0026-895X/10/7706-1037–1046$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 77:1037–1046, 2010

Vol. 77, No. 6
62927/3589990
Printed in U.S.A.

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Amino Acid Substitutions in the Aryl Hydrocarbon Receptor Ligand Binding Domain Reveal YH439 As an Atypical AhR Activator

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Received December 2, 2009; accepted February 17, 2010

ABSTRACT

The aryl hydrocarbon receptor (AhR) is traditionally defined as a transcription factor activated by exogenous polyaromatic and halogenated aromatic hydrocarbon (PAH/HAH) ligands. Active AhR induces genes involved in xenobiotic metabolism, including cytochrome P4501A1, which function to metabolize activating ligands. However, recent studies implicate AhR in biological events that are apparently unrelated to the xenobiotic response, implying that endogenous activation mechanisms exist. Three AhR genes in zebrafish (*Danio rerio*) encode proteins that demonstrate differential activation in response to PAH/HAHs, with the nonresponsive drAhR1a having some sequence divergence from the PAH/HAH-responsive AhRs in the ligand binding domain (LBD). We used these differences to guide the mutagenesis of mouse AhR (mAhR), aiming to generate variants that functionally discriminate between activation mechanisms. We found substitution of histidine 285 in the LBD with tyrosine gave a receptor that could be activated by isopropyl-2-(1,3-dithietane-2-ylidene)-2-[*N*-(4-methylthiazol-2-yl)carbamoyl]acetate (YH439), a potential AhR ligand chemically distinct from classic PAH/HAH-type ligands, but prevented activation by both exogenous PAH/HAH ligands and the endogenous activation mimics of suspension culture and application of shear-stressed serum. The differential response of H285Y mAhR to YH439 suggests that this activator has a novel mode of interaction that tolerates tyrosine at position 285 in the LBD and is distinct from the binding mode of the well characterized PAH/HAH ligands. In support of this, the PAH-type antagonist 3′,4′-dimethoxyflavone blocked mAhR activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin but not YH439. Furthermore, the strict correlation between response to exogenous PAH/HAH ligands and mimics of endogenous activation suggests that a PAH-type ligand may underpin endogenous mechanisms of activation.

The aryl hydrocarbon receptor (AhR), also known as the dioxin receptor, is a basic helix-loop-helix-Per-aryl hydrocarbon receptor nuclear translocator (ARNT)-Sim transcription factor best characterized by activation in response to exogenous polyaromatic and halogenated aromatic hydrocarbon (PAH/HAH) type ligands. Latent AhR is bound by chaperones, which serve to present a ligand-accessible conformation of the ligand binding domain (LBD) and maintain cytoplasmic localization (Mitchell and Elferink, 2009). Xenobiotic PAH ligands such as 3-methylcholanthrene (3MC) and benzo[*a*]pyrene (B[*a*]P), and the prototypical HAH, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) bind to the AhR within the LBD, causing a conformational change that allows nuclear import and exchange of chaperones for the AhR partner ARNT. The AhR/ARNT heterodimer binds xenobiotic-response elements (XREs) in regulatory regions of target genes and associates with coactivators to initiate transcription (Kobayashi et al., 1997; Kumar et al., 1999). PAH activation

This work was supported by the Australian National Health and Medical Research Council and the Australian Research Council through the Centre for the Molecular Genetics of Development.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.109.062927.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; 3′,4′-DMF, 3′,4′-dimethoxyflavone; 3MC, 3-methylcholanthrene; ARNT, aryl hydrocarbon receptor nuclear translocator; B[*a*]P, benzo[*a*]pyrene; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; HAH, halogenated aromatic hydrocarbon; LBD, ligand-binding domain; PAH, polyaromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; YH439, isopropyl-2-(1,3-dithietane-2-ylidene)-2- [*N*-(4-methylthiazol-2-yl)carbamoyl]acetate; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; HEK, human embryonic kidney; wt, wild type; XRE, xenobiotic-response element; TK, thymidine kinase; PAGE, polyacrylamide gel electrophoresis; MAb, monoclonal antibody; FCS, fetal calf serum; IRES, internal ribosome entry site.

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of AhR has long been known to induce gene products involved in xenobiotic metabolism, such as cytochrome P4501A1 (CYP1A1), which metabolizes the activating ligand to an excretable form. However, AhR-null mice show a range of phenotypic changes, including decreased liver size caused by failure of fetal vascular remodeling (Fernandez-Salguero et al., 1995; Lahvis et al., 2000) and decreased fertility (Baba et al., 2005), which presumably are due to endogenous function rather than environmental xenobiotic exposure. Recent evidence suggests that physiological regulation of AhR is complex, with a number of activation modes to control a diverse range of cellular events, implicating context-specific, subtle regulation of receptor function. These may be separable from the xenobiotic metabolic responses previously assumed to be analogous to endogenous function (Furness et al., 2007; McMillan and Bradfield, 2007b; Furness and Whelan, 2009).

Endogenous activation mechanisms are proposed to include endogenous ligands such as heme metabolites, tryptophan metabolites, lipoxin A4, and prostaglandin G_2 (McMillan and Bradfield, 2007b). AhR can be activated by suspension culture or disruption of cell-cell contacts in keratinocytes (Sadek and Allen-Hoffmann, 1994; Ikuta et al., 2004) by a currently unknown mechanism. Shear-stressed serum can activate AhR in rat hepatoma cells via oxidized low-density lipoproteins (McMillan and Bradfield, 2007a), providing a potential link to function in vascular remodeling. AhR also acts as a substrate-specific adaptor component of a novel cullin complex Cul4B(AhR), targeting estrogen receptor-α, androgen receptor, and β-catenin for ubiquitination and degradation (Ohtake et al., 2007; Kawajiri et al., 2009). In addition, cross-talk with estrogen receptor-α/androgen receptor target gene activation indicates positive and negative regulatory roles in this system (Ohtake et al., 2008), potentially revealing a mechanism for decreased fertility in AhR(−/−) animals. Post-translational modifications of AhR affect subcellular localization (Ikuta et al., 2004), DNA binding (Pongratz et al., 1991; Carrier et al., 1992), and target gene activation (Guo et al., 2000) and are hypothesized to regulate developmental activation of AhR. As yet, no definitive evidence to link physiological roles or interaction with ligands with a particular modification has been found.

Functions mediated by activated AhR in a particular cellular context may be induced through binding different ligands specific to each role. Zebrafish (*Danio rerio*) have three AhR genes with various potentials for activation by xenobiotics (Tanguay et al., 1999; Andreasen et al., 2002; Karchner et al., 2005). drAhR2 functions in a xenobiotic-responsive capacity, showing high-affinity TCDD binding and gene activation (Tanguay et al., 1999), and is the primary receptor involved in TCDD toxicity (Prasch et al., 2003). In contrast, drAhR1a is unable to bind or be activated by TCDD or β-naphthoflavone (Andreasen et al., 2002). drAhR1b belongs to the drAhR1 clade and shares 34% sequence identity with drAhR1a. However, unlike drAhR1a, drAhR1b binds TCDD and activates gene expression with efficiency similar to that of drAhR2 (Karchner et al., 2005). We used these differences in *D. rerio* AhR LBD sequences to guide mutagenesis of mouse AhR (mAhR), aiming to generate variants that functionally discriminate between activation mechanisms, including PAH/HAH-type ligands and isopropyl-2-(1,3-dithietane-2-ylidene)-2-[*N*-(4-methylthiazol-2-yl)carbamoyl]acetate (YH439) activation, suspension culture, and application of shear-stressed serum. We found that substitution of histidine 285 with tyrosine gave a receptor that could be activated by the putative atypical ligand YH439 (Lee et al., 1996) but prevented activation by both exogenous PAH ligands and the endogenous mechanisms of suspension culture and application of shear-stressed serum. The absolute correlation between response to exogenous PAH/HAH ligands and endogenous activation of H285Y mAhR implicates a PAH-type ligand for these endogenous mechanisms. Furthermore, the differential response of H285Y mAhR to YH439 suggests that this compound has an activation mode that differs from that of the well characterized PAH and HAH ligands.

