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Complementary DNA cloning and characterization of cytochrome P450 2D29 from Japanese monkey liver

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Abstract

A cDNA was cloned from Japanese monkey liver mRNA by reverse transcriptase–polymerase chain reaction (RT–PCR) using oligonucleotide primers based on the marmoset cytochrome P450 2D19 (CYP2D19) nucleotide sequence. The full-length cDNA encoded a 497 amino acid protein (designated CYP2D29) that is 96, 91, and 88% homologous to human CYP2D6, cynomolgus monkey CYP2D17, and marmoset monkey CYP2D19, respectively. Yeast cells (*Saccharomyces cerevisiae* AH-22 strain) transfected with pGYR1 vectors containing the CYP2D29 cDNA were cultured, and microsomal fractions were obtained. Reduced carbon monoxide-difference spectra and western blot analysis using polyclonal antibodies raised against rat CYP2D2 demonstrated that in yeast cell microsomal fractions, the level of CYP2D29 holoenzyme was similar to that of CYP2D6 holoenzyme. However, western blot analysis indicated that the level of CYP2D29 in Japanese monkey liver microsomes might be much higher than that of CYP2D6 in human liver microsomes. Japanese monkey liver microsomes exhibited much higher activities than did human liver microsomes, expressed as nmol/min/mg protein, for debrisoquine (DB) 4-hydroxylation and bufuralol (BF) 1″-hydroxylation (typical reactions catalyzed by CYP2D6), whereas recombinant CYP2D29 activity, expressed as nmol/min/nmol CYP, was similar to that of CYP2D6 for DB and BF hydroxylation. In kinetic analyses, the K_m value of CYP2D29 for DB 4-hydroxylation was much lower than that of Japanese monkey liver microsomes, whereas the K_m value of CYP2D6 for DB 4-hydroxylation was similar to that of human liver microsomes. In contrast, K_m values for BF 1″-hydroxylation were similar for Japanese monkey and human liver microsomes and yeast cell microsomal fractions expressing recombinant CYP2D29 or CYP2D6. These results suggest that the properties of Japanese monkey CYP2D29 are similar to those of human CYP2D6, but their populations and/or some other factors in liver microsomes may cause the difference in microsomal DB 4-hydroxylase activities between Japanese monkeys and humans.
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Keywords: CYP2D29; CYP2D6; Japanese monkey; *Saccharomyces cerevisiae*; Debrisoquine; Bufuralol

1. Introduction

CYP2D6, one of the major CYP isoforms responsible for drug metabolism in human liver, is involved as a main enzyme in the major metabolic pathways of more than 20% of 250 clinical medicines that are often prescribed [1–4]. In addition, *CYP2D6* is known to show typical genetic polymorphism, and over 50 allelic variants of *CYP2D6* have been reported thus far [5]. Extensive experimental and epidemiological studies have revealed that 5–10% of Caucasians hereditarily lack the normal functions of the CYP2D6 enzyme, resulting in their suffering from serious adverse effects due to low hepatic clearance of medicines that are metabolized mainly by CYP2D6.

Many kinds of CYP enzymes belonging to the CYP2D subfamily have been found in mammals such as rats [6–10], mice [11,12], guinea pigs [13], dogs [14,15], cows [16], and rabbits [17]. Among the vast number of mammalian species, the monkey is the closest phylogenetically

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Abbreviations: CYP, cytochrome P450; DB, debrisoquine; 4-OH-DB, 4-hydroxy-debrisoquine; BF, bufuralol; 1″-OH-BF, 1″-hydroxybufuralol; BTL, bunitrolol; G-6-P, glucose 6-phosphate; fp_2, NADPH-cytochrome P450 reductase; RT–PCR, reverse transcriptase–polymerase chain reaction; and SRS, substrate recognition site.

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to the human, and various members of this species such as marmosets, cynomolgus monkeys, and rhesus monkeys have been used in drug-safety evaluation and biotransformation studies. However, there are clear species differences between monkeys and humans in phase I and phase II drug metabolic reactions [18,19]. Therefore, more detailed studies on the enzymological properties of drug-metabolizing enzymes in monkeys are necessary to understand the species differences between humans and monkeys, and to evaluate the increasing usefulness of various kinds of monkeys as alternative animals in the course of drug development.

Two genes of the CYP2D enzymes, *CYP2D17* in cynomolgus monkeys [20] and *CYP2D19* in marmoset monkeys [21], have been reported. Mankowski *et al.* [20] cloned CYP2D17 cDNA and expressed it in insect cells with human NADPH-dependent CYP reductase. The recombinant CYP2D17 protein showed catalytic properties similar to those of human CYP2D6 for typical reactions such as dextromethorphan *O*-demethylation and bufuralol hydroxylation [20]. Igarashi *et al.* [21] succeeded in cloning CYP2D19 cDNA, but did not report on the catalytic properties of the gene product.

We reported previously that liver microsomal fractions from Japanese monkeys are capable of catalyzing BTL 4-hydroxylation [22], a typical reaction catalyzed by human CYP2D6 [23]. Our results obtained from inhibition studies using antibodies and inhibitors indicated that at a substrate concentration of 10 μM, about half of the BTL 4-hydroxylase activity is due to CYP2D enzyme(s) and the remaining half to another enzyme(s) [22]. The CYP2D enzyme species in Japanese monkey livers remain to be characterized at the gene level.

In the present study, we have cloned the cDNA of a CYP2D enzyme (CYP2D29, named by the P450 Nomenclature Committee), which is clearly different from CYP2D17, in a female Japanese monkey liver and then expressed it in the *Saccharomyces cerevisiae* AH-22 strain. Its enzymatic properties are described and compared with those of CYP2D6.

2. Materials and methods

2.1. Chemicals

DB and 4-OH-DB as hemisulfates were obtained from Hoffmann-La Roche; racemic BF and 1″-OH-BF as hydrochlorides were from the Daiichi Pure Chemical Co.; G-6-P, G-6-P dehydrogenase and NADPH were from the Oriental Yeast Co., Ltd.; cytochrome *c* and phenylmethane sulfonylfluoride were from the Sigma Chemical Co.

