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Identification of a novel C-terminal domain involved in the negative function of the rainbow trout Ah receptor nuclear translocator protein isoform a (rtARNTa) in Ah receptor-mediated signaling

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Abstract

Rainbow trout aryl hydrocarbon receptor (AHR) nuclear translocator isoform a (rtARNTa) has a negative function in AHR-mediated signal transduction. Previous analyses suggest that the negative function is at the level of DNA binding and may be due to the presence of 57 C-terminal amino acids that are strongly hydrophobic. To assess the negative activity of rtARNTa at the molecular level, hydrophobic-rich domains corresponding to amino acids 601–637, 601–631, and 616–631 were analyzed for the ability to affect the function of truncated rtARNT proteins in complementation and gel shift assays. Addition of the hydrophobic-rich domains to these proteins reduced their ability to complement AHR-mediated signal transduction in mouse hepatoma cells by 65–95%. The decrease in function was related to a reduced ability of the AHR · rtARNT complex to bind DNA and not due to a lack of dimerization with AHR. Expression of the hydrophobic-rich domains on Gal4 proteins showed that the C-terminal domain of rtARNTa was unlikely to contain transactivation function; however, the hydrophobic domains reduced the ability of the Gal4 proteins to bind DNA. Immunoprecipitation and mutational experiments indicate that the hydrophobic-rich domains do not interact with the bHLH motif of AHR. Interestingly, immunoprecipitation experiments also revealed that the C-terminal hydrophobic-rich region of rtARNTa could oligomerize *in vitro* in a chimera with the Gal4 DNA binding domain. These findings indicate that the C-terminal hydrophobic amino acids are critical for the negative function of rtARNTa in AHR-mediated signaling and suggest that multiple mechanisms may be involved in the repression of DNA binding.

Keywords: Rainbow trout; ARNT; Gene regulation; Dominant negative; Ah receptor; CYP1A1

1. Introduction

The ARNT protein is a constitutively expressed nuclear transcription factor, that belongs to the bHLH/PAS family of proteins [1], and acts as a dimerization partner in two well-studied stress-induced pathways. The first is AHR-mediated signaling that mediates many of the biological effects of halogenated aromatic hydrocarbons, typified by TCDD [reviewed in Refs. 2 and 3]. The second pathway involves the hypoxia-like factor (HIF-1) to modulate expression of genes such as VEGF, erythropoietin, and numerous glycolytic enzymes [reviewed in Refs. 4 and 5]. In both pathways, ARNT is an essential component of the DNA binding heterodimer.

Similar to other transcription factors, the regulation of ARNT and AHR function is influenced by phosphorylation, hsp90, corepressors/coactivators, nuclear import/export, and degradation [6–14]. In addition, there is growing evidence of the regulation of ARNT and AHR function by other bHLH/PAS proteins that act as transcriptional repressors and dominant negative proteins. For example, mSIM2 has been shown to repress the transcriptional activity of ARNT, and can prevent ARNT from forming functional

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Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; rtARNT, rainbow trout ARNT; mARNT, mouse ARNT; bHLH, basic helix-loop-helix; PAS, PER-ARNT-SIM; AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HIF, hypoxia-inducible factor; GAR-HRP, goat anti-rabbit horseradish peroxidase; TBS, Tris-buffered saline; TTBS, Tris-buffered saline with Tween 20; TBE, Tris, boric acid, EDTA; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum, PCR, polymerase chain reaction; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; TNT, transcription and translation; XRE, xenobiotic response element; and P450, cytochrome P450.

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heterodimers with HIF-1 *in vitro* [15,16]. In addition, the alternatively spliced rainbow trout isoform rtARNTa can bind to mAHR and function as a dominant negative regulator of AHR-mediated signaling [17]. The dominant expression of an alternatively spliced ARNT isoform in the estrogen-negative MDA-MB-231 cell line alters responsiveness to TCDD [18]. Finally, the AHR repressor (AhRR) inhibits AHR function by competing with AHR for dimerization with ARNT and XRE binding [19]. Not surprisingly, the expression of transcriptional repressors and dominant negative acting factors is common among many signaling pathways as a mechanism of altering response to stimuli and regulating gene expression [reviewed in Ref. 20]. Collectively, these findings stress the importance of identifying additional negatively-acting bHLH/PAS transcription factors and understanding their mechanism of action.

Previous studies from this laboratory have reported the identification of two alternatively spliced isoforms of rainbow trout ARNT, named rtARNTa and rtARNTb, that have divergent functions in AHR-mediated signal transduction due to the presence of distinct COOH-ends [17,21]. The rtARNTb protein has a positive function in AHR-mediated signaling, and contains a transcriptionally active glutamine-rich COOH-end similar to mARNT. In contrast, the rtARNTa protein is identical to rtARNTb over the first 533 amino acids but contains a distinct 123 amino acid proline-serine-theonine (PST) rich COOH-end due to alternative splicing of an exon and alteration of the open reading frame [17]. Previous experiments have determined that the negative function of rtARNTa is: (i) due to the formation of rtARNTa · AHR complexes that have reduced affinity for DNA [17,21], (ii) related to the presence of the PST-rich domain and not to the lack of amino acids encoded by the missing exon [21], and (iii) possibly mediated through a hydrophobic domain within the PST-rich sequence [21]. To extend these results and gain insight into the mechanism involved in the negative activity of rtARNTa, experiments were initiated to functionally characterize the hydrophobic amino acid domain of the rtARNTa protein with respect to AHR-mediated signaling and transactivation function. Importantly, rtARNT function was evaluated at the level of dimerization to the AHR, binding of the rtARNT · AHR dimer to the XRE, and induction of endogenous P4501A1 protein in cell culture models.

2. Materials and methods

2.1. Materials

Specific antibodies against mouse AHR protein (A-1A) and rainbow trout ARNT (rt-84) were identical to those described previously [11,17,22]. All antibodies were affinity-purified IgG fractions. Antibodies against GAL4 1–147 (anti-Gal4) were purchased from Santa Cruz Biotechnology. Polyclonal antibodies against the FLAG^{TM} epitope were purchased from the Zymed Corp. Polyclonal rabbit antibodies against rat P4501A1 were purchased from Xenotech. Goat anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP) was purchased from Jackson Immunoresearch. TCDD was a gift from Monsanto. The reporter plasmid pFR-Luc was purchased from Stratagene.

2.2. Buffers

PBS is 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.02% KH_2PO_4 (pH 7.4). 2X gel sample buffer is 125 mM Tris (pH 6.8), 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM DTT, 0.005% bromophenol blue. TBS is 50 mM Tris, 150 mM NaCl (pH 7.5). TTBS is 50 mM Tris, 0.2% Tween 20, 150 mM NaCl (pH 7.5). TTBS+ is 50 mM Tris, 0.5% Tween 20, 300 mM NaCl (pH 7.5). BLOTTO is 5% dry milk in TTBS. 2X lysis buffer is 50 mM HEPES (pH 7.4), 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, 20% glycerol. 5X gel shift buffer is 50 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 50% glycerol. 0.5X TBE is 45 mM Tris-borate, 1 mM EDTA (pH 8.4). MENG is 25 mM MOPS (pH 7.1), 1 mM EDTA, 10% glycerol, 0.02% Naazide. 1X immunoprecipitation (IP) and wash buffer is 25 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 10% glycerol, 50 mM l-histidine.

2.3. Cells and growth conditions

Type II Hepa-1 variants were a gift from Dr. Jim Whitlock, Jr. (Department of Pharmacology, Stanford University). These cells have reduced levels of ARNT and have been used in previous studies to assess rtARNT function *in vitro* [17,22]. The cells were propagated at 37° in DMEM containing 5% FBS. All cells were passaged at 1-week intervals and used in experiments during a 2-month period.

