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CYP1D1, pseudogenized in human, is expressed and encodes a functional drug-metabolizing enzyme in cynomolgus monkey

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ABSTRACT

Cytochrome P450 (P450 or CYP) 1 family consists of the CYP1A, CYP1B, CYP1C, and CYP1D subfamilies. In the human genome, *CYP1A1*, *CYP1A2*, and *CYP1B1* are expressed and encode functional enzymes, whereas *CYP1D1P* (formerly known as *CYP1A8P*) is present as a pseudogene due to five nonsense mutations in the putative coding region. In this study, we identified CYP1D1 cDNA, highly identical (nearly 95%) to human *CYP1D1P* sequence, in cynomolgus monkey, a species frequently used in drug metabolism studies due to its evolutionary closeness to human. The amino acid sequence deduced from cynomolgus monkey CYP1D1 cDNA shared the high sequence identity (91%) with human CYP1D1P (postulated from the gene sequence), and the highest sequence identity (44–45%) with CYP1A1 and CYP1A2 among cynomolgus monkey P450s. CYP1D1 mRNA was most abundantly expressed in liver, followed by kidney, and jejunum. The hepatic expression level of CYP1D1 mRNA was comparable to that of CYP1A1 mRNA and much higher than that of CYP1A2 mRNA. CYP1D1 was barely detectable in immunoblots of cynomolgus monkey liver. Cynomolgus monkey CYP1D1 mRNA was induced in primary hepatocytes with omeprazole. Cynomolgus monkey CYP1D1 protein heterologously expressed in *Escherichia coli* catalyzed ethoxyresorufin *O*-deethylation and caffeine 8-hydroxylation, which CYP1As also catalyze. Finally, no nonsense mutations, corresponding to those found in human *CYP1D1P*, were found in the 20 cynomolgus monkeys and 10 rhesus monkeys used in this study. These results suggest that CYP1D1 plays a role as a functional, drug-metabolizing enzyme in cynomolgus monkey liver.

1. Introduction

Cytochrome P450 (*P450* or *CYP*) is a gene family, consisting of 57 functional genes and 58 pseudogenes in human [1]. The *CYP1* family has diverged in each species during evolution, leading to different member genes in each species, due to gene gain and gene loss. The *CYP1* family in human consists of the *CYP1A* and *CYP1B* subfamilies, including two genes (*CYP1A1* and *CYP1A2*) and one gene (*CYP1B1*), respectively. Additional *CYP1* subfamilies, *CYP1C* and *CYP1D*, were recently found in fish. CYP1D1, identified in killfish and zebrafish [2], shared the highest amino acid sequence identity (55–56%) with CYP1A. *CYP1D1P* (formerly known as *CYP1A8P*) found in human, contains five nonsense mutations in the potential coding region of gene sequence in the genome, and thus is not expected to encode a functional protein. Yet, discovery of functional *CYP1D1* in fish raised the possibility that *CYP1D1* in the genome, without nonsense mutations, could be expressed as a functional enzyme in other species.

The CYP1 family is involved in metabolism of drugs, and human CYP1A1 and CYP1A2 are known to preferentially catalyze ethoxyresorufin *O*-deethylation (EROD) and methoxyresorufin *O*-deethylation (MROD), respectively [3,4]. Human CYP1A2 also catalyzes caffeine *N*-3 demethylation [5]. The CYP1 family is also involved in bioactivation of various environmental promutagens such as polycyclic aromatic hydrocarbons (PAHs), herbicides, and pesticides [6]. PAHs are metabolized into reactive intermediates, which form DNA and protein adducts that cause tumor formation and toxicity [7]. The CYP1 family is strongly induced by PAHs through the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, binding to xenobiotic response elements (XREs) at upstream regulatory regions of *CYP1* genes.

Macaques, including cynomolgus monkey (*Macaca fascicularis*) and rhesus monkey (*Macaca mulatta*), are frequently used in

Abbreviations: AHR, aryl hydrocarbon receptor; CYP, individual forms of cytochrome P450 (EC 1.14.14.1); DMSO, dimethyl sulphoxide; EROD, ethoxyresorufin *O*-deethylation; MROD, methoxyresorufin *O*-deethylation; ORF, open reading frame; P450, general term for cytochrome P450; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SRS, substrate recognition site; XRE, xenobiotic response element.

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biomedical studies such as drug metabolism due to their evolutionary closeness and physiological resemblance to humans. However, some differences in drug metabolism are occasionally noted, which could be partly due to divergence in the genes encoding drug-metabolizing enzymes such as P450s. Indeed, cynomolgus monkey CYP2C76, which we recently identified, has no ortholog in human, and is responsible for differences in pitavastatin metabolism between cynomolgus monkey and human [8]. Furthermore, a part of the differences in drug metabolism could be attributable to genes, such as *CYP1D1P*, that are pseudogenized in human, but might be expressed and functional in macaques.

To assess this possibility, in this study, we identified CYP1D1 cDNA in cynomolgus monkey. The CYP1D1 was characterized by sequence analysis, phylogeny, tissue expression patterns, genome organization, protein expression, and drug-metabolizing assays. The induction profile was also examined using typical human P450 inducers.

2. Materials and methods

2.1. Materials

Pooled hepatic microsomes from male cynomolgus monkeys and humans were purchased from BD-GENTEST (Woburn, MA). Recombinant human CYP1A enzymes were prepared as described [9]. Oligonucleotides were synthesized by Invitrogen (Tokyo, Japan), and fluorescent probes were synthesized by Applied Biosystems (Foster City, CA) and Biosearch Technology Japan (Tokyo, Japan). Caffeine, dexamethazone, 7-ethoxyresorufin, omeprazole, rifampicin, and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Tissue samples and preparation of total RNA and genomic DNA

Tissue samples of brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus were collected from six cynomolgus monkeys (three males and three females from Indochina, 4–5 years of age, 3–5 kg). Liver sample was also collected from two rhesus monkeys (all males from China, 7 years of age, weighing 3–5 kg). RNA was extracted from these tissues as described previously [12]. Genomic DNA was extracted from whole blood samples from 20 cynomolgus monkeys (10 from Indochina and 10 from Indonesia, 4–5 years of age, 3–5 kg) and 10 rhesus monkeys (from China, 7 years of age, weighing 3–5 kg) using the PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. This study was reviewed and approved by the Institutional Animal Care and Use Committee at Shin Nippon Biomedical Laboratories, Ltd. (Kainan, Japan).

