CITATION OF PUBLISHED ARTICLE:

Merson, R. R., S. I. Karchner, D. G. Franks & M. E. Hahn. 2005. Development and characterization of polyclonal antibodies against the aryl hydrocarbon receptor protein family (AHR1, AHR2, and AHR repressor) of Atlantic killifish *Fundulus heteroclitus*. Comparative Biochemistry and Physiology, Part C Toxicology & Pharmacology 142:85-94.

Development and characterization of polyclonal antibodies against the aryl hydrocarbon receptor protein family (AHR1, AHR2, and AHR repressor) of Atlantic killifish Fundulus heteroclitus

Rebeka R. Merson*, Diana G. Franks, Sibel I. Karchner & Mark E. Hahn*

Biology Department

Woods Hole Oceanographic Institution

Woods Hole, Massachusetts USA

*Corresponding authors

Woods Hole Oceanographic Institution

MS #32

Woods Hole, Massachusetts 02543 USA

Phone: (508) 289-3212

Fax: (508) 457-2134

e-mail: rmerson@whoi.edu; mhahn@whoi.edu

Running title Antibodies specific for killifish AHR1, AHR2 & AHRR

Abstract

The aryl hydrocarbon receptor (AHR) and AHR repressor (AHRR) proteins regulate gene expression in response to some halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons. The Atlantic killifish is a valuable model of the AHR signaling pathway, but antibodies are not available to fully characterize AHR and AHRR proteins. Using bacterially expressed AHRs, we developed specific and sensitive polyclonal antisera against the killifish AHR1, AHR2, and AHRR. In immunoblots, these antibodies recognized full-length killifish AHR and AHRR proteins synthesized in rabbit reticulocyte lysate, proteins expressed in mammalian cells transfected with killifish AHR and AHRR constructs, and AHR proteins in cytosol preparations from killifish tissues. Killifish AHR1 and AHR2 proteins were detected in brain, gill, kidney, heart, liver, and spleen. Antisera specifically precipitated their respective target proteins in immunoprecipitation experiments with in vitro-expressed proteins. Killifish ARNT2 co-precipitated with AHR1 and AHR2. These sensitive, specific, and versatile antibodies will be valuable to researchers investigating AHR signaling and other physiological processes involving AHR and AHRR proteins.

Key words

Aryl hydrocarbon receptor (AHR); AHR repressor (AHRR); basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS); fish; halogenated aromatic hydrocarbons (HAH); polynuclear aromatic hydrocarbons (PAH); recombinant protein; teleost

1. Introduction

The aryl hydrocarbon receptor (AHR) is part of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) protein superfamily of environmental sensors, developmental regulators, and co-activators (Crews 1998; Gu et al 2000; Ledent and Vervoort 2001). Some of the most ubiquitous toxic compounds in the environment, including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated biphenyls (PCBs), and some polycyclic aromatic hydrocarbons (PAHs), elicit biochemical and toxic responses through the AHR signaling pathway. Following activation by a ligand such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the AHR dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Reyes et al 1992; Whitelaw et al 1993), and interacts with AHR response elements (AHREs, also known as XREs or DREs) to regulate expression of genes encoding biotransformation enzymes (Negishi and Nebert 1979; Nebert and Gonzalez 1987; Telakowski-Hopkins et al 1988). The AHR-ARNT complex also regulates numerous other genes involved in a variety of physiological processes (Puga et al 2000). A negative regulatory loop in this pathway involves the related AHR repressor (AHRR), which represses AHR mediated gene transcription (Mimura et al 1999; Karchner et al 2002).

Gene and genome duplication events have expanded the vertebrate AHR gene family to include multiple AHR and AHRR genes. The number of AHR genes varies among taxa. For example, one AHR gene (Burbach et al 1992) and one AHRR gene (Watanabe et al 2001) have been identified in mammals, whereas up to six AHR paralogs (Hansson et al 2004; Karchner and Hahn 2004) and two AHRR paralogs (Evans et al 2005) are present in bony fishes. The structural and functional diversity of AHR proteins may confer species- and strain-specific

differences in the sensitivity to toxic AHR ligands (Hahn et al 2005) and it is possible that numerous, possibly diverse, physiological roles are partitioned among multiple AHRs and AHRRs.

The Atlantic killifish Fundulus heteroclitus, distributed in shallow water habitats from Newfoundland to Florida (Able 2002), is a versatile toxicological model used in field and laboratory research to elucidate the impacts and molecular mechanisms of chemical toxicity. Widespread and abundant, Atlantic killifish inhabit highly polluted areas where they are exposed to high concentrations of PCBs and PAHs (Lake et al 1995; Bello 1999; Nacci et al 1999; 2001). This has provided the opportunity to use killifish as a model to study environmental carcinogenesis (Vogelbein et al 1990; Stine et al 2004), endocrine disruption (Dube and McClatchy 2001; Sharpe et al 2004; Boudreau et al 2005; Greytak et al 2005), PAH toxicity (Willett et al 2001; Wassenberg and DiGiulio 2004), and chemically-mediated changes in gene expression (Peterson and Bain 2004; Meyer et al 2005). Killifish also have been used to investigate evolutionary adaptations to environmental change (Schulte 2001; Cohen 2002, Kraemer and Shulte 2004; Oleksiak et al. 2005). Several geographically isolated populations of Atlantic killifish have independently developed heritable resistance to the toxic effects of contaminants in the environment (Prince and Cooper 1995; Van Veld and Westbrook 1995; Elskus et al 1999; Nacci et al 1999; Bello et al 2001). The mechanisms underlying this resistance are not yet well understood, but in most populations, if not all, the AHR signaling pathway is involved (Wirgin and Waldman 2004).

Several killifish genes in the AHR signaling pathway have been characterized, including CYP1A (Morrison et al 1998), ARNT2 (Powell et al 1999), AHR1 and AHR2 (Hahn et al 1997; Karchner et al 1999; Hahn et al 2004), and AHRR (Karchner et al 2002). Both killifish AHR1 and AHR2 bind TCDD and other AHR ligands in vitro and stimulate transactivation of reporter genes regulated by the CYP1A promoter in cultured mammalian cells. The killifish AHRR does not bind AHR ligands, but negatively regulates killifish AHR1 and AHR2 (Karchner et al 2002).

Despite the intensity of research to characterize the AHR signaling pathway in killifish, a major limitation to identifying the physiological roles of the AHR and molecular mechanisms of resistance in this species is the lack of sensitive, specific antibodies to each AHR protein. Here we describe the development and screening of rabbit polyclonal antisera that distinguish among AHR family members from the Atlantic killifish and we examine the utility of these antibodies in AHR research.

2. Materials and Methods

2.1. Chemicals

2,3,7,8-Tetrachloro[1,6-^{3}H]dibenzo-p-dioxin ([^{3}H]TCDD; 35 Ci/mmol, 95% radiochemical purity) was purchased from Chemsyn Science Laboratories (Lenexa, KS), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were from Ultra Scientific (Hope, RI), L-[^{35}S]methionine (>1000 Ci/mmol, in vivo cell labeling grade) was from Amersham Biosciences (Piscataway, NJ). [^{14}C]Ovalbumin was from NEN Research

Products DuPont (Wilmington, DE) and [^{14}C]catalase was synthesized and described by Bello (1999). All other chemicals were from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

2.2. Oligonucleotide primers

Oligonucleotide PCR primers containing restriction sites were custom-synthesized by Integrated DNA Technologies (Coralville, IA) or GibcoBRL Life Technologies/Invitrogen (Carlsbad, CA) (Table 1).

