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The Active Form of Human Aryl Hydrocarbon Receptor (AHR) Repressor Lacks Exon 8, and Its Pro^{185} and Ala^{185} Variants Repress both AHR and Hypoxia-Inducible Factor▽†

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The aryl hydrocarbon receptor (AHR) repressor (AHRR) inhibits AHR-mediated transcription and has been associated with reproductive dysfunction and tumorigenesis in humans. Previous studies have characterized the repressor function of AHRRs from mice and fish, but the human AHRR ortholog (AHRR$_{715}$) appeared to be nonfunctional in vitro. Here, we report a novel human AHRR cDNA (AHRRΔ8) that lacks exon 8 of AHRR$_{715}$. AHRRΔ8 was the predominant AHRR form expressed in human tissues and cell lines. AHRRΔ8 effectively repressed AHR-dependent transactivation, whereas AHRR$_{715}$ was much less active. Similarly, AHRRΔ8, but not AHRR$_{715}$, formed a complex with AHR nuclear translocator (ARNT). Repression of AHR by AHRRΔ8 was not relieved by overexpression of ARNT or AHR coactivators, suggesting that competition for these cofactors is not the mechanism of repression. AHRRΔ8 interacted weakly with AHR but did not inhibit its nuclear translocation. In a survey of transcription factor specificity, AHRRΔ8 did not repress the nuclear receptor pregnane X receptor or estrogen receptor α but did repress hypoxia-inducible factor (HIF)-dependent signaling. AHRRΔ8-Pro^{185} and -Ala^{185} variants, which have been linked to human reproductive disorders, both were capable of repressing AHR or HIF. Together, these results identify AHRRΔ8 as the active form of human AHRR and reveal novel aspects of its function and specificity as a repressor.

The aryl hydrocarbon receptor (AHR) repressor (AHRR) is a basic-helix-loop-helix/Per-AHR nuclear translocator (ARNT)-Sim (bHLH-PAS) protein discovered because of its similarity to the AHR, a ligand-activated transcription factor involved in the response to synthetic aromatic hydrocarbons (48). The AHR and AHRR form a negative regulatory loop that is evolutionarily conserved in vertebrates (32); expression of AHRR is regulated by the AHR, and AHRR acts as a transcriptional repressor of AHR function (1, 32, 48). Like the AHR, AHRR can dimerize with the ARNT, and the AHRR-ARNT complex can bind to AHR-responsive enhancer elements (AHREs). Repression occurs through competition between AHR and AHRR for binding to AHREs (14, 48) as well as through additional mechanisms that do not involve competition for ARNT and are independent of AHRE binding by AHRR (14).

The biological and toxicological functions of AHRR are not well understood, but recent findings suggest that AHRR is involved in human reproductive physiology and in the regulation of cell growth (reviewed in references 20 and 22). A human AHRR (hAHRR) Ala185Pro polymorphism has been associated with altered reproductive development and infertility in men (16, 46, 59, 64) and endometriosis in women (19, 35, 62, 65), but the functional properties of the polymorphic variants have never been assessed. AHRR overexpression inhibits the growth of human tumor cells in culture (30, 56, 68). Conversely, knockdown of AHRR expression enhances cell growth and confers resistance to apoptosis; consistent with this, the *AHRR* gene has been found to be silenced by hypermethylation in a variety of human cancers (71). Based on these and other findings, the AHRR has been proposed to function as a tumor suppressor gene (22, 71).

In order to assess the functions of AHRR and its polymorphic variants and their relationship to human disease, it is important to understand the nature of the transcripts and proteins encoded by the *AHRR* gene, as well as their expression in human tissues and cell lines. An hAHRR cDNA identified in a large-scale screen of cDNAs from brain (50) encodes a protein of 715 amino acids (aa) (referred to here as AHRR$_{715}$). The human *AHRR* gene encoding this protein has been reported to contain 12 exons, the first of which is noncoding (8, 16). Our initial functional analysis of this protein suggested that, unlike AHRRs from mouse, frog, and fish (15, 32, 48, 70), human AHRR$_{715}$ was not an effective repressor of AHR function in vitro. In phylogenetic analyses involving amino acid sequence alignments of multiple vertebrate AHRRs, we identified an 18-aa segment of AHRR$_{715}$ that was absent from all other AHRRs. We therefore hypothesized the existence of an alternative hAHRR form lacking this segment and also hypothesized that this alternative form might exhibit characteristic repressor function.

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Here, we report the identification and cloning of a novel hAHRR cDNA that lacks exon 8 of the original AHRR clone. This new AHRR (AHRRΔ8) is the predominant form expressed in multiple human tissues and human tumor cell lines. We compare the functions of the two AHRR splice variants and provide the first functional mechanistic assessment of the hAHRR Pro185Ala polymorphic variants that have been associated with increased susceptibility to reproductive dysfunction in human populations. We also show that competition for ARNT or AHR coactivators is not involved in the mechanism of AHR repression and that human AHRRΔ8 (hAHRRΔ8) is capable of repressing hypoxia-inducible factor (HIF)-dependent signaling.

MATERIALS AND METHODS

Chemicals and cell lines. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Ultra Scientific (Hope, RI). Dimethyl sulfoxide (DMSO), clotrimazole, cobalt chloride, and 17β-estradiol were obtained from Sigma-Aldrich (St. Louis, MO). COS-7 cells and the human cell lines HepG2, HeLa, MCF-7, Hs578T, and MDA231 were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown according to standard procedures. BP-1 cells were generously provided by J. Russo (Fox Chase Cancer Center, Philadelphia, PA).

Human cDNA panel screening. The expression of AHRR exon 8 in various human tissues was determined by amplifying a partial fragment of AHRR with primers flanking the exon 8 region. Primers hRR-F635 (5′-AGTACTCGGCCT TCCTGACC-3′) and hRR-R816 (5′-CGCCTTCTTCTTCTGTCCAA-3′) were used with 5 μl of cDNAs from adult and fetal human cDNA panels (BD Biosciences, Mountain View, CA) in a 25-μl amplification reaction mixture using AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: 94°C for 10 min, 94°C for 15 s and 60°C for 30 s for 35 cycles, and 72°C for 7 min. The PCR products were resolved on 15% Tris-borate EDTA gels.

Screening of human cell lines for the Pro/Ala polymorphism and the presence of exon 8. Total RNA was isolated from human cell lines MCF-7, Hs578T, MDA231, and BP-1 as described earlier (68). Briefly, cells were frozen and pulverized into a fine powder. Total cellular RNA was isolated using RNAzol as described by the manufacturer (Leedo Medical Laboratories, Houston, TX). RNA was quantified with a spectrophotometer at optical densities of 260 nm and 280 nm. cDNA was synthesized from 2 μg of total RNA using Omniscript reverse transcriptase (Qiagen, Valencia, CA). A partial fragment of AHRR was amplified from cDNA derived from each cell line using primers hRR-F494 (5′-AGG ACTTCTGCCGGCAGCTCC-3′) and RRex8R (5′-CAGCTGCCAAGCCTGT GACC-3′) flanking the region containing the Pro185Ala polymorphism and exon 8. The PCR products were cloned into the pGEM-T vector (Promega), and multiple clones were sequenced for each cell line.

Generation of AHRR plasmid constructs. The pcDNAhAHRR ($AHRR_{715}$) construct was prepared by subcloning the KIAA1234 cDNA (clone fH08618; a gift from Takahiro Nagase, Kazusa DNA Research Institute, Chiba, Japan [50]) into pcDNA3.1, as we described earlier (32). Full-length hAHRRΔ8 was amplified from testes cDNA (human cDNA panel; BD Biosciences, Mountain View, CA) using primers hRR-F39 (5′-GATCATATGCCGAGGACGAT-3′) and hRR-R2227 (5′-GAGCTTGGATGGTGGTCACT-3′) and Advantage DNA polymerase (BD Biosciences). The PCR conditions were as follows: 94°C for 1 min; 94°C for 5 s, 64°C for 10 s, and 68°C for 2.5 min for five cycles; 94°C for 5 s, 62°C for 10 s, and 68°C for 2.5 min for five cycles; 94°C for 5 s, 60°C for 10 s, and 68°C for 2.5 min for 25 cycles; 72°C for 10 min. The amplified product was cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. The insert was cut out of EcoRI and SpeI sites and transferred to the EcoRI and XbaI sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA).

