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Cytochrome P450 1D1: A novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD

J. V. Goldstone^{1,*}, M. E. Jönsson^{1,†}, L. Behrendt¹, B. R. Woodin¹, M. J. Jenny¹, D. R. Nelson², and J. J. Stegeman^{1,*}

¹ Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA, USA

² Department of Molecular Sciences, University of Tennessee, Memphis, TN, USA

Abstract

Enzymes in the cytochrome P450 1 family oxidize many common environmental toxicants. We identified a new CYP1, termed CYP1D1, in zebrafish. Phylogenetically, CYP1D1 is paralogous to CYP1A and the two share 45% amino acid identity and similar gene structure. In adult zebrafish, *CYP1D1* is most highly expressed in liver and is relatively highly expressed in brain. *CYP1D1* transcript levels were higher at 9 hours post-fertilization than at later developmental times. Treatment of zebrafish with potent aryl hydrocarbon receptor (AHR) agonists (3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) did not induce *CYP1D1* transcript expression. Morpholino oligonucleotide knockdown of AHR2, which mediates induction of other *CYP1s*, did not affect *CYP1D1* expression. Zebrafish CYP1D1 heterologously expressed in yeast exhibited ethoxyresorufin- and methoxyresorufin-O-dealkylase activities. Antibodies against a CYP1D1 peptide specifically detected a single electrophoretically-resolved protein band in zebrafish liver microsomes, distinct from CYP1A. CYP1D1 in zebrafish is a CYP1A-like gene that could have metabolic functions targeting endogenous compounds.

Keywords

development; CYP1A; CYP1B; CYP1C; AHR; PCB126; TCDD; zebrafish; embryo; vertebrate toxicology; oxidative biotransformation

Introduction

Cytochrome P450 (CYP) monooxygenases in vertebrate CYP gene families 1, 2 and 3 collectively catalyze transformation of a large but unknown number of substrates, and function in numerous physiological and toxicological processes. There is a great diversity of genes in these CYP families in animals, and the relationships among them and their involvement in particular catalytic functions are still poorly understood. Understanding the susceptibility of organisms to effects of drugs and environmental chemicals is critically dependent on knowledge of these CYP functions and diversity, which could vary between individuals and

* Address for correspondence: John Stegeman, jstegeman@whoi.edu; Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, MA 02534.

† Current address: Department of Environmental Toxicology, Uppsala University, Uppsala, Sweden

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species. This paper concerns the CYP1 family in zebrafish, a premier vertebrate model in developmental biology that is increasingly used in carcinogenesis and toxicological research.

The vertebrate CYP1 family shows greater diversity than once thought [1], with *CYP1A*, *CYP1B* and *CYP1C* gene subfamilies, and a complex evolution. Mammalian CYP1A and CYP1B enzymes act on a variety of common environmental pollutants including carcinogens and promutagens [2]. CYP1A1 and CYP1A2 catalyze the oxidative biotransformation of planar aromatic hydrocarbons (PAH) as well as aryl amines and heterocyclic amines, often resulting in bioactivation to toxic and mutagenic derivatives [3;4]. Similarly, CYP1B1 oxidizes and activates PAHs [5]. Collectively, mammalian CYP1As and CYP1B1 also oxidize various endogenous substrates, among them uroporphyrin [6], estradiol [7], retinoids [8;9], and fatty acids, possibly resulting in formation of regulatory molecules, e.g., eicosanoids [10].

Non-mammalian vertebrates also express *CYP1A* and *CYP1B1* genes [11;12;13;14]. A third CYP1 subfamily, CYP1C, was discovered more recently [15;16]. Two *CYP1C* paralogs are found in fish, and a single *CYP1C* occurs in non-mammalian tetrapods [1]. While substrates have yet to be identified for the CYP1Cs, it is conceivable that they also could catalyze xenobiotic transformations.

Like *CYP1A* and *CYP1B1*, the *CYP1Cs* in zebrafish are induced by agonists for the aryl hydrocarbon receptor (AHR) [17;18]. There are three AHRs in zebrafish (AHR1a, AHR1b, and AHR2; [19]). Both AHR2 and AHR1b bind TCDD, although knockdown of AHR expression with morpholino oligonucleotides indicates that AHR2 is the primary mediator of induction of *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* [17]. Interestingly, the four CYP1 genes differ in the magnitude of their basal expression [17;18]. Inducibility of these CYPs varies as well between organs and developmental stages, suggesting that they may play different roles in susceptibility to AHR agonists in zebrafish [17;18]. However, susceptibility also could depend on involvement of other targets, including other CYP.

Here we report on a fourth CYP1 subfamily in zebrafish, CYP1D. Iterative searches of the zebrafish genome database uncovered a novel CYP1 sequence more closely related to *CYP1A* than to *CYP1B1* or the *CYP1Cs*. Full-length *CYP1D1* was cloned from untreated zebrafish by RT-PCR, and transcript expression was examined during development and in different adult organs. We also examined whether the potent AHR agonists 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) might induce *CYP1D1* expression, and whether AHR2 might be involved in the regulation of *CYP1D1* expression. The CYP1Ds expand our view of the diversity of *CYP1* genes in this important model species, and in the vertebrates generally.