2.3. Molecular cloning and sequencing of CYP1D1 cDNAs

To isolate CYP1D1 cDNA, reverse transcription (RT)-polymerase chain reaction (PCR) was performed using liver total RNAs of cynomolgus and rhesus monkey as described previously [12] with the following modifications. Briefly, RT reactions were carried out at 37 °C for 1 h in a mixture containing 1 μg of total RNA, oligo (dT), and SuperScript II RT reverse transcriptase (Invitrogen). PCR was carried out with RT products as a template using KOD Plus DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's protocol with a thermal cycler (Applied Biosystems). Thermal cycler conditions were; an initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. The primers used, 5′-GAAATGATTCTCAACCTAGCAGTCA-3′ and 5′-GCATAGCTCAGGG GAATAAACTG-3′, were designed to amplify the potential open reading frame (ORF) of the *CYP1D1* sequence found in the rhesus monkey genome. To verify the translation initiation codon of CYP1D1 cDNA, 5′ rapid amplification of cDNA ends (RACE) was carried out with liver total RNA of cynomolgus monkey and rhesus monkey using 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's protocol. The primers for 5′ RACE were as follows: 5′-TACTTGTTTCACCATTTC-3′ for RT reaction, 5′-AGACAGGCACCATGCCAAG-3′ for the initial PCR, and 5′-CGTAAGGTAAGGATGCTCTCCA-3′ for the nested PCR. The PCR products were, after addition of an A-overhang, cloned into pCR2.1 vectors using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The inserts were sequenced using ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

2.4. Sequence analysis

Sequence data were analyzed with DNASIS Pro (Hitachi Software, Tokyo, Japan) and the Genetyx system (Software Development, Tokyo, Japan). Multiple alignments of amino acid sequences were performed using ClustalW. A phylogenetic tree was created by the neighbour-joining method. BLAST (National Center for Biotechnology Information) was used for the homology search. BLAT (UCSC Genome Bioinformatics) was utilized for the analysis of the human and rhesus monkey genome data. The CYP1 amino acid sequences used for the analysis were found in GenBank, including human CYP1A1 (NP_000490), CYP1A2 (NP_000752), CYP1B1 (NP_000095), and CYP2A6 (NP_000753); cynomolgus monkey CYP1A1 (BAA04500) and CYP1A2 (BAA33 789); rhesus monkey CYP1A1 (NP_001035328); Japanese monkey CYP1A2 (BAE16271); marmoset CYP1A2 (BAA33790); dog CYP1A1 (P56590), CYP1A2 (NP_001008720), and CYP1B1 (NP_001153156); rat CYP1A1 (NP_036672), CYP1A2 (NP_036 673), and CYP1B1 (NP_037072); and zebrafish CYP1A (NP_571 954), CYP1B1 (NP_001139180), CYP1C1 (NP_001018446), CYP1C2 (NP_001108321), and CYP1D1 (NP_001007311). Cynomolgus and rhesus monkey CYP1D1 amino acid sequences were deduced from the cDNAs identified in this study. Human CYP1D1P amino acid sequences were predicted from the human *CYP1D1P* sequence without the nonsense mutations.

2.5. Analysis of CYP1D1 exons

To assess if mutations corresponding to human *CYP1D1P* are present in macaques, exon 2 and exon 7 were PCR-amplified using the genomic DNAs of 10 Indochinese and 10 Indonesian cynomolgus monkeys, and 10 Chinese rhesus monkeys. PCR reactions (20 μl) contained 1 ng of genomic DNA, 10 pmol each of primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 1 unit of ExTaq DNA polymerase (Takara, Tokyo, Japan) in a total volume of 20 μl. PCR conditions were as follows: 94 °C for 1 min, followed by 35 cycles of 94 °C for 10 s, 58 °C for 30 s, and 72 °C for 1.5 min; a final extension at 72 °C for 10 min. The primers used were 5′-TTAGTGCTTGATATTAGGGGGTGT-3′ and 5′-ATACCTTACCCACA-CACTGAGAAA-3′ for exon 2, and 5′-TGCAAAAGAAAATTATTGTG-GAAA-3′ and 5′-TGAGTATGCCTGTTTATCTGAAGC-3′ for exon 7. The amplified DNAs were sequenced as described earlier by direct sequencing using the primers, 5′-TGGTGGTTAGAACTGTGAAAA-GAA-3′ and 5′-AATAGCTTGGGTACAGGTCATTGT-3′ for exon 2 and 5′-AAACCCTCTGATTTACGTCACACT-3′ for exon 7.

2.6. Quantitative PCR

Expression of cynomolgus monkey CYP1A1, CYP1A2, and CYP1D1 mRNAs was measured by real-time RT-PCR in brain,

lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus, as previously reported [12] with the following modifications. The PCR was carried out with the ABI PRISM 7500 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. The primers used were 5′-AAACCTTTGAGAAGGGCCACA-3′ and 5′-TCATCCAGCTGCTTCTCCTGA-3′ for CYP1A1, 5′-AGAGGTTCAAGGCCTTCAACC-3′ and 5′-GAGGCTCCAGGAGATGGCT-3′ for CYP1A2, and 5′-TCTCCGCTACCTTCCACTGC-3′ and 5′-CAGCGTATTTGTTGTGGCATAC-3′ for CYP1D1. The probes used were 5′-FAM-CCGGGACATCACAGAC-MGB-3′ for CYP1A1, 5′-FAM-TGCCCTGTTCAAGCA-MGB-3′ for CYP1A2, and 5′-FAM-CGGGAGTTTTATCGGGCCCTGA-BHQ-3′ for CYP1D1. The primers and probes were used at final concentrations of 600 nM and 200 nM, respectively. Expression level was normalized to the 18S ribosomal RNA level based on three independent amplifications.

