A new marmoset P450 4F12 enzyme expressed in small intestines and livers efficiently metabolizes an anti-histaminic drug ebastine

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Running Title Page

Running title: Cloning of four marmoset P450 4F DNAs

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Abbreviations: CYP, individual forms of cytochrome P450 (EC 1.14.14.1); HPLC, high performance liquid chromatography; P450, general term for cytochrome P450; PCR, polymerase chain reaction; PXR, pregnane X receptor; RT, reverse transcription; SRS, substrate recognition site.
Abstract

Common marmosets (*Callithrix jacchus*) are attracting attention as animal models in preclinical studies for drug development. However, cytochrome P450s (P450s), major drug-metabolizing enzymes, have not been fully identified and characterized in marmosets. In this study, based on the four novel *P450 4F* genes found on the marmoset genome, we successfully isolated P450 4F2, 4F3B, 4F11, and 4F12 cDNAs in marmoset livers. Deduced amino acid sequences of the four marmoset P450 4F forms exhibited high sequence identities (87-93%) to the human and cynomolgus monkey P450 4F homologues. Marmoset P450 4F3B and 4F11 mRNAs were predominantly expressed in livers, whereas marmoset P450 4F2 and 4F12 mRNAs were highly expressed in small intestines and livers. Four marmoset P450 4F proteins heterologously expressed in *Escherichia coli* catalyzed the ω-hydroxylation of leukotriene B₄. Additionally, marmoset P450 4F12 effectively catalyzed the hydroxylation of anti-allergy drug ebastine, a human P450 2J/4F probe substrate. Ebastine hydroxylation activities by small intestine and liver microsomes from marmosets and cynomolgus monkeys showed greatly higher values than those of humans. Ebastine hydroxylation activities by marmoset and cynomolgus monkey small intestine microsomes were inhibited (~60%) by anti-P450 4F antibodies, unlike human small intestinal microsomes, suggesting that contribution of P450 4F enzymes for ebastine hydroxylation in small intestine might be different between marmosets/cynomolgus monkeys and humans. These results indicated that marmoset P450 4F2, 4F3B, 4F11, and 4F12 were expressed in livers and/or small intestines, and were functional in the metabolism of endogenous and exogenous compounds, similar to those of cynomolgus monkeys and humans.
Introduction

Common marmosets (*Callithrix jacchus*), New World primates, have attracted much attention as the non-human primate model in various biomedical fields, including drug development, neuroscience, and regenerative medicine due to their genetic closeness to humans, small body size, high reproductive efficiency, and applicability of transgenic technologies (Kishi et al., 2014). Cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*), Old World primates, have been extensively used for evaluating drug candidate compounds, and are important species in preclinical drug development studies.

Cytochrome P450s (P450s), the major drug-metabolizing enzymes consisting of multiple subfamilies, play an important role in the metabolism of endogenous and exogenous compounds. In humans, the P450 4F subfamily consists of seven members, P450 4F2, 4F3A, 4F3B, 4F8, 4F11, 4F12, and 4F22 forms expressed in various tissues, including livers and kidneys (P450 4F2, 4F3B, 4F11, and 4F12), intestines (P450 4F2 and 4F12), myeloid cells (P450 4F3A), seminal vesicles (P450 4F8), and epidermis (P450 4F8 and 4F22) (Cui et al., 2000; Bylund et al., 2001; Christmas et al., 2001; Stark et al., 2003; Kelly et al., 2011) and most of these forms catalyze ω-hydroxylation of eicosanoids, including arachidonic acid, leukotrienes, and prostaglandin (Hsu et al., 2007). These metabolites play essential roles in many biological processes such as inflammatory response, tumor progression, angiogenesis, and blood pressure regulation (Johnson et al., 2015). Additionally, P450 4F12 reportedly metabolizes a number of anti-allergy drugs including ebastine, astemizole, and terfenadine (Hashizume et al., 2002; Eksterowicz et al., 2014). P450 4F2 and/or 4F3B also are responsible for the O-demethylation of the anti-parasitic prodrug pafuramidine and the ω-hydroxylation of fingolimod, oral drug for relapsing multiple sclerosis (Wang et al., 2007;
Jin et al., 2011). These findings suggest that P450 4F enzymes are responsible for endogenous and xenobiotic biotransformations.

P450 4F forms have been identified in various species, including mice (P450 4F13, 4F14, 4F15, 4F16, 4F17, 4F18, 4F37, 4F39, and 4F40), rats (P450 4F1, 4F4, 4F5, 4F6, 4F17, 4F18, 4F37, and 4F40), pigs (P450 4F55), cynomolgus monkeys (P450 4F2, 4F3B, 4F11, and 4F12), rhesus monkeys (P450 4F8, 4F12, and 4F22), and orangutans (P450 4F11 and 4F12). In cynomolgus monkeys, P450 4F2, 4F3B, 4F11, and 4F12 were expressed in livers, kidneys, and/or jejunum (Uno et al., 2007). Cynomolgus monkey P450 4F2, 4F3B, and 4F11 proteins heterologously expressed in *Escherichia coli* metabolized arachidonic acids (Uno et al., 2011). Cynomolgus monkey P450 4F-like enzymes are major catalysts of ebastine hydroxylation in small intestines (Hashizume et al., 2001). However, in spite of the potential importance as a primate model in drug development, marmoset P450 4F forms have not been identified.

