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Induction of cytochrome P450 family 1 mRNAs and activities in a cell line from the frog *Xenopus laevis*

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Abstract

Cytochrome P450 family 1 (*CYP1*) includes four subfamilies of enzymes: *CYP1A, CYP1B, CYP1C*, and *CYP1D*. In many vertebrates, *CYP1A, 1B*, and *1C* expression is induced by agonists of the aryl hydrocarbon receptor, including toxic contaminants such as chlorinated dioxins, coplanar chlorinated biphenyls, and polynuclear aromatic hydrocarbons. Assessed at the level of mRNA, protein, or enzyme activity, *CYP1s* (especially CYP1As) represent potent and popular biomarkers of contaminant exposure in aquatic vertebrates. Alkylated resorufins are synthetic substrates used to detect, quantify, and describe catalytic activities of cytochrome P450s. The ability to oxidize specific resorufin-based substrates can distinguish the catalytic activities of individual *CYP1s. Xenopus laevis*, the African clawed frog, is the most widely employed amphibian model in aquatic toxicology, yet the number, inducibility, and activities of *CYP1s* have not been systematically characterized in this species. Here we report the cloning of cDNAs encoding two new CYP1 family members, *X. laevis CYP1B* and *CYP1C*, along with an integrated assessment of the induction of alkyloxyuresorufin-O-dealkylase (AROD) activities and mRNA expression of four known *X. laevis CYP1s*: *CYP1A6, CYP1A7, CYP1B*, and *CYP1C*. Using XLK-WG, an *X. laevis* kidney epithelial cell line, we determined that EROD (ethoxyresorufin substrate) and MROD (methoxyresorufin) were both induced 3000- to 5000-fold following 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) exposure up to 250 nM, while BROD (benzyloxyresorufin) and PROD (pentyloxyresorufin) activity was not detectable regardless of TCDD treatment. TCDD induced *CYP1A6* and *CYP1A7* mRNAs by 2–3 orders of magnitude, while *CYP1B* and *CYP1C* were unchanged. The more potent AHR agonist, FICZ (6-formylindolo[3,2-*b*]carbazole), induced *CYP1B* up to 10-fold at concentrations between 0.1 and 250 nM, while *CYP1C* induction was less than 3-fold. *CYP1B* mRNA showed the highest constitutive mRNA expression, 5- to 75-fold greater than the other *CYP1* transcripts. Taken together, these results suggest that *CYP1A6* and *CYP1A7* perform the bulk of EROD and MROD activities we observed in these cells. The ability of each *X. laevis* CYP1 to catalyze oxidation of individual resorufin substrates remains to be determined. Correlating *CYP1* mRNA and induced AROD activity is a significant step toward clarifying the biochemical meaning of these biomarkers and the roles of CYP1 enzymes in *X. laevis*. The cell culture approach represents an important complement to the long standing use of frog embryos and tadpoles in toxicological studies, providing a well suited model system for determining the molecular mechanisms underlying the regulation of these important biomarkers of contaminant exposure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2012.02.028.

Conflict of interest

None.

Keywords

Cytochrome P450; CYP1 family; *Xenopus laevis*; FETAX; Ortholog; Aryl hydrocarbon receptor

1. Introduction

Enzymes comprising cytochrome P450 family I (*CYP1s*) bio-transform a wide range of xenobiotics and endogenous compounds. The *CYP1* family is comprised of four sub-families, *CYP1A, CYP1B, CYP1C* and *CYP1D*, paralog groups arising from a common ancestral gene (Goldstone et al., 2009). While *CYP1s* seemingly exist in all vertebrate groups, gene duplication and loss have resulted in distinct complements of functional *CYP1* genes in individual taxa. *CYP1A*s and *CYP1B*s exhibit wide taxonomical representation, but *CYP1C*s have not been detected in mammals, and *CYP1D*s are absent from the bird lineage (Jönsson et al., 2011b) and from many mammals, including humans (Uno et al., 2011). Taxonomic groups also differ in the number and evolutionary origin of individual paralogs within each *CYP1* subfamily. Mammals have two *CYP1A* paralogs, *CYP1A1* and *1A2*, the orthologs of bird *CYP1A4* and *1A5*, respectively (Goldstone and Stegeman, 2006). Most fish harbor a single *CYP1A* (Morrison et al., 1995, 1998), although salmonids (Rabergh et al., 2000) and eels (Mahata et al., 2003) have multiple *CYP1A*s that result from relatively recent, taxon-specific gene duplications. Multiple *CYP1C* paralogs have also resulted from teleost-specific gene duplications (Godard et al., 2005; Goldstone et al., 2007).

CYP1A, 1B, and *1C* expression is induced by agonists of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor (reviewed in Okey, 2007). Potent xenobiotic inducers include planar halogenated aromatic hydrocarbons (PHAH; e.g. TCDD, PCBs) and polynuclear aromatic hydrocarbons (PAH; e.g. benzo(*a*)pyrene), many of which are also *CYP1* substrates. Expression of *CYP1s*, especially *CYP1A*s, is a popular biomarker of aquatic animal exposure to these classes of toxic contaminants, and convenient measures exist to detect mRNA, immunoreactive protein, and enzyme activity (reviewed in Hahn, 2002).