2.7. Primary hepatocyte culture and induction analysis

Cynomolgus monkey primary hepatocytes (two lots derived from different animals) were purchased from KAC (Kyoto, Japan) and cultured as recommended by the supplier. Briefly, the primary hepatocytes (2×10^5 cell/well) were cultured on 24-well cell culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) using the culture medium containing Williams' E with GlutaMAX 1 and the additives purchased from KAC. The primary hepatocytes (2×10^5 cell/well) were treated for 24 h with rifampicin (2 μM), omeprazole (50 μM), or dexamethasone (10 μM), as described previously [10]. These inducers were dissolved in dimethyl sulphoxide (DMSO) 0.1% (v/v). Control hepatocytes were also treated with DMSO 0.1% (v/v). Total RNA was extracted from the hepatocytes using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Expression of CYP1A1 and CYP1D1 mRNAs was measured by real-time RT-PCR as described earlier, in the control and inducer-treated samples. Fold induction was calculated by a comparison of mRNA levels between the control and inducer-treated hepatocytes.

2.8. Preparation of CYP1D1 protein

To characterize the CYP1D1 protein, expression plasmids of cynomolgus monkey CYP1A1, CYP1A2, and CYP1D1 were prepared and protein expression was carried out in *Escherichia coli* as described previously [11,12] with the following modifications. The internal NdeI site of CYP1D1 cDNA was mutated without altering the amino acid residue using the QuikChange XL II kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, since the NdeI site was later used to subclone the PCR products. The primers were 5′-CACGTACAAGATCATCTTGCTACTtTATGATAAGGATCATATCCGAG-3′ and 5′-CTCGGATATGATCCTTATCATAaGTAGCAAGATGATCTTGTACGTG-3′, where lower case letters indicate the nucleotides to be mutated. The N-terminus modification was conducted by PCR with the forward and reverse primers, 5′-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTT-GCCACGGAGTTTCTTCTAGC-3′ and 5′-GCTCTAGACAGGGCTCTCAAGCACCTAA-3′ for CYP1A1, 5′-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTT-TTCTCGGCCACAGAGCTTC-3′ and 5′-GCTCTAGATGGTGTCTTCTTCACTTGATGG-3′ for CYP1A2, and 5′-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTT-CCTGGAGAGGTGACCACTT-3′ and 5′-GCTCTAGATTGCTAGTTAAGTAAATTCAAATCATC-3′ for CYP1D1, respectively. The NdeI and XbaI sites (underlined) in the forward and reverse primers, respectively, were usedto subclone PCR products into pCW vectors that contained human NADPH-P450 reductase cDNA. Membranes were prepared and content of P450 protein and reductase in each membrane preparation was measured as described previously [11,12].

2.9. Enzymatic characterization of CYP1D1

The CYP1D1 recombinant protein, along with CYP1A1 and CYP1A2, were analyzed for EROD [13] and caffeine oxidation [14], as described previously. Briefly, typical reactions (0.25 ml) contained recombinant protein (5 pmol) or human or cynomolgus monkey liver microsomes (0.1–2.0 mg protein/ml), an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 unit/ml glucose 6-phosphate dehydrogenase), and substrate (10 μM 7-ethoxyresorufin or 2 mM caffeine) in 100 mM potassium phosphate buffer (pH 7.4). Reactions were incubated at 37 °C for 10 min and terminated by adding 0.5 ml of methanol. Incubates were centrifuged at $900 \times g$ for 5 min and supernatants were analyzed by high-performance liquid chromatography with a fluorescence or UV detector.

2.10. Immunoblotting

Immunoblotting was performed as described previously [12] with the following modifications. Rabbit anti-CYP1D1 antibody was produced using specific peptides (NH2-SKGRKQLSPPGPWS-COOH) by Thermo Fisher Scientific (Waltham, MA). Cynomolgus monkey CYP1A1, CYP1A2, and CYP1D1 proteins (1 pmol each), and cynomolgus monkey liver microsomes (15 μg or 50 μg) were run on 10% SDS polyacrilamide gels and transferred to Hybond-P filters (GE Hrealthcare, Piscataway, NJ). The filters were immunoblotted with rabbit anti-CYP1D1 (1:1000) and sheep anti-rabbit IgG conjugated with horseradish peroxidase (Surmodics, Eden Prairie, MN). A signal of protein disulfide isomerase (PDI) as a loading control was detected as described previously [12]. A protein band was visualized using the ECL Plus Western blotting detection reagent (GE Healthcare) following the manufacturer's instructions.

3. Results

3.1. Identification of CYP1D1 sequence in the macaque genome

To identify gene sequences highly identical to human *CYP1D1P*, the genome data of rhesus macaque, a species closely related to cynomolgus monkey, was analyzed using BLAT, since the genome sequencing has not been completed in cynomolgus monkey. We successfully found a *CYP1D1P* sequence in macaque chromosome 15, located along with *TMC1*, *ALDH1A1*, and *ANX1*, coinciding well in location and direction with their respective orthologs in human chromosome 9 (Fig. 1). Interestingly, in zebrafish, *ALDH1A1* was

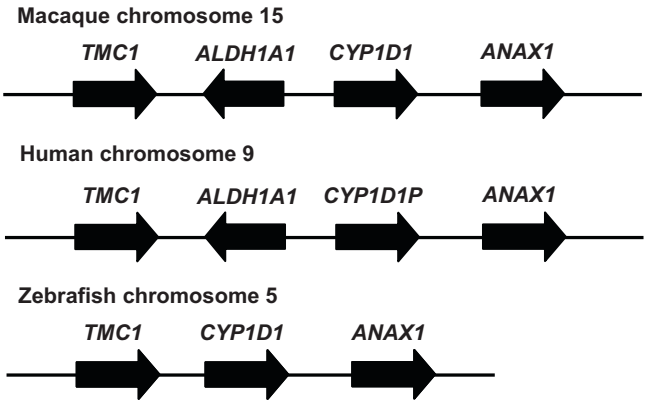

Fig. 1. Genomic structure of macaque *CYP1D1*. The rhesus monkey genome was analyzed using BLAT. *CYP1D1* was located adjacent to *TMC1*, *ALDH1A1*, and *ANX1* in the macaque genome with their directions and locations corresponding well with human orthologs. In zebrafish, *CYP1D1* was also located adjacent to *TMC1* and *ANX1* in the corresponding genome region, but *ALDH1A1* was absent. Size of the genes and the distance between the genes are not proportionate to actual measurement.

