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Enzymatic properties of human CYP2W1 expressed in *Escherichia coli* ☆

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Abstract

The human genome project revealed a new member of the P450 family 2, CYP2W1, which has orthologous form in other vertebrate species, suggesting CYP2W1's significant physiological function. Recently, it was reported that CYP2W1 can metabolize arachidonic acid. In this study, we isolated human CYP2W1 cDNA, and successfully expressed truncated CYP2W1 lacking N-terminal 20 amino acids in *Escherichia coli* cells. In the bicistronic expression system for human CYP2W1 and NADPH-P450 reductase, the formation of blue pigment, indigo, was observed in bacterial cultures. Based on this result, we revealed that CYP2W1 catalyzes the oxidation of indole. In addition, CYP2W1 showed monooxygenase activity towards 3-methylindole and chlorzoxazone. However, no activity was observed towards fatty acids including arachidonic acid. Further analysis using an *E. coli* expression system will reveal substrate specificity of CYP2W1 and why this P450 isoform is universally conserved in vertebrates.

Keywords: CYP2W1; Tissue distribution; Heterologous expression; Indole oxidation

The completion of the sequence of the human genome revealed the presence of 57 different active genes encoding cytochrome P450 and 58 pseudogenes [1]. To date, in families 1 and 2 eight orthologs have been identified: CYP1A1, CYP1A2, and CYP1B1 (family 1); CYP2E1, CYP2R1, CYP2S1, CYP2U1, and CYP2W1 (family 2). The high sequence identity of these CYP orthologs and their evolutionary presence in the Vertebrata indicate a significant physiological function. Until recently, CYP2R1, CYP2S1, CYP2U1, and CYP2W1 were considered as orphan P450s with no known function. Recently, CYP2R1 and CYP2U1 have been demonstrated to have vitamin D hydroxylase and fatty acid hydroxylase activities, respectively [2,3], and CYP2S1 has recently been demonstrated to metabolize retinoic acid [4]. Recent studies showing the fetal-specific expression of CYP2W1 in mice [5] and a much higher expression of CYP2W1 in gastric cancer than in normal tissue suggest that CYP2W1 is involved in development and differentiation. Karlgren et al. [6] suggest that CYP2W1 is potentially an important drug target during cancer therapy because CYP2W1 is specifically expressed in tumors. Thus, information on substrate-specificity of CYP2W1 appears to be not only physiologically but also pharmacologically important. Although Karlgren et al. [6,7] suggested that CYP2W1 can metabolize arachidonic acid, the expression level of CYP2W1 in HEK 293 cells appears too low to reveal its enzymatic properties.

In this study, we tried to express human CYP2W1 in *Escherichia coli* cells to reveal its enzymatic properties, since a lot of microsomal CYPs have been successfully expressed in *E. coli* [8–10]. In addition to full-length CYP2W1, truncated CYP2W1 lacking N-terminal 20 amino acids was examined expecting the increase of its expression level. We also examined co-expression of CYP 2W1with human NADPH-P450 reductase.

☆ *Abbreviations:* CYP, cytochrome P450; Δ2W1, N-terminal truncated CYP2W1; 2W1, full-length CYP2W1; CPR, NADPH-P450 reductase.

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Materials and methods

Materials. A cDNA panel from different human tissues (Multiple Tissue cDNA (MTC™) Panels) was purchased from Clontech (Palo Alto, CA). Primer DNAs were purchased from Proligo Japan KK (Kyoto, Japan) or SIGMA GENOSYS (Hokkaido, Japan). The DNA sequencing kit, BigDye® Teminator v3.1 Cycle Sequencing Kit was purchased from Applied Biosystems (Warrington, England). Pyrobest® DNA polymerase and restriction enzymes were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). Human lung cDNA library No. 104 and human small intestine cDNA library No. 104 were purchased from Takara Bio. Inc. (Shiga, Japan). The expression vector pCWori^{+} was kindly given by Professor M.R. Waterman (Vanderbilt University School of Medicine, Nashville, Tennessee, USA). Recombinant yeast microsomes containing human CYP2A6 were obtained from Sumika Chemical Service (Osaka, Japan). All other chemicals were of the best commercially available grade.

