Published in final edited form as:

Arch Biochem Biophys. 2008 April 15; 472(2): 77–88.

Molecular Determinants of Species-Specific Agonist and Antagonist Activity of A Substituted Flavone towards the Aryl Hydrocarbon Receptor*

E. C. Henry and T. A. Gasiewicz

Department of Environmental Medicine, University of Rochester Medical Center, Rochester NY 14642

Abstract

The aryl hydrocarbon receptor (AhR) mediates the toxicity of dioxins and related xenobiotics. Other chemicals also bind the AhR to elicit either agonist or antagonist responses. Here we used site-directed mutagenesis within the ligand binding domain of murine AhR to probe for specific residues that might interact differentially with the antagonist 3'-methoxy-4'-nitroflavone (MNF) compared with the prototypical agonist TCDD. Reduced ³H-TCDD binding, dioxin-response element (DRE) binding, and transcriptional activity were observed for several point mutants. One mutation, R355I, changed the response to MNF from antagonist to agonist. Notably, Ile is the residue found in the guinea pig AhR, towards which MNF has partial agonist activity in contrast to its strong antagonist activity in mouse. A similar reversal of response to MNF was observed in chimeric AhRs in which the C-terminal region of mAhR was replaced with the guinea pig C-terminal region. These data demonstrate that different amino acids can be important in binding of different AhR ligands and can mediate distinct responses. The ultimate response of the AhR also depends on how other portions of the receptor protein are functionally coupled to the initial ligand binding event.

Keywords

aryl hydrocarbon receptor; point mutation; chimeric receptor; flavone; CYP1A1; TCDD; antagonism

INTRODUCTION

The aryl hydrocarbon receptor (AhR)¹ is a ligand-dependent transcription factor and mediator of toxicity of environmental contaminants such as dioxins, PCBs, and PAHs. It is also necessary for normal development and function of many tissues and organs [1–3]. Its endogenous ligand has not been identified, nor have the pathways of endogenous function been delineated. However, the mechanism of its transcriptional enhancement of AhR-responsive genes, notably

*This work was funded by NIH Grants ES09702 and Center Grant ES01247.

Corresponding Author: Ellen C. Henry, Box EHSC, University of Rochester Medical Center, Rochester NY 14642, e-mail: ellen_henry@urmc.rochester.edu, FAX: 585-256-2591, Phone: 585-275-1776.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

¹**Abbreviations used:** AhR – aryl hydrocarbon receptor; Arnt – aryl hydrocarbon receptor nuclear translocator protein; βNF – β-naphthoflavone; DRE – dioxin responsive element; EMSA – electrophoretic mobility shift assay; MNF – 3'-methoxy-4'-nitroflavone; LBD – ligand-binding domain; 3MC – 3-methylcholanthrene; MCPA – 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide; PAH – polycyclic aromatic hydrocarbon; PCBs – polychlorinated biphenyls; TAD – transcriptional activation domain; TCDF – 2,3,7,8-tetrachlorodibenzofuran.

those encoding several drug-metabolizing enzymes such as certain cytochrome P450s (e.g. CYP1A1) has been well-studied. The binding of ligand such as TCDD initiates a series of molecular events leading to a transcriptionally active AhR conformation that, as a complex with its heterodimeric partner ARNT, is able to bind *cis*-acting dioxin-response elements (DREs) to modulate gene expression. Although TCDD and structurally related xenobiotics are the best-studied and most potent AhR ligands, a large group of both natural and synthetic compounds of great structural diversity has been identified that also can bind the AhR[4]. Some of these substances act to inhibit the response to agonists such as TCDD. Among the more potent of these antagonists are several substituted flavones [5–8] and MCPA [9]. Although these flavones bind the AhR competitively with TCDD, they apparently do not initiate the conformational change in AhR that is necessary to elicit nuclear localization, dimerization with Arnt, and transcriptional activation [7]. The question remains, however, as to how the interaction with the AhR ligand-binding domain (LBD) differs between agonist and antagonist. Considering the multitude of structurally related dioxin-like pollutants to which we may be exposed, an understanding of these differences will have important implications for risk assessment. This information may also help to identify structural constraints for an endogenous ligand.

It is not well understood what precise molecular events are initiated by agonist binding and that lead to the transcriptionally active AhR conformation. However, additional information on the role of ligand may be obtained by using tools such as these antagonists. For example, structure-activity analysis of some flavone derivatives suggested that among 3'-methoxy-4'-substituted flavones, the most potent have an electron-withdrawing group such as NO₂ or N=N=N at the 4' position [6,7]. Thus, unlike TCDD, such molecules might interact electrostatically or through hydrogen bonding with positively charged amino acid(s) within the AhR ligand-binding pocket. This observation provides a starting point for further examination of the interaction between ligand and receptor by comparing activities of AhR containing mutations of specific amino acids.

It has been shown that the wide disparity among species in sensitivity to TCDD toxicity is not simply due to corresponding differences in AhR ligand binding affinity [10]. Although there is no adequate explanation as yet for the qualitative and quantitative species differences in toxicity, it is reasonable to assume that it is partially attributable to qualitative differences in the interaction between ligand and AhR and the consequent ligand-dependent effects on gene expression. We have observed species differences, at least *in vitro*, in AhR response to flavones: certain flavones that are good antagonists of TCDD-induced DRE binding and reporter gene induction in mouse cells have substantial agonist activity in guinea pig cells [8]. Further study of this species difference may offer clues regarding a possible difference in ligand positioning within the ligand-binding pocket that mediates agonism *versus* antagonism, or whether some other domain of the receptor mediates this disparity. Here we report on investigations to determine what region and/or specific amino acids of the AhR may contribute to these differences. Utilizing an hypothesized 3-D model of the AhR ligand-binding pocket [11], we tested the effect of several point mutations within the ligand-binding domain on TCDD binding, DRE binding, and transcriptional activity. We also compared some chimeric AhRs constructed by exchanging domains between mouse and guinea pig AhR. The mutation of R355 in the mouse AhR to the equivalent guinea pig residue resulted in a change in response to the flavone 3'-methoxy-4'-nitroflavone (MNF) from antagonist to agonist. A similar switch in behavior was observed in chimeric AhRs in which the C-terminal region of the mouse AhR was replaced by the guinea pig C-terminal region. Thus, the type of response of the AhR to ligand is determined by interactions between the ligand and specific amino acids within the ligand-binding pocket as well as by how other parts of the receptor protein respond to the ligand binding event.

