NOTE *Toxicology*

Identification and Characterization of CYP2B6 cDNA in Cynomolgus Macaques (*Macaca fascicularis*)

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ABSTRACT. Cytochrome P450 2B6 (CYP2B6), an important drug-metabolizing enzyme, is involved in the metabolism of prescribed drugs in humans. Despite its importance, cDNA for a CYP2B6 ortholog has not been identified and characterized in cynomolgus macaques, which are frequently used in preclinical studies. In this study, cDNA highly homologous to human CYP2B6 was cloned from the cynomolgus macaque liver. This cDNA contained an open reading frame of 491 amino acids and functional domains characteristic for CYP protein, such as substrate recognition sites and a heme-binding region. Cynomolgus *CYP2B6* was expressed predominantly in the liver with some extra-hepatic expression among 10 tissues. Moreover, cynomolgus CYP2B6 revealed activities toward testosterone 16β-hydroxylation and bupropion hydroxylation. These results suggest that cynomolgus CYP2B6 has a functional role in the liver.
KEY WORDS: cloning, liver, cytochrome P450, monkey.

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The human cytochrome P450 2B (*CYP2B*) subfamily contains functional *CYP2B6* and a pseudogene, *CYP2B7P*. CYP2B6 is expressed in the liver and constitutes approximately 3 to 5% of total CYP proteins in human livers [3, 6]. An increasing number of prescribed drugs are metabolized by CYP2B6, including bupropion, cyclophosphamide, diazepam, efavirenz and tamoxifen [6]. Human *CYP2B6* was originally identified as an ortholog to phenobarbital-induced CYP2Bs of rodents, and this induction by this and other drugs is mediated by mechanisms involving the constitutive androstane receptor and pregnane X receptor [16].

Cynomolgus macaques have been used for drug-safety evaluation and biotransformation studies. In this species, cDNAs have been isolated for enzymes in the CYP1–3 subfamilies, which are important for drug metabolism. However, cDNA for CYP2B6 ortholog has not been identified. In this paper, therefore, cynomolgus CYP2B6 cDNA was isolated and characterized by sequence analysis, tissue expression pattern and drug-metabolizing assay.

For isolation and analysis of cynomolgus CYP2B6 cDNA, six cynomolgus macaques (three males and three females) of Indochinese origin were housed under the standard guidelines established at Shin Nippon Biomedical Laboratories (Tokyo, Japan). Tissue samples (brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary and uterus) were collected and snap-frozen in liquid nitrogen to avoid possible RNA degradation as described previously [15]. For each tissue type, tissue samples from the six animals were pooled to prepare the total RNA that was used for the subsequent analyses.

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Abbreviations: CYP, cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcription; SRS, substrate recognition site.

To isolate the cDNA for cynomolgus CYP2B6, reverse transcription (RT) from cynomolgus liver RNA and subsequent polymerase chain reaction (PCR) were performed as described previously [15]. Briefly, PCR was carried out using KOD Plus DNA polymerase (Toyobo, Osaka, Japan) under the following conditions: 20 sec at 95°C, 20 sec at 55°C and 2 min at 72°C for 30 cycles. The primers (5'-CATGGAACTCAGCGTCCTCCT-3' and 5'-GACACTGAATGACCCTGGAA-3') were designed to amplify the coding region of the cDNA based on the human CYP2B6 cDNA sequence (GenBank accession number NM_000767). The resultant PCR products were ligated into the pCR-XL-TUPO vector (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. Sequences of the inserts were determined and analyzed as described previously [15]. We successfully cloned CYP2B6 cDNA from cynomolgus macaque livers and reported its sequence to GenBank (accession number DQ074793). This cDNA contained an open reading frame of 491 amino acids (Fig. 1) with primary sequence structures characteristic for CYP proteins, such as a heme-binding region and six potential substrate recognition sites (SRSs) [4]. Deduced amino acids of cynomolgus CYP2B6 cDNA were highly homologous to rhesus and human CYP2B6 cDNAs (99% and 91%, respectively), higher than those of the dog, rat and mouse (67–79%; Table 1). This indicated the evolutionary closeness of CYP2B6 between macaque species and humans.

