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REGULATION OF THE ARYL HYDROCARBON RECEPTOR PROTEIN LEVELS AND SIGNAL TRANSDUCTION PATHWAYS

A Thesis in

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by

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ABSTRACT

The aryl hydrocarbon receptor is a ligand-activated transcription factor that mediates most of the toxic effects of numerous halogenated and non-halogenated polycyclic aromatic hydrocarbons (e.g., chlorinated dibenzo-*p*-dioxins). In its ligand-bound state, the AhR rapidly accumulates in the nucleus where it dissociates from the hsp90/XAP2 complex and heterodimerizes with ARNT primarily to upregulate genes encoding metabolic enzymes implicated in the carcinogenic activation or detoxification of endogenous and exogenous substances. Interestingly, the AhR protein levels are rapidly depleted after its activation by full agonists in an ubiquitin and proteasome-dependent manner. Given the potential role of the AhR in normal vascular, liver, and immune system development and/or regulation, as well as in mediating the toxicity of numerous HPAH and PAHs, the study of AhR protein level regulation and activation in the cell may be essential to our understanding of the mechanisms of toxicity elicited by persistent AhR activators (e.g., TCDD). The first project in this thesis explored the question of whether the carboxyl-terminus of hsc70 interacting-protein (CHIP) was involved in the regulation of the ligand-mediated degradation of the AhR in an ubiquitin and proteasome-dependent process. CHIP associates with chaperones such as hsp90 and hsc70 and negatively regulates their ability to function as protein folding complexes, causing client proteins (e.g., estrogen receptor) to be degraded through the proteasome. Immunoprecipitates of the AhR revealed that CHIP could also associate with the AhR protein complex at cellular levels and the transient expression of CHIP in cell cultures resulted in AhR protein turnover. Through the use of *in vitro* reconstitution assays,

sucrose gradient fractionation, and RNA silencing methods we established that the E3 ubiquitin ligase CHIP can mediate ubiquitination of the AhR and hsp90. However, CHIP did not seem to regulate the steady-state protein levels of the AhR nor the ligand-mediated degradation of the AhR. Rather, it appears that CHIP is capable of directly mediating ubiquitination of the hsp90 and, perhaps through this mechanism, it may affect the ability of the hsp90 chaperone to protect the AhR from ubiquitination and degradation through the proteasome. A second question explored whether the benzoimidazole-derived anti-asthmatic drugs termed M50354 and M50367 mediated their AhR-dependent therapeutic roles as partial agonists for the AhR and in a non-dioxin responsive element (DRE)-driven process. Previous published work suggested that these substances could not mediate classical DRE-driven gene activation of CYP1A1 to the same degree as established high-affinity AhR ligands. Presumably, these two drugs were also capable of mediating AhR-dependent immunomodulatory functions not entirely elicited by other established AhR ligands, possibly through a non-genomic role of the AhR. However, in contrast to previous observations, we demonstrated that both substances are full but transient AhR agonists. It is currently unknown whether the suggested ability of these drugs to modulate the differentiation of naïve T helper cells in an AhR-dependent manner is through the direct modulation of an immune system associated factor(s) through physical interactions with the ligand-activated AhR and/or through an AhR-regulated DRE-driven gene product. Therefore, this work concludes that further structure-activity relationship analysis is necessary to determine possible role of the liganded AhR in immune system regulation.

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LIST OF ABBREVIATIONS

3-MC, 3-methylcholanthrene
AHH, aryl hydrocarbon hydroxylase
AhR, Aryl hydrocarbon receptor
AIP, aryl hydrocarbon receptor interacting protein (synonym with XAP2)
α-MEM, a-minimal essential medium
α-NF, alpha naphthoflavone
APF-1, ATP-dependent proteolysis factor (synonym with ubiquitin)
ARA9, aryl hydrocarbon receptor associated protein 9 (synonym with XAP2)
ARNT, aryl hydrocarbon receptor nuclear translocator
ATP, adenosine triphosphate
B[a]P, benzo[a]pyrene
βHLH, basic helix-loop-helix
β-NF, beta naphthoflavone
CHIP, Carboxyl terminus of hsc70-interacting protein
COX, cyclooxygenase
CYP, cytochrome P450
CYP1A1, cytochrome P450 1A1
DMSO, dimethyl sulfoxide
DRE, dioxin responsive element
DTT, dithiothreitol
E6-AP,human papilloma virus E6-associated protein (also known as UBE3A)
EDTA, ethylene diaminetetraacetic acid
FBS, fetal bovine serum
GR, glucocorticoid receptor
GSTM1, glutathione S-transferase M1
GST-Ya, glutathione-S-transferase Ya subunit
HAH, halogenated aromatic hydrocarbon
HEPES, 4-(2-hydroxylethyl)-1-piperazineeethanesulfonic acid
HOP, hsp90/hsp70 organizing protein
HPAH, halogenated polycyclic aromatic hydrocarbon
HPAH, halogenated polycyclic aromatic hydrocarbon
hsc70, constitutively expressed 70 kDa heat shock protein
hsp70, inducible 70 kDa heat shock protein
hsp90, 90 kDa heat shock protein
LBD, ligand binding domain
LT, leukotriene
M50354, catalog name for 3-[2-(2-phenylethyl)benzoimidazole-4-yl]-3-hydroxypropanoic acid
M50367, ethyl 3-hydroxy-3-[2-(2-phenylethyl) benzoimidazol-4-yl]propanoate
MENG, 25mM MOPS, 2mM EDTA, 0.02% NaN3 and 10% glycerol (pH=7.5)
MPN, Mpr1p and Pad1p N-terminal regions (protein domain)
Nedd8, neural precursor cell expressed, developmentally down-regulated 8

NF-kB, nuclear factor NF-kappa-B p50 subunit
NQO1, NAD(P)H:Quinone Oxidoreductase 1
P_1-450, synonym for CYP1A1
p23, 23 kDa heat shock protein co-chaperone
PAH, polycyclic aromatic hydrocarbon
PAS, Per-ARNT-Sim protein domain
PBS, phosphate buffered saline
PCI, Proteasome-COP9-initiation factor domain
SDS, sodium dodecyl sulfate
siRNA, small interferring ribonucleic acid
SUMO4, small ubiquitin-like protein 4
TAD, transactivation domain
TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin
Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
TSDS-PAGE, tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ub, ubiquitin
UBB, ubiquitin B
UCHL1, ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
XAP2, hepatitis B virus X-associated protein 2

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Chapter 1

The Aryl Hydrocarbon Receptor: *A Literature Review*

1.1 Background and Historical Perspective.

Organisms are incessantly challenged by hazardous substances of anthropogenic origin. Xenobiotics, such as pesticides, solvents, and many other industrial products are a major source of environmental pollution and public health concern. Throughout history several events of occupational and accidental exposure to halogenated and non-halogenated polycyclic aromatic hydrocarbons (HPAHs and PAHs, respectively) revealed many of the health risks associated with exposure to these chemicals.

In 1949 the accidental release of HPAHs, including TCDD, from Mosanto's chemical plant in Nitro, West Virginia, resulted in several medical cases of chloracne, liver disease, porphyria cutanea tarda, tumors, and alleged exposure-associated deaths [1]. Ironically, in 1957, Dr. Sandermann described the discovery and synthesis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [2], one of the most potent non-genotoxic tumor-promoting substances recognized today. Although not known at the time, TCDD-mediated toxicity is primarily mediated by the aryl hydrocarbon receptor (AhR) and some of its directly regulated genes like CYP1A1 [3, 4]. Unluckily, Sandermann's laboratory assistant 'mysteriously' suffered from chloracne, a painful and disfiguring skin condition, only days after an accidental exposure to TCDD. Despite the earlier association of chloracne with arochlor intoxication [5], it wasn't until Dr. Karl Schulz's courageous act that TCDD could be directly associated with chloracne. Dr. Schulz applied a solution of TCDD on his forearm and confirmed himself that TCDD exposure leads to chloracne [6].

Cumulative research suggests that chronic activation of the AhR by these persistent environmental pollutants may be the major cause of these health complications. For example, deregulation of vitamin A production [7], hormones, and some growth factors such as TGF-β [8] are some of the suspected culprits for the development of TCDD toxicity. In the post TCDD discovery era from 1962 through 1967 the Operation Trail Dust and Operation Ranch Hand conducted by the United States introduced the term chemical warfare in which the compound Agent Orange (AO), known to contain residual TCDD, was utilized to defoliate large forested areas in Vietnam. Since the time, there have been numerous studies discussing the detrimental consequences of AO on both military personnel and the affected populations. However, despite the large number of experimental animal data suggesting TCDD as a potent carcinogen, a direct link to human cancer remains a topic of hot debate due to the lack of proper controls in most studies [9].

TCDD is paradoxically now known to be a trace byproduct in the synthesis of the phenoxy-herbicide 2,4,5-trichlorophenoxyacetic acid (Figure 1-1) [10]. In 1964 Dow Industrials released the first report on the possible dangers of TCDD exposure after several employees who worked in the production of AO developed chloracne. In 1976 a severe industrial accident took place at the Industrie Chimiche Meda Societa Azionaria in Seveso, Italy [11]. Roughly up to kilogram quantities of TCDD and additional TCDD-like chemicals were dispersed into the air with health consequences for the local population [12]. Thousands of animals died and others were sacrificed to prevent further contamination of the food chain. Thanks to the foresight of Dr. Paolo Mocaralli blood

samples were collected and frozen from each patient for future analysis. In subsequent studies, a high proportion of human females were apparently born to couples exposed to highly-contaminated areas in Seveso. However, this assertion continues to be highly debated [13-15]. Yet, a convincing role for AhR ligands and the receptor itself in estrogen receptor (ER) function, degradation, and abnormal sex hormone metabolism has been suggested by relatively recent research [16-20]. Overall, the increased awareness about the imminent dangers of TCDD and polycyclic aromatic compounds prompted the industry to improve their production guidelines to protect their employees from chemical exposure and to minimize the release of harmful substances into the environment. The use of 2,4,5-trichlorophenoxyacetic acid in the production of herbicides has been replaced with dicamba and triclopyr. However, dicamba has been recently labeled as a genotoxic compound based on *in vitro* studies [21], while triclopyr continues to be labeled as a safer alternative given that it is highly polar, readily metabolized and excreted by most organisms [22, 23].

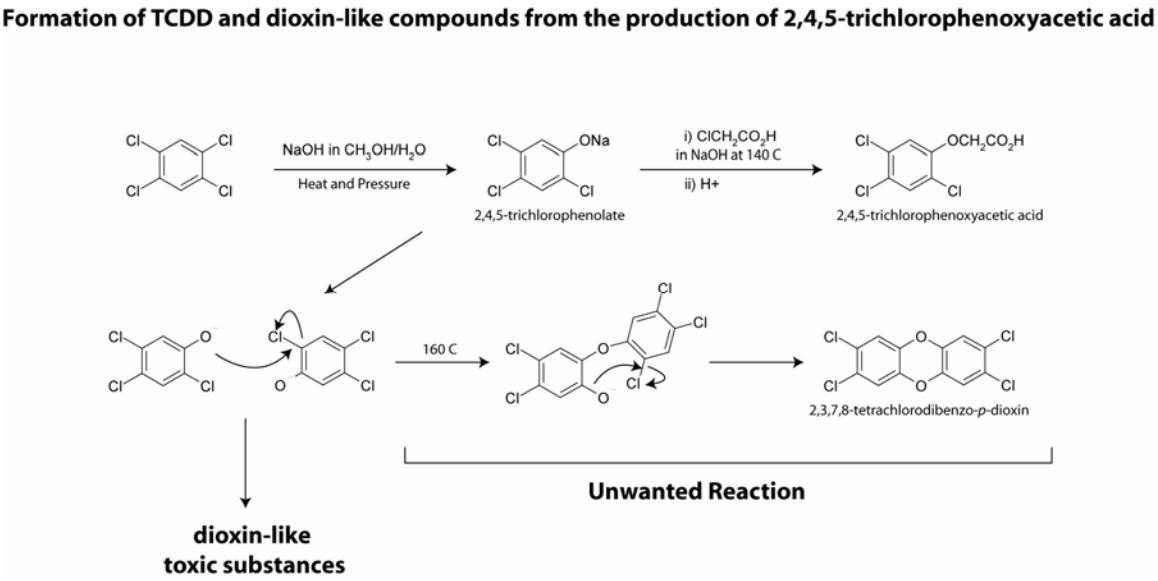


Figure **1-1**: Proposed mechanism for the formation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from the synthesis of 2,4,5-trichlorophenoxyacetic acid.

In recent history, the Ukraine president Viktor Yushchenko became a widely recognized victim of TCDD poisoning (Figure **1-2**) [24]. Severe chloracne skin lesions were prominent on the president's face. In humans, these skin lesions and allergic-like reactions have been associated with polymorphisms in CYP1A1 and GSTM1 genes [25]. Unfortunately, an effective treatment for TCDD poisoning and similar substances has yet to be established, although the use of potato chips fried in the indigestible synthetic fat Olestra™ (Procter & Gamble Company, Cincinnati, Ohio, USA) has shown great promise in this regard [26]. Olestra cannot be absorbed in the gut and therefore it is believed to serve as a carrier of TCDD excreted from the body through biliary secretions and ultimately through feces [27]. The half-life of TCDD in humans is estimated to be around 7 years, although genetic and environmental factors are expected to minimize or prolong its clearance from human tissues.

Figure 1-2: **Acute effects of dioxin poisoning in humans**.

Ukraine president Viktor Yuschenko before (left) and after (right) suspected dioxin poisoning. Severe chloracne is among the acute toxic effects of dioxin exposure in humans, symptoms that may eventually clear away. Chloracne is characterized by painful acne-like lesions and hyperkeratosis. In humans, these skin lesions have been associated with polymorphisms in CYP1A1 and GSTM1 genes [25].

1.2 Identification and cloning of the AhR.

In 1959 the term pharmacogenetics was officially coined by Friedrich Vogel [28]. During the late 1950s it became clear that the efficacy or adverse effects of therapeutical drugs could be often associated with simple inheritable genetic differences (polymorphisms) between individuals. Despite hints dating as far as Garrod's 1906 publication entitled "Inborn Errors of Metabolism [29]", apparently what delayed this realization was the confounding influence of the environment and lifestyle on the phenotypic manifestation of genetic traits as well as the fact that most responses to xenobiotics involved several non-linked genetic loci. Nevertheless, through advances in statistical and genetic analyses it became widely recognized that at least some of these metabolic differences between individuals had simple traceable genetic origins that segregated in Mendelian frequencies.

Similarly, differences in susceptibility to HPAHs, PAHs and other xenobiotics, even between organisms of the same species, could be also traceable to their genetic makeup. These polymorphic genetic loci were recognized to play a role in the controlled expression and/or stability of xenobiotic metabolizing enzymes, receptors, transporters, and perhaps unidentified accessory/regulatory factors. Preceding the identification and cloning of the aryl hydrocarbon receptor (AhR), the upregulation of aryl hydrocarbon

hydroxylase (AHH) activities, as measured *in vitro* by the formation of 3-hydroxybenzo[a]pyrene from the parent compound benzo[a]pyrene (B[a]P), could only be readily detected in liver extracts and some extra-hepatic tissues from so coined 'responsive' mice [30]. The crossing and backcrossing of multiple inbred strains of mice further led to the identification of the *Ah* (aryl hydrocarbon) genetic locus, suspected to control the inducible expression of the spectrally distinct cytochrome P_1-450 (today known as CYP1A1) [31, 32] as well as other AHH activities. This genetic locus segregated primarily as an autosomal dominant trait in specific crosses (e.g., C57BL x DBA mice) where the dominant responsive allele was denoted as Ah^b (in C57BL/6 and B6 strains) and the recessive non-responsive as Ah^d (in DBA/2 strains). As expected, the complex inheritance pattern of AHH responsiveness observed between a wider screen of wild type and inbred mice strains strongly suggested the additional contribution of non-linked loci in the inducibility of AHH/P_1-450 activity [33]. Regardless, the rescue of 'unresponsive' DBA mice with the potent AHH inducer TCDD, for which AHH activity approached that of 3-MC-treated C57BL/6 mice, sparked a new theory: the *Ah* locus encoded a gene product, such as a receptor protein, central to the downstream induction of AHH/P_1-450 [30]. A mutated Ah receptor in the DBA mice strain was suspected as the culprit for a 'failure to recognize the 3-MC signal [34-36]. This hypothesis was validated only after the AhR was cloned. A comparison between the AhR from C57BL and DBA strains of mice indicated that the key differences were an alanine to valine substitution at position 375 (381 in human AhR) in C57BL AhR and a mutation at the stop codon resulted in an elongated carboxyl terminus in the AhR^D allelic variant with reduced affinity for [^{3}H]TCDD [37].

Several research observations reinforced this Ah receptor theory. For example, the peritoneal injection of C57BL/6 mice with [^{3}H]TCDD, resulted in high levels of radioligand retention in the liver, especially when compared to the nearly absent levels in non-responsive DBA/2 strain. Crosses between C57BL/6J x DBA/2J also resulted in offspring (B6D2F$_1$/J) displaying intermediate binding/responses, an observation that correlated with the segregation of a simple autosomal dominant trait controlling AHH inducibility [36]. Extensive *in vitro* and comparative animal toxicology studies emphasizing structure-activity relationships further suggested that a soluble cytosolic receptor protein with varying affinities for TCDD-like compounds was likely responsible for the induction of AHH/P$_1$-450 activity [36, 38-41]. A model started to emerge resembling that of the already established steroid receptor pathway [42]. In brief, the cytosolic Ah receptor, upon the binding of TCDD, would translocate into the nucleus and activate genes controlled by the *Ah* genetic locus. However, until better methods (e.g., immunofluorescence [43-45]) were available, the location of the AhR complex proteins in non-stimulated cells remained a controversial issue for several years [46, 47].

The AHH/P$_1$-450 'inducer/receptor-complex' was later confirmed to undergo translocation into the nucleus of responsive cells following the binding of radioligand, an event that clearly preceded the induction of AHH/P$_1$-450 activity [48, 49]. A comparative study of the non-responsive VERO and HTC mammalian cell culture lines and the responsive H-4-II-E and Hepa-1 also indicated that the translocation of the Ah receptor into the nucleus, as in HTC cells, does not guarantee that AHH/P$_1$-450 induction would

proceed [50]. These results further highlighted that both structural and regulatory genes other than the proposed Ah receptor were likely controlled by the *Ah* locus. Work conducted by Okey et al. 1979 [49], and later revisited by Hanna et al. 1981 [51], identified the presence of a ~6 S (at high ionic strength) and ~9 S (at low ionic strength) receptor complexes, respectively. Both complexes had a measurable [^{3}H]TCDD binding capacity that was virtually absent in extracts from DBA/2N mice. These complexes also displayed sensitivity to proteases and not DNases or RNases. [^{3}H]TCDD binding could be competitively displaced with known P_1-450 inducers but not with established steroid hormones or other classic P-450 activity inducers (e.g., phenobarbital). In addition, only known inducers of P_1-450 (CYP1A1) and unlabeled TCDD could effectively compete for binding to the receptor complex [49]. In the upcoming paragraphs, references are made to a 6 S and 9 S receptor complex form. However, slight deviations from these sedimentation coefficients are stated in several references. For the sake of simplicity a transformed AhR complex is referred to as a ~6 S complex, while the ~9 S complex refers to its non transformed state, which is later found to be primarily composed of two molecules of hsp90 and the immunophilin-like protein XAP2 (also known as AIP and ARA9). Slight variations in the sedimentation coefficients likely result from the approaches used for the *in vitro* studies performed.

The lessons learned about the glucocorticoid receptor family were progressively being applied to the rapidly growing Ah receptor field. For example, the use of 20-30 mM molybdate in sucrose gradient fractionation of the glucocorticoid receptor (GR) was known to stabilize the ligand binding conformation of the GR [52]. The murine and rat

AhRs appeared to benefit partially from this treatment by showing increased thermostability, as well as retaining their ligand binding capacity under high ionic strength [53]. The human Ah receptor appeared to benefit the most, by displaying enhanced stability *in vitro* after molybdate treatment [54, 55].

A key defining moment towards the future cloning and characterization of the Ah receptor was the synthesis of 2-azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin [56], a photoaffinity ligand capable of covalently binding the Ah receptor when excited in the ultraviolet frequency (Figure 1-3). At first, two polypeptides with a mass of 70 kDa and a 95 kDa were suspected to represent the Ah receptor, given that the photoaffinity ligand could be competed off with known Ah agonists from both polypeptides (e.g., TCDD). However, the 70 kDa was shown to be nothing more than a proteolytic fragment of the 95 kDa polypeptide in Hepa-1c1c7 cells [57]. The suspected protease had similar characteristics to the Ca^{+2}-dependent calpain II and was labeled as the culprit of this isolation 'artifact,' although it was not directly established. Hence, the addition of EDTA was recommended to stabilize the AhR during purifications. With the advent of a new molecular photoaffinity probe the AhR field exploded with numerous research findings. For instance, the characterization of several inbred mice strains with the new photoaffinity radioligand lead to the identification of two Ah receptor allelic forms such as the 95 kDa (*Ah*$^{b-1}$ allele*)* from the C57, C58, and MA/MyJ mice strains and the 104 kDa (*Ah*$^{b-2}$ allele*)* from the C3H/HeJ, BALB/cByJ, and A/J strains [58]. Ligand competition-binding protocols with the photoaffinity ligand were being established which helped estimate the binding affinity and characteristics of other suspected Ah receptor

ligands [41, 59]. In 1988 Perdew and Poland described the partial purification of the AhR from C57BL/6J mice [60]. This event led to the eventual purification, N-terminal sequencing, and production of antibodies against the AhR [61, 62]. The generation of antibodies capable of immunoprecipitating the glucocorticoid receptor-associated hsp90 [63-65] and the AhR helped identify the hsp90 as part of the unliganded AhR complex by two different approaches [66, 67].

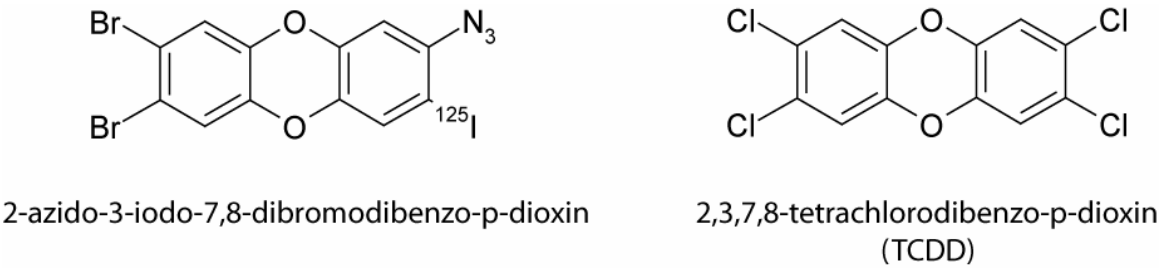


Figure **1-3: Structure of 2-azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin.**

The radiolabeled compound 2-azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin (left) was first synthesized by Poland et al. 1986 [56]. It was eventually used in the identification and isolation of the AhR. The structure of the prototypical high-affinity AhR ligand TCDD is also shown (right).

Yet before the cloning of the AhR, a subunit of the liganded complex was identified and thought to be required for the translocation of the AhR into the nucleus [68, 69]. Briefly, a cell line expressing a 'functional' AhR but uninducible for P_1-450 (CYP1A1) was systematically transfected with a cDNA library. The expression of one of these cDNA constructs restored the translocation of the AhR and the expression of P450IA1. The product of this gene was therefore named ARNT (for Ah receptor nuclear translocator), although the name itself is a misnomer for ARNT is not directly involved in the AhR 'translocation' event *per se*. ARNT was found to share homology with other known *Drosophila* proteins such as Per and Sim and contained a suspected DNA-binding/dimerizing domain termed the basic helix-loop-helix [68]. Together the Per, ARNT, and Sim proteins are the representative members of the PAS domain superfamily (Figure 1-4). PAS domain proteins have been associated with important functions in relaying environmental signals (e.g., light, oxygen, and xenobiotics) to the cell [70] (for an excellent recent review see [71]). The AhR appeared as a unique member in the PAS domain-containing family as it could be activated by exogenous ligands. However, while numerous studies have identified endogenous and exogenous substances capable of activating the AhR, such as photolytic products of tryptophan [72, 73] and indole derivatives [74] found in cruciferous plants, the receptor remains classified as an orphan receptor.

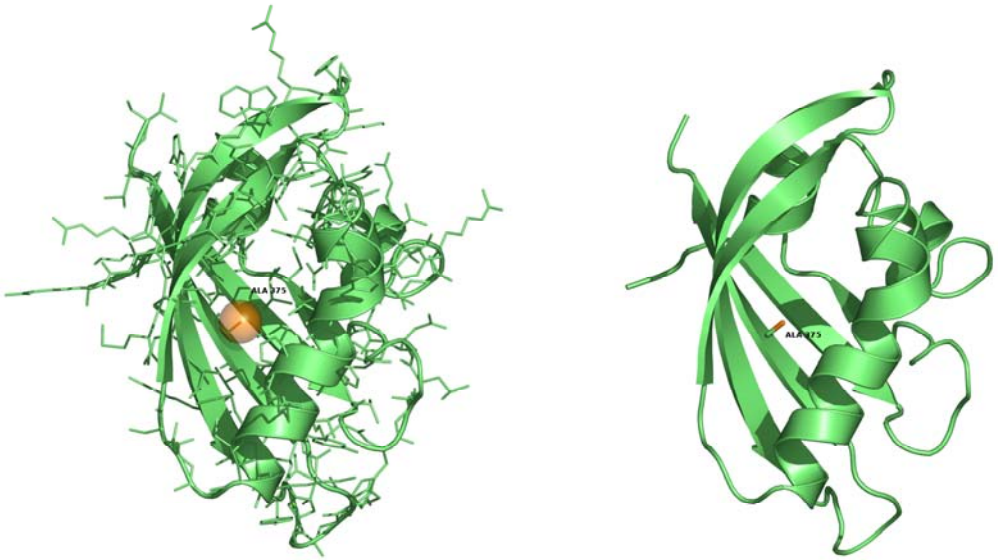


Figure **1-4: Homology-based structure of the mAhR PAS B domain and ligand binding pocket.**

The PAS superfamily of transcription factors are represented by Per, ARNT, and Sim. The homology-based model of the murine AhR PAS B domain (shown above) is characterized by a conserved α/β-fold. Hydrophobic cores in the vicinity of the α/β-fold are suspected binding sites for organic metabolites or ligands (e.g., TCDD). These domains are also required for homo and heterodimerization between PAS-domain transcription factors (e.g., AhR and ARNT). An Ala375Val substitution abolishes ligand binding to the AhR, suggesting the importance of this residue for ligand-mediated activation of the AhR [75]. It is believed that the open architecture of the ligand-binding pocket may allow for the promiscuous interaction of numerous ligands as described in the literature. These structural models were kindly provided by Michael Denison (U.C. Davis)

A distinction between the latent cytosolic 9 S and the activated nuclear 6 S forms of the AhR was later established through chemical crosslinking studies [76, 77]. The AhR complex was found as a tetrameric complex containing the AhR, two molecules of hsp90 and an unidentified ~43 kDa protein (AIP; XAP2) that sedimented at 9 S in sucrose gradients. This tetrameric complex could be transformed into a dimeric 6 S complex in the presence of ligand [77]. The ligand activated 6 S AhR complex was also paradoxically composed of a heterodimer containing the signature 95 kDa Ah receptor and an 'unknown' 85 kDa polypeptide (ARNT).

Given the availability of antibodies and a pre-established AhR purification protocol, the N-terminal sequence of the AhR^{b-1} was determined. This aided the creation of degenerate primers used in the cloning of the AhR^{b-1} gene [78]. The AhR^{b-1} was found to share many features with the previously cloned ARNT. For instance, it contained a bHLH region followed by two 51-amino acid A-B repeats (PAS repeats [70]) and an N-terminal glutamine-rich region [68, 79]. The similarities between the AhR and ARNT, the presence of a bHLH, and previous observations on their interaction with enhancer DNA regions at the CYP1A1 promoter led to the hypothesis that the AhR and ARNT could be heterodimeric partners. In addition, the covalent binding of the photoaffinity ligand to the A-B region suggested that this region could be structurally part of a ligand binding pocket that served as a switch for the transformation of the AhR into a DNA-binding conformation. Finally, the glutamine-rich region was suspected to serve as a transcription activation domain given its presence in other transcription factors [80, 81].

The human AhR was subsequently cloned by the screening of a cDNA library generated from the hepatoma cell line Hep-G2 [55]. In comparison with the murine AhR^{b-1}, the human receptor was 6 kDa larger. Most of the differences between the murine and human receptors were found in the carboxyl terminus, showing about < 60% conservation, while the basic region, the helix-loop-helix, and PAS domains displayed about 100%, 98%, and 87% level of conservation, respectively. A schematic of the murine AhR domains along with the residues important for its interaction with other proteins such as hsp90 and XAP2 is shown in Figure 1-5.