Fig. 2. Multiple alignment of CYP1D1 amino acid sequences. Amino acid sequences deduced from cynomolgus monkey (mf) and rhesus monkey (mm) CYP1D1 cDNAs were aligned with the amino acid sequences postulated from human (h) *CYP1D1P* sequence. The sequences were aligned using ClustalW. The hyphen (-) in the sequence of human CYP1D1P indicates the nonsense mutation. Above the sequences, solid and broken lines indicate the putative substrate recognition sites (SRSs) and heme-binding region, respectively. Asterisks under the sequences indicate identical amino acids.

absent in this region of the genome, although the location and direction of *TMC1*, *CYP1D1*, and *ANX1* were conserved well with human and macaque.

3.2. Isolation of CYP1D1 cDNA from liver

To isolate cDNA corresponding to the *CYP1D1P*-like gene found in the macaque genome, we designed gene-specific primers for amplification of the potential open reading frame (ORF). Using these primers, RT-PCR was performed to isolate CYP1D1 cDNAs from cynomolgus and rhesus monkey liver samples. The isolated cDNAs were 1621 bp long and contained an ORF of 537 amino acids with primary sequence structures characteristic of P450 proteins, including a heme-binding region and putative substrate recognition sites (SRSs) [15] (Fig. 2). Although the forward primer overlapped the putative translation initiation site, it was verified by 5′RACE with cynomolgus and rhesus monkey total liver RNAs. Cynomolgus and rhesus monkey CYP1D1 cDNA sequences were deposited in GenBank under the accession numbers, GU289741 and GU289742, respectively. Cynomolgus monkey CYP1D1, nearly identical to rhesus monkey CYP1D1 (differing in only four amino acid residues), shared a high sequence identity (91%) with amino acid sequences deduced from the human *CYP1D1P* sequence without the nonsense mutations. Cynomolgus monkey CYP1D1 shared the highest sequence identities to CYP1A1 (43%) and CYP1A2 (44%) among cynomolgus monkey P450s (Table 1). Cynomolgus monkey CYP1D1 was much less identical to killfish (49%) and zebrafish (50%) CYP1D1 homologs. Phylogenetic analysis indicated that macaque CYP1D1 was most closely clustered with human CYP1D1P (Fig. 3). These results indicate evolutionary closeness of CYP1D in macaque and human.

3.3. Gene structure of CYP1D1

The exon–intron structure of *CYP1D1* was analyzed by aligning the CYP1D1 cDNA of cynomolgus monkey on the rhesus monkey genome using BLAT. *CYP1D1* spanned nearly 14 kb and contained six exons. The sizes of exons were 87 bp to ≥377 bp, while intron sizes were 80–4821 bp (Table 2). Virtually all introns begin with the dinucleotide GU and end with AG, consistent with the consensus sequences for splice junctions in eukaryotic genes.

3.4. CYP1D1 mRNA expression in cynomolgus monkey tissues

The expression levels of CYP1A1, CYP1A2, and CYP1D1 mRNAs were measured by real-time RT-PCR in cynomolgus monkey tissues: brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus. Among the 10 tissues analyzed, CYP1D1 mRNA was expressed most abundantly in liver, followed by kidney and jejunum, while CYP1A1 and CYP1A2 mRNAs were expressed most abundantly in liver (Fig. 4). CYP1D1 mRNA expression level was comparable to that of CYP1A1 and approximately 21-fold higher than that of CYP1A2 in liver. CYP1A1 and CYP1D1 mRNAs were also expressed in brain, lung, heart, adrenal gland, testis, ovary, and uterus at low levels. CYP1D1 mRNA levels were higher

Table 1
Identity of cynomolgus monkey CYP1D1 to other vertebrate CYP1s in amino acid sequences.

	Amino acid (%)
Human	
CYP1A1	44
CYP1A2	45
CYP1D1P[a]	91
Cynomolgus monkey	
CYP1A1	43
CYP1A2	44
Rhesus monkey	
CYP1A1	43
CYP1D1	98
Japanese monkey	
CYP1A2	44
Marmoset	
CYP1A2	43
Killfish	
CYP1D1	49
Zebrafish	
CYP1D1	50

A homology search was carried out using BLAST.
[a] For human CYP1D1P, amino acid sequence translated from the gene sequence highly identical to cynomolgus monkey CYP1D1 cDNA found in the human genome was used for comparisons.