MATERIALS and METHODS

Chemicals

TCDD was obtained from Cambridge Isotopes (Cambridge MA), and [^3H]TCDD was from Chemsyn Science Laboratories (Lenexa KS). MNF was synthesized by the procedure of Cunningham et al. [12] as previously described. All ligands were dissolved in DMSO.

AhR Constructs

Point mutations were introduced into the expression plasmid for mouse AhR^{b-1} (pcDNA3 β AhR, originally obtained from O. Hankinson) using the QuikChange Site-Directed Mutagenesis Kit following manufacturer's directions (Stratagene, La Jolla CA). Preparation of pcDNA3.1gpAhRwt was described previously [8].

C-terminal chimeric receptors were made by exchanging the C-terminal residues 370–805 of the mAhRwt and the equivalent residues 375–846 of the gpAhRwt. This was achieved by introducing an *FseI* recognition site (which was possible with a silent mutation) into both receptor cDNA sequences, so that cutting the expression plasmids of both species with *FseI* and *Apal* (cut site in the multiple cloning region of pcDNA3 and pcDNA3.1) removed the C-terminal portion of each sequence. This *FseI*-*Apal* fragment from each species was annealed to the N-terminal portion of the other species (still within the vector) after purification by agarose gel electrophoresis. This resulted in pcDNA3-m/gp Δ C and pcDNA3.1-gp/m Δ C expression plasmids (Fig. 1).

Ligand-binding domain (LBD) chimeric receptor constructs were prepared by introducing a recognition site for *HpaI* into both pcDNA3 β AhR (*FseI*) and pcDNA3.1gpAhR (*FseI*) so that both parental plasmids then contained a *HpaI* site and a *FseI* site which bracketed the approximate LBD. Digestion of these mutated constructs with both *HpaI* and *FseI* yielded a LBD fragment of approximately 0.4kb from each species that was purified from the agarose gel and ligated into the remaining fragment (vector minus LBD fragment) from the other species. The mutation that was necessary to form the *HpaI* site caused an amino acid change (Asp to Val); this was remutated to give Asp in the final chimeric constructs. These LBD chimerics (see Fig 1) are referred to as m/gpLBD (mouse AhR with LBD region from guinea pig) and gp/mLBD.

Correct mutations and ligations were confirmed by DNA sequencing of the mutant expression plasmids using Big Dye Terminator DNA sequencing kit (Perkin-Elmer, Branchburg NJ) at the University of Rochester Nucleic Acid Core Facility.

In vitro transcription/translation, ligand binding, EMSA:

Wild type and mutant or chimeric AhRs were synthesized in rabbit reticulocyte lysate using the TNT® system (Promega, Madison WI), mixed with similarly expressed murine Arnt, and diluted with HEDG buffer [25mM Hepes, pH 7.6, 1.5mM EDTA, 1mM DTT, 10% v/v glycerol] for ligand binding, as described previously [13]. After 1.5 – 2 h incubation at room temperature with ligands, aliquots were incubated with herring sperm DNA, 0.08M NaCl, 10mM DTT, and ^{32}P -DRE oligonucleotide and analyzed on a nondenaturing 4% acrylamide gel. Under these conditions, we have found that 10nM TCDD gives maximal DRE binding; for testing flavone antagonism, a less-than-saturating concentration (3nM) was used. Dried gels were visualized and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale CA). For ligand binding analysis, aliquots of the diluted AhR/Arnt mixtures were incubated at room temperature with several concentrations of [^3H]TCDD $\pm$ 150-fold excess TCDF for 1.5–2 h. Specific binding of [^3H]TCDD was determined by the hydroxylapatite method [14]. Data

shown in Fig. 2 are at 4nM [^3H]TCDD; binding curves (not shown) indicated that this was where the specific binding approaches saturation.

Transient transfections

AhR-deficient mouse hepatoma cells (TAO) were grown in Modified Eagle's Medium (MEM) (Mediatech, Herndon VA) with 10% fetal bovine serum. Cells were seeded into 24-well plates at 0.5×10^5 cells/well. The next day triplicate wells were transfected with 0.4 μg AhR plasmid DNA, 0.1 μg p2DLuc (DRE-dependent firefly luciferase reporter plasmid) and 0.015 μg of pRL-TK (*Renilla* luciferase plasmid for normalization) using Geneporter (Gene Therapy Systems, San Diego CA) or FuGene (Roche Applied Science, Indianapolis IN). The following day, the culture medium was changed, and ligands were added in DMSO vehicle for overnight (16–20 h) exposure. Under these conditions, 0.5 – 1nM TCDD gives maximal induction of luciferase. Lysates were prepared for measurement of luciferase activity using the Dual-Luciferase Reporter Assay system (Promega, Madison WI) and a Turner Model TD-20e Luminometer (Turner Designs, Sunnyvale CA). Ratios of firefly (DRE-dependent) luciferase to *Renilla* luciferase were calculated, and results plotted as mean $\pm$ S.D. for the triplicate samples of each treatment.

Western Blot

Aliquots of TNT-expressed samples or lysates from transiently transfected cells were mixed with SDS sample loading buffer and separated by SDS-PAGE using 8% acrylamide resolving gel. Proteins were transferred to a PVDF membrane (Immobilon, Millipore, Bedford MA), and blocked with 5% nonfat milk powder in TBST buffer [50mM Tris base, pH 7.5, 150mM NaCl, 0.2% Tween 20]. Primary antibodies were diluted in 1% nonfat milk powder / TBST and added to membranes at room temperature for at least 1.5 h. Antibodies used were: anti-AhR (Rpt-1, ascites prepared using hybridoma cells provided by Gary Perdew), anti-CYP1A1 (mouse monoclonal; Xenotech, Kansas City KS), and anti-actin (rabbit polyclonal; Sigma, St. Louis MO). Membranes were washed with TBST and incubated another hour at room temperature with the appropriate secondary antibody [HRP-conjugated anti-mouse or anti-rabbit IgG (Jackson Immunoresearch, West Grove PA)]. Proteins were visualized by chemiluminescence using reagents from KPL (Gaithersburg MD).