Materials and Methods

Construction of Expression Plasmids. A plasmid encoding full-length mAhR with an N-terminal 10His-Myc tag and native 3′-untranslated region was constructed from pCMV4mDR (Mason et al., 1994) and pEF/IRES/puro (Hobbs et al., 1998). The resulting plasmid, pEF/His-myc-mAhRfull/IRES/puro, expresses His-myc-mAhR from the EF-1a promoter. The full details of its construction will be published shortly. Mutants K245E, H241Y/H285Y, K297N/G298W/Q299N/L300F, and R369Q/Y372C/Q377H were created using standard overlap extension PCR methods and were subcloned into pEF/His-myc-mAhRfull/IRES/puro. pEF/blank/IRES/puro (Hobbs et al., 1998) was the control empty expression vector. To construct lentiviral expression vectors, the His-Myc-tagged full-length wild-type or mutant mAhR was PCR-amplified from pEF/His-myc-mAhR-full_length series of plasmids with primers 5′-GCTCGGATCCACTAGTCCAG-3′ and 5′-TTGCAGCTCGAGTTATCAACTCTGCACCTTGCTTAGG-3′, introducing unique N-terminal SalI and C-terminal XhoI sites, allowing ligation into SalI/XhoI sites of pENTR1A (Invitrogen, Carlsbad, CA). The wild-type and mutant His-myc-mAhR-full_length fragments were transferred into the lentiviral expression vector pLV416 by standard Gateway cloning (Invitrogen). The control pLV416_empty plasmid was constructed by digesting pLV416 with EcoRI and EcoRV, end-filling with Klenow enzyme, and religating the blunt ends. This step removed most of the CmR gene and the *ccdB* selection gene, which is normally replaced by the inserted DNA sequence during Gateway cloning.

Cell Lines and Cell Culture. Human embryonic kidney (HEK) 293T cells were routinely grown in Dulbecco's modified Eagle's medium (Invitrogen), and MCF-7 cells were grown in RPMI 1640 medium (Invitrogen), each supplemented with 10% fetal calf serum and penicillin/streptomycin [1% (v/v)] and cultured at 37°C with 5% CO_2. Polyclonal stable cell lines were generated by transfecting HEK293T cells with pEF/His-myc-mAhRfull/IRES/puro wt and mutants using Fugene6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. After 48 h, cells were seeded into 10-cm dishes and allowed to recover for 24 h before puromycin selection (2 μg/ml) was applied for 2 weeks. Pools of surviving cells were then cultured in the presence of increasing levels of puromycin, up to 10 μg/ml. Mouse AhR-null hepatocytes [AhR(−/−)] (Murray et al., 2005) were grown in minimum essential medium α medium (Invitrogen), supplemented with 10% fetal calf serum, penicillin/streptomycin [1% (v/v)], 100 nM dexamethasone, and cultured at 34°C with 5% CO_2.

Transient Transfections and Dual Luciferase Assays. The AhR-responsive XRE-thymidine kinase (TK)-luciferase reporter gene construct, pT81-X1X1-luc, and control TK-luciferase, pT81-blank-luc, have been described previously (Berghard et al., 1993). Cotransfection with pRL-TK (Dual Luciferase Reporter method; Promega, Madison, WI) was used to measure relative luciferase activity in transient transfection reporter experiments. Triplicate wells were seeded with the relevant stable cell lines at 1.5×10^5 cells in a 24-well tray and grown for 24 h. Cells were then transiently cotrans-

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fected with 200 ng of pT81-X1X1-luc and 50 ng of pRL-TK using the Fugene6 transfection method (Roche) and incubated for 24 h. Triplicate wells were left untreated (adherent); treated with vehicle alone (0.1% dimethyl sulfoxide; DMSO), 10 μM YH439, 1 μM 3MC, 1 nM TCDD, or 100 nM B[*a*]P; and incubated for a further 16 h. Suspension culture of stable cell lines was performed by aspirating the media and adding 0.05% trypsin/530 μM EDTA in phosphate-buffered saline to dislodge cells from the wells. Cells were resuspended in culture media with the addition of 1.68% methylcellulose to create semisolid media, incubated for 16 h, and harvested as described previously (Monk et al., 2001). Shear-stressed fetal calf serum was prepared by pumping serum through a P1 pump (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) at 10 ml/min for 16 h at room temperature and stored at −80°C until use. This method is an approximation of that used in a previous publication (McMillan and Bradfield, 2007a), which does not require the use of a parallel-plate perfusion chamber and generates serum capable of activating AhR. Cells were treated with serum-free media with the addition of 60% shear-stressed serum and control untreated serum. Lysates were analyzed for luciferase activity using the Dual Luciferase Reporter kit (Promega) according to the manufacturer's instructions and assayed on a 20/20 luminometer (Turner Designs, Sunnyvale, CA).

Immunoblotting and Antibodies. Whole-cell extracts were prepared by lysis into three cell pellet volumes of radioimmunoprecipitation assay buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) in 50 mM Tris, pH 8] quantitated by Bradford analysis, and 50 μg of material was subjected to 7.5% acrylamide SDS-PAGE and then transferred to nitrocellulose membrane. Proteins were detected with anti-Myc MAb 9E10, anti-α-tubulin MAb MCA78G (Serotec, Oxford, UK), anti-AhR MAb RPT1 (Abcam Inc., Cambridge, MA), or anti-Arnt MAb MA1-515 (Affinity BioReagents, Golden, CO) horseradish peroxidase-conjugated secondary antibody and visualized with Immobilon Western chemiluminescent substrate (Millipore Corporation, Billerica, MA).

Lentiviral Production and Infection. Replication-defective viral particles were produced by transiently cotransfecting 293T cells with either pLV416_His-myc-mAhR-full_length lentiviral expression vector or pLV416_empty control vector plus three viral packaging vectors (pCMV-dvrp 8.2, pRSV_Rev, and pMD2.G) in the ratio of 10:6:5:3. The 293T cells were routinely grown in 75-cm^2 flasks to ~90% confluence and transfected with 30 μg of plasmid DNA using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Viral supernatant was harvested 72 h after transfection and collected through a 0.45-μm syringe filter (Sartorius Stedim Biotech, Goettingen, Germany).

Mouse AhR(−/−) hepatocytes (Murray et al., 2005) were seeded in six-well trays at 8×10^5 cells/well 1 day before infection and then infected with relevant viral supernatant [25% (v/v)] in the presence of 8 μg/ml polybrene (Sigma-Aldrich). Twenty-four hours after infection, the viral supernatant was removed, and cells were grown in fresh medium for another day before being expanded into a 10-cm dish. To select for successful integration of His-myc-mAhR-full-length construct, cells were allowed to recover for 24 h and then were treated with 500 μg/ml Geneticin (G418; Invitrogen) for three continuous passages. Protein expression was analyzed by Western blotting using mAhR-specific antibodies.