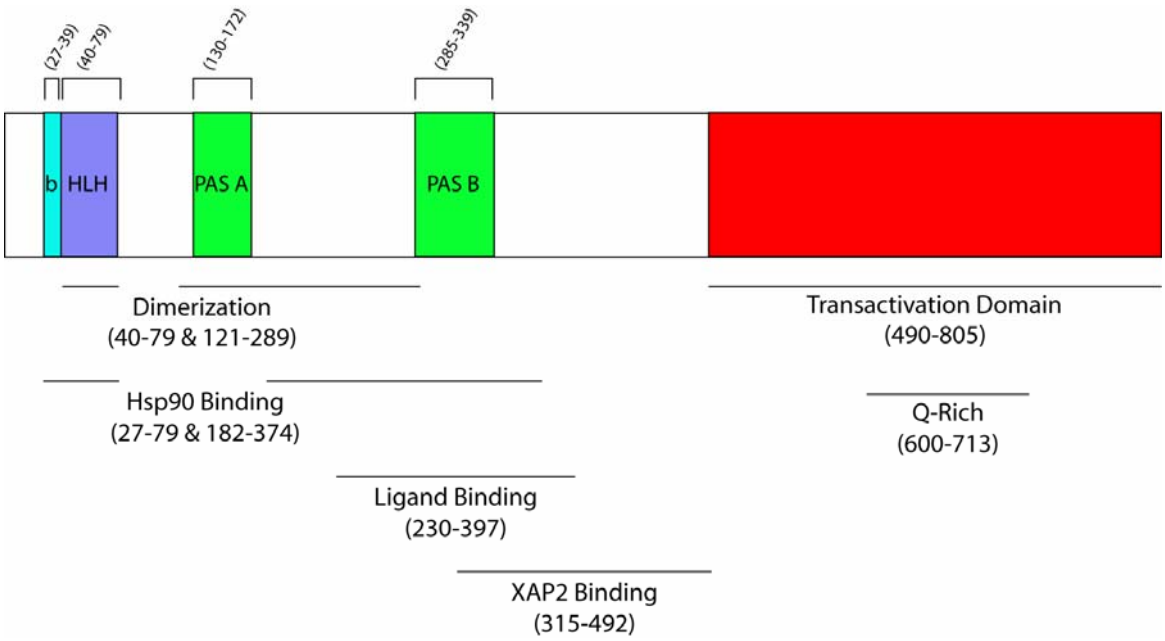


Figure **1-5**: **Functional domains of the murine AhR (drawn to scale).**

The delineation of the domain regions was adopted from several publications [78, 82-85]. The labels 'b' and 'HLH' stand for **b**asic region (DNA binding) and **h**elix-**l**oop-**h**elix domains. The numbers represent the amino acid residues corresponding to each domain or region.

The creation of a photoaffinity ligand and antibodies for the AhR facilitated the use of numerous biochemical approaches that accelerated our understanding of AhR biology. Numerous inbred strains of mice were further characterized with the photoaffinity ligand. The combined work has led to the identification of 4 polymorphic alleles namely the 95-kDa (Ah^{b-1}), 104-kDa (Ah^{b-2}), 105-kDa (Ah^{b-3}), and the 104-kDa (Ah^{d}) allelic variants [86, 87]. Thus, differences between responsive and non-responsive animals to xenobiotics and the low degree of conservation between the mouse and human AhR were quickly being recognized as major obstacles, especially for the extrapolation of animal toxicological data to humans. Nevertheless, animal models have still provided significant insight into the biology of the AhR. For example, the generation and characterization of mice exclusively expressing the human AhR has shown that susceptibility to some TCDD-induced teratogenic effects in mice (e.g., cleft palate) are absent in humanized versus wild type mice [88], while other characteristics such as hydronephrosis are still present. Whether the varying affinities to ligands between these AhR allelic variants or other factors such as their non-conserved transactivation domains and how other transcription factors may interact with the AhR are associated with these differences, will need to be further established in future studies. These observations may suggest that the extrapolation of mice data to humans could be better modeled through 'humanized' mice or help us realize that at least multiple toxic endpoints are possible simply due to allelic variations in the AhR gene. It would perhaps be interesting to explore the use of humanized mice expressing AhR mutants to more precisely determine the physiologically relevant aspects of AhR biology.

1.3 Characterization of AhR and ARNT functional domains.

Following the cloning of the AhR and ARNT, an extensive effort to map their functional and interaction domains was initiated [87, 89-93]. Excellent discussions pertaining to the functional domains of both the AhR and ARNT can be found in [82, 94]. Briefly, through immunoprecipitations of the murine AhR, deletion-mutants of [^{35}S]ARNT were monitored for their ability to interact with the AhR in the presence or absence of TCDD [94]. The bHLH together with the PAS-A and PAS-B domains (amino acids 70-474 of the murine ARNT) were all required for optimal heterodimerization to occur in the presence of TCDD. Deletion of the basic domain of ARNT had virtually no effect on the dimerization process. The ability of the AhR:ARNT heterodimer to bind double stranded DNA oligos containing the consensus XRE sequence also required the basic region of ARNT. Interestingly, an ARNT construct containing only the region from the bHLH through the PAS-A domain was unable to bind its XRE, while still being able to heterodimerize with the AhR. Conversely, a construct containing the bHLH, PAS-A, and PAS-B domains (bHLHAB) was able to restore XRE binding. It was therefore hypothesized that the PAS-B domain may help the ARNT protein fold in a way to maximize the interaction between the basic region of ARNT and DNA. The presence of an oligo containing a xenobiotic responsive element alone did not catalyze the heterodimerization of the AhR with ARNT. Thus, a ligand seems to be required for

AhR:ARNT heterodimerization to occur. Lastly, a construct containing only the bHLH, PAS-A and PAS-B domains could not restore *in vivo* function of ARNT. Therefore, other regions of ARNT were suspected to contribute to its transcriptional activity, namely the region containing amino acids 474 through 627 of the murine ARNT [94].

To characterize the transactivation domains of the AhR and ARNT, chimeras were initially generated with the DNA binding domain of the glucocorticoid receptor [95]. Since the bHLH region of the AhR and ARNT are required for their heterodimerization, this fusion-protein approach seemed like a proper approach to study the individual transactivation potential of the AhR and ARNT. In this study the glucocorticoid DNA binding domain was fused to ARNT and the AhR generating GRDBD-ARNT and GRDBD-AhR chimeras. In general, GRDBD-ARNT chimeras were more transcriptionally active than the GRDBD-AhR variety. Furthermore, in the case of ARNT its transactivation domain potency varied between cell lines, suggesting that cell- and promoter-specific activities are possible for this transcription factor. However, an AhR chimeric construct containing residues 83-593 reduced the ligand-induced activity while a chimeric construct based off residues 594-805 lead to a constitutively active Ah receptor that was as potent as the GRDBD-ARNT on an MMTV promoter. Therefore a region close to the PAS homology/ligand binding region was suspected to impart regulatory control to the AhR. For instance, the construction of a GRDBD-AHR containing residues 340-805 restored the repressive effect and the interaction between the AhR and the hsp90. Based on this and previous observations indicating that an AhR stripped from hsp90 could not bind ligand [96], a possible role for hsp90 in repressing

constitutive AhR activity was suggested. In this model, the hsp90 protein would maintain the AhR in a latent state in the absence of a stimulus. After the binding of a ligand a conformational change would cause the concerted dissociation of AhR from the hsp90 complex, its heterodimerization with ARNT and subsequent activation of target genes.

The use of chimeric AhR and ARNT containing the basic DNA binding domain of GR to study the transactivation domains was later criticized [82]. As a result, a complementary analysis of the AhR domains was performed by Fukunaga et al. 1995 [82], similar to previous domain deletion studies with ARNT [94]. In essence, the ligand binding region of the AhR was mapped to the PAS B domain. Both the bHLH and PAS B domains of the AhR were required for hsp90 binding. While ARNT could enhance the dissociation of the AhR from hsp90, ligand alone was sufficient to facilitate the dissociation of the AhR from hsp90 *in vitro* [82]. Whether the release of AhR from hsp90 in the presence of ligand happens *in vivo* has yet to be fully established although evidence suggests that dissociation of the AhR from hsp90 may only be required for its heterodimerization with ARNT [97]. The regions of the AhR responsible for its interaction with the hsp90 were further mapped to amino acids 1-166 and 289-347 [98], while the use of previously published deletion constructs of the hsp90 [99] suggested that amino acids 272-617 (‘the middle portion’) of the hsp90 were involved in AhR binding [84].

1.4 Basic aspects of AhR-mediated gene regulation.

The nuclear uptake of the Ah receptor was a phenomenon observed after the treatment of C57BL/6J mice and responsive cell cultures with [^{3}H]TCDD [48, 49, 100]. It seemed evident that ligand binding was required for the AhR nuclear translocation event [101], while limited proteolysis of the receptor complex suggested the presence of a DNA binding component of the AhR complex [102]. However, these non-specific DNA binding studies only provided limited information about the DNA motif(s) or response element(s) recognized by the activated AhR. Hence another group took a genomic approach to determine the regions of the P_1-450 (CYP1A1) promoter that contained TCDD responsive elements. HEV (highly expressing variant) cells expressing high levels of the P_1-450 gene were selected for a genomic screen. Several clones containing P_1-450 DNA were screened and one, containing a 2.58 kb fragment, was subcloned upstream of a bacterial chloramphenicol acetyl transferase gene (CAT) reporter [103]. In this manner the 5´-end of the P_1-450 gene was found to contain at least 2 elements that were responsive to several AhR ligands in responsive cell lines and contained a suspected cycloheximide sensitive repressor binding site. Through a deletion analysis of the P_1-450 promoter an AhR/TCDD dependent enhancer region was identified [104, 105]. This enhancer region was later documented as the dioxin responsive element (DRE) [106], although the term XRE (xenobiotic responsive element) or AhRE (aryl hydrocarbon responsive element) has also been used to describe the DNA elements recognized by the

activated AhR. A third DRE was later identified and the consensus DNA sequence for the binding of the AhR to its DRE was suggested [107, 108]. This DRE sequence was suggested to be 5'-TA/TGCGTG-3', a sequence that was present in all 3 DREs described to date. The asymmetrical nature of the DRE also differed from the typical palindromic responsive elements recognized by steroid receptors. The DRE flanking sequences in the enhancer/promoter appeared to be equally important as the subcloning of oligos containing the DRE sequence alone was not sufficient to restore inducible expression of a reporter gene [107, 108]. The core DRE sequence recognized by the AhR complex was further refined through methylation protection and interference studies to 5'-T/GCGTG-3', and four copies of this element were mapped to the P_1-450 promoter [109, 110].

The AhR was initially suspected to bind as a monomer to XRE sequences on double stranded DNA. This was based on the notion that the response elements for steroid receptors were symmetrical (palindromic) and the steroid receptors bound DNA as homodimers. However, several lines of evidence suggested a heterodimeric structure for the DNA-binding AhR complex. Among these was that the transformation of the AhR into a DNA binding complex required a non-ligand binding component [111]. Through crosslinking studies and limited proteolysis studies, a ligand binding component and an accessory component of the activated AhR were identified [112]. Finally, following the cloning of the protein ARNT and the AhR itself, the Hankinson group identified ARNT as a component of the activated AhR in nuclear extracts [113]. In vivo DNA footprinting demonstrated that the AhR bound a DNA element with the sequence 5' CACGCNA/T 3' and another component interacted with a G-rich region [114]. In the same year the

Denison group through gel retardation assays and mutagenesis of DRE-containing oligos were able to derive the consensus sequence GCGTGNNA/TNNNC/G (core sequence is underlined)[115]. In this study some conserved nucleotides 3′ to the core sequence were suspected to contribute to ligand-mediated AhR-ARNT heterodimer DNA binding. ARNT was later found to bind a 5'-GTG-3' half site that resembled that of the E-box element utilized by other members of the helix-loop-helix family of transcription factors [116]. Since nucleotides flanking the core DRE sequence had an impact on AhR complex affinity for its DNA element, the putative XRE was suggested to be 5'-TTGCGTGAGAA-3', where the AhR binds the second 5' thymine and ARNT the third 5′ thymine. While the methods utilized to determine the consensus binding site for the AhR-ARNT heterodimer varied, the combined results highlighted the importance of the residues flanking the core sequence 5′-GCGTG-3′ in the successful binding of the AhR-ARNT heterodimer to this element and to drive gene expression.

1.5 Transcriptional Co-Activators and Co-repressors of the AhR Signal Transduction Pathway.

Most of the knowledge about AhR mediated gene activation has been acquired through extensive studies of the *CYP1A1* proximal promoter. The basal and inducible expression of *CYP1A1* is known to be dependent on several factors, such as the recruitment of the AhR-ARNT heterodimer to DRE elements for its inducible expression. The nearly undetectable basal transcriptional activity is driven by the transcription factor Sp1 through GC boxes, while other basal transcription factors (e.g., TBP and TFIID) are recruited to the TATA box element [117-124].

The transcription factor CBP/p300 was the first coactivator implicated in the activation of AhR-mediated gene regulation through its interaction with the ARNT transactivation domain [125]. The nuclear receptor coactivator SRC-1 was also found to interact with the Q-rich domain of the AhR and enhance its transcriptional activity [126], analogous to the observations seen with the steroid receptor family [127]. In contrast to RIP140 [128], which does not require LxxLL motifs for ligand-dependent interaction with the AhR, LxxLL motifs within SRC-1 were required for its AhR coactivator function [126]. The SRC-1, NCoA-2, and p160 coactivators all belong to a family of approximately 160 kDa proteins serving specific roles promoting an increase in transcriptional output, in part through their associated histone acetylating activities. Given their interaction with other basal transcription machinery proteins such as TFIID

and TBP, these coactivators may serve as adaptors or bridging molecules bringing about the concerted remodeling of chromatin at promoter regions [129]. The p160 family-member NcoA4 was also found to interact with the AhR in the cytoplasm and distribute itself between the cytoplasmic and nuclear compartments in the presence of TCDD, while directly enhancing AhR transcriptional activity [130]. The coactivator 'estrogen receptor associating protein 140' (ERAP 140) and the corepressor 'silencing mediator for retinoic acid and thyroid hormone receptor' (SMRT) can both interact with AhR-ARNT complexes and enhance or repress transcriptional output, respectively [131].

Yet another factor, the retinoblastoma protein (pRb) in its hypophosphorylated state has been implicated in the TCDD induced G1 cell cycle arrest and may serve as an AhR coactivator in the regulation of *CYP1A1* expression [132]. The interaction between the AhR and pRb appears to be driven through pRb association with a LXCXE motif found in the AhR and possibly through pRb interaction with $TAF_{II}250$, which forms part of the TFIID basal-transcription complex [133]. The secondary coactivator and HLH-PAS domain-containing protein CoCoA has also been implicated in AhR-ARNT driven gene expression. This interaction does not appear to require p160 which is considered to be the primary coactivator in steroid receptor-driven gene expression. Thus a direct interaction between CoCoA and the AhR-ARNT heterodimer appears to be responsible for its transcription enhancing properties [134]. The novel coactivator GAC63 also interacts with AHR through its bHLH-PAS domain and is recruited to the proximal CYP1A1 promoter [135].

Interestingly, the unorthodox recruitment of some nuclear receptors to particular promoters, via interaction with other transcription factors and not necessarily their putative DNA-binding elements, is a phenomenon given more attention today thanks to improvements in techniques such as chromatin immunoprecipitation. For example, the estrogen receptor can be recruited to the *CYP1A1* promoter in a dioxin and estrogen dependent manner through its direct interaction with the AhR and transrepresses *CYP1A1* expression [136-138]. Another example involves the mutual repression observed between the AhR and NF-κB, through their apparent direct interaction [139]. This particular interaction has been of great interest given the immunomodulatory effects of many AhR ligands and thus a possible balancing role for the AhR in normal immune system function. Treatment of responsive cells with LPS and TNF-α results in the NF-κB-mediated repression of *CYP1A1* expression, presumably through its interaction with the AhR at the *CYP1A1* promoter [140]. In contrast, RelA and the AhR have also been implicated in the activation of the *c-myc* gene through a direct interaction between these two transcription factors [141]. It thus appears that the promoter context and the availability of a coactivator or corepressor pool in any given cell may ultimately determine the transcriptional outcome of these interactions. Transcriptional activation of the *CYP1A1* gene by the AhR/ARNT heterodimer has been shown to be in part influenced by the Brahma/SWI2-related gene 1 protein [142].Interestingly, *in vitro* studies have shown that both the BRG1/hBRM-Associated Factors (BAF) and the Polybromo-associated BAF (PBAF) nucleosomal remodeling complexes are capable of substituting for each other [143, 144]. However, the differential expression of nucleosomal remodeling-complex subunits, among tissues *in vivo,* has indicated that each

nucleosomal remodeling complexes can perform indispensable functions in a gene context manner [143-145]. Thus *in vitro* generated data, while informative, may not always recapitulate what occurs *in vivo* in a tissue and promoter-specific context. Regardless, *in vitro* data still provides vital information on cellular activities under more tightly controlled conditions. It is therefore critical to test *in vitro* models under different conditions and several cell culture models to ensure consistency of the model being tested. Hence, the role that coactivators and corepressors play in AhR biology remains to be fully established *in vivo*. While an apparent promiscuous interaction between all these factors and the AhR is evident in many *in vitro* culture models, it is likely that these coactivators could serve a more specific function *in vivo*, especially given their tissue specific expression in whole animals. The presence or absence of some of these coactivators or corepressors in a tissue-specific manner would further allow for an exquisite control of AhR-mediated gene regulation and its associated tissue-specific function(s).

1.6 Genes regulated by the AhR.

With the identification of dioxin responsive elements and the cloning of the AhR and ARNT, efforts concentrated towards the identification of novel AhR regulated genes by identifying DREs in their regulatory regions. The list of genes directly and indirectly regulated by the AhR continues to grow quite rapidly, some of which are listed in Table **Table 1.1** . DREs have not been identified in all genes and interspecies expression variability does exist. Yet these genes appear to require a functional AhR complex for their xenobiotic-mediated induction, perhaps through epigenetic mechanisms. While extensive studies have been carried to determine the *in vivo* function of these target genes, many remain poorly characterized. A brief description about the significant aspects of some genes is given below. In general, many of the AhR regulated genes carry important functions in phase I (activation), phase II (conjugation), phase III (excretion) metabolism, as well as in cell cycle regulation (e.g., $p27^{Kip1}$) and differentiation of immune cells through cytokine and hormonal regulation (e.g., erythropoietin). The presence of polymorphisms in the promoter and coding regions for some of these genes and the transcription factors that govern their expression, account for highly complex phenotypic manifestations of metabolic advantages or disorders, as well as increased or decreased sensitivities to toxic xenobiotics. An understanding of these gene networks is therefore crucial to the analysis of AhR-mediated toxicological data.

Table 1.1: **AhR Target Genes**

Metabolic Classification	Gene	Primary Physiological Roles(s)	References
Phase I (Activation)	CYP1A1	Oxidation of primarily planar polycyclic aromatic hydrocarbons.	114
	CYP1A2	Oxidation of aryl and heterocyclic amines.	146-148
	CYP1B1	Role in 7,12-dimethylbenzs[a]anthracene metabolism. Catalytic specificity is unknown.	149,150
	CYP2S1	Oxidation of polycyclic aromatic hydrocarbons.	151
	NAD(P)H:Quinone-oxireductase	Vitamin K -dependent gamma-carboxylation of glutamate residues in prothrombin synthesis	152
Phase II (Conjugation)	UGT1A1	Detoxification of numerous endogenous metabolites and xenobiotics through its UDP-glucoronosyl transferase activity.	153
	Ya subunit of GST (GSTa1)	Reduction of reactive oxygen species (e.g., hydrogen peroxide)	154
Phase III (Excretion)	MDR1 and BRCP	P-glycoprotein involved in the ATP-dependent export of xenobiotics away from cells.	155-156
Other Genes	erythropoietin (Epo)	Hormone produced by the kidneys that stimulates red blood cell production. It also stimulates the production of hemoglobin.	157
	epiregulin	Physiologically induced by angiotensin II, endothelin, and thrombin, it serves as a potent mitogen secreted from vascular smooth muscle cells.	158
	prostaglandin endoperoxide H synthase 2 (a.k.a. COX-2)	Membrane-associated enzymes that carry cyclooxygenase and peroxidase functions in the metabolism of arachidonic acid	161
	AhRR	Possible role in the repression of AhR activity, although the mechanism remains unclear	162
	P27kip1	Cyclin-dependent kinase inhibitor	163

The AhR controls the expression of some cytochrome P-450 genes (e.g., *CYP1A1*) involved in the metabolism of various lipophilic endogenous and exogenous substances. These enzymes of phase I metabolism perform oxidative, reductive, and hydrolytic reactions that transform hydrophobic endobiotics and xenobiotics into more water-soluble substances, resulting in their activation, inactivation, elimination, or associated toxicities. It is the nature of the substance and a number of cellular factors (e.g., metabolic enzyme profiles, transcription factors, etc) what determines whether these substances can be beneficial, detrimental, or innocuous to the organism. Some products of phase I metabolism can also be substrates for AhR-regulated phase II metabolism enzymes. In phase II metabolism, enzymes perform the conjugation of bulky hydrophilic molecules (e.g., glutathione, glucoronides, etc) that may result in the activation or inactivation of many drugs and endobiotics. Together, phase I and II metabolism convert hydrophobic substances into more hydrophilic substances that can also be more easily excreted from the organism. Without increased solubility, both tubular and intestinal re-absorption would prevent efficient elimination of hydrophobic substances from the organism and their accumulation could lead to toxicity. In some instances these metabolic reactions may also lead to the formation of harmful reactive intermediates (e.g., metabolism of benzo[a]pyrene by CYP1B1 as discussed below). Because some of these xenobiotics often induce unique profiles of metabolic enzymes, their combination may also lead to alterations in the metabolism of one another, also known as drug-drug interactions. In phase III metabolism, toxic substances are excreted from the cell primarily through transmembrane ATP-dependent transporters (e.g.,

MDR1) as yet another mechanism of defense against their toxic accumulation. Unfortunately, substances that cannot be readily eliminated from the organism, due to their resistance to metabolic pathways, can lead to severe and persistent activation of numerous cellular pathways and their associated toxic end-points (e.g., cancer) [164].

AhR-Regulated Phase I Metabolism Genes

CYP1A1 – The term 'cytochrome P450' was proposed initially to describe a pigment found in heme containing enzymes having a maximal Soret absorption peak at 450 nm, in their reduced form and bound to carbon monoxide [165]. CYP1A1 was the first identified cytochrome P450 that was inducible by benzo[a]pyrene and 3-methylcholanthrene [30]. Expression of this enzyme relies on the AhR for both its basal and inducible expression [166] and thus it is often used as a marker of AhR activity. CYP1A1 functions in the oxidation and activation of numerous intracellular substances (e.g., 17β-estradiol into 2-hydroxyestradiol), as well as synthetic and natural xenobiotics. The prolonged expression of this gene has been associated with the production of reactive oxygen species (ROS) which triggers the oxidative stress response. Accordingly, the formation of peroxides and other electrophilic byproducts during its catalytic activity may detrimentally react with proteins and DNA [167]. Oxidative stress in turn inhibits CYP1A1 expression. The existence of this regulatory loop is therefore believed to prevent further oxidative damage in the cell caused by prolonged activity of this enzyme [168]. The oxidation of 17-β-estradiol by CYP1A1 results in the formation of the procarcinogen 2-hydroxyestradiol [169] that can covalently bind to DNA. This often

leads to DNA misrepair damage and depurination induced mutations [170]. In addition, CYP1A1 induction has been directly associated with uroporphyria and lethality in mice after a high dose of TCDD [4]. While it is enticing to categorize the expression and activation of this enzyme as a detrimental end-result of xenobiotic intoxication, there is evidence that CYP1A1 null mice can be susceptible to chemical induced toxicity by displaying more pronounced wasting syndrome, severe immunosuppression, and fewer numbers of bone marrow progenitor cells [171]. It is thus important to realize that the interplay between several metabolic enzymes, the reduction/oxidation equilibrium in the cell, and other influencing factors such as diet and genetics ultimately determine the sensitivity displayed by organisms.

CYP1A2 – Unlike, CYP1A1, this enzyme plays a wider role in the metabolic activation and deactivation of many known dietary components, therapeutic drugs [(e.g., imipramine [172] and acetaminophen)], and synthetic xenobiotics. CYP1A2 is constitutively expressed, although exposure to cigarette smoke and halogenated polycyclic aromatic hydrocarbons can lead to high levels of expression in an AhR dependent manner. Some known inducers include the anti-acid omeprazole (commonly known as Prilosec® and Nexium®), the anesthetic phenobarbital, nicotine from cigarette smoke, and the antibiotic used to treat leprosy and tuberculosis, rifampicin. Heterocyclic amines (e.g., 2-amino-3-methyl-imidazo[4,5-f]quinoline) which form during the broiling of high protein-content food, can be metabolized by CYP1A2 into reactive intermediates that can form DNA and protein adducts [173, 174]. Given its ability to metabolize a wide variety of compounds, CYP1A2 is usually a concern in drug design due to potential drug-

drug interactions [175]. The metabolism of caffeine in the liver by CYP1A2 has served as a useful probe for estimating enzymatic activity in humans [176]. Therefore, high activity of CYP1A2, which varies up to 40-fold between individuals [177], has been associated with increased risk for developing various forms of cancer [178]. The CYP1A2*1F polymorphism in a non-coding region of the gene has been associated with impaired expression and thus slow metabolism of caffeine [179]. Therefore, homozygous individuals for the CYP1A2*1F allele may suffer from abnormally high levels of caffeine after consumption, which may lead to myocardial infarction [179, 180]. This enzyme is unlikely involved in the metabolism of sex hormones given that CYP1A2 null mice develop normally and are fertile [181].

CYP1B1 – This enzyme is primarily expressed in extrahepatic tissues, with low expression levels in the liver. Polymorphisms and mutations of this gene have been associated with congenital glaucoma [182], perhaps through its involvement in the metabolism of an unidentified factor (e.g., hormone) required during eye development. CYP1B1, like CYP1A1, has been associated with the formation of reactive metabolites of estrogen (4-hydroxyestradiol), often associated with susceptibility to endometrial cancers [183] and an increased risk for developing mammary tumors [184].

CYP2S1 – This enzyme was recently discovered through database-assisted homology searches [185]. Although inducible by AhR ligands, it is not capable of metabolizing common AhR ligands such as B[a]P and nicotine [186]. A possible role in the metabolism of topical drugs used in the treatment of skin conditions has been proposed

[187]. Interestingly, CYP2S1 is induced by UV radiation possibly through the formation of tryptophan metabolites and also by retinoic acid derivatives, all which are suspected endogenous AhR ligands [72, 188].

NAD(P)H:Quinone-oxireductase (NQO1) – This enzyme has been associated with the activation of vitamin K used in protein gamma-carboxylation [189]. Protein gamma-carboxylation of glutamate residues near the amino termini of some proteins allows them to sequester calcium which helps prevent the formation of conditions such as urolithiasis [190]. Polymorphisms in this gene that render the enzyme inactive have also been associated with increased risk for developing lung cancer due to the accumulation of toxic substances [191].

AhR-Regulated Phase II Metabolism Enzymes

UGT1 family – The *UGT1* genetic locus is comprised of various functional promoters with different first exons through alternative splicing, while exons 2 through 5 are common among the family of 6 members [192]. UGT1A1 expression is tightly controlled during development with nearly absent levels at birth but reaching full expression by at least 3 months of age [193]. Several diseases associated with impaired bilirubin metabolism such as Gilbert's syndrome [194], Crigler-Najjar syndrome type 1 [195], and cholelithiasis in sickle cell anemia patients [196] have been associated with polymorphisms in this gene. Regulation of this gene locus by the AhR appears to involve both electrophile responsive elements and XREs [197]. UGT1A6 was the first member

in the UGT1A family to be identified that plays a primary role in the metabolism of planar phenolic and arylamines. It is primarily expressed in the liver, kidneys and the skin, although it is also found at lower levels in several extrahepatic tissues. Together with UGT1A1, polymorphisms in the UGT1A6 gene have been associated with hyperbilirubinaemia and hyperserotoninaemia, as in Gilbert's disease patients [198, 199]. UGT1A6 plays a role in the metabolism of acetylsalicylic acid (aspirin). The expression of the allotypes UGT1A6*2 and UGT1A6*1 in combination or as homozygous are associated with lower or higher enzymatic activity, respectively [200]. Accordingly, variations in expression between UGT1A6 haplotypes have been linked to changes in the efficacy of various therapeutic drugs such as paracetamol, an analgesic used to treat the symptoms of thalassemia [201].

Ya subunit of GST and GSTA2 – The GST family catalyzes the conjugation of glutathione to electrophilic metabolites, aiding primarily in their detoxification due to improved water solubility. A total of thirteen GST subunits have been identified providing this dimeric enzyme with a high degree of substrate specificity. Five distantly related gene families designated class alpha, mu, pi, sigma, and theta GST encode all cytosolic forms of GSTs. There are also membrane-bound GSTs located in the endoplasmic reticulum as well as the plasma membrane such as leukotriene C4 synthetase [202]. In a study by Friling et al. 1990 the GST-Ya (member of the class alpha) gene was found to be induced by planar aromatic polycyclic hydrocarbons (e.g., benzo[a]pyrene, 3-MC, etc) in an AhR-dependent manner [203]. However, given that the gene is not induced directly by these aromatic compounds in the CYP1A1 deficient cell

line c1 mutant, it appears that GST-Ya induction relies on the formation of PAHs metabolites by generated by CYP1A1. In brief, AhR activation leads to CYP1A1 induction which in turn leads to the formation of electrophilic metabolites suspected in the induction of the Ya subunit through electrophile responsive elements. This electrophile induced oxidative stress by polycyclic aromatic hydrocarbon has also been observed after treatment with several transition metals such as Cd^{2+}, As^{3+}, and Cr^{6+} [204, 205]. However, the mechanisms of how these metals can induce GST-Ya as well as CYP1A1 have not been fully established. Some of the possible mechanisms include the production of heme breakdown products which are known to activate the AhR (e.g., biliverdin and bilirubin [206, 207]), the possible activation of the AhR by oxidative stress-induced arachidonic acid metabolites [208], and the activation of Nrf2 in a protein kinase C-dependent manner [209], are among several other proposed mechanisms [204]. The induction of GSTA2, along with GSTA5, by the chemotherapy drug Oltipraz is thought to play important role in the detoxification of hydrophobic electrophilic derivatives of metabolism, serving a crucial role in the prevention of cellular damage [210].

AhR-Regulated Phase III Metabolism Genes

ATP-binding cassette, subfamily B, member 1 (MDR1) and Breast cancer related protein (BRCP; better known as ABCG2) – These plasma membrane-bound receptors are primarily induced in response to oxidative stress, among other chemical insults, which helps with the efflux of noxious chemicals away from the cell. They have

been implicated in multi-drug resistance in the treatment of several forms of cancer [211, 212]. The induction of these genes in an AhR dependent manner has been debated by several laboratories. It appears that the activation of the AhR by non-metabolizable compounds such as TCDD does not lead to the upregulation of these drug transporters [213]. However, a study demonstrated that MDR1 is induced, perhaps through an indirect mechanism, by two AhR ligands [155]. In brief, the activation of the AhR by metabolizable B[a]P and 3-MC leads to the upregulation of xenobiotic metabolizing enzymes of the AhR battery. It is known that the metabolism of these two xenobiotics results in the formation of reactive intermediates that covalently bind to DNA. These DNA-xenobiotic adducts trigger the p53-dependent DNA repair response that in turn leads to p53 recruitment to elements in the promoter region of MDR1, upregulating its expression. Some recent evidence states that the MDR1 and ABCG2 can be upregulated in response to TCDD [156]. Perhaps these discrepancies are related to variations in response between cell lines examined and/or cell culture conditions utilized.