More than 17 marmoset P450 forms have been identified to date belonging to the P450 1A, 1B, 2A, 2B, 2C, 2D, 2E, 2J, and 3A subfamilies (Igarashi et al., 1997; Schulz et al., 1998; Mayumi et al., 2013; Uehara et al., 2015a; Uehara et al., 2015b; Uehara et al., 2015c; Uehara et al., 2016a; Uehara et al., 2016b; Uehara et al., 2016c). Marmoset P450s have high sequence identities (>85%) to their human P450 counterparts, and most marmoset P450 enzymes generally showed similar substrate specificity to their homologous human P450s, except for P450 2A and 2B enzymes. In this study, we isolated cDNAs of four P450 4F forms from livers. Gene cluster organization, sequence identity, mRNA tissue distribution, and enzymatic characteristics of marmoset P450 4F2, 4F3B, 4F11, and 4F12 were investigated to provide understanding of the metabolism of drugs and fatty acids in marmosets.
Materials and Methods

Chemicals and enzymes

Ebastine, hydroxyebastine, and carebastine were purchased from Almirall-Prodesfarma (Barcelona, Spain). Leukotriene B₄ and 20-hydroxyleukotriene B₄ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bufuralol and 1′-hydroxybufuralol were purchased from Corning Life Sciences (Woburn, MA) and Toronto Research Chemicals (Toronto, Canada), respectively. Coumarin and midazolam were purchased from Wako Pure Chemicals (Osaka, Japan). Resorufin, 7-Ethoxyresorufin, S-warfarin, 7-hydroxywarfarin, and 1′-hydroxymidazolam were purchased from Sigma-Aldrich (Tokyo, Japan). Oligonucleotide primers for polymerase chain reaction (PCR) were synthesized by Sigma-Aldrich. Pooled liver microsomes from marmosets (14 males, sexually mature), cynomolgus monkeys (10 males, 2-7 years old), and humans (74 males and 76 females, 18-82 years old) were purchased from Corning Life Sciences. Pooled intestine microsomes from cynomolgus monkeys (15 males, 4-8 years old) and humans (7 males and 6 females, 18-55 years old) were purchased from Xenotech (Lenexa, KS). Pooled small intestine microsomes from marmosets were prepared from tissue samples of 20 marmosets (10 males and 10 females, >2 years old) as described previously (Uehara et al., 2011). Brains, lungs, livers, kidneys, and small intestines were collected from 20 marmosets at the Central Institution for Experimental Animals (Kawasaki, Japan). Protocols for the animal study were reviewed and approved by the Institutional Animal Care and Use Committee. Anti-human P450 4F antibodies (Hashizume et al., 2001) were donated by Prof. Takanori Hashizume. All other solvents and chemicals used were the highest grade available commercially.
P450 4F cDNA cloning

Marmoset and human P450 4F cDNAs were cloned from liver by reverse transcription (RT)–PCR. Total RNAs were extracted from marmoset and human livers using an RNeasy Mini Kit (Qiagen, Valencia, CA). RT reaction was carried out in a mixture containing 1 µg of total RNA, oligo(dT) primers, and SuperScript III RT reverse transcriptase (Invitrogen, Carlsbad, CA) at 50°C for 60 minutes according to the manufacturer’s instructions. PCR amplification was carried out for 30 cycles; each denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 3 min. PCR primers used were cjCYP4F2 (5rt1) 5′-CAGCAGAAGAGGAGAGGAGG-3′ and cjCYP4F3B (3rt2) 5′-TCAGGGGTCTATTTAGGAAGGG-3′ for marmoset P450 4F2, cjCYP4F3B (5rt2) 5′-GACCTCACCCCTCCCATCCTGTA-3′ and cjCYP4F3B (3rt2) 5′-TCAGGGGTCTATTTAGGAAGGG-3′ for marmoset P450 4F3B, cjCYP4F11 (5rt2) 5′-GACCTCATCCTCCATCCATCC-3′ and cjCYP4F11 (3rt3) 5′-AGGTGGGTGGGTTGATCCGAC-3′ for marmoset P450 4F11, cjCYP4F12 (5rt2) 5′-GAACAAAGCCTGCTCCTGACAG-3′ and cjCYP4F12 (3rt2) 5′-CTATGCAGAGGTGACAGAAGC-3′ for marmoset P450 4F12, hCYP4F2 (5rt1) 5′-GAGAGGAGGTGTTGTCTGGGAC-3′ and hCYP4F2 (3rt1) 5′-TTTAGTGGGGTGTCAGAGTGGG-3′ for human P450 4F2, hCYP4F3B (5rt1) 5′-GGAGGTTGTTGGGACAGG-3′ and hCYP4F3B (3rt2) 5′-TCTTAGGGTAATTCTAGGCC-3′ for human P450 4F3B, hCYP4F11 (5rt2) 5′-ATCCTTTGGCCCTTTCCACTG-3′ and hCYP4F11 (3rt2) 5′-CCCACTCACCTCCCTTTCTT-3′ for human P450 4F11, and hCYP4F12 (5rt1) 5′-GGAGAAGAGGTTGTGTTGGGA-3′ and hCYP4F12 (3rt1) 5′-AAACAGGTGGATGGTTCAGA-3′ for human P450 4F12. All PCR products were...
cloned into pGEM-T easy vector for TA-cloning (Promega, Madison, WI), and then the sequence of each insert was confirmed by DNA sequencing. Cynomolgus monkey P450 4F2, 4F3B, 4F11, and 4F12 cDNAs were identified as described previously (Uno et al., 2007).