Real-time PCR analysis of CYP2W1 expression in human tissues. Primers for human CYP2W1 were designed to ensure specific amplification of a 183 bp fragment of the cDNA. The sequences of these primers were as follows: forward 5′-AGCAGGCTCTGCCCTACACAA-3′ and reverse 5′-GGGTCTGCCACTGTGTCTCATC-3′. Quantitative real-time PCR was performed using SYBR® *Premix EX Taq*™ (Takara). PCR amplifications were conducted in 20 μl reactions using 45 cycles using ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) in triplicate. Following amplification, a dissociation curve was performed on all PCR products to make sure specific PCR products were generated. The real-time PCR results were analyzed using the sequence detection system software vl.2.X (Applied Biosystems). The CYP2W1 expression levels were calculated using comparative CT method.

cDNA cloning and sequencing of human CYP2W1. N-terminal and C-terminal regions of human CYP2W1 were separately cloned from human small intestine cDNA library No. 104 with PCR methods. CYP2 W1 specific primers 5′-ATGGCCCTGCTGCTCTTGCTGTTC CTG-3′ and 5′-GGCACGGACCTCCTCGATCTTGCGCAGGAC-3′ for N-terminal region were used for PCR on the basis of the CYP2W1 cDNA sequence (GenBank Accession No. BC025761). The fragment corresponding to nucleotides 1–726 was amplified with PCR. The PCR amplifications were performed in the presence of 20 mM Tris–HCl (pH 8.3), 10 mM KCl, 6 mM $(NH_4)_2$ SO_4, 2 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mg/ml BSA, 1 μM of forward and reverse primers, 200 μM dNTPs, and 0.5 U of Pyrobest® DNA polymerase under condition that the samples were subjected to 30 cycles of 15 s at 98 °C, 30 s at 71 °C, and 65 s at 72 °C.

CYP2W1-specific primers 5′-CTAGGGCCTGGGCACCGCACAC AGG-3′ and 5′-TTGGGGTCCCCTGGCCTGCAGCTGTT-3′ for C-terminal region were used for PCR on the basis of the human CYP2W1 cDNA sequence (GenBank Accession No. BC025761). The fragment corresponding to 626–1473 was amplified with PCR. The PCR amplifications were performed in the presence of 20 mM Tris–HCl (pH 8.3), 10 mM KCl, 6 mM $(NH_4)_2$ SO_4, 2 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mg/ml BSA, 1 μM of forward and reverse primers, 200 μM dNTPs, and 0.5 U of Pyrobest® DNA polymerase under condition that the samples were subjected to 30 cycles of 15 s at 98 °C, 30 s at 64 °C, and 120 s at 72 °C. (see Fig. 1).

Construction of expression plasmids for human CYP2W1. For the expression of full-length CYP2W1, the nucleotide sequence encoding its N-terminal 5 amino acids was changed to the AT rich sequence without being changed amino acids sequence to expect enhancement of the expression level of CYP2W1 (Fig. 2). For the expression of truncated CYP2W1 lacking N-terminal 20 amino acids, nucleotide sequence encoding its N-terminal 6 amino acids was changed as shown in Fig. 2. Thus, primers 5′-CATATGGCTTTATTGCTTTTGCTGTTCCTGGGC CTCCTGGGG-3′ and 5′-GGCACGGACCTCCTCGATCTTGCGCAG GAC-3′ for N-terminal region of 2W1, and primers 5′-CATATGGCTC AAGATCCTTCTCCAGCTGCCCGGTGGCCCCC-3′ and 5′-GGCA CGGACCTCCTCGATCTTGCGCAGGAC-3′ for N-terminal region of Δ2W1 were used for PCR, respectively. The PCR amplifications were performed in the presence of 20 mM Tris–HCl (pH 8.3), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mg/ml BSA, 1 μM of forward and reverse primers, 200 μM dNTPs, and 0.5 U of Pyrobest® DNA polymerase under condition that the samples were subjected to 35 cycles of 15 s at 98 °C, 30 s at 65 °C, and 60 s at 72 °C.