RESULTS

Point mutations within the ligand-binding domain selectively affect DRE binding of the mouse AhR

Several amino acids within the ligand-binding region of the mouse AhR were mutated and the effect was evaluated by expressing these mutants in a rabbit reticulocyte lysate system (TNT®) to compare their ability to bind [^3H]TCDD, and to bind a consensus DRE sequence in response to TCDD treatment. In most cases, mutations were of residues that are well-conserved across mammalian AhRs, and that are located such that they are hypothesized to interact with ligand based on the 3D model of Procopio et al. [11].

A361 is predicted to be close to A375 that is V375 in low-affinity AhRs such as the DBA ("nonresponsive") mouse or human [15]. A361 and A375 are on adjacent strands of the β -sheet that is modeled to form one side of the ligand cavity [11]. Changing this alanine to the larger but still nonpolar leucine (Fig. 2) or valine (data not shown) essentially eliminated TCDD binding and TCDD-induced DRE binding. This result, analogous to the A375V mutation referred to above, likely reflects steric hindrance by the bulkier Leu or Val residues, so that TCDD either cannot enter the ligand binding cavity and/or cannot orient appropriately to initiate high-affinity binding or AhR activation and dimerization with Arnt. When the adjacent residue, **R362**, was changed to leucine, there was less effect, despite the substitution of a

nonpolar for the charged residue. TCDD binding and DRE binding were reduced compared to AhRwt but the fold induction of DRE binding by TCDD was equal to that of AhRwt. A possible interpretation of this observation is that the R362L mutation allows TCDD access to the binding pocket although it binds with lower affinity due to a modestly changed conformation introduced by the mutation. Additionally, it is likely that the side chain of A361 rather than R362 points toward the ligand binding cavity and therefore more directly determines the ability of ligand to enter and/or bind appropriately to initiate receptor activation.

R333 and **Glu339** are hypothesized to be situated at the entrance to the ligand-binding pocket, and possibly to interact by hydrogen bonding [11]. Breaking of this hydrogen bond by ligand could be involved in receptor conformational change. R333L and to a slightly lesser extent, E339L, showed low TCDD-responsive DRE binding and substantially reduced [³H]TCDD binding, consistent with the assumption that these residues are involved in ligand binding and/or receptor activation (Fig. 2).

The basic and charged **R282** and uncharged but polar **Q377** were suggested to possibly interact with TCDD [11]. Replacement of either of these residues with the nonpolar Leu reduced [³H]TCDD binding and DRE binding, although the calculated fold induction of DRE binding elicited by TCDD was comparable to that for mAHRwt. These mutations may reduce binding although not limiting TCDD access to the ligand binding pocket. Bound ligand appears to still be able to activate these mutant receptors to a DRE-binding conformation. Additionally, **F281** was changed to the bulkier but still nonpolar Trp. This mutant also showed lower DRE binding than AhRwt, but comparable fold inducibility by TCDD. Since response by R282L and F281W seemed to be similar, it is not obvious (unlike with A361 and R362 (see above)) which of these residues likely extends into the ligand binding pocket. Steric hindrance by the bulkier Trp side chain would be expected to interfere with ligand binding, as observed; the fact that its effect was less dramatic than A361L is consistent with the likelihood that F281 is not as centrally located in the ligand pocket. Whether R282 is likely to even contact ligand is not clear from the model; perhaps the modest effect of its mutation reflects its peripheral location and/or the possibility that the side chain of Arg points away from the ligand pocket.

In order to compare selected AhR mutants using transcriptional activity as a somewhat more physiologically relevant endpoint, we transiently transfected the expression plasmids into AhR-deficient hepatoma cells along with the DRE-dependent luciferase reporter gene, p2DLuc, and a normalization reporter plasmid, pRL-TK. TCDD inducibility was reduced compared to AhRwt (Fig. 2C), although the rank order of potency of the mutations differed from their potency of impact on ligand binding and DRE binding. Because luciferase activity was very low in vehicle-treated cells containing AhRs E339L, R333L, and Q377L compared to AhRwt, the calculated fold-induction by TCDD exceeded that for AhRwt; the actual normalized induced activity was only 50% or less of wt levels. A361V showed no transcriptional response to vehicle, TCDD (or MNF).

A species difference in LBD sequence affects ligand interaction and DRE binding

Since we were also interested in the difference between mouse and guinea pig AhR activity, we noted that within the ligand binding region, the two receptors are highly homologous and those residues that differ are generally conservative differences (e.g., a nonpolar residue replaced with another nonpolar). However, **R355** in the mouse sequence aligns with I360 in the guinea pig AhR. The R355 – I360 pair is the only case in which a charged residue (Arg) in one species is a nonpolar residue (Ile) in the other, which is particularly notable given our previous data [7] suggesting a possible interaction of certain flavone antagonists with a positively charged residue. We therefore replaced R355 in mAHR with Ile, and found that this mutant retained good ³H-TCDD binding and DRE binding (Fig. 2A,B).

Although MNF (0.01 – 1 μ M) effectively competes with 3 H-TCDD for binding to TNT-expressed mAhR (Henry, unpublished data), we have found that the DRE-binding response of *in vitro* expressed AhR to several flavonoid compounds is not exactly equivalent to that in cytosolic extracts or whole cells [8] (and Henry, unpublished data). For example, 1 μ M MNF has some agonist activity as assessed by EMSA, unlike the minimal agonism typically seen in mouse cells or cytosolic extracts; at lower concentrations (0.01 – 0.1 μ M), MNF partially inhibits TCDD-induced DRE binding of mAhRwt in reticulocyte lysate (unpublished observations). In contrast, MNF (0.01 – 1 μ M) has little antagonist effect towards TNT-expressed gpAhRwt. Therefore, it was of interest that MNF (1 μ M) treatment of the mAhR-R355I mutant elicited a stronger DRE-binding signal than observed with mAhRwt (Fig 3A). Indeed, in several experiments, MNF was at least as effective as 10nM TCDD. Furthermore, MNF was unable to inhibit TCDD-induced DRE binding of R355I (Fig. 3B). Thus, mutation of this particular residue caused the *in vitro* expressed mAhR to respond in a pattern that is more typical of the gpAhR.