Electrophoretic Mobility Shift Assay Using Cytosolic Extracts. Cytosolic proteins were prepared from cultured mouse hepatocyte cell lines after cell lysis in six volumes of hypotonic buffer [1.5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 1 mM dithiothreitol, and 1× protease inhibitor cocktail (Sigma-Aldrich) in 10 mM Tris-HCl, pH 8.0] for 30 min at 4°C followed by snap-freezing. After thawing on ice, cell debris was pelleted at 14,000*g* for 30 min at 4°C. The supernatant containing the cytosolic extract was collected, and protein was quantitated by Bradford analysis. Electrophoretic mobility shift assay (EMSA) experiments were carried out as described previously (Pongratz et al., 1998) with the addition of ligand to samples of the cytosolic extracts containing 80 μg of total protein.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR. Cultured mammalian cells were seeded at 1.2×10^6 cells/dish in 10-cm dishes and grown at 34°C, 5% for 72 h. Cells were then treated with vehicle alone (0.1% DMSO), 10 μM YH439, 10 nM TCDD, or were resuspended in 10 ml of 1.68% methylcellulose media for 5 h. To extract RNA, cells were lysed in 1 ml of TRI reagent (Ambion, Austin, TX), mixed with 200 μl of chloroform at room temperature for 15 min, and centrifuged at 4°C, 13,000*g* for 15 min. The top aqueous phase, which contains the most cellular RNA, was then further purified with the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Reverse transcription was carried out using 2 μg of RNA at 50°C for 90 min with 200 units of Superscript III RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The levels of specific genes represented in the cDNA products were measured by quantitative real-time PCR (qRT-PCR) using SYBR green PCR master mix and a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA). The qRT-PCR primers used were mCYP1A1_Forward, 5′-ACCTCTTTGGAGCTGGGTTT-3′, and mCYP1A1_Reverse, 5′-GATAGGGCAGCTGAGGTCTG-3′ to amplify CYP1A1 sequences, with mHPRT_Forward, 5′-AGTCCCAGCGTCGTGATTAGC-3′, and mHPRT_Reverse, 5′ CCAAATCCTCGGCATAATG 3′, for the reference gene, hypoxanthine guanine phosphoribosyl transferase. Expression levels of this gene did not vary between untreated, ligand-treated, and suspension-treated conditions. The amplification efficiencies for mHPRT and mCYP1A1 are 88.5 and 91.1%, with amplicon sizes of 85 and 165 base pairs, respectively. All PCR reactions were performed in triplicate.

Results

Homology-Based Targeted Mutagenesis of the Ligand Binding Domain of Mouse AhR. To establish residues with the potential to affect ligand binding, other studies of AhR have focused efforts on modeling the LBD (Procopio et al., 2002; Pandini et al., 2007), direct sequence comparison between species with differing TCDD affinity (Goryo et al., 2007), or a combination of both (Henry and Gasiewicz, 2008; Pandini et al., 2009). Our approach differs from these in that *D. rerio* LBD sequences drAhR1a and drAhR1b are 66% identical but show dramatic functional differences with regard to TCDD binding and inducibility. Alignment of mAhR LBD with drAhR1a and drAhR1b sequences highlighted several positions having amino acids with distinctly different physicochemical properties in drAhR1a relative to the TCDD-responsive AhRs (Fig. 1a). Lysine at position 245 was substituted with glutamic acid (K245E), because alignment with other AhR sequences known to bind TCDD illustrate strict conservation of a basic residue, an arginine or lysine, at this position, and hence, a reversed charge has the potential to affect function. However, Lys245 lies in a linker region that is predicted to be unstructured, and some sequence variability is seen in the surrounding sequence of other fish AhRs (Karchner et al., 2005). Two conservative mutations, H241Y and H285Y, were combined because of their similarity, both in amino acid composition and position at the end of predicted β-strands (Fig. 1a). The sequence comprising residues Lys297, Gly298, Gln299, and Leu300 at the start of the Per-Arnt-Sim core α-helical region had the greatest overall divergence and was substituted with the amino acids found in drAhR1a, with mutations K297N, G298W,

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Q299N, and L300F. Residues 369, 372, and 377 were selected for combined mutagenesis (Triple) because they lie close together in the same predicted β-strand (Yildiz et al., 2005) with substitutions R369Q, Y372C, and Q377H. Several previous studies have focused on mutagenesis of this β-strand and have demonstrated that position 375 has a profound impact on ligand-binding pocket access. Substitution with increasing size of side chain, A375V and A375L progressively reduce the capacity for AhR to be activated by ligand (Pandini et al., 2007), and in the case of A375I, the pocket is believed to be completely closed (Murray et al., 2005). Gln377 has been the target of other mutagenesis studies, based on predicted models of LBD structure in which Gln377 was hypothesized to form hydrogen bonds with TCDD (Procopio et al., 2002) and, in the more recent model from Pandini et al. (2007), predicted to point inward and affect either the steric capacity of the pocket or particular stereoelectronic requirements for ligand association. Thus, this strand has been strongly linked to ligand binding function, and the divergence of sequence in drAhR1 homologs suggested that this region would be a good place to search for ligand selectivity.

Substitutions thus identified were introduced into mAhR by site-directed mutagenesis, generating four clones: K245E, H241Y/H285Y (241.285), K297N/G298W/Q299N/L300F (NWNF), and R369Q/Y372C/Q377H (Triple) (Fig. 1a). The mutant forms of mAhR were stably integrated into HEK293T cells, and expression was examined by immunoblot with antibodies directed against the Myc tag of the mAhR constructs with relative levels confirmed by comparison to α-tubulin levels as the loading control (Fig. 1b). Because activation of AhR can be assessed using a reporter gene assay, and given the similar expression levels of mutant and wt mAhR (Fig. 1b), initial experiments examined mutant mAhR relative to wt receptor activity using the XRE-driven luciferase reporter gene pT81-X1X1-luc (Berghard et al., 1993). We used YH439 (Lee et al., 1996) as a nontoxic, potent activator of the AhR in these assays. Although this thiazolium compound diverges chemically from typical PAH/HAH ligands by exhibiting little aromaticity and having a greater number of heteroatoms, it has a surprising degree of planarity and has considerable geometric overlay with TCDD (Fig. 2). YH439 activates the AhR with kinetics similar to those of PAH/HAH ligands in animal models, in cultured cells, and in cell free protein extracts and is therefore considered a putative atypical AhR ligand (Lee et al., 1996).

The ability of the mutant mAhRs to activate the XRE-driven reporter gene was assessed relative to background levels resulting from activation of endogenous AhR (Fig. 3a).