The expression plasmids for full-length and truncated CYP2W1 were constructed as follows. The PCR fragment encoding the N-terminal region of full-length or truncated CYP2W1 with *Nde*I and *Nco*I sites at each side was subcloned into pUC19 digested with *Nde*I and *Nco*I. The resultant plasmid was digested with *Nde*I and *Nco*I to yield a *Nde*I–*Nco*I fragment. Likewise, the fragment encoding the C-terminal region with *Nco*I and *Hin*dIII was subcloned into pUC19, and digested with *Nco*I and *Hin*dIII to yield a *Nco*I–*Hin*dIII fragment. Both fragments encoding N-terminal

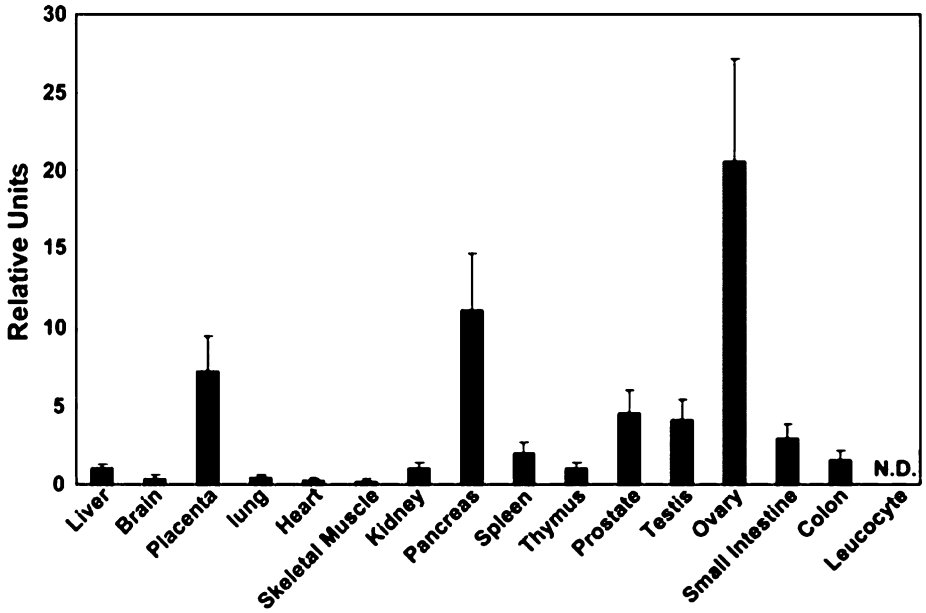

Fig. 1. Tissue distribution of human CYP2W1 mRNA by real-time PCR. The relative levels of CYP2W1 mRNA were determined by real-time PCR. Sixteen different human cDNAs were used as templates for real-time PCR with CYP2W1-specific oligonucleotide probes and SYBR Green PCR master mix. Each unit was compared to that of liver (=1). N.D. represents not detected.

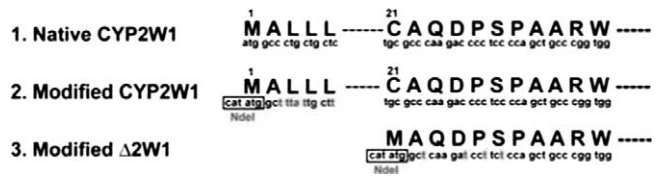

Fig. 2. The N-terminal amino acid and DNA sequences of full-length and truncated CYP2W1 for expression in *E. coli*.

and C-terminal regions were then inserted together into an expression vector pCW digested with *Nde*I and *Hin*dIII. The resultant plasmids for full-length and truncated CYP2W1 were designated as pC2W1 and pCΔ2W1, respectively. Moreover, the *Hin*dIII fragment encoding full-length human NADPH-P450 reductase was inserted into the *Hin*dIII site of pC2W1 and pCΔ2W1 to yield coexpression plasmids, pC2W1hR, pCΔ2W1hR, respectively.