Other plasmid constructs. The pEF-hAHR and mouse AHR-yellow fluorescent protein (YFP) fusion constructs were provided by Gary Perdew (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 M. Denison (University of California, Davis, CA). Rat Cyp1a1-Luc and human XRE.1A1-Luc were obtained from R. Barouki (University of Rene Descartes, France) and S. K. Kim (Seoul National University, South Korea), respectively. Expression constructs for human ARNT and the hypoxia-responsive luciferase reporter, HRE-luc (PL949 [25]), were obtained from C. Bradfield (University of Wisconsin, Madison, WI). The human pregnane X receptor (PXR) expression construct was provided by S. Kliewer (University of North Carolina at Chapel Hill), and the PXR reporter construct (XREM-tk-luc) was obtained from J. Moore (Molecular Discovery Research, GlaxoSmithKline, Research Triangle Park, NC). The human estrogen receptor α (ERα) construct and an estrogen-responsive luciferase reporter (3xERE-TATA-luc) were obtained from D. McDonnell (Duke University Medical Center, Durham, NC). Expression constructs for the receptor coactivators GRIP, CoCoA, and GAC63 were provided by M. Stallcup (University of Southern California, Los Angeles, CA). Src-1a and Src-1e constructs were obtained from E. Kalkhoven (University Medical Center Utrecht, The Netherlands) and M. Parker (Imperial College London, United Kingdom). The p300 expression construct was from Upstate Biotechnologies (Lake Placid, NY).

Transient transfections and luciferase assays. Transient transfections were performed as described earlier (14, 32). Briefly, 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 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 DMSO (0.5%), TCDD (10 nM final concentration), clotrimazole (10 μM final concentration), CoCl (150 μM final concentration), or 17β-estradiol (10 nM final concentration). (For transfections involving 17β-estradiol and ERα, cells were grown in phenol red-free medium with charcoal-stripped serum.) *Renilla* luciferase (pRL-TK or pGL4.74; Promega, Madison, WI) 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 *Renilla* luciferase units. Experiments were repeated multiple times.

AHRR antibody production and Western blots. Polyclonal antibodies to hAHRR (designed to recognize both forms) were raised in two rabbits (21st Century Biochemicals, Marlboro, MA) by coimmunizing the animals with two peptides corresponding to amino acid residues 18 to 31 (LQKQRPAVGAE KSN) and 80 to 101 (FQVVQEQSSRQPAAGAPSPGDS). To avoid cross-reactivity with AHR, the peptides were in regions of the AHRR protein exhibiting low sequence identity with the AHR (see Fig. S1 in the supplemental material). Preimmune serum and serum from six bleeds were collected over the course of several weeks, and antibody titer was tested by Western blotting using hAHRR-transfected COS-7 cell lysates; lysates from COS-7 cells transfected with empty vector served as a control for specificity. COS-7 cells were plated and transfected as described above. Twenty-four hours after transfection, cells were rinsed with phosphate-buffered saline and resuspended in 2× sample treatment buffer. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were blotted onto nitrocellulose. The serum antibody titer for each bleed was tested by blotting with two dilutions (1:250 and 1:1,000). Two affinity-purified polyclonal antibodies were isolated from serum (bleeds 3 thru 6) from a single rabbit by separate affinity purification procedures using the two individual peptides. The affinity-purified antibodies are designated PAb-RR-18-1 (against residues 18 to 31) and PAb-RR-80-2 (against residues 80 to 101). The specificity of the affinity-purified polyclonal antibodies was assessed by blotting against lysates from COS-7 cells transfected with plasmids for hAHRR, human AHR, mouse AHRR, and killifish AHRR. All results reported here (Western blots and immunoprecipitations) were performed using PAb-RR-80-2.

Expression of hAHRR protein in the transient-transfection assays was measured by Western blotting with PAb-RR-80-2 (3 μg/ml), followed by a goat anti-rabbit immunoglobulin G (IgG)−horseradish peroxidase (Upstate/Millipore, Billerica, MA) secondary antibody (1:5,000). The AHRR proteins were then visualized by enhanced chemiluminescence (ECL-Plus; GE Healthcare, Piscataway, NJ).

Coimmunoprecipitation assay. The full-length $AHRR_{715}$, AHRRΔ8, AHR, and ARNT proteins were synthesized by in vitro transcription and translation (TnT; Promega, Madison, WI) in the presence or absence of [^{35}S]methionine (MP Biomedicals, Solon, OH). Five microliters of unlabeled protein was mixed with 15 μl of radiolabeled protein and incubated at room temperature for 2 h. For mixtures containing AHR, TCDD (10 nM) was added. 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 (immunoprecipitation buffer). After two rounds of preclearing with normal mouse IgG and protein G-agarose, 5 μg of specific antibody or 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8% polyacrylamide gel. The gels were dried and visualized by fluorography. hAHRR antibody (PAb-RR-80-2) or normal rabbit IgG was used to precipitate AHRR complexes and nonspecific complexes, respectively. For ARNT complexes, monoclonal ARNT antibody (MA1-515; Affinity BioReagents, Golden, CO) and normal mouse IgG were used.

Subcellular localization of mouse AHR-YFP. COS-7 cells were grown on coverslips in six-well plates. Cells were cotransfected with 350 ng of mouse AHR-YFP and 350 ng of human ARNT expression constructs, with or without 350 ng of hAHRRΔ8 construct, using Lipofectamine 2000 reagent (Invitrogen). Luciferase reporter pGudLuc6.1 (300 ng) and the transfection control pRL-TK (40 ng) were also transfected. 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 a Zeiss Axio Imager.Z1 fluorescence microscope, and Axiovision software was used to collect the images. To confirm the effectiveness of AHRRΔ8 as a repressor under the conditions of the assay, luciferase was measured in a plate of cells run in parallel.

Nucleotide sequence accession numbers. The AHRRΔ8 sequences have been deposited in GenBank, with accession numbers EU293605 (mRNA) and ABX89616 (protein).

RESULTS

Identification and characterization of the major form of hAHRR. In our earlier studies of the evolutionary conservation of AHRR in vertebrates (15, 32), we noticed that the predicted 715-aa protein derived from the original hAHRR cDNA ($AHRR_{715}$ [50]) included an 18-aa segment that had no counterpart (homologous amino acids) in other AHRRs. A more recent phylogenetic analysis involving an alignment of all known (i.e., verified) mammalian, amphibian, and fish AHRR protein sequences confirmed that this unique 18-aa segment is present only in the human $AHRR_{715}$ (Fig. 1A). This segment is located in an otherwise conserved portion of the PAS region downstream of the PAS-A repeat (sometimes referred to as the "intervening region" [10] between PAS repeats) (see Fig. S1 in the supplemental material); it is encoded by a single exon of 54 nucleotides in the human genome (Fig. 1B), corresponding to exon 8 described by others (8, 16). $AHRR_{715}$, the 715-aa protein encoded by the cDNA containing this exon, functioned poorly as a repressor in transient-transfection assays in three different laboratories (unpublished results; see below). We therefore hypothesized that there might be an alternatively spliced AHRR transcript lacking this exon and that the protein encoded by this alternative transcript might have a repressor function like that of AHRRs from other species.

Using primers flanking exon 8, we used PCR to amplify this region of the AHRR transcript from human tissue cDNAs. The primary amplicon was 128 bp, the size predicted for a form lacking exon 8, rather than the 182 bp predicted for the exon 8-containing transcript. Subsequently, we amplified, cloned, and sequenced a full-length cDNA of 2,173 bp with an open reading frame of 2091 bp encoding a predicted AHRR protein of 697 aa. The new cDNA is identical to $AHRR_{715}$ (50), except that it lacks the sequences corresponding to exon 8 and thus has been designated AHRRΔ8. Two polymorphic variants of AHRRΔ8, corresponding to the Ala185Pro single nucleotide polymorphism described earlier (65), were found among the sequenced clones.

To assess the relative expression of $AHRR_{715}$ and AHRRΔ8, we performed PCR analysis on cDNA from human tissues with primers flanking exon 8, designed to produce amplicons of different sizes for the two AHRR variants. A survey of multiple adult and fetal tissues demonstrated that AHRRΔ8 is the predominant, and in most cases only, form of AHRR expressed (Fig. 1C). We also examined the relative expression of $AHRR_{715}$ and AHRRΔ8 and the presence of Ala185Pro polymorphic variants in several human tumor cell lines. As seen with the human tissues, AHRRΔ8 was the predominant form of AHRR expressed in HeLa, HepG2, MCF-7, Hs578T, MDA231, and BP-1 cells (see Fig. S2A and Table S1 in the supplemental material). Although $AHRR_{715}$ transcripts were not detected by using primers flanking exon 8, use of a primer within exon 8 showed that $AHRR_{715}$ transcripts were expressed at low levels in HeLa and HepG2 cells (see Fig. S2B in the supplemental material). Sequencing of AHRR cDNA clones from each of the cell lines confirmed AHRRΔ8 as the predominant expressed form and showed that all of the cell lines except BP-1 are heterozygous for the Ala185Pro polymorphism (see Table S1 in the supplemental material).