Other AhR target genes

Erythropoietin (Epo) – This hormone plays a critical role in the differentiation and maturation of erythroid lineage of bone marrow cells [214]. Interestingly the AhR, and the PAS domain protein Hif-1α, were found to play important roles in the cellular response to low oxygen tension. The AhR can directly induce its expression through its recruitment to several DREs in the *Epo* promoter [157].

Epiregulin (EREG) – Under normal physiological conditions this EGF-related growth factor is expressed after induction by the gonadotropin leuteinizing hormone [215] as well as by unsaturated lysophosphatidic acid and platelet-derived growth factor-BB [216]. In a paracrine and autocrine manner EREG binds to the EGF receptor and related type I receptor tyrosine kinases (e.g., ErbB), which initiates a signaling cascade involving Erk and p38MAPK [216, 217]. The activation of this pathway leads to the upregulation of several genes involved in cellular proliferation and tissue remodeling. Therefore, the uncontrolled expression of EREG has been associated with the progression of cancer and metastasis [218, 219]. EREG induction which is observed after TCDD treatment in an AhR-dependent manner is suspected to contribute, at least partially, to the tumorigenic properties of numerous AhR agonists [158].

ecto-ATP – The gene promoter appears to be constitutively active in Hepa-1c1c7cells and its expression appears to be mostly driven by constitutively occupied GC boxes [220]. While it is induced by AhR ligands, there appears to be no putative DRE element in the promoter region of this gene. Yet its induction by TCDD requires a functional AhR, ARNT, and AhR's transactivation domain [159]. Therefore it is possible that induction of this gene by the AhR requires distant DREs that are yet to be identified or perhaps novel AhR-dependent mechanisms could be involved.

δ-aminolevulinic acid synthase (ALAS1) – Following the expression of this enzyme in the nucleus it is transported into mitochondria where it catalyses the first and

rate-limiting step in heme biosynthesis in all tissues. It catalyzes the condensation of the amino acid glycine and succinyl-CoA (derived from the tricarboxylic acid cycle) in a two-step reaction to form δ-aminolevulinic acid (ALA) in the heme synthesis pathway. The abnormal production of ALA, seen after the induction of ALAS1 by AhR ligands, it is known to exacerbate certain porphyria-related disorders [36, 160]. In addition, the toxic accumulation of ALA, an established carcinogen, can lead to severe renal toxicity [221].

Prostaglandin endoperoxide H synthase 2 (a.k.a. Cyclooxygenase 2; COX2) – In contrast to the constitutively expressed COX1, which is important for maintaining gastrointestinal mucosa integrity, COX2 is mostly undetectable but can be induced by mitogens such as some AhR ligands, phorbol esters, IL-1, or simply as the result of tissue injury [222-225]. It is an early response gene expressed during the inflammatory response and thus several commercially used non-steroidal anti-inflammatory drugs (e.g., aspirin) have been designed to inhibit this enzyme. Interestingly, COX2 expression has been associated with the formation of adenomas in a murine model of adenomatous polyposis coli [226], and its abnormal induction, along with epiregulin, has been associated with increased risk for lung cancer [227].

AhR repressor (AhRR) – The AHRR is induced in response to AhR ligands and competes with the AhR for ARNT by forming apparent 'non-productive' heterodimers [228]. Given its potential role in AhR repression, polymorphisms of this gene such as Pro185Ala have been associated with increased susceptibility to dioxin-induced

micropenis [229, 230]. Polymorphisms at the same locus have also been associated with endometriosis but the evidence is rather weak due to poor controls and study designs [231-233].

CDKN1B (cyclin-dependent kinase inhibitor 1B (a.k.a., $p27^{Kip1}$) – This gene mediates the G1 cell cycle arrest in response to DNA damage, although roles in cellular differentiation have also been well documented [234]. CDKN1B is induced by TCDD in an AhR dependent manner and mediates cell cycle arrest in developing thymocytes and the 5L hepatoma cell [163]. This is among the list of evidence supporting a potential role for the AhR in cell cycle regulation.

1.7 Activation of the AhR by endogenous and exogenous ligands.

The AhR is considered an orphan receptor, given that no putative high affinity endogenous ligand has been identified to date. An endogenous role for the AhR has only been suspected from its ability to control the expression of drug-metabolizing enzymes when stimulated by synthetic substances belonging to the HAHs and PAHs families as well as numerous dietary substances. As such, a large amount of AhR ligands can induce their own metabolism and clearance from the body by inducing drug-metabolizing enzymes of the phase I (oxidation) and phase II (conjugation), and transporters of the phase III (excretion) metabolic pathways [235].

Some widely recognized synthetic inducers of the AhR pathway include compounds like B[a]P, 3-methylcholanthrene, β-naphtoflavone, 2,3,7,8-tetrachlorodibenzo-p-TCDD, 2,3,7,8-tetrachlorodibenzofuran, and 3,4,3′,4,′5-pentachlorobiphenyl [236]. However, numerous studies have suggested that dietary and endogenous substances can also readily activate AhR regulated genes. Some examples include indole-3-carbinol [73], bilirubin [206, 207], curcumin [237], and quercetin [238]. Some ligands can also exert both agonist and antagonist activities at different concentrations such as resveratrol [239] and galangin [240]. It appears logical that the high ligand promiscuity of the AhR may be indicative of its proposed role in the

adaptation of organisms to environmental chemical challenges. On the other hand, a putative endogenous ligand for the Ah receptor has been suspected to exist for numerous reasons. For example, hydrodynamic shearing of cells results in the formation of arachidonic acid metabolites suspected to induce CYP1A1 in an AhR-dependent manner [241]. A rat model of supplemental oxygen treatment for lung insufficiency revealed that hyperoxia treatment of rats led to AhR-dependent CYP1A1 induction [242]. The incubation of epidermal cells in a methylcellulose suspension activated CYP1A1 expression and this effect could be prevented by treatment with the AhR antagonist α-naphtoflavone [243]. UV irradiation could possibly lead to an AhR-dependent CYP1A1 induction by the formation of active tryptophan oxidation products [72]. There are also examples of substances (e.g., omeprazole) that can readily activate the AhR, yet cannot compete with known AhR ligands in competition binding assays [244, 245]. This leaves open the possibility that the AhR could be also activated in a ligand-independent manner. Developmental studies of AhR knockout mice have revealed important roles for the AhR in the development of the liver, closure of the ductus venosus [246], the immune system [247], and the control of many 'ligand-independent' cellular activities [248]. The affinity for exogenous ligands is not conserved between certain mouse strains and other mammalian species. For example, there is a 10-fold difference in affinity between C57BL/6 mice and human AhR [249]. Thus, this lack of conservation may suggest that either a ligand-independent activation of the AhR is important for endogenous AhR function or the ability of a key endogenous ligand to activate the AhR is conserved among species. It also seems plausible that some ligands could be acting through novel receptors that communicate downstream with the AhR either directly or indirectly

through post-translational modifications such as phosphorylation, dephosphorylation, partial proteolysis, ubiquitination, neddylation, SUMOylation, and so on. The release of intracellular calcium, which can serve as a second messenger and as an activator of intracellular calpains has been observed after the treatment of cells with TCDD [223, 250, 251]. Nevertheless, the question of how these events play a key role in AhR mediated signal transduction remains a topic of active research.

1.8 Unliganded AhR Receptor Complex Proteins.

The role for some of the AhR mature complex proteins remains largely unknown. A brief summary about the relevant aspects of each protein is given below. In some cases, these associated proteins have been better characterized in the context of the steroid receptor family. Therefore, some of the proposed roles that each plays on the AhR receptor complex has been extrapolated from steroid hormone receptor data.

Hsp90 – This protein is highly expressed in cells accounting for at least 1% of the total protein pool. Its role in the late folding steps of client proteins (e.g., steroid receptors) allows these to achieve their mature conformation [252]. In addition, the hsp90 stabilizes several receptor proteins in a latent state, thus preventing their constitutive activity in the absence of an agonist or stimuli. The latter has been demonstrated through AhR PAS-B domain-deletion mutants that fail to interact with the hsp90, resulting in a constitutively active AhR and its associated hepatocarcinogenicity [253]. It has been theorized that the hsp90 may mask the nuclear localization signal and/or interaction domains of the AhR, preventing its association with nuclear pore complex proteins (α- and β-importin) and with its heterodimeric partner ARNT, thus preventing its binding to DNA [96, 254]. This rationale is supported by the treatment of cells with molybdate

which prevents the dissociation of hsp90 from the AhR after ligand treatment and thus abolishes its transcriptional activity [97]. In the presence of molybdate the AhR-hsp90 complex is able to translocate into the nucleus but the AhR is unable to heterodimerize with ARNT and bind to its consensus DNA element [97]. Interestingly, it has been suggested that hsp90 binding to the AhR is not required for its ligand binding capacity, at least in some species [255]. However, this particular study is confounded by the observation that no physical separation of the hsp90 and the AhR is performed before the receptor is incubated with the radioligand. Even though the cytosolic extracts are treated with salt concentrations known to displace hsp90 from the AhR, the fact that both proteins are still present in the mixture may allow for transient equilibrium interactions between the hsp90 and the AhR that could allow for 'partial' binding of ligand to transiently formed 9 S mature-complex receptor species. Alternatively, through a hydrophobic effect the ligand may be forced to interact with the AhR. Therefore, it is generally accepted that in the absence of hsp90 the AhR loses its ligand binding ability and it is rapidly degraded through proteolysis.

During heat stress, hsp90 along with other chaperones also assist with the refolding of proteins as well as with the degradation of misfolded or damaged proteins by diverting them through proteolytic pathways (e.g., proteasome and lysosomal pathways) [256-259]. The manner by which proteins are selectively diverted towards degradation versus a refolding pathway is not completely understood. However, it is suspected that this process may be determined by the severity of protein misfolding [260].

XAP2 – The hepatitis virus X-associated protein 2 [261], also known as AIP [262] and ARA9 [263], is an immunophilin-like protein [263] that forms part of the unliganded AhR tetrameric complex. Despite advances in our understanding about this protein, its function remains largely enigmatic. Initial reports proposed that XAP2 may help stabilize the Ah receptor in the cell [84]. In support for this notion, work from Kazlauskas et al. 2000 suggested that the XAP2 may accomplish this effect by preventing AhR ubiquitination and degradation through the proteasome [264].

The XAP2 was also initially implicated in the enhancement of AhR transcriptional activity, perhaps through the modulation of its protein levels [261]. However, subsequent work indicated a repressive role for XAP2 on AhR transcriptional activity [265]. In the latter work, XAP2, by masking the bipartite nuclear localization signal on the AhR (a.a., 13-39), was shown to prevent AhR association with the nuclear pore complex protein β-importin, thus inhibiting the AhR's dynamic nucleocytoplasmic shuttling properties [266]. Differences in how XAP2 interacts with both the human and mouse AhR were later established, which results in the inhibition of nucleocytoplasmic shuttling of the mAhR in the absence of a ligand in cell culture models [267]. In support for a possible role in repression, *in vitro* analyses have suggested that XAP2 is unnecessary for AhR transcriptional activity and that its presence in the AhR complex may be mutually exclusive with p23 [268]. In the latter work, the overexpression of XAP2 also resulted in the repression of AhR activity in cells.

Interestingly, the lowest levels of expression of XAP2 among tissues are seen in the liver, where in turn there is a higher level of AhR protein and activity when compared to other tissues [261, 263]. Surprisingly, a transgenic murine model of XAP2 overexpression in the liver did not reveal any effect on the endogenous levels of the AhR and it had no apparent effect on AhR transcriptional activity as it was expected from *in vitro* data [269]. In the latter work, Hollingshead and Perdew demonstrated that endogenous XAP2, despite its low levels of expression, is sufficient for maximal AhR complex occupancy in the liver. Therefore, it appears that the XAP2-mediated enhancement of murine AhR protein levels is limited to *in vitro* cell culture models. Interestingly, studies addressing the function of the XAP2 at endogenous levels in Hepa-1c1c7 cells further revealed that depletion of XAP2 via siRNA treatment does not result in nuclear accumulation of the AhR [270], suggesting that XAP2 plays no role in the localization of the AhR complex, as previously suggested [271]. This among many other published observations makes it difficult to propose a unifying theory about XAP2 function in the cell and how it relates to AhR activity. What appears consistent is the ability of XAP2 to play a repressive role in AhR transcriptional activity, perhaps through inhibition of AhR transformation and heterodimerization with ARNT [268, 270]. Clearly the *in vivo* role of the XAP2 needs to be further assessed through the use of XAP2 knockout models, shRNA methods, or tissue-specific knockouts. These approaches may help elucidate the endogenous function of XAP2 and its suspected involvement in the AhR signal transduction pathway.

p23 – This protein was first identified by Smith et al. 1990 as a subunit of the unliganded progesterone receptor (PR) and was found to be required for the PR to achieve its ligand binding conformation [272, 273]. As such, p23 acts as a cochaperone of the hsp90 and hsc70 proteins during the folding of client proteins. Accordingly, fractionation studies of rabbit reticulocyte p23 and hsp90 indicated that p23, in an hsp90 dependent manner, could restore baculovirus system-expressed AhR and ARNT heterodimer formation, as well as heterodimer DNA-binding, in a ligand-dependent manner [274]. These results are in agreement with the apparent ability of p23 to enhance the ligand-binding ability of the AhR resulting in higher transcriptional activity [275]. Since XAP2 can displace p23 from AhR complexes and cause repression of AhR transcriptional activity [268], perhaps this indicates a pivotal role for p23 in AhR activity by promoting a competent receptor conformation. Interestingly, p23 along with other molecular chaperones have also been implicated in the disassembly of steroid receptor transcriptional complexes, which restores them to their latent unliganded state [276]. However, the molecular switch(es) controlling these opposing roles have not been fully established.

p23 is also known as ‘prostaglandin E synthase 3’ and, together with prostaglandin-endoperoxide H synthase-1, catalyses a step in the synthesis of prostaglandin E2 from arachidonic acid in response to inflammatory signals, through IL1-β-mediated signaling [277]. The association of this protein with the AhR complex is rather intriguing given the connection of arachidonic acid metabolites with the activation

of the AhR [241]. Perhaps this interaction could be associated with endogenous activation and function of the AhR, although only future research can address this theory.

1.9 Synthesis of the ligand-binding competent AhR complex.

The steps leading to the maturation of the AhR protein after its synthesis, as determined by its ability to achieve a ligand-binding state, have not been fully established. However, given its dependence on the hsc70 and hsp90 chaperones to achieve its native state, it is presumed to follow similar protein-folding steps as the better characterized steroid receptors (e.g., androgen and glucocorticoid receptors). Accordingly, the cochaperone hsp40, which stimulates the ATPase of hsc70, (Ydj1 in yeast; DnaJ in bacteria; and HDJ2 in humans) would bind the nascent AhR polypeptide followed by hsp70/hsc70 [278]. The protein HOP then mediates a non-essential but rate-limiting interaction with hsc70 and hsp90 complexes, bringing about the transition of the AhR-hsc70 complex to the AhR-hsp90 [278-280]. The proteins hsc70, hsp40, and HOP then dissociate from the intermediate complex followed by late recruitment of p23 which stabilizes hsp90 in its ATP-dependent client(e.g., AhR)-bound state [281, 282]. These events are thought to drive the formation of the AhR mature complex, also known as the unliganded latent form of the receptor [84, 261]. Given that XAP2 forms a complex with hsp90 in the absence of the AhR, it is presumed that at the time the hsp90 complex is brought to the foldosome, XAP2 may be already in complex with hsp90 [283]. The putative AhR mature complex is primarily composed of two molecules of hsp90, and the

immunophilin-like protein XAP2 [84, 261]. The ability of p23 to interact with the unliganded AhR complex was established by Kaslauskas et al. 1999 [275]. The existence of other proteins in the AhR complex cannot be excluded given that the methods utilized to determine the tetrameric AhR complex (e.g., crosslinking) may only favor the recovery of proteins that can interact tightly with the AhR.

1.10 Overview and Rationale for Research.

An understanding of AhR protein regulation is important given its potential role in the development of the liver, vascular, and immune systems, as well as in the detoxification or activation of endogenous and exogenous chemicals through the regulation of metabolic enzyme genes [284-287]. Despite significant information describing the important biochemical roles that the AhR plays in the cell, the mechanisms of AhR protein-level regulation have yet to be fully established, which may have an impact in its biological role. Notably, the treatment of cells *in vitro* and *in vivo* with TCDD and other high affinity AhR ligands can significantly reduce the half-life of the AhR from 29 h to 3 h, in a process that can be prevented by pretreatment of cells with proteasome inhibitors [288]. Similarly, geldanamycin, a potent hsp90-disrupting molecule, has been shown to induce AhR degradation in a proteasome-dependent manner [289]. Interestingly, in 1999 a protein termed the carboxyl terminus of hsc70-interacting protein (CHIP) was identified in a yeast two-hybrid screen aimed to discover new hsp90 and hsc70 chaperone-interacting proteins [290]. CHIP was later shown to display E3 ubiquitin ligase activity towards several hsp90 client proteins such as the steroid receptors ER, AR, GR, among a growing number of target proteins that included the chaperone hsc70 [291-295]. Given the direct association and dependence of the AhR on the chaperones hsp90 and hsc70 for its stability and preliminary observations of CHIP-

mediated protein turnover of the AhR in cell culture, the following hypothesis was postulated:

> ‘T**he E3 ubiquitin ligase activity of CHIP regulates the AhR protein levels in an ubiquitin and proteasome-dependent manner and it is involved in the ligand mediated degradation of the AhR’**

Chapter two contains an overview of the ubiquitin and proteasome proteolytic system. Chapter three presents published data [296] on the approach utilized to address the primary hypothesis and discusses the possible role of the E3 ubiquitin ligase CHIP in the regulation of the AhR protein complex.

The second project in this thesis was devoted to the characterization of the anti-asthmatic drugs M50354 and M50367. These two drugs have been implicated in an AhR-dependent modulation of the immune system [297]. Interestingly, the treatment of an animal model of atopic asthma with M50354 or M50367 results in the AhR-dependent skewing of T helper cells of the immune system towards a T_H1 phenotype. This effect reduces disease scores of airway hyperresponsiveness and asthma through an inhibition of IgE synthesis and the T_H2 cytokines IL-4 and IL-5 [298, 299]. The therapeutic effects of M50354 and M50367 were suggested to occur with minimal classical AhR-mediated induction of metabolic enzymes such as CYP1A1, a metabolic enzyme whose expression is driven by several dioxin-responsive elements (DREs) in the promoter region of this gene. Therefore, the following hypothesis was formulated:

"**The AhR-dependent immunoregulatory effects of the weak agonists M50354 and M50367 are mediated through the selective modulation of the AhR signal transduction pathway and does not involve classical DRE-driven responses**"

The fourth chapter demonstrates that, in contrast to previously published observations, both M50354 and M50367 are potent AhR ligands with full agonistic activity, especially at therapeutically relevant doses. The implications for these findings are numerous and are discussed in this chapter. The fifth and last chapter contains a summary of the work presented in this thesis along with conclusions and insights for future experimentation.

Chapter 2

The Ubiquitin-Proteasome Pathway

2.1 Introduction: Protein Quality Control and Disease

Protein degradation in the cell is an essential and highly regulated homeostatic process that functions to maintain an equilibrium between protein synthesis and the concomitant removal of damaged or perhaps no longer needed proteins. For example, without the clearing of the cyclin D kinase inhibitor $P27^{Kip1}$, cells would not divide as its removal is required to release them from G1 cell cycle arrest [300, 301]. Similarly most if not all processes in cells are tightly regulated through specialized proteolytic systems. For instance, cathepsins, caspases, and calpains play roles in the production of active cytokines of the immune system [302], to the regulation of apoptosis [303], and of bone remodeling [304], respectively. Since its discovery in 1949 by Christian de Duve [305], the lysosomal pathway was believed to be the predominant route for protein and organelle recycling in the cell. However, a paradigm shift occurred in 1980 when Ciechanover and colleagues identified a non-lysosomal heat-stable ATP-dependent proteolysis factor 1 (APF-1), today recognized as ubiquitin [306-308].

Ubiquitin (Ub) belongs to a multigene family that includes small proteins such as Nedd8 [309] and SUMO [310], among several others. Like many members of its family, Ub through its single or multiple covalent attachment to other proteins carries important functions in protein trafficking, DNA integrity, repair, and transcription through the

modification of histone H2A and H2B [311-313]. Ubiquitin also plays a role in protein quality control through proteasome-mediated degradation [314, 315], and signaling by the direct activation and inactivation of transcription factors such as NF-kB [316-318]. The importance of ubiquitin and the proteasome is highlighted by numerous diseases in which mutated or absent components of the system can lead to serious clinical conditions. Some well characterized examples include the E3 ubiquitin ligase E6-AP in Angelman syndrome [319, 320], the UCHL1 in familial Parkinson disease [321], SUMO4 in inflammatory diseases such as type I diabetes [322], and UBB in Alzheimer's disease [323], all of which have been associated with the aberrant accumulation of active or misfolded proteins.

Interestingly, three Ub genes have been identified in the human genome with coding regions of 600 nt (Ub A), 1000 nt (Ub B), and 2450 nt (Ub C), as well as some Ub 'pseudogenes' [324-326]. Ub A is the most abundant in human tissues and its mRNA structure comprises a 300-bp encoding a carboxyl terminus precursor sequence, a 3' untranslated region and a poly(A) tail [326]. The Ub A mRNA encodes a single ubiquitin. Ub B is the most prevalent form in porcine and consists of a polyprotein containing three ubiquitin coding repeats followed by an extra cysteine codon in its C-terminus [325]. In addition Ub B contains an intron in the 5′ untranslated region. In Ub C the C-terminal ubiquitin repeat contains an additional valyl residue and this transcript is a polyprotein consisting of 9 repeating units of ubiquitin [327]. Polyproteins from Ub B and Ub C are further processed by ubiquitin isopeptide bond hydrolases (isopeptidases) that cleave the

Gly-Met bond linking these units as well as the Gly-Val bond of the C-terminal repeat [327].

2.2 Proteasome-mediated protein degradation: The 20S and 19S regulatory complexes.

The eukaryotic proteasome, as first coined in 1988 [328], is a large 26S complex composed primarily of a 20S catalytic core and a 19S regulatory complex. The protease was initially isolated by Hough et al 1986 from rabbit reticulocytes as an ATP-dependent proteolytic complex [329]. Crystal structure analysis shows that the 20S core particle is assembled into four stacked heptameric rings, with outer rings composed of 7 unique structural α subunits while the inner two rings contain seven distinct β subunits. Of these seven β subunits, three are catalytically active (e.g., β1, β2, and β5) [330-332]. The 20S catalytic core is dynamic in nature. For example, some β subunits are dynamically interchanged with β1i, β2i, and β5i in response to interferon-γ stimuli [333]. This molecular swap converts *de novo* assembled 'immuno' proteasomes into antigen processing machines [334]. While the number of examples describing the dynamic nature of the proteasome catalytic units is currently limited, more signals controlling their composition are likely to be discovered in future research.

Substrate specificity at the proteasome level is primarily accomplished by the 19S regulator, also termed the PA700 complex, cap, or μ particle [335]. This regulatory complex can be subdivided into a base and a lid complex. At any given time, the 20S core may be associated with one or two 19S regulatory subunits at either or both extremes of the core particle, providing gated entrance into the catalytically active chamber of the 20S particle. The base of the 19S particle contains 6 ATPase subunits termed S1/Rpn2, S2/Rpn1, S4/Rpt2, S6′/Rpt5, S7/Rpt1, S8/Rpt6, S10b/Rpt4, and the widely recognized S5a/Rpn10 (a receptor for Lys48 linked ubiquitin chains [336]), along with three non-ATPase subunits (e.g., Rpn1, Rpn2, and Rpn10). Receptors for ubiquitinated proteins, such as the S5a, recognize hydrophobic patches on Lys48-linked ubiquitin molecules [337, 338]. Once the ubiquitinated substrate associates with the 19S complex, the ATPases located at the base, in a manner analogous to molecular chaperones, are capable of unfolding substrates and channeling them into the proteolytic chamber of the 20S proteasome [339]. In addition, the 19S complex ATPases are thought to stabilize the interactions between the base and the 20S core particle [340]. Interestingly, in the absence of the lid complex, the base of the 19S particle is able to process some proteins without their preconjugation to ubiquitin [340]. However, the lid complex proteins are still required for full processing or editing of ubiquitinated substrates through their associated isopeptidase activity [341]. The PA28 regulatory cap may substitute the constitutive 19S cap complex, or associate simultaneously with the proteasome on alternate ends and it is thought to provide more control over substrate recognition and processing, such as the processing of antigenic peptides in immune cells [342]. Another protein complex with similar structure and function is the COP9

signalosome [343]. COP9 shares homology with the 19S regulatory particle lid and the eukaryotic translation initiation factor 3 through their conserved PCI [344] and MPN domains [345, 346]. The COP9 signalosome associates with the proteasome and is known to interact with multiprotein E3 ubiquitin ligase complexes (e.g., SCF) and catalyze the removal of Nedd8 from Cul1, which results in their inactivation [347]. In summary, the role of the constitutive 19S cap, PA28, and COP9 signalosome is to provide a level of specificity for substrate processing, editing, and degradation by the 20S catalytic particle.

As substrates are degraded by the proteasome, ubiquitin molecules are recycled back into the cellular pool for their reuse. This task is accomplished by ubiquitin carboxypeptidase hydrolases that are associated with the 19S and COP9 complexes. These act by shortening the ubiquitin chains starting at their distal end or hydrolyze ubiquitin chains or subunits from proteins after they are partially degraded by the proteasome [341, 348, 349]. The interaction between all these cellular control systems ensures that proteins are selectively degraded. As discussed in the next section, since ubiquitin can be linked to itself through many of its lysyl residues to form polyubiquitin chains of different architecture, it has been theorized that cells utilize Ub chain structure as signals to determine whether a protein should be degraded or perhaps carry other important functions.

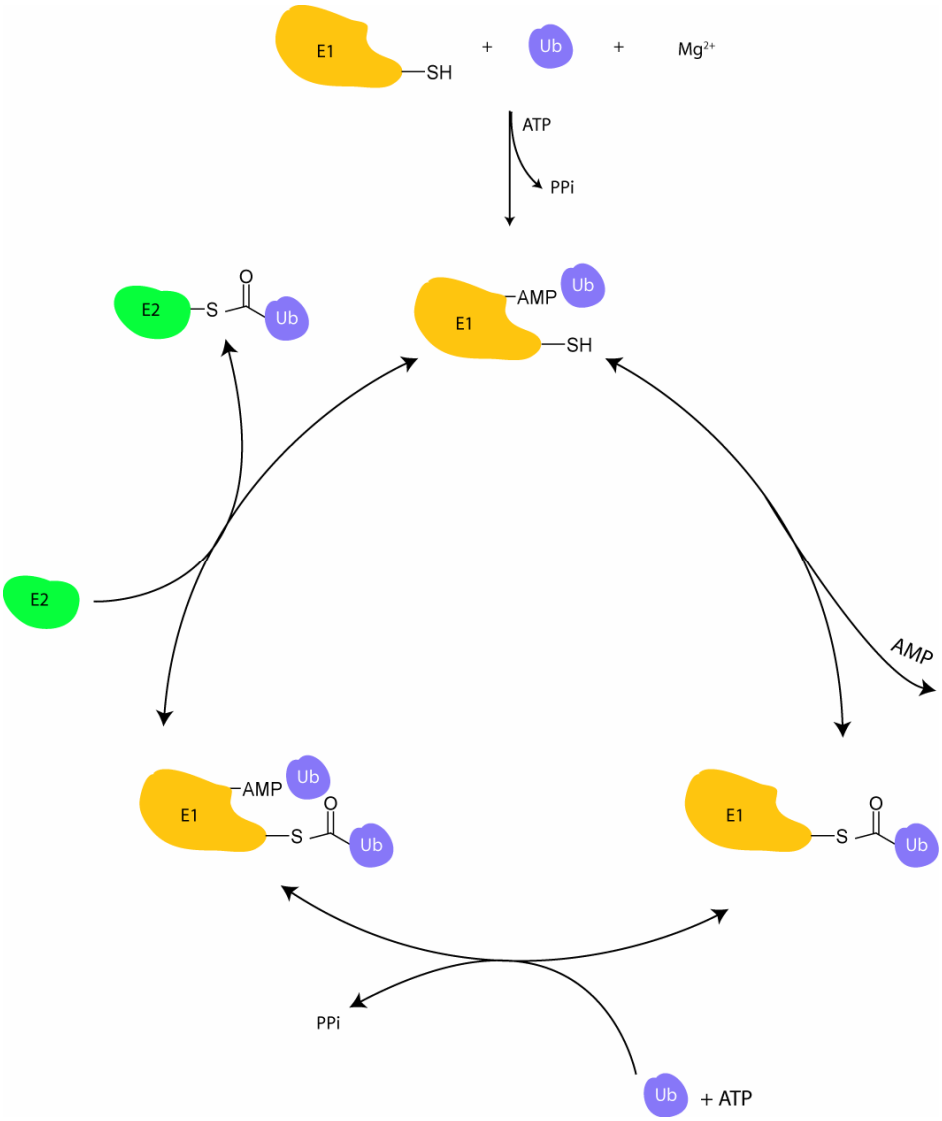
2.3 The Ubiquitin-Conjugation System

Protein ubiquitination is a highly regulated process. In the first step, an ubiquitin-activating enzyme (termed E1) forms a thiol-ester bond with the carboxyl terminus of ubiquitin in an ATP-dependent manner [350, 351] (Figure 2-1), followed by ubiquitin transfer to a conjugating enzyme (E2) [352]. E2s then transfer ubiquitin to substrates by themselves or with the assistance of additional substrate-specificity factors termed ubiquitin ligases (E3) [352, 353] (Figure 2-2). Therefore, an isopeptide bond occurs between the carboxyl terminus of ubiquitin and lysyl residue(s) in the target protein. It has been demonstrated that E3s can also serve as proximal ubiquitin donors (e.g., HECT domain ligases such as E6-AP) or may simply affect the fold of a target protein so that lysyl residues are more readily accessible by E2s (RING domain family of E3s) [354]. A fourth polyubiquitination factor termed E4 has also been described (e.g., Ufd2) [355]. The E4 family is thought to play a role in the assembly of polyubiquitin chains by assisting E3s. However, E4s remain somewhat ill defined [356], especially when some 'E3 ubiquitin ligases', such as the carboxyl terminus of hsc70-interacting protein (described in chapter 3), have been associated with both E3 and E4 activities [357]. However, it is possible that the cooperation between all these ubiquitin ligases may form

dynamic ubiquitination complexes that adapt to numerous substrates and changing cellular conditions. Due to the considerably higher number of E3 ubiquitin ligases in comparison to E2s [358], ubiquitination specificity is primarily achieved by E3s which act as "recognins" bridging the interaction of target substrates with E2s or participating themselves in the conjugation reaction as with the HECT domain family of E3 ligases. For example the E2 UbcH7 has been shown to partner with the E3 ligases Cbl [359], HHARI [360], and E6AP [361], whereas UbcH5 has been shown to associate with the E3 ligases Nedd4 [362] and CHIP [291], among others. Ubiquitination specificity is also achieved through cellular compartmentalization of the E2s and E3s as well as the presence of accessible motifs in target substrates that are recognized by the ubiquitin conjugating system (e.g., KEEE motif) [363]. There appears to be a preference for ubiquitination at loops followed by alpha helices [363]. In general, in a di-lysine sequence, the second lysine is more frequently ubiquitinated and the residue cysteine tends to be excluded from ubiquitination site flanking regions, presumably due to its reactivity and potential interference in the catalysis of isopeptide bond formation [363].