**Sequence analyses of P450 4F forms**

The multiple alignment analysis of amino acid sequences was carried out using Genetyx system (Software Development, Tokyo, Japan). The marmoset genome data was analyzed by BLAT (UCSC Genome Bioinformatics). The amino acid sequence homology with other P450 4F members was analyzed by BLAST (National Center for Biotechnology Information). The phylogenetic tree was constructed by the neighbor-joining method using DNAMIS Pro (Hitachi Software, Tokyo, Japan). The amino acid sequences used were from GenBank; human P450 4A11 (NP_000769), 4F2 (NP_001073), 4F3A (NP_000887), 4F3B (NP_001186137), 4F8 (NP_009184), 4F11 (NP_067010), 4F12 (NP_076433), and 4F22 (NP_775754); orangutan P450 4F11 (NP_001127225) and 4F12 (NP_001126132); cynomolgus monkey P450 4F2 (AAZ29444), 4F3B (NP_001270060), 4F11 (AAZ29446), and 4F12 (NP_001270193); rhesus monkey P450 4F8 (NP_001181472), 4F12 (NP_001181489), and 4F22 (NP_001181468); pig P450 4F55 (NP_001231565); rat P450 4F1 (NP_062569), 4F4 (NP_775146), 4F5 (NP_775147), 4F6 (NP_695230), 4F17 (NP_001178915), 4F18 (NP_001028858), 4F37 (NP_001258281), and 4F40 (NP_001102830); and mouse P450 4F13 (NP_570952), 4F14 (NP_071879), 4F15 (NP_598888), 4F16 (NP_077762), 4F17 (NP_001094915), 4F18 (NP_077764), 4F37 (NP_001093657), 4F39 (NP_796281), and 4F40 (NP_001095058). For marmoset P450 4F forms, the amino acid sequences deduced from cDNAs isolated in this study were used.
Quantitative RT-PCR

The tissue distribution of P450 4F mRNA expression in marmosets was measured by real-time RT-PCR. Total RNAs were extracted from brains, lungs, livers, kidneys, and small intestines using an RNeasy Mini Kit as described earlier. The first-strand cDNAs were reverse transcribed with random primers (Invitrogen) and SuperScript III RT reverse transcriptase (Invitrogen). Real-time PCR was performed with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems) as described previously (Uehara et al., 2015a; Uehara et al., 2015b; Uehara et al., 2015c; Uehara et al., 2016a; Uehara et al., 2016b; Uehara et al., 2016c). The PCR primers used at a final concentration of 400 nM were cjCYP4F2 (5ex2a) 5′-ACACATGGGCGCTGGTCAAC-3′ and cjCYP4F2 (3ex3a) 5′-CTTCAGGAAGCTGTAGAAGACTTG-3′ for marmoset P450 4F2, cjCYP4F3B (5ex2a) 5′-GCCCTTCACCCTCATCATCC-3′ and cjCYP4F3B (3ex3a) 5′-TCAGGAAGCCATAGAAGACTTCATC-3′ for marmoset P450 4F3B, cjCYP4F11 (5ex2a) 5′-CATTTTATGCCACCCTGATACCG-3′ and cjCYP4F11 (3ex3b) 5′-CTTCAGGAAGCCATAGAAGACTTCATC-3′ for marmoset P450 4F11, and cjCYP4F12 (5ex2a) 5′-GCTTGAAGAACTTGACCCAGATATC-3′ and cjCYP4F12 (3ex3a) 5′-AGGAACCTGATGAAGAGCTCG-3′ for marmoset P450 4F12. The PCR conditions were as follows: an initial denaturation for 10 min at 95°C, followed by 40 cycles of 95 °C for 15 s and at 60°C for 1 min. Individual P450 4F mRNA expression was measured in triplicate for each sample using standard curves created by plotting threshold cycle versus the log of the amount of PCR amplicon for each P450 4F form. The expression level of each P450 mRNA was normalized to the level of 18S rRNA measured using Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems).
Heterologous expression of P450 4F proteins