2.4. Recombinant plasmids

Parental vectors containing full-length rtARNTa (pMV-rtARNTa) and rtARNTb (pMVrtARNTb) have been described previously [17,21]. To generate all rtARNT constructs, specific primers and PCR were used to amplify specific C-terminal sequences from the pMVrtARNTa vector. The resulting fragments were cloned into pcrtARNT533 (encodes amino acids 1–533), pcrtARNTEX (encodes amino acids 1–533 and the 123 amino acids of the spliced exon), or pcrtARNTb (encodes the full 723 amino acids of the rtARNTb sequence). These vectors have been detailed previously [21], and the rtARNT domains are schematically represented in Fig. 1A for reference. Thus, the numbers used in the nomenclature in this report represent amino acid residues. The parental sequence is always shown first (i.e. rtARNT533, rtARNTEX, etc.) with the newly ligated sequence following (i.e. rtARNT533/601–631 is a protein containing the 533 N-terminal amino acids followed by

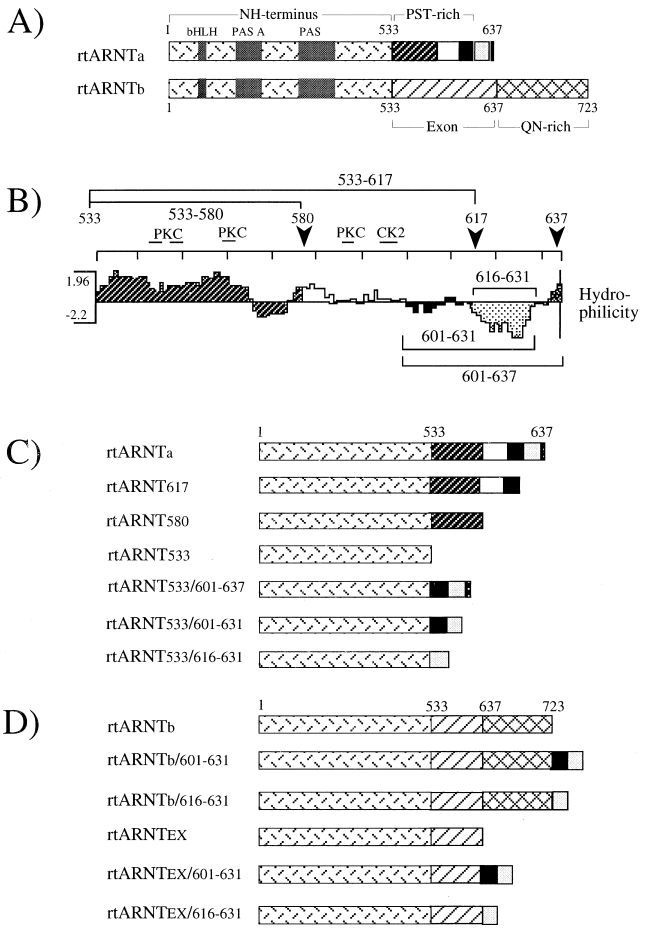

Fig. 1. Schematic diagram of rtARNT isoforms and expression constructs. (A) Domain structure of full-length rtARNTa and rtARNTb. Numbers indicate amino acids. (B) C-terminal domains of rtARNTa showing a hydrophilicity profile. Positive values predict areas that are hydrophilic and have a high probability of being on the surface of the protein. The location of the C-terminal truncations and hydrophobic-rich domains are indicated. (C) Schematic diagram of the constructs used to analyze the function(s) of the C-terminal domain of rtARNTa. (D) Schematic diagram of the constructs used to analyze the function(s) of the C-terminal domain of rtARNTa in the context of rtARNTb.

amino acids 601–631 of rtARNTa). For Gal4 chimeric rtARNT proteins, the pc424 vector that contains the DNA binding region encoded by amino acids 1–147 was used [21]. The nomenclature is the same as detailed above except that the parental sequence is referred to as Gal4 followed by the appropriate rtARNT sequence (i.e. Gal4/601–631 is a protein containing the 147 N-terminal amino acids of the Gal4 protein followed by amino acids 601–631 of rtARNTa). The FLAG™ tagged rtARNTa construct (FLAG™-rtARNTa) was generated by PCR using a 5′ oligonucleotide containing a Kozak and FLAG™ coding sequence [primer 16, Ref. 21], and a 3′ oligonucleotide [primer 2, Ref. 21]. To confirm the correct reading frame and to check for mutations, all constructs were sequenced prior to use.

2.5. In vitro *mutagenesis*

Cysteine 624 of rtARNTa was changed to an alanine residue in the plasmids pcrtARNTa and pcrtARNT533/601–631 to generate rtARNTa/A624 and rtARNT533/601–631A624 by the QuickChange™ *in vitro* mutagenesis kit as detailed by the manufacturer (Stratagene). In addition, leucine 69 and 71 were mutated to alanines in the plasmid pSportM'AHR to generate AHRΔNES as detailed [23]. All clones were sequenced to confirm the presence of the mutation.

2.6. In vitro *expression of ARNT proteins*

The recombinant rtARNT proteins were generated using the TNT™ Coupled Rabbit Reticulocyte Lysate System and the Gal4/rtARNT proteins using the Wheat Germ Extract Kit essentially as detailed by the manufacturer (Promega). Upon completion of the 90-min reaction, samples were either combined with an equal volume of 2X gel sample buffer and boiled for 5 min, or stored at –20° for use in functional studies. The actual concentration of each recombinant protein expressed in each reaction was determined by western blot analysis with the anti-mARNT, rt-84, or anti-Gal4 antibody using standards or cell lysates containing known amounts of endogenous or recombinant protein [11].

2.7. Eukaryotic transfections and reporter gene assays

Approximately 5×10^5 type II cells were plated into 60-mm culture dishes and incubated at 37° for 16–24 hr. One to two micrograms of appropriate plasmid vectors was then transfected into cells with LipofectAMINE™ as detailed by the manufacturer (Gibco). For complementation studies, after a 24-hr recovery, cells were incubated in the presence of 2 nM TCDD or DMSO for an additional 20–40 hr. Cells were harvested from plates by trypsinization, and total cell lysates were prepared as detailed below. For transactivation studies, the cells were also transfected with 1.0 μg of the reporter gene pFR-Luc and 1.0 μg of pSV-β-galactosidase to monitor transfection efficiency (Promega). Cells were allowed to express 24–48 hr after transfection. In these instances, harvested cells were split into two equal fractions. One fraction was used for the preparation of total lysates. The other fraction was resuspended in reporter lysis buffer (Promega), and β-gal and luciferase activities were determined as detailed by the manufacturer (Promega). Triplicate plates were transfected with each plasmid being evaluated, and experiments were completed three times.

2.8. Production of total cell lysates and cytosol

Total cell lysates for western blot analysis were prepared by sonicating cell pellets in 1X lysis buffer and NP-40 as detailed previously [7,11,17,23]. Cytosol was prepared from type II Hepa-1 cells by homogenization in MENG buffer

supplemented with leupeptin (10 μg/mL), aprotinin (20 μg/mL), and phenylmethylsulfonyl fluoride (100 μM). Homogenates were centrifuged at 22,000 *g* for 10 min in a refrigerated microcentrifuge, and aliquots of the supernatants were stored at –70° prior to use in EMSA. Protein concentrations were determined by the Coomassie Blue Plus assay (Pierce) using BSA as the standard.