Polyubiquitin chains can be linked through different lysyl residues in ubiquitin such as K6 [364], K11 [365], K29 [366], K48 (canonical; or putative degradation signal) [314, 367], and/or K63 [368]. As such, polyubiquitin chain linkage is thought to convey a particular signal that determines whether a protein is intended for degradation (e.g., K48 linked polyubiquitin chains), an alteration in its trafficking behavior [369, 370], or even its activation as seen with the transcription factors NF-κB, SPT23, and MGA2 [371-374]. Although commonly believed that Lys48 linked polyubiquitin chains are the minimal

signal for degradation through the proteasome [375], certainly some exceptions exist as with the regulation of the transcription factor Met4 [376]. Interestingly, Met4 has an ubiquitin binding domain that binds the nascent Lys48 linked polyubiquitin chain preventing further chain elongation and its interaction with the proteasome ubiquitin receptors. Therefore, Met4 is not degraded through the proteasome but it is rather maintained in an inactive state through polyubiquitnation [377].



2.1: **Schematic of the proposed mechanism for the activation of ubiquitin by the ubiquitin-activating enzyme** (E1). Adapted from [351].

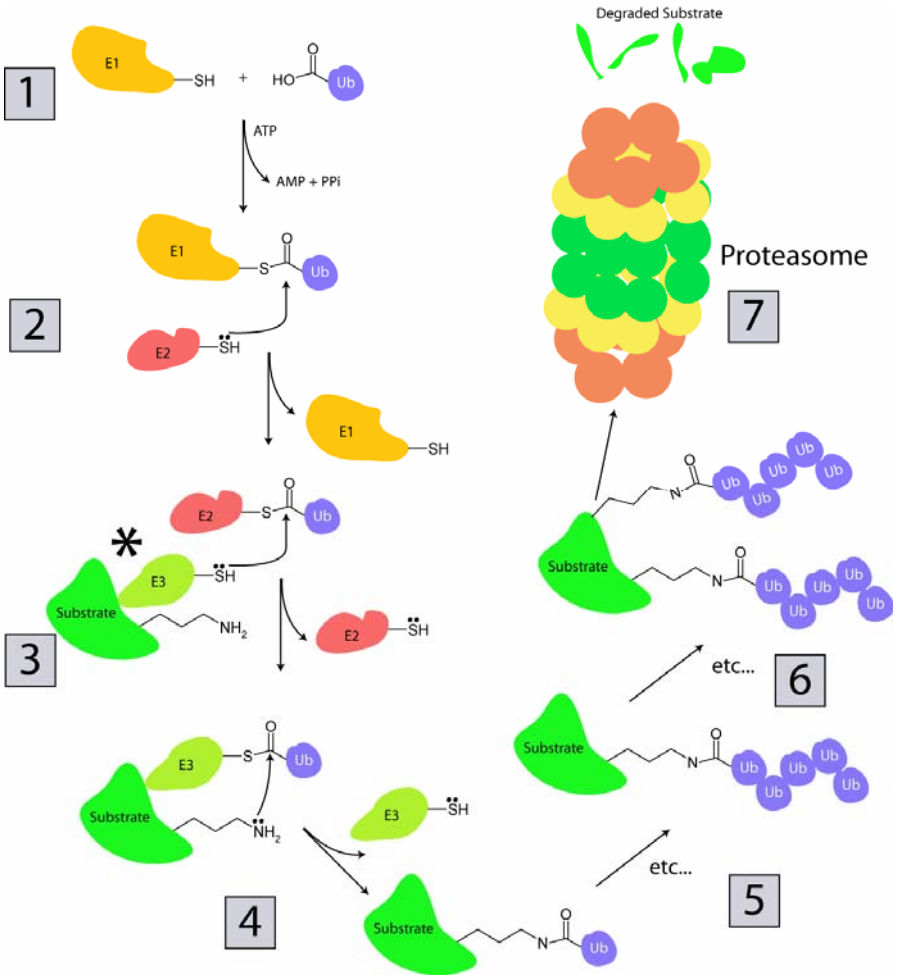


Figure 2.2: **Ubiquitin-dependent proteasome degradation of proteins**. After the activation of ubiquitin (Ub) by E1 [1], the Ub moiety is transferred to an E2 [2] and then to a HECT domain E3 [3]. Some ubiquitin ligases containing the Homologous to E6AP C-Terminus (HECT) domain can be proximal donors of ubiquitin to target substrates [378]. However*, some E3s (e.g., RING domain) may simply serve as substrate specificity factors [379, 380] for E2-mediated ubiquitin transfer (not shown in scheme). After a substrate has been ubiquitinated [4], this process may continue until a polyubiquitin chain is formed at one [5] or more lysyl residues in the substrate [6]. Polyubiquitin chains can be assembled through lysyl residues in ubiquitin itself. An ubiquitin chain of 4 or more units linked through Ub-Lys48 is the suggested minimal signal [375] for proteasome-mediated proteolysis [7].

As discussed in the next chapter, an ubiquitin ligase system responsible for the ligand-induced AhR protein degradation or the maintenance of its steady state levels has not been identified to date, despite strong evidence suggesting its existence. We present first-time direct evidence that the hsc70-associated U-box domain E3 ligase CHIP is capable of mediating AhR and hsp90 ubiquitination and degradation through the proteasome [296]. A potential role for CHIP in the regulation of the AhR and the hsp90 protein levels is discussed ahead.

Chapter 3

CHIP can remodel mature AhR complexes and mediate ubiquitination of both the AhR and the hsp90 *in vitro*.

3.1 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 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, as it did not affect well characterized activities of the AhR nor affected its steady-state protein levels. However, the presence of potential compensatory mechanisms may be confounding this particular observation. Our results suggests a model where the E3 ubiquitin ligase CHIP cooperates with other ubiquitination factors to remodel native AhR-hsp90 complexes and that co-chaperones such as the XAP2 may affect the ability of CHIP to target AhR complexes for ubiquitination.

3.2 Introduction

The AhR is a member of the basic helix-loop-helix PER-ARNT-SIM family and a cytosolic ligand activated transcription factor [381]. 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) [382]. Some reports have also indicated that endogenous and dietary substances can directly activate the AhR [383, 384], although a putative endogenous ligand for the AhR remains unidentified. The mature unliganded AhR complex is primarily composed of a homodimer of hsp90 and at least one molecule of the immunophilin-like protein XAP2 and/or p23 [261, 262, 385, 386]. 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 [383].

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 in primordial follicles, respectively [387, 388]. As a result, the regulation of the AhR protein levels has been the subject of several important studies, as 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 GA [288, 289, 389]. Several findings

have implicated the 26 S proteasome in the ligand and geldanamycin mediated turnover of the AhR, while no direct evidence of AhR ubiquitination has been published. A possible role for the E3 ubiquitin ligase CHIP in the regulation of AhR protein levels has been debated in recent studies [390, 391]. 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 [250]. However, this report did not reconcile observations previously made in ts20 cells expressing a temperature sensitive mutant of the ubiquitin activating enzyme [288]. Therefore, it appears that the AhR may be targeted by both proteolytic pathways.

The 90 kDa heat shock protein (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 [392, 393]. Briefly, *de novo* synthesized hsp90 client proteins are rapidly engaged by the hsc70 and co-chaperone hsp40 in an ATP dependent fashion [394]. As the client continues to fold, the hsc70-client complex interacts with the hsp90 with 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 [395]. 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 [264]. However, the time at which XAP2 joins the AhR complex and its true role remains under investigation [267, 268, 270]. Hsp90 has also been associated with the degradation of some client proteins such as the apoprotein B [396].

The carboxyl terminus of hsc70-interacting protein (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 an ubiquitin and proteasome dependent manner [397]. Some known CHIP protein targets are the GR [290] and mutant p53 [398], in addition to the hsp70 and the hsp90 chaperone proteins themselves, under stressful conditions [291, 295]. CHIP contains a U-box domain homologous to the yeast Ufd2 protein that is thought to participate in the assembly of polyubiquitin chains [356]. The U-box domain is also required for the recruitment of the UBC5 family of ubiquitin conjugating enzymes in the ubiquitination of substrates [291]. 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 [399, 400]. 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 enzyme termed E4.

In a previous report, the ubiquitin ligase CHIP was associated with the turnover of hsp70 and hsp90 protein levels [295]. We now demonstrate through the use of sucrose gradient fractionation methods and *in vitro* experiments with purified components that CHIP can directly mediate both multi-site 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.

3.3 Materials and Methods

3.3.1 Source of Mammalian Expression Constructs.

The pcDNA3/βmAhR and pCI/hAhR-FLAG were generated previously [261]. The pcDNA3/CHIP, pcDNA3/CHIP-*myc*, pcDNA3/CHIPΔE4 (CHIPΔU-box), and pcDNA3/CHIPΔTPR were kindly provided by Cam Patterson (University of North Carolina, Chapel Hill, NC) [291, 401, 402]. The pCI/XAP2 and pCI/G272D-XAP2 were previously generated [261, 268].

3.3.2 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, CHIPΔU-box, and CHIPΔTPR in E. coli, respectively. The human proteins hsp70 and hsp90-α used for *in vitro* ubiquitination assays were purchased from Stressgen Bioreagents (British Columbia, Canada).

3.3.3 Cell culture conditions.

Cells were routinely grown at 37°C and 5% CO_2 in modified Eagle's α-minimum essential medium (Sigma, St. Louis, MO), supplemented with 1000 units/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO), and 8% fetal bovine serum (HyClone, Logan, UT).

3.3.4 Western Blotting.

Proteins were transferred from 6-8% polyacrylamide gels to 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-α) polyclonal rabbit antibodies were generated previously [45]; and the anti-hsp70 MAb and affinity purified rabbit anti-CHIP polyclonal antibodies were purchased from Affinity Bioreagents (Golden, CO); anti-CHIP rabbit polyclonal used in Figure-3.1B and Figure-3.3 were generated previously and a generous gift from C. Patterson (University of North Carolina, Chapel Hill, NC) Ballinger et al. 1999). Proteins were visualized by autoradiography using radioactive ^{125}I-goat anti-rabbit or anti-mouse antibodies, the combination of biotin-conjugated secondary antibodies with

radioactive streptavidin, or by enhanced chemiluminescence using horseradish peroxidase conjugated secondary antibodies, as indicated in the figure legends. The quantification of proteins was performed by phosphor image analysis of radioactive blots.

3.3.5 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 pre-bound to 40 μl of Protein-A resin (Pierce, Rockford, IL) 80 μl 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 500 μl/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, 4 mM EDTA] containing 1% NP-40 and a protease inhibitor cocktail P8340 (Sigma, St. Louis, MO). Cells from up to 2 x 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 100,000g for 45 min at 4°C. The supernatant was collected and a total of 200 μg 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/Rb-IgG resin and incubated at 4°C for 1 h on a platform rocker. The resin was then centrifuged at 1,000g and washed 4 times with MENG buffer containing 150 mM NaCl, followed by once with MENG buffer

alone. The immunoprecipitated proteins were then heated in 2x SDS sample buffer at 95°C for 5 min and separated via 8% TSDS-PAGE [403]. Proteins were visualized by autoradiography using biotin-conjugated secondary antibodies and ^{125}I-streptavidin.

3.3.6 Immunoprecipitation of CHIP-myc from COS-1 cells.

COS-1 cells propagated on 100 mm dishes were transfected with 6 μg pCI/hAhR-FLAG and 3 μg of pcDNA3 (Invitrogen, Carlsbad, CA) or 6 μg of pCI/hAhR-FLAG and 3 μg of pcDNA3/CHIP-*myc* using Lipofectamine™ and Plus™ reagents (Invitrogen), according to manufacturer's instructions. The cells were harvested 24 h post-transfection by trypsinization. Protein extracts were prepared in MENGM buffer (MENG + 20 mM molybdate) + protease inhibitors and 40 strokes in a stainless steel Dounce homogenizer. A total of 500 μg of cell lysate obtained from cells expressing hAhR-FLAG alone or CHIP-*myc* and hAhR-FLAG were independently combined with 80 μL 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, washed 6 times in MENGM with 50 mM NaCl, and twice with MENGM. The immunoprecipitates were combined with 2x 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.

3.3.7 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 non-targeting siRNA #1 (D-001210-01; siCONTROL) using DharmaFECT 4 transfection reagent according to manufacturer's instructions. 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 pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton-X, 0.1% deoxycholate, and 140 mM NaCl. Proteins were resolved by 8% TSDS-PAGE, transferred to PVDF membranes. Protein was detected by autoradiography using biotin-conjugate secondary antibodies and ^{125}I-streptavidin.

3.3.8 Real-time quantitative PCR.

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 manufacturer's instructions. 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 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′.

3.3.9 Transient CHIP and AhR expression experiments.

COS-1 cells at 90% confluence in 60 mm dishes were transfected with 0.25 μg, 0.5 μg, or 1.0 μg of pcDNA3/CHIP along with 1 μg of pcDNA3/βmAhR using the Lipofectamine™ and Plus™ transfection reagents and using manufacturer's instructions (Invitrogen, Carlsbad, CA). For the pcDNA3/CHIPΔU-box and pcDNA3/CHIPΔTPR constructs a total of 1.0 μg was transfected. The total amount of DNA transfected was normalized to 3 μg using pcDNA3, where necessary. The cells were washed 18-20 h post-transfection twice with PBS at 25°C and harvested by trypsinization, collected into 1.5 ml tubes and centrifuged at 100g for 3 min. Cell pellets were each resuspended in 100 μl 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 14,000g for 15 min and the supernatants transferred to a fresh tube. Protein extracts were quantified and a total of 80 μg of protein per sample was combined with 2x gel loading buffer and analyzed by 8%

TSDS-PAGE. Proteins were detected by autoradiography using biotin-conjugate secondary antibodies and ^{125}I-streptavidin.

3.3.10 Sucrose density fractionation of in vitro translated proteins.

In vitro translations were performed using the TNT Quick Coupled Transcription/Translation System following manufacturer's instructions (Promega, Madison, Wisconsin). Briefly, two hundred microliters of *in vitro* translated AhR in the presence of ^{35}S-methionine or methionine were diluted to 300 µl with M/N buffer (MENG buffer without glycerol and EDTA) and subjected to an M/N buffer 10-30% sucrose density-gradient analysis, as previously described [404]. A total of 25 x 200 µl fractions were collected and 10 µl from each fraction were combined with 2x SDS sample buffer, heated at 95°C for 5 min, and resolved by 6% TSDS-PAGE. Proteins were transferred to 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.

3.3.11 *In vitro* ubiquitination assays.

In vitro translated AhR or XAP2 proteins in the presence or absence of ^{35}S-methionine were treated with 50 µg/ml cycloheximide, 100 µM MG-132, and 3 µM ubiquitin aldehyde before their use in reactions. One to two micro-liters of *in vitro*

translated protein or 5-9 μL of sucrose gradient fractionated AhR were used for individual ubiquitination assays. The final concentrations in 20 μl reactions for the CHIP ubiquitination cocktail included 100 nM E1, 1 μM UbcH5a or UbcH5a-DM, 350 nM CHIP, CHIPΔTPR or CHIPΔU-box, 1x 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 μM 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 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, Michigan) 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°C for 1h. The reaction buffer (50 mM HEPES at pH 8.0) was used to normalize reaction volumes and to dilute commercial proteins, where applicable. For reactions carried in the presence of XAP2 or G272D-XAP2, whole *in vitro* translated AhR and XAP2 were combined at the ratio of 1:3 (3:9 μL) and pre-incubated on ice for 10 min before performing the ubiquitination reactions. Reactions were quenched with an equal volume of 2x SDS sample buffer and heated at 95°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}S-methionine labeled proteins were visualized by autoradiography and quantified by phosphor image analysis.

3.3.12 Photo-affinity ligand binding assay.

The sucrose gradient fraction containing the 9 S AhR (non-radioactive) (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 µl with MENGM and transferred to borosilicate glass tubes. A total of 500,000 cpm 2-azido-3-[125I]iodo-7,8-dibromodibenzo-*p*-dioxin was added to the diluted samples and incubated at room temperature for 30 min, followed by 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°C and exposed to 15-W UV lamps (>302 nm) at 8 cm for 4 min. Supernatants were then combined with 2x SDS sample buffer and heated at 95°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.

3.4 Results

3.4.1 CHIP can interact with the AhR at endogenous levels.

The observation that the AhR protein is highly sensitive to hsp90 disruption [289, 405], in addition to the relationship 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 3.1**).

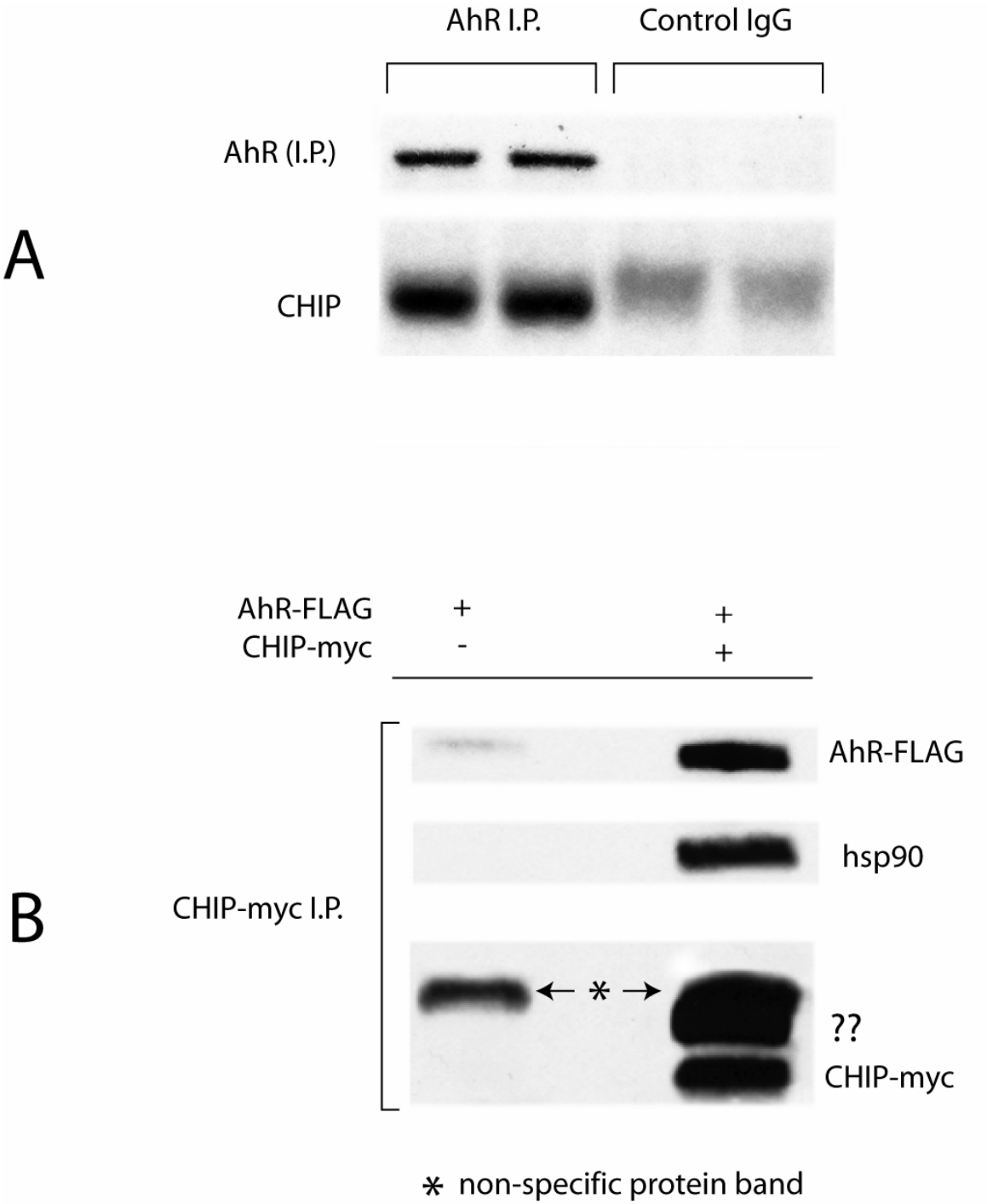


Figure 3.1: **Interaction of endogenous Hepa-1c1c7 AhR with CHIP.**

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

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 background. We also verified if the immunoprecipitation of CHIP could pull down the AhR. However, due to 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-3.1B) when transiently expressed in COS-1 cells, similar to previous observations [391]. 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 [391]. This is due to its lower than expected molecular weight shift and the lack of reactivity to anti-ubiquitin antibodies (data not shown).

3.4.2 Evaluation of AhR protein levels and activity in Hepa-1c1c7 cells following the silencing of CHIP by small interfering RNA.

After establishing 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 3-2A). In addition, the hsp90 protein levels remained unaffected by CHIP downregulation, in agreement with previously published results [390].

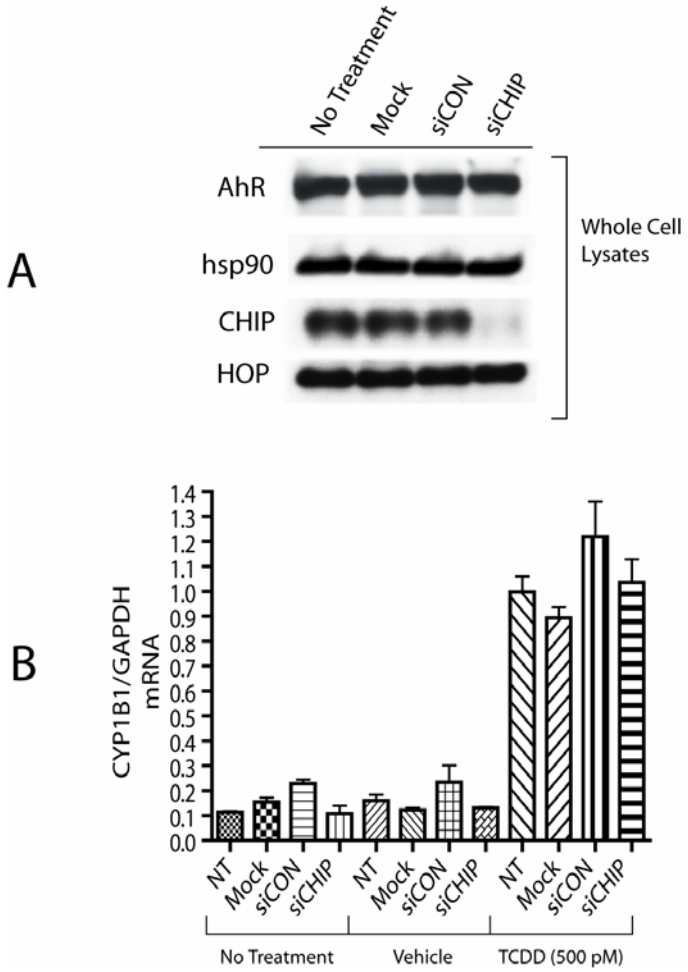


Figure 3-2: **Silencing of CHIP expression in Hepa-1c1c7 and evaluation of AhR transcriptional activity.**

(A) Exponentially growing Hepa-1c1c7 cells were transfected, according to manufacturer instructions (Dharmacon), in 6-well plates with a non-targeting or a targeting siRNA depicted as siCON or siCHIP, respectively. A mock transfection with Dharmafect-4 alone was used to assess for non-specific effects. Whole cell lysates were prepared in RIPA buffer supplemented with protease inhibitors. A total of 40 μg protein per sample was resolved by TSDS-PAGE analysis and visualized as in Figure-3.1A. These results are representative of three independent experiments. (B) Hepa-1c1c7 cells growing on 6-well plates were transfected with a control non-targeting siRNA (siCON) or siCHIP using manufacturer's instructions. After 48 h, cells were treated with vehicle control (DMSO) or treated with 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 3 independent experiments.

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 [390]. 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 cells. The silencing of CHIP had no impact in the transcriptional activity of the AhR (Figure-3.2B). 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 report [390], 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.

3.4.3 CHIP and CHIPΔU-box can equally mediate AhR turnover when transiently expressed in COS-1 cells.

In order 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 3-3) [391]. Unexpectedly, the CHIPΔU-box, which is unable to interact with its cognate ubiquitin conjugating enzyme UBC5 (a,b, and c isoforms) [291] 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 CHIPΔU-box was unable to mediate turnover of the AhR [391] or the dominant-negative effect observed on the cystic-fibrosis transmembrane-conductance regulator [406]. The blot was also probed for HOP to show that the CHIP mediated effect was not global in nature. Lastly, the CHIPΔTPR 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 CHIPΔTPR. This band migrates at the expected molecular weight for CHIP. It is likely that CHIPΔTPR is leading to induction of endogenous CHIP in COS-1 cells by an uncharacterized mechanism. It is important to indicate that CHIP expression in COS-1 cells does not appear to affect the steady-state levels of hsp90 (data not shown and [401]). At this point it was difficult to assess whether CHIP is capable of

directly ubiquitinating the AhR or that it may simply promote AhR degradation by influencing hsp90 function such as influencing the ATP binding state [290]. Due to the complexity of the ubiquitin and proteasome pathways it was necessary to differentiate between the above two possibilities using an *in vitro* approach.

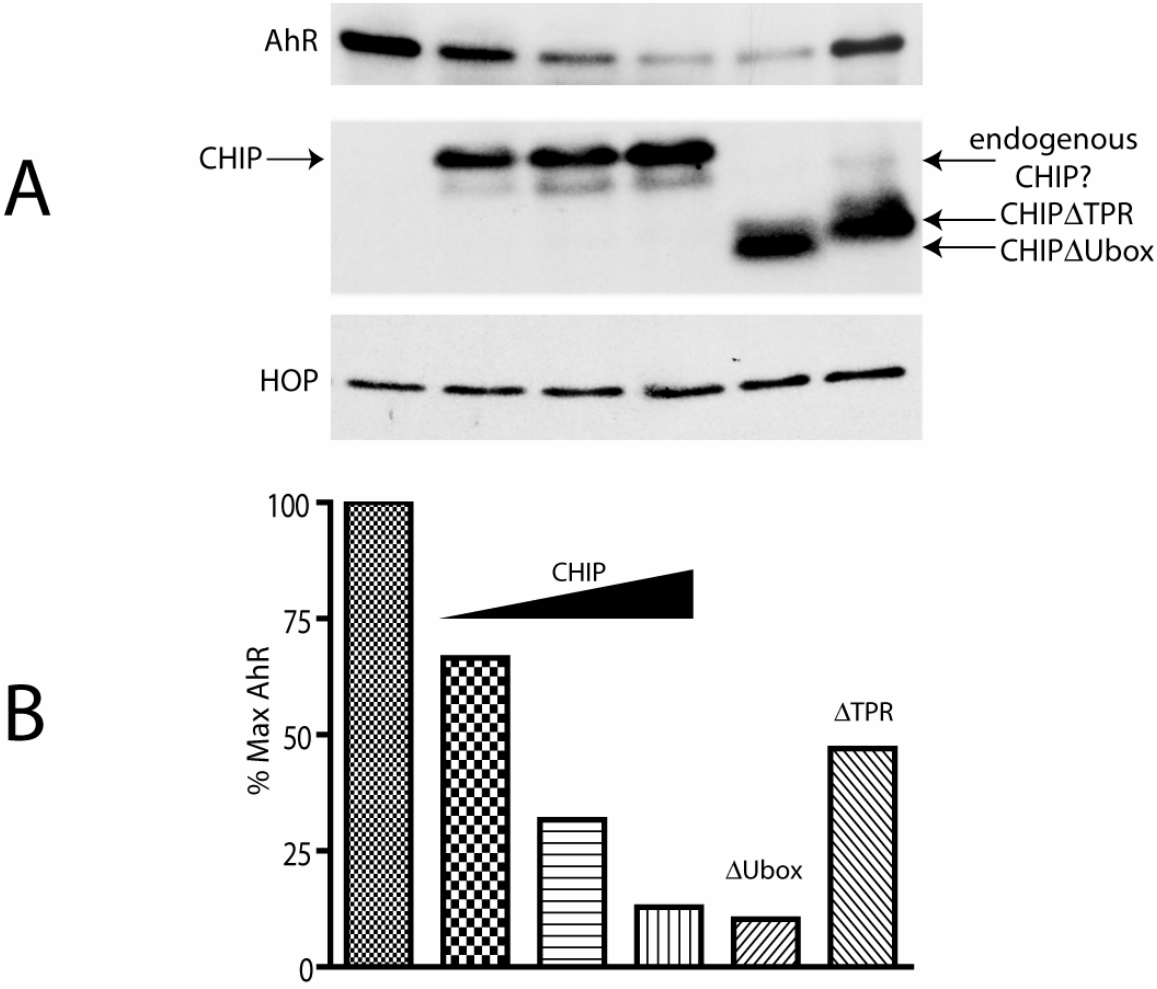


Figure **3-3**: **CHIP and CHIPΔU-box overexpression in COS-1 promotes AhR turnover.**

A, COS-1 cells propagated on 60 mm dishes were transfected with 1 μg of pcDNA3/βmAhR and increasing amounts of pcDNA3/CHIP (0.25, 0.50, and 1.0 μg) and pcDNA3/CHIPΔU-box (1 μg) or pcDNA3/CHIPΔTPR (1 μg). Whole cell lysates were prepared 24 h post transfection in RIPA buffer and proteins resolved and analyzed as in Figure-3.1A. B, The AhR protein bands, shown in A, were quantified by phosphor-image analysis and expressed as percentage maximum of the control treatment (AhR expressed in COS-1 cells by itself).