The tissue distribution of the gene expression was determined by real-time RT-PCR as described previously [15], except that SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) was utilized. The primers were 5'-CAACATCATCTGCTCCATCG-3' and 5'-TGTGTGCCCCAGGAAAGTA-3', and their final concentrations were 200 nM in each reaction. Among the 10 tissues analyzed, the brain, lung, heart, liver, kidney, adrenal gland,

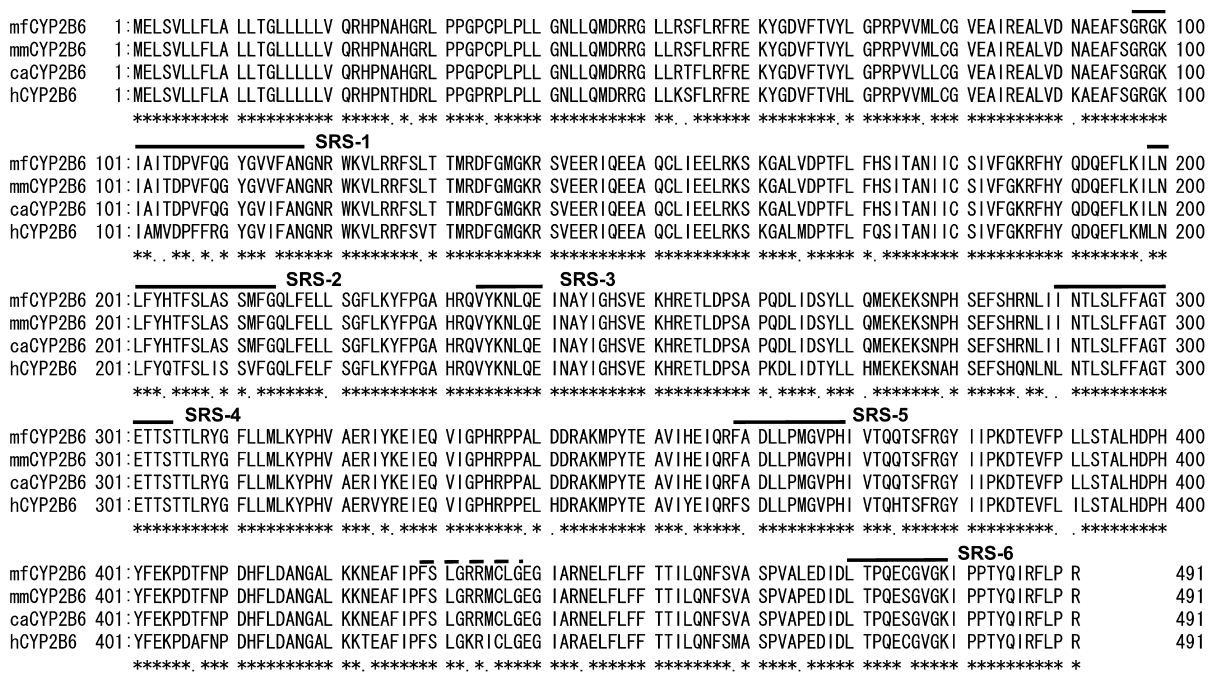

Fig. 1. Comparison of four CYP2B6 amino acids from primates. CYP2B6 sequences are shown for the cynomolgus macaque (mf; *Macaca fascicularis*), rhesus macaque (mm; *Macaca mulatta*), African green monkey (ca; *Cercopithecus aethiops*) and human (h; *Homo sapiens*). Above the sequences, the solid lines describe the six putative SRSs, and the broken lines indicate the heme-binding region. Under the sequences, asterisks show identical amino acids, and dots designate conservatively replaced amino acids.

Table 1. Identities of cynomolgus CYP2B6 to 10 CYP2B enzymes in the cDNA and amino acid sequences

Species	Name	cDNA*	Protein
		%	
Human	CYP2B6	94	91
Rhesus	CYP2B6	99	99
African Green monkey	CYP2B	–	98
Dog	CYP2B11	83	79
Rat	CYP2B1	79	75
	CYP2B2	78	74
	CYP2B3	76	67
	CYP2B12	78	72
	CYP2B15	79	71
	CYP2B21	78	73
Mouse	CYP2b9	79	72
	CYP2b10	79	76
	CYP2b13	76	71
	CYP2b19	79	73
	CYP2b23	78	73

* The African green monkey CYP2B6 cDNA sequence was not found in GenBank.

jejunum, testis, ovary and uterus, cynomolgus *CYP2B6* was expressed predominantly in the liver, while low expression was seen in the brain, jejunum, testis and ovary (Fig. 2). The expression in the liver was approximately 84-fold higher than that in the testis, the tissue that showed the second largest expression level among the tissues examined. Similarly, predominant expression in the liver has been seen for human *CYP2B6* [3].