2.9. Quantitative western blot analysis

Protein samples were resolved by denaturing electrophoresis on discontinuous polyacrylamide slab gels (SDS–PAGE) and were electrophoretically transferred to nitrocellulose as described [7,8,11,17]. Immunochemical staining was carried out with various concentrations of primary antibody (see text and figure legends) in BLOTTO buffer for 1–2 hr at 22°, followed by three washes of TTBS+ for a total of 45 min. The blot was then incubated in BLOTTO buffer containing a 1:10,000 dilution of GAR-HRP (see text and figure legends) for 1 hr at 22° and washed in three changes of TTBS+. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (Amersham). The relative concentrations of rtARNT, P4501A1, Gal4, and actin were determined by computer analysis of the autoradiographs as detailed previously [7,8,11,17]. All exposures analyzed by this method were within the linear range established for each antibody.

2.10. In vitro activation of AHR · ARNT complexes and EMSA

AHR · ARNT complexes were produced by combining approximately 50–80 ng of *in vitro* translated mARNT or rtARNT proteins with 100 μg of type II cytosol in the presence of 10 nM TCDD or 0.5% DMSO at 25° for 2 hr. The amount of each recombinant protein added to each sample was always identical as determined by western blotting of the input protein (as detailed above).

Oligonucleotides XRE-1 (5′-CGGCTCGGAGTTGCGTGAGAAGAG) and XRE-2 (5′-CGGCTCTTCTCACGCAACTCCGAG) were annealed and labeled with [^{32}P]dCTP by Klenow fill in [24]. The double-stranded fragment corresponds to the consensus XRE-1 of the CYP1A1 promoter as previously described [25]. The *in vitro* activated type II cytosol was then incubated at 22° for 15 min in 1X gel shift buffer supplemented with KCl (80 mM) and poly(dIdC) (0.1 mg/mL). In some instances, 0.5 to 1.0 μg of affinity purified anti-mARNT, rt-84, A-1, or preimmune IgG was included in the sample. Then approximately 4 ng of ^{32}P-labeled XRE was added to each sample, and the incubation was continued for an additional 15 min at 22°. The samples were resolved on 5% acrylamide/0.5X TBE gels, dried, and exposed to film.

2.11. EMSA of Gal4/rtARNT proteins

Oligonucleotide Gal4BS (5′-ACTGCTCGGAGGACAGTACTCCGCT) was annealed and labeled with [^{32}P]dATP. The double-stranded fragment corresponds to the consensus Gal4 binding site [26]. The desired *in vitro* expressed Gal4/rtARNT protein was incubated at 22° for 15 min in 1X gel shift buffer supplemented with KCl (120 mM) and poly(dIdC) (0.1 mg/mL). In some instances, 0.5 to 1.0 μg of affinity-purified anti-Gal4 antibody was included in the sample. Then approximately 4 ng of ^{32}P-labeled XRE was added to each sample, and the incubation was continued for an additional 15 min at 22°. The samples were resolved on 5% acrylamide/0.5X TBE gels, dried, and exposed to film.

2.12. Immunoprecipitation

Recombinant ARNT was produced *in vitro* in the presence of [^{35}S]methionine. [^{35}S]Methionine-labeled ARNT was combined in an equal molar ratio with unlabeled (cold) AHR in 1X MENG buffer and activated with 10 nM TCDD for 2 hr at 25°. Protein mixtures were then incubated with 5 μg of affinity-purified antibody in the presence of 1X immunoprecipitation buffer for 1 hr at 22°. The samples were transferred to new tubes, supplemented with 25 μL of protein A-Sepharose CL-4B beads (Pharmacia Biotech, Inc.), and incubated for 1 hr at 22°. Samples were centrifuged at 500 *g* for 2 min at 22°, and the beads were removed to fresh tubes. The beads were washed for a total of 15 min with three changes of IP wash buffer, mixed with 25 μL of 2X gel sample buffer, boiled for 5 min at 95°, and resolved by SDS–PAGE. The gels were incubated with Amplify (Amersham Corp.) for 30 min and exposed to film.

3. Results and discussion

3.1. Functional analysis of rtARNTa hydrophobic domains

Previous studies have demonstrated that the 40 residues making up the COOH-end of rtARNTa encode strongly hydrophobic amino acids, and experiments showed that truncation of these residues restored the ability of rtARNTa to complement AHR-mediated signal transduction in cells [21]. Thus, the studies within this report were designed to determine which amino acids were responsible for the negative function of rtARNTa and to determine the mechanism through which the effect was mediated.

To begin the analysis of these questions, three regions of hydrophobic amino acids contained within the C-terminus of rtARNTa were evaluated in the context of rtARNT for their ability to complement endogenous AHR-mediated signaling in ARNT-defective cells (type II cells). Figure 1 shows a schematic representation of the domains and the nomenclature of the constructs used to express each protein.