2.3. Expression constructs

Full-length Atlantic killifish (Fundulus heteroclitus) AHR1, AHR2, AHRR, and ARNT2 cDNA constructs, referred to respectively as pcDNAFhAHR1*1 and pSPFhAHR1*1, pcDNAFhAHR2, pcDNAFhAHRR, and pcDNAFhARNT2 are described elsewhere (Karchner et al 1999; Powell et al 1999; Karchner et al 2002; Hahn et al 2004). For expression of the amino- and carboxyl-terminal halves of killifish AHR1, AHR2, and AHRR, cDNAs were amplified by polymerase chain reaction (PCR) with Deep Vent polymerase (New England Biolabs, Beverly, MA) or Advantage Polymerase Mix (Clontech, Palo Alto, CA) from the templates pSPFhAHR1*1, pcDNAFhAHR2, and pcDNAFhAHRR using primer combinations 1-33F/1-382R and 1-596F/1-942R, 2-40F/2-397R and 2-559F/2-951+R, and R-11F/R-341R and R-343F/R-675R, respectively. Following sequence confirmation, they were inserted into pQE80/82 (Qiagen) vector in-frame with the N-terminal sequence for six histidine (his) residues and were designated

as pQEFh1N, pQEFh1C, pQEFh2N, pQEFh2C, pQEFhRN, pQEFhRC. The BL21-CodonPlus (RP) E. coli strain (Strategene, La Jolla, CA) was selected for protein expression since this strain contains additional tRNA genes for codons found in killifish AHRs and can be used with the T5 promoter-driven pQE vector.

2.4. Protein production

Transformed bacteria were grown in Luria Broth (LB) medium containing 100 μg/mL ampicillin and 50 μg/mL chloramphenicol at 37 °C in a shaker incubator rotating at 150 rpm until the OD_{600} was 0.6. Proteins expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for 3 h at 37 °C with shaking at 150-240 rpm. Preparative cultures (500-1000 mL) were centrifuged at 4000 x g at 4 °C for 20 min. Bacterial pellets were lysed in 5 mL denaturing buffer (8.0 M urea, 100 mM NaH_2PO_4, 10 mM Tris, 0.1 % Triton X-100, pH 8.0) per gram pellet and sonicated (VirTis VirSonic 475; Gardiner, NY) on ice. Lysates were centrifuged at 10,000 x g for 30 min at 4 °C, assessed visually by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue (CBB) staining. These expressed proteins are referred to as 1NAB 1CAB, 2NAB, 2CAB, RNAB, and RCAB.

2.5. Protein purification

The his-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrices (Qiagen, Valencia, CA) according to manufacturer's instructions. Initially, cleared lysates from small cultures (50-100 mL) were screened for the presence of his-

tagged proteins using Ni-NTA spin columns (Qiagen). Cleared lysates from large cultures (500-1000 mL) were adsorbed onto Ni-NTA agarose (Qiagen) for 30 min with rocking at room temperature and loaded onto a PolyPrep (Bio-Rad, Richmond, CA) column. The resin was washed with a series of denaturing buffers of progressively lower pH (pH 8.0, 6.3, 5.9). Purified proteins were eluted with denaturing buffer pH 4.5. The purified protein solutions were dialyzed overnight into Tris-phosphate buffer using Slide-A-Lyzer Dialysis Cassettes (10,000 Da, Pierce, Rockford, IL) or the buffer exchanged and protein concentrated in a filtering device (Centricon-30, Millipore, Billerica, MA). Proteins were diluted to 1 mg/mL with phosphate-buffered saline (PBS) in preparation for immunization.

2.6. Immunization

Antisera were generated by New England Peptide, Inc. (Gardner, MA). Immediately prior to immunization, sera were collected from research grade SPF New Zealand White rabbits. Two rabbits were immunized with each of the purified E. coli-expressed proteins in complete Freund's adjuvant on days 0, 19, and 40 and were subsequently given 5 boosters of the respective proteins in incomplete Freund's adjuvant. Boosters were given approximately once per month and twice per month blood was collected and coagulated to clear serum. Sera were frozen at –20 °C.

2.7. Affinity purification of antibodies

Protein from antisera was precipitated by ammonium sulfate as described by Harlow and Lane (1988) followed by purification on affinity columns using the AminoLink Plus Immobilization Kit (Pierce). Briefly, 10 mL raw antiserum was centrifuged at 3,000 x g for 30 min at 4 °C. Supernatant was diluted 2:1 with sterile PBS, pH 7.6, precipitated with a saturated ammonium sulfate solution, resuspended and dialyzed in PBS overnight in a Slide-A-Lyzer cassette (10,000 Da). A total of 7.2, 7.5, and 9.8 mg of purified E. coli-expressed 1CAB, 2CAB, and RCAB proteins, respectively, were diluted in sodium citrate coupling buffer (pH 10) and loaded onto columns containing 2 mL AminoLink coupling gel. Columns were processed according to manufacturer's protocol; antibodies were eluted from with ImmunoPure Elution Buffer (Pierce) in 1 mL fractions and neutralized with 50 μL 1M Tris, pH 10.0. Fractions reading 280 nm absorbance of 0.2-0.8 were pooled and stored as a 50% glycerol solution at –80 °C. Defrosted aliquots were preserved in 0.02% sodium azide in PBS.

2.8. Purification of in vitro-expressed proteins

To prepare proteins for screening raw antisera and purified antibodies, full-length killifish AHR1 (pcDNAFhAHR1*1), AHR2 (pcDNAFhAHR2), and AHRR (pcDNAFhAHRR) proteins were expressed in vitro with rabbit reticulocyte lysate using the TNT® Quick Coupled Transcription/Translation System (TNT) (Promega, Madison, WI) following manufacturer's instructions and purified by velocity sedimentation on sucrose density gradients. In vitro-expressed AHR1, AHR2, and unprogrammed lysate (TNT reactions with empty vector, UPL) were incubated overnight with TCDF (Karchner et al 2002). To identify the AHR-containing fractions, additional tubes were prepared with AHR proteins incubated with 2 nM of the

radioligand [^{3}H]TCDD (Tsui and Okey 1981; Karchner et al 1999).

Proteins were loaded on 10-30% sucrose gradients (5 mL in MEEDMG buffer plus protease inhibitors) prepared with the Gradient Master (BioComp, Fredericton, NB Canada). Internal standards, [^{14}C]ovalbumin and [^{14}C]catalase, were included in the UPL tube to estimate sedimentation coefficients (Martin and Ames 1961). Gradients were centrifuged in a vertical tube rotor at 327,398 x g for 2 h at 4 °C, and fractionated (Brandel Gaithersburg, MD) to approximately 120 μL/fraction (0.750 mL/min). Fractions were counted by liquid scintillation; the pattern of specific binding of [^{3}H]TCDD was similar to that reported by Karchner et al (1999). Fractions 10-19 were collected and pooled for each of the TCDF-containing AHR1, AHR2, and UPL gradients processed in parallel to the [^{3}H]TCDD gradients.

Because the AHRR protein does not bind AHR ligands (Karchner et al 2002), we used [^{35}S]methionine to track AHRR in sucrose gradients. AHRR protein (pcDNAFhAHRR) was synthesized in vitro in the presence of [^{35}S]methionine as specified by the manufacturer. To reduce background signal, unincorporated [^{35}S]methionine was removed with NICK® Spin Columns (Pharmacia, Sweden). Labeled AHRR was partially purified on 5-30 % sucrose gradients, fractionated as described for AHR1 and AHR2, and counted by liquid scintillation. A UPL control was synthesized in the presence of [^{35}S]methionine and processed exactly as the [^{35}S]methionine-labeled AHRR. As an internal control, a gradient containing [^{14}C]ovalbumin and [^{14}C]catalase was centrifuged and fractionated in parallel with the AHRR gradients. Fractions exhibiting [^{35}S] signals above that of the UPL (12-32) were collected from the gradients containing in vitro-expressed AHRR protein synthesized with unlabeled methionine.

The pooled fractions were concentrated using Centricon-30 centrifugal devices.