To prepare the recombinant proteins, expression plasmids of marmoset and human P450 4F forms were constructed using pCW vector containing a cDNA of human NADPH-cytochrome P450 reductase as described previously (Uehara et al., 2016c). The N-terminus modification was performed by PCR using the forward and reverse primers containing the restriction sites of the NdeI and XbaI sites (underlined), respectively, including

cjCYP4F2
5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCG-3’ and cjCYP4F2
(3exp2) 5’-TCTAGATTAGCTCAGGGGCTCCA-3’ for marmoset P450 4F2, cjCYP4F2
(5exp1bov) 5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCG-3’ and

 cjCYP4F3B (3exp2) 5’-TCTAGATTAGCTCAGGGGCTCCA-3’ for marmoset P450 4F3B,
cjCYP4F11 (5exp2bov) 5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCG-3’ and

cjCYP4F11 (3exp2) 5’-TCTAGATTACTGTGTTTTCACCC-3’ for marmoset P450 4F11,
cjCYP4F12 (5exp1bov) 5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCG-3’ and cjCYP4F12
(3exp2) 5’-TCTAGATTACTGTGTTTTCACCC-3’ for marmoset P450 4F12, hCYP4F2
(5exp1bov) 5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCGGCG-3’ and

hCYP4F2 (3exp1) 5’-TCTAGATTACTGTGTTTTCACCC-3’ for human P450 4F2,
hCYP4F3B (5exp1bov) 5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCGGCGGCG-3’ and hCYP4F2
(3exp1) 5’-TCTAGATTACTGTGTTTTCACCC-3’ for human P450 4F3B,
cjCYP4F11 (5exp2bov) 5’-CATATGGCTCTGTATTAGCAGTTTTTCTGGGCCTGCGGCGGCGGCGGCGGCG-3’ and
hCYP4F11 (3exp1) 5′-TCTAGATTACTGTGAGTCTTGCCACCCAGG-3′ for human P450 4F11, hCYP4F12 (5exp1bov) 5′-CATATGGCTCTGTTATTAGCAGTTTCTGCGCTGACCCGGTG-3′ and hCYP4F12 (3exp1) 5′-TCTAGATTACTGGAAGCTTACACATTCAGG-3′ for human P450 4F12, and mfCYP4Fs (5exp1bov) 5′-GGAATTCCATATG GCTCTGTTATTAGCAGTTTCTGCGCTGACCCGGTG-3′ and mfCYP4F12 (3exp1) 5′-GCTCTAGAGGTGATGGATACAGAGTCA-3′ for cynomolgus monkey P450 4F12. All PCR products were cloned into pGEM-T easy vectors, and then the inserts were subcloned into pCW vectors using the restriction enzyme sites. Expression plasmids of cynomolgus monkey P450 4F2, 4F3B, and 4F11 were constructed as described previously (Uno et al., 2011). Membrane preparation of E. coli DH5α expressing each recombinant P450 4F protein was carried out as described previously (Yamazaki et al., 2002). The concentration of P450 and NADPH-P450 reductase in each membrane preparation was measured as described previously (Yamazaki et al., 2002).