2.2. cDNA cloning

Total RNA was isolated from female Japanese monkey liver using Isogene (Nippon Gene), and first-strand DNA was made using an RNA PCR kit (Ver. 2.1, Takara Biomedicals) according to the protocols of the manufacturer. A full-length cDNA encoding Japanese monkey CYP2D29 was initially isolated by PCR with the following oligonucleotide primers: 5′-TCCAGGGGTGTCCAGAGGAG-3′ (sense, nucleotide position: −42 to −23) and 5′-GGAACTACCACATTGCTTTA-3′ (antisense, nucleotide position: 1596 to 1615), which were designed based on the nucleotide sequences in the flanking regions of the marmoset CYP2D19 (GenBank Accession No. D29822).

PCR was performed with a TP2000 thermal cycler (Takara Biomedicals) in a reaction mixture (50 μL) containing 1 μL of a reverse transcriptase reaction mixture, 15 pmol each of sense and antisense primers, 0.2 mM each of four deoxynucleotide triphosphates, 1 U of KOD-*plus* DNA polymerase (Toyobo) and 5 μL of KOD-*plus* buffer under the following conditions: after an initial 2-min denaturation at 94°, the reaction was carried out for 35 cycles with a 15-sec denaturation at 94°, a 30-sec annealing at 50°, and a 90-sec extension at 68°. After the RT–PCR product was separated by 1.5% agarose gel electrophoresis, the band with *ca.* 1.5 kb that was visualized by ethidium bromide staining was excised from the gel, and the DNA was extracted and purified with a Prep-A-Gene DNA purification kit (Bio-Rad Laboratories). To obtain the authentic Japanese monkey sequences around the 5′- and 3′-ends of the coding region, the nucleotide sequence of the purified RT–PCR product was determined with a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and the following Cy5-labeled primers designed based on the marmoset CYP2D19 nucleotide sequence: 5′-GGAAGTCCACATGCAGCAGGTTGCC-3’ (antisense, nucleotide position 178 to 202) and 5′-GGCCCAGCCCTCACGGTGTCTTTGC-3′ (sense, nucleotide position 1469 to 1493).

The full-length cDNA was again made using the primers designed based on the nucleotide sequences determined as described above: JM-1, 5′-aaaagcttaaaaaaATGGAGCTAGATGCACTGGTGCCC-3′ and JM-2, 5′-gggaagcttgTTCTAGCGGGGCACAGCACAAAGCTC-3′; the CYP2D29 sequence is shown in capital letters, and the start and stop codons and the *Hin*dIII sites added to facilitate subcloning to pGYR1 are underlined. The six adenines just upstream of the ATG start codon in JM-1 were added to efficiently express the corresponding gene in yeast cells [24]. The PCR product was digested with *Hin*dIII and ligated into the same restriction enzyme site of pBluescript resulting in pBS2D29. Nucleotide sequences of both strands of the pBS2D29 insert were determined using appropriate Cy5-labeled sequence primers as described above. Moreover, the nucleotide sequences of the five additional cDNA clones, separately RT–PCR amplified using the same total RNA sample with primers JM-1 and -2, were also determined with the same results.

Sequence analysis was conducted with the Genetyx-Mac 9.0 software package (GENETYX Software Development Co.). The BLAST program of the Institute for Chemical Research, Kyoto University, was used to determine the homology of the predicted amino acid sequence of CYP2D29 to other CYP2D species.

2.3. Expression of Japanese monkey CYP2D29 in S. cerevisiae

CYP2D29 cDNA cut out with *Hin*dIII from pBS2D29 was subcloned into a yeast expression vector, pGYR1 [24]. The plasmid was used to transform the *S. cerevisiae* AH-22 strain by the lithium acetate method [25]. Cultivation of yeast cells was performed as previously described [26]. After centrifugation of the culture, the pellet was washed twice with buffer A containing 10 mM Tris–HCl (pH 7.5), 2 M D-sorbitol, 0.1 mM dithiothreitol, and 0.2 mM EDTA and digested with zymolyase in buffer A (0.5 mg/mL) for 2 hr at 35° by gently shaking. The pellet obtained after centrifugation at 2000 *g* for 10 min at 4° was washed again with buffer A before being resuspended in buffer B containing 10 mM Tris–HCl (pH 7.5), 0.65 M D-sorbitol, and 0.1 mM EDTA. After sonication in ice 10 times (30 sec each time), the microsomal fraction was prepared by centrifugation (105,000 *g* for 60 min at 4°) and suspended in buffer C containing 0.1 M sodium phosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. Recombinant CYP2D6 was expressed in yeast cells as reported elsewhere [26].

2.4. Assay of CYP2D holo- and apoproteins and assays of fp_2

The microsomal fractions were diluted to a protein concentration of 2 mg/mL with 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, and total holo-CYP contents were measured spectrophotometrically as reduced carbon monoxide (CO) spectra according to the method of Omura and Sato [27] using 91 mM^{-1} cm^{-1} as an absorption coefficient. Appropriate portions of yeast cell microsomal fractions were subjected to SDS–PAGE using a 10% slab gel. Following electrophoresis, proteins on the gel were electronically transblotted to a PVDF membrane, and analyzed by western blotting according to the method of Guengerich *et al.* [28] using polyclonal antibodies raised against CYP2D2 [9]. Relative degrees of staining of CYP2D protein bands on the membrane were assessed with NIH Image (version 1.2) run on a Macintosh 7300/180 computer with a Canon IX-4015 scanner. Yeast cell microsomal fp_2 was assayed by the method of Omura and Sato [27], in which the reduction of cytochrome *c* with NADPH was monitored at 354 nm. Protein concentrations were determined by the method of Lowry *et al.* [29].

2.5. Microsomal fractions from human and Japanese monkey livers

Human liver microsomal fractions [three samples, two male and one female Caucasians, ages ranging from 38 to 65] were supplied by the Human and Animal Bridge Discussion Group (HAB). This study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Okayama University. Liver samples from Japanese monkeys (*Macaca fuscata*, 1.5- to 4-years-old) were supplied by the Primate Research Institute, Kyoto University. Monkey liver microsomal fractions were prepared as described previously [22].