Incorporation of [^{35}S]methionine into TNT products was used to estimate expression efficiency for each AHR construct. AHR1 (pcDNA-FhAHR1*1), AHR2 (pcDNAFhAHR2) and AHRR (pcDNAFhAHRR) TNT products synthesized in the presence of [^{35}S]methionine were resolved by SDS-PAGE. [^{35}S]methionine signals were detected by fluorography and quantified by scintillation counting of the gel band. In separate experiments, we determined that the protein synthesis of the most methionine-rich AHR protein (FhAHR2) is limited when the total concentration of methionine in the TNT reaction is less than 10 μM. Thus, only reactions with methionine content 10 μM or greater were used to calculate protein expression levels. The average expression of FhAHR1, FhAHR2 and FhAHRR proteins were 4.1, 1.6, and 1.1 fmol μL^{-1} TNT lysate, respectively.

2.9. Cytosol protein extracts and expression of killifish AHR protein in mammalian cells

Atlantic killifish were collected from a relatively unpolluted location, Wood Neck Beach, Falmouth, MA USA. Fish were euthanized by cervical transection. Dissected tissues were homogenized in ice-cold buffer plus protease inhibitors and cytosols were prepared as described in Bello et al (2001).

Killifish AHR1, AHR2 and AHRR were expressed in African green monkey kidney fibroblast cells (COS-7) (ATCC #CRL-1651) grown in 48-well plates and transfected with 200 ng of pcDNAFhAHR1*1, pcDNAFhAHR2, or pcDNAFhAHRR as described earlier (Karchner et al

2002; Hahn et al 2004). Wells were washed 24 h after transfection with PBS and cells were disrupted by addition of 25 μL sample treatment buffer.

2.10. Immunoblot analysis

Proteins were resolved by SDS-PAGE and transferred onto 0.45 μm nitrocellulose (Protran, Schleicher and Schuell, Keene, NH). Membranes were blocked for one hour to overnight in Blotto/Tween (5% milk in Tris-buffered saline, 0.1% Tween-20). Membranes were incubated with rocking for one hour at room temperature or overnight at 4 °C with antibodies diluted in Blotto/Tween (see figure captions for primary antibody dilution). Following washing with Tris-buffered saline containing 0.1% Tween-20 (TBST), membranes were incubated for one h with horseradish peroxidase (HRP)-conjugated goat Anti-Rabbit IgM+IgG (H+L chain specific) (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:5000 in Blotto/Tween. Detection of HRP activity was by enhanced chemoluminescence (ECL) onto Hyperfilm ECL (Amersham).

2.11. Immunoprecipitation

Full-length killifish AHR1 (pcDNAFhAHR1*1), AHR2 (pcDNAFhAHR2), and AHRR (pcDNAFhAHRR) proteins were synthesized by TNT with [^{35}S]methionine (20.4 μM final concentration) and unlabeled methionine (20 μM final concentration). TNT reactions diluted in freshly prepared immunoprecipitation (IP) buffer (25 mM HEPES, 1.2 mM EDTA, 200 mM NaCl, pH 7.4 0.1% Nonidet P-40, 10% glycerol), were pre-cleared with normal rabbit serum

(Sigma R-9133) and protein A agarose (6% fast flow, 50% v/v slurry, Sigma P3476), and collected using Handee cup columns (Pierce). AHR1, AHR2, and AHRR were incubated with immune serum or preimmune control serum at 4 °C with end-over-end rocking. Complexes were precipitated with fresh protein A agarose for 1 hour, and washed three times with 500 μL ice-cold IP buffer. Precipitates were boiled in sample treatment buffer (STB; Laemmli buffer, 10% β-mercaptoethanol) and subjected to SDS-PAGE and fluorography as described earlier.

2.12. Coimmunoprecipitation

Full-length killifish ARNT2 (pcDNAFhARNT2) was synthesized by TNT with [^{35}S]methionine; AHR1 and AHR2 were synthesized with unlabeled methionine in the presence of TCDD (final concentration 10 nM) or dimethyl sulfoxide (DMSO, final concentration 2 %). AHR1 and AHR2 were incubated with ARNT at a molar ratio of 3.7:1 and 2.9:1, respectively, diluted to 200 μL in fresh ice-cold IP buffer, and kept overnight at 4 °C. Each protein-protein incubation was divided in half and incubated with immune or control sera for 1 hour at room temperature, followed by incubation with protein A agarose for 1 hour at 4 °C with rocking. Precipitates were washed, and subjected to SDS-PAGE and fluorography as described earlier.

3. RESULTS

3.1. Synthesis and purification of recombinant AHR1, AHR2, and AHRR proteins

To generate antibodies to killifish AHR1, AHR2, and AHRR, two constructs of each target protein, an N-terminal half and a C-terminal half (Fig. 1), were prepared for expression in E. coli. The N-terminal halves of the AHRs are comprised of the bHLH and PAS domains, the domains that are the most highly conserved among taxa. The intent of targeting the N-terminal halves of the AHR and AHRR proteins was to generate antibodies specific to each killifish protein, but with the possibility that they would also recognize the orthologous proteins from other fish species. The rationale for using the C-terminal half of the AHR proteins, which are not well conserved among AHR/AHRR orthologs or paralogs, was to increase the probability of producing antibodies specific for killifish AHR1, AHR2, and AHRR proteins. However, this reduces the likelihood that the antibodies would recognize AHR proteins from other species.

Histidine-tagged killifish AHR1, AHR2, and AHRR proteins were produced by E. coli cultures and purified on nickel-nitrilotriacetic acid columns. Despite several attempts, the N-terminal proteins did not express well in E. coli. Only the C-terminal constructs were expressed at quantities sufficient to purify 2 mg of each protein for immunizations and an additional 7 mg protein to generate affinity columns (Fig. 2).

3.2. Specificity and sensitivity of antisera

The bacterially expressed, C-terminal AHR1, AHR2, and AHRR proteins were used to raise polyclonal antisera (two rabbits per protein). To determine whether the antisera specifically recognize full-length target proteins, we performed western blots with in vitro-expressed full-length AHR proteins that had been purified by velocity sedimentation on sucrose density

gradients. Unprogrammed TNT lysate (UPL) included in the immunoblots controlled for non-specific recognition of proteins present in the rabbit reticulocyte lysate, and identical immunoblots were screened with preimmune sera. Each antiserum recognized its target protein with minimal cross-reactivity to non-specific proteins (Fig. 3).

To evaluate the sensitivity of the antisera, we tested their ability to detect in vitro-expressed, purified proteins over a range of protein concentrations (0.1 to 80 fmol). Each antiserum detected the target protein down to 10 fmol (Fig. 4).

3.3. Applications

3.3.1. AHR protein expression in mammalian cells

Assays involving transient transfection of expression constructs in mammalian cell culture are commonly used to evaluate protein function and to elucidate molecular mechanisms of gene regulation. To determine whether our antibodies recognize AHR and AHRR proteins expressed in COS-7 cells, a cell line frequently used in reporter gene-driven luciferase assays, cells were transfected with constructs encoding full-length killifish AHR1, AHR2, or AHRR, or an empty vector control. Affinity-purified antibodies were used in immunoblot analysis of cell lysates. Exceptionally strong signals were observed from each transfected COS-7 lysate (Fig. 5). There was no cross-reactivity of the antibodies with proteins in lysate of cells transfected with non-target proteins or the empty vector control, providing further support for the conclusion that these antibodies are specific to each AHR or AHRR protein.

3.3.2. AHR protein expression in killifish tissues

To test whether the antisera would detect AHR1 and AHR2 proteins expressed in vivo and to determine the relative expression of these proteins among killifish tissues, cytosol preparations of several tissues were analyzed by immunoblotting. No definitive AHR1 bands were detected in cytosols blotted with raw antisera (not shown). The affinity-purified AHR1 antibodies recognized a protein migrating at approximately the same molecular mass as the positive control in cytosols from all tissues (Fig. 6A). The protein band identified in brain cytosol migrated at a higher molecular mass (~115 kDa) than the bands from other tissues (105 kDa).