AHRRΔ8 and AHRR differ in repressor activity. To compare the functional properties of the original 715-aa AHRR protein ($AHRR_{715}$) and AHRRΔ8, we performed transient-transfection assays in which we measured the ability of the AHRRs to inhibit the TCDD-inducible transactivation of reporter gene construct pGudLuc6.1 mediated by either transfected or endogenously expressed AHR. After transfection with the respective constructs, $AHRR_{715}$ and AHRRΔ8 proteins were expressed at similar levels in COS-7 cells, as assessed by Western blotting using an hAHRR antibody that recognizes both AHRR forms but does not recognize human AHR (Fig. 2A and B). When transfected into COS-7 cells with ARNT in the presence or absence of TCDD, neither AHRR form was able to activate transcription of pGudLuc6.1 (see Fig. S3A in the supplemental material). Thus, hAHRRs lack a function as transcriptional activators, as observed previously for AHRRs from other species (15, 32, 48).

To test the ability of $AHRR_{715}$ and AHRRΔ8 to repress AHR-mediated signaling, each form was transfected into COS-7 cells together with expression constructs for human AHR and ARNT and pGudLuc6.1. In the absence of cotransfected AHRR, AHR and ARNT caused transactivation of the luciferase reporter that was enhanced by TCDD (Fig. 2C). Transfection of the $AHRR_{715}$ expression construct at 50 and 150 ng/well caused little change in the AHR-dependent activation of luciferase expression or its induction by TCDD. In contrast, AHRRΔ8 at 50 or 150 ng/well completely repressed both constitutive (i.e., exogenous ligand-independent) and TCDD-inducible reporter gene activity (Fig. 2C). Similarly, AHRRΔ8, but not $AHRR_{715}$, repressed transactivation by mouse AHR in COS-7 cells (see Fig. S3B in the supplemental material). To evaluate the effect of the AHRRs on endogenously expressed human AHR, $AHRR_{715}$ or AHRRΔ8 was cotransfected with pGudLuc6.1 into HepG2 cells, which express abundant AHR (13). As we saw with the transfected AHRs in COS-7 cells, $AHRR_{715}$ was ineffective as a repressor of the endogenous HepG2 AHR, whereas AHRRΔ8 reduced TCDD-inducible reporter gene activation by 61% (Fig. 2D). AHRRΔ8 also was much more effective than $AHRR_{715}$ at repressing endogenous AHR in MCF-7 cells cotransfected with two different reporter gene constructs (Cyp1a1-Luc and

FIG. 1. Identification of hAHRR splice variant AHRRΔ8 as the major variant expressed in human tissues. (A) Partial amino acid sequence alignment of AHRRs and AHRs from different species. The top sequence is human $AHRR_{715}$; immediately below it is the sequence of AHRRΔ8. Abbreviations: Hs, human; Mm, mouse; Rn, rat; Xl, frog; Fh, killifish; Tr, Japanese puffer fish; Dr, zebrafish; Mt, tomcod. For GenBank accession numbers, see the legend to Fig. S1 in the supplemental material. (B) hAHRR gene structure. Translated exons are shown in black boxes. The first exon and part of the second exon are untranslated. The additional exon (exon 8) is shown in gray. The position of the Pro185Ala polymorphism is marked by an arrow. (C) Survey of adult human tissues for the expression of $AHRR_{715}$ and AHRRΔ8 transcripts. A partial AHRR cDNA fragment was amplified from adult and fetal human cDNAs, using primers flanking exon 8 (shown in gray in panel B). The presence of the exon would have produced a 182-bp PCR product ($AHRR_{715}$), as opposed to the 128-bp product (AHRRΔ8) obtained in all tissues.

XRE.1A1-Luc) (Fig. 2E; see also Fig. S3C in the supplemental material). (The lower percent repression in HepG2 and MCF-7 cells than in COS-7 cells likely reflects the fact that endogenous AHR is expressed in all HepG2 and MCF-7 cells, whereas not all of the cells take up transiently transfected AHRR.) Together, these results demonstrate that the much greater ability of AHRRΔ8 than of $AHRR_{715}$ to repress AHR transactivation is consistently observed for transfected and endogenously expressed AHRs from humans and mice, in three different cell lines, and with AHRE-containing reporter gene constructs derived from three different mammalian species.

AHRRΔ8 and $AHRR_{715}$ differ in ability to interact with ARNT. The 18-aa peptide encoded by exon 8 in $AHRR_{715}$ lies in the ~100-aa "intervening region" downstream of the PAS-A repeat. This region is highly conserved in AHR and AHRR proteins (Fig. 1A; see also Fig. S1 in the supplemental material), and in the AHR, it has been shown to be important for dimerization with ARNT (10, 45). This region also appears to be important for dimerization of AHRR and ARNT, because deletion of this region in the zebrafish AHRRa caused a dramatic reduction in the ability of AHRRa to interact with ARNT (14). To determine whether the presence of the 18-aa peptide within the intervening region of $AHRR_{715}$ affects its ability to dimerize with ARNT, we performed a coimmunoprecipitation experiment in which radiolabeled ARNT was incubated with in vitro-expressed AHRRΔ8 or $AHRR_{715}$ and the complex was immunoprecipitated with affinity-purified antibody against hAHRR, which recognizes both forms. AHRRΔ8 and $AHRR_{715}$ were synthesized to similar levels by in vitro transcription and translation (Fig. 3A). AHRRΔ8