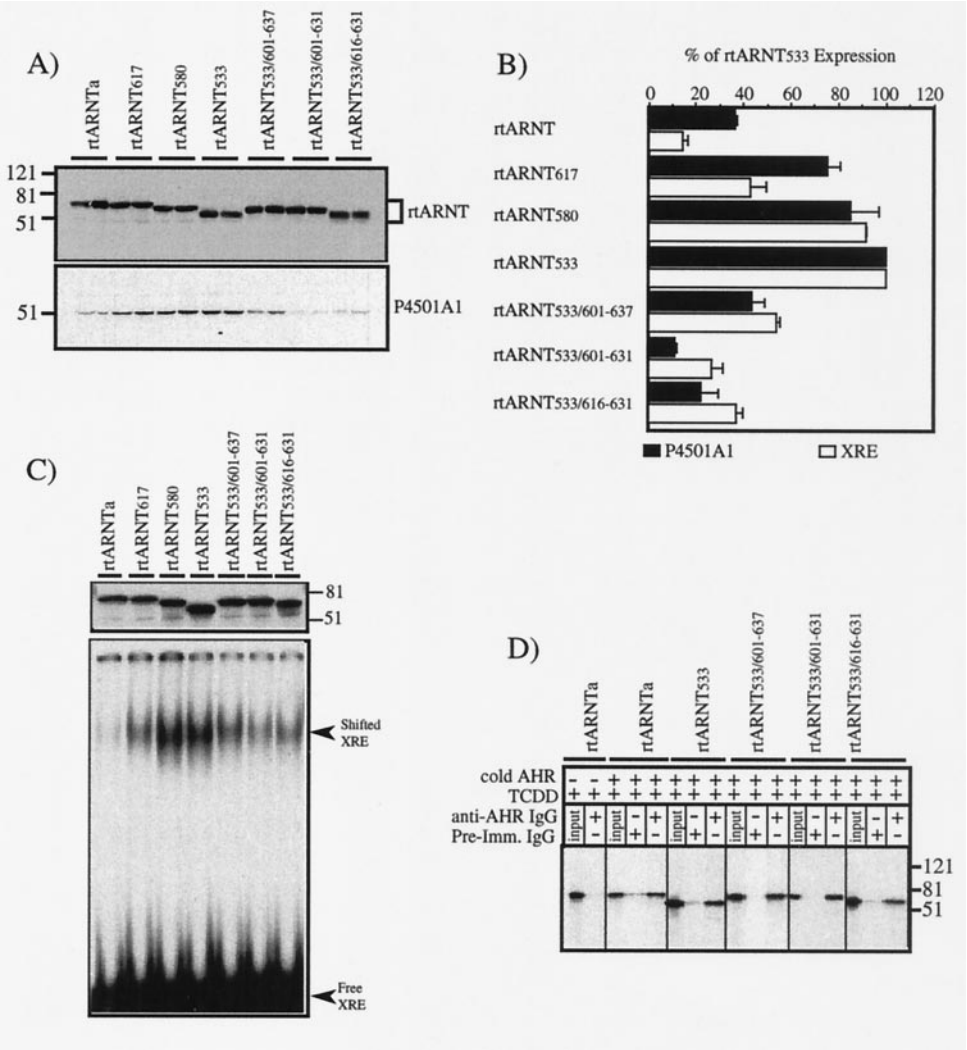

Fig. 2. Analysis of hydrophobic-rich domains in the context of rtARNT533. (A) Triplicate plates of type II cells were transfected with the indicated constructs, treated with TCDD (2 nM) for 16 hr, and harvested. Total cell lysates (18 μg) were analyzed by western blotting with either rt-84 IgG (1.0 μg/mL) or anti-P4501A1 IgG (1:500) and visualized by ECL with GAR-HRP (1:10,000). (B) Composite of P4501A1 expression and XRE binding. The levels of P4501A1, rtARNT protein, and specifically shifted XRE were quantified by computer densitometry. The data are presented as the percentage of P4501A1 expression or XRE binding compared with rtARNT533 (100%). Each bar represents the mean ± SD of at least three independent experiments. (C) One hundred micrograms of type II cytosol was combined with 50 ng of the indicated rtARNT protein. Samples were activated with TCDD (10 nM) for 2 hr at 30°. The specifically shifted band associated with rtARNT · AHR · XRE complex is indicated. The western blot at the top of the EMSA represents an aliquot of the exact samples used for the EMSA stained with the rt-84 antibody as detailed in panel A. (D) Analysis of rtARNT association with mAHR. Equal amounts of ^{35}S-labeled rtARNT and AHR protein were combined, activated with 2 nM TCDD for 2 hr at 30°, and precipitated with 8 μg of A-1 IgG (anti-AHR) or pre-immune IgG. Samples were then resolved by SDS–PAGE, and the dried gels were exposed to film. Only 50% of the input samples were resolved. Sizes are in kDa.

Type II cells were then transfected with identical amounts of each construct, treated with 2 nM TCDD for 16 hr, and total lysates prepared. Then the levels of rtARNT and P4501A1 protein were determined by quantitative western blotting. Figure 2A shows a representative blot, with each lane representing a single dish of transfected cells, while Fig. 2B shows the level of P4501A1 induction normalized to the level of each rtARNT protein and compared with the level induced by rtARNT533.

The results demonstrated that rtARNT533 complements AHR-mediated signaling in comparison to the rtARNTa protein. This finding is consistent with previous studies that have demonstrated that the COOH-end of mammalian ARNT is not required for AHR-mediated gene induction [27,28]. Truncation of 20 (rtARNT617) and 57 (rtARNT580) amino acids from the C-terminal domain of rtARNTa also complemented induction of P4501A1 to at least 70% of the level of rtARNT533. In contrast, cells expressing rtARNT533/601–637 and rtARNT533/616–631 functioned in AHR-mediated signaling to less than 55% of the level of rtARNT533 with the rtARNT533/601–631 showing only about 10% of the activity of rtARNT533 *in vitro*. Thus, these results support the

hypothesis that the C-terminal hydrophobic amino acids between residues 601 and 631 are critical for the negative function of rtARNTa in AHR-mediated signaling.

Previous studies have shown that reduction in the TCDD-inducible expression of P4501A1 in cells transfected with rtARNTa occurs at the level of DNA binding [17,21]. To evaluate the interaction of rtARNT · AHR complexes with DNA, identical amounts of each rtARNT protein were combined with type II cytosol and activated with TCDD. The formation of rtARNT · AHR · XRE complexes was then evaluated by EMSA. A typical assay is shown in Fig. 2C, while the quantified data are presented in Fig. 2B. rtARNTa failed to produce a strong shift, while rtARNT533 functioned in the EMSA consistent with the induction of P4501A1. However, the addition of the hydrophobic-rich domains to rtARNT533 resulted in a decrease in DNA binding that closely paralleled the data from the complementation assay (Fig. 2, B and C). In both complementation and gel shift experiments, residues 601–631 showed the strongest ability to repress the function of rtARNT533. It can also be observed that partial truncation of the hydrophobic domain (i.e. rtARNT617) did not restore positive function to rtARNTa completely. Collectively, these results suggest that the inhibitory function of rtARNTa resides within amino acids 601–631, although it cannot be ruled out that additional amino acids within the N-terminal 533 residues facilitate the ability of the hydrophobic domain to function.

It is possible that the reduced ability of proteins containing the hydrophobic-rich domains to bind the XRE might reflect the failure of rtARNT and AHR to heterodimerize. To evaluate this possibility, identical amounts of ^{35}S-labeled rtARNT protein were combined with unlabeled AHR protein, activated with TCDD, and immunoprecipitated. Figure 2D shows the results from a representative experiment. It can be observed that an antibody specific to the murine AHR was able to effectively bring down each of the rtARNT constructs. Specificity is demonstrated by the lack of ARNT precipitation with pre-immune IgG and in samples lacking AHR. Thus, the impaired DNA-binding activity observed with proteins expressing the hydrophobic domains does not result from deficient dimerization function and implies that the C-terminal hydrophobic amino acids are involved in the negative function of rtARNTa by inhibiting its DNA binding activity.

The previous studies established that the hydrophobic-rich domains could inhibit function when fused to the C-terminus of rtARNT533. Thus, it was pertinent to investigate whether these domains would function in a similar manner when present on the functional rtARNTb protein or in the presence of the spliced exon. Thus, domains 601–631 and 616–631 were expressed at the C-terminus of full-length rtARNTb (rtARNTb/601–631; rtARNTb/616–631) or rtARNTEX (rtARNTEX/601–631; rtARNTEX/616–631). The rtARNTEX protein contains the 533 N-terminal amino acids and the 123 amino acids encoded by the spliced exon found in rtARNTb (all constructs are shown in Fig. 1D). Constructs were then analyzed for their ability to complement AHR-mediated signaling in type II cells, bind the XRE, and form a heterodimer with AHR as detailed above.

Figure 3A shows the results of a typical complementation assay where each lane represents an independent plate of transfected cells. The quantified data are presented in Fig. 3B. Consistent with previous data, both rtARNTb and rtARNTEX were capable of inducing P4501A1 expression in type II cells [17,21]. However, addition of amino acids 616–631 and 601–631 to rtARNTb resulted in approximately 90% reductions in P4501A1 expression when compared with rtARNTb. Similarly, addition of the hydrophobic domains to rtARNTEX resulted in 90–95% reductions in P4501A1 induction compared with levels induced by rtARNTEX.

To confirm that these results were related to reduced levels of DNA binding, identical amounts of recombinant rtARNT protein were combined with type II cytosol, activated, and evaluated by EMSA. Figure 3C shows the results of a typical assay. As expected, rtARNTb and rtARNTEX produced a strong gel shift in the presence of type II cytosol. However, addition of residues 616–631 and 601–631 to either rtARNTb or rtARNTEX resulted in approximately 75–90% reductions in the DNA binding that closely paralleled the results of the complementation assay (Fig. 3A). To confirm that dimerization with AHR was not affected in these studies, co-immunoprecipitation assays were performed for each protein. All rtARNT proteins were precipitated efficiently in the presence of AHR and TCDD showing that dimerization was not affected (data not shown). Thus, these experiments have shown that amino acid domains 601–631 and 616–631 of rtARNTa can negatively affect the function of three different sized rtARNT proteins (rtARNT533, rtARNTEX, and rtARNTb) at the level of DNA binding. Interestingly, the magnitude of repression appeared higher with the rtARNTEX (656 amino acids) and rtARNTb (723 amino acids) proteins than with rtARNT533 (533 amino acids). This may be related to the fact that repression was based on the activity of the full-length rtARNTb protein (as opposed to the rtARNT533) or that the presence of the exon sequence allows more efficient function of the hydrophobic domains.