Analysis of cytosol preparations of brain, gill, heart, kidney, liver, and spleen tissues with raw AHR2 antisera revealed protein bands migrating at the same molecular mass (105 kDa) as the AHR2 positive control (not shown). These bands did not appear in immunoblots with preimmune serum. Affinity-purified AHR2 antisera also detected the AHR2 protein in all tissue cytosols and the positive control (Fig. 6B). However unlike blots incubated with raw antisera, immunoblots analyzed using affinity-purified antibodies had virtually no non-specific protein recognition. Gill cytosol appeared to contain the most AHR2 protein.

3.3.3. Immunoprecipitation and coimmunoprecipitation

Antibodies are commonly used to precipitate proteins and protein complexes to investigate protein-protein interactions. We evaluated the ability of these antibodies to bind native proteins

(not denatured) and precipitate them in vitro. All antisera specifically precipitated their respective target proteins in immunoprecipitation experiments with [^{35}S]methionine-labeled in vitro-expressed proteins (Fig. 7). To determine whether the antisera were able to precipitate the AHR-ARNT complex, coimmunoprecipitation experiments were conducted using unlabeled in vitro-expressed killifish AHR1 and AHR2, and [^{35}S]methionine-labeled killifish ARNT2. AHR1 antiserum, but not preimmune serum, precipitated [^{35}S]ARNT2 in the absence or presence of 10 nM TCDD. Similarly, AHR2 antiserum precipitated [^{35}S]ARNT2 (Fig. 8). Thus, each antiserum is able to immunoprecipitate heterodimeric complexes containing its target protein.

4. Discussion

Investigations of the aryl hydrocarbon receptor signaling pathway in Atlantic killifish and other bony fish models have been hampered by the lack of suitable protein detection reagents. We have developed specific and sensitive polyclonal antisera against Atlantic killifish AHR1, AHR2 and AHRR. Our experiments demonstrate that these antibodies recognize in vitro-synthesized full-length proteins, proteins expressed by a mammalian cell line transfected with killifish AHR or AHRR constructs, and AHR proteins from killifish tissue cytosols. The antisera recognize denatured and native AHR proteins. Here we show their versatility by applying them in a variety of experimental approaches including immunoblot, immunoprecipitation, and coimmunoprecipitation.

With immunoblot analysis we were able to show that both AHR1 and AHR2 proteins are present in all the killifish tissues examined. Karchner et al (1999) reported that killifish AHR1 mRNA

was most highly expressed in heart, brain and ovary with lower expression in liver, kidney, and gill. Our results suggest that the AHR1 protein is more widely expressed than mRNA expression levels would suggest. This incongruence may be due to tissue-specific differences in the stability of AHR1 mRNA or protein, or differences in the AHR status among the fish sampled in the two studies including factors influencing tissue mRNA expression and protein concentration, for example, time of day (Richardson et al 1998). With respect to AHR2 protein, our results showing that AHR2 protein is widely expressed are consistent with the mRNA data of Karchner et al (1999).

We observed differences in the molecular mass of AHR1 proteins in killifish tissue cytosols. The most striking difference occurred with AHR1 in brain cytosol, which appeared slightly larger than the AHR1 detected in the other tissues. Differences in molecular mass of AHR proteins among tissues may be due to splice variants and/or posttranslational processing. Splice variants of AHR1 exist in the killifish, and although one reported variant (Karchner et al 1999) was expressed only in ovary, this does not preclude the existence of other variants. Modulation of AHR activity occurs by posttranslational modifications such as phosphorylation (Park et al 2000) or ubiquitination (Ma and Baldwin 2002); modified AHR proteins migrate at higher molecular masses than unmodified AHR. Thus, our observation of higher molecular mass AHR1 in killifish brain cytosol could represent modified proteins.

The antibodies described in this report will be valuable to researchers investigating the AHR signaling pathway as well as other pathways that interact with AHR proteins. For example, the AHR1 antiserum described here was used to confirm the expression of two AHR alleles in COS-

7 cells transfected with killifish AHR1*1A or AHR1*3A constructs (Hahn et al 2004). In addition, our affinity-purified AHRR antibodies have been used to measure the expression of killifish AHRR in human breast cancer cells transfected with an AHRR construct (Yang et al, 2005).

With these antibody reagents AHR protein dynamics, including tissue- and developmental stage-specific protein expression, protein degradation, and protein-protein interactions, can now be addressed in the killifish model. Importantly, we have shown that these antibodies can be used with in vitro-synthesized proteins as well as proteins derived from whole animals.

Acknowledgements

We thank Drs. Richard Pollenz and Charles Rice for helpful technical discussions. Funding for this research was provided by National Institutes of Health, National Research Service Award (F32 ES05935) from the National Institute of Environmental Health Sciences (RRM), NIEHS Superfund Basic Research Program Grant P42 ES007381 at Boston University (MEH), and the Oliver S. and Jennie R. Donaldson Charitable Trust (MEH and RRM).

References

Able, K. W. 2002. Killifishes. Order Cyprinodontiformes. In: Collette, B. B., Klein-MacPhee, G. (Eds.), Bigelow and Schroeder's Fishes of the Gulf of Maine, third edition, Smithsonian Institution Press, Washington DC, pp. 292-297.

Bello, S.M., 1999. Characterization of resistance to halogenated aromatic hydrocarbons in a population of Fundulus heteroclitus from a marine Superfund site. Ph.D. Dissertation, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution, Woods Hole, MA, USA.

Bello, S.M., Franks, D.G., Stegeman, J.J., Hahn, M.E., 2001. Acquired resistance to Ah receptor agonists in a population of Atlantic killifish (Fundulus heteroclitus) inhabiting a marine superfund site: In vivo and in vitro studies on the inducibility of xenobiotic metabolizing enzymes. Toxicol. Sci. 60, 77-91.

Boudreau, M., Courtenay, S.C., Maclatchy, D.L., Berube, C.H., Hewitt, L.M., Van Der Kraak, G.J., 2005. Morphological abnormalities during early-life development of the estuarine mummichog, Fundulus heteroclitus, as an indicator of androgenic and anti-androgenic endocrine disruption. Aquat. Toxicol. 71, 357-369.

Burbach, K.M., Poland, A., Bradfield, C.A., 1992. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc. Natl. Acad. Sci. U S A 89, 8185-8189.

Cohen, S., 2002. Strong positive selection and habitat-specific amino acid substitution patterns in MHC from an estuarine fish under intense pollution stress. Mol. Biol. Evol. 19, 1870-1880.

Crews, S.T., 1998. Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev. 12, 607-620.

Dube, M.G., MacLatchy, D.L., 2001. Identification and treatment of a waste stream at a bleached-kraft pulp mill that depresses a sex steroid in the mummichog (Fundulus heteroclitus). Environ. Toxicol. Chem. 20, 985-995.

Elskus, A.A., Monosson, E., McElroy, A.E., Stegeman, J.J., Woltering, D.S., 1999. Altered CYP1A expression in Fundulus heteroclitus adults and larvae: a sign of pollutant resistance? Aquat. Toxicol. 45, 99–113.

Evans, B. R., Karchner, S. I., Franks, D. G., and Hahn, M. E. (2005). Duplicate aryl hydrocarbon receptor repressor genes (ahrr1 and ahrr2) in the zebrafish Danio rerio: Structure, function, evolution, and AHR-dependent regulation in vivo. Arch. Biochem. Biophys. 441, 151-167.

Gu, Y.Z., Hogenesch, J.B., Bradfield, C.A., 2000. The PAS superfamily: sensors of environmental and developmental signals. Annu. Rev. Pharmacol. Toxicol. 40, 519-561.

Greytak, S.R., Champlin, D., Callard, G.V., 2005. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (Fundulus heteroclitus): differential expression in fish from polluted and unpolluted environments. Aquat. Toxicol. 71, 371-389.

Hahn, M.E., Karchner, S.I., Franks, D.G., Merson, R.R., 2004. Aryl hydrocarbon receptor polymorphisms and dioxin resistance in Atlantic killifish (Fundulus heteroclitus). Pharmacogenetics 14, 131-143.