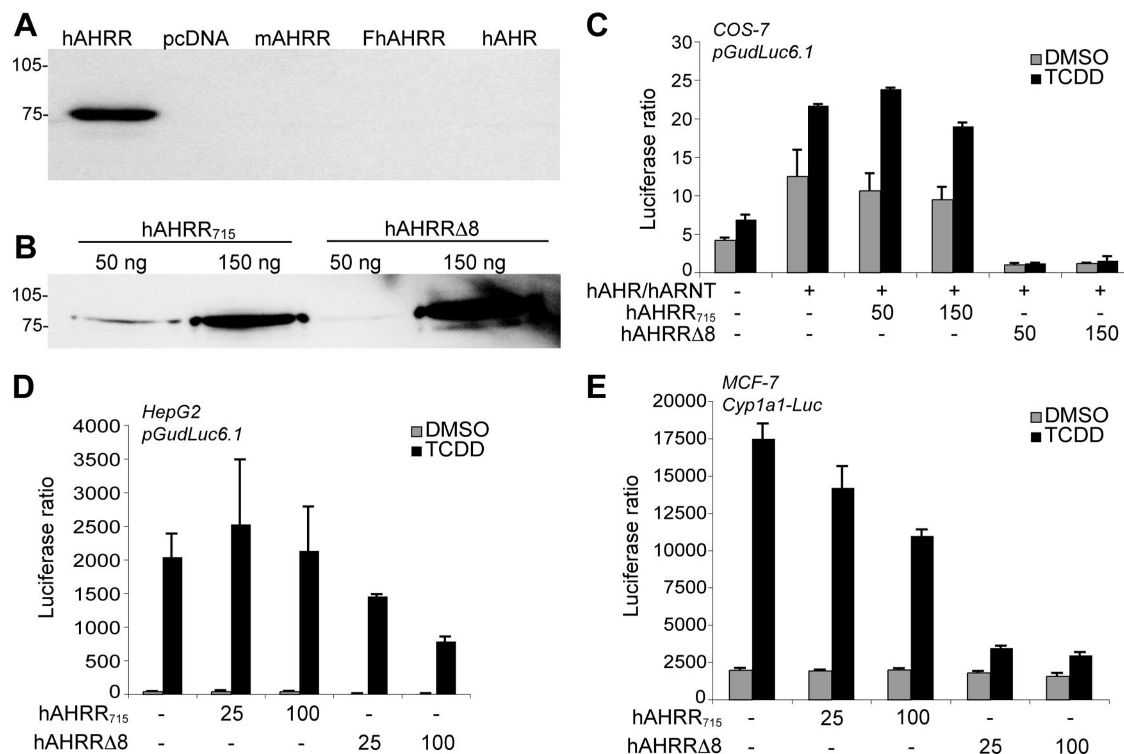

FIG. 2. Repressor activity of hAHRR splice variants $AHRR_{715}$ and AHRRΔ8. (A) The hAHRR antibody does not recognize human AHR or AHRRs from other species. Cell lysates from COS-7 cells transiently expressing the indicated constructs were blotted and probed with antibody PAb-RR-80-2 raised against the hAHRR. (B) In transient-transfection assays in COS-7 cells, $AHRR_{715}$ and AHRRΔ8 are expressed at similar levels. Cell lysates were blotted and probed with the hAHRR antibody. Numbers at left of panels A and B are molecular masses in kilodaltons. (C) Repression of exogenously expressed AHR by $AHRR_{715}$ and AHRRΔ8 in COS-7 cells. COS-7 cells were transfected with human AHR (5 ng), human ARNT (25 ng), and $AHRR_{715}$ or AHRRΔ8 constructs (50 or 150 ng each), along with a luciferase reporter under the control of dioxin response elements (pGudLuc6.1) and a transfection control plasmid expressing *Renilla* luciferase (pRL-TK). Cells were dosed with DMSO or TCDD (10 nM final concentration), followed by a luciferase assay. The results shown are representative of seven independent experiments. (D and E) Repression of endogenous AHR in HepG2 (D) and MCF-7 (E) cells by $AHRR_{715}$ and AHRRΔ8. Cells were transfected with $AHRR_{715}$ or AHRRΔ8 constructs (25 and 100 ng each), along with a luciferase reporter under the control of dioxin response elements (for HepG2, pGudLuc6.1; for MCF-7, Cyp1a1-Luc) and pRL-TK. Cells were dosed with DMSO or TCDD (10 nM final concentration), followed by a luciferase assay. The results shown in panels D and E are representative of two and three independent experiments, respectively.

formed a complex with ARNT that could be specifically and strongly immunoprecipitated by the AHRR antibody (Fig. 3B, lane 3 versus lane 4; see also Fig. S4B in the supplemental material). In contrast, $AHRR_{715}$ did not interact with ARNT (Fig. 3B, lane 1 versus lane 2; see also Fig. S4B in the supplemental material). The results suggest that the presence of the 18-aa peptide encoded by exon 8 disrupts ARNT dimerization, that hAHRR requires ARNT to repress AHR, and that the difference in the repressor function of AHRRΔ8 and $AHRR_{715}$ reflects the inability of the latter protein to associate with ARNT.

Functional comparison of AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} polymorphic variants. An Ala185Pro polymorphism in the hAHRR has been associated with human diseases in several studies (16, 19, 35, 46, 59, 62, 64, 65), but the functional properties of the two variants have never been assessed. Both of these polymorphic variants were present in our pool of AHRRΔ8 clones. To compare their abilities to repress AHR transactivation, we performed transient-transfection assays in which we measured the abilities of AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} to repress AHR- and ARNT-dependent transactivation of pGudLuc6.1 in COS-7 cells. The AHRRΔ8 variants were expressed at similar levels in the transfected cells (Fig. 4A). Both AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} were effective at repressing constitutive (exogenous ligand-independent) and TCDD-inducible expression of pGudLuc6.1. In experiments in which increasing amounts of AHRRΔ8 expression constructs were transfected, the two variants were equally potent at repressing AHR-mediated transactivation of the reporter gene (Fig. 4B and C). We conclude that both AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} are fully functional as repressors of AHR and thus that the Ala185Pro polymorphism does not affect repression of AHR-mediated transcription.

Mechanism of repression of AHR by AHRRΔ8. Mimura et al. (48) showed that mouse AHRR could dimerize with ARNT and bind to AHREs and proposed that the mechanism of repression involved competition between AHR and AHRR for binding to ARNT and for binding to AHRE sequences. Our recent studies using the zebrafish AHRRa provided evidence that competition for ARNT is not an important element of the mechanism of repression and that AHRE binding may contribute to the repression but is not required (14). To assess the role of competition for ARNT in the repression of human AHR by hAHRRΔ8, we performed a series of transient-trans-

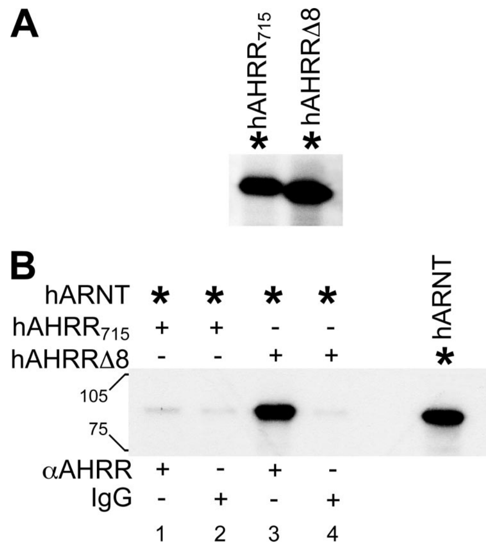

FIG. 3. Human ARNT dimerizes with AHRRΔ8 but not with $AHRR_{715}$. (A) Synthesis of AHRRΔ8 and $AHRR_{715}$ by in vitro transcription and translation in the presence of [^{35}S]methionine, demonstrating that the two proteins were synthesized at similar levels. (B) In vitro-synthesized AHRRΔ8 and $AHRR_{715}$ were each incubated with radiolabeled ARNT and coimmunoprecipitated by either the hAHRR antibody (PAb-RR-80-2) or IgG. The asterisk indicates that ARNT was labeled with [^{35}S]methionine. The *hARNT lane contains an aliquot of the input labeled protein. The results shown are representative of three independent experiments (see also Fig. S4B in the supplemental material). Numbers at left are molecular masses in kilodaltons.

fection experiments to assess the function of AHRRΔ8 under conditions in which ARNT was overexpressed. In experiments in which a single, maximally inducing concentration of TCDD was used to activate AHR, cotransfection of excess ARNT expression plasmid in COS-7 cells failed to prevent the repression of reporter gene expression by AHRRΔ8 (Fig. 5A). To further examine the effect of excess ARNT and to determine whether it could reverse repression at submaximally inducing doses of TCDD, we used HepG2 cells, which express endogenous AHR and ARNT. HepG2 cells were cotransfected with pGudLuc6.1 with or without AHRRΔ8 expression plasmid and with or without ARNT expression plasmid (to supplement endogenous ARNT). AHRRΔ8 was an effective repressor at all concentrations of TCDD and in the presence or absence of extra ARNT (Fig. 5B). The degree of repression was slightly greater at lower concentrations of TCDD (92 to 95% repression at 0.1 nM) compared to 10 nM TCDD (72 to 79% repression) but was independent of the presence of additional ARNT. The failure of excess ARNT to reverse the repression suggests that hAHRRΔ8, like zebrafish AHRRa (14), does not repress by sequestering ARNT away from AHR.

The slight reduction in the degree of repression at higher concentrations of TCDD (Fig. 5B) suggests that AHR and AHRRΔ8 might be competing for some other limiting factor in the cell. We therefore considered the possibility that AHR and AHRRΔ8 might compete for a coactivator that is necessary for transcription ("squelching") (5). Several coactivators have been shown to interact with AHR (2, 3, 6, 11, 34, 38, 69), and some of these could be targets of AHRR. We therefore performed a transient-transfection assay in which COS-7 cells were cotransfected with constructs for AHR, ARNT, pGudLuc6.1, and AHRRΔ8 together with one of several AHR coactivators: steroid receptor coactivator 1 (SRC-1 [NCoA1]; isoforms Src-1a and Src-1e), GRIP1 (NCoA2), CoCoA, GAC63, and p300. The experiments were performed using the smallest amount of AHRRΔ8 needed to repress AHR by 80% (5 ng; Fig. 6A), to ensure that AHRRΔ8 was not present in excess. All of the coactivators enhanced the TCDD-induced expression of the reporter gene. However, none of them reversed the repression of AHR caused by the limiting amount of

