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Repression of Aryl Hydrocarbon Receptor (AHR) Signaling by AHR Repressor: Role of DNA Binding and Competition for AHR Nuclear Translocator

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AHR) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin causes altered gene expression and toxicity. The AHR repressor (AHRR) inhibits AHR signaling through a proposed mechanism involving competition with AHR for dimerization with AHR nuclear translocator (ARNT) and binding to AHR-responsive enhancer elements (AHREs). We sought to delineate the relative roles of competition for ARNT and AHREs in the mechanism of repression. In transient transfections in which AHR2-dependent transactivation was repressed by AHRR1 or AHRR2, increasing ARNT expression failed to reverse the repression, suggesting that AHRR inhibition of AHR signaling does not occur through sequestration of ARNT. An AHRR1 point mutant (AHRR1-Y9F) that could not bind to AHREs but that retained its nuclear localization was only slightly reduced in its ability to repress AHR2, demonstrating that AHRR repression does not occur solely through competition for AHREs. When both proposed mechanisms were blocked (AHRR1-Y9F plus excess ARNT), AHRR remained functional. AHRR1 neither blocked AHR nuclear translocation nor reduced the levels of AHR2 protein. Experiments using AHRR1 C-terminal deletion mutants showed that amino acids 270 to 550 are dispensable for repression. These results demonstrate that repression of AHR transactivation by AHRR involves the N-terminal portion of AHRR; does not involve competition for ARNT; and does not require binding to AHREs, although AHRE binding can contribute to the repression. We propose a mechanism of AHRR action involving "transrepression" of AHR signaling through protein-protein interactions rather than by inhibition of the formation or DNA binding of the AHR-ARNT complex.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and member of the bHLH-PAS protein superfamily (Schmidt and Bradfield, 1996). The AHR is required to mediate the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Fernandez-Salguero et al., 1996; Mimura et al., 1997; Prasch et al., 2003). Many adverse effects of TCDD are caused by AHR-dependent changes in gene expression (Bunger et al., 2003; Nebert et al., 2004). The AHR is located in the cytoplasm as a complex with 90-kDa heat shock protein and aryl hydrocarbon receptor-associated protein (ARA9, also called XAP2 or AIP) (LaPres et al., 2000; Petrulis et al., 2000). Upon binding TCDD, the AHR translocates to the nucleus, dissociates from 90-kDa heat shock protein and ARA9 (Heid et al., 2000), and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Reyes et al., 1992). The AHR-ARNT complex binds to AHR-responsive enhancer elements (AHREs, also called DREs or XREs) in the 5′ regulatory regions of AHR target genes and activates or represses transcription (Mimura and Fujii-Kuriyama, 2003). The mechanism of transcriptional activation by TCDD-activated AHR has been extensively charac-

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; bHLH, basic-helix-loop-helix; PAS, Per-ARNT-Sim; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; AHRE, aryl hydrocarbon receptor-responsive enhancer element; AHRR, aryl hydrocarbon receptor repressor; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; m, mouse; YFP, yellow fluorescent protein; TNT, transcription/translation; EMSA, electrophoretic mobility shift assay; SRC-1, steroid receptor coactivator-1.

terized. However, the negative regulation of the AHR signaling pathway is not as well understood.

There are several mechanisms by which AHR signaling may be down-regulated. One mechanism involves ligand-dependent degradation of AHR protein through a proteasomal pathway (Pollenz, 2002; Wentworth et al., 2004). The resultant decrease in AHR protein causes a reduction in AHR-dependent transcription (Pollenz et al., 1998). Other mechanisms by which AHR-dependent signaling can be reduced involve transcriptional repression of AHR target genes. This may involve competition for cofactors (Gradin et al., 1996; Ke et al., 2001), binding to negative regulatory elements (Boucher et al., 1995), or interference with AHRE binding of the AHR-ARNT complex (Gradin et al., 1993, 1999). Gradin et al. (1993) provided evidence for a protein that interacts with ARNT and binds to AHREs, resulting in nonresponsiveness of human fibroblasts to TCDD treatment. One protein that has been identified and proposed to function by this mechanism is the AHR repressor (AHRR) (Mimura et al., 1999; Haarmann-Stemmann and Abel, 2006). In transient transfections, AHRR represses AHR-dependent transactivation of a reporter construct (Mimura et al., 1999; Karchner et al., 2002; Evans et al., 2005). AHRR expression is induced by a variety of AHR agonists (Mimura et al., 1999; Karchner et al., 2002; Evans et al., 2005). Induction of AHRR by AHR agonists and AHRR-dependent repression of AHR signaling imply that the AHRR forms a negative feedback loop with the AHR.

Mimura et al. (1999) proposed a dual mechanism for AHRR repression of AHR signaling. They showed that the AHRR can form a heterodimer with ARNT and that this complex can bind to AHREs. They hypothesized that the mechanism of repression involved both competition with AHR for binding to ARNT and competition of AHRR-ARNT and AHR-ARNT complexes for binding to AHREs (Mimura et al., 1999). Although suggestive, the results of Mimura et al. (1999) did not establish with certainty the role of ARNT and AHRE binding in the mechanism of repression and whether these two mechanisms are sufficient to explain the loss of AHR signaling in the presence of AHRR. In addition, the relative roles of competition between AHR and AHRR for binding to ARNT or AHREs were not elucidated.

In the present study, we sought to determine the relative contributions of competition for ARNT and AHREs in the mechanism of repression by AHRR. We hypothesized that if repression were occurring primarily through competition for ARNT, then overexpression of ARNT would relieve the repression. However, if the mechanism were primarily through competition for AHREs, then an AHRR that lacks the ability to bind DNA would be unable to repress AHR transactivation. Our results indicate that neither competition for ARNT nor displacement of AHR from AHREs is the sole mechanism by which the AHRR functions as a repressor and that when both proposed mechanisms are blocked, AHRR remains functional. We also show that AHRR does not act by reducing AHR protein levels or interfering with ligand-induced nuclear translocation of the AHR and that the C-terminal half of AHRRs, which is not well conserved and is variable in size across species (Evans et al., 2005), is dispensable for repression. Thus, the N-terminal half of AHRRs, which contains the putative ARNT heterodimerization and DNA binding domains (Fukunaga et al., 1995), is sufficient for repression but through a mechanism that does not require direct binding to AHREs or competition for ARNT.

Materials and Methods

Chemicals. TCDD was obtained from Ultra Scientific (Hope, RI). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO).

Generation of AHRR1 Wild-Type and Mutant Plasmid Constructs. The pcDNA-AHRR1 and pcDNA-AHRR2 constructs encoding full-length zebrafish AHRR1 (*ahrra*) and AHRR2 (*ahrrb*) have been described previously (Evans et al., 2005). A wild-type AHRR1-EGFP fusion protein was generated as follows: a small fragment of AHRR1 that introduced an EcoRI restriction site in place of the stop codon was amplified by polymerase chain reaction using Advantage 2 polymerase (Clontech, Mountain View, CA), the forward primer 5′-GCAGGACTGAAGGCCTGTACG-3′, and the reverse primer 5′-GGAATTCGTGTGTGCAGGTGTGTGTGTCG-3′, and it was subsequently cloned into pGEM-T Easy vector (Promega, Madison, WI). For generation of a full-length AHRR1-EGFP expression construct, this product was cut with SacII and EcoRI and ligated with a fragment from the full-length pcDNA3.1-AHRR1 that was cut with HindIII and SacII. Next, these fragments were ligated together and inserted into EGFP-N2 (Clontech) at the HindIII and EcoRI sites to generate AHRR1-EGFP.

AHRR1-Y9F-EGFP was made by introducing a point mutation converting the tyrosine at position 9 to a phenylalanine. The point mutation was performed by the QuikChange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using AHRR1-EGFP as a template with the forward primer 5′-GGAGACTGTCTTTTCGCCGGGAGGAAGAGG-3′ and the reverse primer 5′-CCTCTTCCTCCCGGCGAAAAGACAGTCTCC-3′. This expression construct was confirmed by sequencing at the University of Maine DNA Sequencing Facility (Orono, ME). A second mutant AHRR1, pcDNA-AHRR1-Y9F, was made by cutting AHRR1-Y9F-EGFP with NotI and SacI and then ligating this product with a fragment of pcDNA3.1-AHRR1 that had been cut with SacI and XbaI. This full-length AHRR1-Y9F was inserted into pcDNA 3.1 at the NotI and XbaI sites to create pcDNA-AHRR1-Y9F.