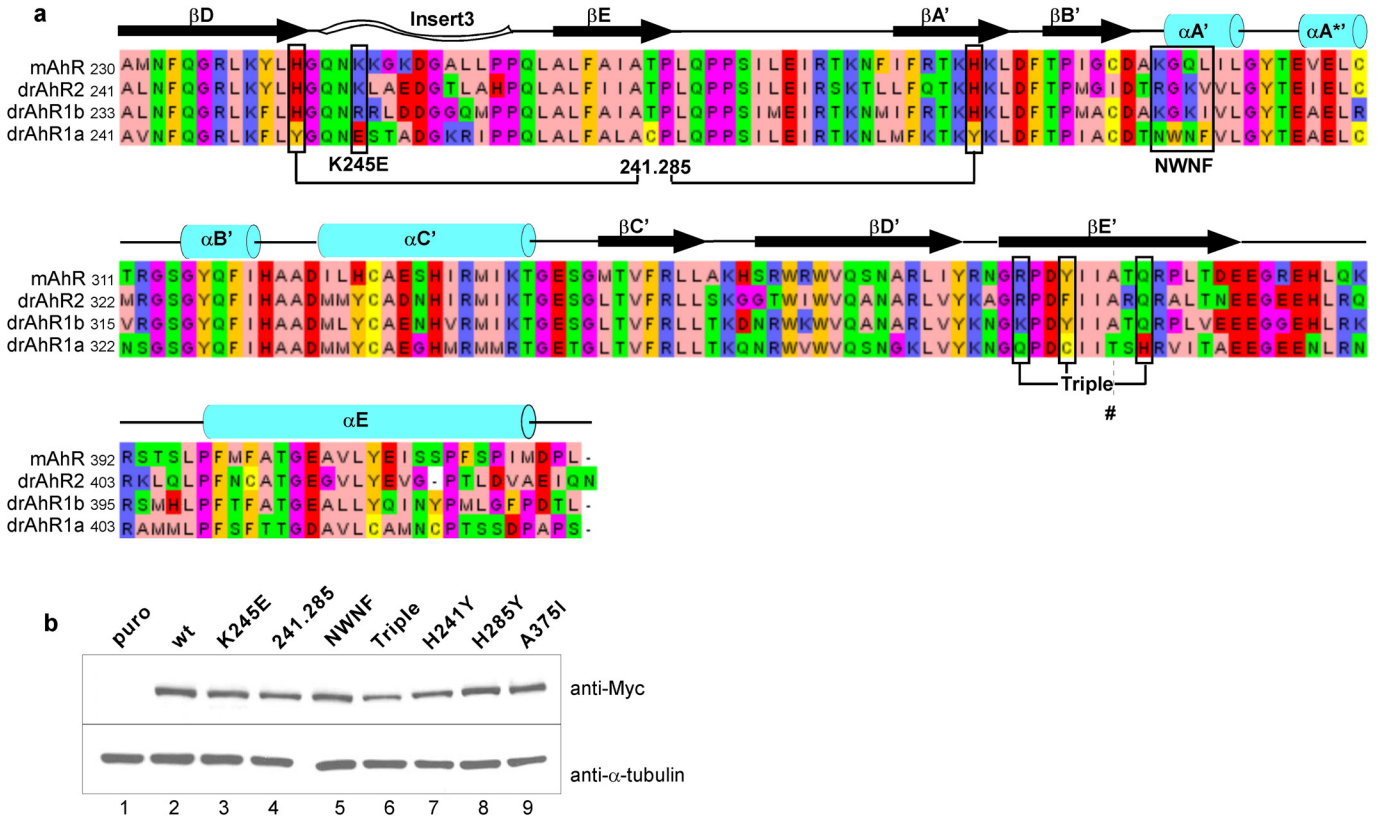

Fig. 1. AhR LBD alignment, site-directed mutagenesis, and expression of wt and mutant mAhR. a, LBD sequences of mouse (mAhR, NP_038492) and zebrafish aryl hydrocarbon receptor 2 (drAhR2, NP_571339), 1b (drAhR1b, NP_001019987), and 1a (drAhR1a, NP_571103) were aligned using ClustalW2. JalView (Waterhouse et al., 2009) was used to present the alignment in which Zappo colors represent the physicochemical properties of side chains (pink, aliphatic; orange, aromatic; blue, positively charged; red, negatively charged; green, hydrophilic; purple, conformationally special; yellow = cysteine). Secondary structures predicted from the structure of *Drosophila melanogaster* Period are represented above the alignment, where an arrow represents a β-strand and a cylinder, an α-helix (Yildiz et al., 2005). drAhR1a and drAhR1b were compared, and side chains with markedly different physicochemical properties were point-mutated in the mAhR LBD, including H241Y H285Y (241.285), K245E, K297N G298W Q299N L300F (NWNF), and R369Q Y372C Q377H (Triple). A375, proposed to be critical for LBD ligand access (Murray et al., 2005) is indicated by #. b, expression of Myc-tagged mAhR wt and mutant constructs from stable HEK293T cell lines. Whole-cell extracts (50 μg) were separated by SDS-PAGE, before immunoblotting for mAhR with anti-Myc or α-tubulin as a loading control.

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The empty reporter construct, pT81-blank-luc, showed no activation above baseline, whereas endogenous AhR activated pT81-X1X1-luc, albeit weakly, in response to YH439, and the latter is henceforth referred to as background activity (Fig. 3a, data sets 1 and 2). Exogenously expressed wt mAhR showed some basal activity (i.e., induction of XRE-driven luciferase activity) in the absence of stimulus (Fig. 3a, data set 3, vehicle and adherent controls) as generally is seen for overexpression of AhR (Murray et al., 2005) and strong inducible activity after treatment with YH439 and suspension culture (Fig. 3a, data set 3).

K245E mAhR showed a slight decrease in activity relative to wt in the reporter gene assay for both YH439 and suspension treatment and reduced basal activity in the untreated controls (Fig. 3a, data set 4). Because all conditions had similarly reduced activity relative to wt, we conclude that mutation K245E is similarly tolerated for YH439 and suspension activation of mAhR. Triple mAhR was expressed at only slightly lower levels than wt mAhR (Fig. 1b, lane 6) but showed overall much reduced activation in response to both YH439 treatment and suspension culture, with a decrease in basal activity also evident (Fig. 3a, data set 6). NWNF, representing mutations K297N, G298W, Q299N, and L300F, although expressed at levels similar to wt mAhR (Fig. 1b), did not show any reporter gene activity above background, and all treatments were similarly affected (Fig. 3, b and c). Because there were no selective differences in activity in response to YH439 and suspension for K245E, Triple, and NWNF mAhR, these mutations do not allow us to distinguish between activation modes and were not analyzed further. The 241.285 mAhR mutant was expressed at levels similar to those of wt mAhR (Fig. 1b, lanes 2 and 4) and was able to activate the reporter gene after treatment with YH439. However, 241.285 completely abrogated suspension activation of mAhR and showed no basal activation (i.e., above endogenous levels) (Fig. 3a, data set 5). In view of the selective activation by YH439 of 241.285 mAhR, although being unaffected by suspension culture, these mutations were of interest.

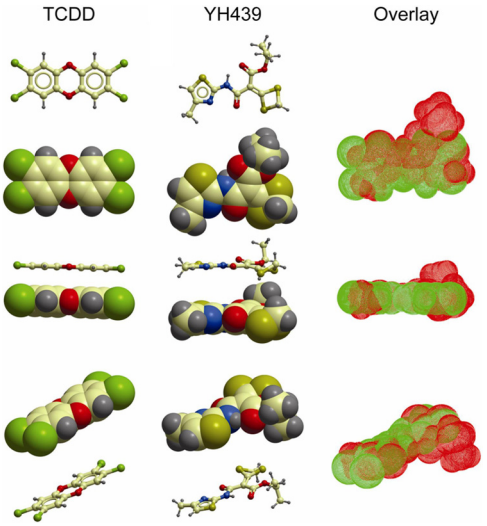

Fig. 2. Comparison of Ah receptor activators. Minimum energy conformations of Ah receptor activators TCDD and YH439 are shown in ball-and-stick and space-filling forms. Traditional pose with ligand flat axis in plane of paper are shown top, flat axis perpendicular to plane of paper at middle, and a partial rotation at bottom. Right, an overlay of space-filling models of TCDD (green) and YH439 (red) in the same orientation as is adjacent.