Measurement of enzyme activities

For ebastine hydroxylation, the incubation mixture (0.25 mL) consisted of 40 pmol/mL recombinant protein or 0.5 mg/mL liver or small intestine microsomes, 0.4-500 μM ebastine, an NADPH-generating system (0.25 mM NADP+, 2.5 mM glucose 6-phosphate, and 0.25 units/mL glucose 6-phosphate dehydrogenase), and 100 mM potassium phosphate buffer (pH 7.4). Reactions initiated by adding the NADPH-generating system, proceeded at 37°C for 30 minutes. For immunoinhibition study, the incubation mixture (0.125 mL) consisted of 12.5 μl of anti-human P450 4F antibodies (40 mg/mL) or non-immune rabbit sera, 40 pmol/mL recombinant protein or 0.5 mg/mL tissue microsomes from marmosets, cynomolgus monkeys, and humans at room temperature for 30 minutes. The reaction was started by adding 1 mM...
ebastine (final concentration, 10 μM) and an NADPH-generating system and continued at 37°C for 30 minutes. Reactions then terminated by addition of acetonitrile (1.0 mL). The resultant solutions were centrifuged at 10000 g for 5 minutes. The supernatants (1.0 mL) were dried using a rotary evaporator under vacuum and then dissolved in 30% acetonitrile (0.2 mL). Samples (50 μL) were analyzed by reversed-phase HPLC on a C18 column (Mightysil, 5 μm, 250 × 4.6 mm; Kanto Chemical) at a flow rate of 1.0 mL/min using a mobile phase gradient consisting of 6.3 mM ammonium acetate (pH4.5) and acetonitrile with monitoring at 256 nm. The gradient elution conditions were 35% acetonitrile (0–3 minutes), 35%–85% acetonitrile (3–25 minutes), 85% acetonitrile (25–30 minutes), and 35% acetonitrile (30–40 minutes). Carebastine, 4-hydroxyebastine, and ebastine were detected with retention times of 12.9, 14.6, and 18.7 minutes, respectively. Leukotriene B₄ 20-hydroxylation activity was determined by the method as described (Jin et al., 1998), with minor modifications. The incubation mixture (0.25 mL) contained 10 pmol of recombinant P450 protein, 30 μM leukotriene B₄, the NADPH-generating system, and 100 mM potassium phosphate buffer (pH7.4). Reactions were started by adding the NADPH-generating system, incubated at 37°C for 20 minutes, and then terminated by addition of 4.0 M HCl (25 μL). The resultant solutions were centrifuged at 10000 g for 5 minutes. Supernatants (50 μL) were analyzed by reversed-phase HPLC on a C18 column (Mightysil, 5 μm, 250 × 4.6 mm) using isocratic elution by methanol/0.5% phosphoric acid (60:40, v/v) at a flow rate of 1.2 mL/min with monitoring at 270 nm. As a result, 20-hydroxyleukotriene B₄ and leukotriene B₄ were detected with retention times of 8.3 and 20.1 minutes, respectively. 7-Ethoxyresorufin O-deethylation, S-warfarin 7-hydroxylation, bufuralol 1’-hydroxylation, and midazolam 1’-hydroxylation by recombinant proteins were measured as described previously (Uehara et al., 2015b). Rates of metabolite formation were determined from standard curves prepared using reference standards. Kinetic parameters for ebastine hydroxylation were estimated from
the fitted curves employing Michaelis-Menten equations using the KaleidaGraph program (Synergy Software, Reading, PA).
Results

Isolation of marmoset P450 4F cDNAs

Using the marmoset genome data, the four new marmoset P450 4F genes, namely P450 4F2, 4F3B, 4F11, and 4F12, were found in the P450 4F gene cluster on chromosome 22 (Fig. 1). To obtain marmoset P450 4F cDNAs, RT-PCR was performed with the gene-specific primers. The four P450 4F cDNAs isolated from livers encoded 520, 520, 524, and 526 amino acids for P450 4F2, 4F3B, 4F11, and 4F12, respectively, which had the characteristic domain of the P450 family including six substrate recognition sites and heme-binding site, similar to human and cynomolgus monkey P450 4F forms (Fig. 2). Amino acid sequences of marmoset P450 4F2, 4F3B, 4F11, and 4F12 showed high degrees of identity (85-93%) to the human and cynomolgus monkey P450 4F counterparts (Table 1). A phylogenetic tree created using the P450 4F forms of seven species showed marmoset P450 4F forms clustered closer to the P450 4F forms of primates, including humans, orangutans, rhesus monkeys, and cynomolgus monkeys than those of dogs, pigs, rats, and mice (Fig. 3).

Tissue distribution of four P450 4F mRNAs in marmosets

To investigate the expression level of marmoset P450 4F mRNAs in brains, lungs, livers, kidneys, and small intestines, real-time RT-PCR was performed using the gene-specific primers. All four marmoset P450 4F mRNAs were expressed in livers where P450 4F3B mRNA was expressed most abundantly among the P450 4F mRNAs, followed by P450 4F11, 4F2, and 4F12. In addition, P450 4F2 and 4F12 were also abundantly expressed in small intestines (Fig. 4) These results indicated that marmoset P450 4F forms were expressed in livers and small intestines, the drug-metabolizing organs.
Enzymatic activities of marmoset P450 4F proteins

To assess the catalytic function of marmoset P450 4F enzymes, hydroxylation of leukotriene B₄ and ebastine by recombinant P450 4F proteins and tissue microsomes were analyzed. All marmoset P450 4F enzymes catalyzed leukotriene B₄ 20-hydroxylation, a typical human P450 4F probe activity (Table 2). In contrast, 7-ethoxyresorufin O-deethylation, S-warfarin 7-hydroxylation, bufuralol 1′-hydroxylation, and midazolam 1′-hydroxylation activities by recombinant proteins were below the detection limit (<0.002, <0.0001, <0.001, and <0.3 nmol products/min/nmol P450, respectively). For ebastine hydroxylation, P450 4F12 enzyme had the highest catalytic activity among the four marmoset P450 enzymes, as it also did in cynomolgus monkeys and humans (Table 3). Kinetic analyses indicated that marmoset P450 4F12 effectively catalyzed ebastine hydroxylation (Vₘₐₓ/Kₘₐₓ, 0.19 mL/min/nmol), similar to those of human and cynomolgus monkey P450 4F forms (Vₘₐₓ/Kₘₐₓ, 0.41 and 0.45 mL/min/nmol, respectively) (Table 4, Fig. 5A). Additionally, marmoset small intestine microsomes more efficiently catalyzed ebastine hydroxylation (Vₘₐₓ/Kₘₐₓ, 44 µL/min/mg), in comparison to cynomolgus monkey and human small intestine microsomes (Vₘₐₓ/Kₘₐₓ, 23 and 19 µL/min/mg, respectively) (Table 4, Fig. 5B). These results showed that marmoset P450 4F enzymes were similarly functional to those of humans and cynomolgus monkeys, and especially, P450 4F12 effectively catalyzed ebastine hydroxylation.