3.4.4 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 [390]. 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 unfractionated *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}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 [404]. A representative sucrose gradient was analyzed by TSDS-PAGE to assess the distribution of the AhR in the fractions collected (Figure 3-4A). In order to demonstrate that the AhR complex is in a competent ligand binding state, it was necessary to employ the use of the AhR photo-affinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin. Sucrose gradient fractionation of *in vitro* translated AhR in the absence of ^{35}S-methionine was performed as in Figure-3.4A 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-3.4C for details), the ability of the AhR to bind ligand was severely impaired (Figure-3.4B). 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 with the 9 S ^{35}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-3.4C). 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 CHIPΔU-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-3.3). However, as such we expected a level of ubiquitination comparable to full length CHIP, given that the CHIPΔU-box was fully capable of promoting AhR degradation in COS-1 cells.

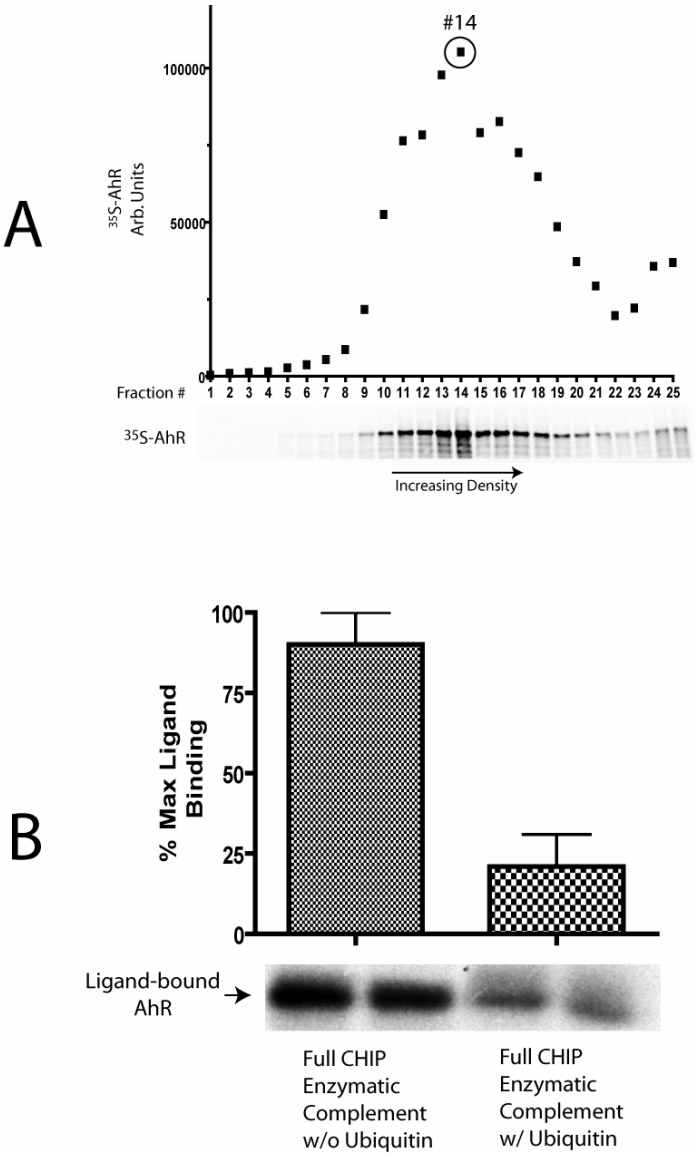


Figure 3-4: **CHIP can mediate ubiquitination of the AhR *in vitro*.**

(A) AhR *in vitro* translated in the presence of ^{35}S-methionine was subjected to a 10-30% sucrose density-gradient analysis as described in the *Experimental Procedures*. A total of 25 x 200 µl fractions were collected. Ten microliters from each fraction were combined with 2x SDS-PAGE sample buffer and resolved by 6% TSDS-PAGE to assess 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) Photo-affinity labeling of the 9 S AhR with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin (see Experimental Procedures for details) was performed after *in vitro* CHIP-mediated ubiquitination of the AhR in the presence or absence of ubiquitin at 30°C for 1 h. Error bars denote the standard deviation.

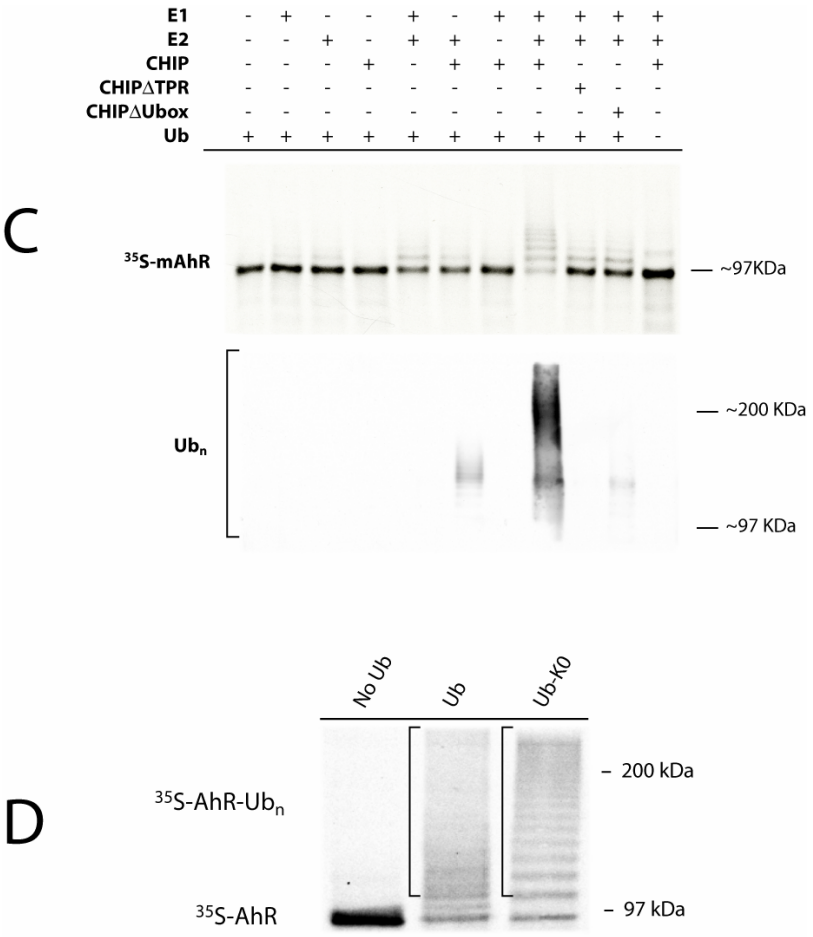


Figure 3-4 (continued) **CHIP can mediate ubiquitination of the AhR *in vitro*.**

(C) As in B, *in vitro* ubiquitination reactions were carried with the AhR from fraction 14, containing the 9 S AhR unliganded receptor [404]. Reactions were incubated for 1 h at 30°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 µl of Protein-A resin with pre-bound rabbit anti-AhR antibody. Immunoprecipitated complexes were then washed 4 times with MENG buffer (MENG, 20 mM molybdate, 150 mM NaCl) and finally combined with 2x 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 *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 as in B. However, the whole reaction products were loaded on a gel and analyzed as in A.

Having 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 (#14) in the presence of the ubiquitin mutant Ub-K0, in order to estimate the number of lysyl residues on the AhR being targeted for ubiquitination. The Ub-K0 mutant is quite useful since 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-3.4D), 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 since 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 [404].

3.4.5 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 [391]. 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 non-fractionated *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 minutes 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 [268]. In brief, the G272D-XAP2 TPR mutant 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 *trans* mechanism. Consistent with this notion and the proposed ability of the XAP2 protein to partially protect the AhR from ubiquitination [264], the XAP2 was capable of protecting a subset of the AhR from CHIP mediated ubiquitination *in vitro* (Figure **3-5**). From three independent experimental replicates the mean non-ubiquitinated AhR values were significantly higher in the presence of XAP2 than reactions carried 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 [407]. Therefore, the magnitude of XAP2 and the AhR expression may vary slightly between reticulocyte lots due to differences in translation efficiencies. Furthermore, the level of XAP2 expression is likely not saturating under these conditions, since the expression of XAP2 in cells has been shown to completely prevent CHIP mediated degradation of the AhR [391].

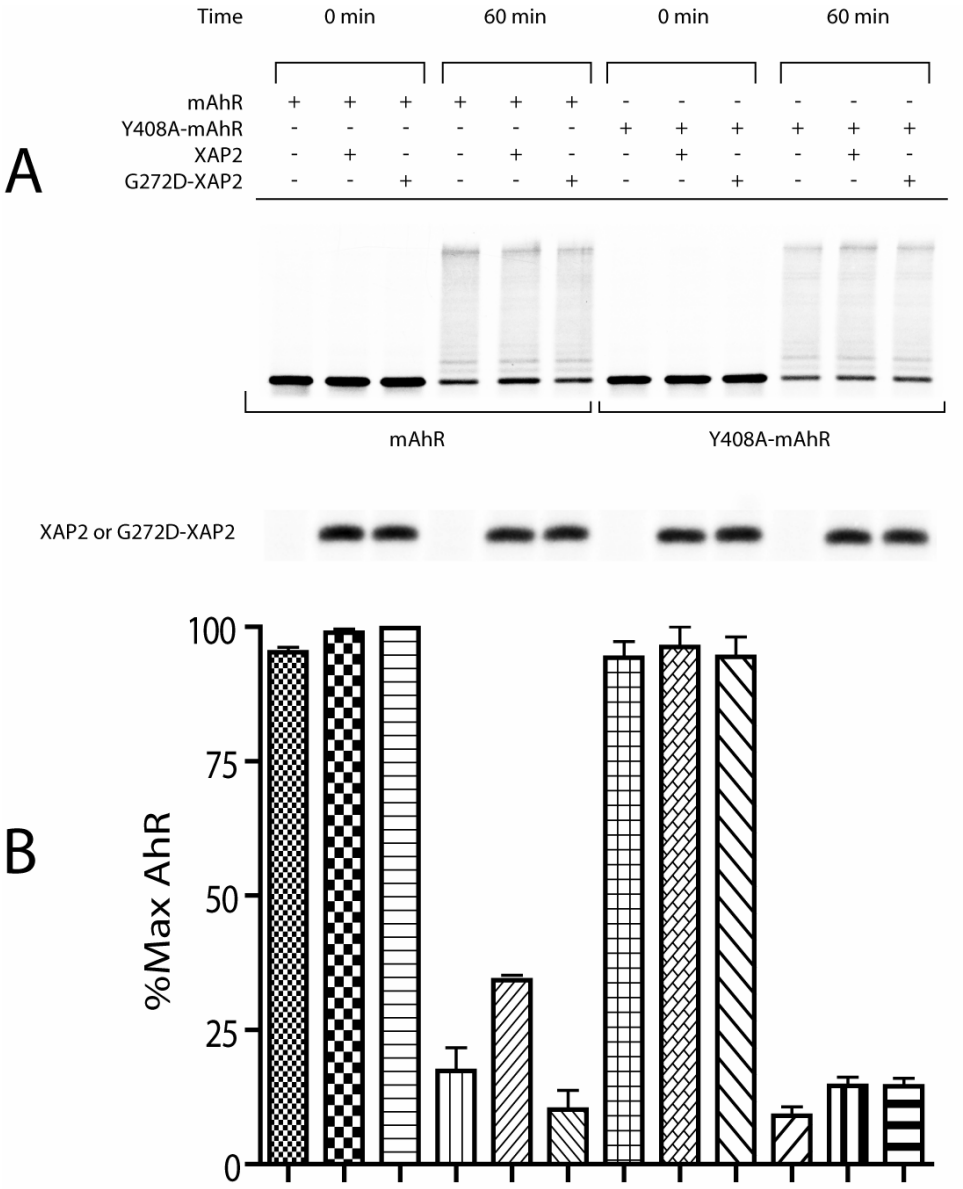


Figure **3-5**: **XAP2 can partially protect the AhR protein from CHIP-UbcH5a mediated ubiquitination.**

(A) Whole *in vitro* translated AhR or Y408A-AhR in the presence of ^{35}S-methionine was used for CHIP-UbcH5a dependent ubiquitination reactions in the presence or absence of radiolabeled XAP2 or G272D-XAP2. Reactions carried were carried at 30°C for 1 h. As a control, reactions in the absence of XAP2 received equal amounts of reticulocyte lysate that had pcDNA3 empty vector used during translation. Reactions were quenched in 2x SDS-PAGE sample buffer and analyzed as in Figure-3.4C. (B) The 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 of G272D-XAP2 (*t*-test p = 0.0014; n=3). Error bars denote standard deviation.

3.4.6 Identification of hsp90 as a direct target for CHIP mediated ubiquitination *in vitro*.

Several <9 S AhR fractions (fractions 10-13; Figure-3.4A) were also evaluated for CHIP mediated ubiquitination of the AhR (data not shown). However, these fractions displayed a high level of ubiquitinating and non-specific 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-3.4A for details) was highly resistant to CHIP mediated ubiquitination (Figure **3-6** A). 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), as it is also a confirmed direct target of CHIP mediated ubiquitination [295]. 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 article indicated that the hsp90-α appeared to be regulated by CHIP, although no direct evidence of CHIP mediated ubiquitination was shown [295]. 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 hsp90 signal also occurred in the absence of E1. However, this is consistent with the modest presence of the ubiquitin activating enzyme in RRL [408] 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 due to some other unanticipated effect *in vitro*.

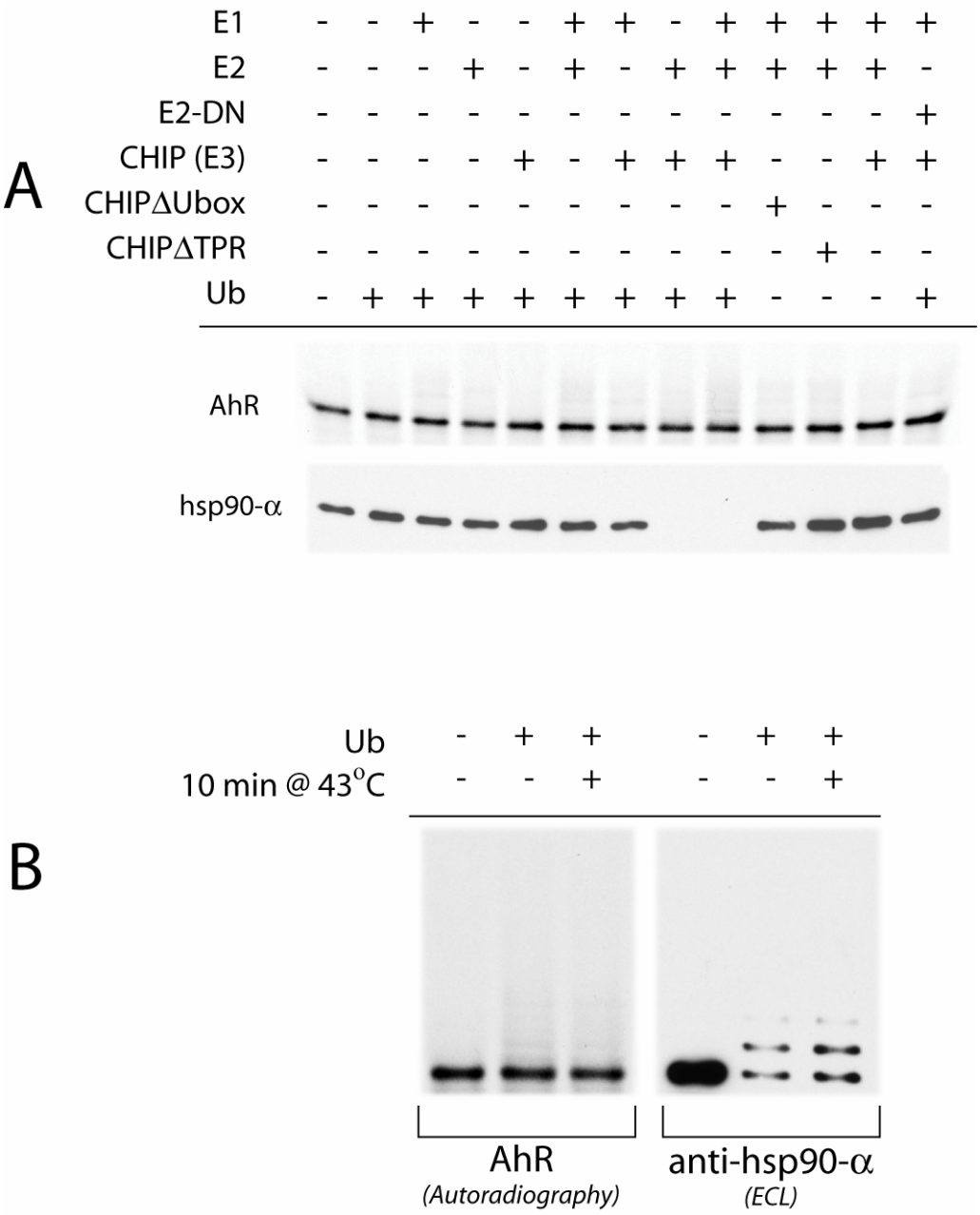


Figure 3-6: **CHIP cannot mediate >9 S AhR ubiquitination yet targets hsp90-α for ubiquitination**.

(A) Ubiquitination reactions were carried and analyzed as in Figure-3.4B 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, CHIP, and in the presence or absence of ubiquitin. Samples containing the >9 S AhR were pre-heated at 43°C for 10 min before used in reactions.

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 [289]. The CHIP mediated ubiquitination of the firefly luciferase enzyme has been previously shown to require its prior denaturation [260, 295]. 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 minutes to stimulate CHIP-UbcH5a mediated ubiquitination of the AhR. However, denaturing heat pre-treatment of the >9 S AhR samples did not enhance the ubiquitination of the AhR by CHIP-UbcH5a complexes (Figure-3.6B). 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 indicate that the AhR is not a direct target of CHIP mediated ubiquitination and perhaps other factors cooperate in this process.

3.4.7 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-3.2A) 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 3-7 A). 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. Since the hsc70 has been shown to be a target of CHIP mediated ubiquitination, it is possible that the reduced hsp90 ubiquitination observed is related to competition between these two substrates for ubiquitination. A non-specific 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 [352]. More importantly, all the CHIP-mediated ubiquitination components were required for hsp90 ubiquitination *in vitro*.

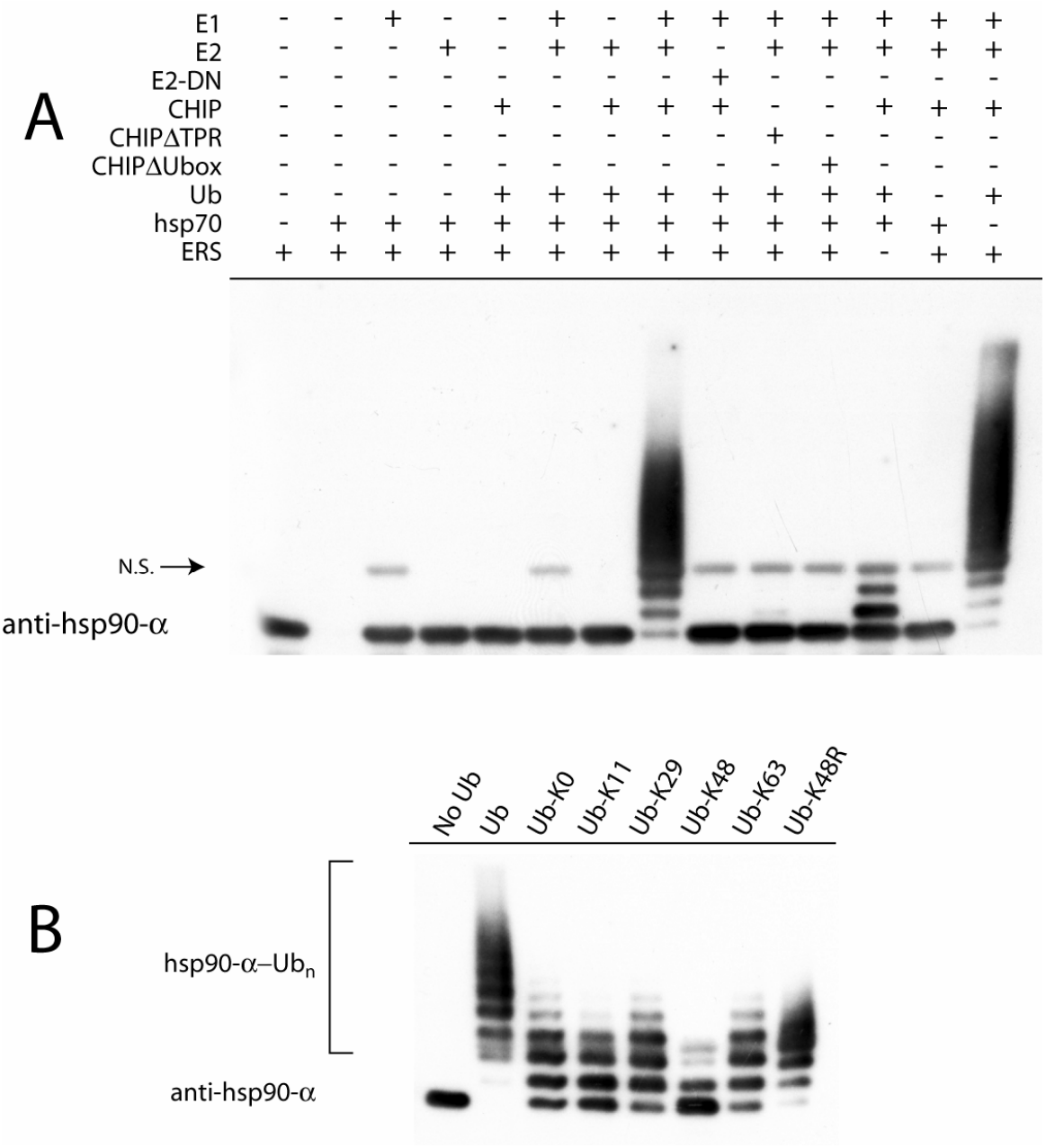


Figure **3-7**: **Ubiquitination of hsp90 by CHIP-UbcH5a *in vitro***.

(A) *In vitro* hsp90-α ubiquitination reactions were performed at 30°C for 1 h (see Materials and Methods for details). Full reactions were quenched with 2X sample buffer and analyzed by TSDS-PAGE. The hsp90 protein was detected as in Figure-3.2A. The arrow with a N.S. caption stands for non-specific protein band. (B) *In vitro* ubiquitination reactions were carried in the absence of ubiquitin, the lysine free Ub-K0, with single-lysine ubiquitin variants (Ub-K11, Ub-K29, Ub-K48, Ub-K63), or the point-mutant K48R-Ub. The hsp90 protein was analyzed as in Figure-3.2A.

Having 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)[375, 399, 409]. Therefore, several ubiquitin mutants were screened to determine which 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-3.7B), giving 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 multi-site ubiquitination. The use of Ub-K0 revealed that at least 6 to 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 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, since 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 [295]. 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 the mono-lysyl ubiquitin variants was mostly comparable to that of Ub-K0. Polyubiquitinated substrates often cannot be readily resolved in a polyacrylamide gel due to the non-linearity of the conjugated molecules and often appear as smears in western blots. Based on this assumption and the modest level of smearing observed with K48R-Ub we conclude that the CHIP-UbcH5a can assemble non-canonical polyubiquitin chains on hsp90. However, it remains unclear whether these ubiquitin chains synthesized on hsp90 are of mixed linkage.

3.5 Discussion

The detection of CHIP in AhR complexes (Figure-3.1) 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-3.2), in agreement with earlier studies [390]. 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. A precedence exists for this idea. For example, the transcription factor p53 is primarily regulated by the Mdm2/hdm2 ubiquitin ligase [410], 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 [411-413]. Since the E3 ligase CHIP has also been proposed to regulate p53 turnover [398], it is likely that CHIP could be just one of many E3 ligase complexes working together to remodel large protein complexes. CHIP has also been associated with the SCF multi-protein ubiquitin ligase complex in the notch-induced degradation of E2A [414] and glycoproteins through the F-

box protein Fbx2 [415]. 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 [416].

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 neither how the CHIP protein levels are regulated in the cell nor 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 towards the CHIP degradation pathway. Furthermore, the certainty that CHIP does not act alone and requires other components such as its cognate E2 (UBC5 family [291]) 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 [260]. This idea is further supported by the observation that $CHIP^{-/-}$ mice develop normally but are sensitive to temperature and environmental stresses [417]. 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 [418]. Although hypothetical, changes in the AhR elicited by post translational modifications of itself or any of its associated proteins (e.g. acetylation [419] and/or phosphorylation [418]) 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 in order 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 CHIPΔU-box could both lead to a dramatic loss of AhR protein levels in COS-1 cells, while CHIPΔTPR only had a modest effect (Figure-3.3). These results highlighted that the U-box domain, which is required for CHIP mediated ubiquitination of its *bona fide* substrates [291, 295], was not required to mediate the turnover of the AhR. This result is also in direct contrast to previous observations where the CHIPΔU-box or CHIPΔTPR had no effect on the protein levels of a nuclear compartmentalized AhR (DR-NLS) [391]. Perhaps the discrepancy is related to the fact that a nuclear localized AhR was used in their studies (DR-NLS) or possibly due to cell line specific idiosyncrasies. Nevertheless, the effect of CHIPΔU-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, by regulating the substrate binding cycle or nucleotide binding state of the hsp90 and hsp70 chaperones as previously suggested [290]. 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-3.4). This result confirms that the ubiquitin ligase CHIP can remodel mature AhR complexes and its activity on the AhR is not due to misfolding of the receptor, often speculated about transient protein over-expression experiments in cells. Furthermore, the use of the ubiquitin mutant Ub-K0 revealed a high degree of multi-site 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 [391]. 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. By shielding the AhR from potential ubiquitin ligases, co-chaperones could provide another level of regulation for the AhR. This mechanism has been suggested for other proteins [399] and is in part supported by our observations on the ability of XAP2 protein to protect the AhR from CHIP-mediated ubiquitination (Figure-3.5). The XAP2 protein is an AhR interacting

partner capable of enhancing the AhR protein levels when over-expressed in cells [405]. 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 [264]. 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 [268]. Most recent observations suggest that the XAP2 may regulate the ligand-mediated transformation of the AhR into transcriptionally active complexes [270]. However, the true function of the XAP2 *in vivo* remains largely enigmatic. Recent studies with transgenic mice exclusively over-expressing 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* [269]. Therefore, although a role in AhR protein stability is evident in cell culture models, the true function of the XAP2 *in vivo* 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-3.6). 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-3.7A), providing an explanation for the CHIP-dependent degradation of the hsp90 observed in recent studies [295]. Since 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 non-canonical polyubiquitin chains on hsp90, while our observations on the limitations of the Ub-K48 (Figure-3.7B) may be of great interest to structural biology laboratories studying the mechanisms polyubiquitin linkage selection by ubiquitin ligases [420, 421]. The implications of our findings are numerous given the importance of ubiquitin chain structure as the ultimate signal determining the fate of polyubiquitinated proteins [375]. 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-α, along with its client proteins. The discovery of hsp90 as a direct target of CHIP mediated ubiquitination is paramount to the understanding of CHIP-mediated regulation of chaperone client proteins.

Chapter 4

Synthesis and Characterization of the aryl hydrocarbon receptor ligands and antiallergic drugs 3-[2-(2-phenylethyl)benzoimidazole-4-yl]-3-hydroxypropanoic acid (M50354) and ethyl 3-hydroxy-3-[2-(2-phenylethyl) benzoimidazol-4-yl]propanoate (M50367)

4.1 Abstract

The aryl hydrocarbon receptor is a ligand-activated transcription factor that mediates most of the toxic effects of numerous chlorinated and non-chlorinated polycyclic aromatic compounds (e.g., benzo[a]pyrene). Studies in AhR null mice suggested that this receptor may also play a role in the modulation of immune responses. Recently two drugs, namely M50354 and M50367 (ethyl ester derivative of M50354), were described as AhR agonists with high efficacy toward reducing atopic allergic symptoms in an AhR-dependent manner by skewing T helper cell differentiation towards a T_H1 phenotype (Negishi et al 2005, J Immunol. 2005;175(11):7348-56). Surprisingly, these drugs were shown to have minimal activity towards inducing classical dioxin responsive element-driven AhR-mediated CYP1A1 transcription. We synthesized and reevaluated the ability of these drugs to regulate AhR activity. In contrast to previously published data, both M50354 and M50367 were found to be potent but transient inducers of several AhR target genes, namely CYP1A1, CYP1B1, UGT1A2, and the recently identified gene epiregulin. M50367 was a more effective agonist than M50354, perhaps accounting for its higher bioavailability in vivo. However, M50354 was capable of displacing an AhR radioligand more effectively than M50367. This is consistent with M50354 being the active metabolite of M50367. In conclusion, two selective inhibitors of T_H2 differentiation are full AhR agonists.