Transcriptional activity of R355I and related mutants

These AhRs were also expressed in Ah receptor-deficient (TAO) cells by transient transfection to compare their transcriptional activity. In this system, the gpAhRwt consistently produced higher constitutive (not shown) and vehicle-induced luciferase activity compared to mAhRwt, as well as correspondingly lower fold-induction by TCDD (Fig. 3C). Luciferase induction by the mR355I mutant resembled the guinea pig pattern — high vehicle-induced activity and low TCDD fold-inducibility. The inverse mutation, gp I360R, showed a pattern of transcriptional activity more like the mAhRwt. Thus, this single amino acid exchange between species produced a reversal of the pattern of their responsiveness. MNF was able to inhibit TCDD-induced luciferase activity in a dose-related manner (data not shown); in contrast to the clear agonist activity of MNF that we have seen in stably transfected guinea pig reporter cells in comparison with very little agonism in mouse cells [8], this disparity in reporter gene induction between species was not consistently observed in these transient transfectants.

We also compared the ability of these mutants to induce the endogenous gene, *CYP1A1*, in the transiently transfected TAO cells. As expected, CYP1A1 protein was generally undetectable in lysates of vehicle-treated cells containing any of the tested AhRs (Fig. 3D). TCDD (0.5nM) strongly induced CYP1A1 in all cells. MNF had little or no inducing effect in cells containing mAhRwt and was able to inhibit induction by TCDD (Fig. 3D, lanes 3,4). In cells transfected with gpAhRwt, on the other hand, CYP1A1 was clearly detectable in MNF-treated cells in the presence or absence of TCDD (Fig. 3D, lanes 7, 8). Interestingly, CYP1A1 was also strongly induced by MNF in cells transfected with mAhR-R355I, again indicating a dramatic change in AhR activity by this one amino acid substitution. The inverse mutant, gp I360R, however, retained some MNF-inducibility of CYP1A1. AhR protein was also analyzed on these western blots to verify that all transfected AhRs were expressed equivalently. Note also that since guinea pig AhR is of larger molecular weight than mouse AhR, the low level expression of endogenous mAhR in the TAO cells is detectable on western blots of lysates from gpAhR transfections.

Several additional mutants at the mR355 site were made to determine the specificity of the isoleucine substitution. R355 was changed to another basic residue (K, Lysine), to the equivalent residue in the human AhR (T, Threonine), or to another nonpolar residue (L, leucine). Initial testing of the *in vitro*-expressed mutant AhRs indicated that all were activated by TCDD to a DRE-binding form (Fig 4A). As previously observed, R355I showed higher than wt DRE binding under all conditions. The other mutant with a nonpolar amino acid substitution (R355L) showed a similar tendency. When these four R355 mutant AhRs were transiently transfected into TAO cells, only R355I showed the high luciferase reporter response

to DMSO vehicle and the consequent low fold-induction by TCDD (though actual induced luciferase level exceeded that for AhRwt (Fig 4B)). Similarly, strong CYP1A1 induction by MNF was consistently detectable only in cells transfected with mR355I (Fig 4C). In the gel shown, weak induction of CYP1A1 by R355L was also observed; although this was not detected consistently, it is notable that this is the amino acid substitution that is most similar to the R355I (nonpolar). Note that transfected AhRs were all expressed approximately equally as detected by western blot. In some cases, TCDD (and not MNF) treatment caused a down-regulation of AhR level (e.g., Fig 4C). This was observed frequently although not consistently (e.g., Fig 3D, 4D), in contrast to the well-documented and consistent TCDD-induced down-regulation of AhR in nontransfected wild-type cells (Hepa1c1c7) even at much lower concentrations of TCDD. This difference is likely an indicator of the limitations of transient transfection as a model system for transcriptional activation.

Additional amino acid switches between mouse and guinea pig AhR were made as potential negative controls to further determine how specific this effect was for R355. The two nearest residues to R355 that differ between species are **S352** (N357 in guinea pig) and **V357** (M362 in guinea pig). The four AhRs containing these switched residues were expressed *in vitro* for testing by EMSA, and transiently transfected into TAO cells to measure reporter gene and CYP1A1 induction by TCDD and MNF. Although the actual luciferase activities measured for these mutants were not identical to those for the corresponding wild type AhRs (not shown), mR355I was the only mouse AhR found to strongly induce CYP1A1 in MNF-treated cells (Fig 4D) and to show the high luciferase activity (relative to mAhRwt) in DMSO and MNF-treated cells as observed previously (data not shown). The gp mutants (N357S and M362V) were similar to gp wt in having lower fold-inducibility of luciferase by TCDD due to higher DMSO-induced levels (data not shown). gpM362V, like gp wt and gpI360R strongly induced CYP1A1 in response to TCDD or MNF (Fig 3D). In several experiments with cells transfected with gpN357S, CYP1A1 induction was weaker in response to either TCDD or MNF and correlated with poor transfection efficiency; we did not further investigate this mutant to verify whether this effect was significant. Thus, the mR355I mutation appears to be unique in its effect on CYP1A1 inducibility by flavone, although it is clearly not the only determinant of response.