CYP1s exhibit distinct but overlapping catalytic specificities for alkyl resorufin fluorescent substrates used in biomarker and kinetic studies (Burke and Mayer, 1983). Studies in mammals established 7-ethoxyresorufin dealkylation (EROD) as a predominantly *CYP1A1* activity, although *CYP1A2* and *CYP1B1* can also utilize this substrate (Burke et al., 1994; Crespi et al., 1997). The MROD assay (7-methoxyresorufin dealkylation) is associated with *CYP1A2* (Burke et al., 1994). Catalytic preferences of the orthologous enzymes in birds and fish suggest that extrapolating these canonical relationships to other animal groups is not necessarily straightforward. While EROD is a specific biomarker of *CYP1A4* activity in chickens (Rifkind et al., 1994; Sinclair et al., 1997), in the common cormorant (*Phalacrocorax carbo*) both *CYP1A4* and *1A5* exhibit substantial EROD activity, and catalytic specificity is instead defined by the high BROD (7-benzyloxyresorufin dealkylation) activity in *CYP1A4* and high MROD activity of *CYP1A5*. (Kubota et al., 2009). In zebrafish *CYP1A* exhibits the greatest rate of EROD activity, although *CYP1B1, 1C1* and *1C2* are substantially active as well. All four enzymes have roughly comparable MROD and BROD activity, while PROD (7-pentoxyresorufin dealkylation) is lacking only in *CYP1B1* (Scornaienchi et al., 2010). CYP1D1 performs all four AROD reactions poorly (Scornaienchi et al., 2010). Overall, it is clear that differences in catalytic preferences between CYP1 isoforms in different animal groups can present complications in the interpretation and comparison of biomarker expression data in toxicological studies.

In contrast to mammals, birds, and fishes, relatively little is known about the activities and expression of *CYP1s* in amphibians. In this study, we sought to characterize important features of the *CYP1* gene family in *Xenopus laevis*, the African clawed frog. *X. laevis* is a long standing model of vertebrate development in which morphological changes from fertilization through metamorphosis are well documented (Nieuwkoop and Faber, 1994). It is also a widely used aquatic model of developmental toxicity, including teratogenesis (e.g. the frog embryo teratogenesis assay – FETAX; American Society of Testing and Materials, 1998) and endocrine disruption (e.g. Degitz et al., 2005). Although *X. laevis* is insensitive to lethality induced by TCDD (2,3,7,8 tetrachlorodibenzo-*p*-dioxin) and other AHR agonists (Gutleb et al., 1999; Jung and Walker, 1997) and *X. laevis* AHRs have relatively low affinity for TCDD (Lavine et al., 2005), this species nonetheless expresses two *CYP1A* mRNAs, *CYP1A6* and *CYP1A7*, which are inducible by 3-methylcholanthrene (Fujita et al., 1999), TCDD (Lavine et al., 2005) and FICZ (6-formylindolo[3,2-*b*]carbazole), a potent candidate endogenous AHR ligand (Laub et al., 2010). Like the numerous other closely related paralogs in *X. laevis, CYP1A6* and *1A7* likely arose from a species-specific genome duplication event approximately 30 mya (Hughes and Hughes, 1993). *Xenopus tropicalis*, a diploid congeneric, harbors only one *CYP1A* gene. One gene each of *CYP1B1* and *CYP1C1* were recently shown to be up-regulated in *X. tropicalis* tadpoles exposed to PCB126 (Jönsson et al., 2011a). As in other vertebrates, expression of the single *X. tropicalis* CYP1D1 was not affected (Jönsson et al., 2011a). Taken together, observations in frogs raise questions about the number, orthology, and inducibility of additional *CYP1s* in *X. laevis*, the much more widely used toxicological model, as well as the correlation between mRNA expression and the inducibility of AROD activities commonly used as biomarkers in aquatic toxicology. The objective of this study was to resolve these questions. Here we report the cloning of *CYP1B* and *CYP1C* cDNAs from this species, as well as induction profiles of AROD activity biomarkers in XLK-WG, a convenient *X. laevis* cell line derived from kidney epithelium.

2. Materials and methods

2.1. Cell culture

XLK-WG and Hepa1c1c7 cells were obtained from the ATCC (Manassas, VA; Martin et al., 1998). XLK-WG cells were maintained at 30 °C with 5% CO_2 in RPMI-1640 medium (ATCC) plus 20% fetal bovine serum (Invitrogen). Hepa1c1c7 cells were maintained at 37 °C with 5% CO_2 in α-MEM medium (Sigma, St. Louis, MO) with 10% fetal bovine serum. Cells were routinely cultured in 75 cm^2 plastic flasks (Greiner). To prevent formation of FICZ from exposure to fluorescent light, media were stored and warmed in dark conditions, including amber and/or foil-wrapped vessels, and culture hood lights remained off during all procedures (Diani-Moore et al., 2006; Oberg et al., 2005).

FICZ (95% purity) was obtained from Biomol (Plymouth Meeting, PA). TCDD (purity 97–99% purity) was obtained from Ultra Scientific (Kingstown, RI).

2.2. Enzyme activity assays

CYP1 activity was measured in whole cells using alkylresorufin-O-deethylase (AROD) assays (Kennedy and Jones, 1994). Substrates used in these studies were 7-ethoxyresorufin (the EROD assay), 7-methoxyresorufin (MROD), 7-benzyloxyresorufin (BROD), and 7-pentyloxyresorufin (PROD). Conditions were similar to those described in Hestermann et al. (2000). XLK-WG and Hepa1c1c7 cells were seeded in 96-well plates (Greiner, black wall/clear bottom and lid) with 250 μl medium (40,000 cells/well) and grown for 24 or 45 h before being dosed with TCDD, FICZ, or DMSO vehicle (0.25%). For XLK-WG cells, exposures ranged from 0.1 nM to 500 nM; for Hepa1c1c7 cells, concentrations ranged from

1 pM to 10 nM. Treatment lasted 3 or 24 h so that the total time for cell growth was 48 h. Cell viability was verified in representative wells by trypan blue staining. Cells were washed with 250 μl/well 1x PBS and subsequently treated with 100 μl/well 2 μM 7-ethoxyresorufin for 30 min. Resorufin was detected using a Gemini EM multi-well fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 530 and 586 nm, respectively. The addition of 75 μl/well fluorescamine (175 mg/ml) in acetonitrile was used to stop the reaction and measure protein content using the excitation and emission wavelengths of 400 and 460 nm, respectively (Kennedy and Jones, 1994). A standard curve used to determine resorufin and protein concentrations was constructed using known concentrations of resorufin and bovine serum albumin (Promega). Alkylresorufin substrates and resorufin were purchased from Anaspec (San Jose, CA).