Hahn, M.E., Karchner, S.I., Shapiro, M.A., Perera, S.A., 1997. Molecular evolution of two vertebrate aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family. Proc. Natl. Acad. Sci. U S A 94, 13743-13748.

Hahn, M.E., Merson, R.R., Karchner, S.I., 2005. Xenobiotic receptors in fish: Structural and functional diversity. In: Mommsen, T.P., Moon, T.W. (Eds.), Environmental Toxicology, vol. 6. Elsevier, Amsterdam, Netherlands, pp. 191-228.

Hansson, M.C., Wittzell, H., Persson, K., von Schantz, T., 2004. Unprecedented genomic diversity of AhR1 and AhR2 genes in Atlantic salmon (Salmo salar L.). Aquat. Toxicol. 68, 219-232.

Harlow, E., Lane, D., 1988. Antibodies: A laboratory manual. CSHL Press, Cold Spring Harbor, NY.

Karchner, S.I., Franks, D.G., Powell, W.H., Hahn, M.E., 2002. Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AHR repressor, AHR1, and AHR2. J. Biol. Chem. 277, 6949-6959.

Karchner, S.I., Hahn, M.E., 2004. Pufferfish (Fugu rubripes) aryl hydrocarbon receptors: unusually high diversity in a compact genome. Mar. Environ. Res. 58, 139-140.

Karchner, S.I., Powell, W.H., Hahn, M.E., 1999. Identification and functional characterization of two highly divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost Fundulus heteroclitus. Evidence for a novel subfamily of ligand-binding basic helix loop helix-Per-ARNT-Sim (bHLH-PAS) factors. J. Biol. Chem. 274, 33814-33824.

Kraemer, L.D., Schulte, P.M., 2004. Prior PCB exposure suppresses hypoxia-induced up-regulation of glycolytic enzymes in Fundulus heteroclitus. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 139, 23-29.

Lake, J.L., McKinney, R., Lake, C.A., Osterman, F.A., Heltshe, J., 1995. Comparisons of patterns of polychlorinated biphenyl congeners in water, sediment, and indigenous organisms from New Bedford Harbor, Massachusetts. Arch. Environ. Contam. Toxicol. 29, 207-220.

Ledent, V., Vervoort, M., 2001. The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis. Genome Res. 11, 754-770.

Ma, Q., Baldwin, K.T., 2002. A cycloheximide-sensitive factor regulates TCDD-induced degradation of the aryl hydrocarbon receptor. Chemosphere 46, 1491-1500.

Martin, R.G., Ames, B.N., 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236, 1372-1379.

Meyer J. N., Volz, D. C., Freedman, J. H., Di Giulio, R. T., 2005. Differential display of hepatic mRNA from killifish (Fundulus heteroclitus) inhabiting a Superfund estuary. Aquat. Toxicol. 73, 327-341.

Mimura, J., Ema, M., Sogawa, K., Fujii-Kuriyama, Y., 1999. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. Genes Dev. 13, 20-25.

Morrison, H.G., Weil, E.J., Karchner, S.I., Sogin, M.L., Stegeman, J.J., 1998. Molecular cloning of CYP1A from the estuarine fish Fundulus heteroclitus and phylogenetic analysis of CYP1 genes: update with new sequences. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 121, 231-240.

Nacci, D., Coiro, L., Champlin, D., Jayaraman, S., McKinney, R., Gleason, T., Munns, W.R.J., Specker, J.L., Cooper, K., 1999. Adaptation of wild populations of the estuarine fish Fundulus heteroclitus to persistent environmental contaminants. Mar. Biol. 134, 9-17.

Nacci, D., Jayaraman, S., Specker, J., 2001. Stored retinoids in populations of the estuarine fish Fundulus heteroclitus indigenous to PCB-contaminated and reference sites. Arch. Environ. Contam. Toxicol. 40, 511-518.

Nebert, D.W., Gonzalez, F.J., 1987. P450 genes: structure, evolution, and regulation. Annu. Rev. Biochem. 56, 945-993.

Negishi, M., Nebert, D.W., 1979. Structural gene products of the Ah locus. Genetic and immunochemical evidence for two forms of mouse liver cytochrome P-450 induced by 3-methylcholanthrene. J. Biol. Chem. 254, 11015-11023.

Oleksiak, M. F., Roach, J. L., and Crawford, D. L. 2005. Natural variation in cardiac metabolism and gene expression in Fundulus heteroclitus. Nat. Genet. 37, 67-72.

Park, S., Henry, E.C., Gasiewicz, T.A., 2000. Regulation of DNA binding activity of the ligand-activated aryl hydrocarbon receptor by tyrosine phosphorylation. Arch. Biochem. Biophys. 381, 302-312.

Peterson, J.S., Bain, L.J., 2004. Differential gene expression in anthracene-exposed mummichogs (Fundulus heteroclitus). Aquat. Toxicol. 66, 345-355.

Powell, W.H., Karchner, S.I., Bright, R., Hahn, M.E., 1999. Functional diversity of vertebrate ARNT proteins: identification of ARNT2 as the predominant form of ARNT in the marine teleost, Fundulus heteroclitus. Arch. Biochem. Biophys. 361, 156-163.

Prince, R., Cooper, K.R., 1995. Comparisons of the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on chemically impacted and nonimpacted subpopulatioons of Fundulus heteroclitus: I. TCDD toxicity. Environ. Toxicol. Chem. 14, 579-587.

Puga, A., Maier, A., Medvedovic, M., 2000. The transcriptional signature of dioxin in human hepatoma HepG2 cells. Biochem. Pharmacol. 60, 1129-1142.

Reyes, H., Reisz-Porszasz, S., Hankinson, O., 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 256, 1193-1195.

Richardson, V.M., Santostefano, M.J., Birnbaum, L.S., 1998. Daily cycle of bHLH-PAS proteins, Ah receptor and Arnt, in multiple tissues of female Sprague-Dawley rats. Biochem. Biophys. Res. Commun. 252, 225-231.

Schulte, P.M., 2001. Environmental adaptations as windows on molecular evolution. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 128, 597-611.

Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C., Van Der Kraak, G.J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (Fundulus heteroclitus) bioassay. Aquat. Toxicol. 67, 203-215.

Stine, C.B., Smith, D.L., Vogelbein, W.K., Harshbarger, J.C., Gudla, P.R., Lipsky, M.M., Kane, A.S., 2004. Morphometry of hepatic neoplasms and altered foci in the mummichog, Fundulus heteroclitus. Toxicol. Pathol. 32, 375-383.

Telakowski-Hopkins, C.A., King, R.G., Pickett, C.B., 1988. Glutathione S-transferase Ya subunit gene: identification of regulatory elements required for basal level and inducible expression. Proc. Natl. Acad. Sci. U S A 85, 1000-1004.

Tsui, H.W., Okey, A.B., 1981. Rapid vertical tube rotor gradient assay for binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin to the Ah receptor. Can. J. Physiol. Pharmacol. 59, 927-931.

Van Veld, P.A., Westbrook, D.J., 1995. Evidence for depression of cytochrome P4501A in a population of chemically resistant mummichog (Fundulus heteroclitus). Environ. Sci. 3, 221-234.

Vogelbein, W.K., Fournie, J.W., Van Veld, P.A., Huggett, R.J., 1990. Hepatic neoplasms in the mummichog Fundulus heteroclitus from a creosote-contaminated site. Cancer Res 50, 5978-5986.

Wassenberg, D.M., Di Giulio, R.T., 2004. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in Fundulus heteroclitus. Environ. Health Perspect. 112, 1658-1664.

Watanabe, T., Imoto, I., Kosugi, Y., Fukuda, Y., Mimura, J., Fujii, Y., Isaka, K., Takayama, M., Sato, A., Inazawa, J., 2001. Human aryl hydrocarbon receptor repressor (AHRR) gene: Genomic structure and analysis of polymorphism in endometriosis. J. Hum. Genet. 46, 342-346.