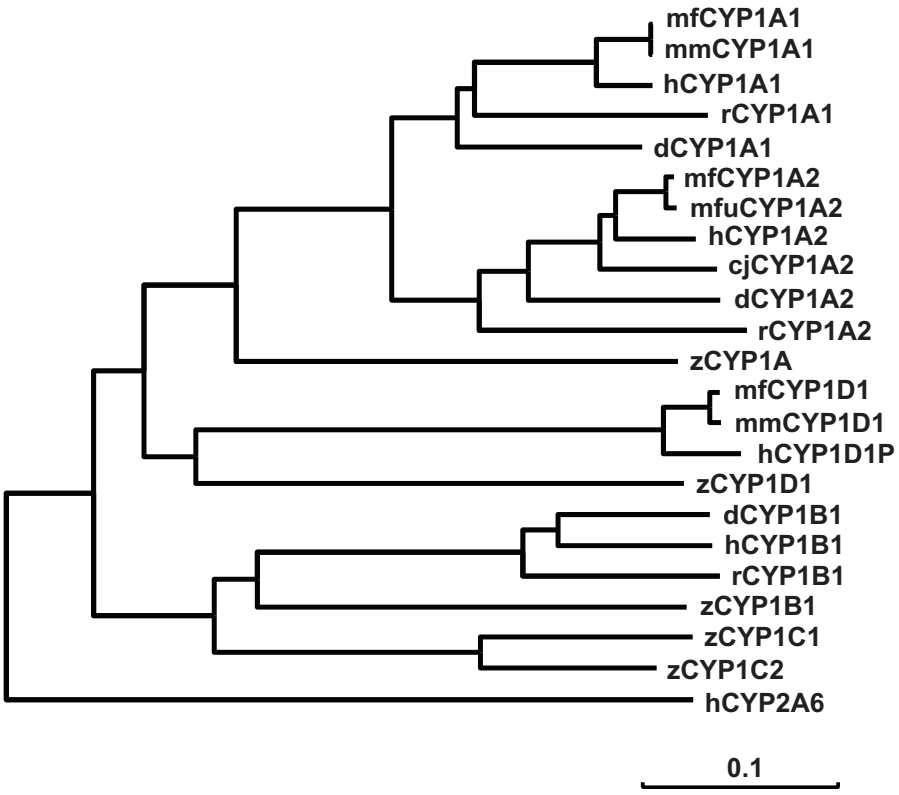

Fig. 3. Phylogenetic tree of CYP1 amino acid sequences. The phylogenetic tree was created by the neighbour-joining method using CYP1A1, CYP1A2, and CYP1D1 amino acid sequences from human (h), cynomolgus monkey (mf), rhesus monkey (mm), Japanese monkey (mfu), marmoset (cj), dog (d), rat (r), and zebrafish (z). Human CYP2A6 was used as outgroup. The scale bar represents 0.1 amino acid substitutions per site.

than CYP1A1 mRNA levels, in all these tissues except lung (data not shown).

3.5. Induction of CYP1D1 mRNA

To see if CYP1D1 is inducible, CYP1D1 mRNA level was measured using total RNAs extracted from the cynomolgus monkey hepatocytes (two lots) that were treated with human P450 inducer (rifampicin, omeprazole, or dexamethazone) dissolved in DMSO. CYP1D1 mRNA levels of control hepatocytes (DMSO-treated) and inducer-treated hepatocytes were compared. The results showed that CYP1D1 mRNA was induced by 1.9-fold ($p < 0.05$) or 3.2-fold ($p < 0.01$) with omeprazole, depending on the lot of the hepatocytes (Fig. 5), but the level of induction was much lower than that of CYP1A1 mRNA (data not shown). CYP1D1 mRNA was also significantly induced by 2.0-fold ($p < 0.05$) with rifampicin in one lot of hepatocytes.

Table 2
Exon–intron boundary sequences of macaque *CYP1D1*.

Exon	Exon size (bp)	3′ splice site	5′ splice site	Intron size (bp)
1	170		GGCACAG**gt**gagcctt	3909
2	839	ctgttttt**ag**GTGATAT	TGATAAG**gt**aaggatt	1856
3	124	tctcctc**ag**GATCATA	GGAGCTG**gt**atgtgac	4821
4	90	ctttttt**ag**GGTTTGA	GAAATTG**gt**aatatgt	80
5	121	tctttaa**ag**ATGGAAA	CACATTG**gt**aagcata	2961
6	87	ctttgtt**ag**TACTACA	ACGATGA**gt**aagtcac	2656
7	≥377	ttcatgc**ag**AACTATT		

Exon–intron structure was determined by mapping the cynomolgus monkey CYP1D1 cDNA sequence on the rhesus monkey genome using BLAT. Exon and intron sequences are indicated in capital and small letters, respectively. The dinucleotide sequence at the highly conserved GU–AG motif is shown as bold lettering with underline.

3.6. Drug-metabolizing activities

To investigate the oxidation potential of CYP1D1, EROD and caffeine oxidation were analyzed using the proteins heterologously expressed in *E. coli* (cynomolgus monkey CYP1A1, CYP1A2, and CYP1D1, and human CYP1A1 and CYP1A2). CYP1D1 activity was observed in EROD, albeit at a lower level than CYP1A1 and CYP1A2 (Table 3). 3-Demethylation of caffeine was mainly catalyzed by

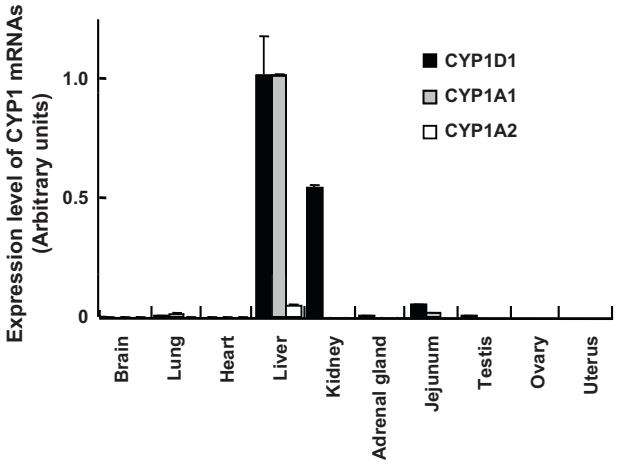

Fig. 4. Expression of CYP1D1 mRNA in cynomolgus monkey tissues. Expression of CYP1D1 mRNA was quantified by real-time RT-PCR in brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus. As a comparison, expression of CYP1A1 and CYP1A2 mRNAs was also measured. Expression level of each mRNA was normalized to 18S rRNA level and values represent the average ± SD from three independent amplifications. For graphic presentation, the level of CYP1D1 mRNA expression in liver was adjusted to 1, with which all other values were compared.