Inhibitory effects of anti-P450 4F antibodies on ebastine hydroxylation

To investigate the involvement of P450 4F enzymes for ebastine hydroxylation in liver and small intestine microsomes, inhibitory effects of anti-human P450 4F antibodies were determined. Anti-human P450 4F antibodies (40 mg/mL) substantially inhibited ebastine hydroxylation activities mediated by recombinant marmoset P450 4F12 (Fig. 6A).
Interestingly, ebastine hydroxylation activities by small intestine microsomes from marmosets and cynomolgus monkeys were inhibited (~60% of control activity) under the same conditions, unlike humans. Additionally, ebastine hydroxylation activities by small intestine microsomes of marmosets were inhibited by anti-P450 4F antibodies up to 40 mg/mL in a concentration-dependent manner (Fig. 6B). In contrast, ebastine hydroxylation activities in liver microsomes from marmosets, cynomolgus monkeys, and humans were weakly inhibited (~40% of control activity) (Fig. 6A). These results indicated that P450 4F enzymes are involved in the metabolism of ebastine in the liver and small intestine in marmosets and cynomolgus monkeys, suggesting an important role of intestinal P450 4F enzymes for ebastine metabolism in marmosets and cynomolgus monkeys.
Discussion

In humans, P450 4F enzymes occupy 15% of total hepatic cytochrome P450 protein levels, involved in the metabolism of endogenous compounds and xenobiotics (Michaels and Wang, 2014). In the previous study, de novo gene expression analysis in marmosets indicated expression of the marmoset P450 4F11-like gene in the liver, and 4F12-like gene in the liver, small intestine, and kidney (Shimizu et al., 2014): however, marmoset P450 4F forms have not been identified and characterized. In this study, we identified four novel P450 4F genes using the marmoset genome data, including P450 4F2, 4F3, 4F11, and 4F12, and the catalytic function of P450 4F proteins heterologously expressed in E. coli was estimated by analyzing leukotriene B4 20-hydroxylation and ebastine hydroxylation. Amino acid sequences of each marmoset P450 4F form were highly identical (85-93%) to the human and cynomolgus monkey P450 4F homologues (Table 1). Phylogenetic analysis showed that marmoset P450 4F forms were evolutionarily closer to human P450 4F forms than P450 4F forms of other non-primate experimental animal species including dogs, pigs, rats, and mice (Fig. 3).

Among the five tissues analyzed, marmoset P450 4F3B and 4F11 mRNAs were expressed abundantly in liver, and P450 4F2 and 4F12 mRNAs were expressed abundantly in liver and small intestine (Fig. 4). In humans, P450 4F3A and 4F3B mRNAs show distinct tissue expression pattern due to tissue-specific alternative splicing; P450 4F3A and 4F3B are predominantly expressed in neutrophils and livers, respectively (Christmas et al., 2001). In the marmoset genome, the P450 4F3A specific exon 3 sequence was found in our preliminary study (data not shown), implying the possibility of P450 4F3 alternative splicing in marmosets, similar to cynomolgus monkeys (Uno et al., 2007). Additionally, human P450 4F2 and 4F12 have been expressed in the liver and small intestine (Bylund et al., 2001;
Christmas et al., 2001), and human P450 4F11 is expressed in liver (Cui et al., 2000). Therefore, tissue distribution of P450 4F mRNAs are similar between marmosets and humans.

Ebastine hydroxylation rates in the liver and small intestine of marmosets and cynomolgus monkeys were remarkably faster than those of humans (Table 4). Moreover, ebastine hydroxylation activities by small intestine microsomes from marmosets and cynomolgus monkeys were inhibited by anti-P450 4F antibodies (Fig. 6A), unlike those of humans, indicating species differences of P450 4F enzymes for ebastine hydroxylation in small intestines among marmosets, and cynomolgus monkeys, and humans. Indeed, the major ebastine hydroxylase in intestinal microsomes is P450 2J2 for humans (Hashizume et al., 2002), but P450 4F12 for cynomolgus monkeys (Hashizume et al., 2001). Marmoset P450 2J2 identified recently was expressed in the liver and small intestine (Uehara et al., 2016b). Investigation of ebastine metabolism catalyzed by P450 4F and 2J enzymes might help further understand the species differences of ebastine hydroxylation in small intestine among marmosets, cynomolgus monkeys, and humans.