Whitelaw, M., Pongratz, I., Wilhelmsson, A., Gustafsson, J.A., Poellinger, L., 1993. Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. Mol. Cell. Biol. 13, 2504-2514.

Willett, K.L., Wassenberg, D., Lienesch, L., Reichert, W., Di Giulio, R.T., 2001. In vivo and in vitro inhibition of CYP1A-dependent activity in Fundulus heteroclitus by the polynuclear aromatic hydrocarbon fluoranthene. Toxicol. Appl. Pharmacol. 177, 264-271.

Wirgin, I., Waldman, J.R., 2004. Resistance to contaminants in North American fish populations. Mutat. Res. 552, 73-100.

Yang, X., Liu, D., Murray, T. J., Mitchell, G. C., Hestermann, E. V., Karchner, S. I., Merson, R. R., Hahn, M. E., Sherr, D. H., 2005. The aryl hydrocarbon receptor constitutively represses c-myc transcription in human mammary tumor cells. Oncogene 24:7869-7881.

Table 1
Details of oligonucleotide primers. Restriction site portion of primer sequences are in lower case letters.

Primer name	Sequences (5' – 3')
1-33F	atggatccGACCGGCTGAATGGTGAACTG
1-382R	gcatgagctcTAGAAGTGGCCTCTGGGTGGCGATG
1-596F	atggatccTGTATTGAGGGAAAGGCATTCTC
1-942R	gcatcagctcTCAGAGGTAGAAGCTGCTGGTGGGTTG
2-379R	gcatgagctcGGTCAAGGCTTTCTGGAATGCAATG
2-40F	atggatccGATCGGCTAAACGGGGAGCTGG
2-559F	atggatccCAGAGGAGCGCCGGGTCCGATCTG
2-951+R	tacagtcgacTCCTCCTGCGGTTGAAG
R-11F	atggatccGGCAGAAAGCGGAGGAAGCCC
R-341R	gcatgagctcGGATGGGGACAAGAAAAGAG
R-343F	gaagatctGCTACGCAGCGAAGCCAACACC
R-675R	gcatgagctcGGACTGGACTTGTTGTGGC

Captions to figures

Fig. 1. Protein fragments used to raise antibodies against killifish AHR1, AHR2, and AHRR.

cDNA fragments encoding these protein fragments were inserted into pQE80 vector in frame with a hexahistidine tag and expressed in E. coli. Numbers above diagrams refer to the amino acid position. Antigen names are in parentheses and the predicted molecular mass of the fusion protein fragment is shown. Phylogenetically conserved and divergent regions of the AHR are labeled. The basic helix-loop-helix (bHLH) domain, and Per-ARNT-Sim (PAS) A and B repeats are identified.

Fig. 2. Purified bacterially-expressed AHR fusion proteins.

Hexahistidine-tagged Atlantic killifish AHR1 (1CAB), AHR2 (2CAB), and AHRR (RCAB) C-terminal proteins were expressed in E. coli and purified on Ni-NTA agarose columns. Proteins (21 μg) were separated on a 4-12% Bis-Tris SDS polyacrylamide gel, and stained with Coomassie brilliant blue.

Fig. 3. Antisera specifically recognize their target AHR proteins expressed in rabbit reticulocyte lysate.

Full-length Atlantic killifish AHR proteins were expressed in rabbit reticulocyte lysate and purified by velocity sedimentation on sucrose density gradients. Five microliters of AHR1 (lane 1), AHR2 (lane 2), and AHRR (lane R) and unprogrammed TNT lysate (lane U) were resolved on 4-12 % Bis-Tris SDS-polyacrylamide gel and immunoblotted as detailed in Methods. Immunoblots were probed with raw antisera and respective preimmune sera; the working

dilution of all antisera is 1:500. Shown are representative immunoblots of 2 experiments with similar results. Top and bottom panels are antisera from two different rabbits using (A) anti-FhAHR1, (B) anti-FhAHR2, and (C) anti-FhAHRR. Arrowheads indicate location of target protein.

Fig. 4. Sensitivity of AHR1, AHR2, and AHRR antisera.

10.0, 1.0, and 0.1 μL of purified in vitro-expressed FhAHR1 (8.2 fmol/μL), FhAHR2 (2.4 fmol/μL), and FhAHRR (1.2 fmol/μL) was separated on 4-12 % Bis-Tris polyacrylamide gel. Positive controls are lysate from AHR1 (1CAB), AHR2 (2CAB) or AHRR (RCAB) expressing bacterial cultures (lanes 1, 2, and R, respectively). The negative control is 10 μL of sucrose density gradient purified UPL (lane U). Shown are representative immunoblots from 3 separate experiments with similar results: (A) AHR1 antisera 1:500, (B) AHR2 antisera 1:500, and (C) AHRR antisera 1:500. Arrowheads indicate in vitro-expressed target protein, asterisk (*) indicates positive control (lysates from protein fragments expressed in E. coli cultures).

Fig. 5. Antisera specifically recognize their target protein expressed in mammalian cell culture.

Whole cell lysates prepared from COS-7 cells 24 h after transfection with 200 ng of FhAHR1 (lane 1), FhAHR2 (lane 2), FhAHRR (lane R), or pcDNA empty vector control (lane EV) were resolved on a 7.5 % SDS-polyacrylamide gel. Positive controls are purified in vitro-expressed (TNT) proteins. Shown are representative immunoblots from 2 experiments with similar results using affinity-purified antisera (A) FhAHR1 (antibody dilution 1:50), (B) FhAHR2 (antibody dilution 1:500), and (C) FhAHRR (antibody dilution 1:500).

Fig. 6. Tissue expression profiles of AHR1 and AHR2 in Atlantic killifish.

Immunoblots of tissue cytosols probed with (A) affinity-purified FhAHR1 antiserum (1:50), and (B) affinity-purified FhAHR2 antiserum (1:1000). Immunoblots are representative of 4 experiments with similar results. Lanes were loaded with cytosolic protein from brain (32 μg), gill (50 μg), heart (25 μg), kidney (50 μg), liver (50 μg), and spleen (50 μg). Positive control lanes contained approximately 41 and 5 fmol purified, in vitro-expressed FhAHR1 and FhAHR2, respectively. Proteins were resolved on 7.5% SDS polyacrylamide gel and blotted as specified in Methods.

Fig. 7. Antisera specifically precipitate Atlantic killifish AHR proteins.

In vitro-expressed [^{35}S]methionine-labeled FhAHR1, FhAHR2, and FhAHRR proteins were immunoprecipitated with raw antisera from two rabbits and preimmune controls (PI) (rabbit number in parentheses). Immunoprecipitated proteins were resolved on 8% SDS polyacrylamide gel and [^{35}S] detected by fluorography. This figure is representative of 4 immunoprecipitation experiments with similar results. (A) FhAHR1 precipitated with anti-AHR1 and anti-AHR2, (B) FhAHR2 precipitated with anti-AHR1 and anti-AHR2, and (C) FhAHRR protein precipitated with anti-FhAHRR.

Fig. 8. AHR antisera precipitate AHR-ARNT complexes in vitro.

In vitro-expressed, [^{35}S]methionine-labeled FhARNT2 was incubated with in vitro-expressed FhAHR1 or FhAHR2, precipitated with pre-immune serum (PI) or raw antisera specific (I) for FhAHR1 or FhAHR2, in the presence or absence of 10 nM TCDD. Proteins were separated on

8% SDS polyacrylamide gel and [^{35}S] detected by fluorography. The low ARNT signal in the anti-AHR2 precipitate is probably due to the relatively low level of pcDNAFhAHR2 expression in the rabbit reticulocyte lysate.