Expression of human CYP2W1 in E. coli DH5α cells. *Escherichia coli* DH5α cells were transformed with each of the expression plasmids mentioned above, and cultured in TB medium containing 0.5 mM δ-amino-levulinic acid, 50 μg/ml ampicillin, and 1.0 mM IPTG for 40–48 h. Membrane fraction was prepared from recombinant *E. coli* cells according to the following procedure. The recombinant *E. coli* cells were harvested by centrifugation at 5000*g* for 5 min, and resuspended in 20 mM potassium phosphate buffer (pH 7.4). After centrifugation at 5000*g* for 5 min, the cells were resuspended in 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonylfluoride (PMSF). Cells were lysed by ultrasonication for 15 min at 0 °C with INSONATOR 201M (Kubota, Tokyo, Japan), and centrifuged at 3000*g* for 20 min. The resulting supernatant was centrifuged at 100,000*g* for 60 min to precipitate the membranes. The pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and stored at −80 °C.

Preparation of anti-CYP2W1 peptide antiserum. To produce human CYP2W1-specific antibody showing no cross-reactivity with other human CYPs, the peptide SLPTTPARAFTMRPRAQAL corresponding to amino acids 466–484 of CYP2W1 was selected. An additional cysteine residue was added to the amino-terminus of the sequence to conjugate the peptide with keyhole limpet hemocyanin (KLH). The synthetic peptide was conjugated to KLH through the amino-terminal cysteine of the peptide by using maleimidobenzoyl-*N*-hydroxysuccinimide. Rabbits were immunized with 0.2 mg of the KLH-peptide conjugates in emulsified Freund's complete adjuvant applied subcutaneously three times at 2-week intervals, and bled 2 weeks after the third injection. The antisera titers were monitored by an ELISA using the synthetic peptide conjugated with bovine serum albumin as a standard.

Western blot analysis. Membrane fractions prepared from recombinant *E. coli* cells were subjected to SDS–PAGE using a 10% gel and transferred to PVDF membrane. Immunodetection was done using an ECL Western blotting detection system (Amersham) with the anti-human CYP2W1 peptide antiserum and horseradish peroxidase-conjugated goat anti-(rabbit IgG) IgG.

Measurement of reduced CO-difference spectra. The reduced CO-difference spectra were measured with Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) according to the following procedure as described previously [11]. The concentration of human CYP2W1 was determined from the reduced CO-difference spectrum using a difference of the extinction coefficients at 446 and 490 nm of 91 $mM^{-1}cm^{-1}$[12].

Pigment production during cultivation. Cultures were sampled at various intervals up to 72 h. Aliquots of culture medium were extracted twice with ethyl acetate, and UV–visible spectra were measured. Indigo and indirubin concentrations were estimated using their authentic standards.

Measurement of indole oxidation by human CYP2W1. Membrane fractions prepared from DH5α/pCΔ2W1hR expressing Δ2W1 and human CPR, and yeast microsomes containing CYP2A6 and yeast NADPH-P450 reductase were used for metabolism of indole. The reaction mixture contains Δ2W1(60–120 nM) or CYP2A6 [13] and 0.5–5 mM indole in a final volume of 0.50 ml of 100 mM Tris–HCl buffer (pH 7.4). The reaction was initiated by the addition of 1 mM NADPH, and the reaction mixture was incubated at 37 °C for 0–60 min. Aliquots of the reaction mixture were extracted with four volumes of chloroform/methanol (3:1 v/v). The organic phase was recovered and dried in a vacuum evaporator centrifuge (Sakuma Seisakusyo, Tokyo, Japan). The resultant residue was solubilized with acetonitrile and applied to HPLC under the following conditions: column, YMC-Pack ODS-AM [4.6 mm × 300 mm] (YMC, Kyoto, Japan); UV detection, 240 nm; flow rate, 1.0 ml/min; column temperature, 40 °C; mobile phase, linear gradient of 20–100% acetonitrile aqueous solution containing 0.01% trifluoroacetic acid. The kinetic parameters, K_m and k_{cat}, were calculated by the nonlinear regression analysis using the KaleidaGraph (Synergy software).