2.6. Assay of drug oxidation activities

DB 4-hydroxylase activity was measured by the HPLC method reported previously [30] with a slight modification. Briefly, a 500-μL incubation mixture in an Eppendorf-type tube (1.5 mL) contained 0.3 to 0.5 mg microsomal protein, 5 mM G-6-P, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH, and 100 μM DB in 100 mM potassium phosphate buffer (pH 7.4). After a 5-min preincubation at 37°, the reaction was started by adding NADPH and continued at 37° for 5 min. After the reaction was stopped by adding 50 μL of 60% $HClO_4$ aqueous solution and vortex mixing, the tube containing the reaction mixture was centrifuged at 5000 *g* for 10 min at room temperature. An aliquot (10 μL) of the supernatant was subjected to HPLC under the conditions described below.

BF 1″-hydroxylase activity was assayed by a similar HPLC method used previously for BTL 4-hydroxylase assay [30,31]. Briefly, a 500-μL incubation mixture in a brown glass conical tube (10 mL) with a stopper contained 0.3 to 0.5 mg microsomal protein, 5 mM G-6-P, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM NADPH, and 100 μM BF racemate in 100 mM potassium phosphate buffer (pH 7.4). After a 5-min preincubation at 37°, reaction was started by adding NADPH and continued at 37° for 5 min. After the reaction was stopped by adding 1 mL of 1 N NaOH aqueous solution and vortex mixing, 1 mL of 1 M sodium carbonate buffer (pH 9.6) and 20 μL of propranolol racemate (100 nmol) (internal standard) were added, and 1″-OH-BF was extracted into 5 mL of ethyl acetate by vigorous shaking. After centrifugation, 4 mL of the organic layer was taken in a new brown glass conical tube, and evaporated to dryness under an N_2 stream. The residue was dissolved in 100 μL of an HPLC mobile phase described below, and an aliquot (10 μL) was subjected to HPLC. In preliminary experiments, linearity was confirmed in product formation with time for each assay condition.

2.7. HPLC conditions

The HPLC conditions were: a Hitachi 655-12A liquid chromatograph equipped with a Hitachi L-7480 fluores-

cence detector, a Rheodyne model 7125 injector, and a Shimadzu C-R3A Chromatopac data processor; column, Inertsil ODS (4.6 × 250 mm, GL Science); mobile phase-A, acetonitrile/20 mM perchloric acid (pH 2.5) (15:85, v/v) for DB 4-hydroxylation; mobile phase-B, acetonitrile/20 mM perchloric acid (pH 2.5) (35:65, v/v) for BF 1″-hydroxylation; flow rate, 1.0 mL/min; detection, fluorescence 219/286 nm (excitation/emission) for DB 4-hydroxylation and 252/302 nm for BF 1″-hydroxylation. Kinetic analyses for DB 4-hydroxylation and BF 1″-hydroxylation were performed using a DB concentration range from 2.5 to 150 μM and a BF concentration range from 10 to 250 μM. Apparent Michaelis constants (K_m) and maximal velocities (V_{max}) were calculated using Lineweaver–Burk plots and least squares analysis.

3. Results and discussion

3.1. Sequence analysis

Fig. 1 shows cDNA nucleotide and deduced amino acid sequences of CYP2D29. The sequences have been registered to GenBank (GenBank Assession No. AF301911). The nucleotide sequence of CYP2D29 showed 97% identity to that of human CYP2D6, and 93 and 89% to those of cynomolgus monkey (CYP2D17) [20] and marmoset monkey (CYP2D19) [21], respectively. The multiple amino acid sequence alignment of CYP2D29 (Fig. 2) with those of cynomolgus monkey CYP2D17 and human CYP2D6 revealed that CYP2D29 is 96 and 91% identical to human CYP2D6 and CYP2D17, respectively. In contrast, CYP2D17 was 93% identical to CYP2D6 (Table 1). Thus, CYP2D29 is closer to human CYP2D6 than to CYP2D17. Goto [32] proposed that there are six SRSs in the amino acid sequence of CYP2D6, which come close to each other in the CYP2D6 conformation, forming its active site cavity where substrates are oxidized. Interestingly, the amino acid sequences of the six SRSs are completely identical between CYP2D29 and CYP2D6.

3.2. Expression in yeast cells

The content of CYP2D29 holoenzyme in yeast cell microsomes was determined with reduced CO-difference spectroscopy (Fig. 3). CYP2D29 showed a Soret peak at 450 nm, the shape of which was similar to that of CYP2D6 expressed in yeast cells (data not shown). The contents of total protein, CYP, and fp_2 in microsomes from yeast cells expressing CYP2D29 or CYP2D6 are summarized in Table 2. The total protein levels of CYP2D29-expressing and CYP2D6-expressing yeast microsomes were almost the same, and the ratio of fp_2 to CYP contents was from 1.3 to 1.9, indicating that the fp_2 protein was at a satisfactory level in each microsomal fraction.

The content of CYP2D29 in microsomal fractions from yeast cells and Japanese monkey livers was also determined by western blot analysis using polyclonal antibodies raised against CYP2D2, and compared with the contents of CYP2D6 in human liver microsomes (Fig. 4). When samples (50 μg protein each) were subjected to the analysis, microsomal fractions from yeast cells expressing recombinant CYP2D29 or CYP2D6, and from Japanese monkey livers gave a clear protein band with a molecular mass of about 50 kDa that cross-reacted with antibodies. However, the protein band in the human liver microsomes was relatively pale and scant compared with those of the other three samples. Relative amounts of the proteins on the membrane were estimated on a microcomputer using NIH-image as follows: human liver microsomes/recombinant CYP2D6/Japanese monkey liver microsomes/recombinant CYP2D29 (0.3:4.4:4.6:6.2). These results indicated that the level of the CYP protein(s) immunochemically cross-reacting with CYP2D2 antibodies is higher in Japanese monkey livers than in human livers.

