# Molecular characterization of the Ah receptor pathway in flounder (*Platichthys flesus*).

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## Introduction

The molecular mechanism of action of PAHs and PHAHs is initiated by binding of the xenobiotic to the cytosolic Aryl hydrocarbon (Ah) receptor. The liganded Ah receptor is transported to the nucleus and binds to a specific DNA sequence, termed dioxin responsive enhancer (DRE) which finally leads to enhanced or inhibited rates of transcription of genes associated with the Ah receptor complex.

The first, most-studied, and best-understood response shown to be mediated by the Ah receptor, is induction of hepatic CYP1A and associated EROD activity. Since induction of EROD activity is one of the earliest responses observed in fish upon exposure to PAHs and PHAHs, the use of EROD activity as biomarker in fish has been advocated[1]. In a previous study, we observed low hepatic CYP1A activity in flounder (*Platichthys flesus*) upon exposure to the commercial PCB mixture Clophen A50[2]. These observations on low responsiveness of induction of EROD activity upon exposure to PCBs seriously hamper the further development and use of EROD activity as a reliable biomarker for exposure of fish to PHAHs and related compounds.

The reasons for this low responsiveness are not fully understood and may either be a consequence of an "inefficient" Ah receptor pathway for induction of CYP1A activity by PCBs, or may be a consequence of interference of PCB congeners with the catalytic activity of CYP1A in flounder. In this study we report on the molecular characterisation of the Ah receptor pathway for CYP1A induction.

## Material and Methods

*Animals, cells and chemicals.* Animals, cells and chemicals were obtained as described by Besselink et al.[3].

*Ah receptor ligand-binding studies.* Specific binding of [$^3$H]-TCDD to hepatic cytosol was measured by velocity sedimentation analysis on sucrose gradient (SGA), hydroxylapatite adsorption analysis (HAP) and protamine sulphate adsorption analysis (PSAA). As described by Besselink *et al.*[3]. Basically, aliquots of flounder hepatic cytosol or HEPA-1 cell lysate were incubated with [$^3$H]-TCDD (2 nM) ± TCDF (200 nM). For SGA, after incubation aliquots were layered

**Table 1** Concentrations of Ah receptor in hepatic cytosol from various species as determined by velocity sedimentation analysis on sucrose gradient (SG), hydroxylapatite (HAP), and potassium sulphate adsorption analysis (PSAA).

| | Ah-receptor concentration | | |
|---|---|---|---|
| | SG (fmol/mg) | HAP (fmol/mg) | PSAA (fmol/mg) |
| European flounder | 3.83 ± 0.31 (5) | 6.86 ± 1.62 (4) | 1.88 ± 0.35 (3) |
| Winter flounder | 19[f] | 4-8[f] | |
| Rainbow trout | 7.5[e] | 5.1 ± 2.6 (4)[e] | |
| Sprague-Dawley rat | 33 (1)[a] | 97 ± 5 (4)[b] | |
| C57BL/6N mouse | 60 ± 12 (8)[a] | 92 ± 10 (4)[b] | |
| RTH-149 | 29[d] | | |
| HEPA-1 | 361.90 (1) | 72.08 (1) | 31.24 (1) |

Values are expressed as mean ± S.E.M. Number of animals in parentheses. Data obtained from [a]: Okey *et al., J. Biol. Chem.*, **1979**, 254, 11636. [b]: Denison *et al., Chemosphere*, **1986**, 15, 1665. [c]: Heilmann *et al., DNA*, **1988**, 252, 954. [d]: Lorentzen and Okey, *Toxicol. Appl. Pharmacol.*, **1990**, 106, 53. [e]: Bank *et al., Eur. J. Pharmacol.-Environm. Toxicol.*, **1992**. 228, 85. [f]: Hahn *et al., Arch. Biochem. Biophys.*, **1994**, 310, 218.

onto linear (10-30%) sucrose gradients, centrifuged in a vertical-tube rotor after which 200 µl fractions were collected and radioactivity measured. For HAP and PSAA, incubated aliquots were added to HAP or PS containing tubes and incubated for 30 minutes. Pellets were resuspended and transferred onto a filter. The filter was washed, dried and and radioactivity was quantified.

*Gel retardation analysis.* Gel retardation analysis using hepatic cytosol from flounder Sprague-Dawley rat and guinea pig, were performed basically as described by Bank *et al.*[6]. The DRE oligonucleotides were synthesised, purified, annealed and radiolabeled[7]. Cytosol (2 mg protein/ml for flounder and 16 mg protein/ml for all other species) was incubated with DMSO (20 µl/ml) ± TCDD (2 nM) for 2 h at 20$^0$C. Poly dIdC was added to aliquots of the incubation mixture and incubated (15 min., 20$^0$C). The incubation was continued for another 15 min. (20$^0$C) after addition of $^{32}$P-labeled oligonucleotides. Protein-DNA complexes were analysed by polyacrylamide gel electrophoresis and autoradiography[3].