LC-MS analysis of the metabolites. Isolated metabolites from HPLC effluents were subjected to mass spectrometric analysis using a Finnegan Mat TSQ-70 with atmospheric pressure chemical ionization, positive mode. The conditions of LC were described below: column; reverse phase ODS column (6 mm × 150 mm) (μBondapak C18, Waters); mobile phase, 80% methanol aqueous solution; flow-rate, 1.0 ml/min; UV detection, 240 nm.

Other methods. Total protein concentration was estimated by the method of Lowry et al. [14], using bovine serum albumin as a standard. The content of NADPH-P450 reductase was estimated from cytochrome *c* reduction activity as described previously [15].

Results

cDNA cloning and sequencing of human CYP2W1

The cloned CYP2W1 cDNA was 1473 bp in length containing one silent mutation at nucleotide position 166 (c → t). Thus, the cloned cDNA encodes 490 amino acids corresponding to the predicted amino acid sequence of human CYP2W1.

Distribution of CYP2W1 mRNA in human tissues

Real-time PCR analysis was performed to determine relative abundance of CYP2W1 in various human tissues. The cDNAs used for amplification were normalized based on the amounts of multiple housekeeping genes. As shown in Fig. 1, relative abundance of CYP2W1 mRNA was observed in ovary, pancreas, prostate, and testis. These results are similar to those reported by Choudhary et al. [5].

Expression of human CYP2W1 in E. coli

Human CYP2W1 was expressed in *E. coli* DH5α cells. Reduced CO difference spectrum of N-terminal truncated CYP2W1 (Δ2W1) in the membrane fraction showed a peak at approximately 455 nm indicating the presence of heme-containing Δ2W1 an active P450 enzyme (Fig. 3), while full-length CYP2W1 (2W1) showed no peak at around 455 nm. The expression level of Δ2W1 in DH5α/pCΔ2W1 and DH5a/pCΔ2W1hR was approximately 100 nmol/L culture. SDS–PAGE analysis of membrane fraction demonstrated the expression of CPR and Δ2W1, although no clear band corresponding to 2W1 was detected (Fig. 4). Western blot analysis confirmed the expression of Δ2W1, while no distinct bands of 2W1 were detected (Fig. 5).

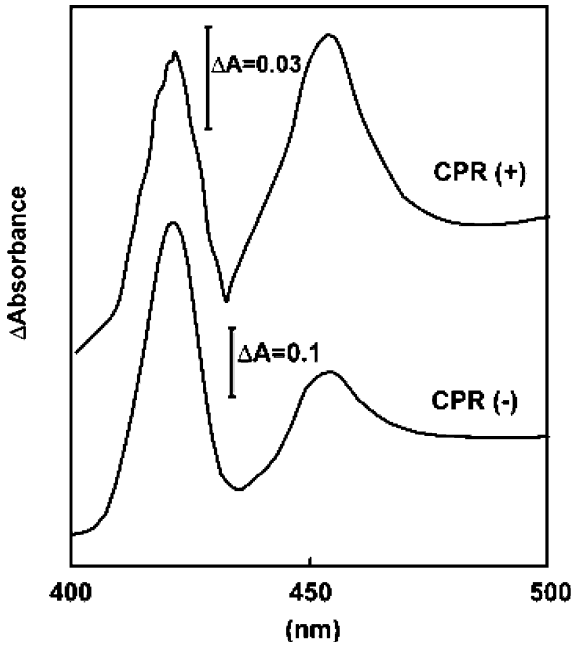

Fig. 3. Reduced CO difference spectra of membrane fractions prepared from the recombinant *E. coli* DH5α cells expressing Δ2W1. CPR(+) indicates coexpression with human NADPH-P450 reductase.