Histidine 285 in mAhR LBD Is Critical for Activation by Endogenous Paradigms but Not for Inducibility by YH439. The mutations H285Y and H241Y were constructed separately in mAhR to determine which amino acid was responsible for the selective activation by YH439. H241Y and H285Y mAhRs stably integrated into HEK293T cells were both expressed at similar levels to wt mAhR (Fig. 1b, lanes 2, 7, and 8) and showed comparable induction of reporter gene activity after treatment with YH439 (Fig. 4a, data sets 5 and 6). H241Y mAhR also had activation potential similar to that of wt mAhR in suspension culture (Fig. 4a, data set 5). In contrast, the H285Y mutation alone was responsible for abrogating suspension culture activation (Fig. 4a, data set 6) and for reducing basal activation of this receptor to endogenous levels (Fig. 4b, compare untreated samples, data sets 2, 4, and 6). It is clear that mutagenesis of His285 to tyrosine did not simply result in a nonfunctional mAhR, because strong activity was maintained after YH439 treatment. The enticing observation that AhR can be activated by oxidized low-density lipoproteins (McMillan and Bradfield, 2007a) prompted us to investigate the response of H285Y mAhR to this activation mechanism. In our hands, shear-stressed FCS applied at 60% of the total culture medium showed strong activation of XRE-driven reporter gene in the wt mAhR line, exceeding that of the suspension culture (Fig. 4c, data set 3). However, in contrast, H285Y mAhR showed no activation

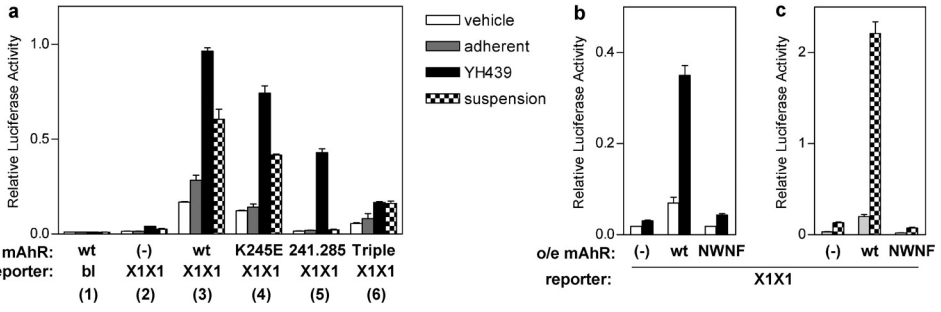

Fig. 3. Effects of mAhR LBD-targeted mutagenesis on inducibility of an AhR-responsive reporter gene. Mutant 241.285 shows abrogated suspension activity. 293T cell lines stably expressing wt or mutant forms of mAhR from the expression vector pEF/His-myc-mAhRfull/IRES/puro (o/e mAhR) (a); K245E, H241Y/H285Y (241.285), and R369Q/Y372C/Q377H (Triple) (b and c); K297N/G298W/Q299N/L300F (NWNF) were cotransfected with pT81-X1X1-luc (X1X1) or pT81-blank-luc (bl) reporter plasmids and RL-TK control plasmid for 24 h and then treated with vehicle alone (DMSO), YH439, or adherent or suspension culture as indicated for 16 h. The 293T cell line stably transfected with the pEF/blank/IRES/puro control expression vector is shown as (−). Relative luciferase activity was determined as described under *Materials and Methods*. Data are mean ± S.D. of transfections performed in triplicate and are representative of greater than three independent experiments.

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above background with application of shear-stressed serum or culture in suspension (Fig. 4c, data set 4). We also analyzed the proposed ligand pocket blocking mutation A375I in these assays; like H285Y mAhR, A375I mAhR showed no activation above background with these endogenous mechanism mimics (Fig. 4c, data set 5).

YH439, Unlike Prototypical PAH/HAH Ligands, Has a Novel Activation Mode that Tolerates Mutation of Histidine 285 to Tyrosine. Given the capacity of H285Y mAhR to categorically discriminate between YH439 and endogenous activation by suspension culture and in response to shear-stressed serum, an analysis of typical PAH- and HAH-type ligands was undertaken, and the response was assessed by reporter gene activity. Endogenous AhR in the 293T cell line showed some activity after treatment with the HAH TCDD and PAHs 3MC and B[*a*]P (Fig. 4d, data set 2). Exogenous expression of wt mAhR gave induction of luciferase reporter gene activity with the addition of these three ligands and with YH439 treatment. In contrast, mutant H285Y mAhR had no activity after treatment with PAH/HAH- type ligands and, as seen before, no basal activity, but it showed selective inducibility upon treatment with YH439 (Fig. 4d, data sets 3 and 4). This demonstrates that typical PAH/HAH ligand activation of AhR does not tolerate the relatively conservative mutation at position 285 of histidine to tyrosine. As discussed above, A375 seems to be critical for ligand binding pocket access (Pandini et al., 2007), and substitution with isoleucine has been shown to abrogate TCDD inducibility and prevent the basal constitutive activation of exogenous mAhR (Murray et al., 2005). Therefore, A375I mAhR was included in these assays to allow comparison with the effects of H285Y. We saw some YH439-induced activation of A375I mAhR above background, supporting the suggestion that YH439 binds the LBD in a manner distinct from TCDD and predicted endogenous ligands (Fig. 4d, data set 5). The observation that YH439 induced the activity of A375I mAhR was reduced relative to wt mAhR suggests that this compound still requires some access to the modeled PAH/HAH binding pocket. We conclude that H285Y mAhR does not respond to suspension culture, shear-stressed serum, or PAH/HAH-type ligands, and this activation profile reflects that seen for the proposed ligand binding pocket blocker A375I (Fig. 4, c and d).

These assays were performed at a concentration of YH439 (10 μM) in which the highest induction of target gene CYP1A1 has been identified (Lee et al., 1996), and the PAH/HAH ligands were used at concentrations that are saturating for wt AhR (Harper et al., 1991; Okey et al., 1994). Therefore, we considered the possibility that H285Y was a weakly activatible mutant mAhR and that the activation we observed was simply due to a vast excess of YH439. Reporter gene assays performed over a concentration range of YH439 indicated that H285Y mAhR responded in the same manner as wt mAhR to YH439 over the range 0.1 to 10 μM (Fig. 5a). Furthermore, both wt and H285Y mAhRs were maximally induced, and to similar levels, at the standard concentration