*RT-PCR and Southern blotting.* Flounder and Fundulus hepatic total RNA and subsequent polyA+ RNA were isolated as described by Besselink et al.[3]. RT-PCR was performed using purified flounder (3 µL) and Fundulus (1 µl) polyA+ RNA[3]. Aliquots of the RT-PCR reaction were run on 2% agarose gels, stained with ethidium bromide, and photographed. RT-PCR products were analysed by Southern blotting as described by Besselink et al.[3]. The RT-PCR products from four individuals were pooled, cloned into pCNTR (5 prime → 3 prime, Inc.) and sequenced.

## Results

*Ah receptor binding studies.* The concentrations of Ah receptor in HEPA-1 cells and flounder hepatic cytosol calculated from SGA, HAP and PSAA are given in Table 1. Flounder Ah receptor levels ranged from 1.88 to 6.86 fmol/mg protein dependent on the assay used. These levels are much lower than Ah receptor concentrations in rodent species and HEPA-1 liver cells.

*Gel retardation analysis.* Autoradiograms of interaction of flounder, rat, and guinea pig cytosolic Ah receptor and DRE-containing oligonucleotides in the presence or absence of TCDD are shown in Fig. 1. Rat and guinea pig autoradiograms reveal a TCDD-inducible (see arrow), representing binding of TCDD-AhR complex to $^{32}$P-labeled DRE-containing oligonucleotides. In contrast to rat and guinea pig, no TCDD-inducible protein-DRE complex was observed in flounder under the same experimental conditions.

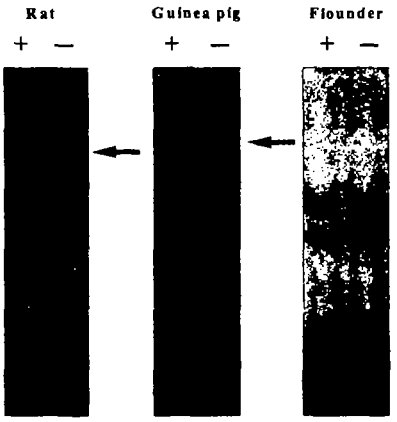


**Fig. 1** Autoradiograms of liganded cytosolic Ah receptor and DNA complexes of rat, guinea pig and flounder, analysed by gel retardation analysis. Hepatic cytosol was incubated in the absence (-) or presence (+) of 20 nM TCDD, followed by addition of $^{32}$P-labeled DRE oligonucleotide as described under Material and Methods. Arrows indicate the position of TCDD-induced protein-DNA complex.

*RT-PCR and DNA sequencing.* RT-PCR of Fundulus and 4 individual flounder poly A+ RNAs led to a detectable band of approximately 690 bp (data not shown). Southern blotting of the flounder RT-PCR products and hybridisation using degenerate oligonucleotide AhR-J2u revealed a single band of 690 bp for each individual (data not shown). The RT-PCR products were cloned and sequenced. The deduced amino acid sequence of the flounder RT-PCR product was 59-60% identical to the PAS domain of mammalian AhRs (Fig. 2) and 61% identical to Fundulus AhR. Interestingly the flounder sequence shared greater amino acid identity (75%) with the *Fundulus* AhR-2 sequence identified recently[5,8].

## Discussion

The present results show for the first time the positive identification of the AhR in the European flounder (*Platichthys flesus*). Evidence for the presence of the hepatic cytosolic AhR in flounder was provided using three AhR binding assays. Although the concentrations of Ah receptor detected in flounder (~ 4 fmol/mg protein) using the described [$^3$H]TCDD binding studies, was in the same order of magnitude as found in other species of fish, it must be noted that the Ah receptor levels were very low as compared to Ah receptor levels in mammalian hepatic cytosolic tissue (~50-300 fmol/mg protein).

Additional evidence for the presence of the cytosolic AhR in flounder liver was provided by amplification of flounder AhR cDNA using RT-PCR. The size of the PCR product was 690 bp, identical to recently identified *Fundulus* AhR-2 cDNA[5,8]. The specificity of the flounder AhR cDNA was established by Southern blotting. Sequencing of the flounder 690 bp product revealed that its deduced amino acid sequence was more related to *Fundulus* AhR-2 sequence (75 % identical) than to *Fundulus* AhR-1 sequence (61 % identical), which is closer to mammalian AhRs than AhR-2[5,8].