Two C-terminal deletion mutants were constructed. To make pcDNA-AHRR1Δ270-550, a fragment of AHRR1 was amplified by the forward primer 5′-GACTACATCCATGTGGACGACC-3′ and the reverse primer 5′-TCTAGAACGGCAGCAGGGGA-3′, with a stop codon introduced at position 270, using pcDNA-AHRR1 as a template. This product was cloned into pGEM-T easy vector, cut with SacI and XbaI, and then ligated with wild-type AHRR1 cut with NotI and SacI into pcDNA3.1. To make pcDNA-AHRR1Δ189-550, a fragment of AHRR1 was amplified by the forward primer 5′-GCTGATGTCGGAGAAGTCAAACC-3′ and the reverse primer 5′-TCTAGAACTCCTCCGCTGTGC-3′, with a stop codon introduced at position 189, using pcDNA3.1-AHRR1 as a template. This product was cloned into pGEM-T easy vector, cut with PstI and XbaI, and then ligated with wild-type AHRR1 cut with NotI and PstI into pcDNA3.1. To make pcDNA-AHRR1-Y9FΔ189-550, a fragment of AHRR1 was amplified by the forward primer 5′-GCTGATGTCGGAGAAGTCAAACC-3′ and the reverse primer 5′-TCTAGAACTCCTCCGCTGTGC-3′, with a stop codon introduced at position 189, using pcDNA-AHRR1 as a template. This product was cloned into pGEM-T easy vector, cut with PstI and XbaI, ligated with AHRR1-Y9F cut with NotI and PstI and inserted into pcDNA3.1.

Other Plasmid Constructs. Expression vectors for zebrafish AHR2 (pBK-CMV-zfAHR2) (Tanguay et al., 1999), ARNT2b (pBK-CMV-FlagzfARNT2b) (Tanguay et al., 2000), and ARNT1c (pBKCMV-zfARNT1c) (Prasch et al., 2006) were provided by Dr. R. Tanguay (Oregon State University, Corvallis, OR) and Dr. R. Peterson (University of Wisconsin, Madison, WI). The zebrafish ARNT2b-Flag cDNA was subcloned into pcDNA3.1. The mouse AHRR expression construct (pcDNA-mAHRR) was derived from plasmid pBSKmAhRR (Mimura et

al., 1999) (a gift from Y. Fujii-Kuriyama, University of Tsukuba, Japan) as described previously (Karchner et al., 2002). The mouse AHR-YFP fusion construct (Petrulis et al., 2000) was provided by Dr. Gary Perdew (The Pennsylvania State University, University Park, PA). The plasmid pGudLuc 6.1, which contains the firefly luciferase reporter under the control of a mouse mammary tumor virus promoter regulated by four AHREs from the murine CYP1A1 promoter, was a gift from Dr. M. Denison (University of California, Davis, CA) (Karchner et al., 2002). Expression constructs for human ARNT and mouse ARA9 (LaPres et al., 2000) were obtained from Dr. C. Bradfield (University of Wisconsin).

Transient Transfections and Luciferase Assays. Transient transfections were performed as described previously (Evans et al., 2005). In brief, COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with fetal calf serum (10% final concentration) at 37°C under 5% CO_2. Cells were plated at 3×10^4 cells/well in 48-well plates. Transfections of DNA with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) were carried out in triplicate wells 24 h after plating. Approximately 300 ng of DNA was complexed with 1 μl of Lipofectamine 2000, and then it was added to cells; the amount of DNA used for each expression construct is listed in the figure legends. The total amount of DNA was kept constant by adding in empty vector. Five hours after transfection, cells were exposed to 0.5% DMSO or TCDD (10 nM final concentration). *Renilla reniformis* luciferase (pRL-TK; Promega) was used as the transfection control. Cells were lysed 19 h after dosing, and luminescence was measured using the Dual Luciferase Assay kit (Promega) in a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). The final values are expressed as a ratio of the firefly luciferase units to the *R. reniformis* luciferase units.

Western Blots. COS-7 cells were plated and transfected as described above. Five hours after transfection, cell lysates were prepared in 2× sample treatment buffer. Fifty microliters of each cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose. In vitro (TNT)-expressed ARNT2b served as a positive control. For ARNT2, blots were probed with a 1:4000 dilution of MA1-515 (Affinity BioReagents, Golden, CO), a monoclonal antibody directed against the C-terminal amino acids of human ARNT, which are conserved in zebrafish ARNT2. For detection of AHR2, blots were probed with 1:1000 dilution of an AHR2 antibody directed against amino acids 816 to 1027 (Wentworth et al., 2004). For AHRR1 and AHRR1-Y9F, blots were probed with affinity-purified antiserum (5 μg/ml) raised against amino acids 285 to 300 of zebrafish AHRR1. A monoclonal actin antibody (Sigma-Aldrich) was used at 1:5000 dilution as the internal control. Blots were subsequently probed with goat anti-mouse IgG horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) secondary antibody (1:10,000) for ARNT2 and actin, and goat anti-rabbit IgG horseradish peroxidase (Upstate/Millipore Corporation, Billerica, MA) secondary antibody (1:5000) for AHR2 and AHRR1. The blots were then visualized by enhanced chemiluminescence.

Electrophoretic Mobility Shift Assay. TNT Quick-Coupled Reticulocyte Lysate systems (Promega) were used to synthesize proteins following manufacturer's directions. Complementary oligonucleotides containing the mouse CYP1A AHRE sequence (5′-GATCTGGCTCTTCTCACGCAACTCCG-3′) (the core AHR binding site is underlined) were labeled with ^{32}P using T4 polynucleotide kinase (Promega). The end-labeled AHRE was purified using a Centrispin-20 column (Princeton Separations, Adelphia, NJ). In vitro-synthesized AHRR1, mutant AHRR1s, or mouse AHRR was mixed with ARNT2b in equal amounts and incubated for 30 min with 50,000 cpm of labeled AHRE and buffer [final concentrations: 20 mM HEPES, pH 8.0, 130 mM sodium chloride, 5 mM dithiothreitol, 0.1% bovine serum albumin, 2.5 mM $MgCl_2$, 5% glycerol, and 2 μg of poly(dI-dC)]. A 200 times excess of either unlabeled AHRE or mutant AHRE (5′-GATCTGGCTCTTCTCACACAACTCCGGATC-3′) (mutated bases are underlined) was added to determine the specificity of DNA binding. An AHRR-specific antibody (Merson et al., 2006) was used for supershift experiments. Mixtures were run for 1.5 h at 200 V on 5% nondenaturing polyacrylamide gel that had been prerun for 30 min at 200 V. Gels were dried and exposed to film.

Coimmunoprecipitation Assay. The full-length AHRR1, AHRR1Δ270-550, AHRR1Δ189-550, and AHRR1-Y9FΔ189-550 proteins were synthesized by in vitro transcription and translation (TNT; Promega) in the presence of [^{35}S]methionine (GE Healthcare Bio-Sciences, Piscataway, NJ). Zebrafish ARNT2 was synthesized unlabeled. Five microliters of unlabeled protein was mixed with 15 μl of radiolabeled protein and incubated at room temperature for 2 h. The mixtures were adjusted to 25 mM HEPES, 200 mM NaCl, 1.2 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40, pH 7.4 with protease inhibitors (IP buffer). After two rounds of preclearing with normal mouse IgG and protein G agarose, 5 μg of monoclonal ARNT antibody (MA1-515; Affinity BioReagents) or normal mouse IgG was added and incubated for 2 h, followed by precipitation with protein G agarose overnight. The beads were washed two times with IP buffer, boiled in sample treatment buffer, and subjected to SDS-polyacrylamide gel electrophoresis on an 8 or 12% gel.

Subcellular Localization of AHRR1 and AHRR1-Y9F. Hepa1c1c7 mouse hepatocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in α-minimal essential medium supplemented with 10% fetal bovine serum at 37°C. Hepa1c1c7 cells were plated at 3×10^5 cells/well in six-well plates, with each well containing a coverslip. Eight hundred nanograms of EGFP-N2, AHRR1-EGFP, or AHRR1-Y9F-EGFP was transfected into cells using Lipofectamine 2000 reagent (Invitrogen). Cells were washed twice with 2 ml of 1× phosphate-buffered saline. Coverslips were then removed and placed on glass slides and examined with a fluorescent Axiovert S100 microscope (Carl Zeiss, Jena, Germany) with a fluorescein isothiocyanate short pass filter set. A Hamamatsu digital camera with the OpenLab 2.25 software (Improvision, Coventry, UK) was used to collect images.

Subcellular Localization of mAHR-YFP. COS-7 cells were grown on coverslips in six-well plates. Cells were cotransfected with 500 ng of mouse AHR-YFP, 500 ng of human ARNT, with or without 500 ng of AHRR1 and 1000 ng of mouse ARA9 constructs using Lipofectamine 2000 reagent (Invitrogen). Cells were dosed with DMSO or TCDD (10 nM final concentration) 5 h after transfection. Twenty-four hours after transfection, cells were washed with 1× phosphate-buffered saline and fixed in 4% formaldehyde. The coverslips were inverted onto slides and mounted with Vectashield hard-setting mounting medium (Vector Laboratories, Burlingame, CA). Cells were visualized using an Axio Imager.Z1 fluorescence microscope (Carl Zeiss), and Axiovision software (Carl Zeiss) was used to collect the images.