Statistical analysis of all dose-response studies was completed using Prism 4.0b (GraphPad, San Diego, CA). EC_{50} values were determined via non-linear regression of the fractional response (Poland and Glover, 1975), constraining the background response to 0 and the maximal response to 1. All assays in XLK-WG cells were replicated three to four times, and Hepa1c1c7 experiments were replicated at least twice.

2.3. RNA extraction

Prior to exposure, cells were seeded in 25 cm^2 flasks (Greiner) and grown to near confluence. Cells were exposed to graded concentrations of TCDD or FICZ dissolved in dimethylsulfoxide (DMSO) for 24 h or 3 h, respectively. For XLK-WG cells, exposures ranged from 0.1 nM to 500 nM; for Hepa1c1c7 cells, concentrations ranged from 1 pM to 10 nM. Control cells were exposed to an equal volume of DMSO (0.25% final concentration). Total RNA from each flask was extracted using QIAshredder spin columns and RNeasy kits (Qiagen, Valencia, CA) as directed by the manufacturer.

2.4. Amplification and cloning of X. laevis CYP1B and CYP1C

Partial cDNAs encoding *CYP1B* and *CYP1C* were amplified by RT-PCR using the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems) with degenerate primers. Degenerate primers were designed based on conserved sequences of several vertebrate *CYP1s* (Table S1). Predicted sequences of *X. tropicalis CYP1B* and *CYP1C* were used to reduce degeneracy where possible, and inosine was inserted in select positions where 4-fold degeneracy remained (Wilkie and Simon, 1991). Reverse transcription of 1 μg total RNA was primed with random hexamers as directed by the manufacturer. PCR conditions were: 95°/10′; 43 cycles of [95°/15″; 50°/30″; 72°/60″]; 72°/7′. RT-PCR products were cloned into pGEM-T Easy (Promega) and sequenced. Blastx searches revealed that partial cDNAs for both *CYP1B* and *CYP1C* had been isolated.

Partial cDNA sequences were used to design gene-specific primers (Table S2) for the amplification of 5′ and 3′ ends of each cDNA using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA) under the following cycling conditions: 94 °C/2 min; 30 cycles of [94°/30″; annealing/30″; 72°/3′]; 72 °C/7′. Annealing temperatures varied with primer sequence.

Full length contiguous sequences were determined for each cDNA with the phred/phrap/cross-match algorithms in MacVector Assembler (MacVector, Cary, NC) using 3–10 clones of each overlapping PCR product. The existence of each contiguous DNA sequence was verified by amplifying each as a single PCR product encompassing the entire open reading frame using primers in Table S3.

Sequencing was performed by Retrogen (San Diego, CA) or by the University of Maine DNA Sequencing Facility (Orono, ME).

2.5. Sequence alignment and phylogenetic analysis

The translated *CYP1B* and *CYP1C* amino acid sequences were aligned with amino acid sequences for other CYP1 family proteins using CLUSTAL-W (Thompson et al., 1997) within MacVector 10.0.2. The Neighbor Joining algorithm (Saitou and Nei, 1987) with 100 bootstrap samplings was used to construct a phylogenetic tree. Sequences used in the phylogenetic analysis are indicated in Table S4.

2.6. Quantitative PCR

Total RNA was extracted as described above and treated with DNase using the Turbo DNAfree kit (Ambion). DNase-treated RNA (10 ng/PCR reaction) was converted to cDNA using random hexamers and Taqman Reverse Transcription Reagents (rMoMuLV reverse transcriptase; Applied Biosystems, Foster City, CA) before being quantified using Power SYBR Green PCR Master Mix (Applied Biosystems) with specific primers (Operon Biotechnologies). PCR conditions were the following: 95° C/10′; 50 cycles of [95° C/15 s, 60 °C/1 min]. Relative expression was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) in SDS v1.4 software (Applied Biosystems). Error is expressed as the range of possible relative expression values determined by the standard error of $\Delta\Delta C_t$. β-Actin was used as the endogenous control. Primer sequences for each transcript are indicated in Table S5.

3. Results

3.1. Inducibility of AROD biomarkers in XLK-WG cells

The existence of *X. laevis CYP1A6* and *1A7* genes was reported by Fujita et al. (1999), and the inducibility of their mRNAs by several AHR agonists has been amply demonstrated (e.g. Jelaso et al., 2003; Laub et al., 2010; Lavine et al., 2005). Less well examined have been the AROD activities associated with these or other frog *CYP1s*. Here we used XLK-WG, a kidney epithelial cell line (Martin et al., 1998), to demonstrate that EROD activity is induced by TCDD. Consistent with our previous observations (Laub et al., 2010), EROD induction in frog cells was three orders of magnitude less responsive than in Hepa1c1c7 (Fig. 1A; Table 1), a widely studied mouse hepatoma cell line. This difference is consistent with the low affinity of *X. laevis* AHRs for TCDD (Lavine et al., 2005). The mouse cells also exhibited around 5-fold greater maximal reaction rate than XLK-WG (Fig. 1B and C). We next examined the TCDD-inducibility of MROD activity in the same cell lines. TCDD was a dramatically less potent MROD inducer in the frog cells than in the mouse cells (more than 5000-fold; Fig. 2A, Table 1). The two cell lines exhibited less than 3-fold difference in the maximum reaction rates (Fig. 2B and C). Induction of BROD and PROD, activities observed previously in zebrafish CYP1 enzymes (Scornaienchi et al., 2010), was not detected in our assays with TCDD-treated XLK-WG cells (detection limit <0.5 pmol/mg protein/min; data not shown).