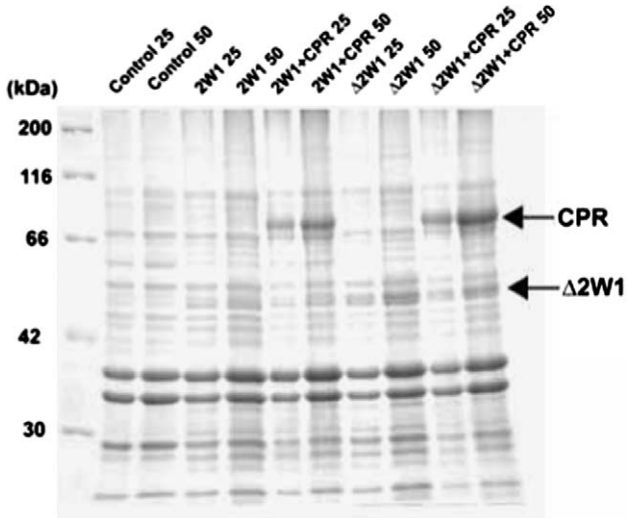

Fig. 4. SDS–PAGE analysis of the membrane fractions prepared from the recombinant *E. coli* DH5α cells expressing full-length (2W1) or truncated (Δ2W1) CYP2W1, with or without human NADPH-P450 reductase (CPR). The arrows indicate the putative migrating points of CPR and Δ2W1, respectively. Numbers 25 and 50 mean the amounts of the applied protein (μg).

Observation and characterization of blue cultures

In the bicistronic expression system for Δ2W1 and CPR, the appearance of bluish cultures was observed, while no blue pigment was seen in cultures only expressing Δ2W1. The blue color was kept in the bacterial pellet upon centrifugation of cultures, and membranes isolated by ultracentrifugation of bacteria were also colored blue. Gillam et al. [16,17] had reported that most of human P450s in family 2 catalyze the pigment formation. Thus, the same reactions appeared to occur in the bicistronic expression system for Δ2W1 and CPR. UV–visible spectrum of the blue extracts from the CYP2W1 cultures was similar to that of complex of indigo and indirubin (data not shown). HPLC analysis of the culture extract showed the peaks with the same retention times as authentic standards of isatin, oxindole, indirubin, and indigo (Fig. 6).

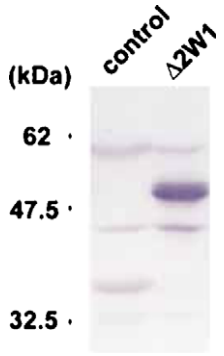

Fig. 5. Western blot analysis of truncated CYP2W1 (Δ2W1). Control and Δ2W1 indicate the control DH5α/pCW and DH5α/pCD2W1hR cells expressing both Δ2W1 and CPR, respectively.

Measurement of indole oxidation by human CYP2W1

Metabolism of indole by recombinant human CYP2W1 (Δ2W1) was examined. Fig. 7 shows HPLC profile of indole and its metabolite by Δ2W1. The retention time of the metabolite was identical to that of authentic standard of oxindole. In addition, LC-MS analysis revealed that the molecular weight of the metabolite was 133, identical to that of authentic oxindole (data not shown). These results strongly suggest that the metabolite is oxindole.

Kinetic analysis of oxidation of indole to oxindole by recombinant human CYP2A6 and Δ2W1 was carried out (Table 1). The k_{cat} value of CYP2W1 was half of that of CYP2A6, and the K_m value of CYP2W1 was 30 times higher than that of CYP2A6.