Materials and Methods

Fish husbandry

The TL (Tupfel/Long fin mutations) wild-type strain of zebrafish were used for all experiments, and were maintained as previously described [17]. Fertilized eggs were obtained from multiple group breedings from tanks of 30 female and 15 male fish. Embryos were reared as described previously [18]. Procedures used in these experiments were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

Cloning of CYP1D1

A predicted gene with homology to zebrafish *CYP1* genes was initially identified by BLAST searches of the zebrafish genome. Subsequent hidden Markov model searches (Hmmer v2.3.2 [20]) of the ENSEMBL protein predictions with the PFAM p450 protein model confirmed the

presence of an additional CYP1-like gene prediction. Primers were designed to amplify the ENSEMBL predicted transcript (ENSDART00000051565).

Year old sexually mature male and female zebrafish were killed and gill, liver, kidney, eyes, heart and gut were removed. Total RNA was extracted from individual tissues using STAT 60 RNA isolation reagent (Tel. Test Inc. Friendswood, TX). RNA was then reverse transcribed with the Omniscript reverse transcriptase kit (Qiagen, Valencia, CA) using random hexamers and the RNasin RNase Inhibitor (Promega, Madison, WI). cDNA was pooled from all tissues. *CYP1D1* was amplified with the Advantage 2 PCR kit (Clontech, Mountainview CA) using gene specific primers (Supplemental Table 1, Operon Biotechnologies, Huntsville, AL). *CYP1D1* cDNA was gel purified, ligated into the pGEM-TEZ (Promega) plasmid vector, and clones were used to transform TOP10 cells (Invitrogen, Carlsbad, CA). Plasmids were isolated using QIAprep Miniprep kits (Quiagen) and were sequenced by MWG Biotech (Highpoint, NC) using standard protocols

Sequence analyses

Gene structures and specific pattern searches for degenerate versions of the consensus xenobiotic receptor element (XRE) were mapped using GCG (v. 10.3; Accelrys, San Diego, CA). Syntenic relationships were examined using ENSEMBL. Phylogenetic relationships were investigated using maximum likelihood (RAxML-7.0.3; [21]) and Bayesian techniques (MrBayes v 3.1.2; [22]). MrBayes estimates posterior probabilities using Metropolis-Hastings coupled Monte Carlo Markov chains (MC³). We performed MC³ estimates with uninformative prior probabilities using the WAG model of amino acid substitution [23] and prior uniform gamma distributions approximated with four categories (WAG+I+Γ). Four incrementally heated, randomly seeded Markov chains were run for 5×10⁶ generations, and topologies were sampled every 100th generation. Burnin value was set to 10⁶ generations. The WAG substitution model using the categories approximation (PROTMIXWAG [24]) was used for RAxML analyses. 1000 bootstrap replicates were performed. Promoter region searching was performed using MatInspector [25] and GCG. Synonymous and nonsynonymous substitution rates were estimated with PAML (v 3.15; [26]).

CYP1D1 expression by real time PCR

Total RNA was extracted using RNA STAT 60 and then treated with the TURBO DNase-free kit (Ambion, Austin, TX). cDNA was synthesized using Omniscript reverse transcriptase. Zebrafish *CYP1D1* primers, 5'-ATCGTCCAAGAGATAGATAACCAAG-3'(forward) and 5'-TGGTGAATGGCATGTAGGAC-3' (reverse), and primers for *CYP1A*, *ARNT2* and *β-actin* cDNA [17] were obtained from Operon Biotechnologies Inc. Real-time PCR was performed according to procedures previously described [17]. To ensure that a single product was amplified, melting curve analysis was performed on the PCR products at the end of each run and only data that showed one major melting peak were used.

Relative expression of *CYP1A* and *CYP1D1* mRNAs was calculated for each reaction according to Livak and Schmittgen [27]. PCR efficiency (E) was determined by the LinRegPCR program [28]. As reference genes we chose to use *ARNT2* in embryos and juveniles and *β-actin* in adults, as described previously [17]. Basal expression was calculated using E^{ΔCt} and expression in PCB126-exposed fish (at 3 and 57 dpf) was calculated using the E^{ΔΔCt}-method with the mean value in corresponding controls as a calibrator (i.e., E^{ΔCt PCB126} / E^{ΔCt control}) [27]. Outlying values were excluded based on Grubbs test [29]. Differences in *CYP1* gene expression between PCB126-exposed and control groups were determined by one-way ANOVA followed by Dunnet's test. Prism 4 (GraphPad Software Inc., San Diego, CA) was used for statistical examinations.

CYP1D1 gene expression was studied in four different experiments, in order to examine temporal changes during development, organ distribution, responsiveness to AHR agonists, and involvement of AHR in *CYP1D1* regulation by morpholino knock-down.

Temporal changes during development—The expression of *CYP1D1* and *CYP1A* (for comparison) was examined in unexposed developing zebrafish. Fertilized eggs were kept in 0.3× Danieau's solution (28 °C) and sampled every day for seven days, i.e., over 9–178 hours post-fertilization (hpf). Juvenile fish were sampled at 57 dpf. Three replicates of embryos or larvae (25 per replicate), or juveniles (1 fish per replicate) were sampled, frozen in liquid nitrogen and then stored at -80°C.