Table 1
Identities of the nucleotide and deduced amino acid sequences of four primate CYP2D enzymes

	CYP2D6	CYP2D29	CYP2D17	CYP2D19
CYP2D6		97.3	94.1	91.6
CYP2D29	96.0		92.6	89.4
CYP2D17	93.4	90.5		91.0
CYP2D19	90.7	87.7	91.1	

Upper-right values: percentage identities of the nucleotide sequences. Lower-left values: percentage identities of deduced amino acid sequences.

Table 2
Levels of microsomal protein, CYP2D enzymes, and NADPH-cytochrome P450 reductase in yeast microsomal fractions

	Total protein content (mg/mL)	CYP content[a] (pmol/mg protein)	fp_2[b] (IU/mg protein)	Ratio[c] (fp_2/CYP)
rCYP2D6	44.6	46.7	0.262	1.87
rCYP2D29	67.9	47.2	0.180	1.30

Data are typical of results from two different experiments.
[a] Determined by reduced CO-difference spectroscopy.
[b] Determined by the reduction of cytochrome *c*.
[c] Estimated on the assumption that pure fp_2 has the activity of 20 IU/mg protein.

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                                                                CCGGGGTGTCCAGAGGAGCCCAGGGGGCAGTAAGGCAGCA   40

    ATGGAGCTAGATGCACTGGTGCCCCTGGCTGTGACAGTGGCCATCATCCTGCTCCTGGTGGACCTGATGCACCGGCGCCAACGC  124
  1 M  E  L  D  A  L  V  P  L  A  V  T  V  A  I  I  L  L  L  V  D  L  M  H  R  R  Q  R

    TGGGCGGCACGCTACCCACCAGGCCCCCTGCCACTGCCCTTCCTGGGCAAACTGCTGCATGTGGACTTCCAGAACACACCATAC  208
 29 W  A  A  R  Y  P  P  G  P  L  P  L  P  F  L  G  K  L  L  H  V  D  F  Q  N  T  P  Y

    TGCTTCGACCAGTTGCGGCGCCGCTTCGGGGACGTGTTCAGCCTGCAGCTGGCCTGGACGCCGGTGGTCGTGCTCAATGGGCTG  292
 57 C  F  D  Q  L  R  R  R  F  G  D  V  F  S  L  Q  L  A  W  T  P  V  V  V  L  N  G  L

    GCGGCCGTGGCCGAGGCGCTGGTGACCCACGGCGAGGACACCGCCGACCGCCCGCCTGTGCCCATCACCCAGATCCTGGGTTTT  376
 85 A  A  V  A  E  A  L  V  T  H  G  E  D  T  A  D  R  P  P  V  P  I  T  Q  I  L  G  F

    GGGCCGCGTTCCCAAGGGGTGTTCCTGGCGCGCTATGGCGCCTCGTGGCGCGAGCAGAGGCGCTTCTCCGTCTCCACCTTGCGC  460
113 G  P  R  S  Q  G  V  F  L  A  R  Y  G  A  S  W  R  E  Q  R  R  F  S  V  S  T  L  R

    AACTTGGGCCTGGGCAAGAAGTCGGGGACCCAGTGGGTGACCGGGGAGGCCGCCTGCCTTTGTGCCGCCTTCGCCAACCACTCC  544
141 N  L  G  L  G  K  K  S  G  T  Q  W  V  T  G  E  A  A  C  L  C  A  A  F  A  N  H  S

    GGACGCCCCTTTCGCCCCAACGGTCTCTTGGACAAAGCCGTGAGCAACGTGATCGCCTCCCTCACCTGCGGGCGCCGCTTCGAG  628
169 G  R  P  F  R  P  N  G  L  L  D  K  A  V  S  N  V  I  A  S  L  T  C  G  R  R  F  E

    TACGACGACCCTCGCTTCCTCAGGCTGCTGGACCTAGCTCAGGAGGGACTGAAGGAGGAGCCGGGCTTTCTGCGCGAAGTGCTG  712
197 Y  D  D  P  R  F  L  R  L  L  D  L  A  Q  E  G  L  K  E  E  P  G  F  L  R  E  V  L

    AATGCTGTCCCCGTCCTCCTGCATATCCCAGCGCTGGCTGGCAAGGTCCTACGCTTCCAAAAGGCTTTCCTGACCCAGCTGGAT  796
225 N  A  V  P  V  L  L  H  I  P  A  L  A  G  K  V  L  R  F  Q  K  A  F  L  T  Q  L  D

    GAGCTGCTAACTGAGCACAGGATGACCTGGGACCCAGCCCAGCCCCCCCCGAGACCTGACTGAGGCCTTCCTGGCAGAGATGGAG  880
253 E  L  L  T  E  H  R  M  T  W  D  P  A  Q  P  P  R  D  L  T  E  A  F  L  A  E  M  E

    AAGGCCAAGGGGAACCCTGAGAGCAGCTTCAATGATGAGAACCTGCGCATAGTGGTGGCTGACCTGTTCTCTGCCGGGATGGTG  964
281 K  A  K  G  N  P  E  S  S  F  N  D  E  N  L  R  I  V  V  A  D  L  F  S  A  G  M  V

    ACCACCTCGACCACGCTGGCCTGGGGCCTCCTGCTCATGATCCTACATCCGGATGTGCAGCGCCGTGTCCAACAGGAGATCGAC 1048
309 T  T  S  T  T  L  A  W  G  L  L  L  M  I  L  H  P  D  V  Q  R  R  V  Q  Q  E  I  D

    GACGTGATAGGGGCTGTGTGGCGTCCTGCGTTGGGTGAACAGGCTCACATGCCCTACACCCCTGCCGTCAAGCATGAGGTGCAG 1132
337 D  V  I  G  A  V  W  R  P  A  L  G  E  Q  A  H  M  P  Y  T  P  A  V  K  H  E  V  Q

    CGCTTTGGGGACATCGTCCCCCTGGGTGTGACCCATATGACATCCCGTGACATCGAAGTACAGGGCTTCCGCATCCCTAAGGGA 1216
365 R  F  G  D  I  V  P  L  G  V  T  H  M  T  S  R  D  I  E  V  Q  G  F  R  I  P  K  G