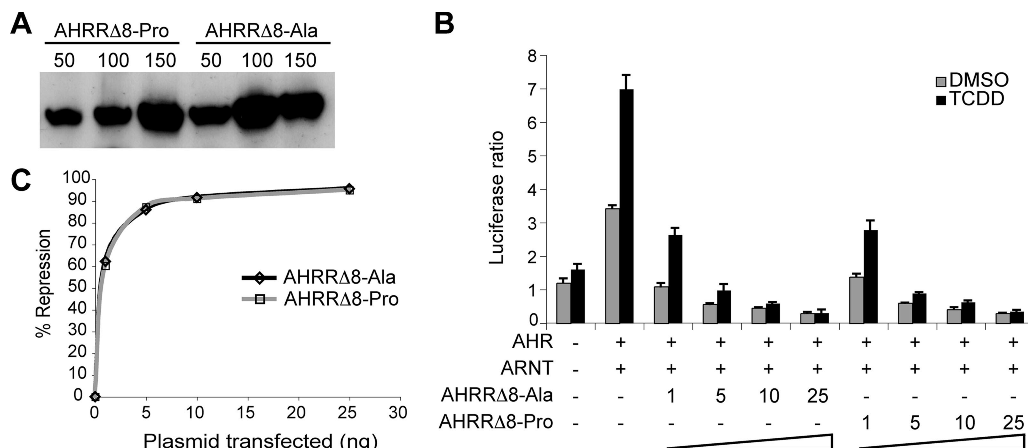

FIG. 4. Repression of AHR transcriptional activation by AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} polymorphic variants of the hAHRR. (A) In transient-transfection assays in COS-7 cells, AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} are expressed at similar levels. COS-7 cells were transfected with 50, 100, or 150 ng of the hAHRR constructs. The cell lysates were blotted and probed with an antibody against the hAHRR. (B) COS-7 cells were transfected with human AHR (5 ng), human ARNT (25 ng), and AHRRΔ8-Ala^{185} or AHRRΔ8-Pro^{185} constructs (1, 5, 10, and 25 ng), along with pGudLuc6.1 and pRL-TK. Cells were dosed with DMSO or TCDD (10 nM final concentration), followed by a luciferase assay. (C) The data for TCDD-inducible luciferase activity in panel B were expressed as percent repression in comparison to the "AHR-plus-ARNT only" group. AHRRΔ8-Ala^{185} and AHRRΔ8-Pro^{185} repressed AHR-ARNT transactivation of pGudLuc6.1 to the same extent. The results shown are representative of three independent experiments.

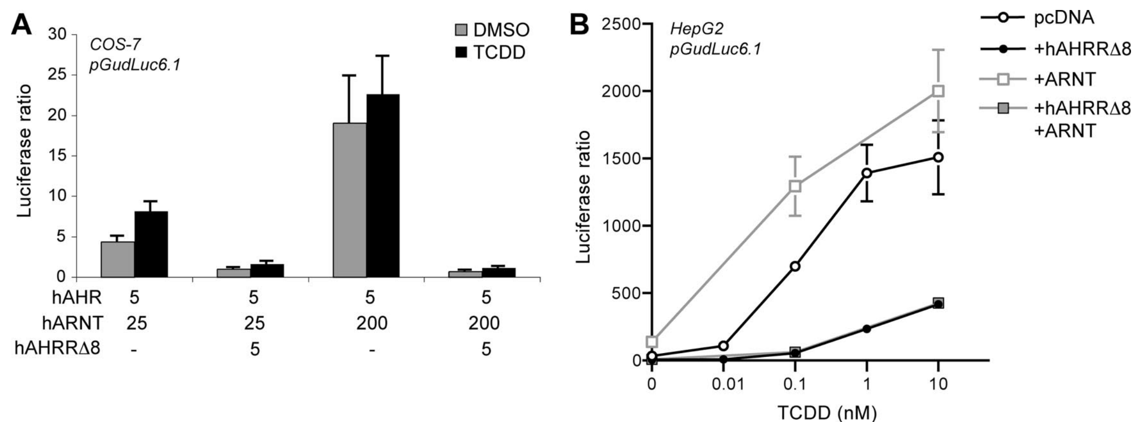

FIG. 5. Effect of ARNT overexpression and increasing TCDD concentrations on the repression of AHR by AHRRΔ8. (A) COS-7 cells were transfected with human AHR (5 ng) and human ARNT (25 and 200 ng) with or without AHRRΔ8 (5 ng). The presence of excess ARNT did not rescue the repression of AHR by AHRRΔ8. The results shown are representative of three independent experiments. (B) HepG2 cells were transfected with the luciferase reporter pGudLuc6.1 with or without additional ARNT (50 ng). Cells were dosed with DMSO or increasing concentrations of TCDD, followed by luciferase assays. Cotransfection of AHRRΔ8 (100 ng) caused repression of the endogenous AHR. The extent of repression was unaffected by the presence of additional ARNT.

AHRRΔ8 (Fig. 6B). We conclude that AHRRΔ8 does not act by competing with AHR for these specific coactivators.

Another possible mechanism of repression is the direct interaction of AHRRΔ8 with the AHR, which might block nuclear translocation of the AHR or prevent it from interacting with essential coregulatory proteins. There are a number of repressors, including the bHLH-PAS protein IPAS (inhibitory PAS protein, an HIF-3α splice variant), that inhibit transcrip-

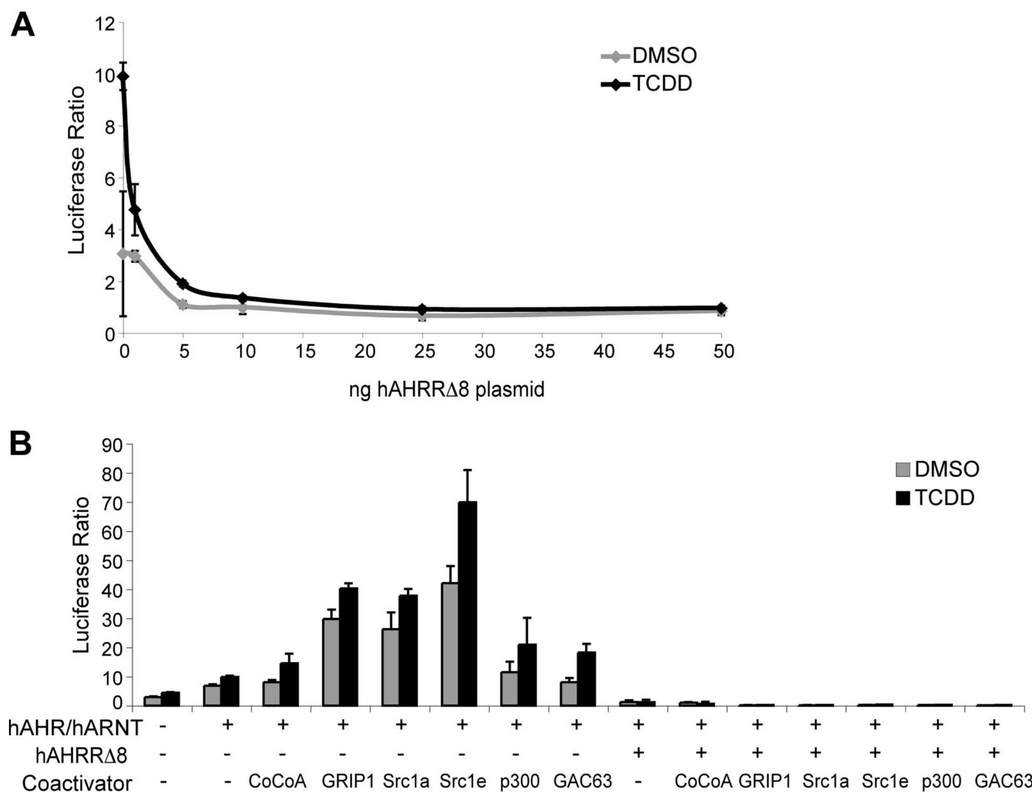

FIG. 6. Overexpression of AHR coactivators does not rescue repression by AHRR. (A) Determination of the minimal amount of AHRRΔ8 needed to repress AHR by 80%. COS-7 cells were transfected with 5 ng each of human AHR and ARNT constructs and increasing amounts (1, 5, 10, 25, and 50 ng) of the hAHRRΔ8 construct, along with the pGudLuc6.1 luciferase reporter and pRL-TK. Cells were dosed with DMSO or TCDD (1 nM), followed by luciferase assays. (B) COS-7 cells were transfected with 5 ng of human AHR and 25 ng of human ARNT, with and without 5 ng of hAHRRΔ8 construct and 200 ng each of the different receptor coactivators. The results shown are representative of three independent experiments.