In the present study, we were able to demonstrate TCDD-inducible protein-DRE binding in hepatic cytosol from rat and guinea pig but not in flounder hepatic cytosol . The absence of TCDD-inducible protein-DNA complex may be due to low levels of Ah receptor in flounder or instability of flounder Ah receptor[6]. With respect to the levels of cytosolic Ah receptor, these were low in flounder hepatic cytosol as compared to cytosolic Ah receptor levels in mammals. This might have its influence on the absence of TCDD-inducible protein-DNA complex, but other possibilities can not be ruled out.

In conclusion, the presented data suggest that the Ah receptor pathway is present in flounder. Therefore, our observations on low responsiveness of flounder hepatic EROD activity

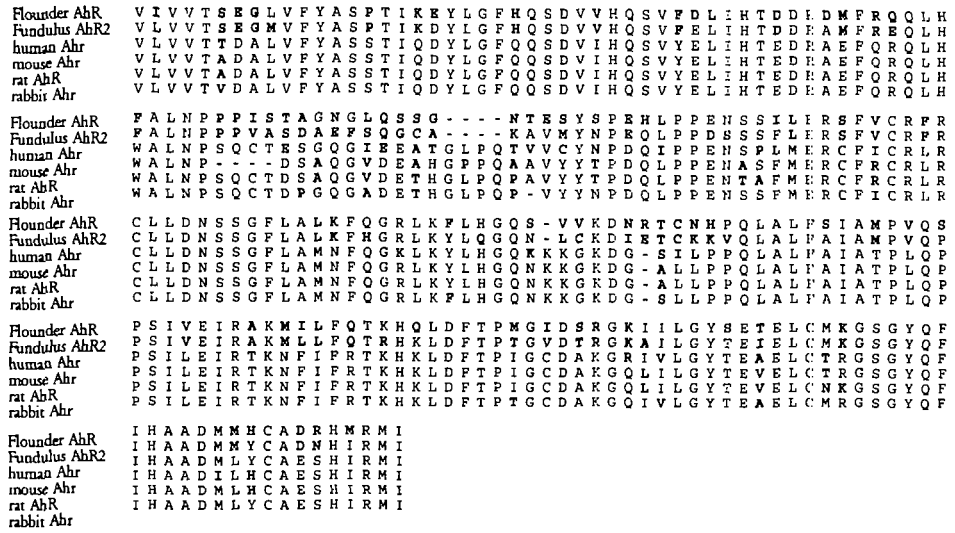


**Fig. 2** Deduced amino acid sequence of flounder RT-PCR product aligned with partial amino acid sequences (PAS domain) of other vertebrate Ah receptors. Amino acid sequences were aligned using ClustalW(1.6). GenBank accession numbers of the sequences used are: flounder AhR (AF034412); Fundulus AhR2 (U29679); human AhR (L19872); mouse AhR (M94623); rat AhR (U09000); rabbit AhR (D38226). Amino acids that are identical in three or more of the sequences are boxed and shaded. Similar amoni acids are in bold type.

upon exposure to PCBs are most probably not a result of an "inefficient" Ah receptor pathway for induction of CYP1A activity by PCBs.

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## References

[1]Bucheli, T.D. and Fent, K., *Crit. Rev. Environ. Sci. Technol.*, 1995, **25**, 201.

[2]Besselink, H.T., Beusekom, S. van, Roex, R., Vethaak, A.D., Koeman, J.H. and Brouwer, A., *Environ. Pollut.*, 1996, **92**, 267.

[3]Besselink, H.Y., Denison, M.S., Hahn, M.E., Karchner, S.I. and Brouwer, A., Toxicol. Sci., 1998, in press

[4]Besselink, H.T., E. van Santen, W. Vorstman, A.D. Vethaak, J.H. Koeman and Brouwer, A., *Environ. Toxicol. Chem.*, 1997, **16**, 816.

[5]Hahn, M.E. and Karchner, S.I., *Biochem. J.*, 1995, **310**, 383.

[6]Bank, P.A., Yao, E.F., Phelps, C.L., Harper, P.A. and Denison, M.S., *Eur. J. Pharmacol.-Environm. Toxicol. Pharmacol Section.*, 1992, **228**, 85.

[7]Denison, M.S., Fisher, J.M. and Whitlock, J.P. Jr., *Proc. Natl. Acad. Sci.*, 1988, **85**, 2528

[8]Hahn, M.E., Karchner, S.I., Shapiro, M.A. and Perera, S.A., *Proc. Natl. Acad. Sci., U.S.A.*, 1997, **94**:13743.