Results

Role of ARNT in the Mechanism of Repression. The AHRR has been hypothesized to repress AHR transactivation by sequestering ARNT or through competition between AHRR-ARNT and AHR-ARNT complexes for binding to AHREs (Mimura et al., 1999). It is unclear under what circumstances ARNT concentrations might be limiting in the cell (Pollenz et al., 1999). We sought to determine whether competition between AHR and AHRR for a potentially limited pool of ARNT was the primary mechanism of repression. Because the balance between levels of AHR, AHRR, and ARNT is important for these experiments, we performed preliminary studies to optimize the amounts of transfected expression constructs. First, we determined the lowest amount of ARNT2b needed for maximal TCDD-inducible luciferase expression. Increasing concentrations of ARNT2b expression construct were transfected into COS-7 cells along with an AHR2 expression construct and a luciferase reporter gene under control of AHREs (pGudLuc6.1). The monoclonal

antibody MA1-515, previously shown to recognize teleost ARNT2 (Powell et al., 1999), was used to confirm that increased amounts of transfected DNA resulted in increasing ARNT2b protein expression in cell lysates (Fig. 1A). Maximal luciferase expression was consistently seen at between 5 and 25 ng of ARNT2b construct (Fig. 1B, lanes 4 and 5).

To determine whether an excess of ARNT2b could restore AHR2 transactivation that had been inhibited by AHRR, we cotransfected AHR2 and AHRR1 (5-ng) expression constructs along with increasing amounts of ARNT2b plasmid. The amount of AHRR1 construct used in this experiment was the lowest amount needed to repress greater than 80% of AHR2 transactivation (Evans et al., 2005). This ensured that AHRR1 was not present in excess, so that transfection of additional amounts of ARNT2b would rescue the repression if competition for ARNT were involved in the mechanism. As we showed previously (Evans et al., 2005), AHRR1 repressed AHR2-dependent transactivation of a luciferase reporter gene (Fig. 1B). Increasing the amount of ARNT2b did not reverse the AHRR1-dependent repression of luciferase expression (Fig. 1B, lanes 9–14). Likewise, overexpression of ARNT2b was not able to overcome the repression produced by AHRR2, a paralog of AHRR1 (Fig. 1C, lanes 9–14). We also tested the ability of the recently identified zebrafish ARNT1c (Prasch et al., 2006) to restore AHR transactivation that had been repressed by AHRR1. However, as we saw with ARNT2b, ARNT1c did not reverse the repression of AHR2 by AHRR1 (data not shown). These results indicate that competition for ARNT is not the mechanism by which AHRR represses AHR-dependent transactivation.

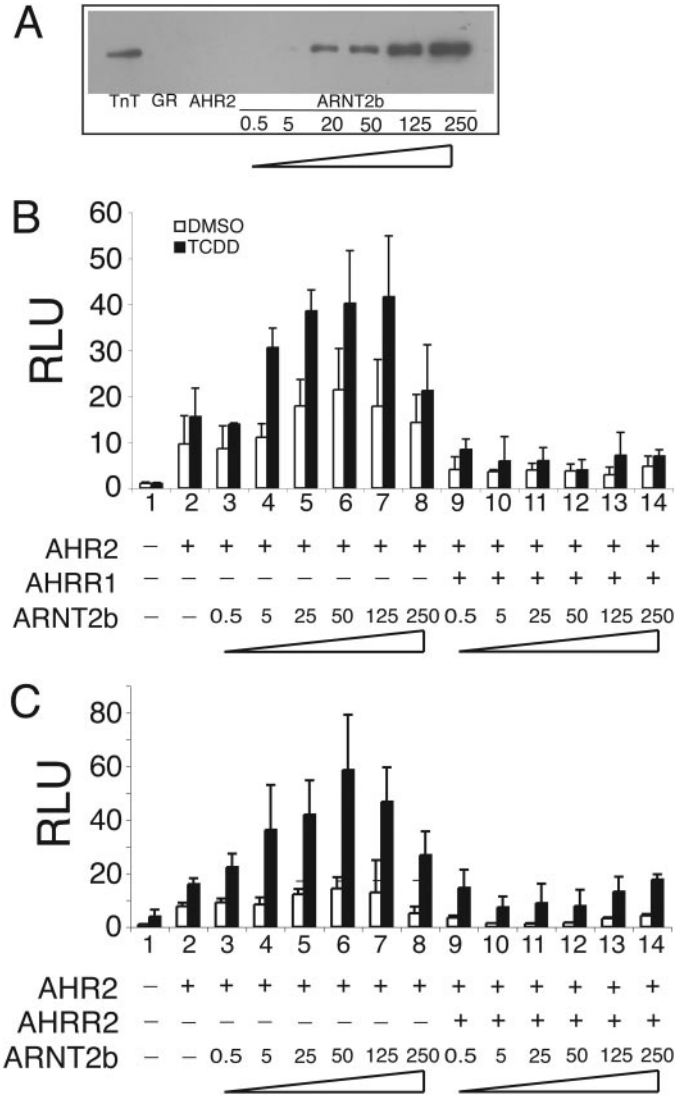

Fig. 1. Overexpression of ARNT2b does not reverse the repression of AHR2-dependent transactivation by AHRR1 or AHRR2. COS-7 cells were transfected with pGudLuc6.1 and pRL-TK. A, Western blot analysis confirmed the presence of increasing amounts of ARNT2b protein in cells transfected with increasing amounts of ARNT2b expression construct. The mouse monoclonal antibody to human ARNT, MA1-515 (Affinity BioReagents) was used. ARNT2b synthesized by in vitro transcription and translation was run as a positive control. GR corresponds to cells transfected only with the two luciferase constructs and an empty expression construct. B and C, zebrafish AHR2 (5 ng), pcDNA 3.1-AHRR1 (5 ng) (B) or pcDNA 3.1-AHRR2 (5 ng) (C) and increasing amounts of ARNT2b expression constructs were cotransfected into cells as indicated in the figure. Cells were treated with 0.5% DMSO or 10 nM TCDD. Firefly luciferase activity was measured and normalized to the transfection control *R. reniformis* luciferase. Results are representative of at least two independent experiments. [The apparent low responsiveness (-fold induction) of the reporter gene is commonly seen in transient transfection assays in which AHR is transfected along with a reporter gene; it may reflect some constitutive activation of AHR when overexpressed from a plasmid, leading to higher basal reporter gene expression and thus lower -fold inducibility; Fukunaga and Hankinson, 1996.].

Role of AHRR DNA Binding in the Mechanism of Repression. After determining that competition for ARNT was not the mechanism of repression, we investigated the second proposed mechanism, competition for binding to AHREs. To do this, we sought to construct an AHRR1 mutant that was defective in AHRE binding. The tyrosine at position 9 of AHR has been shown to be important for AHRE binding (Bacsi and Hankinson, 1996; Fukunaga and Hankinson, 1996). Minsavage and Gasiewicz showed that a point mutation changing this tyrosine of mouse AHR to a phenylalanine resulted in a 80% reduction in the ability to bind AHREs and transactivate a luciferase reporter construct (Minsavage et al., 2003) without affecting any other AHR functions (Minsavage et al., 2004). Alignment of various AHRR and AHR sequences showed that this tyrosine residue, along with the majority of the basic domain, is conserved among AHRs and AHRRs (Fig. 2A). We therefore constructed AHRR1-Y9F containing a point mutation substituting a phenylalanine for tyrosine at position 9.

To determine whether the DNA binding ability of the AHRR1-Y9F protein had been disrupted, we synthesized wild-type AHRR1, AHRR1-Y9F, mAHRR, and ARNT2b by in vitro transcription and translation and used them in an EMSA. An AHRE derived from the promoter of the mouse CYP1A gene was used as a probe, because this AHRE also drives the luciferase reporter used in the transient transfection experiments; mAHRR was used as a positive control because it has been shown previously to bind to AHREs (Mimura et al., 1999). In the absence of ARNT2b, none of the repressors could bind to the AHRE oligonucleotide (Fig. 2B, lanes 2–4). When ARNT was added, both mAHRR and AHRR1 bound to AHREs; this binding was inhibited in the presence of an excess of unlabeled AHRE oligonucleotide but not by an excess of oligonucleotide in which the AHRE had been mutated, demonstrating the sequence specificity of the interaction (Fig. 2B, lanes 12–14 and lanes 6–8 for mAHRR and AHRR1, respectively). An antibody against AHRR caused a supershift, suggesting that the AHRR1 protein was part of the AHRE-protein complex. In contrast to the wild-

type AHRR1, AHRR1-Y9F was unable to bind to the AHRE oligonucleotide, indicating that the point mutation disrupted DNA binding (Fig. 2B, lane 9). The loss of the ability of AHRR1-Y9F to bind AHREs seemed to be complete, in contrast to the 80% loss of AHRE binding for the Y9F mutant of mouse AHR (Minsavage et al., 2003) but similar to the complete loss of AHRE binding after a Y9A mutation of AHR (Bacsi and Hankinson, 1996; Fukunaga and Hankinson, 1996).

To confirm that the point mutation did not disrupt the ability of AHRR1-Y9F to localize to the nucleus, we performed transient transfections of Hepa1c1c7 cells with constructs expressing EGFP, AHRR1-EGFP, or AHRR1-Y9F-EGFP. The EGFP expression construct was equally distributed in the cytoplasm and the nucleus (Fig. 2C). In contrast, both the wild-type AHRR1-EGFP and AHRR1-Y9F-EGFP were localized to the nucleus (Fig. 2C). These results indicate that the point mutation disrupted the ability of the mutant AHRR to bind to AHREs without affecting its ability to translocate to the nucleus.