Figure 1

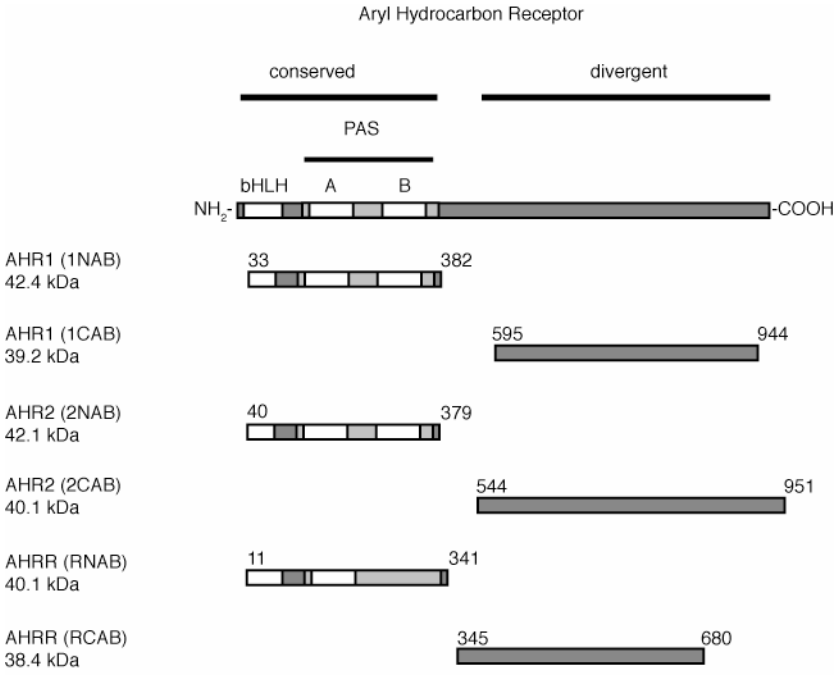

Aryl Hydrocarbon Receptor
conserved
divergent
PAS
bHLH
A
B
NH2-
-COOH
AHR1 (1NAB)
42.4 kDa
33
382
AHR1 (1CAB)
39.2 kDa
595
944
AHR2 (2NAB)
42.1 kDa
40
379
AHR2 (2CAB)
40.1 kDa
544
951
AHRR (RNAB)
40.1 kDa
11
341
AHRR (RCAB)
38.4 kDa
345
680