Mouse-guinea pig chimeric AhRs

In addition to investigating individual residues within the ligand-binding domain, we made several chimeric AhRs (Fig. 1A) with the goal of identifying whether the differential response to flavone between mouse and guinea pig as observed in cells [8] could be localized to the highly-homologous ligand-binding domain or the less-homologous C-terminal region. The chimeric AhRs were initially expressed *in vitro* in rabbit reticulocyte lysate and tested by EMSA. Although the fold induction by TCDD was rather small compared to that historically seen in cytosolic extracts, the wild-type *in vitro* expressed AhRs showed induction of DRE binding at 3 and 10nM TCDD. Unlike the lack of agonist activity of MNF when mouse cell cytosol is used, MNF (1μM) induced mAhRwt-DRE binding in this system, although the level of induced binding was typically less than that produced by 10nM TCDD. In contrast, induction of **gp**AhRwt-DRE binding by MNF was equal to or greater than by 10nM TCDD (Fig. 5A). Chimeric AhRs in which the LBD was the mouse sequence (m/gpΔC, or gp/mLBD) were found to behave more like mAhRwt, i.e., elicited DRE binding by MNF was less than that elicited by TCDD. The activity of those chimerics containing the gp LBD (gp/mΔC, m/gpLBD) more resembled gpAhRwt, i.e., induction of DRE binding by MNF exceeded that elicited by TCDD (Fig. 5A). These data suggested that DRE binding activity was determined more by the source species of the ligand-binding domain, whereas swapping the C-terminal regions had less impact.

Upon transfection of the chimeric AhRs into TAO cells along with p2Dluc and pRL-TK reporter genes, the pattern of transcriptional response to TCDD was more dependent on the identity of the C-terminal part of the chimeric AhR (Fig. 5B) i.e., m/gp Δ C responded more like gpAhRwt whereas gp/m Δ C responded more like mAhRwt. Response to MNF was more variable among experiments. In initial experiments using GenePorter transfection reagent, only one of the LBD chimerics, namely m/gpLBD, consistently showed substantial TCDD-responsiveness, whereas the gp/mLBD showed little luciferase inducibility, although the protein was clearly expressed and CYP1A1 was inducible. However, in subsequent transfections using FuGene, gp/mLBD was expressed well (in fact to higher levels than m/gpLBD) (Fig 5C), did show TCDD-responsiveness, although reporter gene induction was low. Induction of CYP1A1 by the chimeric AhRs was more reproducible and differences among AhRs were more distinct than for luciferase induction. The ability of MNF to induce CYP1A1 protein was predominantly dependent on the C-terminal portion of the AhR being of guinea pig origin (Fig. 5C). This is in contrast to the results for DRE binding in which the ligand binding domain of the chimeric AhRs was the more important determinant. The response (reporter gene and CYP1A1) to MNF+TCDD cotreatment was equivalent to MNF alone and is not shown in Fig 5. In control TAO cells transfected with empty pcDNA3.1 vector or left untransfected, the low levels of endogenous AhR are detectable (Fig 5C, last four lanes), and this endogenous AhR is TCDD-inducible (as shown by detectable CYP1A1 protein and decrease in AhR level).

DISCUSSION

Among the LBD point mutants, there was a broad range of effect upon TCDD binding and transcriptional activity, from a modest decrease to a total elimination of responsiveness. One residue in particular was identified to have a role in distinguishing agonist *versus* antagonist response to the flavone MNF. Although MNF is an antagonist towards mAhRwt, when Arg355 was changed to Ile (the equivalent residue in the guinea pig AhR), the mAhR retained responsiveness to TCDD, but also was activated by MNF to induce CYP1A1. We have previously found that MNF has agonist activity toward the gpAhR, so it was especially notable that this particular mutation in mAhR changed its response to MNF to resemble gpAhR (Fig 3). The 355 residue is rather variable among other species, being Ala in rabbit, Thr in human, Arg in hamster and rat as well as mouse, and Gln and Tyr respectively in the non-ligand-binding AhRs of *Drosophila* and *C. elegans*. Although we have not investigated responsiveness of rabbit, hamster, or rat AhR towards MNF, human hepatoma cells respond similarly to murine cells (Henry, Zhou, Gasiewicz, unpublished data). Additionally, mutation of mR355 to Thr, Lys (basic residue), or Leu (nonpolar residue) did not have the effect of changing response to MNF from antagonist to agonist (Fig. 4). The Ile residue seems to be unique to the guinea pig and unique in its effect on mAhR response to MNF.

It could be hypothesized that the charged arginine (R355) constrains the positioning of MNF with its electronegative nitro substituent in such a way that normal agonist-induced transformation of mAhR is inhibited. When replaced by the nonpolar Ile, MNF binding may become less constrained, and AhR activation enabled. R355I also showed a higher degree of responsiveness to vehicle and/or to other activators within cells or culture medium, consistent with a more “sloppy” ligand binding pocket. R355I mutation had less effect on binding and activation by the high-affinity and uncharged TCDD. These observations support our previous hypothesis [7] that the electronegative center in potent antagonist flavones interacts, perhaps by H-bonding, with a positively charged residue to inhibit AhR activation. For several steroid hormone receptors, distinct residues have been identified whose mutation differentially alters interaction with agonist *versus* antagonist; additionally, single residue differences between species have been noted that account for species-specific action of ligand [16–20]. Our results suggest that the murine AhR residue R355 may have an analogous function.

It is not clear from proposed models of AhR LBD [11,21] whether the R355 side chain is predicted to extend into the ligand binding pocket, although this would be consistent with the above hypothesis. It is also possible that the charged side chain may be directed outside of the ligand pocket to contact some nearby portion of the AhR protein. In this case, it may help in maintaining the ligand pocket shape, and/or in “communication” between the LBD and, for example, amino acid(s) within the TAD. Our data do not allow us to distinguish between these possibilities. However, this residue clearly has a role in differential response to TCDD and MNF, and partially accounts for the difference between mouse and guinea pig AhR in response to this flavone.