Next, we determined whether the AHRR1-Y9F was capable of repressing AHR-dependent transactivation. We performed transient transfections in COS-7 cells with expression constructs for AHRR1-EGFP, AHRR1-Y9F-EGFP, or EGFP together with constructs for AHR2, ARNT2b, and pGudLuc6.1. EGFP alone (expressed from the vector that was used to create the fusion proteins) had no effect upon constitutive or TCDD-inducible, AHR2-dependent induction of luciferase activity (Fig. 3A, lane 4 versus lane 3). Transfection of a small amount (5 ng) of AHRR1-EGFP or AHRR1-

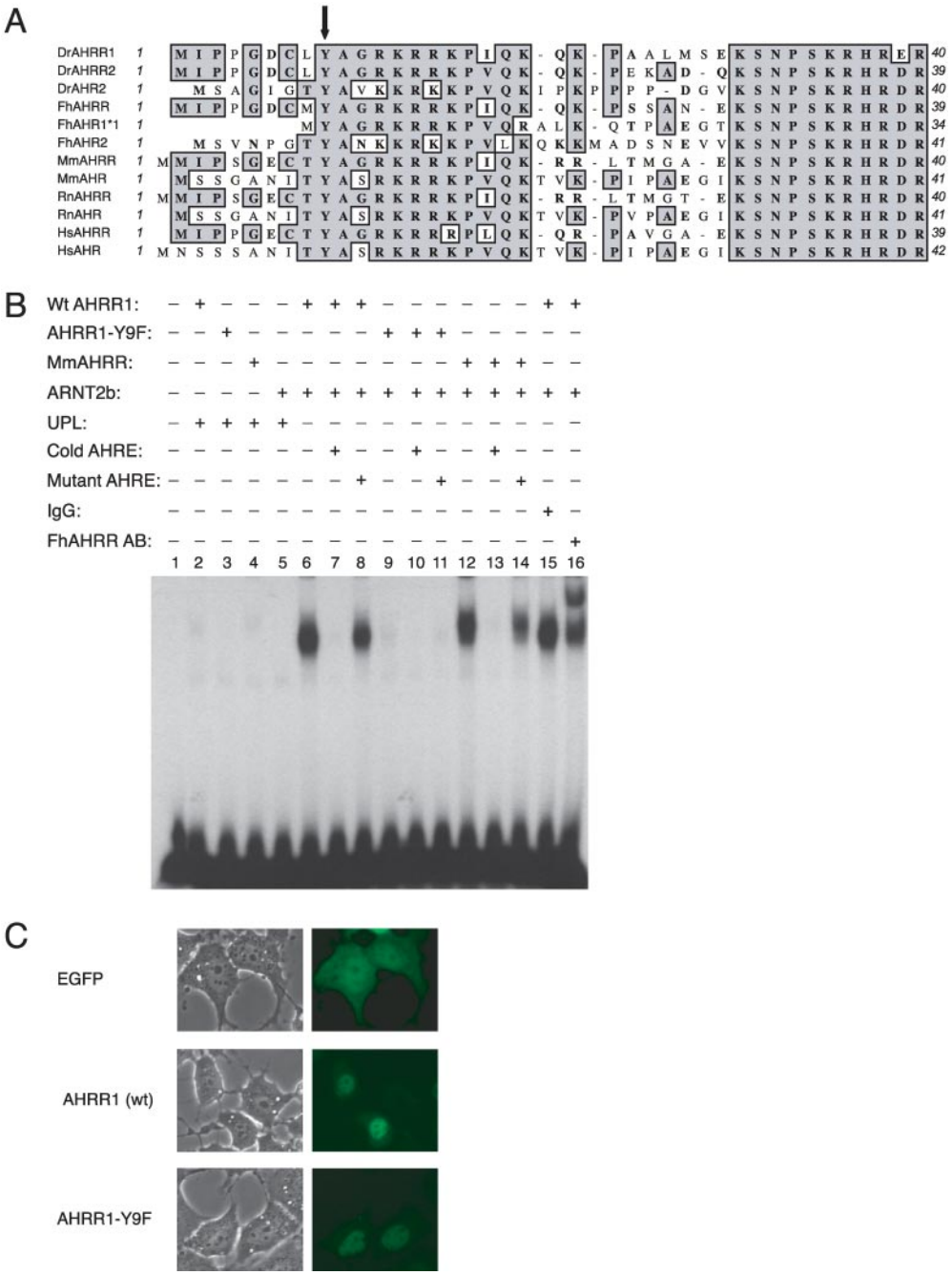

Fig. 2. Effects of Y9F mutation on AHRE binding and nuclear localization of AHRR1. A, alignment showing that the tyrosine at position 9 is conserved among AHRs and AHRRs. B, Y9F mutation disrupts DNA binding in AHRR1. Wild-type pcDNA 3.1-AHRR1, pcDNA 3.1-AHRR1-Y9F, and mAHRR were in vitro translated using the TnT rabbit reticulocyte system and incubated with similarly expressed ARNT2b in the presence of ^{32}P-labeled mouse AHRE probe. Mixtures were then run on a nondenaturing gel. C, Y9F mutation does not affect the nuclear localization of AHRR1. Hepa1c1c7 cells were transiently transfected and visualized 12 h after transfection by fluorescence microscopy. Results are representative of two independent experiments. UPL, unprogrammed lysate. GenBank accession numbers for the sequences used are as follows: zebrafish AHRR1 (AY928203), zebrafish AHRR2 (AY928204), killifish AHRR (AF443441), mouse AHRR (AB015140), rat AHRR (NP_001019456), human AHRR (AB033060), human AHR (L19872), mouse AHR (M94623), rat AHR (NM_013149), killifish AHR1*1 (AF024591), killifish AHR2 (U29679), and zebrafish AHR2 (AF063446). Prefixes used are as follows: Dr, *Danio rerio*; Fh, *F. heteroclitus*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; and Hs, *Homo sapiens*.