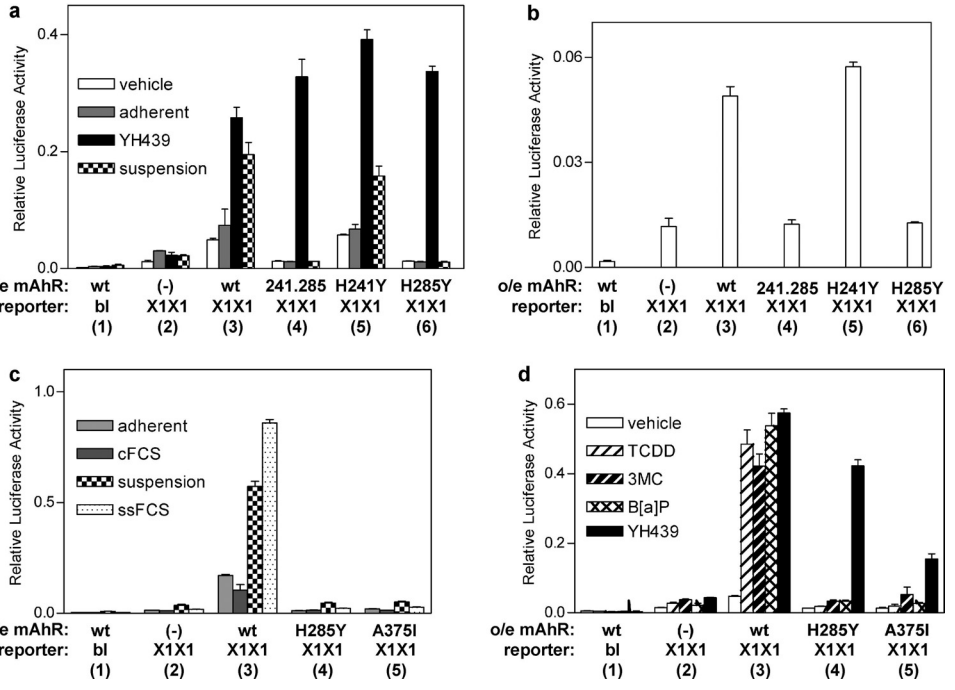

Fig. 4. Characterization of mAhR LBD mutant H285Y 293T cell lines stably expressing wt or mutant forms of mAhR from the expression vector pEF/His-myc-mAhRfull/IRES/puro (o/e mAhR) were cotransfected with pT81-X1X1-luc (X1X1) or pT81-blank-luc (bl) reporter plasmids and RL-TK control plasmid for 24 h and then treated as indicated. Relative luciferase activity was determined as described under *Materials and Methods*. Data are mean ± S.D. of transfections performed in triplicate and are representative of more than three independent experiments. a, mAhR LBD mutant H285Y cannot be activated by suspension treatment, shows no background activity, and maintains full YH439 activation. Treatment of mAhR double-mutant 241.285, mutants H241Y alone or H285Y alone with vehicle (DMSO), 10 μM YH439, or adherent or suspension culture for 16 h. b, an enlarged representation of the background activity (vehicle control) for the cell lines shown in a. c, mAhR mutant H285Y and A375I are not activated by shear-stressed FCS or suspension treatment. To examine the effect of H285Y and the proposed ligand binding pocket blocking mutation A375I (Murray et al., 2005) on endogenous activation states, cell lines expressing mutant forms of mAhR were left untreated (adherent), grown in suspension culture, or cultured in 60% untreated (cFCS) or shear-stressed FCS (ssFCS) for 16 h. d, mAhR mutant H285Y cannot be activated by typical PAH compounds 3MC, B[*a*]P, and HAH compound TCDD. H285Y and A375I were tested for inducibility by PAH/HAH-type ligands or YH439. Cell lines were treated with vehicle (0.1% DMSO), 1 nM TCDD, 1 μM 3MC, 100 nM B[*a*]P, or 10 μM YH439 for 16 h.

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of YH439, (i.e., 10 μM). Reduced response was detected at very low YH439 (0.01 μM) for the H285Y mutant relative to wt mAhR, suggesting that H285Y mAhR may have somewhat reduced affinity for and/or activation response to YH439. To address the possibility that H285Y mAhR was unresponsive to PAH ligands in our assays because of reduced affinity for all ligands rather than being selectively activated by YH439, we compared both H285Y mAhR and the PAH/HAH ligand pocket blocking mutant A375I mAhR with wt mAhR for response to very high concentrations of TCDD (Fig. 5b). At concentrations up to 100 nM TCDD, in contrast to wt mAhR, neither H285Y nor A375I mAhR showed activation in reporter gene assays, demonstrating that the H285Y mutation does not simply reduce affinity for the PAH/HAH class of ligand typified by TCDD.

To investigate the effect of the H285Y mutation on inducibility of an endogenous target gene in response to mAhR activation, wt, H285Y, and A375I mAhR constructs were stably integrated into mouse AhR(−/−) hepatocytes using lentiviral-mediated infection. Expression levels of mAhR were similar across the stable cell lines, compared by Western blotting with α-tubulin loading controls (Fig. 6a). Activation of the prototypical AhR target gene CYP1A1 in these cell lines, measured by qRT-PCR, showed a dramatic difference between wt and mutant mAhRs. As expected, CYP1A1 expression was strongly induced by mAhR in response to YH439, TCDD, and suspension culture (Fig. 6b). In contrast, only YH439 but not TCDD or suspension treatment of H285Y mAhR was able to induce CYP1A1 expression (Fig. 6b, inset). The relative induction of CYP1A1 by wt and mutant mAhR in response to the activating stimuli YH439, TCDD, and suspension culture mediated was consistent with the effects seen in reporter gene assays. However, there was a marked difference (>10-fold) between wt and H285Y mAhR in the basal level of activation of the endogenous target gene in these cell lines resulting simply from exogenous expression of the receptor (Fig. 6b and inset, vehicle control), and this difference was reflected in levels of induction by YH439. In addition, consistent with the reporter gene assay data, YH439 but not TCDD treatment or suspension culture was able to induce CYP1A1 expression in cells harboring the A375I mAhR receptor (Fig. 6c). This supports the notion of a novel activation mode for YH439 that is distinct from the classic PAH/HAH type of ligand or putative endogenous ligand induced by suspension treatment.

Next, we looked at the effect of the H285Y and A375I substitutions on binding of mAhR to DNA containing an XRE derived from the mCYP1A1 promoter in EMSAs in vitro

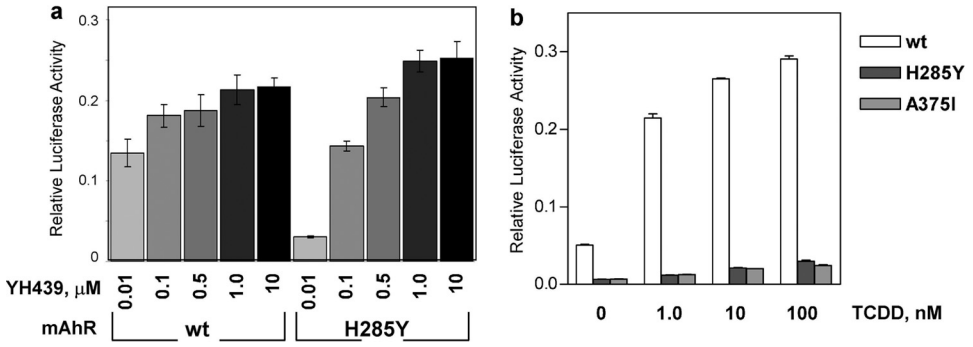

Fig. 5. H285Y mAhR is not a broad-specificity low-affinity receptor. 293T cell lines stably expressing wt or mutant forms of mAhR from the expression vector pEF/His-myc-mAhRfull/IRES/puro were cotransfected with pT81-X1X1-luc reporter and RL-TK for 24 h followed by treatment with vehicle (DMSO), YH439 (a), or TCDD (b) for 16 h at the concentrations indicated. a, wt and H285Y mAhR are similarly induced over a concentration gradient of YH439. The inducibility of H285Y mAhR relative to wt mAhR by YH439 was assessed using a gradient activation reporter gene assay, in which the highest concentration, 10 μM, was that used in all other experiments. Data are mean ± S.D. of transfections performed in triplicate, adjusted for basal activity of exogenous mAhR in the untreated lines, and representative of more than three independent experiments. b, H285Y and A375I mAhR are unresponsive to very high concentrations of TCDD. wt, H285Y, and A375I mAhR were assayed for activation of the reporter gene in response to increasing concentrations of the prototypical HAH ligand TCDD. The highest concentration, 100 nM, was 100-fold greater than that used in Fig. 4d. Data are mean ± S.D. of transfections performed in triplicate and are representative of two independent experiments.

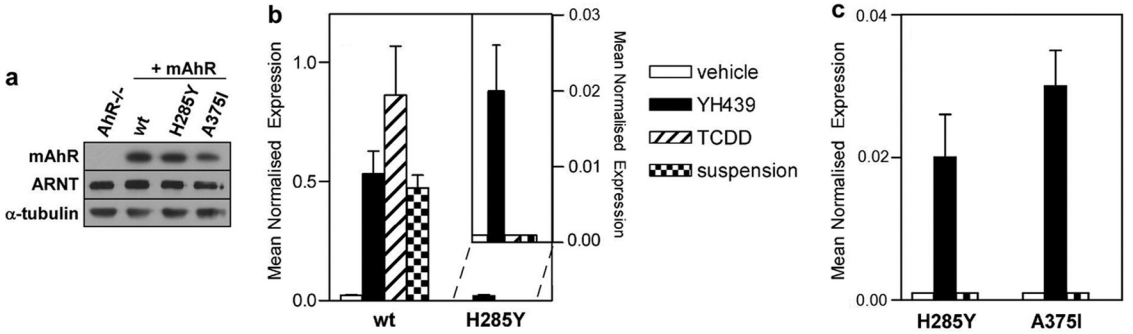

Fig. 6. mAhR mutants H285Y and A375I activate CYP1A1 expression in hepatocytes only in response to YH439. wt, H285Y, and A375I mAhR constitutively expressed in AhR(−/−) hepatocytes from integrated lentiviral vectors were analyzed for their ability to induce CYP1A1 mRNA in response to treatments. a, protein levels of mAhR wt, H285Y, and A375I. Whole-cell extracts (50 μg of protein) were separated by SDS-PAGE, before immunoblotting for mAhR, ARNT, or α-tubulin as a loading control. AhR(−/−) represents lines having integration of pLV416_empty plasmid into the parental AhR null cell line. b and c, cell lines expressing wt, H285Y, or A375I mAhR were treated with vehicle alone (DMSO), 10 μM YH439, 10 nM TCDD, or suspension culture as indicated for 5 h, and induced levels of CYP1A1 mRNA were determined by qRT-PCR. b, inset, H285Y mAhR data with enlarged scale. Data are mean ± S.E. from three independent experiments.