Organ distribution—In this study untreated adult fish were used. The fish were killed by decapitation, body weight recorded, and sex determined. The body weights were 0.6±0.1g in males and 1.1±0.2g in females (mean±SD). Liver, gut, kidney, heart, brain, eye, and gill were removed. Organs from four fish of the same sex were pooled to obtain sufficient amounts of cDNA for the analyses, resulting in three pools of male and three pools of female samples for each organ. The dissected organs were preserved in RNAlater (Ambion).

Response to AHR agonists—Groups of fertilized zebrafish eggs (33±5) were placed in 10-cm glass Petri dishes containing 30 ml of 0.3× Danieau's solution (28°C). At 9 hpf, stock solutions of PCB126 (Cambridge Laboratories Inc., Andover, MA) in acetone or acetone alone (controls) were added to the Danieau's solution, yielding nominal PCB126 concentrations ranging from 0.3 to 100 nM (including 100 ppm acetone) or 100 ppm acetone alone. After 24 hours exposure, the dosing solutions were replaced with fresh 0.3× Danieau's solution and the embryos were held for another 48 hours with renewal of the Danieau's solution after 24 hours. Groups of PCB126- and solvent-exposed embryos/larvae were sampled at 80 hpf, frozen in liquid nitrogen, and then stored at -80°C.

For TCDD exposure, embryos were placed in glass petri dishes with no more than three embryos per milliliter of 0.3× Danieau's and then exposed to 0.1% dimethyl sulfoxide (DMSO) or 2 nM TCDD (including 0.1% DMSO) for 1 h, starting 6 hpf. After exposure, the embryos were washed three times in fresh 0.3× Danieau's, then placed in Petri dishes with 25 ml fresh 0.3× Danieau's and held as above. Danieau's solution was renewed at 24 hpf. At 48 hpf, three or four replicates of 20 pooled embryos were collected from each group, frozen in liquid nitrogen and then stored at -80°C until RNA isolation.

To evaluate the response of juveniles to the AHR agonist PCB126, groups of six 55 day old fish were placed in two cling-wrap-covered glass beakers filled with 1 liter of continuously aerated zebrafish system water (28°C). Aliquots of PCB126 dissolved in acetone or acetone only were added to two of the beakers, yielding nominal concentrations of 100 nM PCB126 (including 100 ppm acetone) or 100 ppm acetone. After 24 hours of exposure, all solutions were replaced with fresh system water and the fish were held for another 24 hours, at which time they were frozen individually in liquid nitrogen then stored at -80°C.

Gene knock-down with morpholino antisense oligonucleotides—Morpholino antisense oligonucleotides targeted to zebrafish aryl hydrocarbon receptors were used to determine whether AHR2 might be involved in expression of *CYP1D1*. AHR2-MO, targeted to the start site, (5'- TGTACCGATACCCGCCGACATGGTT-3') [30;31] and the standard negative control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3'), were obtained from Gene Tools (Philomath, OR). The morpholinos were fluorescein-tagged for screening purposes to guarantee that only successfully injected embryos were used for the subsequent experiments. Morpholinos were injected at approximately 3.3 ng per embryo into 2 to 4-cell stage embryos (i.e., by 1 hpf) as previously described [18], resulting in approximately 3.3 ng

of morpholino per embryo. Injection volumes were calibrated as before [18]. At 3 hpf, embryos were sorted, eggs that were damaged or not developing were removed, and the remaining embryos screened for homogeneous fluorescence distribution.

Four groups of 25 embryos successfully injected with the two MOs (AHR2-MO, and control-MO), and four groups of non-injected embryos, were exposed to carrier (100 ppm of acetone) or 30 nM PCB126 in carrier for 24 hours (starting 9 hpf), as previously described [18]. After 24 hours of exposure, all solutions were replaced with 0.3× Danieaus's. At 48 hpf all embryos in a group were pooled, frozen in liquid nitrogen, and then stored at -80°C.

Heterologous expression of CYP1D1

CYP1D1 was heterologously expressed in yeast as a fusion protein with a V5 epitope and a 6 histidine tag at the C-terminus of the protein. The open reading frame of *CYP1D1* was PCR amplified from cDNA using a high fidelity polymerase with proofreading function (Pfu Ultra II, Stratagene) using gene specific primers designed by the procedures described in the p-ENTR-D-TOPO cloning kit (Invitrogen). PCR products were purified and cloned into the pENTR-D-TOPO vector and amplified in TOP10 cells according to the manufacturer's protocol. Positive clones grown on selective plates were picked and grown overnight in LB media + kanamycin (50 µg/ml). Plasmids were purified from the overnight cultures using the Perfect mini-prep kit (Eppendorf, Hamburg, Germany). Plasmids confirmed by sequencing (MWG) to contain correctly oriented insert, were directionally recombined into the pYES-DEST52 destination vector (Invitrogen), which has in-frame C-terminal V5 and His tags, using the Gateway LR-Clonase enzyme mix (Invitrogen). The purified pYES-DEST52 plasmids containing the CYP1D1 insert were used to produce functional yeast expression clones with the *Saccharomyces cerevisiae* W(R) strain, which were made competent using the S.c. EasyComp Transformation Kit (Invitrogen) according to the manufacturer's protocol. The W(R) yeast strain has been engineered to express human CYP-oxidoreductase (CYPOR) for use in heterologous CYP expression studies [32]. A pYES-DEST52 plasmid containing Arabidopsis β glucuronidase (p-GUS) was used as a transformation and expression control, and microsomes from yeast transformed with this plasmid were used as controls for catalytic activity.

