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Cytochrome P450 20A1 in zebrafish: Cloning, regulation and potential involvement in hyperactivity disorders

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ABSTRACT

Cytochrome P450 (CYP) enzymes for which there is no functional information are considered "orphan" CYPs. Previous studies showed that CYP20A1, an orphan, is expressed in human hippocampus and substantia nigra, and in zebrafish (*Danio rerio*) *CYP20A1* maternal transcript occurs in eggs, suggesting involvement in brain and in early development. Moreover, hyperactivity is reported in humans with chromosome 2 microdeletions including *CYP20A1*. We examined CYP20A1 in zebrafish, including impacts of chemical exposure on expression. Zebrafish CYP20A1 cDNA was cloned, sequenced, and aligned with cloned human CYP20A1 and predicted vertebrate orthologs. CYP20A1s share a highly conserved N-terminal region and unusual sequences in the I-helix and the heme-binding CYP signature motifs. *CYP20A1* mRNA expression was observed in adult zebrafish organs including the liver, heart, gonads, spleen and brain, as well as the eye and optic nerve. Putative binding sites in proximal promoter regions of *CYP20A1*s, and response of zebrafish CYP20A1 to selected nuclear and xenobiotic receptor agonists, point to up-regulation by agents involved in steroid hormone response, cholesterol and lipid metabolism. There also was a dose-dependent reduction of *CYP20A1* expression in embryos exposed to environmentally relevant levels of methylmercury. Morpholino knockdown of *CYP20A1* in developing zebrafish resulted in behavioral effects, including hyperactivity and a slowing of the optomotor response in larvae. The results suggest that altered expression of *CYP20A1* might be part of a mechanism linking methylmercury exposure to neurobehavioral deficits. The expanded information on CYP20A1 brings us closer to "deorphanization", that is, identifying CYP20A1 functions and its roles in health and disease.

1. Introduction

In animals, enzymes of the cytochrome P450 (CYP) superfamily (Nelson, 2009) catalyze oxidation reactions as well as reductions and rearrangements with a vast array of endogenous and exogenous compounds, often with high regio- and stereo-selectivity (Lamb and Waterman, 2013). These activities serve a wide range of physiological and toxicological functions. In vertebrate genomes, the number of protein-coding *CYP* genes range from around 40 to more than 100, in 19 gene families (Nelson et al., 2013). While functions are known for many human CYPs, the physiological substrate(s) and function of a substantial number of vertebrate CYPs remain unknown. In humans, these so-called "orphan P450s" notably include CYP4F22, CYP4V2 and CYP20A1 (Stark and Guengerich, 2007). In the model species zebrafish (*Danio rerio*), there are few CYPs for which function has been shown. Functions can be inferred for many CYPs in gene families 5–51, based on activities of known mammalian orthologs, yet predicted function has been confirmed in few cases (e.g., the lanosterol 14α-demethylase CYP51) (Morrison et al., 2014). In gene families 1–3, there has been much lineage specific expansion, resulting in co-orthologs, most with unknown function (Kubota et al., 2013). Thus, many zebrafish CYPs still are properly considered orphans, including CYP20A1.

Abbreviations: AHR, aryl hydrocarbon receptor; AR, androgen receptor; *arnt2*, aryl hydrocarbon receptor nuclear translocator 2; *CYP*, cytochrome P450; DES, diethylstilbestrol; dpf, days post-fertilization; *ef1α*, elongation factor 1 alpha; ER, estrogen receptor; ERR, estrogen-related receptor; FXR, farnesoid X receptor; GR, glucocorticoid receptor; HNF4, hepatocyte nuclear factor 4; hpf, hours post-fertilization; LXR, liver X receptor; MeHg, methylmercury; MO, morpholino; OMR, optomotor response; PCB, polychlorinated biphenyl; PPAR, peroxisome-proliferator-activated receptor; PR, progesterone receptor; PXR, pregnane X receptor; qPCR, real-time quantitative PCR; RAR, retinoic acid receptor; ROR, RAR-related orphan receptor; RXR, retinoid X receptor; SF1, vertebrate steroidogenic factor; TF, transcription factor; TL, Tupfel Long-fin zebrafish strain.