4.2 Introduction

Atopy is an inherited tendency to develop chronic allergic responses that manifest themselves in the form of asthma, eczema, and anaphylaxis, among other predominantly T lymphocyte helper type 2 (T_H2) -driven disorders[1]. The etiologies of these allergic conditions have not been fully established, although polymorphisms in genetic loci coding for proteins involved in immune system function are often reported [422-425]. Biological parameters such as increased IgE production, eosinophilia, GATA-3 expression, increases in IL-4, IL-5, IL-10, and tumor necrosis factor-alpha cytokine production are among a complex array of markers for T_H2-driven immune responses. Immune system disorders are often classified into T_H1 and T_H2-biased responses based on the expression profiles of numerous cellular markers and cytokines of $CD4^+$ T helper cells. However, while this Mosmann paradigm [426] has proven useful and simplifies how immunological diseases are analyzed and classified, its application to other immune cells, namely $CD8^+$ lymphocytes, is still occasionally challenged by complex signaling and phenotypic manifestations that cannot be fit into the T_H1-T_H2 paradigm [427, 428]. Regardless, T_H2 driven conditions such as the acute relapse symptoms of atopic asthma and airway hyperresponsiveness are commonly treated with steroid drugs, such as the glucocorticoid receptor agonists dexamethasone and prednisone [429]. Unfortunately, these drugs have global immunosuppressive properties towards both T_H1 and T_H2 driven

clinical conditions and can also become ineffective during their prolonged use [430]. In addition, some individuals using these therapeutic agents may suffer from opportunistic infections and mood disorders [431, 432]. As a result, the search for drugs capable of specifically preserving the T_H1-driven innate immune system response, while skewing naïve T_H differentiation away from T_H2 phenotypes, may hold promise in the treatment of atopic allergic diseases and similar T_H2-driven conditions.

In 1999 Kato and colleagues published the identification of a novel benzoimidazole derived drug ethyl 3-hydroxy-3-[2-(2-phenylethyl) benzoimidazol-4-yl]propanoate (M50367) (Figure 4-1) capable of significantly reducing disease scores of experimentally induced asthma and airway hyperresponsiveness in mice [299]. This compound was shown to target important signal transduction pathways leading to airway hyperresponsiveness and asthma, by blocking IL-4 (e.g., important for IgE class switching) and IL-5 production (e.g., important for the homing of eosinophils at inflammation sites), respectively [299]. M50367 was also capable of enhancing production of the T_H1 cytokine INF-γ in bronchoalveolar lavage fluid and cultured spleenocytes and had no effect on IL-2 expression (e.g., important for T_H1 driven responses). Consequently and in contrast to prednisone, M50367 does not suppress the innate immune system but it is active in ameliorating both OVA and DNP Ascaris-induced asthma and airway hyperresponsiveness in mice [299]. Furthermore, no weight loss in mice or toxicity to cultured spleenocytes could be observed *in vitro*, which is in contrast to some of the side effects exerted by prednisone. Later studies suggested that, following an oral administration of M50367, the compound's ethyl ester group is

removed through hydrolysis to yield the compound M50354, which was coined the 'active metabolite' of M50367 [298]. The target cells for M50354 and M50367 were subsequently suggested to be differentiating naïve T_H cells, since none of the drugs affected mature T_H1 or T_H2 cell functions [298]. The mechanisms by which M50367 and M50354 elicited these immunomodulatory activities remained at least partially unknown until they were identified as ligands for the AhR, a receptor whose expression levels are also modulated during the differentiation program of naïve T_H cells [297].

The AhR is a basic helix-loop-helix and PAS domain transcription factor and an orphan receptor that mediates most of the toxic effects elicited by several halogenated (HPAHs) and non-halogenated polycyclic aromatic compounds (PAHs) [388]. In its unliganded state, the AhR is found in the cytoplasmic compartment primarily as a tetrameric complex composed of two molecules of hsp90 and the immunophilin-like protein XAP2 [77, 261, 433]. After the association of a ligand with the AhR, the tetrameric complex rapidly translocates into the nucleus where it heterodimerizes with ARNT and dissociates from the hsp90 dimer and XAP2. The transformed AhR-ARNT heterodimer then binds xenobiotic responsive elements with the consensus sequence 5′-TNGCGTGA-3′ and upregulates target gene expression [115]. Activation of the AhR leads to the upregulation of phase I, II, and III metabolism gene products that are important for the clearance and/or activation of various endogenous and exogenous substances. However, chronic exposure to high affinity AhR ligands such as TCDD, may lead to toxic end-points such as immune system suppression, hydronephrosis, and porphyric disorders [434-437]. The mechanism by which the AhR is responsible for some

of these conditions remains largely unknown but it has been associated with the expression of some of its target genes such as CYP1A1 [4]. Furthermore, repression of the NF-κB and AP1 signaling pathways by the AhR signaling pathway has also been documented [139, 438-440]. Even though suspected to exist, no endogenous high-affinity ligand has been identified for the AhR that would make clear its role in normal physiologic homeostasis. Yet the AhR can bind numerous synthetic and naturally occurring substances that serve as agonists or antagonists such as indole-3-carbinol [73], bilirubin [206], curcumin [237], quercetin [238], resveratrol [239], galangin [240], and the synthetic compound α-naphthoflavone [441], among others. In addition, cellular shear stress has been shown to induce phospholipase D activity, resulting in the formation of phosphatidic acid that activates phospholipase A2 (PLA2). PLA2 activity then results in the release of arachidonic acid which is further metabolized into CYP1A1 inducing metabolites and therefore suspected AhR ligands [241]. Clearly further work is necessary to establish how the AhR is activated under normal physiological conditions.

Perhaps one of the key observations linking the AhR with normal immune system function came from the generation of AhR knockout mice ($AhR^{-/-}$). The spleen and lymph nodes of $AhR^{-/-}$ mice display a reduced presence, or perhaps recruitment, of T lymphocytes during the first 10 weeks after birth [247]. Consequently, some of these mice succumb to opportunistic infections during this early period. However, mice that do survive show normal T lymphocytes numbers by 10-12 weeks of age but again suffer a loss of cells to ~50% of the wild type levels later in life [247]. An underlying problem possibly associated with delayed migration and maturation and/or altered cytokine

expression in $AhR^{-/-}$ immune cells or supportive tissues was hypothesized to account for these abnormalities. Others have challenged this evidence as being limited to the particular strain of mouse utilized since other $AhR^{-/-}$ mice display normal antigen-mediated immune responses [442]. However, a recent study on $AhR^{-/-}$ mice revealed that these mice exhibit abnormally elevated expression of both T helper cell 1 (T_H1) and T helper cell 2 (T_H2) cytokines in OVA sensitized AhR null mice [297]. Therefore, although the mechanisms have not been fully established, evidence suggests that the AhR could play a balancing role in the immune system.

When M50367 and M50354 were identified as AhR ligands, the authors highlighted that these drugs could only partially induce CYP1A1 activity when compared to other established high affinity AhR ligands [297]. In addition, these compounds appeared to exhibit unique AhR-driven gene expression profiles when compared to other AhR ligands. These results were rather surprising and captured our interest due to a lack of planarity in these molecules, a characteristic that is typical of most high affinity AhR ligands (e.g., TCDD). Analogous to the effects seen for selective modulators of the steroid receptors, namely the glucocorticoid receptor, peroxisome proliferator activated receptors, and the liver X receptors and their role in the modulation of immunological responses (discussed in [443]), the M50367 and M50354 compounds were suggested to be selective AhR modulators, driving receptor-dependent immunological changes without significant induction of CYP1A1 [297]. In this study, the synthesis and further characterization of M50367 and M50354 within the AhR signal transduction pathway is reported. In contrast to published reports, we demonstrate that both M50367 and M50354

behave as transient but full AhR agonists, at previously indicated therapeutically-relevant doses [298, 299]. The transient but significant nature of M50354 and M50367 AhR-driven gene expression may have accounted for the underestimation of this response, due to the methods and time-frame chosen for analyses by previous authors [297]. The implications of these findings, on the potential use of these drugs in the treatment of atopic allergic diseases, are further discussed.

4.3 Materials and Methods

Chemistry.

4.3.1 Synthesis of 3-[2-(2-phenylethyl) benzoimidazole-4-yl]-3-hydroxypropanoic acid (M50354) and ethyl 3-hydroxy-3-[2-(2-phenylethyl) benzoimidazol-4-yl]propanoate (M50367).

All solvents and reagents were used as purchased, without further purification, unless noted otherwise. Anhydrous tetrahydrofuran (THF) was prepared by distillation from sodium and benzophenone. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts are expressed in ppm with respect to TMS (tetramethylsilane) as the internal standard. Column chromatography was performed on a silica gel 60Å 70-230 mesh or flash on 230-320 mesh. For thin-layer chromatography aluminum plates pre-coated with silica gel 60 F_{254} (0.2 mm) were used.

4.3.1.1 2-(3-Phenylethyl)benzimidazole-4-carboxylic acid.

To a solution of 2,3-diaminobenzoic acid (7.8 g, 51.3 mmol) in 120 ml of dimethylacetamide (11 g, 82.08 mmol, 1.6 eq.) of 3-phenylpropionaldehyde was added slowly with stirring followed by potassium ferricyanide (33 g, 0.1 mol, 2 eq.). Mixture

was heated and stirred at 60 °C under N_2 for 12 h. The deep blue solid was filtered out, washed with ethyl acetate, washings were combined with filtrate and the solvents removed *in vacuo*. Residue was purified by silica gel flash column chromatography using $CHCl_3$ with increasing amount of EtOH (2-20%) to give 11.5 g (40%) of acid intermediate **3 (**see Figure 4-2**)** as an off-white solid. Mp 220 °C (sample was recrystallized from $CHCl_3$: MeOH); ^{1}H NMR (acetone-d_6) δ 3.21-3.25 (m, 2H), 3.32-3.36 (m, 2H), 7.15-7.19 (m, 1H), 7.27-7.35 (m, 5H), 7.86 (d, J = 8.0 Hz, 1H), 7.90 (d, J = 8.0 Hz, 1H).

4.3.1.2 Ethyl 3-oxo-3-[2-(β-phenylethyl)-1H-benzimidazole-4-yl]propionate.

A mixture of acid **3** (4 g, 15.8 mmol) and thionyl chloride (80 ml) was refluxed for 3 h. Benzene (250 ml) was then added and the solid material was filtered off. It was washed twice with both benzene and Et_2O and dried in vacuo to yield 4.1 g of acid chloride **4** as a white powder. To a solution of monoethyl malonate (2.6 g, 20 mmol, 2.5 eq.) in anhydrous THF (45 ml) under N_2 was added a few milligrams of 2,2'-bipyridyl as an indicator. The solution was cooled to -70 °C and *n*-BuLi (2.5 M in hexanes, 16 ml, 40 mmol) was added dropwise allowing the temperature to rise to -5 °C at the end of the addition. Mixture was maintained at this temperature for 10 min. observing persistence of pink indicator color. It was then recooled to -65 °C and the acid chloride **4** suspension (2 g, 8.0 mmol) in THF (20 ml) was added dropwise over 5 min. After 1.5 h at -65 °C, the mixture was diluted with Et_2O (90 ml), acidified to pH 2-3 with ~60 ml of 1 N HCl, and

the phases separated. Water phase was alkalized to pH 8 with 10 N NaOH, extracted either, combined extracts dried over $MgSO_4$ and evaporated. The crude product was purified by flash column chromatography (gradient: hexanes/ethyl acetate from 5 to 50% v/v) to give 1.4 g of pure β-ketoester **5** as a white solid. Workup of organic (Et_2O) phase yielded additional 0.4 g of **5**; combined yield 1.8 g (72%). Mp 100-101 °C; ^{1}H NMR ($CDCl_3$) δ 1.27 (t, *J* = 7.0 Hz, 3H), 3.22-3.25 (m, 2H), 3.28-3.32 (m, 2H), 4.11 (s, 2H), 4.26 (q, *J* = 7.0 Hz, 2H), 7.24-7.28 (m, 3H), 7.32-7.36 (m, 3H), 7.74 (d, *J* = 7.5 Hz, 1H), 8.01 (d, *J* = 8.0 Hz, 1H).

4.3.1.3 Ethyl 3-hydroxy-3-[2-(β-phenylethyl)-1H-benzimidazole-4-yl]propionate.

To a solution of ketoester **5** (0.95 g, 2.83 mmol) in 20 ml of 95% EtOH was added dropwise a solution of sodium borohydride (44 mg, 1.16 mmol) in EtOH (5 ml) at room temperature. Progress of the reaction was monitored by TLC (hexanes/ethyl acetate 1:1). After 1.5 h min., next portion of $NaBH_4$ (10 mg, 0.264) in EtOH (1.5 ml) was added and a half of this amount was added again after 1.5 h. The reaction was continued for another 1 h, after which time no starting material was detected (TLC). The solution was poured into 70 ml of water, acidified to pH 5-5.5 with 25% AcOH, extracted with ethyl acetate and dried ($MgSO_4$). The extract was stripped of solvent to leave glassy solid which, after flash chromatography (hexanes/ethyl acetate, gradient from 10-30% of ethyl acetate) gave 0.82 (86%) of pure **1** as a white solid. Mp 123-124 °C; ^{1}H NMR ($CDCl_3$) δ 1.32 (t, *J* = 7.0 Hz, 3H), 2.87 (d, *J* = 6.5 Hz, 2H), 3.17-3.21 (m, 2H), 3.23-3.27 (m, 2H), 4.24 (q, *J*

= 7.0 Hz, 2H), 5.50 (t, *J* = 6.5 Hz, 1H), 7.02 (d, J = 7.5 Hz, 1H), 7.19 (dd, J = 8.0 and 7.5 Hz, 1H), 7.24-7.34 (m, 6H), 7.56 (br d, *J* = 7.0 Hz, 1H).

The enantiomers of **1** were separated by HPLC using Pirkle Covalent (S,S) Whelk-O1 column 250 x 4.6 mm #786101 (Regis Technologies Inc.). Hexanes/ethanol (92:8) + 8 mM ammonium acetate was used as a mobile phase in isocratic mode (flow rate: 1.5 ml/min.). Baseline separation was achieved and fractions corresponding to the peaks at 22 and 24 min respectively were collected. Each of them contained single enantiomer as yet chiroptically unassigned.

4.3.1.4 3-Hydroxy-3-[2-(β-phenylethyl)-1H-benzimidazole-4-yl]propionic acid.

To a solution of **1** (0.2 g, 0.591 mmol) in MeOH (3 ml) was added 0.2 N LiOH in 3:1 MeOH/H_2O (6 ml). Reaction progress was monitored by TLC (4:1 ethyl acetate/MeOH). After 40 min. of stirring at room temperature, 50 ml of water was added and the pH was brought to 5-5.5 with 25% AcOH. Mixture was extracted with ethyl acetate, extract dried ($MgSO_4$) and solvent was removed to give 0.12 g (66%) of **2** as a white solid. Mp 110-112 °C; ^{1}H NMR (acetone-d_6) δ 2.78 (dd, *J* = 15.6 and 9.2 Hz, 1H, -CH_2-), 2.94 (dd, *J* = 15.6 and 3.3 Hz, 1H, -CH_2-), 3.17-3.21 (m, 2H), 3.23-3.26 (m, 2H), 5.58 (dd, *J* = 9.0 and 3,5 Hz), 7.11-7.21 (m, 3H), 7.25-7.30 (m, 4H), 7.39 (d, *J* = 8.0 Hz, 1H).

Biology.

4.3.2 Antibodies

The primary antibodies utilized included mouse anti-AhR monoclonal IgG_1 (RPT1) (Affinity BioReagents), rabbit anti-XAP2 IgG [444], rabbit anti-CYP1A1 IgG (H-70) (Santa Cruz), and mouse anti-HOP IgG (F5) (David Toft, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN) were used to detect the AhR, XAP2, CYP1A1, and HOP proteins, respectively.

4.3.3 Cell Culture

Cells were maintained in modified Eagle's α-minimum essential medium (Sigma, St. Louis, MO), supplemented with 1000 units/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO), and 7% fetal bovine serum (HyClone, Logan, UT) and kept in a humidified incubator at 37°C and 5% CO_2. Growth medium was replaced every two days.

4.3.4 Cell culture treatments to determine AhR transcriptional activity.

A thousand-fold working stocks of TCDD (10 μM, 0.5 μM, etc.), B[a]P [10 mM], M50354 [10 mM], and M50367 [10 mM] were prepared in DMSO (Sigma). Unless otherwise noted in figure legends, cells were dosed by adding each compound directly into cell culture plates, mixed, and incubated for the stated period in the figures and legends. The amount of DMSO never exceeded 0.1%. Notice that TCDD and B[a]P are very toxic substances [445]. In addition, toxicological information for M50354 and M50367 has yet to be established. Therefore, these compounds, including the solvents and reactants utilized to synthesize them, should be treated as potentially harmful and must be disposed according to toxicological waste-disposal guidelines established by your institution.

4.3.5 Western Blots.

Proteins were resolved by TSDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA) using standard procedures. Membranes were pre-incubated with blocking buffer [10 mM monobasic sodium phosphate mono hydrate, 150 mM sodium chloride, 0.5% (v/v) Tween 20 (Sigma, St. Louis, MO), and 3% (m/v) bovine serum albumin fraction V (EMD Chemicals Inc., San Diego, CA)] for 1 h. Primary antibodies were diluted in wash buffer [10 mM monobasic sodium phosphate mono hydrate, 150 mM sodium chloride, 0.5% (v/v) Tween 20 (Sigma, St. Louis, MO), and

0.1% (m/v) bovine serum albumin fraction V (EMD Chemicals Inc., San Diego, CA)] and incubated for an additional 1 h at room temperature. Blots were washed 3 times in a 30 minute period. A 1 h incubation with biotin-conjugated secondary antibodies followed by a 15 min incubation with [^{125}I]-streptavidin. Blots were washed and proteins were visualized by autoradiography. The relative protein levels were determined by phosphor image analysis.

4.3.6 Real-time PCR analysis.

Total mRNA was isolated using Sigma's TRI® Reagent mRNA isolation reagent (Sigma, St. Louis, MO) and amplified using the High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA). The level of CYP1A1, CYP1B1, UGT1A2, and epiregulin mRNA were assessed by real-time qPCR using the MyIQ single-color PCR detection system (BioRad, Hercules, CA) and the iQ SYBR Green supermix (BioRad). The primers utilized are shown on Table **4-1.**

Table **4-1**: Real-time PCR Primers

Gene	Species	Forward Primers	Reverse Primers
CYP1A1	human	CAG CTG CAT TTG GAA GTG CTC ACA	AGA GGT TGG CCA CTT TGA CCC TTA
CYP1B1	human	TGC CTG TCA CTA TTC CTC ATG CCA	ATC AAA GTT CTC CGG GTT AGG CCA
GAPDH	human	TCG ACA GTC AGC CGC ATC TTC TTT	ACC AAA TCC GTT GAC TCC GAC CTT
Epiregulin	human	GAG GAG GAT GGA GAT GCT CTG	CAC TGG ACT CTC CTG GGA TAC A
CYP1A1	mouse	AGA GGT TGG CCA CTT TGA CCC TTA	TGA CTA TGC TGA GCA GCT CTT GGT
CYP1B1	mouse	TTC CTA GAG CTG CTC AGC CAC AAT	GAA CGA AGT TGC TGC AGT TGC GGT
GAPDH	mouse	TCA ACA GCA ACT CCC ACT CTT CCA	ACC CTG TTG CTG TAG CCG TAT TCA
UGT1A2	mouse	AAG GCT TTC TGA CCA CAT GGA	GGC AAA TGT ACT TCA GGA CCA GAT

4.3.7 Luciferase reporter gene experiment.

The previously established DRE-driven reporter cell lines HG40/6 [446] and H1L1.1c2 [447], derived from the Hep-G2 and Hepa-1c1c7 cell lines, respectively, were utilized to monitor AhR transcriptional activity. Two days before treatments with ligands, cells were fed with complete medium containing no antibiotics. Ligands were added at the specified concentrations for the given period as stated in the figure legends. After treatments luciferase activity was measured using Promega's (Madison, WI) Luciferase Assay System following manufacturer's instructions.

4.3.8 Determination of CYP1A1 activity directly in cells.

CYP1A1 activity in Hepa-1c1c7 cells was measured using Promega's (Madison, WI) P450-Glo™ microsomal assay with the CYP1A1-specific substrate luciferin-CEE. Briefly, cells grown on 12-well plates were dosed with the appropriate CYP1A1 inducing agent (e.g., TCDD) for 6 h and/or 24 h. The growth medium was then discarded and 500 µl of fresh serum-free DMEM containing 8 µl of luciferin-CEE was added to each well and incubated for an additional 3 h. CYP1A1 activity was then determined according to manufacturer's instructions using a Turner TD-20e luminometer (Turner BioSystems, Sunnyvale, CA).

4.3.9 Competition ligand binding experiments.

Hepa-1c1c7 or HaCaT cytosolic extracts were prepared in MENGM buffer [16.2 mM 3-(N-morpholino)-propanesulfonic acid sodium salt, 10 mM free acid 3-(N-morpholino)-propanesulfonic acid, 0.02% (m/v) sodium azide, 10% (m/v) glycerol, 4 mM EDTA, and 20 mM molybdate]. Briefly, a 150 μl of cytosol at 2 mg/ml transferred to 12x75 mm borosilicate glass tubes. Samples were pre-incubated with cold competitor for 10 minutes at room temperature prior to the addition of radioligand. A total of 0.13 pmol 2-azido-3-[^{125}I] iodo-7,8-dibromodibenzo-*p*-dioxin (photoaffinity ligand) was added per sample and incubated at room temperature for 30 min in the presence or absence of competitor. The samples were finally exposed to 15-W UV lamps (>302 nm) at 8 cm for 4 min. Sixty microliters of each supernatant (120 μg) were then combined with 5X Laemli buffer containing β-mercaptoethanol, heated at 95°C for 5 min, and analyzed by TSDS-PAGE. Resolved proteins were then transferred to PVDF membranes and autoradiographs were generated. The protein bands were quantified via phosphor image analysis.

4.4 Results

Chemistry

4.4.1 Synthesis of M50367 and M50354.

During the course of this study the anti-allergic agent M50367 [Ethyl 3-hydroxy-3-[2-(β-phenylethyl)-1*H*-benzimidazole-4-yl]propionate (**1**)] and its active metabolite M50354 **[**3-Hydroxy-3-[2-(β-phenylethyl)-1*H*-benzimidazole-4-yl]propionate (**2**)] were synthesized (Figure **4-1)** . The synthetic strategy is outlined in Figure **4-2**. In the initial step, suitably substituted 2,4-benzimidazole derivative was synthesized adopting a published method with modification [448]. Oxidative cyclization of 2,3-diaminobenzoic acid and 3-phenylpropionaldehyde utilizing potassium ferricyanide as an oxidant gave 2-(3-Phenylethyl)benzimidazole-4-carboxylic acid (**3**) with moderate yield. Acid chloride **4** prepared by standard procedure using thionyl chloride was without further purification converted to β-ketoester **5** [449] by condensation with monethyl malonate. Finally, reduction of **5** with $NaBH_4$ in ethanol furnished the desired ester **1** which upon hydrolysis [450] gave metabolite **2**. In an attempt to obtain pure enantiomers, **1** was derivatized with auxiliary chiral reagent (N,N-dimethyl-L-phenylalanine) thus creating diastereomeric mixture of amino esters. The diastereomers were separated on flash silica column and characterized by ^{1}H NMR spectroscopy. However, efforts to restore original secondary hydroxyl functionality from amino ester protection were unsuccessful. Secondary reactions might have occurred leading to other products, nature of which was not

investigated. Required separation and purification of unmodified enantiomers of **1** was finally achieved by enantioselective HPLC on Whelk-O1 chiral stationary phase column.

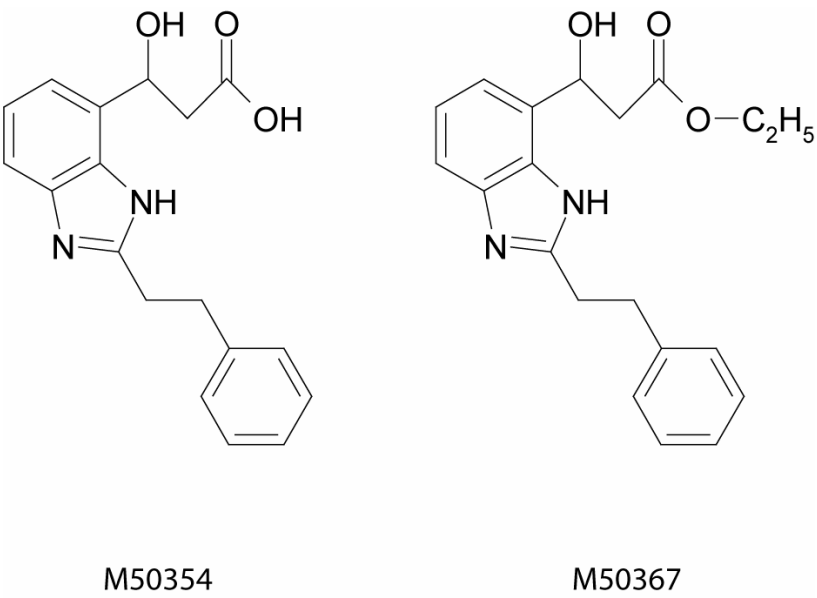


Figure 4-1: **Structure of M50354 and M50367.**

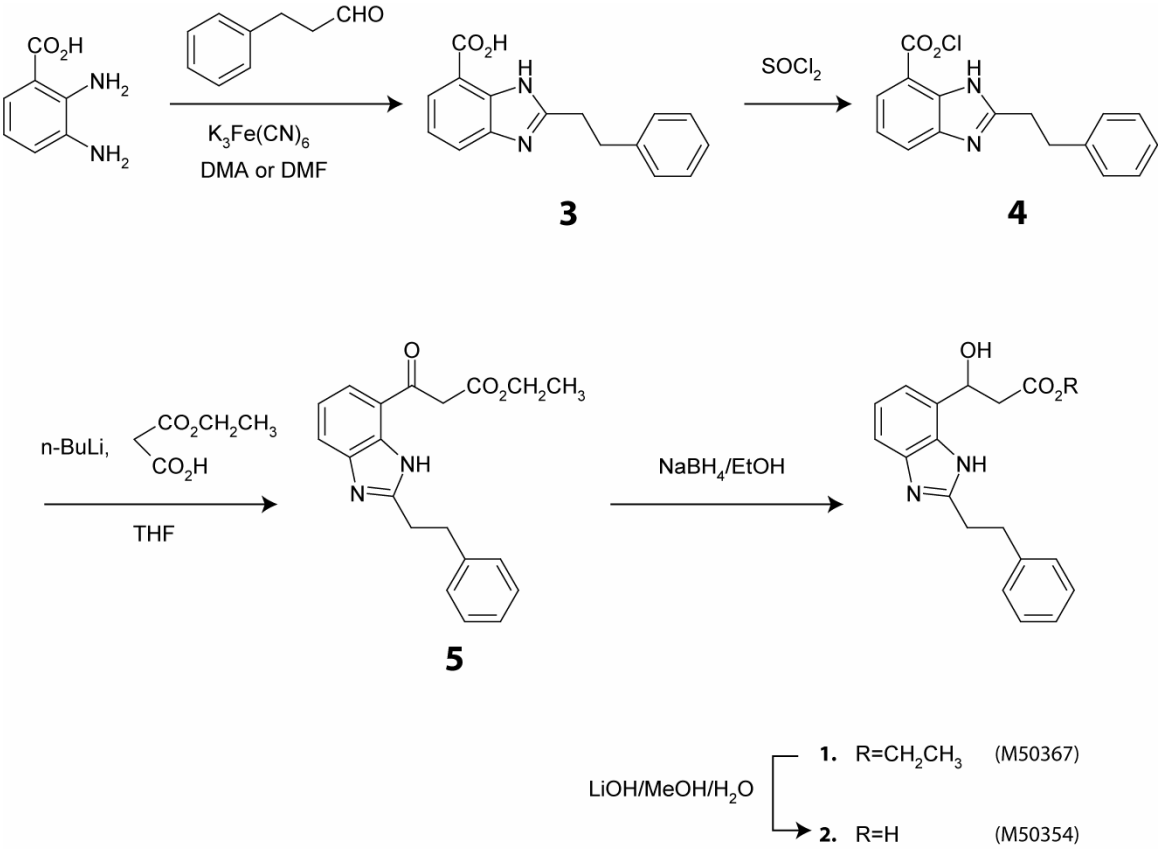


Figure 4-2: **Schematic for Synthesis of M50354 and M50367.**

Biology.

4.4.2 M50367 and M50354 Exhibit significant AhR Agonistic Activity.

The recently published work of Negishi and colleagues suggested that two potentially useful anti-asthmatic drugs, namely M50354 and M50367, had immunomodulatory activities that were dependent on the functional expression of the AhR [297]. These substances were identified as AhR ligands in competition ligand

binding experiments, although their ability to induce classic AhR-dependent CYP1A1 gene expression was considerably lower when compared to relatively potent AhR ligands (e.g., β-naphthoflavone and 3-methylcholanthrene). In order to further assess the ability of M50354 and M50367 to activate the AhR, the Hepa-1c1c7-derived mouse cell line H1L1.1c2 that stably expresses a dioxin responsive element-driven luciferase reporter gene was utilized to determine the extent of AhR activation by M50354 and M50367 versus the prototypical high affinity AhR ligand TCDD. In contrast to previous observations [297] this assay revealed that both M50354 and M50367 could induce significant activity (Figure **4-3**A). In addition, consistent with the higher *in vivo* effects of M50367 [298, 299], the M50367 compound was more active than M50354 in this gene reporter test.