The other mAhR amino acids for which we made point mutants are conserved among mammalian species, and were predicted to have some importance in ligand binding. Several (e.g. R282L, Q377L, F281W) partially reduced ligand- and DRE-binding but were not critical inasmuch as the mutants retained significant TCDD-responsiveness. A361 is within a β -strand that is near the ‘entrance’ to the ligand pocket and adjacent to the parallel strand containing the A375 residue whose mutation to Val is associated with low-affinity binding by mouse *d*-allele and human AhRs [15]. A361L and A361V essentially lost TCDD binding and transcriptional activation, likely by steric hindrance of ligand entrance or positioning in the ligand pocket by the larger Leu or Val. In contrast, the more modest effect of mutation of the adjacent R362 implies that its side chain likely points outside of the pocket. Interestingly, the A361V mutation more completely abolished TCDD responsiveness than reported by Pandini et al. [21] for A375V, which is located farther into the binding pocket. Although a possible H-bond between R333 and E339 was predicted to respond to ligand entry and possibly initiate a conformational change [11], our results indicate that while these residues are important, they alone are not critical to receptor activation, since the mutants with Leu at either of these sites retained significant, though clearly reduced, TCDD inducibility. A more recent modeling of the 3D structure [21] does not predict this interaction and indeed suggests that the side-chain of M334 faces the ligand pocket and that E339 points out of the pocket, although their E339A showed partial loss of TCDD and DRE binding activities [21]. These authors also tested mutations of two other residues on the helical connector, namely I332P, and M334E and M334A. The former two mutations eliminated binding, which is perhaps not surprising given the substitution of Pro and the charged Glu for the nonpolar wild type residues. The more conservative mutation, M334A, only partially decreased TCDD binding and DRE binding activity. Together with our results for R333L, situated between these residues, these observations suggest that the helical connector is a critical structural component of the ligand pocket, and mutation of any of its residues (whether or not they are thought to project into the actual ligand pocket) impacts ligand binding. It is also important to note that we selected amino acids for mutation based on expectation that these mutations would impact ligand binding. However, it is also likely that the resultant structural changes in the LBD cause ligand-dependent and ligand-independent changes in interaction between LBD and other parts of the AhR protein as well as in AhR interactions with other proteins such as chaperones, coactivators, and/or corepressors. We did not further evaluate the effects of these mutations on AhR kinetics, stability, and protein-protein interactions due to the limitations of the transient transfection model. It is recognized that such factors could at least partially account for the less-than-perfect correlation between relative potency of the several mutants in decreasing ligand binding and decreasing transcriptional activity (Fig 2).

There are a number of other reports in which the AhR LBD is probed by determining the effect of selected point mutations. In the rat AhR, Y320 is homologous to mAhR-Y316 which is modeled to be located in one of the loops rather than within the actual ligand pocket [11] and hence is unlikely to contact ligand directly. Backlund et al. (2004) [22] reported that the rat AhR-Y320F retained wild type TCDD binding/activation, but activation by three low-affinity ligands was lost. Goryo et al. (2007) [23] mutated mAhR residues that are conserved only in

ligand-dependent AhRs. One of these (F318A) lost responsiveness to agonist (TCDD, 3MC, β NF), although more structurally conservative mutants (F318Y, F318W, and F318L) retained some responsiveness. Notably, the ability of the mutant F318L to induce reporter gene or nuclear translocation was dependent on ligand used: although response to TCDD and β NF was lost, this mutant retained responsiveness to 3MC. Alanine mutants of the neighboring I319 and H320 also lost ligand responsiveness. The residue equivalent to murine I319 in the tern AhR is V325, which along with A381, accounted for the reduced TCDD binding affinity, reduced transactivation, and reduced sensitivity to HAH toxicity in terns compared to chickens, in which these residues are Ile and Ser respectively [24]. The I319 residue of mAHR is also one of four identified by Pandini et al. [21] that have smaller side chains than the corresponding residues in the non-ligand-dependent PAS protein HIF-2 α and which they conclude provide a larger “empty” space for ligand in the AhR. Together, these results indicate that numerous amino acids, including those hypothesized not to actually contact ligand, contribute to ligand binding and AhR activation, and the choice of mutant residue relative to wild type affects the functional impact of the mutation. Furthermore, the impact of certain mutations depends on the ligand used, indicating that structurally different ligands are accommodated by interacting with different key residues which in turn may result in different receptor conformations and transcriptional capabilities. Ultimately, a better understanding of these events will depend on obtaining the crystal structure of liganded (with diverse ligands) and ligand-free AhR. Crystal structure analyses of other receptors and proteins has yielded insight into mechanisms of, for example, conformational changes due to ligand accommodation, and protein-protein interactions [25,26].

The behavior of the chimeric AhRs indicates that the amino acid difference within the LBD between mouse and guinea pig is not the only factor contributing to species differences in response to ligands. These experiments suggested that the C-terminal portion of AhR was more important in determining the mouse *versus* guinea pig pattern of induction of CYP1A1 and reporter gene. In chimerics containing the C-terminal region from guinea pig, MNF elicited CYP1A1 induction regardless of the species of N-terminal and LBD. When the C-terminal region was from mouse, MNF antagonized the TCDD-elicited induction of CYP1A1 even if the LBD was from guinea pig. This was in contrast to the greater importance of the LBD in determining *in vitro* DRE binding. This disparity is perhaps not unexpected, since the C-terminal region includes the TAD, which has little importance in DRE recognition but mediates interaction with other proteins that are necessary for initiating gene transcription. The mechanism by which ligand binding transduces signaling to the rest of the receptor is unknown. However, it is apparent that both the LBD and C-terminal regions together contribute to the transformation process that ultimately results in enhanced (or repressed) transcription of target genes. These chimeric AhRs indicate that there are species differences in the inter-domain interactions. Our data for gpAhRs must be interpreted with the caveat that both *in vitro* expressed and transiently transfected gpAhRs are interacting with mouse Arnt and mouse cofactors since the appropriate guinea pig components are unavailable. However, our results are at least qualitatively consistent with the species differences observed between mouse and guinea pig cells [8].

Our current data indicate that relatively subtle differences in AhR sequence/structure between species can produce different responsiveness to ligands, including whether a ligand elicits agonist or antagonist effects. Although the highest potency agonists for AhR have a relatively restricted size and shape [27,28], the ligand binding pocket does accommodate a diversity of chemicals [4]. Most of these bind with lower affinity than TCDD, and most may be more readily metabolized/inactivated than is TCDD. Nonetheless, our data are consistent with the additional possibility that these diverse chemicals are accommodated by different residues, that in turn mediate subtly different receptor conformations that may differ qualitatively in their ability to modulate transcription of particular genes. Published studies of several different ligands are

consistent with this possibility, without ruling out involvement of other factors [29–31]. We recently reported that a potential endogenous AhR ligand, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, is a potent agonist in cell extracts, cultured cells, and intact animals, but does not cause the toxicity associated with TCDD [32]. Whether this might be due to differing conformations elicited and subsequent different gene batteries regulated by these ligands remains to be determined.

