Sequence Characterization and Dioxin-Responsiveness of an Aryl Hydrocarbon Receptor (AHR) from the Salamander, *Ambystoma mexicanum*

Jenny Shoots with mentor Wade Powell - Summer Science 2013

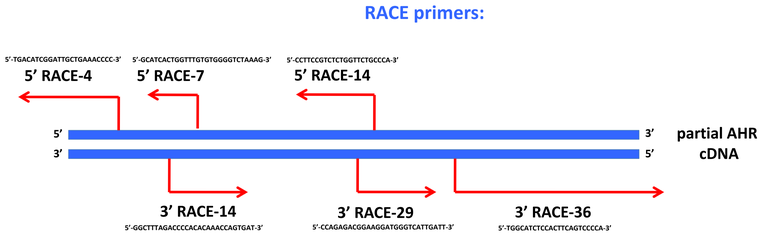
Abstract

The aryl hydrocarbon receptor (AHR) forms part of a transcription complex upon activation by xenobiotic ligands such as TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), a toxic environmental contaminant. Frogs exhibit low sensitivity to TCDD compared to other vertebrates, which is attributed to the weak affinity of frog AHRs to TCDD. This lowered affinity has been traced to specific amino acids within the ligand-binding domain of the frog AHR (Odio *et al,* 2013). In this study, we seek to determine whether low TCDD affinity is unique to frog AHRs or a property shared by other amphibian groups. We cloned an AHR cDNA sequence from the Mexican axolotl salamander (*Ambystoma mexicanum*) via RT-PCR with degenerate primers and RACE-PCR. Phylogenetic analysis indicates that the salamander AHR is of the AHR1 lineage, as are the AHRs of frogs. The *A. mexicanum* AHR possesses the same amino acids in the ligand-binding domain that confer lowered TCDD affinity to frog AHRs. Based on this sequence characterization, we predict that salamanders, like frogs, are relatively insensitive to TCDD. A reporter gene assay determining the ability of the *A. mexicanum* AHR to regulate the expression of target genes has been optimized, and preliminary results suggest that the salamander AHR does not induce TCDD-dependent gene expression as readily as a mammalian AHR, which binds TCDD with high affinity.

Methods

- **Cloning of a partial AHR cDNA:** A 600 bp portion of the *A. mexicanum* AHR was amplified via RT-PCR using degenerate primers (sense 5'-CGGGATCCGAYTAYCTIGGITTYCARCA-3'; antisense 5'-GCTCTAGACATICCRCTYTCICCIGTYTT-3') designed to target conserved regions of the vertebrate AHR amino acid sequence. This amplicon (Figure 1a) was cloned into the pGEM-T Easy vector and sequenced.

- **Obtaining the full length AHR sequence:** Using the partial cDNA sequence, AHR-specific primers were designed to target the 5' and 3' ends of the cDNA for use in RACE (Rapid Amplification of cDNA Ends) PCR. The 5' and 3' amplicons (Figure 1b) were cloned into the pGEM-T Easy vector and sequenced. The clones were aligned together with the partial cDNA, providing the entire AHR sequence (Figure 2).



-**Phylogenetic analysis:** The predicted amino acid sequence of *A. mexicanum* AHR was aligned with the protein sequence of other vertebrate species to construct a phylogeny (Figure 3).

- **Constructing an AHR expression vector**: The open reading frame of the *A. mexicanum* AHR was synthesized by Epoch Life Sciences and subcloned into the pCMVTNT expression vector.

- **Transactivation assay:** The responsiveness of the *A. mexicanum* AHR to TCDD was characterized with a reporter gene assay measuring its ability to induce the expression of target genes. The AHR expression construct was co-transfected with Lipofectamine 2000 into COS-7 cells along with an ARNT expression construct, a firefly luciferase reporter plasmid, and a *Renilla* luciferase transfection control plasmid. For a comparison with other AHRs, cells were also transfected with a frog AHR (*Xenopus laevis* AHR1β) or a chimeric AHR (*X. laevis* AHR1β with ligand binding domain of mouse AHR). After transfection, cells were exposed to graded concentrations of TCDD. Transactivation by the AHR was determined by lysing the cells and measuring luminescence using the Dual Luciferase Kit (Promega) and a TD- 20/20 luminometer (Figure 5).