W(R) yeast were cultured and harvested by the procedures described by Pompon et al [32]. Briefly, transformed yeast were grown in SGAI medium for 24 hours. One volume of this culture was then transferred into 50 volumes of YPGE complete media and grown overnight. To induce protein expression, this culture was inoculated with 10% (by volume) with sterile filtered galactose. After 15 h the yeast cells were harvested and pelleted by centrifugation (1500 × g for 5 min). Resulting pellets were washed with distilled water, and resuspended in degassed TSG buffer containing 1.0 mM dithiothreitol (DTT) and protease inhibitor cocktail (1X) (Sigma) and mechanically disrupted using the BeadBeater (BioSpec Products, Inc., Bartlesville, OK). The resuspended cells were disrupted and microsomes were prepared as previously described [33]. Microsomal pellets were resuspended in TEG buffer with 1 mM DTT and were stored in LN2 until use. Microsomal protein concentration was determined using the Pierce bicinchoninic acid (BCA) assay kit with BSA as the standard.

Additional confirmation of CYP1D transformation of yeast was performed by subcloning yeast cultures on selective plates, picking of 5 colonies which were grown and induced with galactose as described above, and the resulting yeast cultures were used for microsomal preparations, with an aliquot of each being reserved for plasmid extraction. Plasmids extracted from these yeast were used to retransform TOP10 bacteria for amplification, isolation and sequence analysis to confirm CYP1D1 sequence in cultures used for microsomal preparation and catalytic assay.

Catalytic Assay

Ethoxyresorufin-O-deethylase (EROD) and methoxyresorufin-O-demethylase (MROD), activities were measured in microsomes from yeast expressing zebrafish CYP1D1, using previously published procedures (Hahn et al, 1996). Microsomes from yeast transformed with pGUS were used as controls for catalytic activity.

Immunoblot of CYP1D1 protein in zebrafish liver microsomes

Forty male zebrafish were exposed to 0.02 % DMSO (control) or to 100 nM PCB126 added in 0.02 % DMSO for 24 h at room temperature, followed by 24 h in charcoal filtered water. Fish were killed and livers were pooled within groups and microsomal fractions were prepared as previously described [34]. Microsomal protein concentration was determined using the Pierce bicinchoninic acid (BCA) assay kit with BSA as the standard.

Microsomal proteins and prestained molecular weight standards (Precision Plus, BioRad) were resolved on 10% acylamide gels (PAGEgel) and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences) for immunoblotting as previously described [35]. Membranes were blocked with 5% non-fat dry milk in TRIS-buffered saline (TBS), and then incubated at room temperature with shaking for 1 h each with either 1 µg/ml affinity purified rabbit polyclonal antibody (21st Century Biochemicals, Marlboro MA) to a C-terminus peptide inferred from the sequence of zebrafish CYP1D1, or with 3 µg/ml mouse monoclonal antibody to scup CYP1A. Anti-CYP1A blots were then incubated with 1:1000 diluted goat antibody to mouse IgG labelled with IRDye 800 (LI-COR Biosciences, San Diego, CA) and anti-CYP1D1 blots were incubated with 1:1000 diluted goat antibody to rabbit IgG labelled with IRDye 800 (LI-COR). Antibody solutions were made up in TBS containing 5% non-fat dry milk and 0.1% Tween 20. Between incubations, membranes were rinsed thoroughly with distilled water and then washed 3X for 5 min with TBS with 0.1 % Tween 20. After the final washes, blots were transferred into TBS and then scanned using the Odyssey Infrared fluorescent dual laser scanner (LI-COR). Signal was quantified for CYP1D1 and the molecular weight standards using 700 nm laser excitation and for CYP1A and CYP1D1 using 800 nm laser excitation.

Yeast microsomes were immunoblotted as above for liver microsomes, and were also blotted with mouse monoclonal antibody to the V5 epitope (Invitrogen). IRDye labelled secondary antibodies were used for immunodetection of yeast expressed CYP1s as described.

Results

Gene structure

Searching the zebrafish genome with a combination of BLAST and HMM disclosed a novel sequence closely related to known CYP1 sequences in zebrafish. The full-length transcript was cloned from RNA pooled from developmental stages and several different adult tissues of Tupfel/Long fin (TL) zebrafish. The amino acid sequence predicted for the cloned transcript differed from the Ensembl genome prediction derived from the Tübingen strain (ENSDARG00000035569) in 4 positions (TL/Tübingen - L21F, S90T, G129E, and T176A; Figure 1). The full, unmasked CYP coding region shares 54% nucleotide identity and 45% amino acid identity with zebrafish CYP1A, greater than the identities with other zebrafish CYP. However, the amino acid sequence differed from the known zebrafish CYP1 sequences enough to warrant separate classification, as CYP1D1 (GenBank: FJ416496).

Phylogenetic analyses of zebrafish CYP1D1 shows that it is related to CYP sequences predicted from two other fish species, medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatus*) [36], and that these sequences form a distinct subfamily that clusters together with the CYP1As, in a clade that is distinct from the CYP1B/CYP1C clade (Figure 2). Using both

Bayesian and maximum likelihood techniques, we obtained strong support for a distinct CYP1D subfamily and a CYP1A/CYP1D clade.