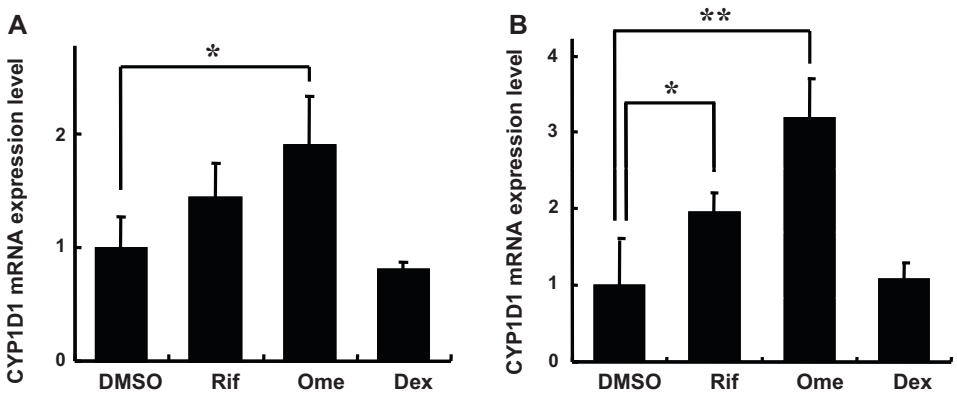

Fig. 5. Induction of CYP1D1 mRNA in primary hepatocytes of cynomolgus monkey. CYP1D1 mRNA was measured by real-time RT-PCR in the cynomolgus monkey hepatocytes that were treated with P450 inducer, rifampicin (Rif), omeprazole (Ome), or dexamethazone (Dex). Two lots of the hepatocytes (A and B), derived from different animals, were analyzed. The CYP1D1 mRNA level in the inducer-treated hepatocytes was compared with that of the control hepatocytes treated with solvent (0.1% DMSO). For graphic presentation, the value of the control (DMSO) was adjusted to 1, with which all other values were compared. CYP1D1 mRNA was significantly induced with omeprazole in both lots of the hepatocytes, and with rifampicin in one lot of the hepatocytes. $^{*}p < 0.05$; $^{**}p < 0.01$.

CYP1A1 while 8-hydroxylation was mediated by CYP1A1 and CYP1A2, or by CYP1D1 to less extent, in contrast to the cases using human enzymes. These results indicate that CYP1D1 is a functional enzyme and, at least partly, shares metabolic property with CYP1As.

3.7. Immunoblotting

Protein expression in liver microsomes was investigated using a polyclonal antibody raised against cynomolgus monkey CYP1D1. Analysis of blots using cynomolgus monkey CYP1A1, CYP1A2, and CYP1D1 proteins revealed two distinct bands; a large band in CYP1D1 and a small band in CYP1A1 and CYP1A2 (Fig. 6). The large band can be used as an indicator of CYP1D1 protein. A strong signal was not detected, using this antibody, in cynomolgus monkey liver microsomes, but large and small bands, although faint, were visibly appreciable with the naked eye. It should be noted that a similar faint band was detected even with a larger amount of liver microsomes (50 μg). These bands could correspond to CYP1D1 and CYP1As (CYP1A1 and CYP1A2), respectively (Fig. 6), and suggest that CYP1D1 is expressed at a level barely detectable in cynomolgus monkey liver.

3.8. Genotyping of CYP1D1 exons

Human *CYP1D1P* contains five nonsense mutations, three (c.128G > A, c.424C > T, and c.618T > G) in exon 2 and two (c.1441C > A and c.1545C > A) in exon 7. To determine if nonsense mutations corresponding to those in human *CYP1D1P* are present in macaque *CYP1D1*, *CYP1D1* exon 2 and exon 7, from 20 cynomolgus monkeys (10 Indochinese and 10 Indonesian) and 10 rhesus monkeys, were sequenced. All these animals, unlike humans, did not have the nonsense mutations in exon 2 and exon 7 (Fig. 7).

Table 3
Drug-metabolizing activity of CYP1D1 as compared with CYP1A1 and CYP1A2 in cynomolgus monkey and human.

	Ethoxyresorufin *O*-deethylation	Caffeine	
		3-Demethylation	8-Hydroxylation
Cynomolgus monkey			
CYP1A1	4.8	0.44	0.16
CYP1A2	2.1	<0.01	0.18
CYP1D1	0.33	<0.01	0.04
Liver microsomes	0.086	0.07	0.03
Human			
CYP1A1	12.1	0.04	0.04
CYP1A2	0.27	0.19	0.02
Liver microsomes	0.020	0.03	0.01

nmol/min/nmol P450 (recombinant protein), nmol/min/mg protein (liver microsomes). Metabolic assays were carried out using substrate (10 μM 7-ethoxyresorufin or 2 mM caffeine), and 20 nM of the recombinant protein or 0.1–2 mg protein/ml of liver microsomes (cynomolgus monkey and human) as described in Section 2.

4. Discussion

Human *CYP1D1P*, a pseudogene, does not encode a functional protein due to five nonsense mutations in the potential coding region. By analyzing rhesus monkey genome data, we found a gene sequence highly identical to human *CYP1D1P*. Using this sequence information, we isolated CYP1D1 cDNA from cynomolgus and rhesus monkey livers. The deduced amino acid sequences of these macaque CYP1D1s were highly identical to amino acid sequences predicted from the human *CYP1D1P* sequence (without nonsense mutations).

In this study, phylogenetic analysis showed that CYP1D1 was more closely related to CYP1As than CYP1Bs (or CYP1Cs in zebrafish) in all seven species tested, human, cynomolgus monkey,

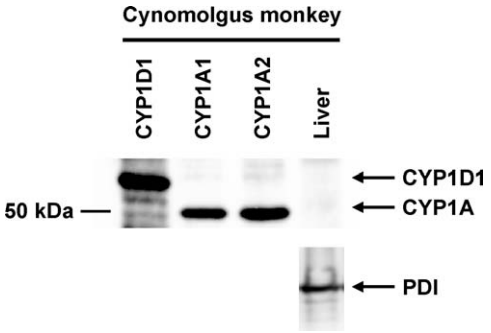

Fig. 6. Expression of CYP1D1 protein in liver microsomes. The recombinant proteins (1.0 pmol of P450/lane) of cynomolgus monkey CYP1A1, CYP1A2, and CYP1D1 or cynomolgus monkey liver microsomes (15 μg) were analyzed by immunoblotting using the antibody raised against cynomolgus monkey CYP1D1. The analysis using the recombinant proteins of CYP1A1, CYP1A2, and CYP1D1 indicated that a large band, seen in CYP1D1 but not in CYP1A1 or CYP1A2, could be used as an indication of CYP1D1 protein. The analysis using cynomolgus monkey liver microsomes indicated that expression of CYP1D1 was barely detected with the naked eye. PDI (protein disulfide isomerase) used as a loading control was detected in the liver microsomes.