3.2. Cloning and phylogenetic analysis of CYP1B and CYP1C from X. laevis

To begin understanding the relationship between AROD activities and expression of *X. laevis* CYP1 mRNAs, we sought to clone cDNAs encoding additional CYP1 family members expressed in XLK-WG cells. We designed degenerate primers using an alignment of multiple *CYP1B* and *1C* amino acid sequences, carefully considering the nucleotide sequences of *X. tropicalis CYP1s* to reduce primer degeneracy. We successfully isolated partial cDNAs encoding single orthologs of *CYP1B* and *CYP1C*. Remaining sequence of each cDNA was determined by 5′ and 3′ RACE. To verify its existence in the cells, each open reading frame was amplified as a single RT-PCR product (data not shown) and sequenced in full. The orthology of the encoded proteins was confirmed by phylogenetic analysis. *CYP1A6, CYP1A7, CYP1B*, and *CYP1C* appear in appropriate monophyletic

groups with orthologs from other species (Fig. 3). Perhaps surprisingly, the frog *CYP1B* and *1C* amino acid sequences bore greater resemblance to their respective orthologs in zebrafish than in chicken, another tetrapod (Tables 2 and 3). This divergence may reflect different selective pressures in the adaptation to terrestrial vs. aquatic habitats. Although zebrafish *CYP1C*1 and *1C2* are co-orthologs of *X. laevis CYP1C* (Fig. 3), the frog protein shares a slightly greater degree of identity with *CYP1C*1 (Table 3). *CYP1B* and *CYP1C* cDNA sequences have been deposited in GenBank with accession numbers JN089388 and JN089389.

3.3. CYP1 mRNA expression and inducibility by AHR agonists

We next used qRT-PCR to examine the inducibility of each *X. laevis CYP1* mRNA. After 24 h of exposure to TCDD, there was no evidence of *CYP1B* or *CYP1C* expression beyond the constitutive level (data not shown), while the substantial induction of *CYP1A6* and *1A7* at relatively high concentrations (data not shown) was consistent with our previously published observations (Laub et al., 2010; Lavine et al., 2005). We thus turned to FICZ, an endogenous AHR agonist that displays much greater potency than TCDD with both *X. laevis* (Laub et al., 2010) and mammalian AHRs (Wincent et al., 2009). Because FICZ is readily metabolized by XLK-WG and other cell lines, exposure time was reduced to 3 h to preserve the sensitivity of the response (Laub et al., 2010; Wincent et al., 2009). A representative experiment is shown in Fig. 4. FICZ induced *CYP1A6* mRNA more than 700-fold, and the maximal induction of *CYP1A7* was even greater (Fig. 4A and B). *CYP1B* mRNA was also clearly induced, although the degree of induction was dramatically lower (~10-fold, vs. 700- to 1500-fold for the *CYP1A*s; Fig. 4C). Surprisingly, *CYP1C* mRNA induction was barely detectable, even at the high concentrations of FICZ used in these experiments.

Fig. 4 depicts induction of each *X. laevis CYP1* mRNA relative to its own constitutive expression. To compare mRNA levels between paralogs, we repeated the experiments using a single 96-well plate and normalized expression to *CYP1C*, the least abundant transcript. A representative experiment is show in Fig. 5. Presuming equal efficiency of each primer set, these experiments reveal that *CYP1B* mRNA was 5- to 75-fold more abundant than the other transcripts (Fig. 5A). In contrast, *CYP1A7* mRNA was most highly expressed following FICZ treatment (Fig. 5B). The degree to which mRNA expression relates to actual protein expression will require the development of specific antibodies for each protein. Meanwhile, it seems reasonable to hypothesize that *CYP1C* makes a minimal contribution to induced EROD and MROD activities, while *CYP1A6* and *1A7* likely contribute the bulk of these activities in XLK-WG cells. Although *CYP1B* mRNA is expressed at relatively high levels in untreated cells, these cells exhibit very little constitutive AROD activity (Figs. 1 and 2), suggesting a minimal role for the encoded enzyme.

4. Discussion

CYP1 induction represents an extensively used biomarker of exposure of vertebrates to contaminant ligands of the AHR (Hahn, 2002). In this study we sought to identify inducible CYP1 genes from *X. laevis*, the most widely employed amphibian model in aquatic toxicology, and to discern the relationship between the induction of *X. laevis CYP1s* at the mRNA and enzyme activity levels.

4.1. CYP1 gene multiplicity and evolution

We identified two new members of the CYP1 family: *X. laevis CYP1B* and *CYP1C*. These complement the previously described *CYP1A6* and *1A7* (Fujita et al., 1999). Sequencing of multiple clones strongly indicated that only one form each of *CYP1B* and *CYP1C* were

present. *X. laevis* is a pseudotetraploid species (Hughes and Hughes, 1993), and it is perhaps surprising that we did not isolate duplicate forms of these genes. However, we have no reason to believe that our degenerate primers would favor one closely related paralog over another, and a similar approach taken by our lab successfully amplified multiple paralogs of both AHRs and both ARNTs in this species (Lavine et al., 2005; Rowatt et al., 2003). Furthermore, teleosts were also subject to lineage specific genome duplications (Hoegg et al., 2004; Meyer and Van de Peer, 2005), yet zebrafish (and other teleosts) have only one copy each of *CYP1A, 1B*, and *1D* (Godard et al., 2005; Goldstone et al., 2009). Thus, the existence of multiple *CYP1B*s and *CYP1C*s in *X. laevis* is not a foregone conclusion, and the paralogs resulting from the historic genome duplication in this species (Hughes and Hughes, 1993) are either not expressed in this cell line or lost altogether as functional genes. Until the search for *CYP1B* and *1C* paralogs is exhaustively concluded, we have refrained from assigning a number to the names of these genes, as has been done for their *X. tropicalis* orthologs *CYP1B1* and *1C1*.