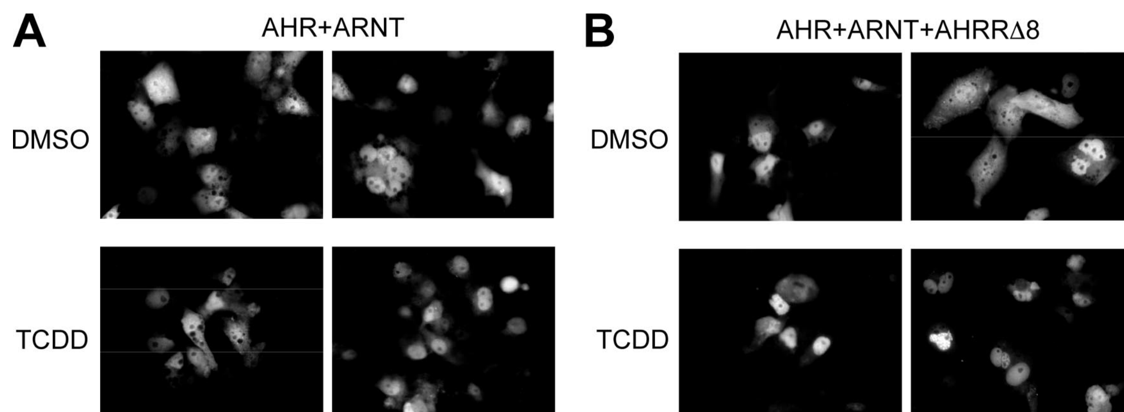

FIG. 7. AHRRΔ8 does not inhibit TCDD-induced nuclear localization of AHR. COS-7 cells were transfected with 350 ng each of a mouse AHR-YFP fusion construct and human ARNT without (A) or with (B) 350 ng of the hAHRRΔ8 construct. Cells were dosed with DMSO or TCDD (1 nM) for 6 h. After being fixed in formaldehyde, the cells were mounted onto slides and viewed under a fluorescence microscope (Zeiss Imager.Z1). For each treatment group, two representative images are shown.

tion factor function through direct protein-protein interactions (41). To test the ability of AHRRΔ8 to interfere with nuclear translocation of AHR, we cotransfected AHRRΔ8 into COS-7 cells together with expression constructs for human ARNT and a mouse AHR-YFP fusion protein (51), followed by exposure of the cells to DMSO or TCDD. In DMSO-treated cells, AHR-YFP was localized to both the cytoplasm and nuclei, as noted previously for transiently transfected cells (39, 51). Exposure to TCDD resulted in nearly all AHR-YFP becoming nuclear (Fig. 7A). Cotransfection of AHRRΔ8 had no effect on these patterns of AHR-YFP distribution (Fig. 7B). Thus, hAHRRΔ8, like zebrafish AHRRa (14), does not inhibit the nuclear localization of AHR.

To directly test the hypothesis that AHR and AHRRΔ8 can physically interact, we performed a series of coimmunoprecipitation experiments using in vitro-expressed proteins and specific antibodies or nonspecific IgG. In one set of experiments, we tested the ability of hAHRRΔ8 to interact with human and mouse AHRs; for comparison, AHR-ARNT and AHRRΔ8-ARNT interactions also were assessed. After incubation of ARNT with ^{35}S-labeled AHR, antibodies against ARNT strongly and specifically coimmunoprecipitated labeled AHR, as expected (Fig. 8, lane 1 versus lane 2). Similarly, when AHRRΔ8 and labeled ARNT were incubated together, affinity-purified antibodies against hAHRR coimmunoprecipitated labeled ARNT (Fig. 8, lane 5 versus lane 6; also Fig. 3). When AHRRΔ8 and labeled human AHR were incubated together, antibodies against hAHRR coimmunoprecipitated labeled human AHR (Fig. 8, lane 3 versus lane 4). Although the bands were less intense than those obtained from pulldown of labeled ARNT and the difference between anti-AHRR and IgG is modest, this difference was observed consistently in multiple experiments and was statistically significant (see Fig. S4A and B in the supplemental material). In addition, a similar interaction was shown to occur between AHRRΔ8 and labeled mouse AHR (see Fig. S4B in the supplemental material). Importantly, anti-AHRR did not pull down labeled AHR in the absence of AHRRΔ8 (not shown), i.e., the antibodies were specific for AHRR (see also the Western blot in Fig. 2A).

In the experiments shown in Fig. 8, lanes 3 and 4, ARNT was not present in the incubation of AHR and AHRRΔ8. It is possible that the interaction of AHRRΔ8 with AHR might be enhanced if either AHRRΔ8 or AHR or both are present as dimers with ARNT. To test the hypothesis that ARNT can influence the interaction between hAHRRΔ8 and AHR, we performed a coimmunoprecipitation experiment with human AHR (labeled) plus AHRRΔ8 plus ARNT. Antibodies to ARNT specifically coimmunoprecipitated radiolabeled AHR when incubated with AHR plus ARNT plus AHRRΔ8 (see Fig. S4A, lane 8 versus lane 9, in the supplemental material). The amount of precipitated AHR was similar to that seen in the absence of AHRRΔ8 (see Fig. S4A, lane 8 versus lane 1, in the supplemental material), suggesting that the presence of AHRRΔ8 did not inhibit the ability of AHR to dimerize with ARNT. Similarly, anti-AHRR coimmunoprecipitated radiolabeled AHR in the presence of AHRRΔ8 and ARNT (see Fig. S4A, lane 7 versus lane 9, in the supplemental material). The similar intensities of the immunoprecipitated AHR bands in

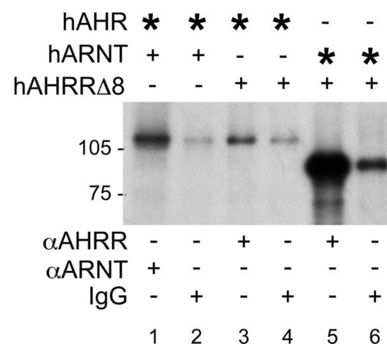

FIG. 8. Coimmunoprecipitation of in vitro-synthesized proteins. Proteins were synthesized by in vitro transcription and translation and incubated with radiolabeled ARNT or AHR. Protein complexes were immunoprecipitated with either the hAHRR antibody (PAb-RR-80-2) or an ARNT antibody (MA1-515). IgG was used to detect nonspecific complexes. The asterisks indicate proteins that were labeled with [^{35}S]methionine. The results shown are representative of at least three independent experiments. Additional results and a summary are provided in Fig. S4 in the supplemental material. Numbers at left are molecular masses in kilodaltons.