3.2. Transactivation potential of rtARNTa hydrophobic domains

The C-terminal ends of mammalian ARNT, rainbow trout rtARNTb, and other bHLH proteins have been shown to contain transcription activation domains (TADs) rich in glutamine [21,27–30]. In addition, many transcription factors including rainbow trout AHR have potent TADs rich in proline, serine, and threonine (Pollenz RS, unpublished observations). Previous studies were unable to assign transcriptional function to the PST-rich COOH-end of rtARNTa

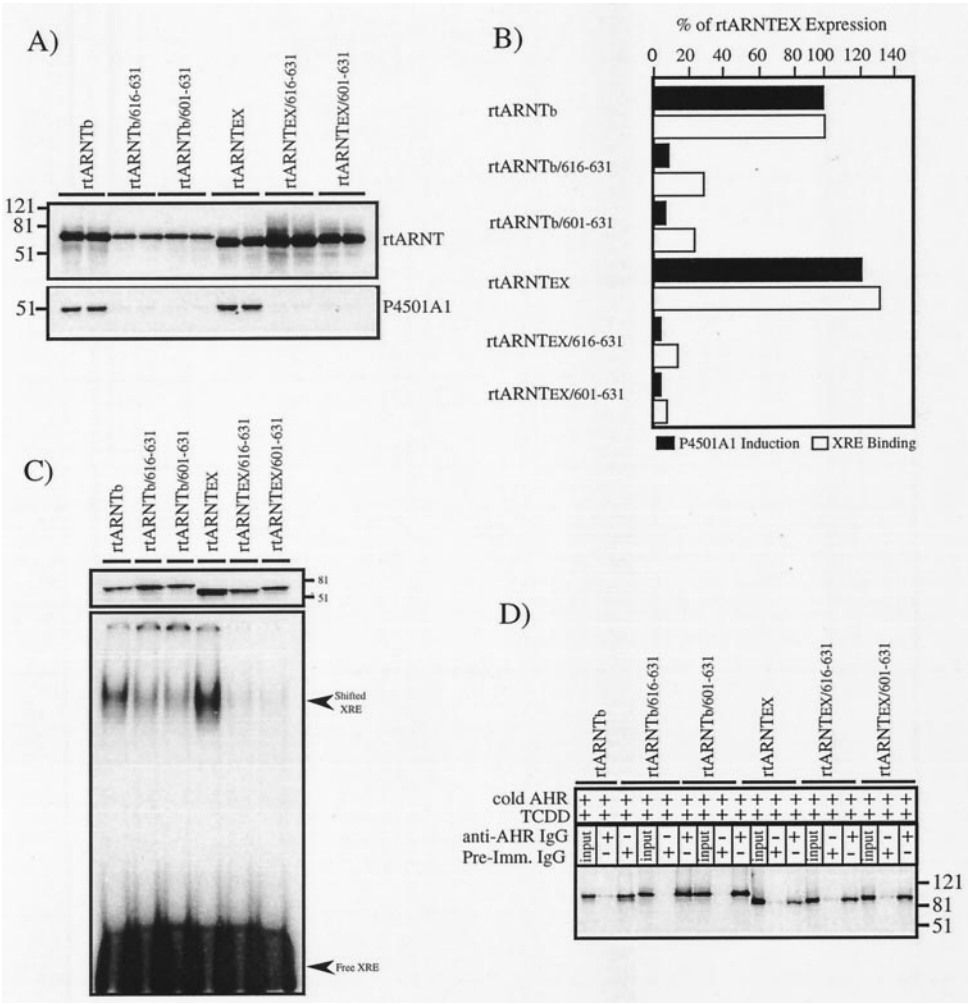

Fig. 3. Analysis of hydrophobic-rich domains in the context of rtARNTb and rtARNTEX. (A) Triplicate plates of type II cells were transfected with the indicated constructs, treated with TCDD (2 nM) for 16 hr, and harvested. Total cell lysates (18 μg) were analyzed by western blotting with either rt-84 IgG (1.0 μg/mL) or anti-P4501A1 IgG (1:500), and visualized by ECL with GAR-HRP (1:10,000). (B) Composite of P4501A1 expression and XRE binding. The levels of P4501A1, rtARNT protein, and specifically shifted XRE were quantified by computer densitometry. The data are presented as the percentage of P4501A1 expression or XRE binding compared with rtARNTb (100%). Each bar represents the mean ± SD of at least three independent experiments. (C) One hundred micrograms of type II cytosol was combined with 50 ng of the indicated rtARNT protein. Samples were activated with TCDD (10 nM) for 2 hr at 30°. The specifically shifted band associated with rtARNT · AHR · XRE complex is indicated. The western blot at the top of the EMSA represents an aliquot of the exact samples used for the EMSA stained with the rt-84 antibody as detailed in panel A. (D) Analysis of rtARNT association with mAHR. ^{35}S-Labeled rtARNT and AHR protein were combined at a 1:1 ratio, activated with 2 nM TCDD for 2 hr at 30°, and precipitated with 8 μg of A-1 IgG or pre-immune IgG. Samples were then resolved by SDS–PAGE, and the dried gels were exposed to film. Only 50% of the input samples were resolved. Sizes are in kDa.

because it failed to bind DNA in the Gal4 system [21]. However, these studies were performed with the entire COOH-end of rtARNTa and contained the inhibitory hydrophobic amino acids.

To test whether the PST-rich portion of the COOH-end contained transactivation function that might be masked by the hydrophobic domain, portions of the C-terminal end of rtARNTa were fused to Gal4 DNA binding domain and assayed for transactivation and DNA binding. To evaluate the transcriptional activity of the chimeric proteins, each construct was transfected into the type II cells with the reporter gene, pFR-Luc. Luciferase activity was normalized to the level of each chimeric Gal4 protein, and a representative experiment is presented in Fig. 4A. As a control, the Gal4/QN construct was used. This construct expresses a protein that contains the Gal4 DNA binding domain fused to the transcriptionally active QN-rich COOH-end of rtARNTb [see Fig. 1A and Ref. 21]. The results show that even though each Gal4 protein was expressed in the assay efficiently, all the proteins containing sequence from rtARNTa showed minimal levels of transactivation compared with Gal4/QN.

To confirm that the reduced level of transactivation by the various rtARNTa chimeras was not the result of reduced DNA binding, each Gal4/rtARNT chimera was expressed *in vitro* and then evaluated by EMSA using an oligonucleotide containing the Gal4 binding site. Figure 4B shows the expression of each protein using an anti-Gal4 antibody. A