CYP1D genes comprise the remaining CYP1 subfamily. A *CYP1D* ortholog exists in *X. tropicalis* (Jönsson et al., 2011a) and likely in *X. laevis* as well. Because CYP1D is not induced by AHR agonists in other species (Goldstone et al., 2009; Jönsson et al., 2011a, 2007; Zanette et al., 2009), we did not search for *X. laevis CYP1D* sequences in this study.

4.2. Induction of CYP1B and CYP1C mRNAs: X. laevis vs. X. tropicalis

Although *CYP1A6* and *1A7* mRNAs are strongly induced by TCDD in XLK-WG cells, we were unable to detect induction of *CYP1B* and *CYP1C*, except by the more potent AHR agonist, FICZ. In contrast, Jönsson et al. (2011a) demonstrated that PCB126 (>100 nM) induced both *CYP1B1* and *CYP1C*1 in *X. tropicalis* prometamorphic tadpoles. The WHO TEF value for PCB126 is only 0.1 (van den Berg et al., 1998). It is thus surprising that expression of these genes was not responsive to TCDD in the *X. laevis* cell line. This discrepancy could reflect differences in the PCB126 affinity of AHRs from each species, species-specific differences in promoter structure and/or chromatin conformation, or tissue-specific effects. Previous studies in our lab using transactivation assays in COS-7 cells (M.E. Kalnoske and W.H. Powell, unpublished data) demonstrated the capacity of *X. laevis* AHR1α and AHR1β to mediate PCB126-induced transcriptional activation of luciferase under control of the mouse CYP1A1 enhancer region (Garrison et al., 1996). In zebrafish kidney, neither *CYP1C1* nor *1C2* is strongly induced by PCB 126 (Zanette et al., 2009), suggesting that low inducibility of *CYP1C* in XLK-WG cells may reflect similar kidney-specific regulation in *X. laevis*.

4.3. CYP1 mRNA vs. AROD expression

Based on parallel expression patterns of mRNA and enzyme activities, we suggest that *CYP1A6* and/or *CYP1A7* perform the bulk of EROD and MROD induced by AHR agonists in XLK-WG cells. Heterologous expression will be necessary to determine the catalytic preferences of the individual enzymes. Meanwhile, examination of the amino acid sequences at positions homologous to the active site residues of orthologous enzymes allows development of some hypotheses. Valine at position 382 of human CYP1A1 [within putative sequence recognition site 5 (SRS-5; Gotoh, 1992)] is associated with alkyl resorufin substrate specificity. *CYP1A2* contains leucine at this position, and the reciprocal mutation of each enzyme switches the catalytic preference for ethoxy- or methoxyresorufin (Liu et al., 2004). *X. laevis CYP1A6* is identical to CYP1A1 with valine at this position (V390), while *CYP1A7* contains methionine (Fig. 6 and Figure S1). *CYP1A7* thus resembles chicken *CYP1A5*, which reportedly exhibits low AROD activity (Rifkind et al., 1994; Sinclair et al., 1997; Verbrugge et al., 2001), suggesting that *CYP1A7* may be similarly inactive with these substrates. Kubota et al. (2009) made a similar argument to explain the disparity in AROD

activities exhibited by *CYP1A5* in cormorants and chickens. Compared with *CYP1A4*, chicken *CYP1A5* preferentially metabolizes arachadonic acid epoxygenation and uroporphyrinogen oxidation (Rifkind et al., 1994; Sinclair et al., 1997). If methionine at this position contributes to catalytic preference for physiologically relevant substrates in *X. laevis CYP1A7*, it could represent an interesting case of convergent evolution, since *CYP1A7* and *CYP1A5* arose from different gene duplication events during vertebrate evolution (Goldstone and Stegeman, 2006 and Fig. 3).

5. Conclusions

In this study, we characterized the inducibility of EROD, MROD, BROD, and PROD activities in XLK-WG, a *X. laevis* cell line. These activities are biomarkers of CYP1 expression induced in vertebrates by exposure to a wide range of contaminants, including PHAH and PAH. We also cloned cDNAs encoding two CYP1 family members, *CYP1B* and *CYP1C*, comparing their induction by AHR agonists to the previously identified family members, *CYP1A6* and *CYP1A7*. Our observations demonstrate that *CYP1A6* and *1A7* mRNAs are much more strongly induced than *CYP1B* and *CYP1C* and thus suggest the hypothesis that the corresponding enzymes are likely responsible for the bulk of induced EROD and MROD activity in this cell line. Characterizing induced CYP1 transcripts and AROD activities represents a significant step toward clarifying the relationship of these mRNA and enzyme biomarkers of contaminant exposure in *X. laevis*, the most commonly used amphibian model system in aquatic toxicology. The tissue culture approach represents a valuable complement to the long standing use of frog embryos and tadpoles in toxicological studies, providing a well suited model system to determining the detailed molecular mechanisms underlying the regulation of these toxicologically important genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CYP1	cytochrome P450 family I
PHAH	planar halogenated aromatic hydrocarbons
PAH	polynuclear aromatic hydrocarbons
PCB	polychlorinated biphenyl
AHR	aryl hydrocarbon receptor
EROD	7-ethoxyresorufin-O-deethylase
MROD	7-methoxyresorufin-O-deethylase
BROD	7-benzyloxyresorufin-O-deethylase
PROD	7-pentyloxyresorufin-O-deethylase
AROD	7-alkyloxyresorufin-O-deethylase
FETAX	frog embryo teratogenesis assay – *Xenopus*

