G272D-XAP2 mutant. No significant changes could be detected for the Y408A-AhR in the presence or absence of either XAP2 or G272D-XAP2. As expected, this process required the direct interaction of XAP2 with the AhR complex. In addition, the XAP2 was not a target for CHIP-mediated ubiquitination, indicating that CHIP-mediated ubiquitination of the AhR was specific. It should be noted that XAP2 and the AhR are not present in rabbit reticulocyte lysates (41). Therefore, the magnitude of XAP2 and the AhR expression may vary slightly between reticulocyte lots because of differences in translation efficiencies. Furthermore, the level of XAP2 expression is likely not saturating under these conditions, because the expression of XAP2 in cells has been shown to completely prevent CHIP-mediated degradation of the AhR (15).

*Identification of hsp90 as a Direct Target for CHIP-Mediated Ubiquitination in Vitro.* Several <9 S AhR

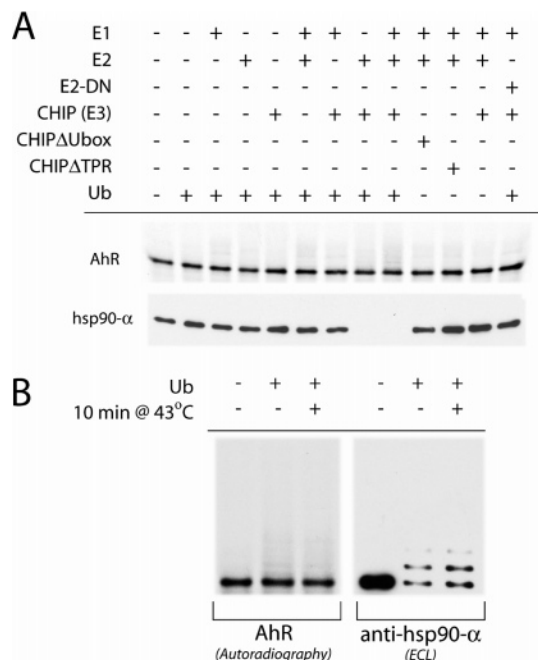


FIGURE 6: CHIP cannot mediate >9 S AhR ubiquitination yet targets hsp90-α for ubiquitination. (A) Ubiquitination reactions were carried out and analyzed as in Figure 4B, except that >9 S AhR (fraction 15) was used and whole reactions were resolved through SDS-PAGE analysis. The AhR was detected by autoradiography, while hsp90-α was probed with anti-hsp90-α antibody and detected by the ECL method. (B) *In vitro* ubiquitination reactions were performed as in A with the full complement of E1, UbcH5a, and CHIP and in the presence or absence of ubiquitin. Samples containing the >9 S AhR were preheated at 43 °C for 10 min before being used in reactions.

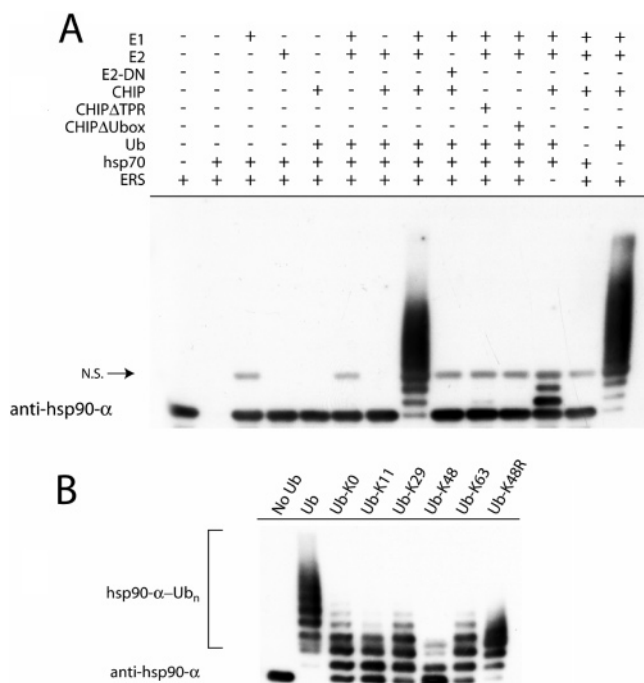
fractions (fractions 10–13; Figure 4A) were also evaluated for CHIP-mediated ubiquitination of the AhR (data not shown). However, these fractions displayed a high level of ubiquitinating and proteolytic activity. For example, the addition of ubiquitin and UbcH5a alone was sufficient for nearly full ubiquitination of the AhR. In contrast, we found that the >9 S AhR (fractions 15–18; see Figure 4A for details) was highly resistant to CHIP-mediated ubiquitination (Figure 6A). We initially theorized that this resistance could be due to the AhR being found in a stable folded state with the hsp90, although the results from the 9 S AhR experiment would argue against this premise. Therefore, we probed our blots for the chaperone hsp70 (data not shown), because it is also a confirmed direct target of CHIP-mediated ubiquitination (29). However, the levels of hsp70 were undetectable in these higher density fractions. Nevertheless, it is still worth noting that CHIP was able to mediate ubiquitination of the hsp70 protein in all fractions where hsp70 (i.e., <9 S hsp70) could be readily detected (data not shown). Interestingly, a recent paper indicated that the hsp90-α appeared to be regulated by CHIP, although no direct evidence of CHIP-mediated ubiquitination was shown (29). Further supporting these observations, we probed our blots for hsp90 with rather surprising results. The hsp90 protein was completely undetectable in reactions containing the full complement of enzymes required for CHIP–UbcH5a-mediated ubiquitination. Therefore, it was evident that the reaction could still proceed efficiently and the resistance of the AhR to ubiquitination had other possible explanations. Evidently, the loss of the hsp90 signal also occurred in the absence of E1.