Figure 2

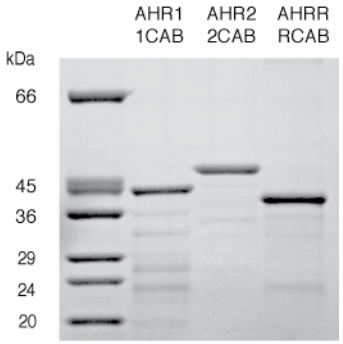


Figure 3

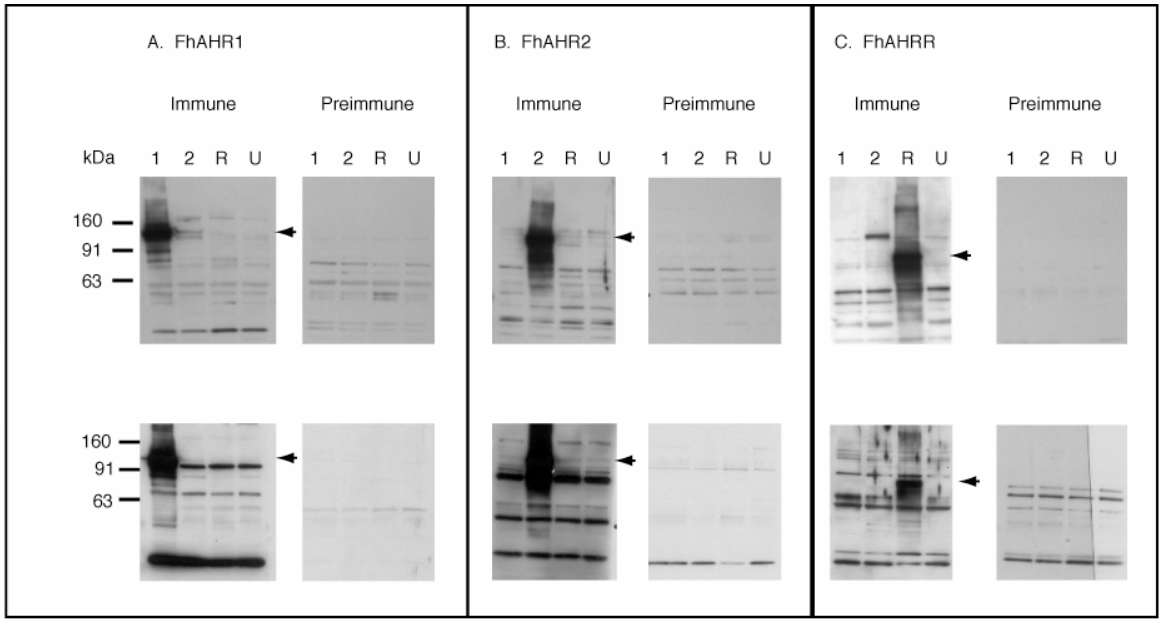

A. FhAHR1
B. FhAHR2
C. FhAHRR
Immune
Preimmune
kDa
1 2 R U
160
91
63

Figure 4

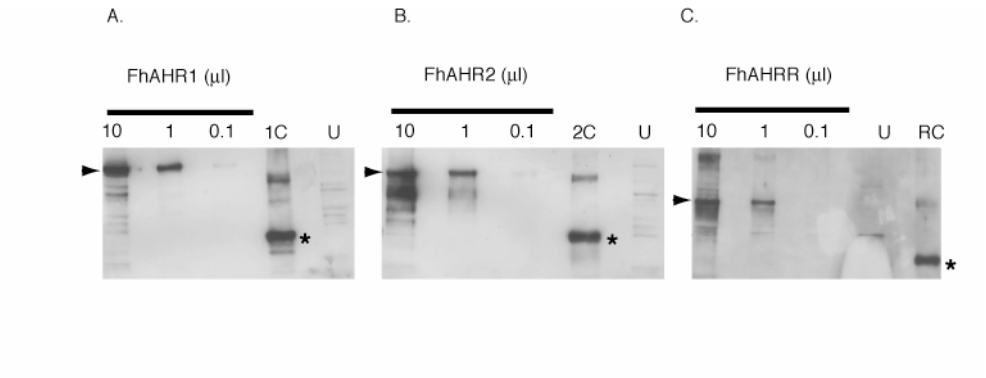

A.
FhAHR1 (µl)
10
1
0.1
1C
U
B.
FhAHR2 (µl)
10
1
0.1
2C
U
C.
FhAHRR (µl)
10
1
0.1
U
RC

Figure 5

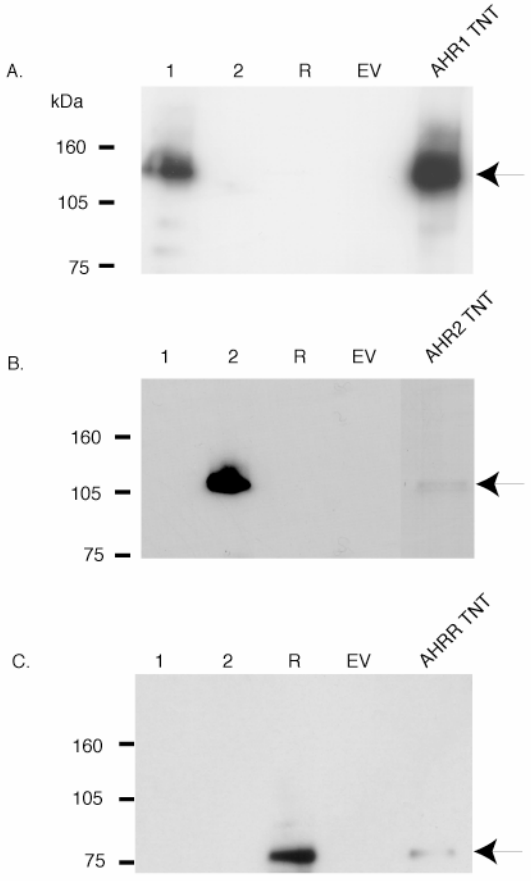

A.
1
2
R
EV
AHR1 TNT
kDa
160
105
75
B.
1
2
R
EV
AHR2 TNT
160
105
75
C.
1
2
R
EV
AHRR TNT
160
105
75

Figure 6

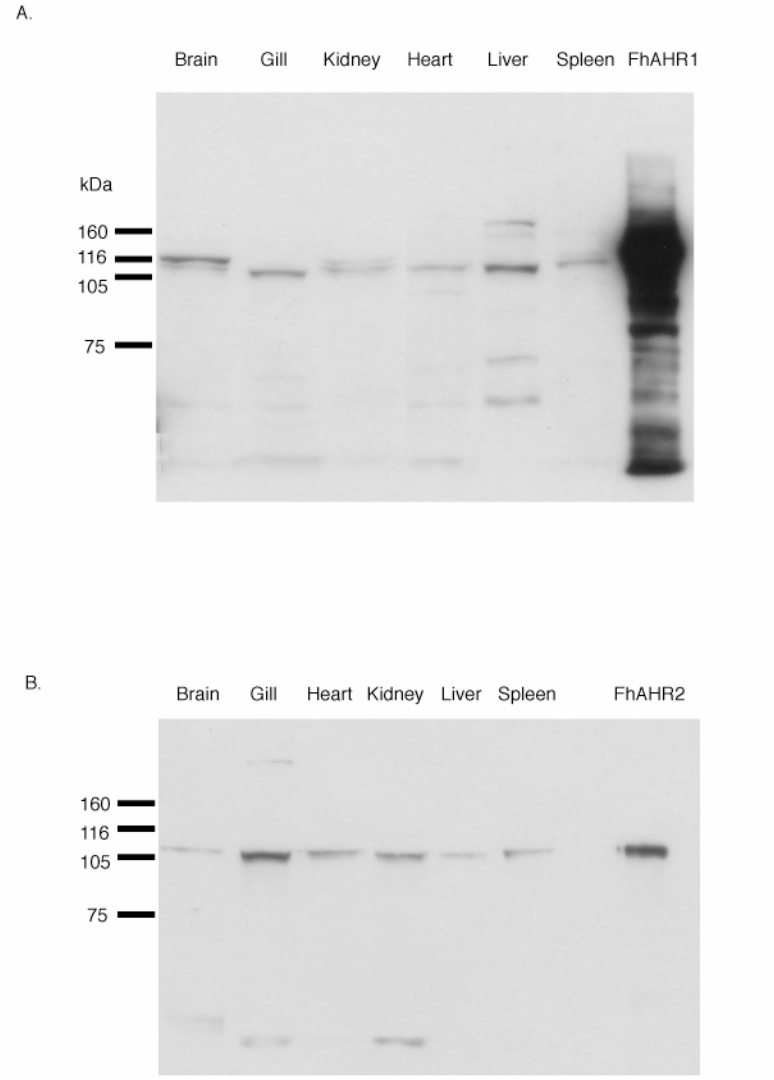

A.
Brain
Gill
Kidney
Heart
Liver
Spleen
FhAHR1
kDa
160
116
105
75
B.
Brain
Gill
Heart
Kidney
Liver
Spleen
FhAHR2
160
116
105
75

Figure 7

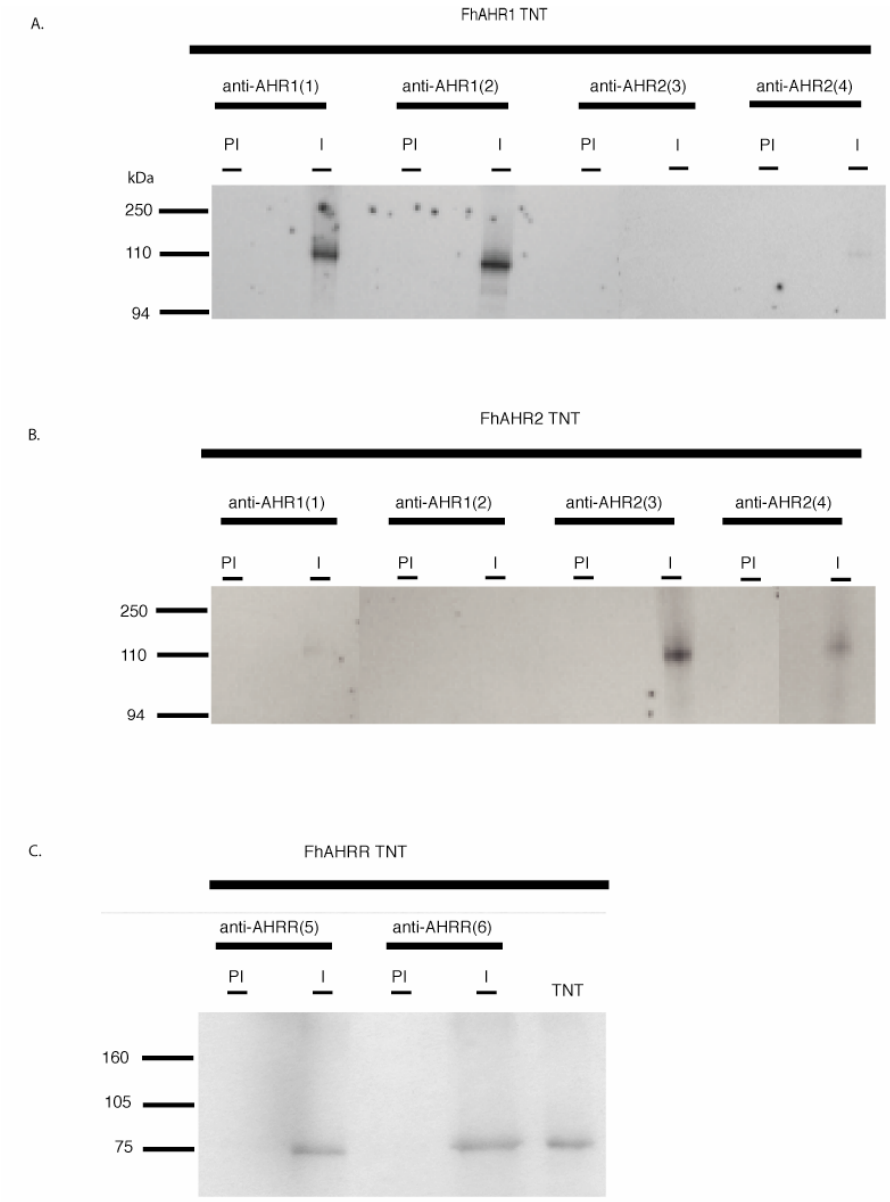
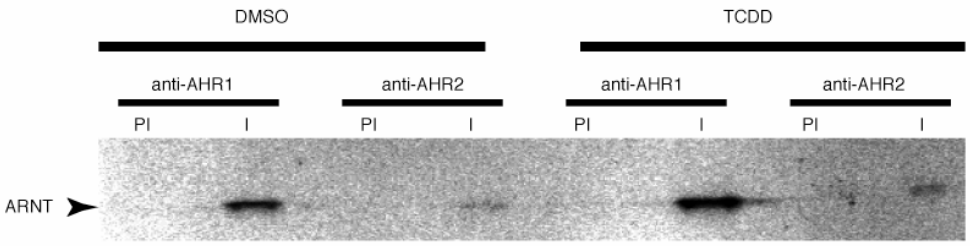

A.
FhAHR1 TNT
anti-AHR1(1)
anti-AHR1(2)
anti-AHR2(3)
anti-AHR2(4)
PI
I
kDa
250
110
94
B.
FhAHR2 TNT
C.
FhAHRR TNT
anti-AHRR(5)
anti-AHRR(6)
TNT
160
105
75

Figure 8


DMSO
TCDD
anti-AHR1
anti-AHR2
anti-AHR1
anti-AHR2
PI
I
PI
I
PI
I
PI
I
ARNT