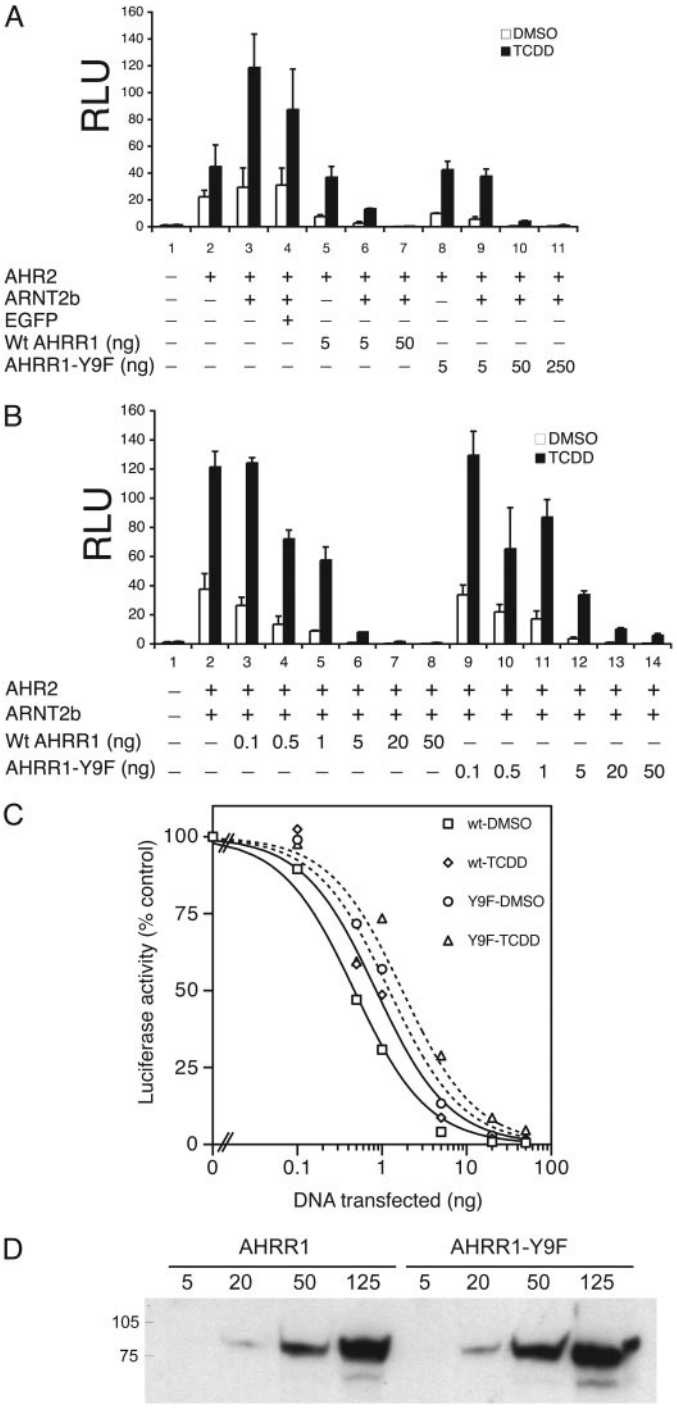

Fig. 3. AHRR1-Y9F is a functional repressor of AHR-dependent transactivation. A, inhibition of AHR2-dependent transactivation by AHRR1 and AHRR1-Y9F. COS-7 cells were transfected with pGudLuc6.1, and pRL-TK. AHR2 (5 ng), ARNT2b (5 ng), and AHRR1-EGFP or AHRR1-Y9F-EGFP expression constructs were also cotransfected into cells as indicated in the figure. Cells were dosed with 0.5% DMSO or 10 nM TCDD. Firefly luciferase activity was measured and normalized to the transfection control *R. reniformis* luciferase. B, both the wild-type and the mutant Y9F repressor inhibit AHR2-dependent transactivation in a concentration-dependent manner. COS-7 cells were transfected with expression constructs and exposed to DMSO or TCDD, and luciferase activity was measured, as described in text. Results are representative of at least two independent experiments. C, inhibition of luciferase activity as a function of amount of transfected AHRR1-EGFP or AHRR1-Y9F-EGFP DNA. Firefly luciferase activity was expressed as percentage of control, where control equals luciferase activity in the absence of AHRR construct, after treatment with DMSO or TCDD (e.g., lane 2 in B). Results of two experiments, each done in triplicate, were averaged. Curves were fitted, and IC_{50} values determined using Prism (GraphPad Software, San Diego, CA). IC_{50} values (and 95% confidence intervals) were as follows: AHRR1/DMSO, 0.47 ng (0.30–0.73); AHRR1/TCDD, 0.85 ng (0.65–1.12); AHRR1-Y9F/DMSO, 1.22 ng (0.81–1.81); and AHRR1-Y9F/TCDD, 1.74 (1.12–2.70). Lines represent the fitted curves for wild-type AHRR1 (solid lines) and AHRR1-Y9F (dashed lines). D, expression of transfected AHRR1-EGFP and AHRR1-Y9F-EGFP constructs in COS-7 cells. Cells were transfected with the indicated amounts of AHRR1 or AHRR1-Y9F expression plasmid, along with expression constructs for AHR2 (5 ng), ARNT2 (25 ng), pGudLuc6.1 (20 ng), and pRL-TK (3 ng). Cell lysates were analyzed by Western blot using affinity-purified antiserum against AHRR1.

Y9F-EGFP plasmids did not repress AHR2 transactivation in the absence of exogenous ARNT (Fig. 3A, lanes 5 and 8 versus lane 2), as we reported previously for AHRR1 (Evans et al., 2005). In the presence of ARNT2, both AHRR1-EGFP and AHRR1-Y9F-EGFP repressed constitutive and TCDD-inducible luciferase activity (Fig. 3A, lanes 6 and 9 versus lane 3), with >95% repression at 50 ng of either AHRR1-EGFP or AHRR1-Y9F-EGFP (Fig. 3A, lanes 7 and 10 versus lane 3). Both repressors inhibited AHR-dependent luciferase induction in a concentration-dependent manner (Fig. 3B). However, AHRR1-Y9F was slightly less effective as a repressor, with IC_{50} values (concentrations of plasmid resulting in 50% repression) that are 2.0-fold (TCDD-treated cells) and 2.6-fold (DMSO-treated cells) greater than those for the wild-type AHRR1 (Fig. 3C), despite similar levels of AHRR1 and AHRR1-Y9F protein expression in the cells (Fig. 3D). To ensure that the results were not influenced by the presence of the EGFP fusion, AHRR1-Y9F was subcloned into pcDNA 3.1; this expression construct behaved similarly to the AHRR1-Y9F-EGFP expression construct (data not shown). These results show that, despite the inability to bind to AHREs, the mutant repressor remains effective at repressing AHR transactivation. Thus, AHRE binding is not required for repression of AHR signaling by AHRR, although it may contribute to the repressive effect, as indicated by the slight difference in effectiveness between AHRR1 and AHRR1-Y9F.

Because AHRR1-Y9F was still a functional repressor, we investigated whether overexpression of ARNT2 could reverse the repression caused by this protein. We performed a transient transfection in which constructs for AHR2 and either AHRR1 or AHRR1-Y9F were cotransfected into COS-7 cells together with increasing amounts of ARNT2b expression construct. The increased levels of ARNT failed to reverse repression when either AHRR1 or AHRR1-Y9F was present (Fig. 4, lanes 9–20). The inability of ARNT overexpression to reverse repression caused by AHRR1-Y9F indicates that AHRR can repress AHR function by a mechanism that is independent of both competition for ARNT and AHRE binding.

AHRR Does Not Reduce AHR Protein Levels or Block AHR Nuclear Translocation. The foregoing results indicate that the repression of AHR by AHRR involves mechanisms in addition to those originally proposed by Mimura et al. (1999). Previous research has shown that the AHR protein is degraded after treatment with an AHR agonist (Pollenz, 2002; Wentworth et al., 2004). We hypothesized that the AHRR might repress AHR signaling by stimulating the degradation of the AHR. To test this hypothesis, we cotransfected AHR2 and AHRR1 into COS-7 cells, and the cell lysates were probed with a polyclonal antibody to AHR2 (Wentworth et al., 2004). Transfection of increasing amounts

of AHRR1 did not cause a loss of AHR2 protein (Fig. 5A). In contrast, there was a slight but reproducible increase in AHR2 at the highest amount of AHRR1 transfected. The mechanism for this increase is not clear. One possibility is that the reduced AHR2 transactivation subsequent to repression by AHRR leads to reduced proteolytic degradation of the AHR; there is evidence that at least one mechanism of AHR degradation requires the AHR to first go through a cycle of transactivation (Ma and Baldwin, 2000; Pollenz et al., 2005; R. Pollenz and E. Dougherty, unpublished observations). Regardless of the mechanism involved in the observed increase, the results clearly show that AHRR does not stimulate AHR degradation; thus, this is not the mechanism of repression.

To investigate whether AHRR1 repressed AHR signaling by interfering with AHR nuclear translocation, AHRR1 was cotransfected into COS-7 cells together with an expression construct for an AHR-YFP fusion protein (Petrulis et al., 2000), followed by exposure of the cells to DMSO or TCDD. In DMSO-treated cells, AHR-YFP was localized to both cytoplasm and nuclei, as noted previously for transiently transfected cells (LaPres et al., 2000; Petrulis et al., 2000). Exposure to TCDD resulted in most, if not all, AHR-YFP becoming nuclear (Fig. 5B). Cotransfection of the cochaperone ARA9/XAP2 caused retention of AHR-YFP in the cytoplasm of DMSO-treated cells, but it did not prevent nuclear localization in the presence of TCDD (Fig. 5B), as noted by others (LaPres et al., 2000; Petrulis et al., 2000). Cotransfection of AHRR1 in the presence or absence of ARA9/XAP2 had no effect on these patterns of AHR-YFP distribution. Similar results were obtained using AHR2 transfection and Western blotting to detect AHR2 protein in nuclear and cytoplasmic fractions (data not shown). Thus, AHRR1 does not inhibit the nuclear localization of AHR.

AHRR1 C Terminus Is Not Required for AHR Repression. To further investigate the mechanism of repression and to determine the domains of AHRR1 protein that are required for repression of AHR signaling, we examined the role of the C-terminal portions of AHRR1. The C terminus of AHR contains the transactivation domain (Fukunaga et al., 1995). Therefore, we hypothesized that the C terminus of the AHRRs might possess the domain responsible for repression. To test this hypothesis, we created two C-terminal deletion mutants of AHRR1. The first deletion mutant (AHRR1Δ270-550) was truncated after the portion of the AHRR PAS domain that is conserved in AHRs and AHRRs (Fig. 6A). This intervening region between the PAS-A and PAS-B repeats has been shown to be important for the ability of AHR to dimerize with ARNT (McGuire et al., 2001; Chapman-Smith et al., 2004). The second deletion mutant (AHRR1Δ189-550) was made by deleting most of the PAS domain, including the intervening region, while retaining the PAS-A repeat (Fig. 6A). An EMSA was then performed to determine whether these deletion mutants were able to bind to AHREs. Like the wild-type AHRR1, AHRR1Δ270-550 was able to bind to AHREs in an ARNT-dependent and AHRE-sequence-specific manner (Fig. 6B, lanes 6–11). In contrast, AHRR1Δ189-550 was unable to bind to AHREs (Fig. 6B, lanes 12–14).