However, this is consistent with the modest presence of the ubiquitin-activating enzyme in RRL (42), and it is likely co-sedimenting with the AhR in these fractions. Even though it was logical to believe that CHIP was mediating ubiquitination of the hsp90, it is not possible to differentiate between whether the absence of detectable hsp90 was due to ubiquitination or some other unanticipated effect *in vitro*.

Despite the hsp90 observation, it was still mystifying how the AhR could be resistant to ubiquitination, given its known high dependence on hsp90 for stability (13). The CHIP-mediated ubiquitination of the firefly luciferase enzyme has been previously shown to require its prior denaturation (29, 43). This observation provided initial clues about the possible role of CHIP in the ubiquitination and degradation of misfolded proteins in the cell. We therefore briefly exposed our >9 S AhR fractions at 43 °C for 10 min to stimulate CHIP–UbcH5a-mediated ubiquitination of the AhR. However, denaturing heat pretreatment of the >9 S AhR samples did not enhance the ubiquitination of the AhR by CHIP–UbcH5a complexes (Figure 6B). In contrast, hsp90 protein ubiquitination could still be detected. It was also clearly evident that our hsp90-α antibody cannot properly recognize highly ubiquitinated hsp90-α. This result highlights the first direct evidence of hsp90-α ubiquitination by a known ubiquitin ligase complex *in vitro*. These results do not rule out the possibility of CHIP mediating the turnover of the AhR but may simply indicate that the AhR is not a direct target of CHIP-mediated ubiquitination.

**Reconstitution of CHIP–UbcH5a-Mediated Ubiquitination of the hsp90-α with Purified Components *In Vitro*.** Given the absence of measurable effects on the hsp90 protein levels after the silencing of CHIP expression (Figure 2A) and the possibility that CHIP could be indirectly mediating ubiquitination of the hsp90 in RRL, as suspected for the AhR, it was critical to determine if the reaction could be reconstituted with purified components alone. As expected for a direct target of CHIP-mediated ubiquitination, the minimal CHIP/UbcH5a complex was fully capable of ubiquitylating the hsp90 protein *in vitro* (Figure 7A). The hsp70 protein was initially added to the reactions. However, it was clearly dispensable for this reaction to take place. Furthermore, in the absence of hsp70, ubiquitination of the hsp90 occurred more readily. A nonspecific band was also apparent approximately at 110 kDa that correlated with the addition of the 110 kDa ubiquitin-activating enzyme (E1). A minor background activity could be detected in the absence of the ATP-regenerating system (ERS). This is likely due to a small amount of contaminating ATP derived from the isolation of these enzymes (44). All of the CHIP-mediated ubiquitination components were required for hsp90 ubiquitination *in vitro*.