Finally, differences in relative TCDD binding affinity or in DRE binding between gpAhR and other species do not appear to fully account for the profound sensitivity of the guinea pig to TCDD toxicity [10,33]. However, it may be that the less restrictive gpAhR ligand-binding pocket in conjunction with specific sequences within the gpAhR TAD allow for an increased efficiency of transcriptional response. To verify this possibility, it will be essential to determine how ligand binding communicates with other domains to elicit a transcriptionally active conformation. It is also possible that the less restrictive gpAhR binding pocket accommodates a different set of endogenous ligands, and/or that metabolic pathways producing endogenous ligands (e.g. from tryptophan [34]) may differ quantitatively or qualitatively between guinea pig and other species. Clearly, further work is necessary to identify endogenous ligands and answer interesting questions regarding how they differ from toxic xenobiotic ligands in their regulation of AhR signaling pathways.

ACKNOWLEDGMENTS

We thank Ann Colasurdo for valuable technical help, particularly with transient transfection experiments, and Steve Dertinger for preparation of anti-AhR ascites.

REFERENCES

1. Fernandez-Salguero PM, Pineau T, Hilbert DM, McPhail T, Lee SST, Kimura S, Nebert DW, Rudikoff S, Ward JM, Gonzalez FJ. *Science* 1995;268:722–726. [PubMed: 7732381]
2. Fernandez-Salguero PM, Ward JM, Sundberg JP, Gonzalez FJ. *Vet Pathol* 1997;34:605–614. [PubMed: 9396142]
3. Lahvis GP, Pyzalski RW, Glover E, Pitot HC, McElwee HK, Bradfield CA. *Mol. Pharmacol* 2005;67:714–720. [PubMed: 15590894]
4. Denison MS, Nagy SR. *Annu. Rev. Pharmacol. Toxicol* 2003;43:309–334. [PubMed: 12540743]
5. Lu Y-F, Santostefano M, Cunningham BDM, Threadgill MD, Safe S. *Biochem. Pharmacol* 1996;51:1077–1087. [PubMed: 8866830]
6. Gasiewicz TA, Kende AS, Rucci G, Whitney B, Willey J. *Biochem. Pharmacol* 1996;52:1787–1803. [PubMed: 8986142]
7. Henry EC, Kende AS, Rucci G, Totleben MJ, Willey J, Dertinger SD, Pollenz RS, Jones JP, Gasiewicz TA. *Molec. Pharmacol* 1999;55:716–725. [PubMed: 10101030]
8. Zhou J-G, Henry EC, Palermo CM, Dertinger SD, Gasiewicz TA. *Molec. Pharmacol* 2003;63:915–924. [PubMed: 12644593]
9. Kim S-H, Henry EC, Kim D-K, Kim Y-H, Shin KJ, Han MS, Lee TG, Kang J-K, Gasiewicz TA, Ryu SH, Suh P-G. *Mol. Pharmacol* 2006;69:1871–1878. [PubMed: 16540597]
10. Gasiewicz TA, Rucci G. *Mol. Pharmacol* 1984;26:90–98. [PubMed: 6087120]
11. Procopio M, Lahm A, Tramontano A, Bonati L, Pitea D. *Eur. J. Biochem* 2002;269:13–18. [PubMed: 11784293]
12. Cunningham BDM, Threadgill MD, Groundwater PW, Dale IL, Hickman JA. *Anticancer Drug Design* 1992;7:365–384.
13. Henry EC, Gasiewicz TA. *Mol. Pharmacol* 2003;63:392–400. [PubMed: 12527811]
14. Gasiewicz TA, Neal RA. *Anal. Biochem* 1982;124:1–11. [PubMed: 6289695]
15. Ema M, Ohe N, Suzuki M, Mimura J, Sogawa K, Ikawa S, Fujii-Kuriyama Y. *J. Biol. Chem* 1994;269:27337–27343. [PubMed: 7961644]

16. McDonnell DP, Clemm DL, Imhof MO. *Seminars in Cancer Biology* 1994;5:327–336. [PubMed: 7849261]
17. Wijayaratne AL, McDonnell DP. *J. Biol. Chem* 2001;276:35684–35692. [PubMed: 11473106]
18. Tirona RG, Leake BF, Podust LM, Kim RB. *Mol. Pharmacol* 2004;65:36–44. [PubMed: 14722235]
19. Terouanne B, Nirde P, Rabenoelina F, Bourget W, Sultan C, Auzou G. *Mol. Pharmacol* 2003;63:791–798. [PubMed: 12644579]
20. Ekena K, Katzenellenbogen JA, Katzenellenbogen BS. *J. Biol. Chem* 1998;273:693–699. [PubMed: 9422719]
21. Pandini A, Denison MS, Song Y, Soshilov AA, Bonati L. *Biochemistry* 2007;46:696–708. [PubMed: 17223691]
22. Backlund M, Ingelman-Sundberg M. *Mol. Pharmacol* 2004;65:416–425. [PubMed: 14742684]
23. Goryo K, Suzuki A, Del Caprio CA, Siizaki I, Kuriyama E, Mikami Y, Kinoshita K, Yasumoto K, Rannug A, Miyamoto A, Fujii-Kuriyama Y, Sogawa K. *Biochem. Biophys. Res. Commun* 2007;354:396–402. [PubMed: 17227672]
24. Karchner SI, Franks DG, Kennedy SW, Hahn ME. *Proc. Natl. Acad. Sci. USA* 2006;103:6252–6257. [PubMed: 16606854]
25. Kallen J, Lattmann R, Beerli R, Blechschmidt A, Blommers MJJ, Geiser M, Ottl J, Schlaeppi J-M, Strauss A, Fournier B. *J. Biol. Chem* 2007;282:23231–23239. [PubMed: 17556356]
26. Harris SF, Shiau AK, Agard DA. *Structure* 2004;12:1087–1097. [PubMed: 15274928]
27. Poland A, Greenlee WF, Kende AS. *Ann. N. Y. Acad. Sci* 1979;320:214–230. [PubMed: 110192]
28. Waller CL, McKinney JD. *Chem. Res. Toxicol* 1995;8:847–858. [PubMed: 7492734]
29. Gouedard C, Barouki R, Morel Y. *Mol. Cell. Biol* 2004;24:5209–5222. [PubMed: 15169886]
30. Gouedard C, Barouki R, Morel Y. *Arterioscler Thromb Vasc Biol* 2004;24:2378–2383.
31. Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu H-Y, Laine J, Sakai T, Korsmeyer SJ, Casper RF, Sherr DH, Tilly JL. *Nat. Genet* 2001;28:355–360. [PubMed: 11455387]
32. Henry EC, Bemis JC, Henry O, Kende AS, Gasiewicz TA. *Arch Biochem Biophys* 2006;450:67–77. [PubMed: 16545771]
33. Henry EC, Rucci G, Gasiewicz TA. *Biochemistry* 1989;28:6430–6440. [PubMed: 2551374]
34. Bittinger MA, Nguyen LP, Bradfield CA. *Mol. Pharmacol* 2003;64:550–556. [PubMed: 12920190]