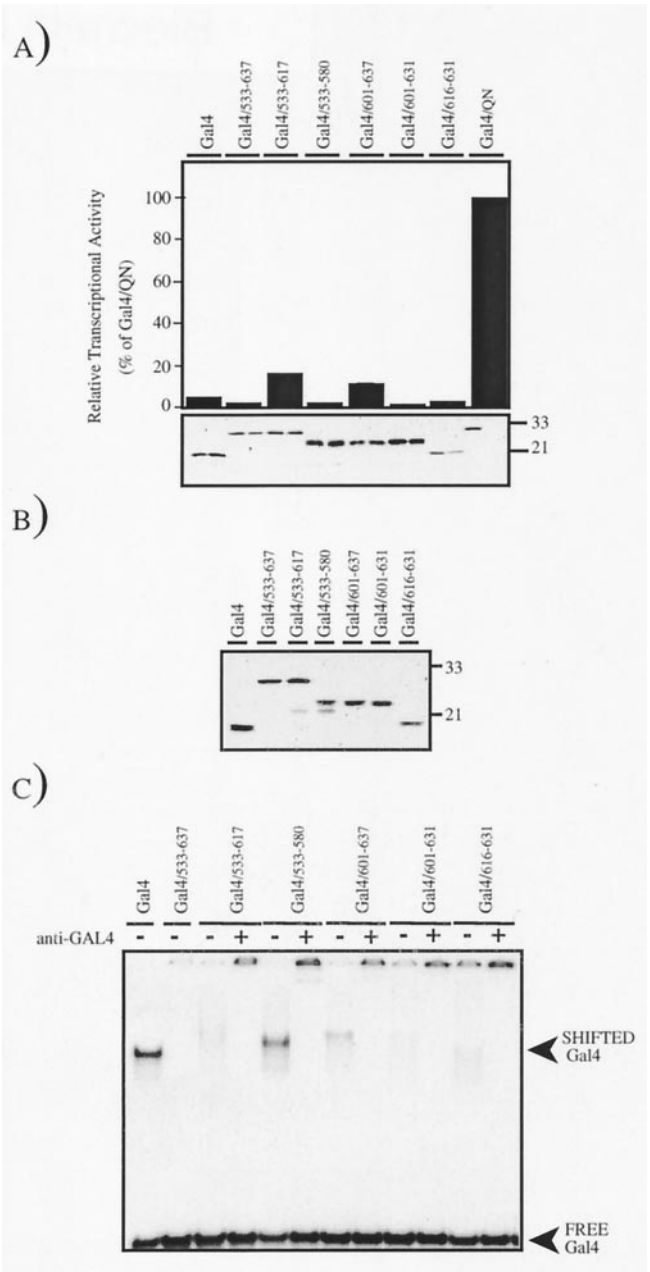

Fig. 4. Analysis of transactivation domains within regions of rtARNTa. (A) The indicated C-terminal domains of rtARNTa and rtARNTb were ligated to the Gal4 DNA binding domain (refer to Fig. 1 for details of the rtARNT domains). Constructs were co-transfected into 3–6 plates of type II cells with a luciferase reporter vector (pFR-LUC) and a β-galactosidase expression vector (pSV-β-gal). Cells were split into two fractions. One fraction was analyzed for luciferase and β-galactosidase activity. The other fraction was used to generate total cell lysates for analysis of Gal4/rtARNT protein expression. The level of luciferase activity was normalized to the expression Gal4/rtARNT protein. (B) Western blot analysis of Gal4/rtARNT protein expression used for EMSA. The blot was stained with anti-Gal4 antibody followed by GAR-HRP (1:10,000) and visualized by ECL. (C) Identical amounts of recombinant protein were subjected to EMSA with the Gal4 oligonucleotide. Shifted bands can be observed at different mobilities that correlate with the size of the Gal4/rtARNT protein. The specificity of the shifted bands is demonstrated in lanes 4, 6, 8, 10, and 12, where addition of anti-Gal4 antibody to the reaction resulted in loss of the band.

representative EMSA using the Gal4 oligonucleotide is shown in Fig. 4C. The Gal4 protein (amino acids 1–147) served as a control and produced a strong shift. Importantly, proteins containing the entire COOH-end of rtARNTa (Gal4/533–637) failed to bind DNA, as has been reported previously [21]. However, deletions removing a portion of the hydrophobic residues (from Gal4/533–617) restored DNA binding slightly, while complete truncation of the hydrophobic residues (Gal4/533–580) restored DNA binding properties. Consistently, the chimeric proteins containing residues 601–631 and 616–631 bound DNA very weakly as compared with Gal4 or Gal4/533–580. Specificity of the assay is demonstrated by the loss of the bands when antibodies against Gal4 protein were added prior to the addition of the Gal4 oligonucleotide (Fig. 4C; lanes 4, 6, 8, 10, and 12). Thus, the results from these experiments indicate that the PST-rich region between amino acids 533 and 580 does not appear to exhibit transactivation function in type II cells. Collectively, these findings suggest that rtARNTa is unlikely to provide transactivation function as observed for rtARNTb [21] and mammalian ARNT proteins [27–30]. This finding makes it unlikely that rtARNTa can provide positive transactivation function to AHR-independent pathways.

3.3. Interactions of hydrophobic domains with AHR

Studies next focused on how the rtARNTa might be impacting DNA binding of the AHR · ARNT dimer. One possible mechanism could involve inappropriate dimerization of the C-terminal domain of rtARNTa with the bHLH region of AHR. To test this hypothesis, leucine 69 and leucine 71 of the HLH domain of AHR were mutated to alanine residues, and the protein was termed AHRΔNES. These mutations have been shown to inhibit the ability of AHR to dimerize with ARNT by greater than 90% [23]. Thus, the formation of heterodimers between AHRΔNES and rtARNTa or rtARNT533 proteins was analyzed by co-immunoprecipitation. The results in Fig. 5 show that rtARNTa clearly forms a complex with wild-type AHR, as demonstrated previously [17]. However, neither rtARNT533 nor rtARNTa associated with the AHRΔNES under the assay conditions used (Fig. 5). rtARNT533 was used as a control since it contains the N-terminus common to rtARNTa but lacks the COOH-end containing the hydrophobic amino acids (see Fig. 1). These results provide important information as they show that the hydrophobic region of rtARNTa is not acting as an additional dimerization domain for association with the AHR. This result implies that AHR and rtARNTa are dimerizing properly through the HLH domain and that the negative impact of the hydrophobic domain occurs through a different mechanism.

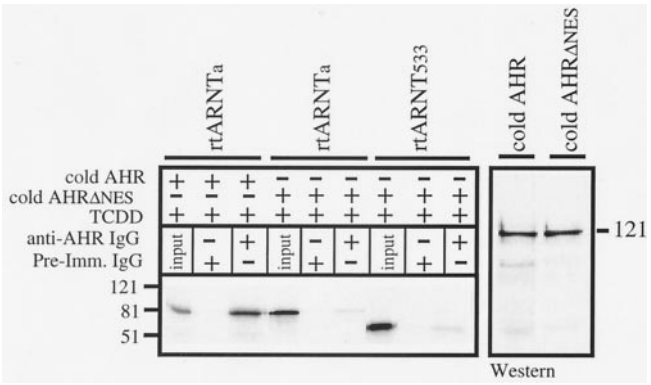

Fig. 5. Analysis of AHR association with hydrophobic-rich domains of rtARNTa. Wild-type AHR or AHRΔNES protein was combined in an equal molar ratio with ^{35}S-labeled rtARNT, activated with 2 nM TCDD for 2 hr at 30°, and precipitated with 8 μg of A-1 IgG or pre-immune IgG. Only 50% of the input samples were resolved so as not to overwhelm the gel. Sizes are in kDa. The western blot on the right shows the expression of the unlabeled AHR and AHRΔNES proteins used in the assay stained with the A-1 antibody (1 μg/mL).