Discussion

Recently, Nelson et al. [1] reported that human CYP2W1 gene has some orthologous genes, mouse CYP2W1, dog CYP2W1, and cattle CYP2W1. They also predicted that orthologous genes are specific for endogenous substrates, whereas non-orthologous genes are likely to act on foreign substrates. They also suggested that human CYP2W1 is evolutionary older or has mutated more rapidly than the rest of the CYP2 genes in the light of phylogenetic analyses, indicating that human CYP2W1 might have been responsible for physiologically significant metabolism. The physiological and toxicological roles of CYP2W1 are still unknown. But human CYP2W1

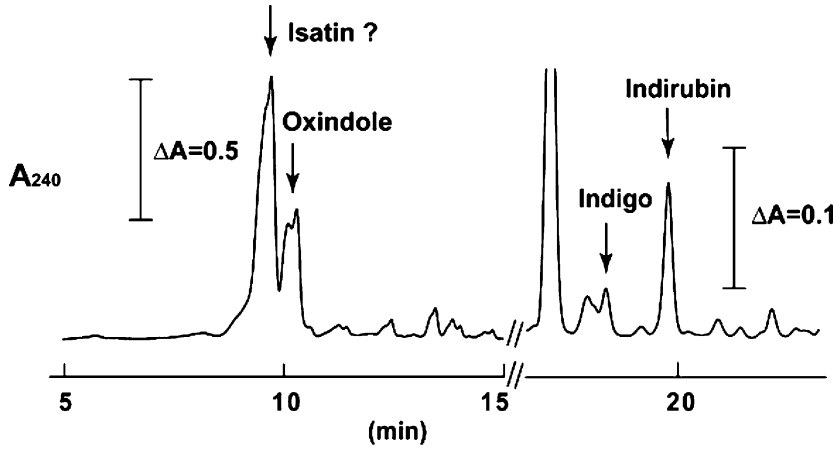

Fig. 6. HPLC profile of the extracts from the culture of the recombinant *E. coli* cells expressing Δ2W1 and CPR.

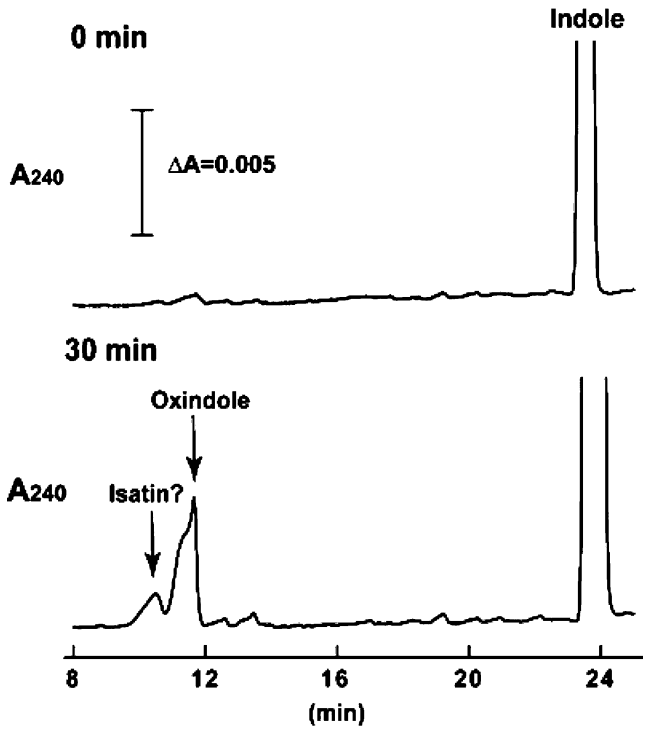

Fig. 7. HPLC profiles of indole and its metabolites formed in the membrane fractions containing Δ2W1 and CPR.