FICZ	6-formylindolo[3,2*b*]carbazole
TCDD	2,3,7,8 tetrachlorodibenzo-*p*-dioxin

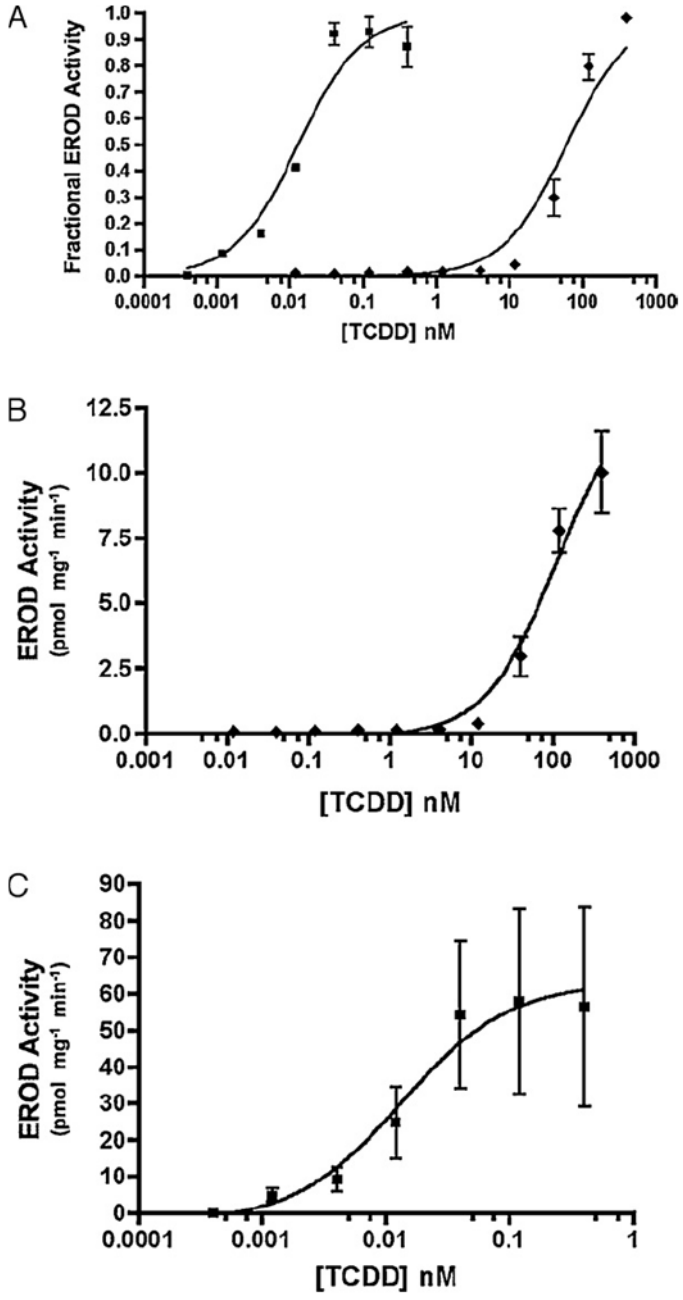

Fig. 1.

TCDD-induced EROD activity in XLK-WG (frog) and Hepa1c1c7 (mouse) cells. (A) Fractional induction of EROD activity in Hepa1c1c7 (mouse; squares) and XLK-WG (frog; diamonds). Fractional activity is normalized to the maximum value in each dose response, which is defined as 1.0. (B) Actual EROD activity in XLK-WG cells. (C) Actual EROD activity in Hepa1c1c7 cells. Error bars indicate standard error. $n = 2–4$.