**The Ubiquitin Ligase Complex CHIP–UbcH5a Can Assemble Noncanonical Ubiquitin Chains on hsp90.** After we established that the hsp90-α could be directly ubiquitinated by the CHIP–UbcH5a complex, it was important to test the nature of the ubiquitin chains assembled on hsp90-α. The architecture of polyubiquitin chains is often dictated by the lysyl residue within ubiquitin itself used for chain elongation (polyubiquitination) and can serve as a distinct signal in protein trafficking of membrane receptors, DNA repair, as well as the proteasomal degradation of some proteins (i.e., K48-linked chains) (32, 45, 46). Therefore, several ubiquitin mutants were screened to determine which



**FIGURE 7:** Ubiquitination of hsp90 by CHIP-UbcH5a *in vitro*. (A) *In vitro* hsp90-α ubiquitination reactions were performed at 30 °C for 1 h (see the Experimental Procedures for details). Full reactions were quenched with 2× sample buffer and analyzed by TSDS-PAGE. The hsp90 protein was detected as in Figure 2A. The arrow with a N.S. caption stands for the nonspecific protein band. (B) *In vitro* ubiquitination reactions were carried out in the absence of ubiquitin, the lysine-free Ub-K0, with single-lysine ubiquitin variants (Ub-K11, Ub-K29, Ub-K48, and Ub-K63), or the point-mutant K48R-Ub. The hsp90 protein was analyzed as in Figure 2A.

residues within ubiquitin itself were being utilized by the CHIP/UbcH5a complex to assemble ubiquitin chains on hsp90. Notably, wild-type ubiquitin was used by CHIP/UbcH5a to form polyubiquitinated hsp90 species (Figure 7B), given the characteristic smearing associated with long ubiquitin chains conjugated to substrates. As expected, the synthesis of polyubiquitin was halted in the presence of Ub-K0 and a clear protein ladder could be observed, consistent with multiple-site ubiquitination. The use of Ub-K0 revealed that at least 6–7 lysyl residues were primarily targeted by CHIP/UbcH5a for ubiquitination in this purified system. Similarly, the use of K11, K29, and K63 all resulted in the formation of varying amounts of ubiquitinated hsp90 species but not beyond the levels achieved with Ub-K0 and with apparent varying kinetics. The use of K48 should have resulted, at least, in the formation of this minimal number of ubiquitinated hsp90 species, because it could theoretically still participate in the initial ubiquitination step. Instead, we observed that the use of K48 severely impaired hsp90 ubiquitination, similar to the observations made recently on the hsc70 ubiquitination by CHIP (29). Given that the mutation of several or all lysyl residues on ubiquitin could alter the ubiquitin structure and functionality, we also tested the ubiquitin point-mutant K48R. Consistent with this notion, the use of K48R restored hsp90 ubiquitination to levels at least comparable to the other mono-lysyl-containing ubiquitin mutants and the Ub-K0. A typical but modest level of smearing could also be detected with K48R-Ub, indicating the possibility of chain elongation. Interestingly, regardless

of the mutant ubiquitin used, the level of ubiquitination achieved with all of the mono-lysyl ubiquitin variants was mostly comparable to that of Ub-K0. Polyubiquitinated substrates often cannot be readily resolved in a polyacrylamide gel because of the nonlinearity of the conjugated molecules and often appear as smears in Western blots. On the basis of this assumption and the modest level of smearing observed with K48R-Ub, we conclude that the CHIP-UbcH5a can assemble noncanonical polyubiquitin chains on hsp90. However, it remains unclear whether these ubiquitin chains synthesized on hsp90 are of mixed linkage.