1. Construction of chimeric Ah receptors

Diagrams of the C-terminal (ΔC) and ligand-binding domain (LBD) chimeric receptors, showing equivalent amino acid numbers of the exchanged fragments. Restriction enzymes *HpaI* and *FseI* were used to cut the expression plasmids of both mouse and guinea pig AhRwt where shown, as described in Materials and Methods.

A

2. Effect of point mutations within the mAhR ligand-binding domain

A. Analysis by EMSA. Mutant mAhRs were expressed in rabbit reticulocyte lysate, mixed with similarly expressed mArnt, incubated with DMSO vehicle (D) or TCDD at 3 and/or 10nM (T_3 , T_{10}). Aliquots were then mixed with 32 P-DRE oligo as described in Materials and Methods and subjected to nondenaturing electrophoresis. The dried gel was visualized and bands were quantified by phosphorimager. Numbers below each lane are the calculated fold induction by TCDD relative to DMSO for each construct (ratio T/D), and the ratio of AhR•DRE band for each mutant compared with equivalent band for mAhRwt (mutant/wt). Gel shown is representative of at least 2 separate experiments for each mutant. For some mutants, a lane of untreated sample (U) is also shown.

B. Ligand binding analysis. In vitro expressed mAhRs and mArnt were mixed and aliquots were incubated with 3 H-TCDD $\pm$ 150-fold excess unlabeled TCDF. Specific binding was determined using the hydroxylapatite assay. Data shown are for 4nM 3 H-TCDD and are representative of two independent experiments.

C. Transactivation activity of selected mutants. AhR-deficient TAO cells were transiently transfected with DRE-dependent luciferase reporter, the indicated AhR plasmid, and a normalization reporter, pRL-TK. Triplicate wells were treated with DMSO, 1nM TCDD, or

1 μ M MNF and luciferase activities measured after 18–20 h. Mean $\pm$ S.D. are shown from one of two independent experiments.

3. The mutant mAhR-R355I has altered responsiveness to flavone MNF

A. In vitro expressed mAhRwt and R355I were mixed with similarly expressed mArnt, treated with DMSO (D), 1 μ M MNF (M), or 10nM TCDD (T), and analyzed by EMSA and visualized by phosphorimager. Bands were quantified and numbers below each lane are the calculated fold induction by TCDD or MNF relative to DMSO.

B. In vitro expressed AhRs/Arnt were treated with 3nM TCDD and MNF at 0, 0.01, 0.1, or 1 μ M, then analyzed by EMSA. DRE-bound AhR was quantified by phosphorimager and expressed as a percent of binding at 3nM TCDD alone.

C. Transactivation by mR355I and inverse mutant gpAhR-I360R compared with wild type AhRs. TAO cells were transiently transfected with the indicated AhR plasmids, DRE-dependent luciferase reporter plasmid, and normalization pRL-TK plasmid. Luciferase activities were measured 18–20h after treatment with DMSO, 0.5nM TCDD, or 1 μ M MNF. Mean ratios (firefly : *renilla* luciferase activities) $\pm$ S.D. from triplicate wells are shown; data are representative of at least 3 separate experiments.

D. Western blot analysis of AhR and CYP1A1 in cell lysates from transiently transfected TAO cells as described in C.

4. Comparison of other mutations at R355 residue of mAhR

A. EMSA analysis of in vitro expressed AhRs was performed as described in Materials and Methods. AhR•DRE bands were quantified by phosphorimager, and data (representative of two separate experiments) are shown normalized to AhRwt treated with DMSO.

B. Transactivation activity of AhR mutants transiently transfected into TAO cells. Data shown are ratios of Firefly:*Renilla* luciferase activities measured in triplicate wells for each condition. Mean $\pm$ S.D. are shown; data are representative of 3 separate experiments.

C. Western blot analysis of AhR and CYP1A1 in lysates from cells transiently transfected with R355 mutant mAhRs and treated with DMSO, 1nM TCDD, or 1 μ M MNF.

D. Western blot analysis of AhR and CYP1A1 levels in lysates from cells transiently transfected with mAhRs and gpAhRs containing point mutations at nearest residues to mR355 that differ between species. Transfected cells were treated with DMSO, 1nM TCDD, 1 μ M MNF, or MNF and TCDD for 18–20h.

C

5. Functional analyses of chimeric AhRs

A. EMSA analysis: In vitro expressed chimeric AhRs were mixed with similarly expressed mARNT and incubated with DMSO, 10nM TCDD, or 1μM MNF. AhR•DRE bands were quantified by phosphorimager to calculate the fold-induction relative to DMSO. Data are expressed as the mean of the difference in fold induction (TCDD – MNF), ± S.D. for 3–4 separate experiments.

B. Transactivation activity of wild type and chimeric AhRs transiently expressed in TAO cells. Ratios of Firefly : *Renilla* luciferase activities shown (mean ± S.D. for triplicate wells) are representative of at least 3 separate experiments.

C. Western blot analysis of AhR and CYP1A1 in lysates of TAO cells transiently transfected with indicated AhRs or the empty vector (pcDNA3.1). Un = untransfected and untreated cells.