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The sole member of the CYP20 family, CYP20A1, is found in a single copy in human, zebrafish and other vertebrate genomes sequenced to date. The catalytic function of CYP20A1 is unknown. Recombinant human CYP20A1 has been tested for activity (Stark et al., 2008), however, no oxidation reaction was detected with several endogenous compounds (steroids, fatty acids, neurotransmitters) or exogenous chemicals (i.e., terfenadine, clotrimazole) that are substrates for some other CYPs. Although limited information is available on this orphan P450, it was found to be relatively highly expressed in human hippocampus and substantia nigra (Stark et al., 2008). These two brain regions are associated with learning and memory, and are involved in neurodegenerative diseases (Wirdefeldt et al., 2011; Zhou et al., 2008). High levels of *CYP20A1* transcript occur also in unfertilized eggs (Goldstone et al., 2010) and in notochord (Thisse and Thisse, 2004) of developing zebrafish, and during embryonic development of mouse (Choudhary et al., 2003).

An important observation derives from conditions associated with a microdeletion on chromosome 2 in humans. Patients diagnosed with microdeletions in the 2q33 chromosome region where *CYP20A1* is located variously show psychomotor retardation, hyperactivity and bouts of anxiety, among other conditions (Balasubramanian et al., 2011; Tomaszewska et al., 2013). The observations together suggest participation of CYP20A1 early in vertebrate development, and possible involvement in brain functions and behavior. However, these possibilities have not been examined experimentally.

In the present study, we employed zebrafish to examine features of *CYP20A1* that could bear on its functions. Zebrafish CYP20A1 was cloned and sequenced, and the inferred primary structure was compared to that of the cloned human enzyme, and to CYP20A1 coding sequences found in vertebrate genomes. We determined the organ distribution of transcript in adult zebrafish, and analyzed the transcriptional responses to agonists for several nuclear receptors and the aryl hydrocarbon receptor (AHR) that are prominent in regulating expression of a number of *CYP* genes in vertebrates (Honkakoski and Negishi, 2000). The latter analysis was complemented by a search for putative binding sites recurring in proximal promoters of vertebrate *CYP20A1* genes. We also conducted studies of *CYP20A1* mRNA expression in zebrafish embryos with the potent environmental toxicant methylmercury (MeHg). Finally, early-developing zebrafish were screened for morphological and behavioral effects resulting from knockdown of *CYP20A1* expression using morpholine-substituted oligonucleotides. The results together provide new information and perspective on the regulation and function of CYP20A1, including possible involvement in neurobehavioral disorders, and the effects of chemicals linked to such disorders.

2. Material and methods

2.1. Animals

Experiments were conducted on early-developing and sexually mature zebrafish (*Danio rerio*) of the Tupfel Long-fin (TL), Tropical 5D and AB strains, and were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution, Oregon State University and Universite Catholique de Louvain, respectively. The associated Animal Welfare Assurance Numbers are respectively A3630-01, A3229-01 and 1458701. Fish were euthanized by immersion in a bath of tricaine methanesulfonate (MS-222). Fish maintenance and breeding procedures were previously described (Jonsson et al., 2007a, 2007b).

2.2. cDNA cloning and sequencing

Primers were designed in the proximal 5′ and 3′ untranslated regions of zebrafish *CYP20A1* transcript [GenBank: NM_213332.1]. Primer sequences were 5′-CTGATGGTCATTGTAGACG-3′ (F) and 5′-TCATGGAT GTTGGAGTGG-3′ (R). We used the Advantage 2 PCR kit from Clontech (Mountain View, USA) to amplify the coding sequence from TL zebrafish liver cDNA with 1 μM of each primer; the thermal profile was: 94 °C for 1 min, [94 °C for 30 s, 58 °C for 3 min] for 35 cycles, and 68 °C for 7 min. PCR products were purified with the QIAquick Gel Extraction Kit from Qiagen (Valencia, USA) and cloned into pGEM-T Easy from Promega (Madison, USA) as per kit instructions. Mach-1 competent cells from Invitrogen (Carlsbad, USA) were transformed, and, following overnight incubation of selected ampicillin-resistant white colonies in liquid Luria-Bertani medium with antibiotic, plasmids were isolated with the QIAprep Spin Miniprep Kit from Qiagen (Valencia, USA). Three clones were sent to Eurofins MWG Operon (Louisville, USA) for forward and reverse sequencing with T7 primers (1 μg of plasmid DNA), and the full-length sequence was then assembled and aligned with [GenBank: NM_213332.1] using MacVector 12.0.2 (Oxford Molecular Group, Madison, USA).