Results

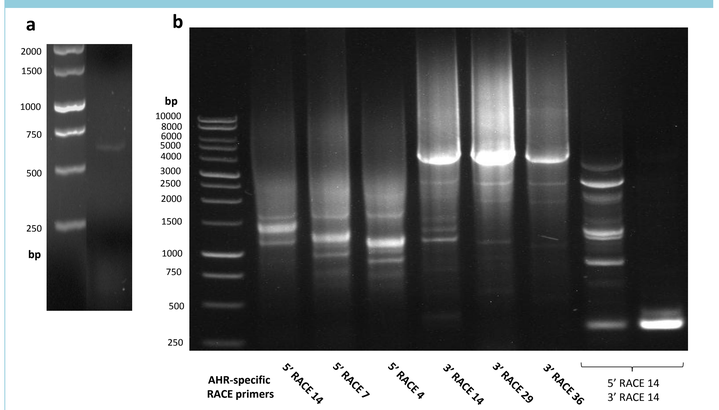


Figure 1. RACE primers amplified 5' and 3' regions of *A. mexicanum* AHR transcript. a) Partial AHR cDNA amplified by RT-PCR with degenerate primers. **b)** cDNA with an adaptor sequence at either the 5' or 3' end was generated via RT-PCR using the SMARTer RACE cDNA Amplification Kit (Clontech). PCR to amplify AHR cDNA was performed using an AHR-specific primer within the known partial cDNA sequence and a primer targeting a RACE adaptor sequence. The last two lanes contain positive control reactions using two RACE primers.

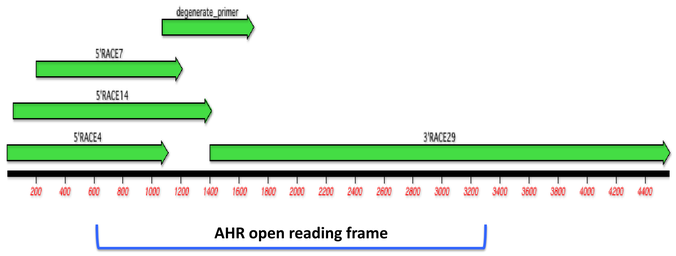


Figure 2. RACE-PCR amplified entire AHR transcript. The clones isolated by degenerate primer RT-PCR and RACE-PCR were aligned in MacVector. Comparison to other vertebrate AHR nucleotide sequences indicated that the entire *A. mexicanum* AHR mRNA was sequenced. The bracket denotes the translated portion of the AHR transcript.

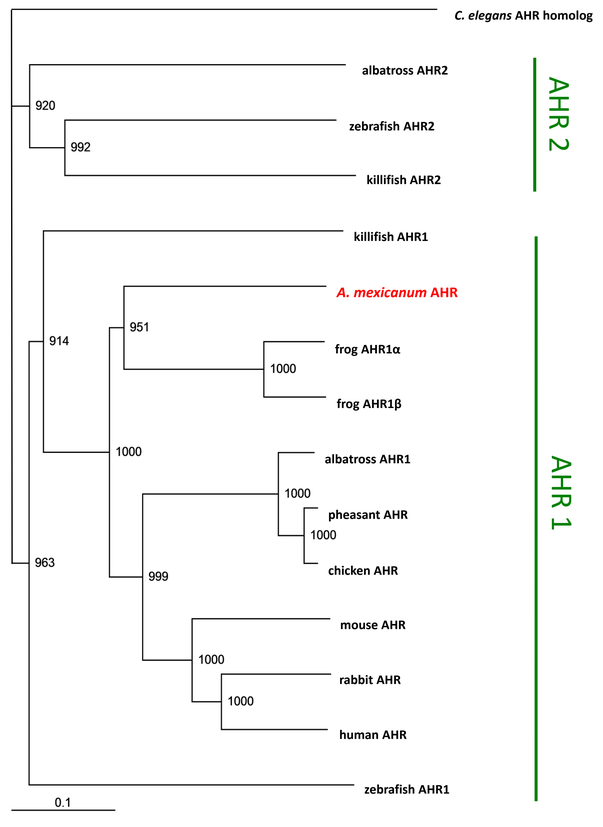


Figure 3. Salamander AHR is a member of the AHR1 lineage. The predicted AHR amino acid sequence was aligned with other vertebrate AHRs in ClustalX2. A tree was inferred by the Neighbor-Joining method, and rooted with the AHR homolog of *C. elegans* as the outgroup. Only one AHR paralog was identified in *A. mexicanum.*

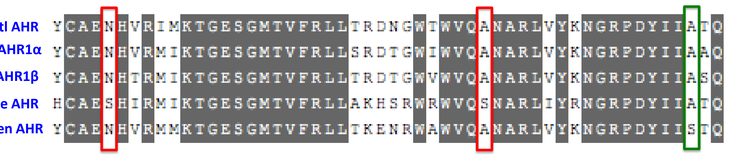


Figure 4. Salamander AHR contains the same amino acids that confer low dioxin affinity to frog AHRs. Protein sequences from amphibian and mouse AHR ligand binding domains. Shaded boxes indicate sequence identity. The red boxes highlight the two residues that protrude into the ligand binding domain, as identified by homology modeling, and are different in the mouse and frog AHRs. They underlie the different binding affinities of frog and mouse AHRs to TCDD. The residues in the green box are important for determining the binding affinity of mammalian and bird AHRs (Odio *et al,* 2013).