Given the relationship between CYP1A and CYP1D1, we examined the amino acid sequence similarity between zebrafish CYP1D1 and zebrafish CYP1A, using the BLOSUM62 scoring matrix (which gives values different than simple identity). There was 58.5% similarity over the full coding region. However, the degree of similarity varies substantially along the length of the proteins (Figure 3). Interestingly, the putative substrate recognition sites (SRS; [37]) showed pronounced variation in the similarity scores between CYP1D1 and CYP1A. SRS4 and SRS5 were highly similar between CYP1D1 and CYP1A, while SRS2 and SRS3 exhibit very low sequence similarity. SRS6 shows an elevated similarity between CYP1D1 and CYP1A relative to flanking sequence, but no such feature is evident in SRS1.

Zebrafish *CYP1D1* is located on chromosome 5 and is flanked by the genes *TMC2* and *ANXA1A*, whereas *CYP1A* is located on chromosome 18 and is flanked by *DTWD1* and *TRPM7*, which are not homologous to the genes flanking *CYP1D1*. However, the gene structure of zebrafish *CYP1D1* is identical to that of *CYP1A*, with six coding exons and one non-coding exon, and conserved exon boundaries (Figure 4).

An analysis of the 10 kb upstream promoter region of *CYP1D1* identified two putative consensus core xenobiotic response elements (XREs; KNGCGTG [38;39]) in the proximal promoter immediately upstream of the transcriptional start site (Figure 4). In contrast, *CYP1A* has 22 core XREs within 10kb of the transcriptional start site. However, the presence of a core XRE sequence may not be sufficient to confer function to a putative XRE, and we did not find any regions in the *CYP1D1* promoter that match the more extended position-specific scoring matrix of ZeRuth and Pollenz (TYGCGTGMVMD [39]), which they found in functional XREs. Other possible response elements found in the proximal promoter region of *CYP1D1* include those for PPAR/RXR, ROR α , and NRF1 (NFE2L1), but not RAR/RXR. Interestingly, the proximal promoter region is enriched in putative neural-specific promoter elements (MYT1, NGFIC, NEUROD1) relative to the number of those elements in the promoter region of *CYP1A*.

CYP1D1 transcript levels in untreated zebrafish

Expression of *CYP1D1* was examined in embryonic and juvenile stages and in adult organs. Developmental expression of *CYP1D1* measured with real-time PCR shows that *CYP1D1* transcript levels are two to three times higher at 9 hpf than at later times and stages (Figure 5). There was, however, an apparent increase around hatching, at day 3, and then a decline (Figure 5). Similar levels of transcript expression were observed in larvae at 6 days post-fertilization and juveniles at 57 dpf. In contrast, *CYP1A* expression was lower during early development, including at 9 hpf, and increased after hatching.

Real-time PCR analysis detected expression of *CYP1D1* in all tissues examined from untreated adult zebrafish, *i.e.* liver, brain, gut, gill, kidney, eye, and heart (Figure 6). The relative levels of *CYP1D1* expression appear to be much higher in liver than in other organs. Similarly, *CYP1A* was much more highly expressed in liver than other organs examined. Compared with other organs, the brain showed much a higher level of *CYP1D1* expression relative to the expression level of *CYP1A*, which was very low.

Response to AHR agonists PCB126 and TCDD

To determine whether AHR agonists might induce *CYP1D1*, we exposed zebrafish embryos and juveniles to the potent AHR2 agonist PCB126 at 100 nM in the water. This concentration has been shown to produce morphological defects including edema in embryos and to strongly

induce expression of other *CYP1* genes [17;18]. In contrast to the strong induction of *CYP1A*, 100 nM PCB126 did not induce *CYP1D1* expression relative to the carrier controls either at 3 dpf or 57 dpf. At 3 dpf, embryos exposed to 100 nM PCB126 had levels of expression of *CYP1D1* and *CYP1A* that were 0.7 ± 0.4 and 215 ± 20 times the carrier control, respectively. In juveniles, i.e., at 57 dpf, expression levels of *CYP1D1* and *CYP1A* were 1.4 ± 0.4 and 70 ± 6 times the carrier control, respectively (Figure 7A).

To determine whether lower doses of PCB126 might affect *CYP1D1* expression, a PCB126 dose-response experiment was carried out in embryos (Figure 7B, C). There was no induction of *CYP1D1* transcript expression detected at any dose of PCB126, although there appeared to be a decline at the higher doses of PCB126. In contrast, there was an expected dose-dependent induction of *CYP1A*. The EC_{50} value for PCB126 induction of *CYP1A* was 1.7 nM, consistent with our previous observations [17; 18].

To test whether an even more potent AHR agonist might induce *CYP1D1* transcript, we exposed embryos to TCDD at 2 nM, a dose that strongly induces *CYP1A* [17;18]. Exposure of embryos to TCDD at 2 nM did not increase the expression of *CYP1D1* (data not shown).