    ACGACACTCATCACCAACCTGTCATCGGTGCTGAAGGATGAGGCCGTCTGGGAGAAGCCCTTCCGCTTCCACCCCGAACACTTC 1300
393 T  T  L  I  T  N  L  S  S  V  L  K  D  E  A  V  W  E  K  P  F  R  F  H  P  E  H  F

    CTGGATGCCCAGGGCCACTTTGTGAAGCCGGAGGCCTTCCTGCCTTTCTCAGCAGGCCGCCGTGCATGCCTCGGGGAGCCCCTG 1384
421 L  D  A  Q  G  H  F  V  K  P  E  A  F  L  P  F  S  A  G  R  R  A  C  L  G  E  P  L

    GCCCGCATGGAGCTCTTCCTCTTCTTCACCTCCCTGCTGCAGCACTTCAGCTTCTCGGTGCCCACTGGACAGCCCCGGCCCAGC 1468
449 A  R  M  E  L  F  L  F  F  T  S  L  L  Q  H  F  S  F  S  V  P  T  G  Q  P  R  P  S

    CACCATGGTGTCTTTGCTTTCCTGGTGAGCCCATCCCCCTATGAGCTTTGTGCTGTGCCCCGCTAGAATGGGTACCTAGTCCCA 1552
477 H  H  G  V  F  A  F  L  V  S  P  S  P  Y  E  L  C  A  V  P  R

    GCCTGCTCCCTAGCCAGGGGCCCTGATGTACAA (1585)
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Fig. 1. Nucleotide and deduced amino acid sequences of Japanese monkey CYP2D29. The numbers of the deduced amino acid and nucleotide sequences are shown on the left and right sides, respectively.

3.3. Drug oxidation activities

Fig. 5 shows the activities of DB 4-hydroxylase (at 100 μM, top panels A and B) and racemic BF 1″-hydroxylase (at 100 μM, bottom panels C and D) in microsomal fractions from yeast cells and Japanese monkey and human livers. The left panels (A and C) show total activity expressed as nmol/min/mg protein, and the right panels (B and D) show the specific activity in nmol/min/nmol CYP. In terms of nmol/min/mg protein, DB 4-hydroxylase

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2D29       1:MELDALVPLAVTVAIILLLVDLMHRRQRWAARYPPGPLPLPFLGKLLHVDFQNTPYCFDQ  60
2D6        1:MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVDFQNTPYCFDQ  60
2D17       1:MELDALVPLAVTVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVDFKNTPYCFDQ  60
2D19       1:MGLDALVPLAVTVAIFVLLVDLMHRRQRWAARYPPGPMPLPGLGNLLHVDFQNTPNSFNQ  60
             * * ******* ***  ******************** *** ** ****** ***  * *

2D29      61:LRRRFGDVFSLQLAWTPVVVLNGLAAVAEALVTHGEDTADRPPVPITQILGFGPRSQGVF  120
2D6       61:LRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTADRPPVPITQILGFGPRSQGVF  120
2D17      61:LRRRFGNVFSLQLAWTPVVVLNGLAAVREALVTCGEDTADRPPVPINQVLGFGPRSQGVF  120
2D19      61:LRRRFGDVFSLQLAWTPVVVLNGLEAVREALVTRGEDTADRPPVPITEMLGFGPHSQGLF  120
             ****** ***************** ** ***** ************   ***** *** *

2D29     121:LARYGASWREQRRFSVSTLRNLGLGKKSGTQWVTGEAACLCAAFANHSGRPFRPNGLLDK  180
2D6      121:LARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFANHSGRPFRPNGLLDK  180
2D17     121:LARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFTDQAGRPFRPNSLLDK  180
2D19     121:LARYGPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEATYLCAAFADHAGRPFRPNGLLDK  180
             *****  *********************  **** **  *****    ******* ****

2D29     181:AVSNVIASLTCGRRFEYDDPRFLRLLDLAQEGLKEEPGFLREVLNAVPVLLHIPALAGKV  240
2D6      181:AVSNVIASLTCGRRFEYDDPRFLRLLDLAQEGLKEESGFLREVLNAVPVLLHIPALAGKV  240
2D17     181:AVSNVIASLTYGRRFEYDDPRFLRLFDLTHEALKEESGFLREVLNAIPLLLRIPGLAGKV  240
2D19     181:AVSNVIASLTCRRRFEYNDPCLLRLLDLTMEGLKEESGLLREVLNAIPVLLRIPGLAGKV  240
             **********  ***** **  *** **  * **** * ******* * ** ** *****

2D29     241:LRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRIVVA  300
2D6      241:LRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRIVVA  300
2D17     241:LRSQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNEENLRMVVA  300
2D19     241:LRSQKAFLAQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKTKGNPESSFNDENLHLVVA  300
             ** ***** ********************************* ********* ***  ***

2D29     301:DLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGAVWRPALGEQAHMPYTPAVK  360
2D6      301:DLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQAHMPYTTAVI  360
2D17     301:DLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQARMPYTTAVI  360
2D19     301:DLFSAGMVTTSITLAWGLLLMILHPDVQRRVQQEIDDVIGRVRRPEMGDQTYMPYTTAVI  360
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2D29     361:HEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLSSVLKDEAVWEKPFRFHPEHF  420
2D6      361:HEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLSSVLKDEAVWEKPFRFHPEHF  420
2D17     361:HEVQRFGDIVPLGVTHMTSRDIELQGFLIPKGTTLFTNLSSVLKDEAVWEKPFRFHPEHF  420
2D19     361:HEVQRFADIVPLGVTHMTSRDIEVQGFLIPKGTTLFTNLSSVLKDEANWEKPFRFHPEHF  420
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2D29     421:LDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSVPTGQPRPSHHGV  480
2D6      421:LDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFTSLLQHFSFSVPTGQPRPSHHGV  480
2D17     421:LDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFTCLLQRFSFSVPAGQPRPSHHGV  480
2D19     421:LDAQGRFVKPEAFLPFSAGRRACLGEPLARMELFLFFTCLLQRFSFSVPAGQPRPSPHGV  480
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2D29     481:FAFLVSPSPYELCAVPR                                            497
2D6      481:FAFLVSPSPYELCAVPR                                            497
2D17     481:FAFLVTPSPYELCAVPR                                            497
2D19     481:FAFLVTPSPYELCAVPR                                            497
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Fig. 2. Alignment of four CYP2D enzymes from primates. CYP2D6 is from a human (GenBank Accession No. M33388), CYP2D17 from a cynomolgus monkey (No. U38218), and CYP2D19 from a marmoset monkey (No. D29822). Substrate recognition sites are marked with solid lines, and the heme-binding domain is noted with a broken line. Key: (∗) conserved amino acid residue among four CYP enzymes.