3.4. Oligomerization potential of rtARNTa

The previous studies ruled out a direct interaction of the hydrophobic domain of rtARNTa with the AHR; however, the possibility exists that the hydrophobic residues might mediate dimerization or oligomerization of rtARNT. Oligomerzation of rtARNT could generate a multi-protein complex that might prevent access of the basic region of AHR or rtARNT with DNA. One mechanism for oligomerization could entail the formation of disulfide bonds between ARNT molecules or with cysteine residues of other proteins. Sequence analysis revealed that residue 624 of rtARNTa was a cysteine and that there were no cysteine residues within the exon sequence of the C-terminal end of rtARNTb [17]. To investigate the contribution of C624 to the negative function of rtARNTa, the residue was changed to an alanine by *in vitro* mutagenesis to produce rtARNT/A624 and rtARNT533/601–631A624. Each construct was then evaluated for complementation of P4501A1 induction in type II cells with the appropriate control constructs, as detailed previously. Figure 6 shows a representative experiment. rtARNT533 was able to complement the induction of P4501A1, whereas rtARNTa was not. Importantly, the mutation of C624 to alanine in the rtARNTa protein did not result in increased levels of P4501A1 induction (Fig. 6, A and B). Likewise, the addition of domain 616–631 to rtARNT533 reduced the function of this protein as detailed earlier, and the mutation of C624 to alanine did not restore function to the level of rtARNT533. In addition, neither of the A624 mutants showed an increase in the level of DNA binding when this was evaluated by the *in vitro* EMSA assay (Fig. 6, B and C). Thus, these results imply that cysteine 624 has minimal influence on the inhibitory function of the hydrophobic domain.

Since the above results do not rule out the possibility that the entire hydrophobic region might serve as a domain for protein–protein interaction, co-immunoprecipitation studies were performed using Gal4 chimeras containing the hydrophobic domain. To generate the Gal4 chimeric proteins, C-terminal domains from rtARNTa were fused to Gal4 DNA binding domain (1–147) as previously detailed [21]. The ^{35}S-labeled Gal4, Gal4/533–637 (entire C-terminal domain), Gal4/533–580, and Gal4/601–631 proteins were incubated with unlabeled recombinant rtARNTa at a ratio of 1:1 and 3:1, and co-immunoprecipitation was performed using an antibody specific to the N-terminus of rtARNT. Thus, the only way ^{35}S-labeled proteins might be precipitated is for the C-terminal domains to associate. A western blot of the unlabeled rtARNTa protein is presented to show the integrity of the sample, and specificity is demonstrated by the lack of precipitation with pre-immune IgG (Fig. 7). In addition, specificity is shown by the lack of precipitation with the rt-84 antibody in samples lacking unlabeled rtARNTa. To serve as a positive control, ^{35}S-labeled rtARNTa was precipitated with the rt-84 antibody.

The data in Fig. 7A show that ^{35}S-labeled Gal4 is not precipitated in the presence of rtARNTa. This is important as it demonstrates that rtARNTa does not interact directly with this protein. In addition, Gal4/533–580, which lacks the hydrophobic residues, failed to associate with rtARNTa. However, samples containing the C-terminal hydrophobic amino acids (Gal4/533–637 and Gal4/601–631) were precipitated with the rt-84 antibody in the presence of rtARNTa at both ratios used. Since the ^{35}S-labeled Gal4 chimeras lack a bHLH/PAS region, these results suggest that the C-terminus, specifically the hydrophobic rich region of rtARNTa, is capable of forming a protein–protein interaction with itself *in vitro.* This finding implies that oligomerization through this domain may be the possible mechanism for reduced DNA binding in Gal4 constructs containing the C-terminal hydrophobic amino acids [Fig. 4 and Ref. 21].

Whereas the previous studies illustrated that the C-terminal end of rtARNTa is capable of oligomerization when fused to Gal4, it was important to determine whether this type of interaction could occur between full-length rtARNTa proteins. To test whether rtARNTa proteins could associate through the C-terminal end, a FLAG amino acid tag was added to the N-terminal domain of rtARNTa (FLAG-rtARNTa). ^{35}S-Labeled rtARNTa was then incubated with unlabeled FLAG-rtARNTa in a ratio of 1:1 or 3:1, and co-immunoprecipitation was performed using an antibody specific to the FLAG epitope. A western blot showing the expression of the unlabeled FLAG-rtARNTa used in the assay is shown. To serve as a positive control, ^{35}S-labeled FLAG-rtARNTa was precipitated with anti-FLAG IgG. Specificity is shown by the lack of precipitation with pre-immune IgG. Figure 7B shows the results of a typical assay. The data show that ^{35}S-labeled rtARNT533 and FLAG-rtARNTa do not exhibit a high level of dimerization. This indicates that there is minimal association of the different rtARNT proteins through the bHLH/PAS re-

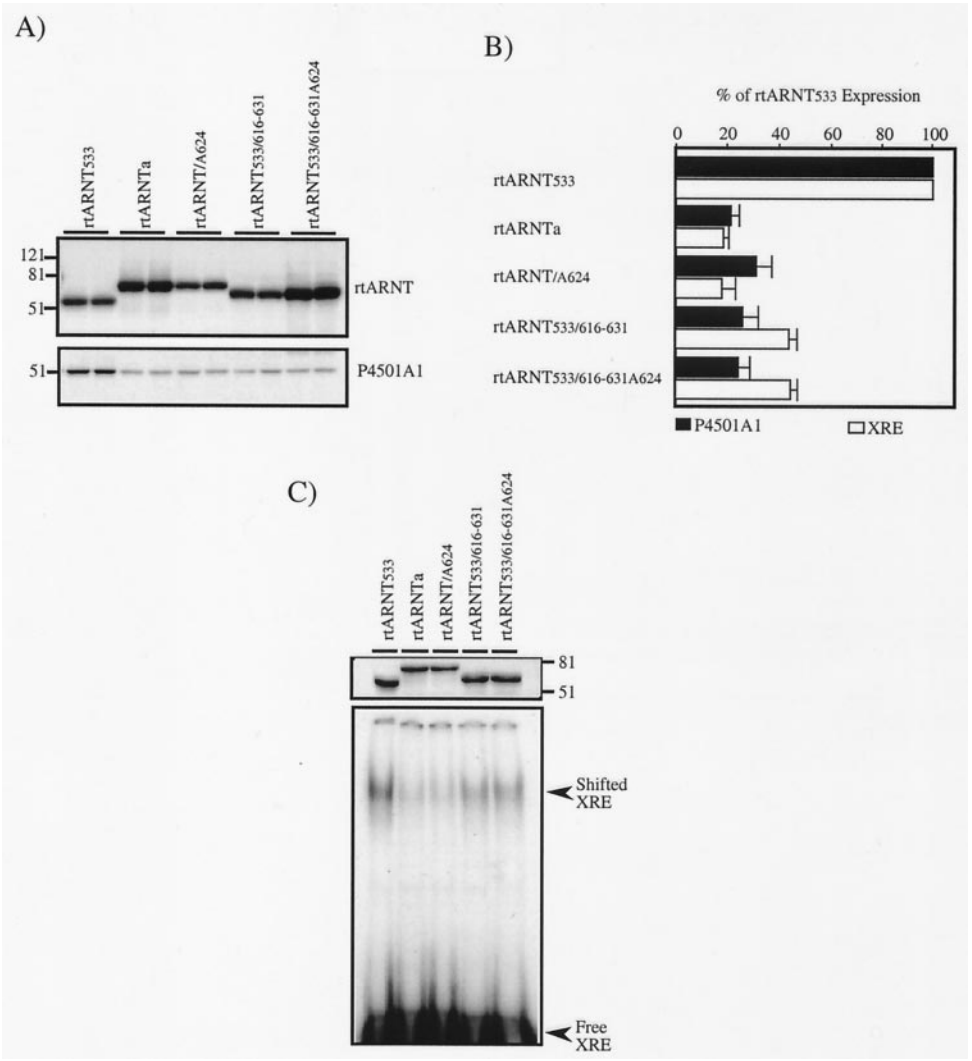

Fig. 6. Analysis of mutagenesis of cysteine 624 within the C-terminus of rtARNTa. (A) Triplicate plates of type II cells were transfected with the indicated constructs, treated with TCDD (2 nM) for 16 hr, and harvested. Total cell lysates (18 μg) were analyzed by western blotting with either rt-84 IgG (1.0 μg/mL) or anti-P4501A1 IgG (1:500), and visualized by ECL with GAR-HRP (1:10,000). (B) Composite of P4501A1 expression and XRE binding. The levels of P4501A1, rtARNT protein, and specifically shifted XRE were quantified by computer densitometry. The data are presented as the percentage of P4501A1 expression or XRE binding compared with rtARNT533 (100%). Each bar represents the mean ± SD of at least three independent experiments. (C) One hundred micrograms of type II cytosol was combined with 50 ng of the indicated rtARNT protein. Samples were then activated with TCDD (10 nM) for 2 hr at 30° and analyzed by EMSA. The western blot at the top of the EMSA represents an aliquot of the exact samples used for the EMSA stained with the rt-84 antibody, as detailed in panel A.