The metabolic activity of cynomolgus CYP2B6 was measured using the protein heterologously expressed in *E. coli*. For protein expression, a bicistronic plasmid carrying cynomolgus CYP2B6 and human NADPH-CYP reductase cDNAs was generated as described previously [5, 15], except that modification of the N-terminus was accomplished by PCR using the primers 5'-GGAATTC CATATGGCTCTGTTATTAGCAGTTTTTCTTGCACTC CTTACAGGCCTC-3' and 5'-GCTCTAGACTTCAGCGG GGCAGGAA-3', which contained the NdeI and XbaI sites (underlined), respectively. These restriction enzyme sites were utilized for subcloning of the amplified products into the vector. With the resultant plasmid, protein expression was carried out in *E. coli* according to a method described elsewhere [5, 15]. From the *E. coli* cells, membrane fractions were prepared as described by Sandhu *et al.* [12]. The content of the CYP protein and NADPH-CYP reductase in the fraction was determined as reported by Omura and Sato [10] and Phillips and Langdon [11], respectively. The membrane fractions prepared were employed to measure drug-metabolizing activity of cynomolgus CYP2B6 using testosterone and bupropion as substrates.

For measurement of testosterone 16β-hydroxylation, the reaction mixtures (0.5 m*l*) containing 100 mM potassium phosphate buffer solution (pH 7.4), cynomolgus CYP2B6 protein (5 pmol/m*l*), ^{14}C-testosterone (100 μM) and NADPH-regenerating system (BD Biosciences, San Jose,

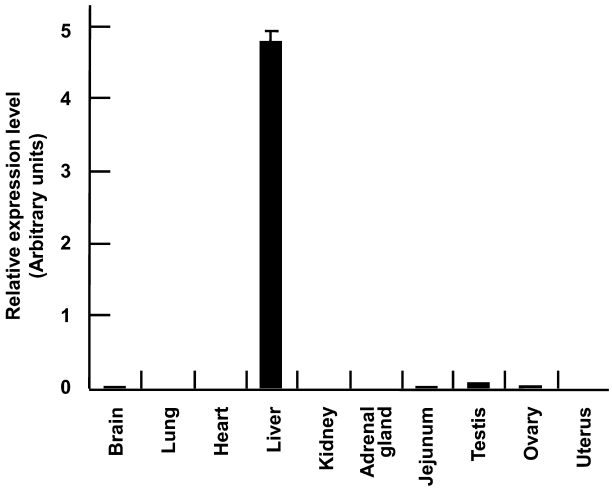

Fig. 2. Expression of cynomolgus *CYP2B6* in 10 different tissues. Using 18S rRNA levels as relative standards, the relative expression level of cynomolgus *CYP2B6* was determined by real-time RT-PCR. The tissues analyzed were the brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary and uterus. For each tissue, pooled samples from the six animals were used to prepare total RNA. Each value represents the average ± S.D. from three independent amplifications.

CA, U.S.A.) were incubated at 37°C for 20 min and subsequently quenched by addition of ice-cold acetonitrile (0.25 m*l*). The resultant mixture was centrifuged (10,000 × g, 4°C, 10 min), and the metabolites in the supernatant were analyzed using an HPLC system (Shimadzu, Kyoto, Japan) equipped with an SCL-10A system controller, two LC-10AD pumps, and an SIL-10AD autoinjector with a chilled sample tray maintained at 4°C. The analytical column was a reverse-phase Hypersil ODS-5 (150 × 4.6 mm, 5 μm particle size; Chemco Scientific, Osaka, Japan). The mobile phase consisted of solvent A [43.0:1.1:55.9 (v/v/v) methanol/acetonitrile/water] and solvent B [75.0:1.9:23.1 (v/v/v) methanol/acetonitrile/water] eluted at a flow rate of 1.5 m*l*/min with a linear gradient from initially 0 to 100% solvent A over 25 min and then returned to the original starting conditions. Testosterone and its metabolites were analyzed with a Ramona Star radioactivity detector (Raytest, Straubenhardt, Germany) and quantified using the GINA star software (Raytest).

