# Specific residues of the aryl hydrocarbon receptor from the frog *Xenopus laevis* confer low responsiveness to TCDD and FICZ

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## Abstract

The aryl hydrocarbon receptor (AHR) mediates the toxic effects of environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by binding these ligands and translocating them to the nucleus of cells. Although this pathway is conserved among species, the *Xenopus laevis* (African clawed frog) is remarkably insensitive to TCDD toxicity. This variation in TCDD toxicity results from differences in specific amino acids within the ligand binding domain (LBD) of the AHR, which in turn affect its affinity for ligands. We sought to identify the residues within *X. laevis* AHR1b associated with low TCDD responsiveness using transactivation assays. Specifically, although the frog AHR binds TCDD with >20 fold-lower affinity than the mouse AHR$^{b-1}$, only two amino acid side chains, which point directly into the LBD, differed between the two species. When A354, was changed to serine, the homologous mouse residue, the $EC_{50}$ for TCDD decreased more than 15-fold. When N325, was changed to serine, $EC_{50}$ declined 3-fold. The combined mutations resulted in a 22-fold decline in $EC_{50}$, from 18.75 nM to 0.83 nM, a large but incomplete recovery of TCDD responsiveness. These findings support the hypothesis that the low responsiveness of AHR1b in transactivation assays is substantially attributable to low affinity binding of TCDD.

We also examined one candidate endogenous ligand of the AHR and 6-formylindolo[3,2-*b*] carbazole (FICZ) in order to determine whether the frog to mouse residue changes affected the responsiveness of the AHR to FICZ. We found that when the frog AHR is made to resemble mouse AHR$^{b-1}$, several of the residues that affect TCDD binding, especially A354S, also increase FICZ responsiveness. These findings suggest that TCDD and FICZ have similar binding mechanisms within the AHR.

## Background

- 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is toxic environmental contaminant. Its effects may include cancer, developmental toxicity, hepatic necrosis, anemia and even death (Huff *et al.* 1980)
- The aryl hydrocarbon receptor (AHR) mediates the molecular response that leads to TCDD toxicity in vertebrates.
- The African clawed frog (*Xenopus laevis*) is extremely insensitive to TCDD. *X. laevis* AHR binds TCDD with 20 fold lower affinity than the mouse AHR$^{b-1}$ (Lavine *et al.* 2005).
- 6-formylindolo[3,2-*b*]carbazole (FICZ) is a tryptophan photoproduct that forms intracellularly and appears to play a role in the UV response (Fritsche *et al.* 2007).
- The AHR also binds FICZ, and the TCDD-insensitive frog AHR conserves its FICZ responsiveness, which supports FICZ candidacy as an AHR endogenous ligand (Laub *et al.* 2010).

## AHR Pathway

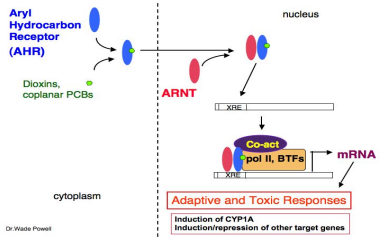



**Figure 1. The AHR Pathway.** The AHR binds to ligands in the cytoplasm and translocates in the nucleus where it forms a heterodimer with ARNT. The AHR/ ARNT heterodimer then acts as a transcription factor and induces the expression of CYP1A and other target genes (XMEs; Prasch *et al.* 2003).

## Objectives

1) To identify which amino acids confer low TCDD responsiveness to the *Xenopus laevis* AHR by replacing predicted frog amino acids with the corresponding mouse amino acids and measuring the resulting AHR responsiveness.
2) To determine whether these amino acids have similar effects on FICZ binding.

## Homology Model of the AHR

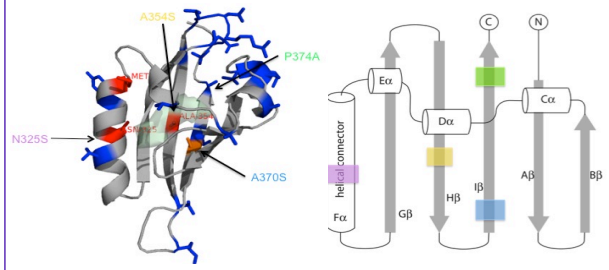



**Figure 2. Homology modeling of the Frog AHR1β ligand binding domain.** Residues that are different from the mouse AHR are shown in blue (outside the binding cavity) and in red (inside the binding cavity). A370S is depicted in orange (it is a chicken mutation rather than a mouse mutation). A homology model of AHR1β LBD (residues 273-379) was generated with MODELLER v. 8.1 (http://www.salilab.org/) using NMR structures of HIF-2α (PDB_ID: 1P97) and ARNT (PDB_ID: 1X0O) as a template. Sequence identities with the templates are medium-low (about 26% with HIF-2α and about 21% with ARNT). Therefore, it is expected that the backbone atomic coordinates are quite reliable, whereas there is uncertainty on the side-chain conformations. An internal binding cavity of 491 cubic angstroms (green shade) was predicted using the CASTp program (http://sts.bioengr.uic.edu/castp/).