activity of the Japanese monkey liver microsomal fraction was 16 times higher than that of human liver microsomal fraction (Fig. 5A). The activity of the microsomal fraction (JMLM-29) of the Japanese monkey liver, from which CYP2D29 was cloned, was similar to those of three different microsomal fractions from Japanese monkeys (data not shown). Recombinant CYP2D29 from Japanese monkeys, however, exhibited similar activity to that of recombinant human CYP2D6 in terms of nmol/min/nmol CYP (Fig. 5B).

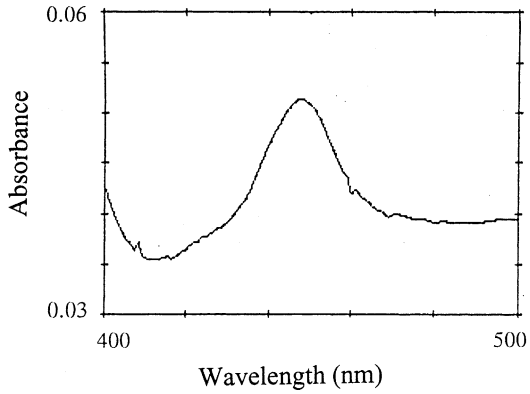

Fig. 3. A reduced CO-difference spectrum of CYP2D29 expressed in yeast microsomes. The spectrum was measured in 20 mM Tris-acetate buffer (pH 7.2) containing 20% glycerol and 0.4% Emulgen 911. The microsomal protein concentration was 0.5 mg/mL. No absorption around 450 nm was observed for the control microsomes from yeast cells transformed with the vector only. Data are typical of results from two different experiments.

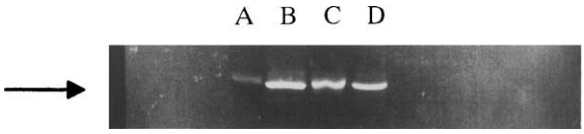

Fig. 4. Western blot analysis of microsomes from human (A) and Japanese monkey (C) livers, and from yeast cells expressing CYP2D6 (B) and CYP2D29 (D). Fifty micrograms of each protein sample was applied to each well for SDS–PAGE. Proteins were then transblotted to a PVDF membrane and immunochemically probed with polyclonal antibodies raised against rat CYP2D2 as described in Section 2. An arrow indicates the mobility of CYP2D29. Data are typical of results from two different experiments.

Likewise, in BF 1″-hydroxylation, Japanese monkey liver microsomes showed much higher activity (nmol/min/mg protein) than human liver microsomes (Fig. 5C). In this case, however, recombinant CYP2D29 possessed activity (nmol/min/mg protein) similar to that of Japanese monkey liver microsomes, which is different from the profile of DB 4-hydroxylation described above. Recombinant CYP2D29 was also found to have activities similar to recombinant CYP2D6 in terms of nmol/min/nmol CYP (Fig. 5D). In this index, K_m values were similar among Japanese monkey and human liver microsomes and the yeast cell microsomal fraction expressing the recombinant enzymes (Table 3, lower part).

3.4. Kinetics

Kinetic studies were performed for DB 4-hydroxylation by Japanese monkey liver microsomes and CYP2D29-expressing yeast cell microsomes, and compared with

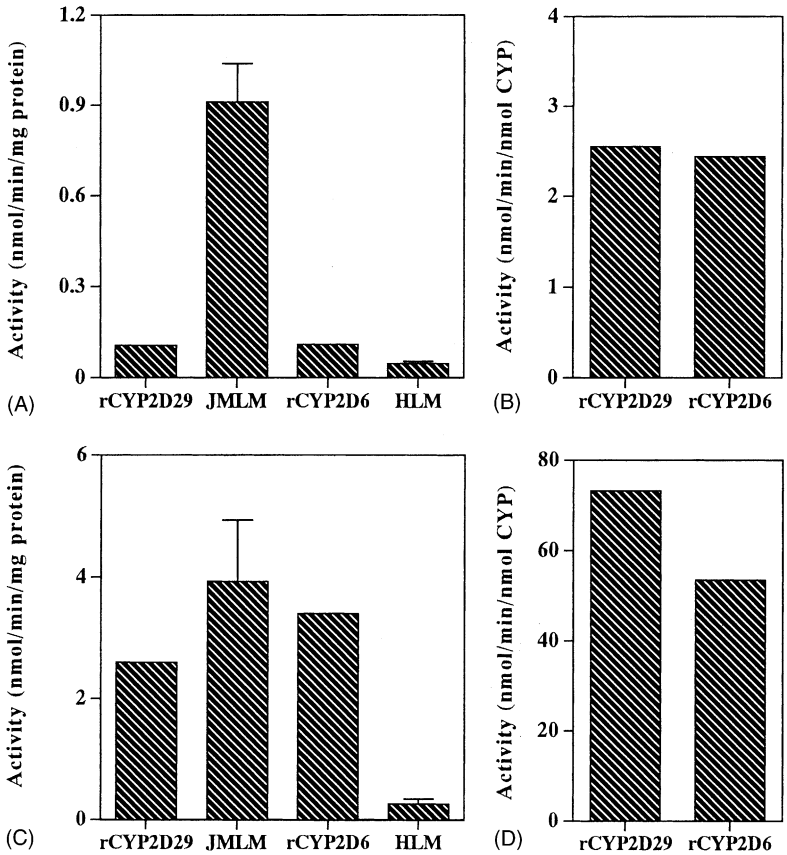

Fig. 5. Comparison of DB 4-hydroxylase and BF 1″-hydroxylase activities of recombinant CYP2D29 and Japanese monkey liver microsomes with those of recombinant CYP2D6 and human liver microsomes. (A) and (B), DB 4-hydroxylation; (C) and (D), BF 1″-hydroxylation. A substrate concentration of 100 μM was chosen for both assays. Values for liver microsomes are means ± SD (N = 3), and those for recombinant CYP enzymes are the means of duplicate determinations.