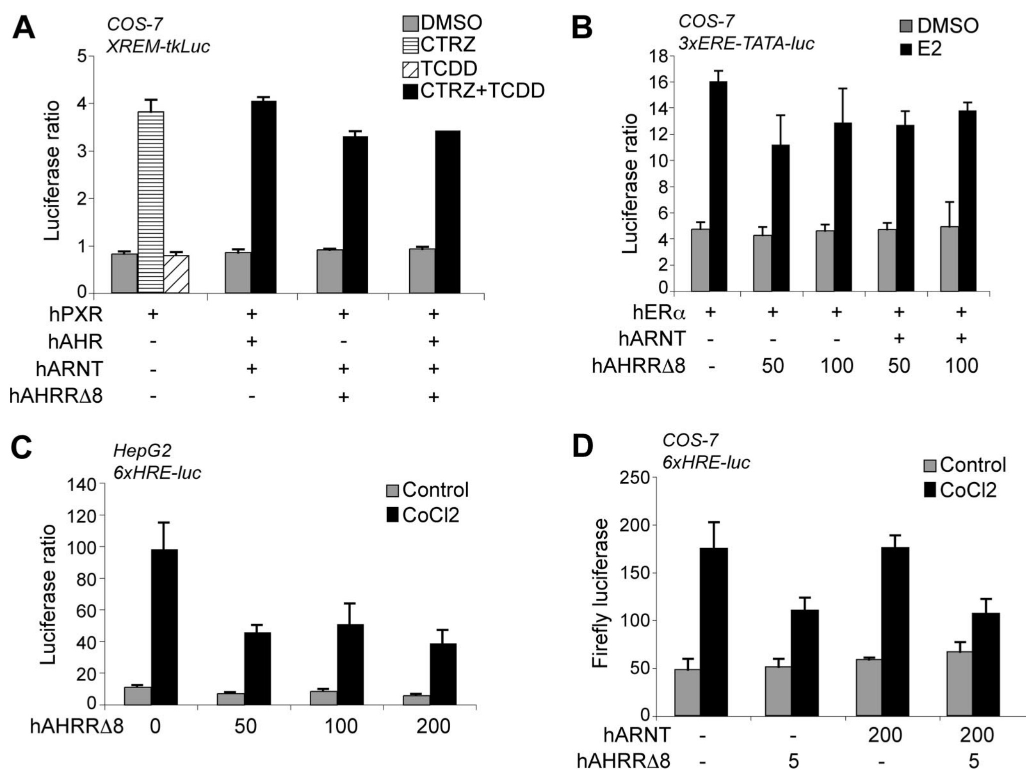

FIG. 9. Specificity of repression by AHRRΔ8. (A) PXR. COS-7 cells were transfected with the human PXR construct (100 ng) and the luciferase reporter XREM-tkLuc (50 ng) with or without AHRRΔ8 (100 ng), AHR (5 ng), and/or ARNT (25 ng). Cells were dosed with clotrimazole (CTRZ) (10 μM final concentration) with or without TCDD (10 nM), followed by a luciferase assay. (B) ERα. COS-7 cells were transfected with the human ERα construct (20 ng) and the luciferase reporter 3×ERE-TATA-luc (50 ng) with or without AHRRΔ8 (50 and 100 ng) and with or without ARNT (25 ng). Cells were dosed with 17β-estradiol (E2) (10 nM final concentration), followed by a luciferase assay. (C and D) HIF. (C) HepG2 cells were transfected with increasing amounts of AHRRΔ8 (0, 50, 100, and 200 ng) and the hypoxia-responsive reporter 6×HRE-luc (10 ng). Cells were dosed with cobalt chloride (CoCl) (150 μM final concentration), followed by a luciferase assay. (D) COS-7 cells were transfected with 6×HRE-luc (10 ng), along with AHRRΔ8 (0 or 5 ng) and ARNT (0 or 200 ng). Cells were dosed with cobalt chloride (CoCl) (150 μM final concentration), followed by a luciferase assay. The results shown in each panel are representative of three or four independent experiments.

lanes 3 (AHR plus AHRRΔ8) and 7 (AHR plus AHRRΔ8 plus ARNT) suggest that the interaction between AHR and AHRRΔ8 is ARNT independent.

Specificity of repression by AHRRΔ8. The AHRR was originally named based on its ability to repress transactivation by AHR (48). However, the specificity of AHRR as a repressor is poorly understood. To test the hypothesis that AHRRΔ8 acts as a general repressor of transcription, we performed a series of experiments to determine whether AHRRΔ8 could repress transactivation by other transcription factors.

We first tested whether AHRRΔ8 could repress transactivation by two nuclear receptors, PXR and ERα. In transient-transfection assays in COS-7 cells, AHRRΔ8 did not inhibit the constitutive or clotrimazole-induced transactivation of reporter gene expression by PXR (Fig. 9A). AHRRΔ8 also did not repress PXR when ARNT or AHR was cotransfected or when cells were treated with TCDD in addition to clotrimazole (Fig. 9A and data not shown). Similarly, AHRRΔ8 had no effect on the estradiol-induced expression of an ERα-dependent reporter gene in COS-7 cells (Fig. 9B). Because ERα is known to interact with AHR, and therefore, AHRR might require AHR to repress ERα, we also performed experiments in which AHR and ARNT were cotransfected along with AHRRΔ8. However, even in the presence of AHR, AHRRΔ8 had no effect on the ERα-dependent transactivation of the reporter gene (data not shown). In these experiments, parallel analyses with the same cells confirmed the ability of AHRRΔ8 to fully repress AHR-ARNT-dependent transactivation of pGudLuc6.1 (not shown), demonstrating the effectiveness of AHRRΔ8 under these conditions.

We next assessed the ability of AHRRΔ8 to repress transactivation by another subfamily of bHLH-PAS proteins, the HIFs. In response to cellular hypoxia, the HIF-α subunits HIF-1α and HIF-2α act as dimers with ARNT (also called HIF-1β) to regulate gene expression though hypoxia-responsive elements (HREs) (26, 63). HepG2 cells, which express endogenous HIF-1α and HIF-2α (4, 28), were transfected with the HIF-responsive, HRE-regulated reporter gene construct PL949 (25) in the presence or absence of the AHRRΔ8 expression construct and exposed to the hypoxia mimic $CoCl_2$ (9) or vehicle. $CoCl_2$ caused a ninefold induction of luciferase activity. Cotransfection of AHRRΔ8 reduced the induction response by ~50% (Fig. 9C). AHRRΔ8 also repressed HRE-dependent transcription in COS-7 cells (Fig. 9D). As we saw for AHR, $AHRR_{715}$ exhibited little or no repressor activity with HIF (data not shown). Overexpression of ARNT in these

cells did not relieve the repressive effect of AHRRΔ8 (Fig. 9D), suggesting that AHRRΔ8 was not acting simply to sequester ARNT away from HIFs. The two polymorphic variants of AHRRΔ8 (Pro^{185} and Ala^{185}) repressed HIF to the same extent (see Fig. S5 in the supplemental material). Together, these results with PXR, ERα, and HIF demonstrate that AHRRΔ8 is not a general repressor of transcription but that the targets of AHRRΔ8 repression are not limited to AHR.

DISCUSSION

The AHRR is an evolutionarily conserved repressor of AHR signaling that has been studied in rodents, amphibians, and fish. However, despite reports linking this protein to tumor cell growth and reproductive dysfunction in human populations, the structural and functional properties of the hAHRR have not been widely investigated and thus are not well understood. We report here the identification and functional characterization of the major, active isoform of the hAHRR, as well as an alternatively spliced form that lacks the ability to bind ARNT and is much less active as a repressor of AHR. We provide the first functional assessment of the Pro185Ala polymorphism and present new information concerning the molecular mechanism of repression. We also demonstrate that the specificity of AHRR-mediated repression is broader than just AHR, extending at least to another set of bHLH-PAS proteins, the HIFs.

AHRRΔ8 is the predominant, and functionally active, AHRR isoform in human tissues and cells. The identification of AHRRΔ8, which lacks one exon compared to the originally described hAHRR cDNA (50), indicates that the AHRR transcript undergoes alternative splicing to generate at least two mRNAs. Our analyses show that AHRRΔ8, encoding a 697-aa AHRR isoform, is the major AHRR form expressed in a variety of human tissues and cell lines. The original AHRR transcript, which encodes a 715-aa protein, was identified in a brain cDNA library (50). The brain and developing nervous system are known to be enriched in alternatively spliced gene products (12, 40). Consistent with the possibility that the larger, exon 8-containing AHRR form is alternatively spliced, exon 8 possesses several features typical of conditional exons (33). For example, the splice recognition sequences surrounding exon 8 are relatively weak compared to those of the other AHRR exons (see Fig. S6 and Table S2 in the supplemental material). In addition, exon 8 is relatively short, is preceded by a long intron, and is symmetrical (preserving reading frame) (33).

Analysis by comparative genomics sheds light on the evolutionary history of exon 8. A VISTA global alignment shows that the genomic sequences corresponding to exon 8 occur in primates but not in other mammals or other vertebrate groups (birds, amphibians, and fish) (see Fig. S7A in the supplemental material), as suggested by our earlier alignment of amino acid sequences. Closer examination of the primate sequences shows a nonsense mutation in the macaque and an altered splice acceptor sequence in the orangutan, suggesting that exon 8 is not expressed in these other primates (see Fig. S7B in the supplemental material). Thus, this exon may have been utilized in an ancestral primate species and has subsequently been inactivated in some primates but retained (but poorly expressed) in humans.

Most previous reports of *AHRR* gene structure in humans have included exon 8 (see references 8 and 16 but also reference 65). However, in the few studies to examine the function of the hAHRR protein, it has not always been clear which form was being investigated (21, 29, 30, 71). The substantial difference in repressor activity between the two forms described here (Fig. 2) highlights the importance of knowing which AHRR protein is being expressed in any experimental system. AHRRΔ8 was the major form present in the several human cell lines (representing three different tissues) that we studied, but there could be other cell lines in which the longer form predominates.