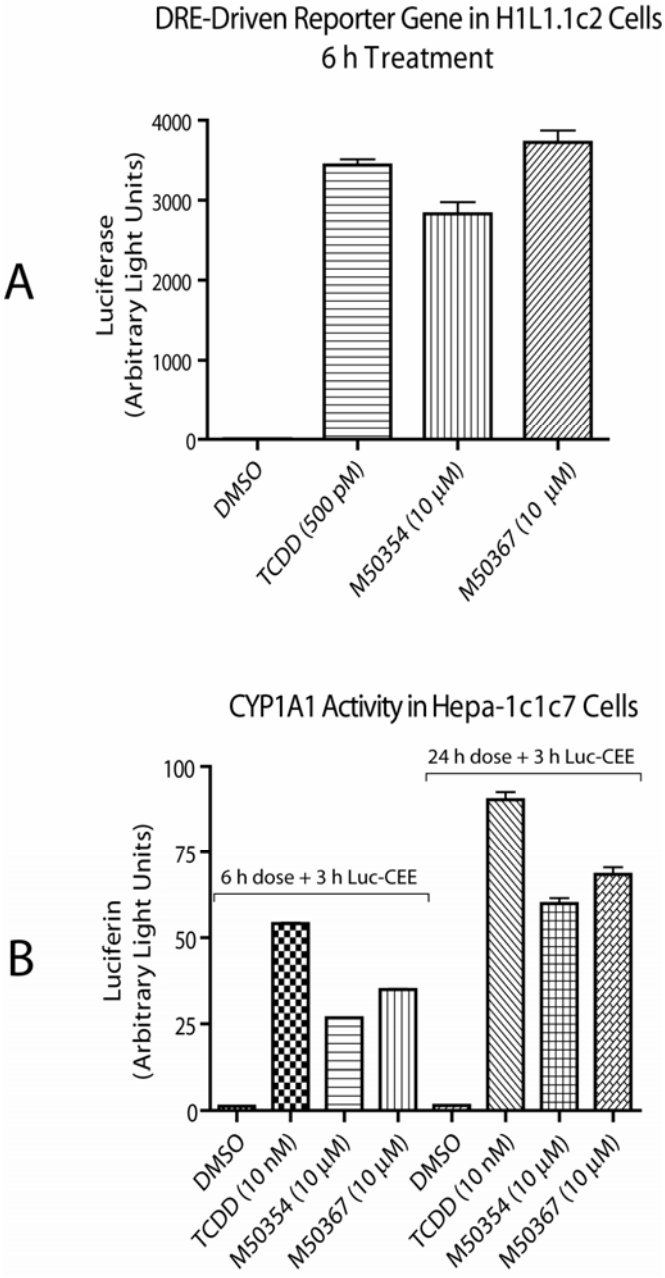


Figure 4-3: **M50354 and M50367 mediated AhR transcriptional activity**.

H1L1.1c2 cells were treated with vehicle (DMSO), TCDD (500 pM), M50354 (10 μM), or M50367 (10 μM) for 6 h. Vehicle concentration never exceeded 0.1% in all treatments. Cells were harvested and luciferase enzymatic activity was measured. **B**. Hepa-1c1c7 cells were treated with TCDD (10 nM), M50354 (10 μM), or M50367 (10 μM) for 6 h or 24 h. CYP1A1 enzymatic activity was measured using Promega's P450-Glo™ Assay with Luciferin-CEE as a substrate, according to manufacturer's instructions. For both A and B, all samples were run in triplicates and the results are representative of three independent experiments.

However, since reporter gene data alone may not completely recapitulate the induction of an endogenous AhR target gene, Promega's P450-Glo™ Luciferin-CEE assay was utilized to detect CYP1A1 enzymatic activity directly in Hepa-1c1c7 cells. Interestingly, both M50367 and M50354 were capable of inducing a highly significant level of CYP1A1 catalytic activity, especially when compared to a saturating dose of TCDD (Figure 4-3B). Consistent with the reporter data, M50367 could induce CYP1A1 enzymatic activity to a higher degree than M50354.

4.4.3 Characterization of M50367 enantiomers.

The approach utilized for the synthesis of both M50354 and M50367 compounds results in the formation of a racemic mixture of stereoisomers. M50367 and M50354 enantiomers could be readily detected via the appropriate HPLC analysis migrating as two equimolar peaks. For simplicity, we will refer to each enantiomer as the 22 min peak 1 (P1) and 24 min peak 2 (P2). A method was designed for the separation of M50367 enantiomers, given that it was imperative to determine whether there was a stereospecific requirement for each M50367 enantiomer to function as an AhR agonist or even possibly as an antagonist. However, since M50367 displayed higher activity in our luciferin-CEE based CYP1A1 assays and the indication that M50367 has a higher therapeutic effect *in vivo* (e.g., mice) [297], we pursued the characterization of the M50367 enantiomer pair alone. Consistent with the results obtained utilizing the racemic mixture of M50367

(Figure 4-3), each M50367 enantiomer at 10 μM could individually induce similar reporter activity in H1L1.1c2 cells in a time course reporter experiment (Figure **4-4**A). However, the extent of induction was less than a saturating dose of TCDD. Regardless, as predicted from the H1L1.1c2 reporter cell line results (Figure 4-3) and Negishi's report [297], the induction of reporter activity approached near basal expression by 24 h, while TCDD-induced reporter activity remained relatively high (Figure 4-4A). Both enantiomers were also tested in the human cell line HG40/6 that stably expresses a DRE-driven luciferase reporter gene. Interestingly, in the HG40/6 reporter line there was a modest increase of reporter activity displayed by the P2 enantiomer (Figure 4-4B). This higher reporter activity seen for P2 in HG40/6 cells was also consistent with higher CYP1A1 mRNA levels in the same cell line (Figure 4-4C). Given the absence of this effect in the H1L1.1c2 cell line, it was important to test whether such differences could be recapitulated on an endogenous AhR target gene and in other cells. Hence, CYP1A1 mRNA expression was also analyzed via real-time PCR in the wild type murine Hepa-1c1c7 and the human keratinocyte-derived cell line HaCaT. Just as in the reporter cell line H1L1.1c2 (Figure 4-4A), these experiments revealed no differences in CYP1A1 mRNA induction by the M50367 enantiomers (Figure 4-4D and 4-4E). Furthermore, while neither M50354 nor M50367 could activate the reporter gene comparably to a saturating dose of TCDD in HG40/6 and H1L1.1c2 cells (Figure 4-4A and 4-4B), both M50367 enantiomers could readily induce CYP1A1 mRNA expression to levels comparable to a saturating dose of TCDD in Hepa-1 and HaCaT cells at 4 h post treatment (Figure 4-4D and 4-4E). Notably, when either of the M50367 enantiomers was administered at 1 μM, they approached the activity of a saturating dose of TCDD in

Hepa-1 cells and to a lesser degree in HaCaT cells. At 10 μM both P1 and P2 induced CYP1A1 mRNA levels to the same level as a saturating dose of TCDD at 4 h.

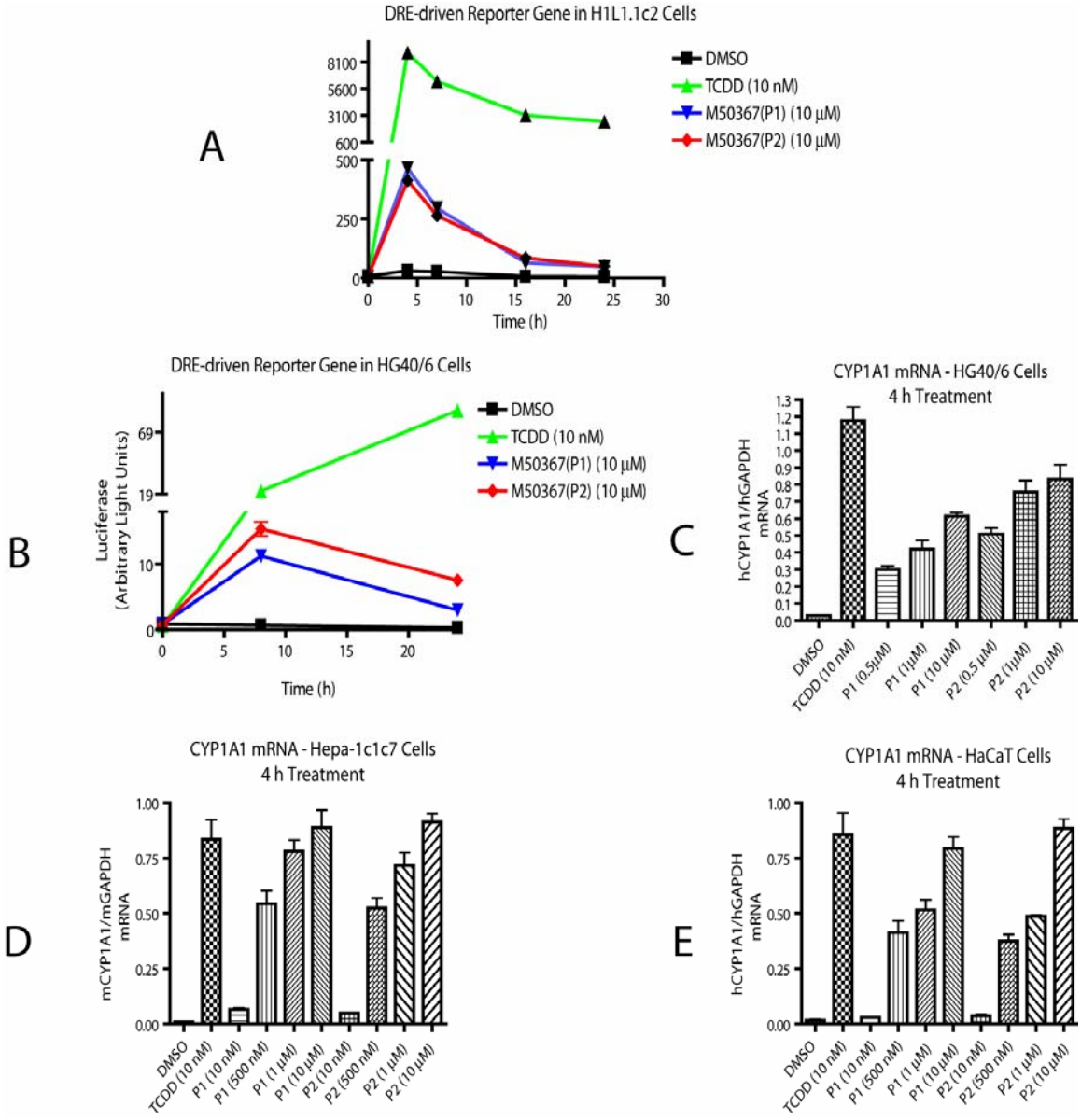


Figure 4-4: **Characterization of M50367 enantiomers.**

A, DRE-driven luciferase reporter gene experiments were performed as in Figure 4-3A, except that cells were treated at the concentrations stated in the figure and samples were analyzed at the 0, 4, 8, 16 , and 24 h time points. Culture medium was replaced with fresh medium containing each compound at the stated concentrations. B, Performed as in A, except samples were collected at 0, 8, and 24 h. **C**, **D**, and **E**, following the treatment cells with each compound at the indicated concentrations and a 4 h incubation period, mRNA was isolated using the Tri-reagent (Sigma) RNA isolation method and analyzed by RT-PCR analysis. The quantities of mRNA were normalized to GAPDH mRNA values. All experimental samples were run in triplicates and the results are representative of three isolated experiments.

Having established that both M50367 enantiomers could readily activate CYP1A1 gene expression, we examined their ability to modulate expression of some known AhR target genes such as CYP1B1, UGT1A2, and the recently associated gene epiregulin [158] in Hepa-1c1c7 and/or HaCaT cells. Consistent with M50367's AhR full agonistic activity on CYP1A1 expression, at 10 μM both enantiomers could readily activate other AhR target genes to levels comparable of a saturating dose of TCDD (Figure **4-5**). Interestingly, the concentration of M50367 enantiomers required to elicit maximal induction of these genes varied between cell lines and in a gene specific manner.

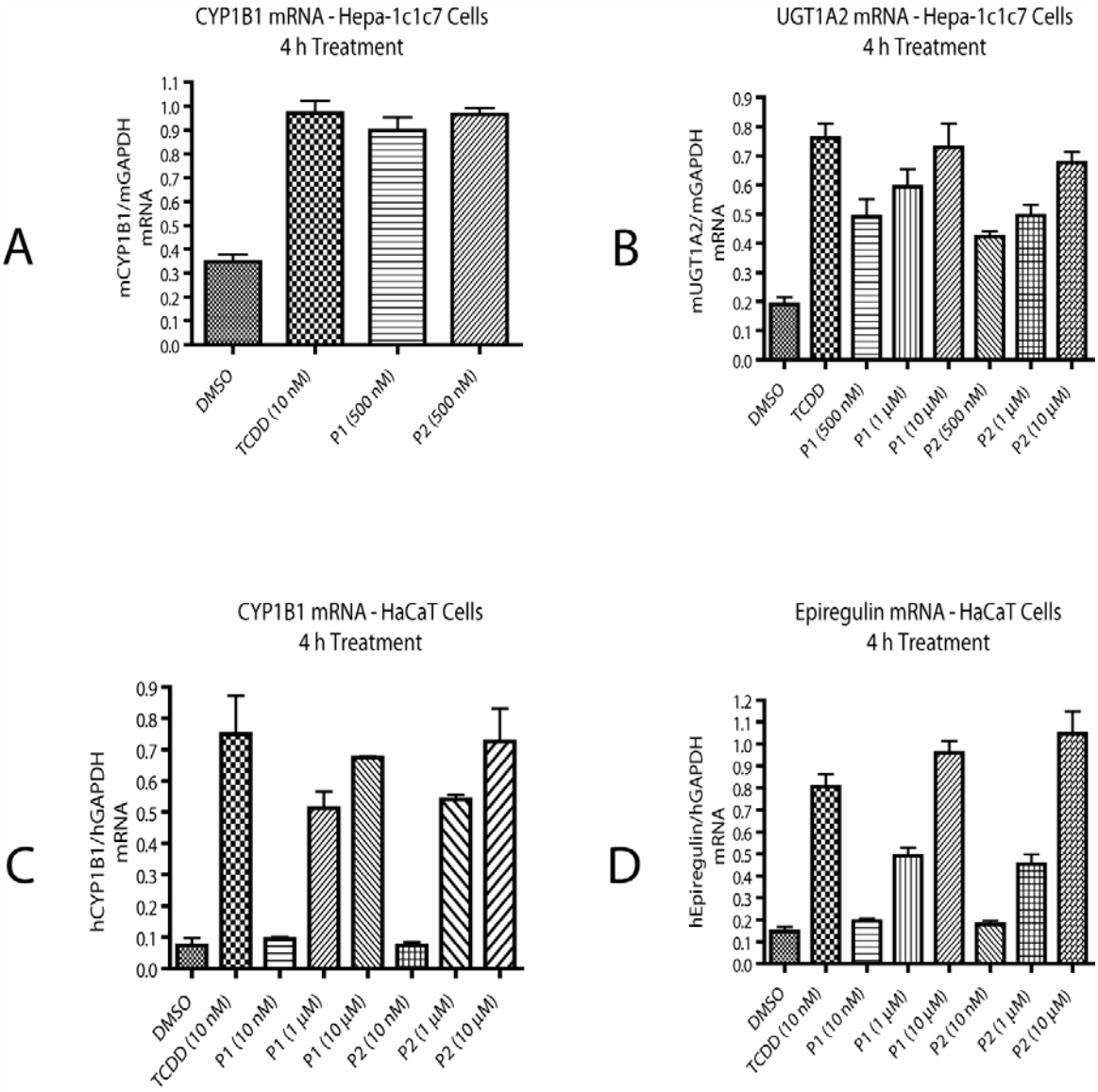


Figure 4-5: **M50367 activates other AhR regulated genes.**

For **A**, **B**, **C**, and **D**, the mRNA levels were determined as in Figure 4-4C and 4-4E. All experimental samples were run in triplicates and results are representative of two isolated experiments.

4.4.4 M50367 and M50354 can compete with 2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin for binding to the AhR in a dose-dependent manner.

The ability of M50354 and M50367 compounds to displace [^{3}H]-M50354 were initially provided in Negishi's report [297], although the experiment was limited to one saturating concentration and provided no information on the relative affinity of M50354 and M50367 for the AhR. Competition-binding experiments were carried with the AhR photoaffinity radioligand 2-azido-3-[^{125}I]-iodo-7,8-dibromodibenzo-*p*-dioxin to determine the relative affinity of the M50354 and M50367 compounds for the AhR and to clearly demonstrate that these compounds are direct AhR ligands. Therefore, cytosolic extracts from Hepa-1c1c7 cells were utilized for competition ligand binding experiments with the photoaffinity ligand. These results indicated that the M50354 was capable of displacing the radioligand to a higher degree than M50367 at 46% versus 24.1% at 10 μM and 90.4% versus 74.5% at 100 μM, respectively (Figure **4-6**). As expected, β-naphthoflavone and α-naphthoflavone could readily displace the radioligand. As a negative control, an equal dose with the estrogen receptor ligand 17β-estradiol was unable to significantly displace the radioligand at 100 μM. Finally, M50367 and M50354 could compete specifically with the radioligand and thus are *bonafide* AhR ligands.

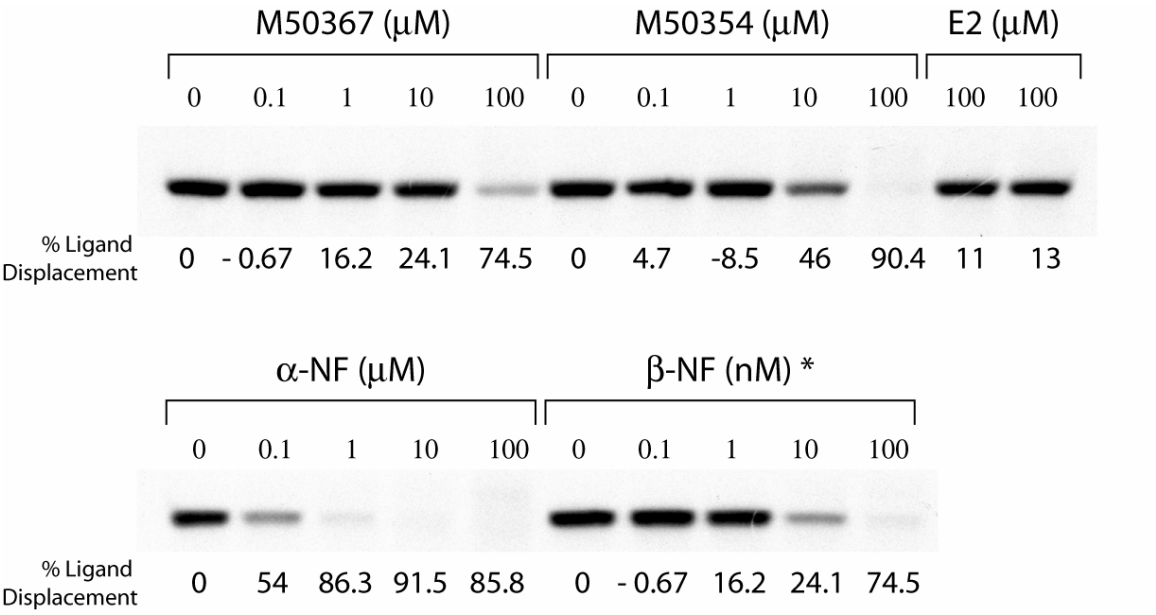


Figure **4-6**: **M50354 and M50367 are direct ligands for the AhR**.

Hepa-1c1c7 cytosol was pre-incubated with each competing ligand at the indicated concentrations for 10 minutes at 25 C followed by a 30-min incubation with the photoaffinity radioligand also at 25 C. Samples were then exposed to 15-W UV lamps (>302 nm) at a distance of 8 cm for 4 min. Sixty microliters of each supernatant (120 μg protein) were then combined with 5X SDS sample buffer supplemented with β-mercaptoethanol and heated at 95°C for 5 min. Proteins were resolved by TSDS-PAGE, transferred to PVDF membranes, and autoradiographs were generated. The protein bands were quantified via phosphor image analysis. *Notice 1000-fold lower concentration for β-NF.

4.4.5 M50354 and M50367-promoted AhR protein turnover and their effect on the temporal expression of CYP1A1 protein.

A key aspect of AhR activation by high affinity ligands is the rapid loss of AhR protein to proteasome-mediated degradation [288, 389]. To look for potential differences between these two drugs versus known AhR ligands, their ability to induce AhR protein degradation was examined. Comparably to TCDD and B[a]P, M50367 was capable of causing a high degree of AhR protein turnover after a 6 h incubation period in Hepa-1c1c7 cells (Figure 4-7). The M50354 compound was also capable of inducing AhR degradation although to a lesser extent than M50367. In summary, the consistent ranking order for each agent in causing AhR protein degradation within a 6 h period was TCDD > M50367 > B[a]P > M50354. However, the level of CYP1A1 protein expression was comparable in the TCDD, M50367, and B[a]P experimental samples at 6 h, with lower but significant levels induced by M50354.

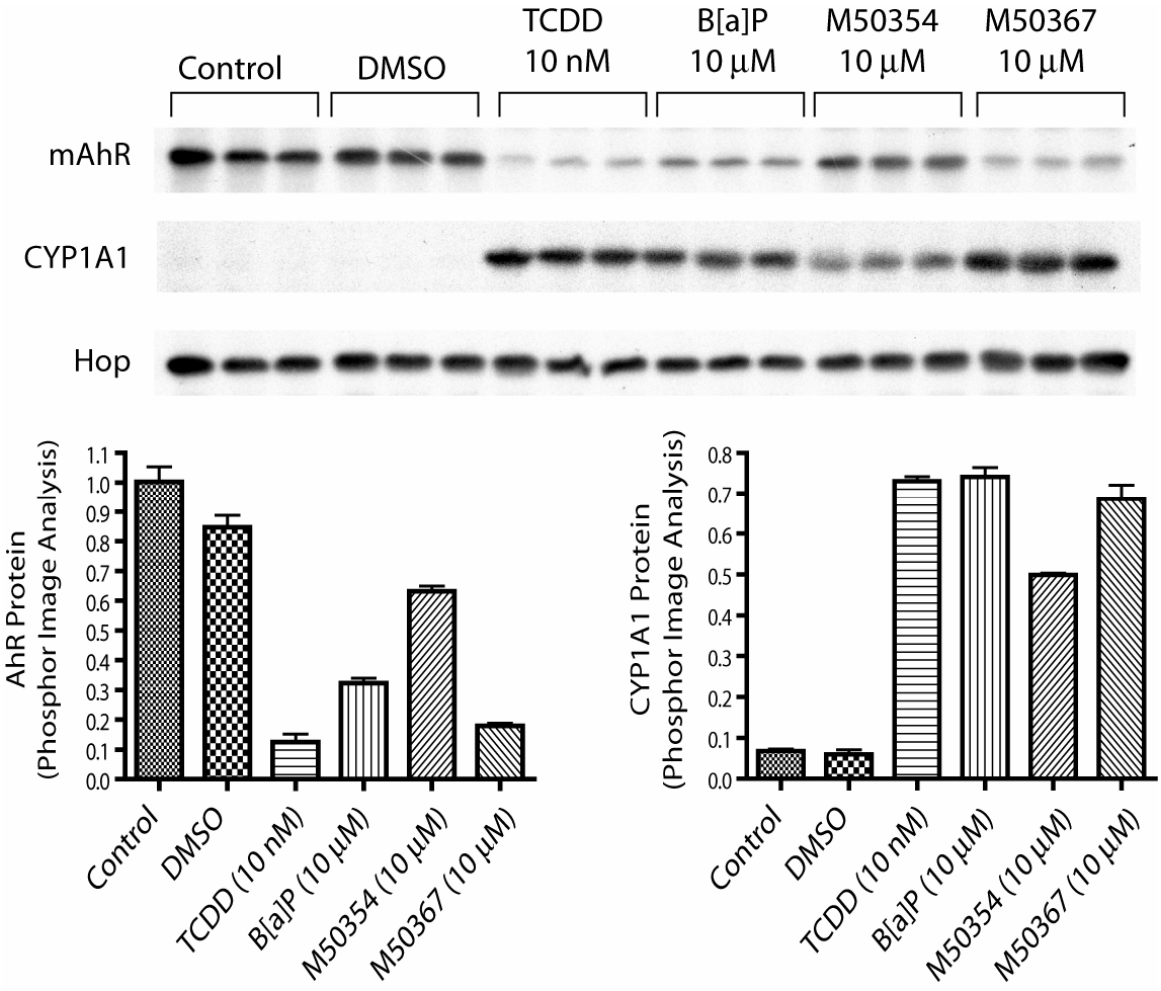


Figure 4-7: **M50354 and M50367 induce AhR protein turnover**.

Following a 6 h treatment with TCDD (10 nM), B[a]P (10 µM), M50354 (10 µM), or M50367 (10 µM), whole cell extracts were prepared in RIPA buffer, proteins were resolved by TSDS-PAGE, and blotted into PVDF membranes. Primary antibodies mouse monoclonal IgG_1 RPT1 (Affinity BioReagents), rabbit IgG H-70 (Santa Cruz), and mouse IgG F5 (kind gift from David Toft) were used to identify the AhR, CYP1A1, and HOP proteins, respectively. To quantitatively detect proteins these were labeled with biotin-conjugated secondary antibodies and [^{125}I]-streptavidin. The relative protein levels were determined by phosphor image analysis and normalized to HOP protein levels, which were not affected by treatments. Proteins were visualized by autoradiography.

Having established that M50367 and M50354 can cause a high degree of AhR protein turnover, we wanted to determine whether CYP1A1 protein upregulation was transient in nature. Therefore, Hepa-1c1c7 cells were pulsed with each compound for a total of 6 h and 36 h. However, the 6 h treatment group (Group A), just like the 36 h (Group B), was also harvested at the 36 h time point and protein levels were analyzed. As expected, the level of CYP1A1 expression seen with TCDD at 36 h for Group A was comparable to Group B, perhaps accounting for its non-metabolizable nature and high affinity for the AhR (Figure **4-8**). For Group A, measurable differences in CYP1A1 protein expression could only be observed for TCDD, B[a]P, and M50367. Thus even after the M50354-dependent induction of CYP1A1 seen in our 6 h experiment (Figure 4-7), the levels of CYP1A1 expression had returned to basal levels by 36 h in Group A (Figure 4-8). Surprisingly the level of CYP1A1 expression remained relatively high for M50367 for Group B and unexpectedly even above B[a]P. Finally, an approximately two-fold CYP1A1 protein expression was seen for M50354 in Group B.

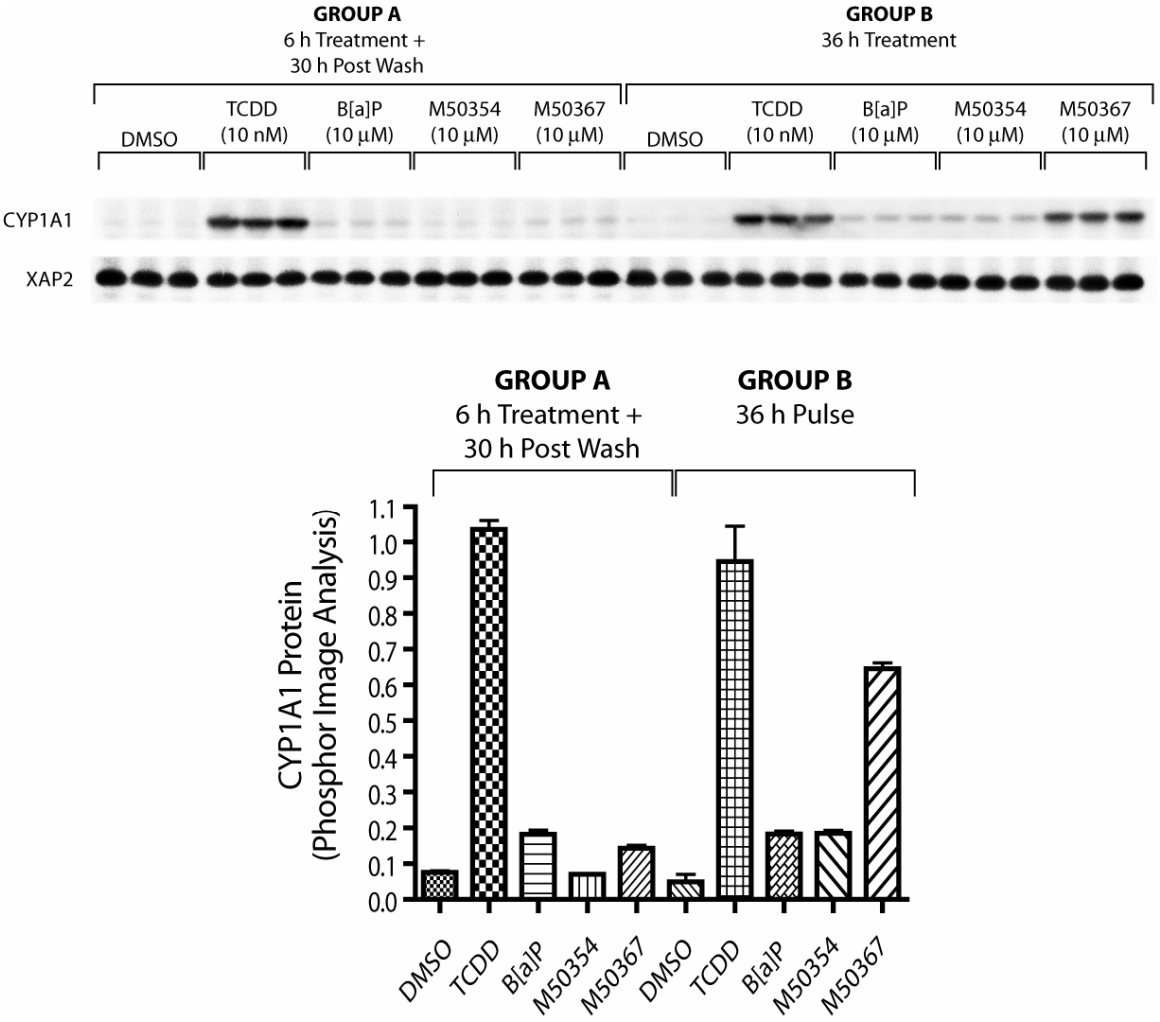


Figure 4-8: **CYP1A1 induced activity after a short and long exposure to M50354 and M50367**

Hepa-1c1c7 cells were pulsed with TCDD (10 nM), B[a]P (10 μM), M50354 (10 μM), or M50367 (10 μM) for a total of 6 h or 36 h. The 6 h samples (Group A) were washed with PBS three times and fresh medium was administered and continued to incubate for the remaining 30 h period. The quantification of CYP1A1 and XAP2 protein expression was determined after 36 h, following the same protocol as in Figure 4-7, except CYP1A1 values were normalized to XAP2 protein levels, which were also not affected by the treatments.