Besides ebastine, human P450 4F enzymes are involved in the metabolism of other drugs. Human P450 4F12 enzyme metabolizes other anti-allergy drugs, astemizole and terfenadine, human P450 2J2 and 3A4 substrates (Hashizume et al., 2002; Eksterowicz et al., 2014). Enteric P450 4F2 and/or 4F3B enzymes catalyze O-demethylation of an anti-parasitic prodrug, pafuramidine (Wang et al., 2007). Indeed, O-demethylation of pafuramidine in human intestine microsomes has been reportedly inhibited 92% of total metabolic activity by anti-P450 4F2 antibodies, whereas the antibodies against P450 3A4/5 and 2J2 showed moderate inhibition (~28%). Additionally, P450 4F2 and/or 4F3B enzymes have reportedly catalyzed $\omega$-hydroxylation of fingolimod, the major elimination pathway (Jin et al., 2011).
ω-Hydroxylation of fingolimod in human liver microsomes has been also inhibited 93% of total metabolic activity by anti-P450 4F2 antibodies, differing from little or no effects by antibodies of P450 2D6, 2E1, and 3A4. P450 4F11 also effectively metabolizes an antibiotic, erythromycin (Kalsotra et al., 2004). On the basis of high sequence identities of amino acids (>87%), marmoset P450 4F enzymes might also metabolize numerous drugs, similar to those of humans. Further analysis of P450 4F forms for enzymatic characteristics including substrate specificity is needed to understand the P450 4F-dependent metabolism in marmosets.

In humans, P450 4F enzymes are involved in the ω-hydroxylation of many eicosanoids such as arachidonic acid, leukotriene B4, and prostaglandins (Hsu et al., 2007), and nutrients such as phylloquinone (vitamin K1) (Edson et al., 2013) and tocopherol (vitamin E) (Sontag and Parker, 2002) and also the substrate selectivity of each P450 4F enzyme differs among the P450 4F enzymes. P450 4F8 and 4F12 mainly catalyze ω2- and ω3-hydroxylation of eicosanoids (Bylund et al., 2000; Bylund et al., 2001), unlike the other P450 4F enzymes (Jin et al., 1998; Kikuta et al., 1998; Christmas et al., 2001). A glycine residue (Gly-328) in SRS-4 of P450 4F8 and 4F12 would be likely to contribute to the position specificity for ω2- and ω3-hydroxylation of arachidonic acid (Stark et al., 2005). Marmoset and cynomolgus monkey P450 4F12 without any glycine residues in SRS-4 might implicate different position specificity for the hydroxylation of arachidonic acid from human P450 4F12, which may affect processes for activation and deactivation of numerous eicosanoid signaling pathways involving important physiological functions. Therefore, it is of great interest to investigate the metabolism of various eicosanoids by marmoset P450 4F enzymes in the future.

In humans, the P450 4F genes are transcriptionally regulated by nuclear receptors. Indeed, the sequence-specific binding of pregnane X receptor (PXR) in P450 4F12 gene
increased in the presence of rifampicin (Hariparsad et al., 2009). Additionally, the basal P450 4F12 expression level in human hepatocytes decreased (~80%) by siRNA knockdown of PXR. Therefore, human P450 4F12 gene is most likely regulated by PXR, indicating the possibility that P450 4F12 induction by PXR ligands leads to drug-drug interactions toward the metabolism of P450 4F12 substrates (e.g. ebastine, astemizole, and terfenadine). Additionally, P450 4F11 gene expression is also negatively regulated by nuclear factor κB, in human liver carcinoma cell line (HepG2) (Bell and Strobel, 2012). These results, together with the similar P450 4F mRNA distribution between marmosets and humans, suggest that marmosets and humans share, at least partly, transcriptional regulation and induction of P450 4F genes. Therefore, marmosets are potentially a suitable model for preclinical safety testing in drug development.

In conclusion, we found four novel marmoset P450 4F genes using the marmoset genome data, and successfully isolated P450 4F2, 4F3B, 4F11, and 4F12 cDNAs from marmoset livers. Amino acid sequences of the four marmoset P450 4F forms showed high sequence identities (85-93%) with the homologous P450s of cynomolgus monkeys and humans, and in phylogenetic analysis each P450 4F was more closely related with the human counterpart, compared to other experimental animal species, such as mice, rats, and pigs. Four P450 4F mRNAs were abundant in livers and/or small intestines in marmosets, similar to humans. Enzyme assays indicated that all four marmoset P450 4F enzymes were functional, and especially, marmoset P450 4F12 effectively catalyzed ebastine hydroxylation. Additionally, inhibition assay using anti-P450 4F antibodies indicated that P450 4F enzyme might play an important role for ebastine hydroxylation by marmoset small intestine microsomes. These findings indicated that marmoset P450 4F enzymes expressed in livers and/or small intestines were involved in the metabolism of endogenous and exogenous compounds.
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Authorship contribution

Participated in research design: Uehara, Uno, and Yamazaki.