Table 3
Kinetic parameters for debrisoquine (DB) 4-hydroxylation and bufuralol (BF) 1″-hydroxylation by recombinant CYP2D6 and CYP2D29 expressed in yeast cells, and in human and Japanese monkey liver microsomes

	K_m (μM)	V_{max} (nmol/min/mg protein)	V_{max} (nmol/min/nmol CYP)	V_{max}/K_m
DB 4-hydroxylation				
rCYP2D29	7.7	0.131	2.78	0.017
JMLM	160.6 ± 73.3	2.82 ± 1.78		0.020
rCYP2D6	15.2	0.161	3.45	0.011
HLM	25.4 ± 12.5	0.059 ± 0.002		0.003
BF 1″-hydroxylation				
rCYP2D29	5.49	2.73	88.15	0.49
JMLM	13.5 ± 3.42	4.68 ± 1.74		0.35
rCYP2D6	13.9	4.12	57.89	0.30
HLM	8.88 ± 1.64	0.24 ± 0.04		0.027
rCYP2D17[a]	1.0	2.0		2.0
CMLM[a]	8.8 ± 2.3	1.9 ± 1.74		0.22

Abbreviations: rCYP2D29, recombinant CYP2D29; HLM, human liver microsomes; JMLM, Japanese monkey liver microsomes; and CMLM, cynomolgus monkey liver microsomes. For rCYP2D6 and rCYP2D29, each value represents the mean of duplicate determinations. For HLM and JMLM, each values represent the mean ± SD (N = 3).

[a] Data from Mankowski *et al.* [20].

the results of human liver microsomes and recombinant CYP2D6. Fig. 6 shows Lineweaver–Burk plots for DB 4-hydroxylation by microsomal fractions of the Japanese monkey liver (left panel), from which CYP2D29 cDNA was cloned, and of yeast cells expressing CYP2D29 (right panel). The reaction profiles exhibited by all of the microsomal fractions were analyzed to be monophasic under the conditions used, and Table 3 summarizes the kinetic parameters calculated. The K_m and V_{max} values of recombinant CYP2D29 were close to those of human recombinant CYP2D6. However, the K_m value of CYP2D29 was one-twentieth that of Japanese monkey liver microsomes (160.6 μM), and one-third that of human liver microsomes (25.4 μM). In the kinetics for BF 1″-hydroxylation, the K_m and V_{max} values of recombinant CYP2D29 were also similar to those of recombinant CYP2D6. In contrast to the results of DB 4-hydroxylation, the K_m value of recombinant CYP2D29 (5.49 μM) was close to that of Japanese monkey liver microsomes (13.5 μM) in this index.

3.5. Inhibition studies

To assess to what extent the CYP2D enzymes, including CYP2D29, contribute to DB 4-hydroxylation in Japanese monkey liver, inhibition studies were conducted using quinidine, the typical inhibitor of human CYP2D6, and rabbit antiserum raised against rat CYP2D2 [9]. The microsomal fraction used in both experiments was obtained from the liver from which CYP2D29 cDNA was cloned. Fig. 7A shows that quinidine added to the

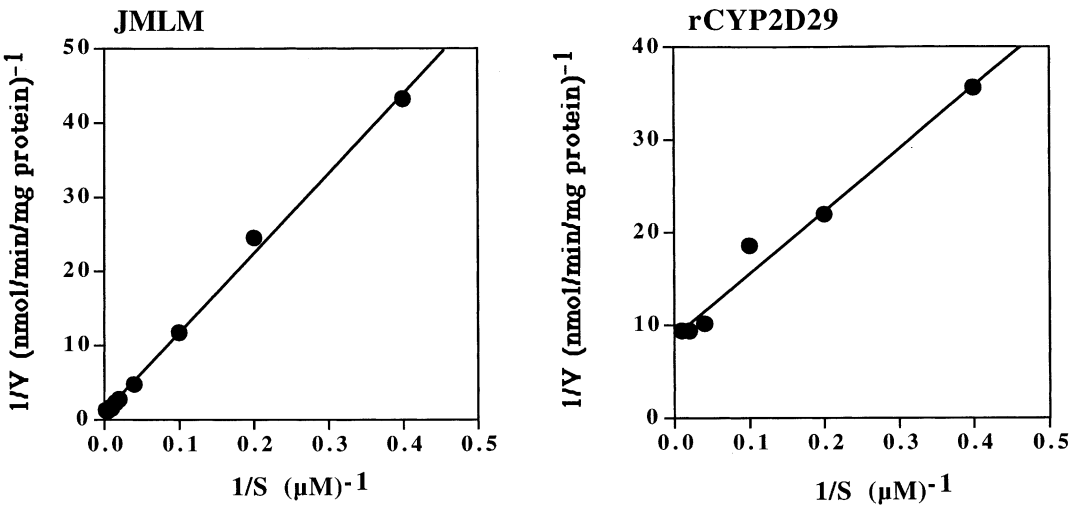

Fig. 6. Lineweaver–Burk plots for DB 4-hydroxylation by microsomal fractions of Japanese monkey liver from which CYP2D29 cDNA was cloned (left panel) and of yeast cells expressing CYP2D29 (right panel). Kinetic parameters were calculated as follows: K_m 123.8 μM and V_{max} 1.143 nmol/min/mg protein for Japanese monkey liver microsomes; K_m 7.7 μM and V_{max} 0.131 nmol/min/mg protein for recombinant CYP2D29. Data are typical of results from two different experiments.