4.5 Discussion

The anti-asthmatic properties of the drug M50367 and its active metabolite M50354 were revealed in a study designed to identify novel substances capable of reducing allergic disease scores in a mouse model of atopic asthma and airway hyperresponsiveness [298, 299]. It was established that these compounds were capable of promoting a shift in T-helper cell balance towards a T_H1 phenotype, while promoting a reduction in IgE production and eosinophil infiltration at sites of inflammation. The AhR became a prime suspect in this M50354/M50367-elcited T_H1 shift, given the ability of both compounds to induce CYP1A1, a gene directly regulated by the AhR [166], and the absence of a therapeutic effect in AhR null mice [297]. Paradoxically, these drugs were coined as mild CYP1A1 inducers [297]. As shown in Figure 4-3**,** these two substances were potent inducers of CYP1A1 activity in Hepa-1c1c7 cells, well beyond what was appreciable through reporter assays. However, these discrepancies are reconciled from our DRE-driven reporter gene-model results (Figure 4-4A and 4-4B), in which a time-course experiment in HG40/6 and H1L1.1c2 cells with M50367 enantiomers revealed that reporter gene expression had returned to near basal levels by 24 h, while induction by TCDD remained highly elevated (Figure 4-4A and 4-4B). If this time-point in a reporter-gene experimental approach was considered alone, as in a previous report [297], the ability of M50367/M50354 to activate the AhR would be clearly underestimated.

Furthermore, our survey of some AhR target genes CYP1B1, UGT1A2, and epiregulin, further illustrated that all were readily activated by either of the M50367 enantiomers (Figure 4-5). These results are all consistent with the notion of M50367 as a full AhR agonist at the dose and time frames examined, while in comparison M50354 exhibited similar activity (Figure 4-3B). Given that M50354 is the active metabolite [298], these minor differences are likely the result of M50367 having higher bioavailability in cells as stated previously [298, 299]. An interesting aspect of these results is the lower reporter gene expression (Figure 4-5) and higher CYP1A1 activity (Figure 4-3B) observed by 24 h. However, these discrepancies could be related to differences in the half-life of the firefly luciferase and CYP1A1 proteins. As a result, induced metabolic enzymes (e.g., CYP1A1) would remain in the cell well beyond the initial rise and fall of AhR transcriptional activity as seen in reporter experiments, which is also consistent with the combined results shown in Figure 4-7 and Figure 4-8.

Interestingly, while no difference could be established between the M50367 enantiomers in their ability to induce CYP1A1 and CYP1B1 mRNA expression in Hepa-1c1c7 and HaCaT cells (Figure 4-4 and Figure 4-5), the evaluation of the Hep-G2-derived HG40/6 reporter cell line revealed that P2 was modestly more active than P1 in both reporter and real-time PCR experiments (Figure 4-4B and 4-4C). While the biological significance or importance of this result remains to be further established, it indicates that variations in the cell-specific magnitude of responses are theoretically possible for each enantiomer. This is consistent with the absence of this effect in HaCaT cells, which are also of human origin (Figure 4-3E). The possibility that the P1

enantiomer could be metabolized more readily than the P2 in a cell specific manner is a feasible scenario. Future studies may further assess whether these differences can be observed in a tissue-specific manner *in vivo*.

Competition binding experiments established that M50354 could displace the photoaffinity ligand more readily than M50367 (Figure 4-6). This result is consistent with previous work suggesting that M50354 was the active metabolite of M50367 [297]. Negishi's conclusion was based on the observation that shortly after an oral dose with M50367, the compound M50367 could not be detected in plasma but only as its metabolite M50354 [298]. It is important to note that while M50354 displaced the photoaffinity radioligand more readily than M50367 (Figure 4-6), this is not sufficient evidence to suggest that M50354 is the 'only' active metabolite. Clearly α-naphthoflavone, a partial AhR antagonist and agonist can displace the radioligand more readily than either M50354 or M50367, yet both M50354 and M50367 were more active at inducing CYP1A1 and reporter activity than α-naphthoflavone at 10 μM in 6 h treatments. In addition, M50367 had profound and long lasting effects in cells than M50354 (Figure 4-8). Perhaps differences in the ability of these compounds to transverse the plasma membrane and activate the AhR may account for the reduced activity of M50354 in our experiments when compared to M50367 (Figure 4-3). However, it is also probable that M50367 is simply not hydrolyzed into M50354 as readily in cells as it is *in vivo* [298]. The slow hydrolysis of M50367 into M50354 in cell culture may then account for extended activation of the AhR, as seen with the induction of CYP1A1 in Figure 4-8. It was also evident that at the concentrations of 1 μM and 10 μM the level of radioligand

displaced was not as high as expected, especially since, at these concentrations, a high level of AhR-driven gene expression could be detected in cell culture experiments. It is possible that these discrepancies are associated with the ligand binding pocket structure of the AhR *in vitro* being suboptimal for the association of M50354 and M50367. Perhaps in its 'ideal' native state, as in cells, the AhR could bind both M50354 and M50367 more readily and thus drive target gene expression as seen in cell culture experiments. However, the metabolism of these compounds into metabolites with higher affinity for the AhR is also a possibility. Another aspect we examined was whether these substances can cause AhR protein turnover. This was important since perhaps the reported actions of M50354 and M50367 could have AhR-dependent immunomodulatory functions without affecting AhR protein levels. However, consistent with the ability of high-affinity AhR ligands to induce degradation of the AhR [288, 451], treatment with either M50367 or M50354 could induce AhR turnover (Figure 4-7). Interestingly, the higher the turnover observed between each ligand tested did not correlate with the level of CYP1A1 inducibility. For example, whereas B[a]P could induce AhR degradation to a lesser degree than M50367 in the time-frame examined, the level of CYP1A1 protein induction was still comparable. This result suggests that AhR degradation and transcriptional activity are differentially regulated.

As seen in Figure 4-8, the analysis of CYP1A1 expression after a 6 h pulse or 36 h pulse with each compound revealed that M50367 had greater potency and lasting effects than M50354 on the expression of CYP1A1 protein, when analyzed at the 36 h time point. While M50354 and M50367 could both induce CYP1A1 activity to a similar

degree in a 6 h dose analysis (Figure 4-3B), noticeably after 36 h, M50367 had a profound effect on CYP1A1 protein expression (Figure 4-8). Given that chronic expression of CYP1A1 and other AhR target genes has been implicated in carcinogenesis [452], perhaps our results would indicate that M50354 may be a more favorable candidate to use *in vivo.* However, the therapeutic index for these substances has yet to be determined. Therefore, future toxicological data in animals is required to fully address these questions.

A potential caveat for the use of these drugs (M50354 and M50367) in the treatment of human atopic allergic diseases is warranted by the data presented in this report. The chronic activation of CYP1A1 and other AhR target genes by TCDD and several polycyclic aromatic hydrocarbons have often been associated with several toxic end-points. Some examples include the formation of reactive endogenous metabolites (e.g., catechol estrogens) that are capable of forming DNA and protein adducts account at least for some of their carcinogenic properties [169, 170]. Accordingly, the activation of AhR target genes by these M50354 or M50367 would be of concern given the chronic nature of atopic allergic diseases and the potential need for continuous administration of these drugs during therapy. The results presented here illustrate that M50354 and M50367 are transient but potent inducers of key AhR target genes. While certain compounds can be activated into toxic derivatives, the role of AhR-induced metabolic enzymes (e.g., CYP1A1) in the detoxification of some compounds *in vivo* has also been documented. For example, whereas disruption of CYP1A1 protein expression can protect against B[a]P induced toxicity and lethality, its absence can also promote the formation

of DNA adducts by B[a]P [453]. As previously suggested, although high-affinity AhR ligands exert pleiotropic immunosuppressive effects on the production of various antibody isotypes and cytokines [454], M50367 preferentially affected T_H2-specific cytokine and antibody production [297]. Therefore, our report does not necessarily negate the potential use of these drugs or derivatives for the treatment of atopic allergic diseases. Perhaps the transient nature of AhR transcriptional activation observed with M50354 and M50367 may not result in a toxic end-point, especially if these compounds are more readily metabolized into harmless and excretable substances *in vivo*. Similarly, the drug omeprazole (Prilosec), which has been used for many years in the treatment of gastric ulcers and acid reflux disease, is also an inducer of CYP1A1 activity [244, 455]. Yet its therapeutic value has been indicated for severe acid reflux conditions. The observations made by Negishi *et al* 2005 on the profile of cytokines and antibodies affected by M50367 treatment deserve further investigation. Since these drugs are able to fully induce AhR-mediated microsomal activity in our tests (Figure 4-3B, Figure 4-7, and Figure 4-8), their structures may still allow the AhR to achieve an unusual conformation that selectively modulates specific signaling cascade branches of the immune system. Alternatively, the metabolism of this compound by AhR induced metabolic enzymes may activate it into a modulator of other signaling pathways (e.g., immune system branch).

We have established that both M50354 and M50367 are potent but relatively transient inducers of direct AhR target genes, which is in contrast to a previous publication [297]. Given the suitability of M50354 for a diverse series of chemical modifications, this compound may be an invaluable tool for quantitative structure-activity

relationship (QSAR) studies. With specific measurable immunological parameters already established [297-299, 456] (e.g., specific cytokines, transcription factor activation and so on), perhaps more insights into AhR cellular functions could be obtained with these compounds serving as molecular backbones in future QSAR studies.

Chapter 5

Summary and Conclusions

Several research groups [288, 389, 457-460] have made significant efforts to understand AhR protein regulation in the cell, given its role in mediating many of the toxic effects elicited by some chlorinated and non-chlorinated polycyclic aromatic hydrocarbons [388, 461]. It is believed that AhR degradation in the cell may be an important homeostatic process to prevent the uncontrolled or constitutive expression of metabolic enzymes (e.g., CYP1A1, CYP1B1), which can have detrimental consequences to the organism (e.g., cancer, hormone disruption, etc) [462]. Despite substantial evidence suggesting that the AhR is regulated in an ubiquitin- and proteasome-dependent manner [288, 389, 451, 458, 463], no ubiquitin ligase had been associated with AhR ubiquitination nor convincing evidence of AhR ubiquitination had been demonstrated in the field. The first project in this thesis addressed the hypothesis of whether the hsc70-associated E3 ubiquitin ligase CHIP was involved in the ligand-mediated attenuation of the AhR protein levels through the proteasome. Given the preliminary observations that 1) CHIP could be immunoprecipitated with the AhR complex at endogenous levels, 2) its transient expression in cells could mediate AhR turnover, and 3) CHIP's well established E3 ubiquitin ligase activity towards many steroid receptors, it was logical to hypothesize that CHIP was associated with AhR protein level regulation in the cell. In chapter 3 we established for the first time compelling evidence of AhR ubiquitination promoted by the CHIP E3 ubiquitin ligase complex. However, a role for CHIP in the regulation of ligand-mediated degradation of the AhR or the control of its steady-state protein levels could not be established. Experimentally, the latter statement is supported by the knock-down of CHIP through siRNA methods, which had no appreciable impact on AhR steady state protein levels nor affected well characterized AhR activities[296 3338], which is also

consistent with CHIP$^{-/-}$ mice data[390]. Consequently, the ubiquitin ligase system(s) responsible for the ligand-mediated and steady-state maintenance of the AhR protein levels were not established by this thesis. Nevertheless, the interaction of CHIP with the AhR complex especially within the context of endogenous cellular levels remains intriguing. It seems plausible that the association of CHIP-mediated AhR degradation may be exclusive to protein overexpression models, especially since CHIP interacts with the carboxyl terminus of both hsc70 and hsp90. Accordingly, it is possible that an excess of CHIP expression in the cell could simply compromise the stability of many chaperone client proteins by affecting the substrate binding cycle of hsp70 and dhsp90 through CHIP overloading [401]. However, the level of specificity achieved *in vitro* with purified and partially purified components may also suggest that, while we do not understand how this process may be regulated in the cell, CHIP-mediated degradation of the AhR may occur under a permissible yet to be established condition. It is also theoretically possible that other ubiquitin ligase systems may be compensating for the loss of CHIP in the cell. For example, the transcription factor p53 is regulated by multiple, often redundant, ubiquitin-mediated degradation pathways, some of which include CHIP itself (discussed in chapter 3). Similarly, CHIP may simply be one redundant molecule in the regulation of AhR protein levels and that of many already described steroid receptors in the literature [293, 401, 418, 464, 465]. Our *in vitro* ubiquitination assays suggested that other entities, perhaps other E3 ubiquitin ligases or adaptor proteins, may work in concert with CHIP to mediate AhR ubiquitination and degradation [296]. Therefore, it seems attractive to suggest that by utilizing the *in vitro* ubiquitination approach established for the AhR, it may now be possible to screen a series of well characterized ubiquitin conjugation

enzymes (E2s) for their ability to promote AhR ubiquitination and degradation through the proteasome. This approach is rather simple and would help to narrow down the search for an AhR-specific ubiquitin conjugation system(s). The ubiquitin ligases identified in this manner would need to be further validated *in vivo*, either through gene knockout models or the more practically through a siRNA approach. Yet it is important to consider that much remains to be learned about the mechanisms of substrate selection by CHIP and the regulation of CHIP protein levels. Perhaps when such information becomes available its role in the AhR protein complex could be reexamined with *in vivo* models.

The study of AhR protein level regulation remains a challenging area of investigation. For example, much controversy exists in that this process appears to be regulated in a cell-specific manner, through different proteolytic systems (e.g., proteasome and calpain [250, 460]), and possibly by more than one ubiquitin-dependent process. The cellular localization of the AhR also appears to influence receptor stability as well as the substances inducing AhR destabilization (e.g., TCDD vs. GA). For instance, the treatment of Hepa-1c1c7 and Hep-G2 cells with leptomycin B, which blocks nuclear export of the AhR, was shown to significantly reduce the ligand-mediated degradation of the AhR, suggesting that nuclear export of the AhR is required for its ligand-mediated degradation by the cytosolic proteasome [389, 466]. Interestingly, it has also been shown that ligand mediated degradation of the AhR can be blocked by actinomycin D (AD) and cycloheximide (CHX), while geldanamycin-induced AhR protein degradation is not inhibited by the same treatments and in the same cell lines [416]. Intriguingly, both ligand-mediated and GA-induced AhR degradation are

proteasome dependent processes but evidently appear to be regulated through different mechanisms [288, 289]. The transactivation domain of the AhR also appears to impart regulatory control over AhR turnover through the proteasome. For example, ligand induced degradation of deletion mutants of the AhR such as the AhR_{1-500} and AhR_{1-640}, could not be blocked by AD or CHX treatment [416]. Therefore, factors affected by AD or CHX treatment require a functional AhR TAD to impart AhR protein stability. In addition, blocking of ligand-mediated degradation of the AhR by AD required 'sufficient' ARNT expression, as AD was ineffective in cells expressing low amounts of ARNT [416]. These studies strongly suggest that the regulation of the AhR protein levels may be controlled by multiple factors, perhaps through interaction domains (e.g., TAD) and partner proteins (e.g., ARNT). Whether any of these factors are related to the CHIP-mediated ubiquitinating activity we detected in our *in vitro* ubiquitination experiments of the AhR remains to be explored. An adaptation of the protocol established in chapter 3 for *in vitro* ubiquitination of sucrose gradient fractionated AhR could now be exploited to search for cellular fractions containing active components responsible for each of the AhR degradation pathways explored previously by Pollenz [463]. In the event that no sucrose gradient fraction contains all the factors necessary to recapitulate each of the events observed by Pollenz, the systematic combination of gradient fractions along with purified components of the ubiquitin-proteasome system could be tested. This approach could yield valuable information that may lead to the identification of key components required for AhR ubiquitination and degradation *in vivo*. A similar approach was utilized recently to identify enzymes involved in the ubiquitination of the ER by a novel AhR-associated ubiquitin ligase complex [20]. For example, sucrose gradient fractionation

along with an immunoprecipitation approach was utilized to isolate AhR complexes with different sedimentation properties. The factors associated with the AhR were then analyzed through mass spectrometry and their sequences were matched to known components of the ubiquitin-proteasome pathway (e.g., CUL4B).

Much could be done to possibly identify the ubiquitin ligase system(s) associated with the ligand-mediated proteasomal degradation of the AhR or the maintenance of its steady-state protein levels. The systematic use of siRNA methods to knock-down expression of ubiquitin conjugating enzymes (E2s), especially those known to be promiscuous, could more decidedly help identify biologically-relevant ubiquitin conjugation systems associated with AhR turnover. The UbcH4/UbcH5 family would be the first logical target, given that it collaborates with CHIP and other well characterized E3 ligases such as E6AP and Nedd4 and its addition to crude rabbit reticulocyte lysates could trigger AhR ubiquitination in the absence of exogenous added CHIP[296, 467]. Subsequent use of siRNA knockdown methods to target specific E3 ligases known to associate with the E2 identified would help narrow down the ubiquitin conjugation system involved. Finally, once an *in vivo* or cell culture candidate(s) is identified it could be tested for its ability to ubiquitinate the AhR *in vitro* and thus confirm its direct role in the ubiquitination and possible degradation of the AhR. Existing information on interacting partners for each E2 could be used to narrow down the screen for potential E3s involved in AhR regulation. A useful list of these E2 ligases along with their E3 interacting partners was published in a review article[400] by the ubiquitin expert Dr. Cecile Pickart, who regrettably passed away recently due to a cancer-related illness. I

believe these approaches could help identify the ubiquitin conjugation system(s) responsible for AhR protein regulation in the cell. Once candidates are identified, a simple *in vitro* ubiquitination approach as established in chapter 3 could decisively confirm the association of such ubiquitination system with AhR protein-level regulation. A caveat exists with the use of a siRNA approach to target E2s. It is possible that by affecting the expression of a multifaceted enzyme more than one signal transduction pathway could be compromised leading to confounding results. Nervetheless, once a candidate is discovered the use of other methods such as in vitro ubiquitination and interaction assays such as immunoprecipitations could be utilized to further validate that a given enzyme is directly involved in AhR regulation. Another complication is that while we provide evidence of AhR ubiquitination, this does not confirm that the ligand-mediated and proteasome-dependent degradation of the AhR occurs in such fashion, even when the expression of ubiquitin mutants suggests otherwise [391]. Interestingly, the enzyme ornithine decarboxylase (ODC) is degraded through the proteasome in a non-ubiquitin but antizyme-1 (AZ1)-dependent manner [468]. It appears that AZ1, a protein important for the regulation of polyamine synthesis (e.g., spermidine) in the cell [469], is recognized by receptors in the proteasome in a manner that is similar to polyubiquitin chain recognition [468]. Similarly, the transcription factor Smad1 is not only regulated by AZ1 but also through ubiquitination [470]. Therefore, the ubiquitination of other AhR complex proteins or the theoretical interaction of the AhR with factors, perhaps similar to the interaction between AZ1 and ODC, may be sufficient to target the AhR for degradation. These ideas should be considered when evaluating future AhR protein regulation experimental data.

The discovery of hsp90 as a direct target for CHIP-mediated ubiquitination was perhaps the most surprising information gathered in this thesis. When CHIP was discovered it was described as an hsc70 interacting protein that negatively regulated the substrate binding cycle of this chaperone [290]. Whereas CHIP was capable of mediating hsc70 ubiquitination as well, no direct link to its degradation could be established at the time [291]. In 2006 Qian and colleagues demonstrated that CHIP could mediate turnover of heat shock induced hsp70 and hsp90 in a process that reestablishes the steady state levels of these important chaperones [295]. Our work suggested that perhaps CHIP accomplishes this hsp90 protein level regulation through an ubiquitination-dependent process and through the use of non-canonical ubiquitination of the hsp90. This CHIP-mediated regulation of hsp90 through ubiquitination may help explain why its overexpression in cells is known to affect negatively most hsp90 clients examined to date. It is interesting to note that while CHIP could be previously associated with hsp90 turnover, the knockdown of CHIP under steady-state conditions had no impact on hsp90 protein levels [296]. Therefore, intracellular changes involving the raise in hsp90 levels caused by heat shock stress seem to regulate this hsp90 CHIP-mediated protein degradation process [295]. This leads to the question: what is the nature of this signal? Some possible insights were published by Rees and colleagues. For example, recognition of the androgen receptor by the CHIP ubiquitin ligase has been suggested to depend on its phosphorylation status [418]. A simple search in PubMed on phosphorylation dependent ubiquitination also shows hundreds of proteins that are regulated in such manner (e.g., inhibitor I kappa-B alpha [471]). Hsp90 is known to be phosphorylated at

two serine residues by casein kinase II [472] and at two threonines by the DNA-dependent protein kinase [473]. Furthermore, the phosphorylation status of the hsp90 seems important for the modulation of its chaperone functions [474]. As a result, it seems logical to test whether these mutations would have any impact in CHIP mediated ubiquitination and degradation of the hsp90 after its heat shock induction or whether these mutations in hsp90 have any effect on CHIP mediated degradation of its client proteins.

Some interesting insights were acquired through the use of ubiquitin mutants for our *in vitro* ubiquitination reconstitution assays of the hsp90. These mutants are often utilized as proof of chain assembly through a particular lysyl residue within ubiquitin on target proteins. However, there appears to be a serious problem with their use, especially without proper controls. Mutations in ubiquitin could have serious implications for its recognition and proper use by ubiquitin conjugation pathway enzymes (UCPE)[364]. With the use of mutant ubiquitin variants it is normally assumed that if a particular chain is not assembled it is due to the fact that such chains could not be formed by the UCPE under investigation. However, just like some ubiquitin surface residues, besides lysyl residues, are important for the recognition of ubiquitinated substrates by the 19S cap of the proteasome, the generation of multiple lysyl ubiquitin mutants, even with conservative mutations, may affect how these could be utilized by the UCPE and processed by the proteasome. Notably, when we utilized the ubiquitin mutant K48, which has all lysyl residues mutated except K48, no ubiquitination of hsp90 could be detected. Other laboratories have used such information to state that the UCPE under study cannot

assemble the type of ubiquitin chain expected, as in the hsp70 example shown by Qian et al. 2006 [295]. However, when the zero lysyl ubiquitin was utilized, multiple ubiquitinated forms of the hsp90 could be observed, consistent with multiple site ubiquitination [296]. These results highlighted the fact that the K48 ubiquitin, used commonly to demonstrate non-canonical ubiquitination of proteins, could be defective to the point where it cannot be used for UCPE-mediated conjugation of itself to hsp90 [296] or hsc70 [295]. It is likely that the mono lysyl ubiquitin (K48) achieves an unusual conformation that prevents its use by the ubiquitin activating or conjugating enzymes. However, this thesis does not establish whether the problem lies in the inability of E1 to activate ubiquitin for transfer to the E2 or any other enzymatic steps. Intriguingly, a recent publication suggested that CHIP can assemble ubiquitin chains with the K48 ubiquitin mutant and the E2 UbcH5 [467]. Therefore, it appears that, depending on the substrate, some UCPEs may be unable to utilize the K48 ubiquitin mutant for conjugation as in the CHIP-mediated ubiquitination of the hsp90. These issues could be related to stereochemical limitations of the ubiquitin mutants for conjugation on a substrate-specific manner. Therefore, analogous to the use of dideoxynucleotides or deoxynucleotides lacking a 3'-OH group required for the formation of a phosphodiester bond in DNA sequencing, the use of monolysyl ubiquitin mutants could be utilized in combination to determine ubiquitin chain architecture on CHIP target proteins.

In chapter 4 we described the synthesis and characterization of the anti-asthmatic drugs M50354 and M50367. Interestingly, these drugs were originally presented as marginal inducers of direct AhR target genes, while being efficacious towards reducing

disease scores for atopic allergic disorders [297]. An unorthodox endogenous role for the AhR in the immune system was therefore implied by this published work. However, we found that both M50354 and M50367 were potent but relatively transient inducers of AhR transcriptional activity, perhaps due to their rapid metabolism [298]. These two drugs also mediated AhR protein turnover which is typical of potent AhR ligands [457]. The main discrepancies between the original work [297] and the data presented in chapter 4 reside in the methods utilized and more importantly the time-frame examined. Our finding raises concerns towards the use of these drugs in the treatment of allergic disorders in humans. Certainly the most important yet missing information is whether the use of these drugs in test animals would have the same carcinogenic effects as other known AhR ligands, such as the polycyclic aromatic compound B[a]P and TCDD. However, given the observation that these anti-allergic drugs are potent inducers of DRE-driven AhR target genes and still display 'selective' higher efficacy towards ameliorating atopic asthma disease scores, more investigation is warranted. Mouse models in which the endogenous AhR has been replaced with the human homolog of the gene (often termed 'humanized mice') may therefore provide additional insights, especially since these two receptors have affinities for common ligands that can vary by at least 10-fold and certain regions such as the transactivation domain are less conserved between the human and mouse species [249].

An inevitable question arises from the work presented in chapter 4: is the AhR directly involved in the modulation of the immune system? In addition, is this a truly endogenous function of the AhR? It has been reported that AhR null mice suffer from

deregulated T_H1 and T_H2 branches of the immune system [297]. Therefore, on the surface it does appear that the AhR may be at least playing a balancing role in modulating immunological responses. Interestingly, the substance D-pinitol, which does not resemble the characteristics of AhR ligands, has been shown to suppress similar allergic inflammation pathways as the anti-allergic drugs that were examined in this thesis [475, 476]. It is therefore tempting to speculate that the effects of M50354 and M50367 could be mediated through their metabolism into substances capable of modulating the immune system as opposed to the AhR being directly involved in the regulation of some immune system related genes, such as physically altering their transcription (e.g., possible transrepression of *GATA-3* [297]). Perhaps the role that the AhR plays in this process could be to upregulate metabolic enzymes that would activate both M50354 and M50367 into active suppressants of naïve T_H differentiation. However, it is also possible that D-pinitol could be regulating the production of an endogenous AhR ligand and thus having a similar effect as the M50354 and M50367 compounds. Consequently, it would be interesting to evaluate whether D-pinitol has the same reported [475, 476] immunomodulatory functions in $AhR^{-/-}$ mice. Another possibility is that the activation of the AhR by M50354 and M50367 may lead to estrogen receptor degradation. For example, in the presence of a high affinity ligand like TCDD, the AhR has been suggested to form a multi-protein complex with the estrogen receptor and, apparently acting as an E3 ubiquitin ligase, mediates ER protein degradation through the proteasome [20]. Recent publications suggested that sex hormones may play an important role in the development of allergic inflammation in mice [477, 478]. Therefore, it is tempting to hypothesize that at least some of the mechanisms of action of M50354 and M50367 could

be related to AhR mediated modulation of the estrogen receptor, perhaps through ER protein turnover. This hypothesis could be addressed by first determining whether the treatment of cell cultures with M50354 or M50367 leads to ER protein turnover, in an AhR dependent manner. While the downregulation of ER by M50354 and M50367 in an AhR dependent manner would not provide direct proof of its role in the effects of these drugs, existing evidence on the role of the ER and sex hormones in the modulation of immune responses would at least suggest that such a relationship may exist.

The work of Negishi should be reevaluated as they claimed that the effects of the M50354 and M50367 compounds were 'exclusive' to naïve T helper cells in an AhR dependent manner and that $AhR^{-/-}$ mice have deregulated T_H1 and T_H2 immune responses [297]. Given that the developmental stages of all $CD8^+$ T cells were not fully evaluated by previous work [297-299] it would be attractive to explore whether the timing of dose during the development of several immune cells (e.g., natural killer cells, dendritic cells, etc) would have an effect in their biological roles or maturation. Since the AhR ligands B[a]P and 3-MC have been implicated as inhibitors of dendritic cell development and function [440, 479-481], it would be important to test whether these anti-allergic drugs would have a similar effect on these cells and thus determining whether their therapeutic value is truly limited to naïve T_H cells, as stated previously [297]. Finally, if the AhR plays a direct role in regulating the immune system, then perhaps the ability to easily modify the chemical structures of M50354 and M50367 could be used effectively in quantitative structure-activity analysis (QSAR) of the AhR ligand binding pocket, along with the chemical and functional characterization of various immune cells. With the

availability of a transgenic mouse expressing a DNA-binding mutant of the AhR (Perdew, unpublished data), further insights could be obtained about the role of the AhR in modulating the immune system branch without DRE-driven gene expression. Under ideal circumstances these QSAR studies utilizing M50354 as a backbone may even yield information to suggest a physiologically important endogenous ligand for the AhR.

Appendix

Research Publications and Sources of Financial Support

Portions of this thesis were reproduced in part or in full with permission from the publisher of the following peer-reviewed publications:

Morales J.L., Krzeminski J., Amin S., Perdew G.H.. Characterization of the Antiallergic Drugs 3-[2-(2-Phenylethyl) benzoimidazole-4-yl]-3-hydroxypropanoic Acid and Ethyl 3-Hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl]propanoate as Full Aryl Hydrocarbon Receptor Agonists. *Chemical Research in Toxicology*. 21(2):472-82. Reprinted with permission from the American Chemical Society © 2008

Beischlag, T.V., **Morales, J.L**., Hollingshead, B.D., and Perdew, G.H. Aryl hydrocarbon receptor complex and the control of gene expression. *Critical Reviews in Eukaryotic Gene Expression,*18(3) pp. 207-215. Reprinted in part with permission from Begell House, Inc. © 2008

Morales, J.L., Perdew, G.H.. 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*. *Biochemistry* 2007**,** 46:610-621. Reprinted with permission from the American Chemical Society © 2007

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PUBLICATIONS

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