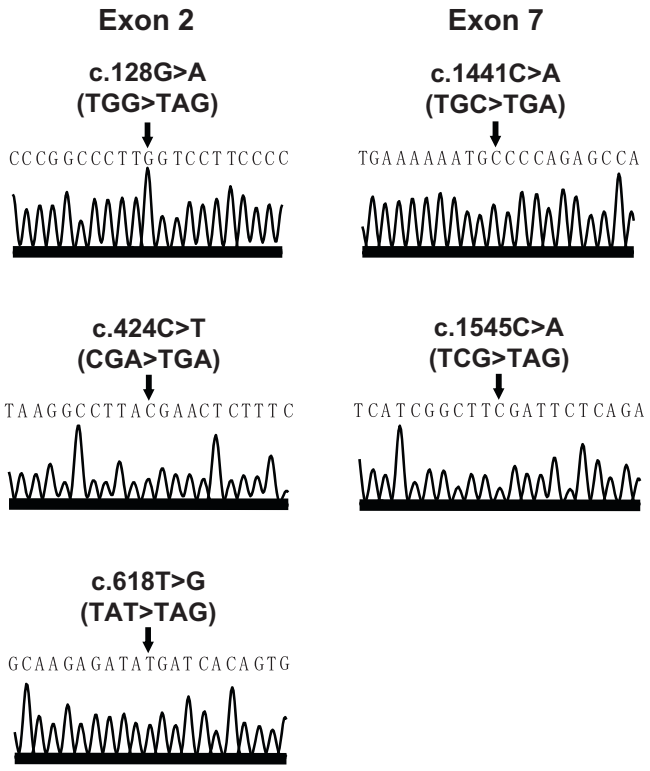

Fig. 7. Analysis of *CYP1D1* exons in cynomolgus and rhesus monkeys. The presence of the nonsense mutations corresponding to those of human *CYP1D1P* (c.128G > A, c.424C > T, and c.618T > G in exon 2, and c.1441C > A and c.1545C > A in exon 7) was assessed by direct sequencing of exon 2 and exon 7. None of the nonsense mutations or other deleterious mutations was found in 10 Indochinese cynomolgus monkeys, 10 Indonesian cynomolgus monkeys, and 10 rhesus monkeys. The figure shows a representative of the *CYP1D1* chromatograms, which was obtained from a cynomolgus monkey.

rhesus monkey, marmoset, dog, rat, and zebrafish. Similar phylogenetic relationships were also found in zebrafish and killfish, which led to the hypothesis that *CYP1A*/*CYP1D* and *CYP1B*/*CYP1C* diverged from different common ancestral genes [16,17]. The results of this study suggest that this hypothesis might also be true in mammals, including primate species.

In the macaque genome, *CYP1D1* was located adjacent to *TMC1*, *ALDH1A1*, and *ANX1*, and the directions and arrangement of these genes corresponded well with the respective human orthologs. Similarly, *CYP1D1* homologs were also found in chimpanzee, orangutan, dog, and bovine genomes with similar genomic organization, but were not found in mouse and rat (Uno Y, unpublished data), indicating that the genome structure of *CYP1D1* is conserved in some mammalian species, but *CYP1D1* has been lost in others. *CYP1D1* was also identified in killfish and zebrafish [2,18]. Interestingly, zebrafish *CYP1D1* was located along with *TMC1* and *ANX1* in the genome, but *ALDH1A1* was absent from this region of the genome. Similarly, killfish and medaka also lacked *ALDH1A1* in this region of the genome, and thus *ALDH1As* were not conserved in teleosts [19]. A similar gene organization of *CYP1D1* indicated that *CYP1D1* is evolutionarily conserved from fish to human, but has been lost or pseudogenized in some species.

Of the 10 tissues analyzed, CYP1D1 mRNA was expressed most abundantly in liver, followed by kidney and jejunum. Expression level of CYP1D1 mRNA in liver was comparable to that of CYP1A1 mRNA. And, expression level of CYP1A2 mRNA was much lower than that of CYP1A1, as has been shown previously [20], indicating that CYP1D1, along with CYP1A1, might be a major CYP1 in liver. In jejunum and kidney, CYP1D1 mRNA expression levels were higher than that of CYP1A1 or CYP1A2, and thus CYP1D1 might play a role in these tissues (e.g. the first-pass effect in jejunum). CYP1D1 mRNA was also expressed in adrenal gland, testis, ovary, and uterus, indicating that CYP1D1 might be involved in the metabolism of endogenous substrates, such as sex hormones.

Cynomolgus monkey CYP1A1 mRNA was expressed in liver as well as extra-hepatic tissues, while cynomolgus monkey CYP1A2 mRNA was expressed predominantly in liver. In human, CYP1A1 is expressed in the extrahepatic tissues, and CYP1A2 is expressed predominantly in liver. These results indicate a similar tissue expression pattern for CYP1A1 and CYP1A2 between cynomolgus monkey and human, except for CYP1A1 hepatic expression. Expression levels of CYP1As in liver appear to be much lower in cynomolgus monkey than in human, since expression of cynomolgus monkey CYP1A proteins are barely detectable [21,22]. Basal expression of CYP1As in liver might be regulated differently in the two species.