2.3. Secondary structure prediction

Amino acid sequences of CYP20A1 proteins were retrieved from the Ensembl database (Flicek et al., 2013) and aligned with MacVector 12.0.2 software (Oxford Molecular Group, USA) (see Supplemental Fig. S1 for sequence identifiers). Note that the protein sequence inferred from the TL zebrafish transcript we cloned was fully identical to that predicted from the genome [Ensembl: ENSDARP00000003222]. The specific motifs of CYP20A1 secondary structure (i.e. α-helices, β-strands, transmembrane domain, signal peptide) were predicted with two different online softwares, and overlapping predictions were chosen to construct the consensual secondary structure of the orphan P450. The α-helices were predicted with NetSurfP 1.1 (Petersen et al., 2009) and Psipred 3.0 (Bryson et al., 2005) at a cut-off of 80%. Putative β strands were localized with PredictProtein (Rost et al., 2004) and BETApro (Cheng and Baldi, 2005) under default settings. Hypothetical transmembrane domains were identified with PredictProtein (Rost et al., 2004) and TMHMM 2.0 (Krogh et al., 2001) at a cut-off of 80%. The possible presence of a signal peptide in the N-terminal portion of CYP20A1 was investigated with pSORTII (Nakai and Horton, 1999) and SignalP 4.1 (Petersen et al., 2011) under default settings. The locations of the P450 signature motifs (i.e., I-helix, K helix and heme-binding region) were analyzed based on the information available in reviews (Sezutsu et al., 2013; Werck-Reichhart and Feyereisen, 2000). The motifs specific to zebrafish and human CYP20A1 protein also were compared to those of all other zebrafish and human protein-coding CYPs available in the Ensembl database (data not shown). A subset of the zebrafish CYPs was used as a basis for the comparison of CYP20A1 signature motifs shown in Table 1. The sequence identifiers for the selected non-CYP20 isoforms are shown in Supplemental Table S6.

Table 1
P450 signature motifs in CYP20A1 and other selected CYPs in zebrafish.

P450 isoform	I-helix motif [G/AGXE/DTX]	K helix motif [EXXR]	Heme binding motif [FXXGXR/HXCXG]
CYP20A1	AGCVIT	ETVR	F-SGSQACPE
CYP1A	AGFDTI	EIFR	FGLGKRRCIG
CYP2AA2	AGTDTT	EIQR	FSLGPRACLG
CYP3C1	GGYETT	ESMR	FGLGPRNCIG
CYP5A1	AGYETS	ESLR	FGAGPRSCVG
CYP7A1	ASQGNT	EAMR	FGSGVTKCPG
CYP7C1	ASVGNT	ESLR	FGSGATQCPG
CYP8A1	VTQGNA	ETLR	WGTEDNLCPG
CYP8B1	ASQGNT	ETLR	WGAGTTMCPG
CYP8B2	ASQGNT	ETLR	WGAGTTMCPG
CYP8B3	ASQGNT	ETLR	WGAGTTMCPG
CYP11C1	GGVDTT	ETLR	FGFGSRQCVG
CYP39A1	ASLANA	EAIR	FGGGKNQCPG
CYP51	AGQHTS	ETLR	FGAGRHRCIG

Underlined residues in some CYP isoforms (including CYP20A1) are those differing from the classical P450 signature motif reported in the heading of each column. See sources in Table S6.