Prediction: Salamanders are relatively insensitive to TCDD toxicity.

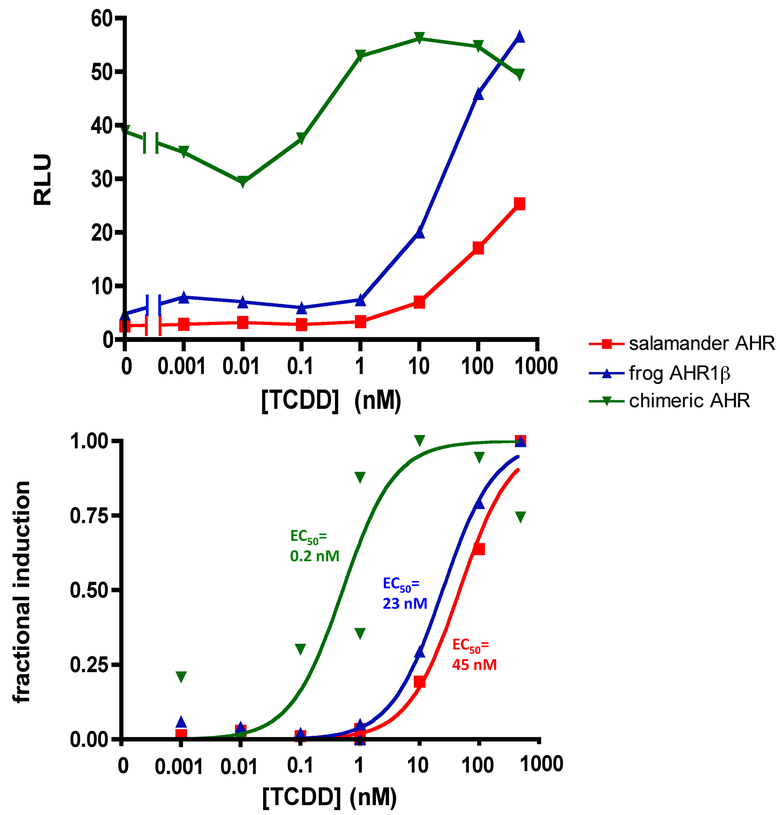


Figure 5. Salamander AHR has low responsiveness to TCDD. COS-7 cells were co-transfected with an AHR expression construct and a reporter plasmid containing luciferase downstream of mouse CYP1A enhancer and promoter regions. CYP1A enzymes are target genes of the activated AHR. Cells were treated with DMSO or the indicated doses of TCDD 5 hr after transfection. Transactivation by the AHR after 18 hr is given by relative luciferase units (RLU) which represents the ratio of firefly luciferase luminescence to that of *Renilla*, a constitutively expressed control. RLUs were then normalized to the maximal value of each AHR, which was assigned a fractional induction level of 1. The fractional induction curves are non-linear regressions constrained within 0 to 1, used to calculate EC_{50}. The 45 nM EC_{50} of *A. mexicanum* AHR (R^2= 0.98) indicated that it was the least responsive to TCDD, compared to the chimera (0.2 nM; R^2=0.88) and frog AHR (23 nM; R^2=0.99).

Conclusion

The salamander AHR exhibits reduced responsiveness to TCDD than a mouse-like AHR in its ability to induce target genes. Our characterizations predict that salamanders, like frogs, are relatively insensitive to TCDD toxicity. Future studies include determining the binding affinity of the *A. mexicanum* AHR to TCDD, and measuring its responsiveness to FICZ, an endogenous AHR ligand.

References:

Lavine JA, Rowatt AJ, Klimova T, Whitington AJ, Dengler E, Beck C, and WH Powell. 2005. Aryl hydrocarbon receptors in the frog *Xenopus laevis:* two AhR1 paralogs exhibit low affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicol Sci 88: 60-72.

Odio C, Holzman SA, Denison MS, Fraccalvieri D, Bonati L, Franks DG, Hahn ME, and WH Powell. 2013. Specific ligand binding domain residues confer low dioxin responsiveness to AHR1β of *Xenopus laevis.* Biochem doi:10.1021/bi301722k.

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