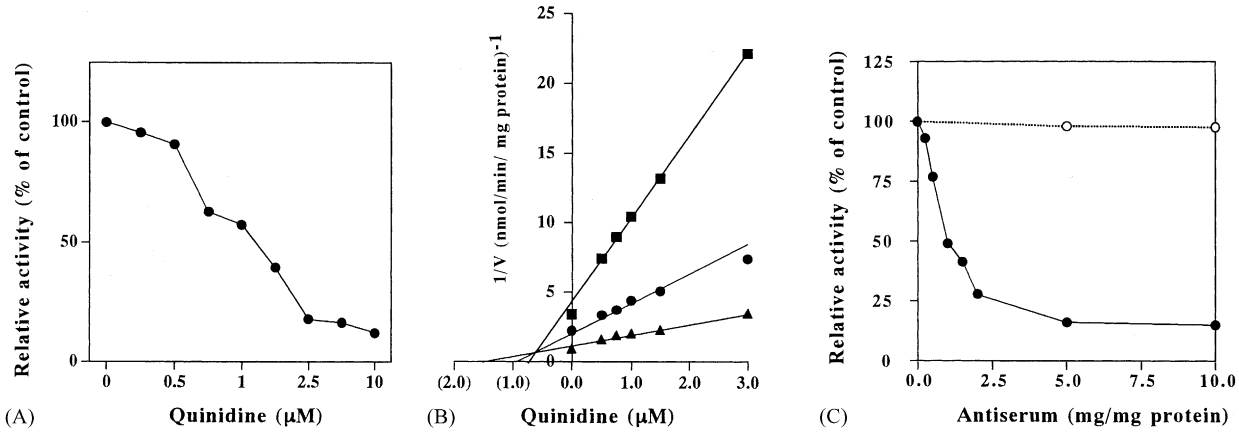

Fig. 7. Evidence for the involvement of CYP2D enzyme(s) in DB 4-hydroxylation in Japanese monkey liver microsomes. (A) An inhibition profile by quinidine. (B) Dixon plots for the inhibition by quinidine. (C) An immunotitration curve with polyclonal antibodies raised against rat CYP2D2. Each point is the mean value of duplicate determinations. The substrate concentration used in experiments (A) and (C) was 100 μM. In (B), (■) 50 μM debrisoquine; (●) 100 μM debrisoquine; and (▲) 250 μM debrisoquine. In (C), (●) antiserum added; and (○) control serum added.

incubation medium inhibited DB 4-hydroxylation in Japanese monkey liver microsomes in a concentration-dependent manner, and approximately 90% of the activity was inhibited at the highest quinidine concentration (10 μM). Fig. 7B shows Dixon plots for the inhibition of DB 4-hydroxylation by quinidine. Quinidine was found to inhibit DB 4-hydroxylation competitively with a K_i value of 0.53 μM. At a DB concentration of 100 μM, the antiserum inhibited 85% of the activity at most (Fig. 7C). If some CYP enzyme(s) other than CYP2D29 contributes to DB 4-hydroxylation in Japanese monkey liver microsomes, the enzyme(s) should be sensitive to both quinidine and antibodies raised against CYP2D2. From these results, it is possible that the CYP enzyme(s) involved in DB 4-hydroxylation in Japanese monkey livers is one belonging to the CYP2D subfamily. Moreover, in Japanese monkey liver microsomes CYP2D29 [and its isoform(s)] may be mainly responsible for DB 4-hydroxylation at a substrate concentration of 100 μM or less.

3.6. General discussion

In the present study, the cDNA encoding CYP2D29 in Japanese monkey livers has been cloned successfully in *Escherichia coli*, and the CYP2D29 protein expressed in yeast cells was characterized. In the two typical indices examined (the oxidation of DB and BF), the total drug-oxidizing activity (expressed as nmol/min/mg protein) of Japanese monkey liver microsomes was higher than that of human liver microsomes. However, the specific activity (capacity) of the recombinant CYP2D29, expressed as nmol/min/nmol CYP, in which the content of the CYP holoenzyme was determined on the basis of the reduced CO-difference spectrum, was found to be similar to that of recombinant CYP2D6. In the western blot analysis using polyclonal antibodies raised against rat CYP2D2, a protein band in Japanese monkey liver microsomes was stained to a greater extent than that in human liver microsomes. These results suggest that the content of CYP2D29 in Japanese monkey liver microsomes may be higher than that of CYP2D6 in human liver microsomes, resulting in the difference in the activity expressed per milligram protein.

In the kinetic analyses, the K_m value of recombinant CYP2D29 for DB 4-hydroxylation was much lower than that of the Japanese monkey liver microsomes, whereas the K_m values for BF 1″-hydroxylation were similar. These results suggest two possibilities. First, it is possible that CYP2D29 is not the major enzyme responsible for the oxidations of DB and BF in monkey liver microsomes. That is, another CYP2D enzyme having a high K_m of approximately 150 μM for DB 4-hydroxylation and approximately 10 μM for BF 1″-hydroxylation may also be involved in the reaction as a major enzyme. Compared with another major enzyme with a high K_m, the contribution of CYP2D29 with a low K_m to DB 4-hydroxylation is not great enough to give biphasic kinetics. Second, although yeast cells have been used as the expression system of mammalian CYP enzymes, there are various differences in the cellular components between yeast and mammalian hepatocytes. Some cellular components of yeast cells including fp_2, cytochrome b_5, and lipids may affect the kinetic parameters. We are conducting further studies to explore these possibilities.

In summary, we have cloned the cDNA of a novel CYP2D enzyme, CYP2D29, from Japanese monkey livers and deduced its amino acid sequence. Some catalytic properties of the recombinant CYP2D29 expressed in yeast cells have also been described. To the best of our knowledge, CYP2D29 is the CYP2D most homologous to the human CYP2D6 enzyme, i.e. there is a difference of only 20 amino acid residues between CYP2D29 and CYP2D6. In this context, CYP2D29 can be thought to be one of the CYP2D6 variants. Detailed comparative studies on the catalytic properties of CYP2D29 and CYP2D6 in the future may give us important keys for understanding the oxidation mechanism of various drugs as substrates of CYP2D6.

Acknowledgments

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