Table 1
Comparison of kinetic parameters for conversion of indole to oxindole between CYP2W1 and CYP2A6

	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1}M^{-1}$)
CYP2W1	2.0 ± 0.27	4.53 ± 0.30	461 ± 49
CYP2A6	4.02 ± 0.62	0.152 ± 0.046	$27{,}800 \pm 7800$

The k_{cat} and K_m values represent means ± SD from three separate experiments.

transcripts are more abundant in extrahepatic tissues (such as ovary, pancreas, placenta, prostate, and testis) than in hepatic tissue, suggesting CYP2W1's important function in the extrahepatic tissues. Recently, Du et al. [18] reported that the CYP2W1 gene is expressed in human epidermal keratinocyte cultures differentiated for 6 days. Skin is the largest and most accessible drug-metabolizing organ. Therefore, CYP2W1 may play an important role in metabolizing in cutaneous tissues. On the other hand, since mouse CYP2W1 showed fetal-specific expression [5], and that human CYP2W1 was expressed much higher in gastric cancer than in normal tissues [6], CYP2W1 activity might be involved in development and differentiation.

Recently, Karlgren et al. [6,7] suggested that CYP2W1 has monooxygenase activity toward arachidonic acid. However, the reported activity of CYP2W1 expressed in HEK293 cells appears uncertain, because control cells harboring an empty vector have a considerable activity toward arachidonic acid. Thus, we attempted to overexpress CYP2W1 in *E. coli* to examine its enzymatic properties. Generally, to overexpress microsomal P450s in *E. coli*, their N-terminal 20–30 hydrophobic amino acids should be deleted or changed. Expectedly, N-terminal 20 amino acids-truncated CYP2W1 (Δ2W1) was expressed at a high level while full-length CYP2W1 was not successfully expressed. Because extensive studies about genetically modified P450s indicated that the N-terminal deletion of microsomal P450s does not affect their enzymatic activities, it is reasonable to consider that Δ2W1 has nearly the same enzymatic properties as full-length CYP2W1.

We examined the metabolism of arachidonic acid by CYP2W1 expressed in *E. coli*. However, no metabolites were observed. Based on the detection limit, CYP2W1 activity toward arachidonic acid is less than 0.02 mol/min/mol P450. In addition, no activities for such fatty acids as docosahexaenoic acid, lauric acid, and retinoic acid were observed. The fact that CYP2W1 transcript was abundant in the genital organs such as ovaries, placenta, prostate, and testes suggests that CYP2W1 is involved in the metabolism of sex hormones. However, no CYP2W1 activities for sex hormones such as progesterone, estrone, estradiol, and testosterone were observed. On the other hand, we found bluish pigment formation in the culture of the recombinant *E. coli* cells expressing both Δ2W1 and human

NADPH-P450 reductase, suggesting that indigo was formed in the recombinant *E. coli* cells as reported by Gillam et al. [16,17]. These results also suggest that CYP2W1 can convert indole to oxindole or isatin. Expectedly, CYP2W1 showed monooxygenase activity toward indole, demonstrating that the CYP2W1 expressed in *E. coli* cells is an active form. Recently, Adachi et al. [19] reported that indirubin is a potent aryl hydrocarbon receptor ligand. Thus, it might be possible to assume that CYP2W1 produces a ligand of Ah receptor to regulate development and/or differentiation, although further studies are needed. The production and analysis of CYP2W1 knockout mice will help clarify the physiological role of CYP2W1. Recently, Karlgren et al. [6] reported a cancer-specific expression of CYP2W1, and suggested that CYP2W1 is potentially useful as a molecular marker for diagnosis and cancer therapy using a CYP2W1-specific prodrug. Thus, it appears that CYP2W1 is not only physiologically but also pharmacologically important.

By using an *E. coli* expression system, we successfully overproduced CYP2W1, and revealed its reliable substrate. Based on the results that CYP2W1 also showed monooxygenase activity for chlorzoxazone and 3-methylindole, compounds with indole ring are potent candidates for the substrates of CYP2W1. The coexpression system for CYP2W1 and CPR appears quite useful because whole cell culture produces a large amount of metabolites just as indirubin and indigo. Further analysis using the coexpression system will reveal the substrate specificity of CYP2W1 and why this P450 isoform is universally conserved in vertebrates.

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