Morpholino oligonucleotide knock-down of AHR2

To test further whether the AHR might be involved in regulating expression of *CYP1D1* in embryos, we analyzed *CYP1D1* transcript in embryos that were injected at the 2-4-cell stage with morpholino oligonucleotides (MOs) designed against *AHR2*, with and without exposure to PCB126 or TCDD. *AHR2* was selected because it regulates zebrafish *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* [17;18]. MOs targeting the translational start site of *AHR2* had no effect on *CYP1D1* expression in zebrafish embryos, whether embryos were exposed to the carrier or to PCB126 (Figure 8), or to 2 nM TCDD (data not shown). In the same groups of embryos, treatment with the *AHR2*-MO resulted in 80-90% inhibition of TCDD or PCB126-induced expression of *CYP1A*, *1B1*, *1C1*, and *1C2* [18].

Zebrafish CYP1D1 protein expression

To assess whether *CYP1D1* transcript generated functional protein, we expressed CYP1D1 as a fusion protein with a C-terminal V5 and histidine tag in W(R) yeast, which also had been engineered to express CYP-oxidoreductase [32]. Electrophoretic resolution and immunoblot analysis of microsomes from yeast transformed with the *CYP1D1* plasmid confirmed the presence of a protein that carried the V5 epitope tag (data not shown). EROD and MROD activities were assessed in multiple microsomal preparations from yeast transfected with *CYP1D1*, as compared to yeast transfected with p-GUS and yeast transfected with a similar plasmid construct containing zebrafish *CYP1A*. EROD and MROD activities measured in 5 microsomal preparations of the CYP1D1-expressing yeast averaged 0.4 ± 0.2 , and ranged up to 1.0 pmol/min/mg total protein for EROD, and 0.2 ± 0.1 and up to 1.0 pmol/min/mg total protein for MROD. The EROD and MROD activities are very low; however under the same conditions neither activity could be detected in the pGUS control yeast microsomes. By comparison, EROD and MROD activities measured in microsomes similarly prepared from yeast expressing zebrafish CYP1A were 86 ± 2 pmol/min/mg total protein and 56 ± 1 pmol/min/mg total protein, respectively.

Microsomal fractions prepared from CYP1D1 and CYP1A-expressing yeast, from control (pGUS) yeast and from liver of control and PCB-treated zebrafish were immunoblotted with affinity-purified rabbit polyclonal antibodies against a C-terminal peptide of zebrafish CYP1D1 and with mouse monoclonal antibody 1-12-3 to scup CYP1A, which is cross reactive with CYP1A of zebrafish [40]. Rabbit anti-zebrafish-CYP1D1 antibody reacted specifically with CYP1D1 expressed in yeast, but not with proteins in microsomes from CYP1A-expressing

yeast (Figure 9). Anti-CYP1D1 antibody also recognized a single electrophoretic band in zebrafish liver microsomes (Figure 9), resolving above the CYP1A band. The immunodetected liver microsomal CYP1D1 was at similar levels in control and PCB126-treated fish, while as expected CYP1A was profoundly induced in PCB126-treated fish.

Discussion

The cloning and expression of zebrafish *CYP1D1* expands the *CYP1* family in this important biomedical model to four subfamilies and five genes, *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2* and *CYP1D1*. Zebrafish *CYP1D1* has an exon-intron structure identical to that of zebrafish *CYP1A*, very different from those of *CYP1B1* and the *CYP1Cs* (which have 2 and 1 exons, respectively). Phylogenetic analysis places zebrafish CYP1D1 together with predicted CYP1D1 sequences from two other fish [36], and places the CYP1D subfamily in a common clade with the CYP1As. Thus, within the vertebrate CYP1 gene family, the CYP1As and CYP1Ds together constitute one clade, while the CYP1Bs and CYP1Cs constitute another clade (Figure 2). This suggests a common ancestral origin for CYP1A and CYP1D1 and that these subfamilies diverged from one another subsequent to divergence of the CYP1B/CYP1C clade from the CYP1A clade.

An important question concerning new gene sequences is whether functional protein is expressed in vivo. We addressed this by demonstrating that zebrafish CYP1D1 could be expressed in yeast, and that it was catalytically active with both ethoxyresorufin and methoxyresorufin. Moreover, immunoblot analysis of liver microsomes with polyclonal antibodies to a CYP1D1 peptide indicated that CYP1D1 protein is expressed in vivo in zebrafish. Rates of EROD activity by CYP1D1 were very low relative to those of zebrafish CYP1A (40-150 pmol/min/mg, this study and [33]) or human CYP1A1 (150-160 pmol/min/mg [41]) expressed in yeast. In biomarker studies, EROD activity is often attributed to CYP1A and used to indicate exposure of fish to AHR agonists. Even though EROD rates were low, the expression of CYP1D1 protein in control zebrafish liver microsomes suggests that CYP1D1 should be examined in other species, as under conditions of low level exposure CYP1D1 might contribute to EROD activity usually attributed to CYP1A.

While detection of EROD and MROD activities suggests some catalytic similarity between CYP1D1 and CYP1A, it is likely that functionality differs between the two. Rates of EROD and MROD in yeast microsomes were much lower for CYP1D1 than for CYP1A. Analysis of similarity scores within the deduced amino acid sequence of zebrafish CYP1D1 and CYP1A revealed intriguing similarities and differences in the putative SRSs thought to be important in CYP substrate specificity. Specifically, SRS2 and SRS3 in zebrafish CYP1D1 are very dissimilar from those in zebrafish CYP1A; residues in these regions have been predicted to influence substrate specificity of fish CYP1A in comparison to mammalian CYP1A1 [42]. In contrast, SRS4, SRS5, and SRS6 exhibit higher than average similarity scores between zebrafish CYP1D1 and zebrafish CYP1A. These three SRSs have been shown to be highly conserved among CYP1A1 orthologs [43], implying importance in establishing substrate specificity, demonstrated for SRS5 in human CYP1A1 [44]. How the differences or similarities in the various SRS may affect substrate recognition differences between CYP1D1 and CYP1A is not yet known. Studies to identify other substrates of CYP1D1 are underway.