## DISCUSSION

The detection of CHIP in AhR complexes (Figure 1) suggested that CHIP could regulate the AhR protein levels through its well-characterized ubiquitin ligase activity. Surprisingly, the silencing of CHIP expression in Hepa1c1c7 did not have an apparent impact in the AhR protein levels or its transactivation potential (Figure 2), in agreement with earlier studies (14). From these results, it can be concluded that CHIP is not an essential component for these aspects of AhR biology, at least in the cell lines and conditions examined thus far. However, this result alone does not rule out CHIP as a potential regulator of AhR protein levels, whether directly or indirectly. At this time, it is rather difficult to determine whether these observations are not confounded by possible compensatory mechanisms in the cell. Evidently, the transcription factor p53 is primarily regulated by the Mdm2/hdm2 ubiquitin ligase (47). However, existing evidence suggests that other ubiquitin ligases can also mediate p53 turnover in cells and can compensate for the loss of Mdm2. Notably, the E3 ligase complexes of COP1, ARF-BP1, and Pirh2 are also known to regulate p53 transcriptional activity and protein levels under specific cellular conditions (48–50). Because the E3 ligase CHIP has also been proposed to regulate p53 turnover (28), it is likely that CHIP could be just one of many E3 ligase complexes working together to remodel large protein complexes. The CHIP protein has also been associated with the SCF multiprotein ubiquitin ligase complex in the notch-induced degradation of E2A (51) and glycoproteins through the F-box protein Fbx2 (52). Whether the AhR could be regulated in a manner similar to p53 by multiple E3 ligases remains to be determined, but it is a strong possibility, especially with current evidence implicating the transactivation domain of the AhR and its DNA binding in distinct degradation pathways (53).

Experiments based on siRNA or gene-knockout approaches are useful but have their own inherent limitations, especially when inadequate information is available about a cellular pathway. It is important to consider that there is currently no clear evidence demonstrating either how the CHIP protein levels are regulated in the cell or the mechanisms of CHIP activation or selection of its substrates. Certainly, a simple change in the stoichiometry of the AhR complex chaperones or co-chaperones may shift the equilibrium toward the CHIP degradation pathway. Furthermore, the certainty that CHIP does not act alone and requires other components such as its cognate E2 [UBC5 family (30)] and ubiquitin provides yet another opportunity for extensive regulation of this process, and it should be considered when analyzing its role *in vivo* or in cells under normal endogenous levels. The current consensus is that CHIP selects its targets

by recognizing partially or fully denatured proteins with the assistance of the hsp90 and hsp70 chaperones (43). This idea is further supported by the observation that CHIP<sup>-/-</sup> mice develop normally but are sensitive to temperature and environmental stresses (54). Nevertheless, the denaturation of a protein or alterations in their conformation are two broad terms describing events that can be technically elicited by numerous conditions such as pH changes, the association and dissociation of interacting proteins such as co-chaperones, mutations, temperature changes, energy availability, post-translational modifications, nutrients, and so on. A study suggested that phosphorylation may play a role in the recognition of the androgen receptor by CHIP (55). Although hypothetical, changes in the AhR elicited by post-translational modifications of itself or any of its associated proteins [e.g., acetylation (56) and/or phosphorylation (55)] may prompt the activation of CHIP and its cognate E2 ubiquitin-conjugating enzymes to modulate these complexes via hsp90 or hsp70. It is logical that the disruption of CHIP expression alone by gene-knockout or RNA-silencing methods may not inherently be sufficient to reveal its potential role in the regulation of the AhR and similar hsp90 client proteins. Therefore, it remains critical to determine the mechanisms of CHIP activation in the cell to determine the true role of CHIP in the AhR complex.

Some insights on the ability of CHIP to mediate degradation of the AhR were gathered in our study. For example, the expression of CHIP or CHIP $\Delta$ U-box could both lead to a dramatic loss of AhR protein levels in COS-1 cells, while CHIP $\Delta$ TPR only had a modest effect (Figure 3). These results highlighted that the U-box domain, which is required for CHIP-mediated ubiquitination of its *bona fide* substrates (29, 30), was not required to mediate the turnover of the AhR. This result is also in direct contrast to previous observations where the CHIP $\Delta$ U-box or CHIP $\Delta$ TPR had no effect on the protein levels of a nuclear compartmentalized AhR (DR-NLS) (15). Perhaps the discrepancy is related to the fact that a nuclear localized AhR was used in their studies (DR-NLS) or possibly because of cell-line-specific idiosyncrasies. Nevertheless, the effect of CHIP $\Delta$ U-box on the AhR protein levels in our experiments strongly suggested that other factors could be cooperating with CHIP to mediate the AhR degradation in COS-1 cells. Furthermore, we theorized that the binding of CHIP to the AhR-hsp90 could be leading to AhR turnover in a manner analogous to geldanamycin, that is, by regulating the substrate-binding cycle or nucleotide-binding state of the hsp90 and hsp70 chaperones as previously suggested (27). The above model is further supported by our *in vitro* ubiquitination assays of sucrose-gradient-fractionated AhR complexes discussed ahead.