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(Pongratz et al., 1998). To eliminate any effects of the mutations on nuclear translocation, mAhR was transformed in vitro by the addition of TCDD or YH439 to cytosolic extracts prepared from the lentiviral hepatocyte cell lines expressing wt or mutant receptor before testing DNA binding ability. As expected, both TCDD and YH439 were able to induce wt mAhR transformation and promote subsequent DNA binding (Fig. 7). Consistent with the reporter gene and qRT-PCR assays, DNA binding was detected for the mAhR H285Y and A375I mutants only in the presence of YH439, albeit at reduced levels, and neither mutant mAhR was converted to an active DNA binding form by TCDD. This difference in ligand selection clearly was not due to protein levels, because all stable lines expressed similar levels of mAhR and the dimerization partner Arnt (Fig. 6a). The reduced DNA binding induced by YH439 for H285Y and A375I mAhR relative to wt mAhR is consistent with the extent to which each receptor was able to activate the endogenous target gene CYP1A1 in the hepatocyte lines. However, this effect does not contribute significantly to the response to YH439 detected in reporter gene assays, which presumably reflects the higher concentration of both exogenous mAhR and DNA binding sites in the reporter gene experiments. In addition, immunohistochemistry experiments with anti-AhR antibody showed a marked reduction of ligand-induced nuclear translocation of mutant H285Y mAhR, and to a lesser extent A375I mAhR, compared with wt receptor (data not shown). This reduced nuclear traffic is consistent with the marked reduction in the basal activity of the mutant receptors in both reporter gene and CYP1A1 activation levels.

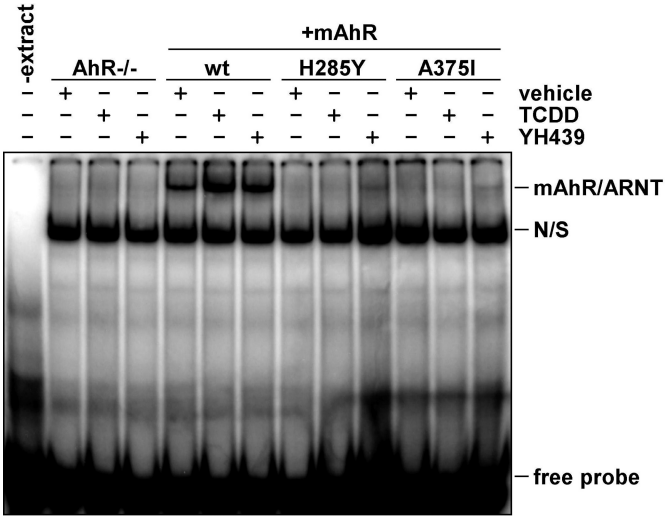

Fig. 7. DNA binding ability of wt, H285Y, and A375I mAhR YH439. Cytosolic extracts from AhR(−/−) hepatocytes constitutively expressing wt, H285Y, or A375I mAhR from integrated lentiviral vectors were treated in vitro with vehicle alone (0.1% DMSO), 10 nM TCDD, or 10 μM YH439 and then analyzed for binding to XRE DNA in EMSAs. AhR(−/−) represents integration of empty vector into the parental AhR-null cell line. The mobility of mAhR/ARNT/DNA complex and unbound DNA is indicated (mAhR/ARNT and free probe), −extract, no added extract; N/S, nonspecific species; the data shown are representative of three independent experiments.

The specific AhR antagonist 3′,4′-dimethoxyflavone (3′,4′-DMF) is a PAH compound and thus is structurally related to TCDD (Lee and Safe, 2000). We predicted that if the mode of activation of YH439 with the AhR LBD is mechanistically distinct from binding by PAH/HAH compounds, 3′,4′-DMF would not antagonize the induction of AhR activity by the structurally different compound YH439. For both endogenous hAhR in the MCF-7 breast cancer cell line and our 293T cell line constitutively expressing exogenous mAhR, 3′,4′-DMF inhibited TCDD-dependent reporter gene activation, as expected. In contrast, although YH439 induced the reporter gene to a level comparable with TCDD treatment in both cell lines, cotreatment with 3′,4′-DMF gave no reduction in activation in response to YH439, presumably because of a lack of direct competition between the PAH compound 3′,4′-DMF and YH439 for sites in the AhR LBD (Fig. 8). Neither YH439 nor TCDD had any effect on the reporter gene in cell lines expressing a constitutively active mAhR, in which the LBD has been deleted (Lees and Whitelaw, 1999), suggesting that YH439 induction of AhR is, in fact, working through the LBD (data not shown).

Discussion

We have used existing knowledge of drAhR homologs 1a and 1b to perform a targeted mutagenesis screen of the mAhR LBD to discover mutants that could discern discrete activation mechanisms. We sought to determine whether abrogating exogenous ligand binding would affect all PAH/HAH-type ligands, the atypical activator YH439, and endogenous activation states mimicked by suspension culture and application of shear-stressed FCS. Past mutagenesis experiments provide proof-of-principle for this approach and have yielded mutant mAhR, which could be selectively activated by a particular exogenous ligand. For example, 3MC can activate F318L-substituted AhR, whereas β-naphthoflavone and TCDD cannot (Goryo et al., 2007). However, past LBD mutagenesis screens have simply used basal activity to indicate whether mutants were competent for endogenous activation. Our analysis is distinct from these investigations in that we have specifically set out to explore the effects of mutations on endogenous activation of mAhR. Furthermore, we included a chemically atypical AhR activator for a more

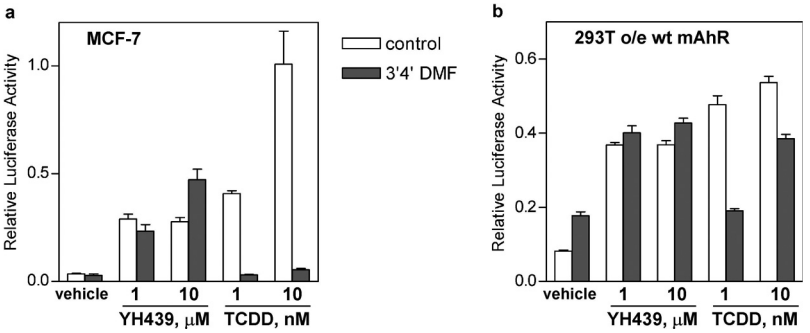

Fig. 8. The PAH-type antagonist 3′,4′-DMF blocks mAhR induction by TCDD but not YH439. Cell lines MCF-7 (a) or 293T (b) stably expressing wt mAhR were cotransfected with pT81-X1X1-luc and RL-TK control plasmid for 24 h and then treated with 10 μM 3′,4′-DMF together with vehicle alone (DMSO), YH439, or TCDD as indicated for 16 h. Relative luciferase activity was determined as described under *Materials and Methods*. Data are mean ± S.D. of transfections performed in triplicate and are representative of two independent experiments.