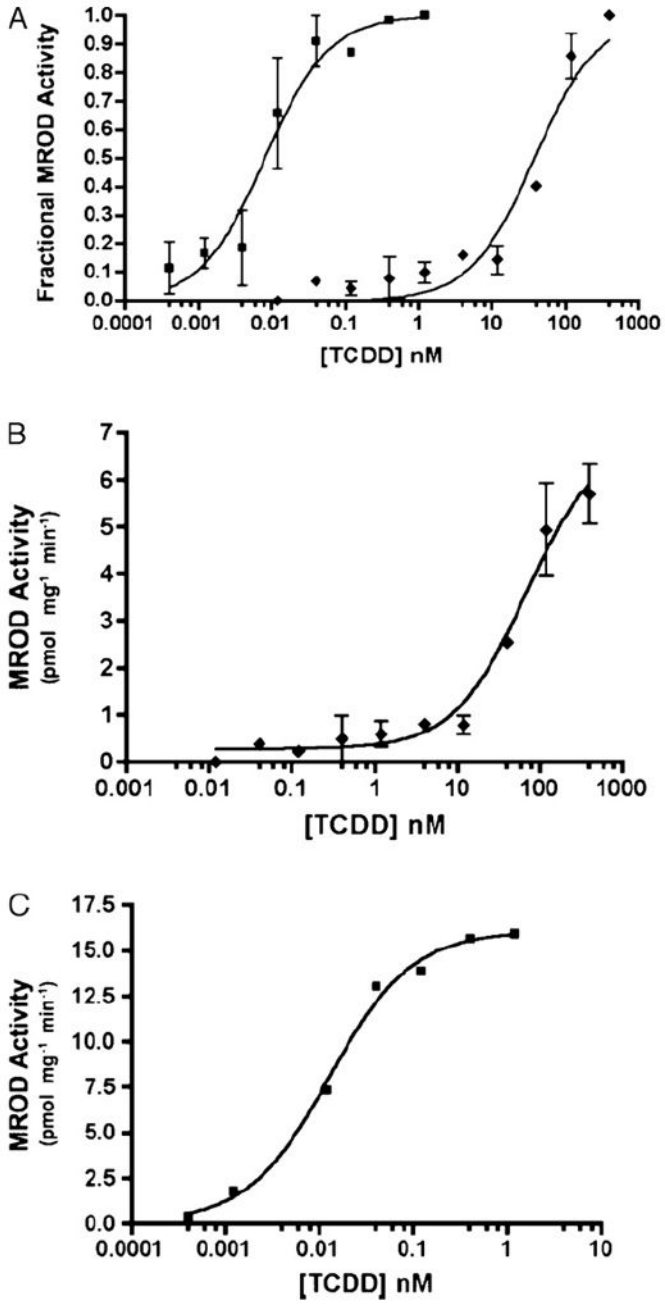

Fig. 2.

TCDD-induced MROD activity in XLK-WG (frog) and Hepa1c1c7 (mouse) cells. (A) Fractional induction of MROD activity in Hepa1c1c7 (mouse; squares) and XLK-WG (frog; diamonds). Fractional activity is normalized to the maximum value in each dose response, which is defined as 1.0. (B) Actual MROD activity in XLK-WG cells. (C) Actual MROD activity in Hepa1c1c7 cells. Error bars indicate standard error. $n = 2–4$.

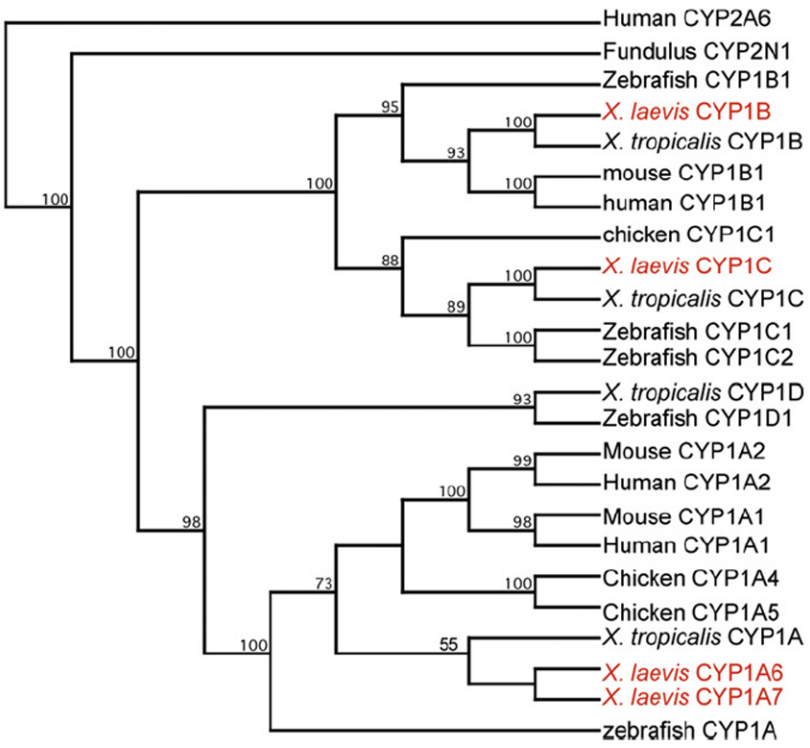

Fig. 3.

Phylogenetic analysis of *X. laevis* CYP1 sequences. Amino acid sequences of the indicated proteins were aligned, and a neighbor-joining tree was inferred using Clustal W. Numbers at the branch points represent the bootstrap values based on 100 samplings. Accession numbers of the sequences are found in Table S4 (online supplemental material).

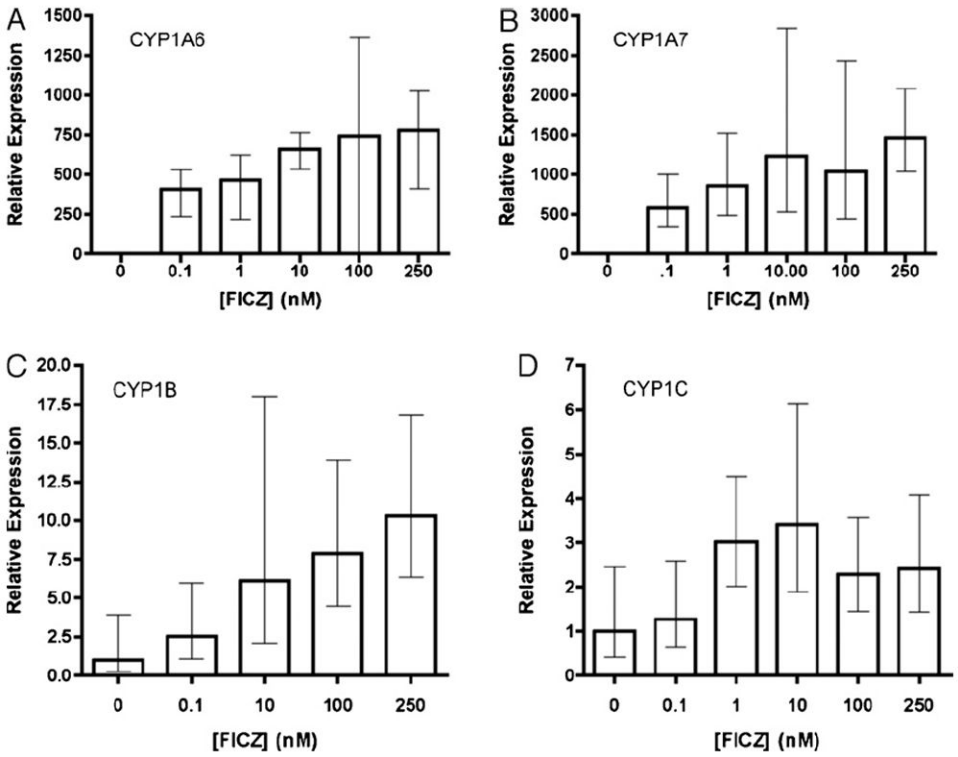

Fig. 4.