## Luciferase Reporter Gene Assay

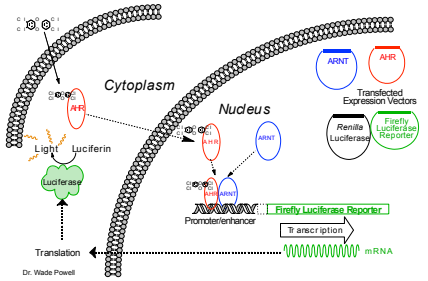



**Figure 3. The luciferase reporter gene assay.** AHR, ARNT, the firefly luciferase reporter, and the control renilla luciferase were transfected into COS-7 cells. When activated by a ligand, the AHR translocates to the nucleus where it induces the expression of luciferase. Increased luciferase intensity indicates increased AHR responsiveness to the ligand.

## Cell Transfection

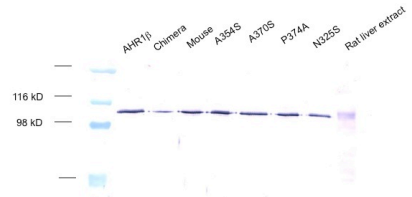
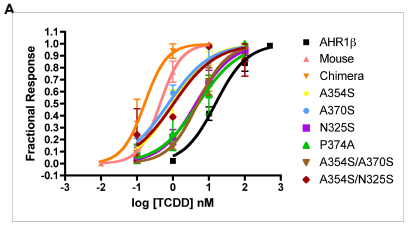
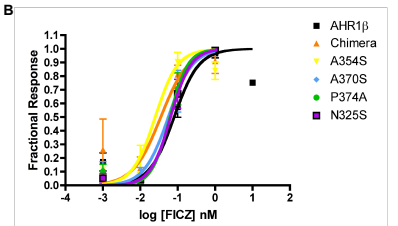



**Figure 4. Expression of AHR constructs in COS-7 cells.** COS-7 cells were plated in 48 well plates and incubated at 37 ºC. Twenty-four hours after plating, cells were transfected in triplicate wells with the labeled AHR construct and incubated for eighteen hours. Cells were lysed using passive lysis buffer (Promega) and 15 µL of lysate (5 from each triplicate well) was used for the western blot of each AHR construct. Protein was visualized with an anti-AHR rabbit polyclonal antibody (1:500, Enzo) and an anti-rabbit IgG secondary antibody derived from goat (1:1000, Sigma). Rat liver extract was used as a positive control of AHR expression. The AHR is 110 kD in size.

## Mutant AHR Responsiveness to TCDD and FICZ




| AHR | EC50 (nM) | Fold Difference |
|---|---|---|
| AHR1β | 18.65 | - |
| Chimera | 0.2003 | ~ 90 |
| Mouse | 0.5037 | ~ 37 |
| A354S | 1.241 | ~ 15 |
| N325S | 5.697 | ~ 3 |
| A354S/ N325S | 0.8282 | ~ 22 |




| AHR | EC50 (nM) | Fold Difference |
|---|---|---|
| AHR1β | 0.2062 | - |
| Chimera | 0.0367 | ~ 7 |
| A354S | 0.02164 | ~ 10 |
| N325S | 0.08245 | ~ 2 |
| P374A | 0.07152 | ~ 3 |

**Figure 5. Responsiveness of AHR constructs to TCDD (A) and FICZ (B).** Cos-7 cells were transfected with frog AHR, mouse ARNT, pGudluc 6.1 (reporter construct), pTK-RL (transfection control construct) ) (as described in Lavine *et al.* 2005). Cells were then dosed with graded concentrations of TCDD or FICZ in DMSO, incubated for 18 hours, and luciferase activity was measured using the Dual Luciferase Assay Kit (Promega) in a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). Overall responsiveness was measured in Relative Luciferase Units (RLU), a ratio of firefly luciferase activity to control *Renilla* luciferase activity. Fractional response of the compiled RLU data from two or more experiments was normalized to the maximum response for each AHR. EC50s were calculated by taking the average EC50 from two or more experiments.

## Conclusions

- These data indicate that the A354S mutant substantially restores the responsiveness of the frog AHR to TCDD. The N325S mutant marginally increases responsiveness.
- The aggregate of the data suggests that the effects of the A354S and N325S mutants on AHR responsiveness is roughly additive; however, more experiments are needed to confirm this result.
- The difference in FICZ responsiveness between frog and mouse AHRs is smaller than that observed for TCDD. These findings are consistent with previous work (Laub *et al.*, 2010) and support the candidacy of FICZ as an endogenous ligand of the AHR.
- Several of the residues that affect TCDD binding also increase FICZ responsiveness when the frog AHR is made to resemble mouse AHR$^{b-1}$ at those positions. The A354S change seems especially potent, which suggests that FICZ and TCDD may have similar binding mechanisms within the AHR.

## References

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