Omeprazole induces expression of human CYP1A1 in an AHR-dependent manner [23]. Omeprazole also induces cynomolgus monkey CYP1A1 mRNA expression in the primary hepatocytes [10]. In this study, omeprazole induced CYP1D1 mRNA expression in macaque primary hepatocytes, but to a much lesser extent than CYP1A1 mRNA. In zebrafish, CYP1D1 mRNA expression was not induced by potent AHR agonists (3,3′,4,4′,5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) [17], but was expressed abundantly in early embryos, suggesting that CYP1D1 might play a developmental role, metabolizing endogenous substrates [18]. In zebrafish, *CYP1D1* has 2 putative XREs in the upstream regulatory region, whereas AHR-responsive genes *CYP1A*, *CYP1B*, *CYP1C1*, and *CYP1C2* contained 12, 6, 4, and 6 XREs, respectively. A search for XREs found 1 putative XRE in the 5′-flanking region (approximately 10 kb) of *CYP1D1* (Uno, Y, unpublished data), possibly supporting the weak induction of macaque *CYP1D1* by omeprazole.

In cynomolgus monkey, CYP1D1 catalyzed EROD, but less efficiently than CYP1A1 or CYP1A2. MROD catalysis by the CYP1A2 T124S mutant in human decreased by 80% from wild-type activity, while EROD and MROD catalysis by the CYP1A1 equivalent reciprocal mutant (S122T) increased [24], indicating the importance of threonine in this position of CYP1A protein to metabolize a substrate. Amino acid residue in the corresponding position of CYP1D1 is serine, which partly explains a low activity of CYP1D1 in EROD. In human, CYP1A1 and CYP1A2 have clear preferences for EROD and MROD, respectively [3,4], and amino acid changes of V382L in CYP1A1 and L382V in CYP1A2 show interchanged specificities [24]. The importance of residue 382 for substrate binding has been suggested for human CYP1A1 [25] and CYP1A2 [26]. Residue L382 in CYP1D1 might also account for the low catalytic efficiency of EROD by CYP1D1.

Species differences are important in drug metabolism, because animal data need to be extrapolated to humans. Macaques share very similar metabolic properties with humans, but differences are noted occasionally. Previously, we showed that such species differences were partly accounted for by CYP2C76, which does not have human orthologs [8]. Cynomolgus monkey CYP1D1 metabolized caffeine in this study. In human, caffeine 3-demethylation (a major pathway) is mediated by CYP1A2 [5], while caffeine 8-hydroxylation is mediated by CYP2A6 and CYP1A2 [14]. In cynomolgus monkey, CYP1D1, together with CYP1A2, catalyzed caffeine 8-hydroxylation, but not caffeine 3-demethylation. Instead, cynomolgus monkey CYP1A1 catalyzed caffeine 3-demethylation. Therefore, minimal caffeine 3-demethylation in cynomolgus monkey liver [27], might be accounted for by a relatively low CYP1A1 expression in cynomolgus monkey liver, as shown by this study and previous studies [21,22,27].

Caffeine 7-demethylation to theophylline has been reported to be the major pathway in cynomolgus monkey [27] and rhesus

monkey [28]. It is not known which P450s account for 7-demethylation in these monkey species, but inhibition of this reaction by furafylline and α-naphthoflavone, potent CYP1A inhibitors, indicates that CYP1A-like proteins are involved in this reaction [29], which would give an important insight into species difference in drug metabolism. In cynomolgus monkey, CYP1A1 mRNA is expressed much more abundantly than CYP1A2 mRNA in liver; raising the possibility that this 7-demethylation might be catalyzed by CYP1A1. Alternatively, CYP1D1 might be involved in this reaction, considering a comparable expression level of CYP1D1 mRNA to CYP1A1 mRNA and enzymatic properties of CYP1D1 shared with CYP1As (both CYP1D1 and CYP1As catalyzed EROD and caffeine 8-hydroxylation). However, in caffeine metabolic assays using CYP1D1 protein, theophylline formation was below the detection limit under our current experimental conditions (unpublished data), which suggests a minor role of CYP1D1 in caffeine 7-demethylation, considering that theophylline is formed from caffeine through 7-demethylation. CYP1D1 presents another possibility to account for such species differences, since the macaque ortholog of human *CYP1D1P* (a pseudogene) is expressed and functional in liver. Further investigation on the metabolic properties of CYP1D1 using various P450 substrates, will help to assess the importance for such species differences.

P450s are known to be polymorphic in human. Macaque *P450s* might also be polymorphic as evidenced by metabolic assays [30] and identification of numerous genetic variants in *P450s*, including *CYP2C76* and *CYP3As* [31,32]. In human *P450s* such as *CYP2A6* and *CYP2D6*, the entire genes are lost or their protein functions are inactivated by nonsense mutations in some individuals (see http://www.imm.ki.se/CYPalleles/). Human *CYP1D1P* was pseudogenized by five nonsense mutations, which were not found in the animals genotyped in this study. In the course of this analysis, genetic variants were found in exon 2 and exon 7, including non-synonymous variants, which might influence the metabolic activity of CYP1D1. Functional characterization of these and other variants will help reveal the genetic diversity of *CYP1D1* and the functional properties of the CYP1D1 protein variants.

In conclusion, we identified a new CYP1, CYP1D1, highly identical to human CYP1D1P in cynomolgus and rhesus monkeys. Cynomolgus monkey CYP1D1 shared the highest sequence identity of amino acids with CYP1A1 and CYP1A2 among cynomolgus monkey P450s. CYP1D1 mRNA was expressed most abundantly in liver and its hepatic expression level was comparable with CYP1A1 mRNA. Cynomolgus monkey CYP1D1 partially shares metabolic properties with CYP1As, as it catalyzed EROD, which is generally catalyzed by CYP1As. CYP1D1 mRNA was induced with omeprazole, but at a much lower level than CYP1A1 mRNA. Finally, the 20 cynomolgus and 10 rhesus monkeys analyzed did not possess the nonsense mutations equivalent to those of human *CYP1D1P*. These results suggest the functional role of CYP1D1 as a drug-metabolizing enzyme in macaques.

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