Alternative splicing has been reported for several bHLH-PAS proteins, including AHR (7), BMAL (27, 58), HIF-3α (18, 42, 44), SIM2 (47), and ARNT (36). In some cases, the alternatively spliced products have been shown to possess distinct functional properties (42, 44, 47, 52, 53). Similarly, we show here that the alternatively spliced forms of hAHRR differ in their ability to act as repressors. In the original (longer) $AHRR_{715}$ protein, the presence of an 18-aa insertion (compared to AHRRΔ8 and AHRR orthologs in other species) causes a dramatic loss in the ability to repress both AHR and HIF. The insertion occurs in a region of the AHRR protein that is highly conserved among vertebrate species and that corresponds to the conserved, ~100-aa "intervening region" of the AHR between the PAS-A and PAS-B repeats. In the AHR, this intervening region is important for dimerization with ARNT (10, 45). Similarly, deletion of this region of the zebrafish AHRRa causes a dramatic loss in ARNT dimerization (14). Our coimmunoprecipitation data demonstrate that disruption of this region with the 18-aa insertion eliminates the ability of AHRR to dimerize with ARNT. The fact that repressor activity is lost concomitantly with loss of ARNT binding suggests that it is the AHRR-ARNT complex, rather than AHRR alone, that is the active repressor. However, it is also possible that the loss of repression activity in the presence of the additional 18 aa is the result of other structural changes and is unrelated to the ability to bind ARNT. Additional studies will be needed to establish conclusively whether repression by AHRRΔ8 requires dimerization with ARNT.

AHRRΔ8 Pro^{185} and -Ala^{185} variants are functionally indistinguishable. Recent epidemiological studies (reviewed in reference 22) have linked a Pro185Ala polymorphism in the hAHRR (65) to human reproductive disorders, including endometriosis (35, 62) and male reproductive abnormalities such as micropenis and oligospermia (16, 46, 59, 61, 64). Fujita et al. (16) suggested that the Pro185 allele might be a hypomorphic allele with a weaker inhibitory effect on AHR. However, there are no previous reports in which the functions of the AHRR-Pro^{185} and AHRR-Ala^{185} variants have been compared. Here, we found that these two variants are qualitatively and quantitatively indistinguishable in their abilities to repress AHR and HIF (Fig. 4; see also Fig. S5 in the supplemental material). It is possible that in other contexts these two variants function differently. However, we note that the residue of the AHRR protein corresponding to this Pro185Ala polymorphism is not well conserved, even among mammals, and it occurs in a highly variable region of the protein (see Fig. S1 in the supplemental

material). Thus, it seems more likely that this single nucleotide polymorphism (SNP) is not of functional significance. Other explanations for the apparent association between this SNP and reproductive disorders should be considered. For example, the Pro185Ala SNP may be in linkage disequilibrium either with noncoding SNPs that affect AHRR expression or with SNPs at a nearby locus that plays a role in reproductive processes (22).

Mechanism of repression. Although bHLH-PAS proteins are generally thought of as transcriptional activators, several are known to act as repressors: SIM2 (49, 66), NPAS1 (60), HIF-3α (41, 44, 67), and (in some circumstances) AHR (43, 55, 68). The mechanisms of repression are varied and include competition for ARNT (49, 66, 67), formation of abortive complexes with the target transcription factor (41, 44), displacement of DNA-bound transcription factor complexes (17, 37, 49), and displacement of coregulatory proteins (24, 43).

The mechanism by which AHRR represses AHR signaling has been assumed to involve sequestration of ARNT away from AHR combined with competition between AHR-ARNT and AHRR-ARNT complexes for binding to AHRE sequences, as originally proposed (48). However, recent studies with two zebrafish AHRR paralogs (AHRRa and AHRRb) have indicated that (i) sequestration of ARNT complexes is unlikely to be involved in the mechanism of repression and (ii) competition for AHRE binding may contribute to repression but is not the sole mechanism involved (14). The results presented here for hAHRRΔ8 provide additional evidence that sequestration of ARNT does not play a role in the mechanism of repression. Overexpression of ARNT had no effect on the ability of AHRRΔ8 to repress AHR (Fig. 5), as we showed earlier for the zebrafish AHRRs (14). Thus, AHRRΔ8 differs from SIM2 and SIM2s, which repress AHR by competing for ARNT (47, 66).

Another possible mechanism of repression, squelching (competition for coregulatory proteins such as coactivators), is well known to occur with nuclear receptors (5). Squelching has also been demonstrated in interactions involving bHLH-PAS proteins. For example, repression of E2F by the AHR (43) and repression of HIF signaling by p53 (57) both were rescued by overexpression of p300. In contrast, we found that overexpression of several coregulatory proteins known to interact with AHR (CoCoA, GRIP1, SRC1, p300, and GAC63) had no effect on the ability of AHRRΔ8 to repress AHR signaling, suggesting that competition for these proteins is not involved in the mechanism of repression. Conceivably, other AHR coactivators (2, 23) could be targets for AHRRΔ8, or repression might involve simultaneous competition for multiple coactivators.

We also considered the possibility that AHRRΔ8 could interact directly with AHR, similar to the way in which some alternatively spliced products of the HIF-3α locus (18) are able to repress HIF-1α by forming an abortive complex (41, 44). hAHRRΔ8 did not interfere with the TCDD-dependent nuclear translocation of AHR, as we showed earlier for zebrafish AHRRa (14). Interestingly, in coimmunoprecipitation assays we found a modest but reproducible interaction between AHRRΔ8 and AHR. This interaction was neither enhanced nor inhibited by inclusion of ARNT in the incubation, suggesting that AHRRΔ8 can interact with both the AHR monomer and the AHR-ARNT dimer. AHRRΔ8 does not require ARNT in order to interact with AHR, because we saw such interactions in the absence of ARNT (Fig. 8, lane 3); in addition, both AHRRΔ8 and $AHRR_{715}$ (which does not dimerize with ARNT [Fig. 3]) were able to associate with AHR (see Fig. S4B in the supplemental material). Conceivably, both ARNT dimerization and association with AHR are required for repression to occur. Nevertheless, while our results may be suggestive, it is clear that the consequences of the AHRR-AHR interaction and its role in the mechanism of repression will require further investigation.

AHRRΔ8 specificity extends beyond AHR. The name "AHR repressor" implies a specificity of AHRR for AHR, but this has not yet been established. We therefore sought to determine whether the hAHRR is capable of repressing other transcription factors in addition to AHR. AHRRΔ8 inhibited the $CoCl_2$-stimulated transcription of the hypoxia-responsive reporter PL949 in two different cell lines (Fig. 9C and D). This inhibition of HIF signaling provides a possible explanation for the results of Zudaire et al. (71), who found that silencing of AHRR enhanced the angiogenic activity of A549 cells in a directed in vivo angiogenesis assay. Thus, one role of AHRRΔ8 may be to modulate HIF signaling and its downstream consequences such as angiogenesis. The AHRRΔ8-HIF interaction provides a mechanism for cross talk between AHR and hypoxia signaling; for example, AHR ligands may regulate HIF-dependent responses through AHR-dependent induction of AHRRΔ8 and subsequent inhibition of HIF signaling. The ability of AHRRΔ8 to repress both AHR and HIF also provides a possible mechanism for the tumor suppressor activity of AHRR (71), in which it may simultaneously limit the activity of these two transcription factors, both of which are overexpressed in a variety of human cancers (54, 56).

In contrast to the AHRRΔ8 inhibition of HIF-dependent signaling, we found that neither PXR nor ERα was repressed by AHRR. The result with ERα is in contrast to data obtained by Kanno et al. (31), who reported recently that hAHRR bound to ERα and repressed ERα-mediated transactivation of a reporter gene. We cannot yet explain the differences in results, but one possibility is that AHRR-ERα interactions are cell specific; our data were obtained in COS-7 cells, whereas Kanno et al. (31) performed their assays in HepG2 and MCF-7 cells.

Regardless of the explanation for these differences, both the results of Kanno et al. (31) and our data on AHRRΔ8-HIF interactions suggest that AHRR is more than simply a repressor of AHR but rather has interactions that reach beyond the AHR pathway to affect other signaling pathways. Such broad interactions are consistent with the emerging view of AHRR as an important regulatory protein with pleiotropic effects on cell growth and differentiation, including a possible role as a tumor suppressor (22, 30, 56, 68, 71). Understanding these interactions of the hAHRR and their biological significance will be an important goal of future research. The results presented here provide a foundation for that research by identifying AHRRΔ8 as the major active form of hAHRR, providing the first functional assessment of the polymorphic AHRR variants that have been linked to human reproductive disease, and providing new insight into AHRR's function as a repressor of multiple cellular signaling pathways.

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