For the first time, we show direct ubiquitination of the unliganded mature AhR protein by a known ubiquitin ligase complex *in vitro* (Figure 4). This result confirms that the ubiquitin ligase CHIP can remodel mature AhR complexes and its activity on the AhR is not due to the misfolding of the receptor, often speculated about transient protein overexpression experiments in cells. Furthermore, the use of the ubiquitin mutant Ub-K0 revealed a high degree of multisite ubiquitination and polyubiquitination of the AhR, providing a direct explanation for the high level of CHIP-mediated degradation of the AhR when expressed in COS-1 cells and Hepa-1 (15). It should be realized that the AhR ubiquitination

process is likely highly regulated in the cellular context by the controlled expression, localization, and activation of the CHIP ubiquitination cascade components. Furthermore, the interaction of the AhR with its co-chaperones could also determine whether the AhR can be ubiquitinated or not. When the AhR is shielded from potential ubiquitin ligases, co-chaperones could provide another level of regulation for the AhR. This mechanism has been suggested for other proteins (32) and is in part supported by our observations on the ability of the XAP2 protein to protect the AhR from CHIP-mediated ubiquitination (Figure 5). The XAP2 protein is an AhR-interacting partner capable of enhancing the AhR protein levels when overexpressed in cells (39). The nature of this phenomenon appears to be due to the unprecedented ability of XAP2 to protect the AhR from ubiquitin-dependent degradation through the proteasome (21). The XAP2 also appears to repress the ligand-mediated activation of the AhR by displacing p23 from the mature AhR complex in transient cell expression systems (23). Most recent observations suggest that the XAP2 may regulate the ligand-mediated transformation of the AhR into transcriptionally active complexes (22). However, the true function of the XAP2 *in vivo* remains largely enigmatic. Recent studies with transgenic mice exclusively overexpressing XAP2 in the liver strongly suggested that the endogenous XAP2 was sufficient for maximal occupancy of the AhR complex and its overexpression had no impact on the levels of the endogenous AhR or the transcriptional output of the AhR *in vivo* (57). Therefore, the true function of the XAP2 remains largely enigmatic and will require further investigation.

Our ability to uncouple CHIP-mediated ubiquitination of the AhR from the hsp90 provided vital clues into the mechanisms of CHIP-mediated ubiquitination and degradation of the AhR (Figure 6). Given that the >9 S hsp90 could always be ubiquitinated by CHIP, while efficient ubiquitination of the AhR was limited to specific sucrose-gradient fractions, it appears that CHIP may work in concert with an AhR-ubiquitinating factor (AhRUF) to ubiquitinate the AhR. The reasons for these dramatic differences in AhR ubiquitination observed, even between adjacent fractions, are not clear. It also remains unclear whether this AhRUF is an AhR-specific ubiquitin ligase(s) or simply a factor(s) required for direct CHIP-UbcH5-mediated ubiquitination of the AhR. We believe that by establishing a reproducible assay for the ubiquitination of the AhR, our work may help future studies aimed to identify proteins capable or needed for direct ubiquitination of the AhR. We have established that the CHIP-UbcH5 ubiquitination complex is capable of directly ubiquitinating the hsp90 protein *in vitro* (Figure 7A), providing an explanation for the CHIP-dependent degradation of the hsp90 observed in recent studies (29). Because the hsp90 protein is known to interact with over 100 proteins, in addition to the fact that most CHIP-proposed ubiquitination targets are known to interact with the hsp90, our findings may be of great interest to multiple laboratories aiming to elucidate the mechanisms of CHIP-mediated degradation of their protein of interest.

We determined that the CHIP-UbcH5a can assemble noncanonical polyubiquitin chains on hsp90, while our observations on the limitations of the Ub-K48 (Figure 7B) may be of great interest to structural biology laboratories studying the mechanisms of polyubiquitin linkage selection

by ubiquitin ligases (58, 59). The implications of our findings are numerous, given the importance of the ubiquitin chain structure as the ultimate signal determining the fate of polyubiquitinated proteins (45). Therefore, it is now crucial to investigate how ubiquitination of the hsp90 by CHIP may impact its trafficking, activity, and/or the degradation of the hsp90- $\alpha$ , along with its client proteins. The discovery of hsp90 as a direct target of CHIP-mediated ubiquitination is paramount to the understanding of the CHIP-mediated regulation of chaperone client proteins.

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