The lack of AHRE binding by AHRR1Δ189-550 suggested that this deletion mutant may be incapable of dimerizing with ARNT2. To test this hypothesis, coimmunoprecipitation experiments were performed using in vitro expressed proteins and an antibody against ARNT (Fig. 6C). Anti-ARNT was able to coprecipitate full-length AHRR1, whereas nonspecific IgG had a minimal effect (Fig. 6C, lane 1 versus lane

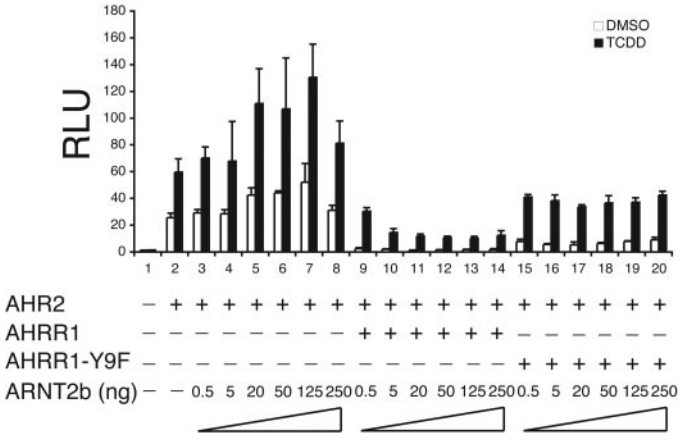

Fig. 4. Overexpression of ARNT fails to reverse repression by AHRR1-Y9F. COS-7 cells were transiently transfected as described in Fig. 1. In brief, 5 ng of AHRR1-EGFP or AHRR1-Y9F-EGFP was cotransfected with AHR2 and increasing amounts of ARNT2b. Cells were dosed with 0.5% DMSO or 10 nM TCDD, and firefly luciferase activity was measured and normalized to transfection control. Results are representative of three independent experiments.

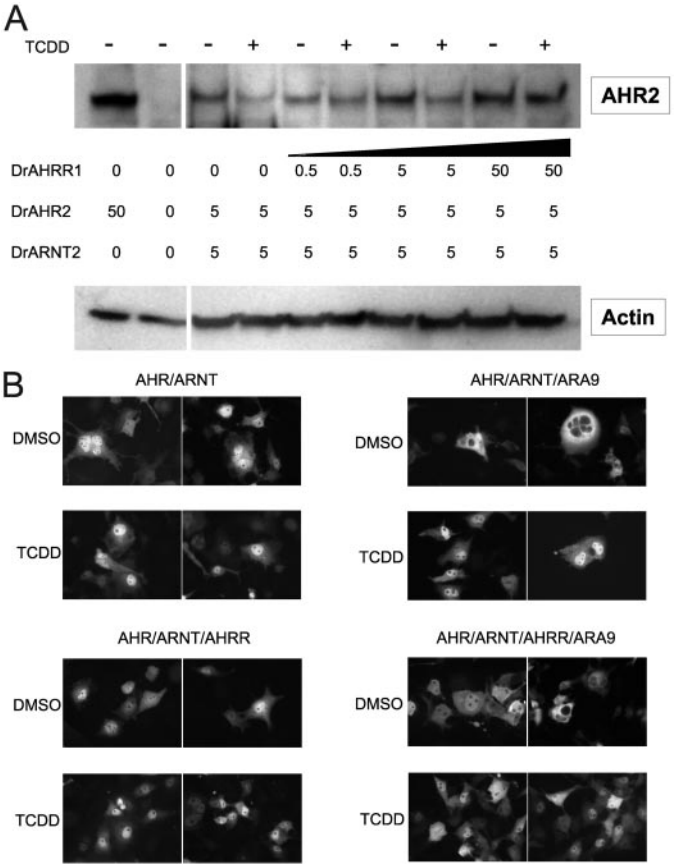

Fig. 5. AHRR1 does not reduce AHR2 protein levels or block nuclear translocation. A, COS-7 cells were transiently transfected with the indicated constructs and dosed with DMSO or TCDD. The transfected cell lysates were analyzed by a Western blot using an AHR2 antibody. An actin antibody was used as a control on the same lysates. B, COS-7 cells were cotransfected with mouse AHR-YFP, human ARNT, with or without AHRR1 and mouse ARA9 constructs. Cells were prepared as described under *Materials and Methods*, and they were visualized using an Axio Imager.Z1 fluorescence microscope (Carl Zeiss).

2). Precipitation of AHRR1 did not occur in the absence of ARNT (data not shown). Like full-length AHRR1, AHRR1Δ270-550 was strongly and specifically coimmunoprecipitated by anti-ARNT (Fig. 6C, lane 3 versus lane 4). In contrast, anti-ARNT and nonspecific IgG both produced faint bands of AHRR1Δ189-550 after immunoprecipitation (Fig. 6C, lane 5 versus lane 6). There was a slight difference in intensity between the anti-ARNT and IgG lanes, which was very small in comparison with the strong immunoprecipitation signals obtained with AHRR1 and AHRR1Δ270-550. We conclude that AHRR1Δ189-550 did not interact with ARNT2, or interacted only very weakly, consistent with its lack of binding to AHREs in the presence of ARNT2b (Fig. 6B). This result also is consistent with results of studies on AHR showing that the intervening region between the PAS-A and PAS-B repeats is important for ARNT dimerization (McGuire et al., 2001; Chapman-Smith et al., 2004).

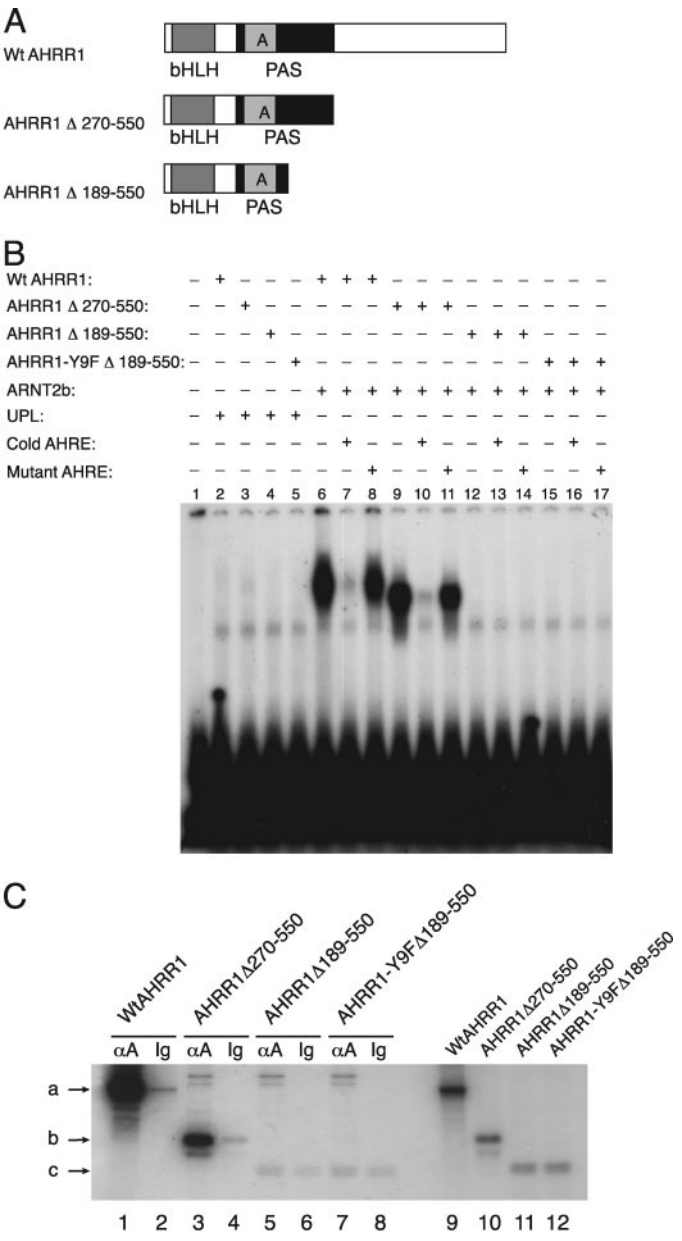

Fig. 6. Effect of AHRR1 C-terminal deletions on AHRE binding and dimerization with ARNT2. A, schematic representation of AHRR1 C-terminal deletion mutants used in this study. Functional domains are listed. B, electrophoretic mobility shift assay. Wild-type pcDNA3.1-AHRR1, pcDNA3.1-AHRR1Δ270-550, pcDNA3.1-AHRR1Δ189-550, and pcDNA3.1-AHRR1-Y9FΔ189-550 were in vitro translated using the TNT rabbit reticulocyte system and incubated with similarly expressed ARNT2b in the presence of ^{32}P-labeled mouse AHRE probe. Mixtures were then run on a nondenaturing gel. C, full-length or truncated mutant constructs of AHRR1 were synthesized in the presence of [^{35}S]methionine, incubated with unlabeled zebrafish ARNT2, and then coimmunoprecipitated using an ARNT antibody (αA) or normal mouse IgG (Ig). Immunoprecipitated products were separated on a 12% acrylamide gel and visualized by fluorography. Arrows indicate the position of wild-type AHRR1 (a, 61.2 kDa) and its deletion variants (b, AHRR1Δ270-550, 30.0 kDa; c, AHRR1Δ189-550, 21.3 kDa). Lanes 1 to 8 are products immunoprecipitated with anti-ARNT (lanes 1, 3, 5, and 7) or IgG (lanes 2, 4, 6, and 8). Lanes 9 to 12 are aliquots of the supernatant after immunoprecipitation. Results are representative of two independent experiments. The anti-ARNT antibody did not immunoprecipitate AHRR1 in the absence of ARNT2 (data not shown).