To prepare the reaction mixtures for bupropion hydroxylation, bupropion (160 mM) was preincubated in 78 mM sodium-potassium phosphate buffer solution (pH 7.4) with cynomolgus CYP2B6 protein (10 pmol/m*l*) at 37°C for 5 min. The reaction was initiated by addition of an NADPH-regenerating system (BD Biosciences) in a total volume of 0.5 m*l*. After incubation at 37°C for 20 min, ice-cold acetonitrile (250 m*l*) was added to stop the reaction. The reaction mixtures were mixed well with 250 μ*l* of internal standard solution (0.1 μmol/*l*) and centrifuged (7,500 × g, 4°C, 10 min). The metabolites in the supernatant were first separated by the HPLC system described above. The analytical column was a reverse-phase Hydrosphere C18 (150 × 2.0 mm, 5 μm particle size; YMC, Kyoto, Japan). The mobile phase consisted of solvent A (0.1% formic acid solution) and solvent B (0.1% formic acid in acetonitrile solution). The initial mobile phase conditions (95% solvent A) were held for 3 min at a rate of 0.2 m*l*/min, followed by a step gradient from 5 to 80% solvent B. The conditions were held for 3 min and then returned to the original starting conditions. The HPLC eluent was introduced into the mass spectrometer, an API4000 (Applied Biosystems), via electrospray ionization. The m/z values of the precursors to the product ions were m/z 256→139 for hydroxybupropion and m/z 249→192 for the internal standards.

The measurements indicated that cynomolgus CYP2B6 exhibited the activity of 20.9 and 32.8 nmol/min/nmol CYP for testosterone 16β-hydroxylation and bupropion hydroxylation, respectively, for which human CYP2B6 also shows activities. Cynomolgus CYP proteins in the CYP1A, CYP2A, CYP2C, CYP2E and CYP3A subfamilies showed no or only minute activities toward these reactions (data not shown), indicating the possibility that, among the cynomolgus CYPs, cynomolgus CYP2B6 has specificity for testosterone 16β-hydroxylation and bupropion hydroxylation. Bupropion hydroxylation has been used as a marker reaction for human CYP2B6; however, its feasibility for cynomolgus CYP2B6 has not been investigated. In this study, substantial activity was found for cynomolgus CYP2B6 toward bupropion hydroxylation, raising the possibility that bupropion can be used as a probe drug for investigation of cynomolgus CYP2B6.

A previous study reported that P450 CMLa, a purified protein from the cynomolgus macaque liver, also shows substantial activity toward testosterone 16β-hydroxylation [9]. Using antibodies raised against P450 CMLa, CYP2B6 cDNA has been isolated in rhesus macaques [8], a closely related species to cynomolgus macaques. The high sequence identity (99%) to this rhesus CYP2B6 cDNA and the activity toward testosterone 16β-hydroxylation suggest that P450 CMLa corresponds to a protein product of cynomolgus CYP2B6. It should be noted that the rhesus ortholog of human CYP2B6 was originally designated as CYP2B30 (or CYP2B17 [8]). In this paper, however, both rhesus and cynomolgus orthologs of human CYP2B6 are regarded as CYP2B6 based on consultation of the P450 nomenclature committee (http://drnelson.utmem.edu/cytochromeP450).

Although the substrate specificity of cynomolgus CYP2B6 appears to be similar to human CYP2B6, previous studies show that CYP2B6 activity is higher in cynomolgus macaques than humans as demonstrated by measurement of pentoxyresorufin *O*-dealkylation [14, 17] and 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase [1]. In the latter case, *K*m is similar between cynomolgus macaques and humans, whereas *V*max is much higher in cynomolgus macaques than in humans [1], indicating that the higher activity can be partly explained by the difference in *V*max.

Moreover, a higher hepatic CYP2B6 content in cynomolgus macaques as compared with humans [14] might also contribute to the higher CYP2B6 activity of cynomolgus macaques. Further investigation of cynomolgus CYP2B6 with additional CYP2B substrates, including pentoxyresorufin and 7-ethoxy-4-trifluoromethylcoumarin, should help to better understand CYP2B6-mediated drug metabolism in cynomolgus macaques. The CYP2B6 protein prepared in this study is useful for such experiments.

Cynomolgus CYP2B-like protein cross-reactive with rat CYP2B1 antibodies has been induced in the liver by phenobarbital treatment [2]. Similarly, CYP2B protein has also been induced in the liver and brain of African green monkeys by treatment with phenobarbital or nicotine, with the latter being to a lesser extent [7, 13]. These results indicate that phenobarbital also induces expression of CYP2B proteins in primates *in vivo*. It is of great interest to see if this induction mechanism occurs at transcriptional level in the cynomolgus macaque brain and liver, which can now be easily assessed by the real-time RT-PCR method developed in this study. The involvement of CYP2B6 in bupropion metabolism suggests that induction of CYP2B6 by phenobarbital treatment may increase the metabolism of bupropion and possibly other centrally acting drugs in microenvironments within the brain.

In this study, cynomolgus CYP2B6 cDNA highly homologous to human CYP2B6 was identified and characterized. A high sequence identity and resemblance in the tissue expression pattern and drug-metabolizing properties suggest a functional similarity of CYP2B6 between cynomolgus macaques and humans.

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