gion under the conditions used in this assay. However, when ^{35}S-labeled rtARNTa and FLAG-rtARNTa were combined, the ^{35}S-labeled rtARNTa was precipitated to a higher degree (based on the level of input rtARNTa) than that seen in the samples with the ^{35}S-labeled rtARNT533. Thus, these data suggest that the full-length rtARNTa proteins can associate with each other in this *in vitro* assay beyond the dimerization observed through the HLH region. These results are consistent with the Gal4 data (Fig. 7A), although it is important to note that it appears that the oligomerization contributed by the C-terminal domain is reduced in the full-length proteins. One explanation for these results is that the Gal4 studies assayed the association of chimeric ARNT that contained only 182–215 amino acids in length compared with the 637 amino acids for the full-length rtARNTa. Thus, the structural conformation of the larger full-length rtARNTa protein may reduce the level of oligomerization between the hydrophobic C-terminal domains and only contribute minimally to the negative function of rtARNTa *in vivo.*

In conclusion, nearly all families of transcription factors contain members that supply some form of dominant negative activity. The most well studied are the various bHLH Id isoforms that appear to function in aspects of muscle differentiation in concert with MyoD [31,32]. In contrast, within the bHLH/PAS family of transcription factors, the presence of proteins with negative activity on signaling is only now becoming evident. In this report, studies have

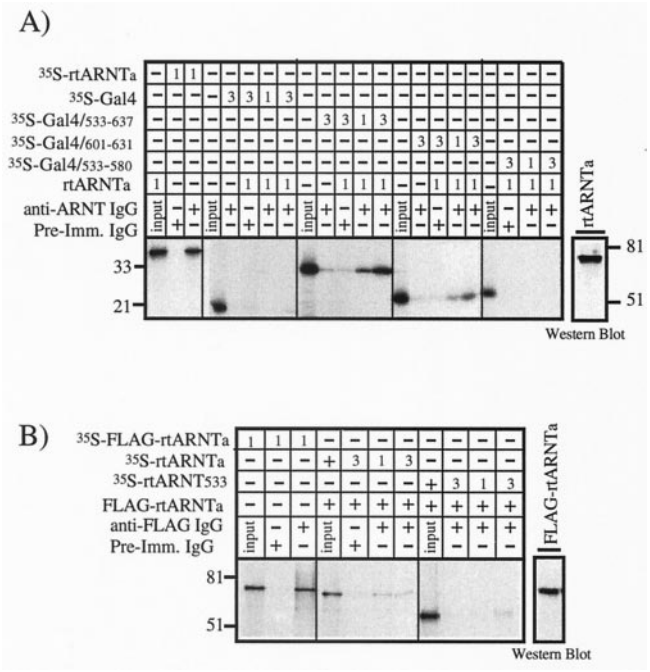

Fig. 7. Oligomerization of the hydrophobic-rich domain of rtARNTa. (A) Each indicated ^{35}S-labeled Gal4 construct was combined in an equal molar ratio of 1:1 and 3:1 with unlabeled rtARNTa, activated with 2 nM TCDD for 2 hr at 30°, and precipitated with 8 μg of rt-84 IgG or pre-immune IgG. To serve as a positive control, ^{35}S-labeled rtARNTa was also precipitated with rt-84 (8 μg) or pre-immune IgG. Only 50% of the input samples were resolved so as not to overwhelm the gel. Sizes are in kDa. The western blot on the right shows the expression of the unlabeled rtARNTa used in the assay stained with the rt-84 antibody (1 μg/mL). (B) Analysis of oligomerization between full-length rtARNTa proteins. ^{35}S-Labeled rtARNTa or ^{35}S-labeled rtARNT533 was combined in a ratio of 1:1 and 3:1 with unlabeled FLAG-rtARNTa, incubated with 2 nM TCDD for 2 hr at 30°, and precipitated with 10 μg of anti-FLAG IgG or pre-immune IgG. ^{35}S-Labeled FLAG-rtARNTa was precipitated to serve as a positive control. A western blot is shown on the right, indicating the expression of the unlabeled FLAG-rtARNTa used in the assay stained with anti-FLAG IgG (2 μg/mL).

focused on the negative function of the rtARNTa isoform in AHR-signal transduction. The results demonstrated that the hydrophobic region within the C-terminal domain is capable of inhibiting the DNA binding of rtARNT proteins and the heterologous Gal4 DNA binding domain. Importantly, the studies show that the ability of the hydrophobic domain to affect the function of rtARNT in AHR-mediated signal transduction is not due to a failure of ARNT to dimerize with the AHR and that the hydrophobic domain does not directly associate with the HLH motif. These findings are consistent with studies of other negatively acting transcription factors that have shown that the C-terminal end is responsible for altering gene expression by direct repression of transcriptional activation or by inhibiting DNA binding of transcription factors [33]. However, the interaction of rtARNTa with the AHR and the mechanism by which DNA binding is repressed have additional components. Indeed, immunoprecipitation assays revealed that Gal4 constructs containing the hydrophobic rich domain could associate with rtARNTa. This finding suggests that the hydrophobic domain can serve as a site for oligomerization *in vitro*. This finding provides a possible mechanism for the reduced binding of the Gal4 constructs containing the hydrophobic domains as these constructs may form multimers that alter the proper conformation required for DNA binding of Gal4.

So how might rtARNTa exert a negative function on AHR-mediated gene regulation? One possibility is through changes in intramolecular folding of AHR and or ARNT during dimerization. Hydrophobic amino acids are most likely to be on the inside of the protein; thus, the location of the hydrophobic domain in the C-terminus suggests that it is likely that the C-terminus folds inward. Such a structural conformation may mask the DNA binding domain or prevent its access to the DNA. Inhibition of DNA binding by intramolecular folding has been described in other proteins. For example, the masking of the N-terminal DNA binding activity by a C-terminal fragment has been reported in *Xenopus* B-Myb [34]. Another possible mechanism whereby rtARNTa may produce a negative function is through oligomerization. It remains possible that oligomerization between the C-terminal domains of rtARNTa proteins can occur following the dimerization of ARNT with AHR, and that such a complex might have reduced affinity for DNA. The validity of this idea is supported by results presented in this report and the finding that oligomerization through a novel C-terminal domain has been proposed as a mechanism for the reduced DNA binding activity of the transcription factor NF1-X [35].

However, regardless of the mechanism whereby rtARNTa mediates its effect, data in this report support rtARNTa as a negative regulator of AHR-mediated signaling and implicate the C-terminal hydrophobic amino acids as a novel repressor domain. Since the genomic structure of the mouse ARNT gene contains 8 exons within the C-terminal coding region [36], it is likely that alternative splice variants of ARNT may exist in mammals as well. Given the importance of ARNT in numerous signaling pathways [37,38], the identification of additional negative acting proteins, their patterns of expression, and the interaction of rtARNTa with other bHLH/PAS proteins remain key areas of future research.

Acknowledgments

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