To determine whether the two deletion mutants were functional repressors, transient transfection assays were performed as described under Transient Transfections and Luciferase Assays. AHRR1Δ270-550 was as effective a repressor at 5 and 50 ng as the wild-type AHRR1 (Fig. 7A, lanes 5 and 6 versus lanes 3 and 4). AHRR1Δ189-550 was also able to repress AHR transactivation, although repression seemed to require higher concentrations of plasmid (50 ng) (Fig. 7A, lanes 7 and 8 versus lane 2). Complete repres-

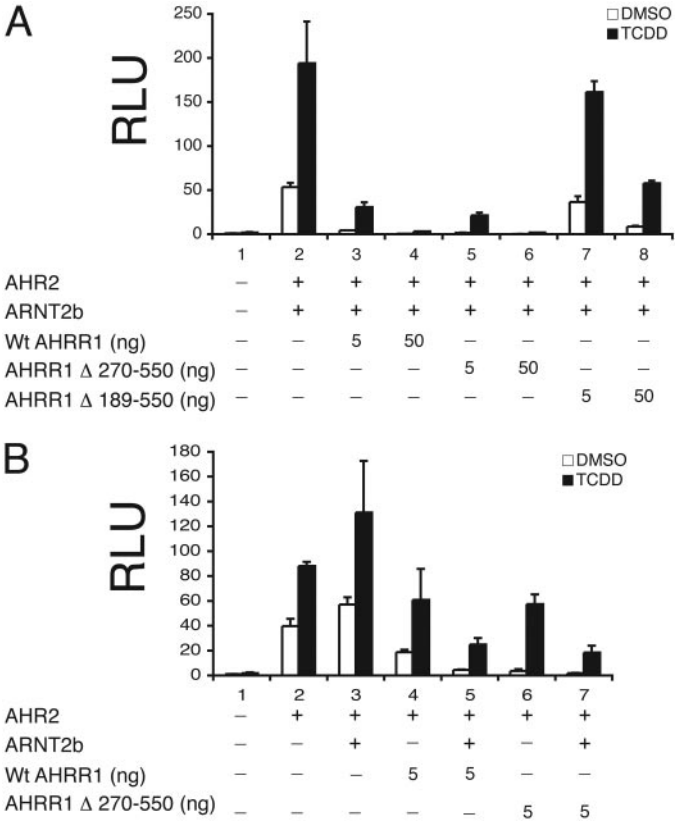

Fig. 7. C terminus of AHRR1 is not required for repression. A, Both pcDNA3.1-AHRR1 deletion mutants repress AHR2. COS-7 cells were transiently transfected as described in Fig. 3 with pcDNA3.1-AHRR1Δ270–550 and pcDNA3.1-AHRR1Δ189-550. B, addition of exogenous ARNT enhances repression by pcDNA3.1-AHRR1Δ270-550. Results are representative of two independent experiments. Expression levels of AHRR1Δ189-550 and AHRR1-Y9FΔ189-550 proteins in the cells have not been measured because the peptide against which the AHRR1 antibody is directed is not present in these proteins. However, AHRR1, AHRR1-Y9F, AHRRΔ270-550, and AHRR1Δ189-550 all express at similar levels by in vitro transcription and translation reactions, suggesting that levels of expression in COS-7 cells are unlikely to differ dramatically.

sion of AHR occurred when 250 ng of AHRR1Δ189-550 was used (data not shown). These results indicate that the C-terminal 362 amino acids of the AHRR1 are not required for repression and therefore that the primary functional domains involved in repression are in the N-terminal portion of the protein that includes the bHLH and PAS-A domains. The apparent difference in the amount of AHRR1Δ270-550 and AHRR1Δ189-550 constructs required to repress AHR signaling (Fig. 7A) suggests that amino acids in the region 189 to 269 may also contribute to the mechanism of repression.

To determine whether the repression by AHRR1Δ189-500 was independent of DNA binding, as we found for the full-length AHRR1 (Figs. 2 and 3), we made a point mutation substituting phenylalanine for tyrosine at position 9 (AHRR1-Y9FΔ189-550) as described for the wild-type AHRR1. Like AHRR1Δ189-550, AHRR1-Y9FΔ189-550 did not interact with ARNT2 (Fig. 6C, lane 7 versus lane 8). An EMSA confirmed that AHRR1-Y9FΔ189-550 did not bind DNA (Fig. 6B, lanes 15–17). Transient transfections show that the double mutant repressor behaved like AHRR1Δ189-550. No repression was observed when 5 ng of either mutant repressor was cotransfected into cells (Fig. 8A, lanes 7 and 5 versus lane 2), but cotransfection of 50 ng of either AHRR1Δ189-550 or AHRR1-Y9FΔ189-550 resulted in a 65% reduction in constitutive luciferase activity and a 70% reduction in TCDD-inducible activity (Fig. 8A, lanes 8 and 6 versus lane 2). Complete repression occurred with 250 ng of AHRR1-Y9FΔ189-550 (data not shown). These results further demonstrate that AHRE binding is not necessary for repression of AHR signaling by full-length AHRR1 or its N-terminal 188-amino acid fragment.

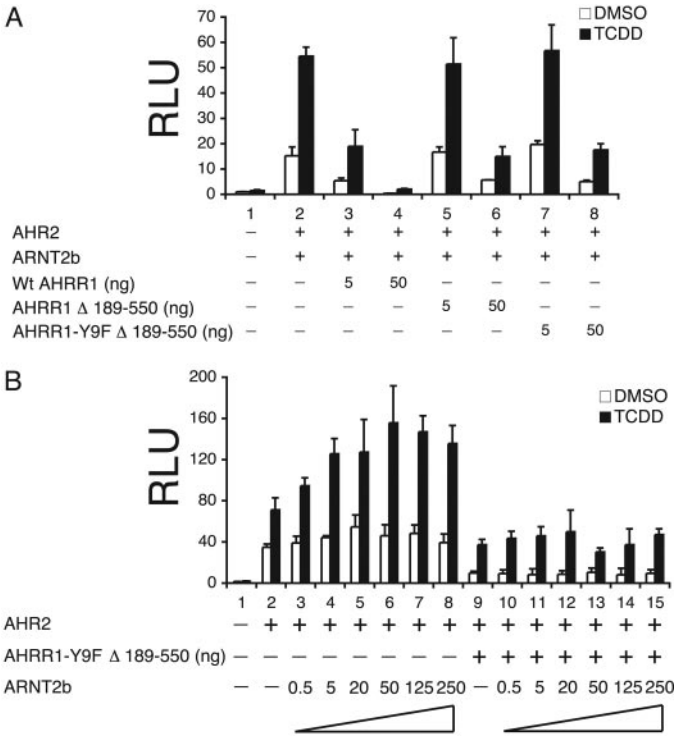

Fig. 8. Repression of AHR2-dependent transactivation by an AHRR1 double mutant. A, pcDNA3.1-AHRR1-Y9FΔ189-550 behaves similarly to pcDNA3.1-AHRR1Δ189-550. Transient transfections were performed as described in Fig. 3, with the addition of pcDNA3.1-AHRR1-Y9FΔ189-550. B, overexpression of ARNT2b does not reverse repression of AHR by the double mutant AHRR1. COS-7 cells were transiently transfected as described in Fig. 1 but with the addition of pcDNA3.1-AHRR1-Y9FΔ189-550. Results are representative of at least two independent experiments.

To determine whether the repression by the AHRR1-Y9FΔ189-550 double mutant involved competition for ARNT, another transient transfection was performed with 50 ng of AHRR1-Y9FΔ189-550 and increasing concentrations of ARNT2b. Increasing the levels of ARNT failed to reverse repression when the double mutant repressor was cotransfected with AHR2 (Fig. 8B, lanes 9–14). These results further demonstrate that AHRE binding, competition for ARNT, and the C-terminal portion of AHRR1 are not required for AHRR-dependent repression of AHR signaling.

Discussion

The AHR is necessary for the biochemical and toxic effects of TCDD (Fernandez-Salguero et al., 1996; Prasch et al., 2003), which occur through changes in gene expression (Bunger et al., 2003; Mimura and Fujii-Kuriyama, 2003). To better understand how the AHR mediates these effects, an understanding of the negative regulation of the AHR is necessary. The AHRR represses AHR transactivation in vitro, suggesting that this repression could be important in the regulation of AHR-dependent gene expression in vivo, as demonstrated recently (Yang et al., 2005). It has been proposed (Mimura et al., 1999) that the mechanism by which the AHRR represses AHR signaling involves competition with the AHR for binding to ARNT or AHREs. Here, we demonstrate that neither competition for ARNT nor competition for binding to AHREs is the sole mechanism by which AHRR acts to repress AHR. We also show that, when both proposed mechanisms are eliminated through use of an AHRR that cannot bind to AHREs combined with overexpression of ARNT, AHRR is still able to act as a repressor. From these data, we conclude that there must be additional mechanism(s) by which the AHRR acts to inhibit AHR-dependent signaling.