The sequence similarities and gene structures indicate a close evolutionary relationship between *CYP1D1* and *CYP1A*. However, unlike *CYP1A*, zebrafish *CYP1D1* is not induced by PCB126 or TCDD. There also is a relatively high level of expression of *CYP1D1* during early development in the zebrafish, which morpholino knockdown studies show does not involve regulation by AHR2. The high level of expression in early development and the lack of induction in embryos by potent halogenated AHR agonists distinguish regulation of

CYP1D1 from that of other *CYP1s* in zebrafish, and suggests that *CYP1D1* could have endogenous functions.

The highest relative levels of *CYP1D1* expression occurred at the earliest time-point examined (9 hpf). The major activation of zygotic transcription occurs during the midblastula transition, at about 3.5 hpf, although some maternal transcripts have been shown to persist up to the segmentation stage (10 hpf) [45]. The initial high levels and subsequent decline of *CYP1D1* transcript expression to levels that were similar in later embryos and juveniles (at 57dpf) suggests that the early high levels could include some contribution of maternal transcript, or that *CYP1D1* plays a role in early development. However, further work will be required to examine these possibilities. In contrast, a recent study of *CYP1* gene expression in developing zebrafish showed that basal expression of *CYP1A* increases steadily during the first three weeks (from 8 hpf), peaking at day 21 at approximately 10 times the 3-dpf level and decreasing thereafter [18]. The maximal basal expression of *CYP1B1* occurs before hatching, i. e., at 24–48 hpf, while the expression of the *CYP1Cs* reaches a maximum around hatching, at 72–96 hpf.

In a high through-put in situ hybridization screen of expressed zebrafish genes, Thisse et al [46] identified a number of anatomical structures in the developing embryo that hybridized to the expressed probe zgc:92205. We determined that the sequence of this clone is identical to that of *CYP1D1*. Structures identified as expressing this clone (*CYP1D1*) at 19–24 hpf include the somites, portions of the cardiovascular system, the hypochord, and the floor plate. At 48 hpf, expression was detected in the liver, the neurocranium, and the floor plate. This expression of *CYP1D1* in the hypochord, neurocranium, and floorplate inferred from in situ hybridization [46] suggests there could be functional role(s) for *CYP1D1* in the developing nervous system. The expression in adult brain also suggests a possible neural involvement of *CYP1D1*. Consistent with that possibility, we found that the proximal promoter region of *CYP1D1* is enriched with putative neural-specific promoter elements (MYT1, NGFIC, NEUROD1), relative to *CYP1A*. In adult zebrafish, both *CYP1A* and *CYP1D1* exhibited a much higher basal expression in liver than in other organs, and we previously observed a stronger expression of *CYP1B1*, *CYP1C1*, and *CYP1C2* in heart and eyes than in other organs [17]. Whether there are overlapping or distinct enzymatic functions of the various *CYP1s* in these organs is unknown, as at present, there is little information on the substrate specificities of zebrafish *CYP1s*. Studies are currently underway to assess the substrate specificities of the five zebrafish *CYP1s*.

CYP1D1 differs from all of the other zebrafish *CYP1* genes in not being inducible by the potent AHR agonists PCB126 or TCDD. Zebrafish *CYP1A* and *CYP1B1* genes have numerous consensus XREs (22 and 6, respectively) in their promoter regions [17], a number of which have been demonstrated to be functional [38;39]. The *CYP1Cs* have fewer (4) XREs than do *CYP1A* or *CYP1B1*, and although the function of these XREs has not been determined, the *CYP1Cs* are inducible in embryos by AHR agonists [17;18]. The lack of putative functional XREs, based on the more stringent approach of Zeruth and Pollenz [39], helps explain the lack of *CYP1D1* induction by PCB126 or TCDD. The two core KNGCGTG motifs in the proximal *CYP1D1* promoter do not match the extended XRE sequence shown to be functional with respect to TCDD-induced trans-activation [39]. This suggests that *CYP1D1* may play a limited role, if any, in adaptive response to dioxin-like compounds in zebrafish. In contrast to the other *CYP1* family members, the lack of induction by PCB126 and TCDD indicates that distinct regulation, and presumably distinct functions, have evolved for the *CYP1D* subfamily. At present we cannot exclude possible induction by other compounds, via other xenobiotic receptors and response elements. Whether *CYP1D1* expression is induced by agonists for other xenobiotic receptors (e.g. PXR or PPAR) is under examination.

The properties of *CYP1D1* invite questions about the occurrence of orthologues of this gene in other vertebrates. Comparison of the *CYP1D1* sequence with human *CYP* sequences

revealed similarity to a previously identified human CYP pseudogene on chromosome 9, *CYP1A8P*. Preliminary studies uncovered substantial shared synteny between the zebrafish *CYP1D1* and human *CYP1A8P*, indicating that the two genes are orthologous. In light of this, the name of the human pseudogene has been changed to human *CYP1D1P*. The phylogenetic distribution of *CYP1D1* is under investigation.