FICZ Induction of CYP1 mRNAs in XLK-WG cells. Near confluent cells were treated for 3 h with DMSO or the indicated concentrations of FICZ. Levels of the indicated transcript were determined by qPCR and calculated using the $2^{-\Delta\Delta C_t}$ method. Error bars define the range of fold induction defined by one standard error of the mean $\Delta\Delta C_t$. C_t values were normalized to the endogenous control β-actin. Experiments were repeated at least three times; a representative result is presented. (A) *CYP1A6*, (B) *CYP1A7*, (C) *CYP1B*, (D) *CYP1C*.

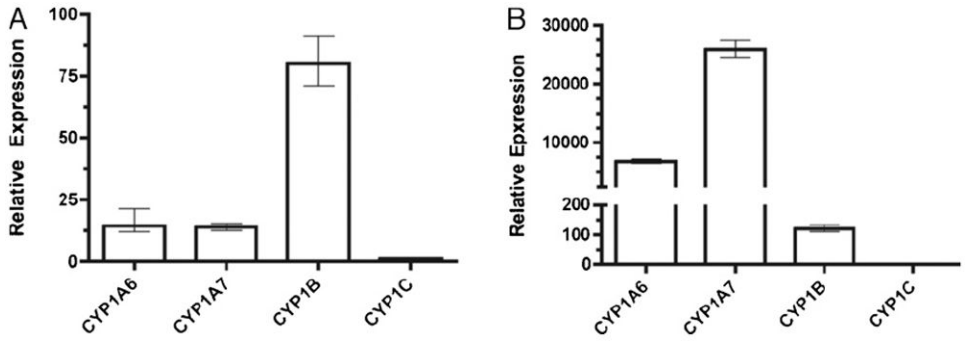

Fig. 5.

Relative expression of *CYP1s* in XLK-WG cells. Near confluent cells were treated for 3 h with (A) DMSO or (B) 250 nM FICZ. Levels of the indicated transcript were determined by qPCR and calculated using the $2^{-\Delta\Delta C_t}$ method. Error bars define the range of fold induction defined by one standard error of the mean $\Delta\Delta C_t$. C_t values were normalized to the endogenous control β-actin. Relative expression was normalized to *CYP1C*, the least abundant transcript. Experiment was repeated three times; a representative result is presented.

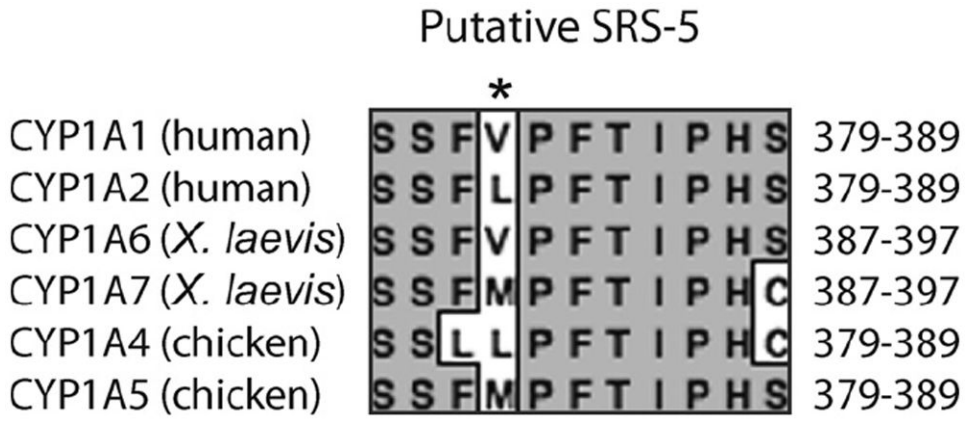

Fig. 6.

Amino acid alignment of putative SRS-5 from human, frog, and chicken CYP1As. Positions of included residues are indicated at right. *Position of variability that impacts AROD substrate preference in human CYP1A1 and 1A2. See Figure S1 (on-line supplemental material) for a complete amino acid alignment of these proteins.

Table 1

Twenty-four hour TCDD treatments on amphibian and mammalian cell lines exhibit distinct EC_{50} values in EROD and MROD assays (half-maximal induction of each activity). Variability expressed as standard error. n = 2–4 per assay.

	XLK-WG	Hepa1c1c7
EROD EC_{50} (nM)	73.7 ± 8.3	0.019 ± 0.006
MROD EC_{50} (nM)	96.1 ± 25.3	0.017 ± 0.002

Table 2

Percent amino acid identity between *Xenopus* spp., chicken (*Gallus gallus*), and zebrafish (*Danio rerio*) *CYP1B* proteins.

	***X. tropicalis* 1B1**	***G. gallus* 1B1**	***D. rerio* 1B1**
X. laevis 1B	81	36	50
X. tropicalis 1B1		38	52
G. gallus 1B1			35

Table 3

Percent amino acid identity between *Xenopus* spp., chicken (*Gallus gallus*), and zebrafish (*Danio rerio*) *CYP1C* proteins.

	***X. tropicalis* 1C1**	***G. gallus* 1C1**	***D. rerio* 1C1**	***D. rerio* 1C2**
X. laevis 1C	91	54	60	52
X. tropicalis 1C1		54	59	53
G. gallus 1C1			50	44
D. rerio 1C1				69