Mimura et al. (1999) reported that the mouse AHRR can form a heterodimer with ARNT, and they suggested that AHRR represses AHR in part through competition with AHR for the recruitment of ARNT. Competition for ARNT has also been suggested as a mechanism involved in the inhibition of AHR signaling that occurs through other bHLH-PAS factors, such as hypoxia inducible factor-1α (Gradin et al., 1996) and single-minded 1 (Probst et al., 1997; Woods and Whitelaw, 2002). The idea that ARNT can be limiting for AHR signaling has been questioned by Pollenz et al. (1999), who demonstrated that the amount of ARNT sequestered by hypoxia inducible factor-1α was only a small fraction of the total ARNT pool in mouse hepatoma cells. In contrast, Woods and Whitelaw (2002) showed that the inhibition of AHR signaling caused by SIM1 and SIM2 in transient transfection assays could be overcome by cotransfection with excess ARNT, suggesting that ARNT can be limiting for AHR signaling in some situations or cell types. In addition, a recent study using mice harboring a hypomorphic *Arnt* allele indicated that reduced levels of ARNT can attenuate some—though not all—effects of TCDD, suggesting that ARNT may be limiting for some AHR-dependent responses in vivo (Walisser et al., 2004).

In light of these results, we reasoned that if the proposed mechanism of competition between AHR and AHRR for ARNT is correct, then increased expression of ARNT should

reverse the AHRR-dependent repression of AHR transactivation in transient transfection assays. Our results indicate that an over-expression of ARNT2 protein is unable to restore AHR signaling in the presence of either AHRR1 or AHRR2 (Fig. 1). Although these results do not preclude a role for ARNT binding in the mechanism of repression under some circumstances, they demonstrate that competition for ARNT is not the mechanism by which AHRRs inhibit AHR-dependent gene expression. Consistent with this, addition of exogenous ARNT2 enhanced the repressive effect of AHRRs at low levels of AHRR transfection (Figs. 3A and 7B), suggesting that ARNT2 may be involved in AHRR function even if its sequestration is not the mechanism of repression. For example, repression by AHRR may occur at least in part through formation of an AHRR-ARNT complex. In other situations, the ability of AHRR to function as a repressor may be ARNT-independent. The ability of AHRR1Δ189-550 to repress AHR supports this idea. This repressor is unable to bind to AHREs despite an intact DNA binding domain (Fig. 5), most likely because it interacts with ARNT2 only weakly (Fig. 6C). Thus, this deletion mutant seems to repress AHR by a mechanism that may be independent of ARNT2. However, we cannot exclude the possibility that the weak interaction of this mutant AHRR protein with ARNT may contribute to its ability to repress AHR.

Recent studies have shown that ARNT2 is not required for the developmental toxicity of TCDD in zebrafish (Prasch et al., 2004) and that a novel ARNT isoform, ARNT1c, is the functional dimerization partner of AHR2 (Prasch et al., 2006). In our experiments, both ARNT2b and ARNT1c were equally able to support TCDD-inducible transactivation by AHR2, but neither isoform could reverse the repression by AHRR1, even when overexpressed. We conclude that AHRR1 represses AHR through a mechanism that does not involve competition for either of these ARNT isoforms.

AHRRs bind to DNA in an AHRE sequence-specific manner, as reported by Mimura et al. (1999) for the mouse AHRR and shown for AHRR1 in the present study. This suggested that a second potential mechanism of repression was through competition for AHREs (Mimura et al., 1999). We hypothesized that if binding to DNA is necessary for the repressor to function, then a mutant AHRR that lacks the ability to bind to AHREs would be ineffective. We made a point mutation in AHRR1 that disrupted AHRE binding without affecting nuclear localization. Despite the loss of AHRE binding ability, this AHRR1 mutant (AHRR1-Y9F) retained its ability to repress AHR signaling. Thus, the results presented here show that AHRE binding is not necessary for repression by AHRR.

Although the results obtained with AHRR1-Y9F show that DNA binding is not *required* for repression, binding of an AHRR-ARNT complex to AHREs may *contribute* to the overall repression. A comparison of results obtained with wild-type AHRR1 and AHRR1-Y9F shows that the enhancement in repression by the addition of exogenous ARNT is dependent on DNA binding (Fig. 3A, lanes 5 and 6 versus lanes 8 and 9) and that AHRR1-Y9F was slightly less effective than AHRR1 (Figs. 3 and 4), suggesting that competition between AHR-ARNT and AHRR-ARNT complexes for AHRE binding is one mechanism of repression. However, in our experiments AHRE binding was responsible for only a fraction of the repressor activity, and AHRR1-Y9F remained a potent repressor, capable of >95% repression of AHR transactivation (Figs. 3 and 4). In addition, overexpression of ARNT with AHRR1-Y9F failed to reverse AHRR-dependent repression (Figs. 4 and 8), showing that under circumstances in which both components of the proposed mechanism are abrogated, AHRR retains its ability to repress AHR signaling. Thus, the dual mechanism proposed previously (Mimura et al., 1999) does not fully explain the ability of AHRR to repress AHR transactivation.

Cotransfection of the AHRR with AHR did not reduce the levels of AHR protein in the presence or absence of TCDD (Fig. 5). These results suggest that the mechanism of repression by AHRR does not involve an enhancement of AHR turnover. Likewise, expression of the *Fundulus heteroclitus* (killifish) AHRR (Karchner et al., 2002) in mammary tumor cells (Yang et al., 2005) does not reduce steady-state levels of endogenous AHR protein (D. Sherr, unpublished observations). Transfection of AHRR also did not interfere with the nuclear localization of AHR after TCDD exposure. Thus, the mechanism of repression by AHRR does not seem to involve reduced AHR protein levels or localization.

The results using the C-terminal deletion mutants of AHRR1 show that the C terminus is not necessary for repression. We also show that two C-terminal deletion mutants that cannot bind to DNA, AHRRΔ189-550 and AHRR1-Y9FΔ189-550, are still functional repressors (Figs. 7 and 8). However, compared with the wild-type AHRR1, both of these mutant AHRRs required transfection of higher amounts of expression construct (250 versus 50 ng) to reduce activity to basal levels. Amino acids in the region 189 to 269 may contribute to the repression caused by the full-length AHRR. Alternatively, mutant AHRR proteins might be expressed at a lower level compared with wild-type AHRR1. The inability of the AHRRΔ189-550 mutants to bind to ARNT2 and AHREs and the failure of ARNT overexpression to reverse the repression indicate that the mutant AHRRs must be acting by mechanisms other than those proposed by Mimura et al. (1999). Overall, our results show that the N-terminal 188 amino acids of AHRR1 are sufficient to repress AHR signaling and that this repression does not require AHRE binding, does not involve competition for ARNT, and may occur at least in part through an ARNT-independent mechanism.

Our results suggest that the mechanism of AHRR action may involve "transrepression" (i.e., repression via protein-protein interactions with promoter-bound transcription factors) (Pascual and Glass, 2006), rather than or in addition to DNA binding. Mimura et al. (1999) reported that repression was lost when increasing amounts of AHR were transfected into cells, suggesting that competition for an AHR-interacting factor is involved in the mechanism of repression. One possibility is that the AHR and AHRR compete for a limiting coactivator that is necessary for transcription ("squelching"). One such coactivator is the steroid receptor coactivator-1 (SRC-1); SRC-1 interacts directly with a ligand bound AHR and increases AHR-dependent reporter activity (Kumar and Perdew, 1999). However, in preliminary experiments, overexpression of SRC-1 in COS-7 cells failed to reverse AHRR repression (S. Karchner, B. Evans, and M. Hahn, unpublished results). Other AHR coactivators have been identified (Carlson and Perdew, 2002) and could be targets of AHRR. Another possible mechanism is the direct interaction of

AHRR with the AHR, which might prevent the AHR from interacting with essential coregulatory proteins.

The experiments reported here were conducted in mammalian cell culture using a murine reporter gene construct and fish AHR/ARNT/AHRR proteins. The functional compatibility of AHR signaling components among vertebrates and the use of mammalian cells to study fish AHR-related proteins are well established (Tanguay et al., 2000; Karchner et al., 2002, 2005; Pollenz et al., 2002; Evans et al., 2005) and fish AHRRs can repress mammalian AHRs (Karchner et al., 2002; Evans et al., 2005; Yang et al., 2005). In addition, we have repeated some of these experiments using the human AHRR, with similar results (S. Karchner, M. Jenny, and M. Hahn, manuscript in preparation). Together, this suggests that the results obtained in cell culture have relevance in vivo. Nevertheless, it will be important to confirm and extend these findings to endogenous genes and to determine the relative roles of ARNT dimerization and AHRE binding in a physiological context.

In summary, we have shown that the AHRR functions as a repressor independently of competition for binding to ARNT or AHREs and by a mechanism that does not require the C-terminal portion of the protein. Our data strongly suggest that competition between AHR and AHRR for ARNT is not involved in the mechanism of repression. We also show that competition between AHR-ARNT and AHRR-ARNT complexes for AHRE binding may play a role in repression but that this mechanism, alone or in combination with a mechanism involving competition for ARNT, does not fully explain the repressor function of AHRR. A plausible additional mechanism is one involving transrepression. Future research to test the transrepression hypothesis and to define the protein-protein interactions involved will further our understanding of the negative regulation of AHR signaling by AHRR.

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