In summary, we have cloned and expressed a new zebrafish CYP1 that is structurally similar to CYP1A. Zebrafish *CYP1D1* transcript is highly expressed during early development and is expressed in embryonic and adult neural tissues as well as in liver, heart and gill and other organs. CYP1D1 protein is expressed in zebrafish, but unlike all other vertebrate *CYP1* genes examined to date, *CYP1D1* expression does not appear to be regulated by the AHR (AHR2) that mediates induction of other CYP1 genes in zebrafish. As a CYP1A-like gene that appears not to be induced by AHR agonists, CYP1D1 may possess functions with endogenous substrates, which could represent physiological substrates of the CYP1 family.

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Abbreviations used

AHR

aromatic hydrocarbon receptor

ARNT

aryl hydrocarbon receptor nuclear translocator

Ct

threshold cycle

CYP

	cytochrome P450
dpf	days post-fertilization
hpf	hours post-fertilization
MO	morpholino oligonucleotide
PCB126	3,3',4,4',5-pentachlorobiphenyl
SRS	substrate recognition site
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TL	Tupfel/Long fin
XRE	xenobiotic response element

Figure 1.

Deduced amino acid sequence alignment of zebrafish CYP1D1, human CYP1D1P, and zebrafish CYP1A showing nonsynonymous substitutions in the cloned CYP1D1 as compared to the genomic sequence (boxed) and the location of the putative substrate recognition sequences (underlined). Dots indicate identity in zebrafish CYP1A and human CYP1D1P relative to zebrafish CYP1D1. Inserted nucleotides in the CYP1D1P pseudogene are indicated by numbers.

Figure 2.

Phylogenetic tree of selected CYP1 amino acid sequences showing the relative position of CYP1D within the vertebrate CYP1A subclade. Numbers at nodal points are support values derived from Bayesian phylogenetic and maximum likelihood bootstrap analyses (5×10^6 generation and 1000 replicates, respectively; Bayes/ML support). Additional sequences presented in this phylogenetic tree include predicted sequences for medaka and stickleback CYP1B1, CYP1C1, CYP1C2, and stickleback CYP1A. Previously analyzed sequences include CYP1A, CYP1B, and CYP1C from *X. tropicalis*, and CYP1B and CYP1C from *G. gallus* [1].

Figure 3.

Zebrafish CYP1D1 and CYP1A similarity scores mapped across the amino acid sequence (ten residue running average of the BLOSUM62 score). Shown also are the location of the putative substrate recognition sites (SRS) in yellow and the secondary structure of the CYP1s (alpha-helices in red and beta-sheets in blue).

Figure 4.

Comparison of the gene structure for zebrafish *CYP1D1* and *CYP1A*. Expressed exons are black boxes, while striped boxes indicate untranslated regions. Shown also are the location of calculated XRE sequences (KNGCGTG); note that not all of the XRE sequences indicated for *CYP1A* have been shown to be active [39].

Figure 5.

Gene expression of *CYP1D* and *CYP1A* in developing zebrafish. Samples were taken during the first week of development (9–178 hours post fertilization; 25 individuals per replicate) and at 57 days post fertilization (1 per replicate). Data are presented with the mean relative expression level $E^{\Delta C_t} \pm$ standard error of the mean ($n=3$).

Figure 6. Organ distribution of *CYP1D1* and *CYP1A* gene expression in adult zebrafish. Data are presented as the mean relative expression level $E^{\Delta C_t} \pm$ standard error of the mean ($n=3$). Abbreviations: liver, L; brain, B; heart, H; gut, Gu; gill, Gi; kidney, K; eye, E.

Figure 7.

Relative expression of *CYP1D* and *IA* (fold control) following exposure to (A) 100 nM PCB126 in 57 days post-fertilization zebrafish, or (B) and (C) 0–100 nM PCB126 in zebrafish embryos (3 days post-fertilization). Statistical differences between controls and exposed groups were examined by Student's *t* test (A) and one-way ANOVA followed by Dunnett's (Figure 6B-C) multiple comparisons test (B and C); ** and *** indicates significant difference from controls at a level of $p < 0.01$ and $p < 0.001$, respectively ($n=3$).

Figure 8.

Relative expression of *CYP1D1* in acetone (carrier) or 100nM PCB126-treated 48 hpf zebrafish embryos (denoted “Ctrl” and “PCB126”) injected with an AHR2 morpholino (AHR2), a negative control morpholino, or not injected. No statistical differences were observed with oneway ANOVA in the morpholino treatments ($n=25$). No = not injected.

Figure 9.

Immunoblots of CYP1D1 in zebrafish hepatic microsomes (“liver”) and transformed yeast microsomes. (A) Immunoblot developed with anti-CYP1A MAb 1-12-3 (see Methods). (B) Immunoblot developed with affinity purified anti-zebrafish CYP1D1 polyclonal antibody. Yeast microsomal loadings are for equal levels of signal developed with mouse monoclonal antibody to the V5 epitope. The second bands of higher electrophoretic mobility in yeast microsomes could be the result of proteolytic degradation or alternative start sites for translation.