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comprehensive understanding of the functional effects of any given mutation.

H285Y mAhR Identifies a Novel Activation Mode for YH439. Our analysis showed that substitution of histidine 285 for tyrosine in the LBD of mAhR generated a receptor that was selectively activated by YH439 in both reporter gene assays (Fig. 4) and on the endogenous target gene CYP1A1 (Fig. 6). In addition, A375I, theorized to block access to the ligand binding pocket (Murray et al., 2005), showed a similarly selective capacity to be activated only by YH439. These results imply that YH439 has a novel activation mode that does not require histidine 285 or full access to the ligand binding pocket according to the PAH/HAH-ligand paradigm (Procopio et al., 2002; Murray et al., 2005; Pandini et al., 2007, 2009), which is permissive of the H285Y mutation. This is supported by our observation that the AhR antagonist 3′,4′-DMF, which is a PAH-type compound, was able to compete with the prototypical HAH ligand TCDD but not with YH439 to inhibit the activation of wt AhR (Fig. 8). YH439 contains a larger number of heteroatoms (S, N, O) than typical AhR ligands, thus providing more opportunities for hydrogen bonding and electrostatic interaction with amino acids in the LBD. Hence, YH439 may represent a physiologically relevant new class of AhR activator, presumably a new class of ligand, and H285Y mAhR seems a good diagnostic tool in reporter gene assays for the classification of this type of binding event.

Our data show that in vitro transformation of mAhR, in response to YH439, to a species competent for DNA binding was reduced for the H285Y and A375I substitutions (Fig. 7). We also saw reduced nuclear localization in response to YH439 for both of these mutant receptors (data not shown). These observations are consistent with the reduced activation of the reporter gene at the lowest YH439 concentrations for H285Y mAhR (Fig. 5) and together suggest that although the H285Y and A375I substitutions are permissive of activation by YH439, both mutant receptors respond to this compound with reduced potency relative to wt mAhR. The disparity between the level of response as measured in reporter gene assays and the ability to activate the CYP1A1 gene in an endogenous context, relative to wt mAhR, is most likely a concentration effect. The presence of multiple copies of the XRE target DNA binding site of mAhR/ARNT in the reporter gene assays, combined with the higher expression levels of mAhR from integrated pEF/His-myc-mAhRfull/IRES/puro than the lentiviral constructs (data not shown), was clearly sufficient to overcome the effects of this reduced affinity completely for H285Y and partially for A375I mAhR.

Assessment of PAH/HAH-type ligand inducibility of wt, H285Y, and A375I mAhR identified that H285Y could not be activated by 3MC, B[*a*]P, or TCDD. This demonstrates that typical PAH and HAH ligand activation of AhR does not tolerate the relatively conservative mutation of histidine at position 285 to tyrosine. His285 is predicted to point into the ligand binding pocket in a recent structural model of the LBD (Pandini et al., 2007), and mutation of His285 to alanine prevents TCDD binding, and consequent DNA binding (Pandini et al., 2007), in agreement with our finding of a requirement for histidine 285 for PAH/HAH binding.

Implications for Endogenous PAH/HAH-Type Ligands for AhR. As with the H285Y substitution, mAhR with the proposed ligand binding pocket blocking mutation, A375I, similarly failed to respond to suspension culture, shear-stressed serum, and PAH/HAH-type ligands. Both of these mutations could also prevent the commonly seen basal activation of the overexpressed receptor in untreated cells (Fig. 4) that is seen in a number of reporter studies (Murray et al., 2005; Goryo et al., 2007). Our data support the hypothesis, as has been proposed in the past (Chang and Puga, 1998; Murray et al., 2005), that endogenous ligand(s) are present in cells in culture that can activate wt mAhR. Such ligands cannot interact with the blocked ligand binding pocket of A375I and are not permissive of a tyrosine at position 285. Furthermore, the mechanism by which shear-stressed serum can activate mAhR probably involves a ligand similar to those responsible for the basal activation of exogenous overexpressed AhR and induced by suspension culture. More specifically, it seems plausible that these endogenous activation states are mediated by endogenous ligand(s) structurally similar to the PAH class of xenobiotic ligands, typified by 3MC, and B[*a*]P. Given that H285Y mAhR responds similarly to each of these treatments, we suggest that such endogenous ligands have similar binding pocket requirements that do not tolerate the mutation of histidine 285 to tyrosine to induce AhR activity. In addition, given that H285Y mAhR could be activated by YH439 but that all other modes of activation we tested were completely abrogated, we hypothesize that endogenous ligands involved in suspension culture, shear-stressed serum, and basal activation access and bind to the ligand binding pocket in a manner distinct from YH439.

What remains to be established is whether mutations in the LBD have differential impacts on other AhR target genes that may be cell context-specific or, indeed, whether mutations alter the efficiency of AhR in its functional role as an adaptor protein in the Cul4B(AhR) ubiquitin ligase context. We predict the analysis of mutations in the context of different ligand species, and assessment of these less well characterized aspects of AhR function will provide different results compared with simple TCDD binding and CYP1A1 promoter activation. Our observations suggest the potential for a new class of activator/ligand that may invoke alternate regulation of AhR to control diverse functional roles, seemingly unrelated to PAH metabolism. Furthermore, mAhR mutant H285Y may have application as a diagnostic tool to aid in defining the binding mode of the expanding list of AhR-activating agents.

LBD Structural Elements Helix αA′ and strand βE′ Are Important for Ligand Binding Functions. For the NWNF substitution in mAhR, in which expression levels were similar to wt mAhR (Fig. 1b, lanes 2 and 6), mutation of residues 297 to 300 may have altered protein folding. Position 297 commonly has a conserved basic residue ahead of the start of helix αA′ (Fig. 1a). Insertion of tryptophan and phenylalanine at the first and third positions of this helix represents a dramatic change in size, in which there are typically small polar or nonpolar side chains. Hence, these mutations may have altered the structure of this helix to abolish the activation of the mutant mAhR. These are the first reported mutations in this predicted helix and demonstrate the importance of this region for mAhR function.

The mutations R369Q, Y372C, and Q377H (Triple) lie within a β-strand of the LBD known to be important for PAH/HAH ligand binding and containing the critical residue

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Ala375, discussed above. Previous studies show the Q377L substitution in AhR causes decreased DNA binding, decreased TCDD binding (70–75% relative to wt), and a concomitant decrease in TCDD-induced reporter gene activity (Henry and Gasiewicz, 2008). In the case of Q377A AhR, DNA binding is reduced to ~50% and TCDD binding to ~30% relative to wt, although no target gene activity was assessed (Pandini et al., 2007). Hence, the composition of this strand seems intrinsically linked to LBD function. Given the effects of mutagenesis of Gln377 to alanine (Pandini et al., 2007) and leucine (Henry and Gasiewicz, 2008), changing this residue to histidine in our study is hypothesized to alter the steric and potentially the stereoelectronic properties of the ligand binding pocket. Our Triple mAhR mutant receptor was largely unresponsive to all activation modes, although being expressed at only slightly lower levels than wt mAhR, and we conclude that this region of the LBD is critical for activation by both typical PAH/HAH ligands, the chemically distinct compound YH439, and for the response to activating signals in the endogenous activation mechanism represented by suspension culture.

Acknowledgments

We thank Dr. Simon Barry, University of Adelaide, for the lentiviral expression plasmid pLV416; Prof. Gary Perdew, Pennsylvania State University, for the AhR(−/−) hepatocyte cell line; Prof. C. K. Shim, Seoul National University and Yuhan Research Institute (Korea), for the gift of YH439; and Dr. J. Simms for assistance with the preparation of Fig. 2.

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