Conducted experiments: Uehara, Uno, Yuki.

Contributed new reagents or analytic tools: Inoue and Sasaki.

Performed data analysis: Uehara, Uno, and Yamazaki.

Wrote or contributed to the writing of the manuscript: Uehara, Uno, and Yamazaki.
References


Hashizume T, Mise M, Matsumoto S, Terauchi Y, Fujii T, Imaoka S, Funae Y, Kamataki T,


Uehara S, Murayama N, Nakanishi Y, Zeldin DC, Yamazaki H, and Uno Y (2011)


Footnotes

Shotaro Uehara and Yasuhiro Uno equally contributed.

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Legends for figures

**Fig. 1.** *P450 4F cluster in marmosets, macaques, and humans.*

Marmoset P450 4F genes were located adjacent to *PGLYRP2* and *RAB8A* in the genome region corresponding to human and macaque P450 4F genes. Solid arrows indicate the expressed genes containing a complete open reading frame (ORF). Shaded arrows indicate pseudogenes with a nearly complete ORF. Shorter shaded arrows indicated pseudogenes lacking most part of an ORF. Open arrows indicate the genes with a complete or nearly complete ORF, but their expression has not been reported. Sizes of the genes and the distance between the genes are not proportionate to actual measurement.

**Fig. 2.** *Multiple alignment of P450 4F amino acid sequences.*

Marmoset (cj) P450 4F2, 4F3B, 4F11, and 4F12 amino acid sequences were aligned with those of the cynomolgus monkey (mf) and human (h) homologs. Substrate recognition sites and heme-binding motif are shown by solid and broken lines, respectively. Amino acids identical and relatively conserved are indicated by asterisks and dots, respectively.

**Fig. 3.** *A phylogenetic tree of P450 4F amino acid sequences in various species.*

A phylogenetic tree was created by the neighbor-joining method using P450 4F amino acid sequences of marmoset (cj), human (h), orangutan (ora), cynomolgus monkey (mf), rhesus monkey (mm), pig (pig), rat (r), and mouse (m). Marmoset and human P450 4F forms are shown in boxes. Human P450 4A11 was used as an outgroup. A scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position.

**Fig. 4.** *Quantification of P450 4F mRNA levels in marmoset tissues.*

Expression levels of marmoset P450 4F2, 4F3B, 4F11, and 4F12 mRNAs were
normalized. The normalization of target gene expression levels were performed with 18S rRNA level in each tissue. Each datum represents the mean ± standard deviations from triplicate determinations.

Fig. 5. Ebastine hydroxylation activities by recombinant marmoset, cynomolgus monkey, and human P450 4F12 proteins and small intestine microsomes from marmosets, cynomolgus monkeys, and humans.

Kinetic parameters for ebastine hydroxylation catalyzed by marmoset (circles), cynomolgus monkey (triangles), and human (squares) P450 4F12 proteins heterologously expressed in *E. coli* (A), and small intestine microsomes (B) from marmosets (circles), cynomolgus monkeys (triangles), and humans (squares).

Fig. 6. Effects of P450 4F antibodies on ebastine hydroxylation in recombinant P450 4F enzymes and liver and intestine microsomes from marmosets, cynomolgus monkeys, and humans.

Recombinant P450 4F12 proteins (80 pmol/mL) or liver/ intestine microsomes (0.50 mg/mL) were pre-incubated with anti-P450 4F antibodies (40 mg/mL) for 30 min at room temperature, and then incubated with 10 µM ebastine in the presence of an NADPH-generating system for 30 minutes at 37°C (A). Values represent the mean ± standard deviations of triplicate determinations. For marmosets, cynomolgus monkeys, and humans, ebastine hydroxylation activities with non-immune serum (100% as the control) by small intestine microsomes were 163, 208, and 21 pmol/min/mg protein, and by liver microsomes were 63, 267, and 94 pmol/min/mg protein, respectively. MLM, marmoset liver microsomes; MIM, marmoset small intestine microsomes; CLM, cynomolgus monkey liver microsomes; CIM, cynomolgus monkey small intestine microsomes; HLM, human liver microsomes; HIM, human small
intestine microsomes. Inhibitory effects of anti-P450 4F antibodies (0-80 mg/mL) on ebastine hydroxylation by marmoset small intestine microsomes were investigated (B). Data are means of duplicate determinations within 15% of ranges over mean values.
Table 1

Identities of the deduced amino acid sequences of four marmoset P450 4F enzymes

<table>
<thead>
<tr>
<th>Species</th>
<th>P450 form</th>
<th>Marmoset P450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4F2</td>
</tr>
<tr>
<td>Human</td>
<td>4F2</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>4F3B</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>4F11</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>4F12</td>
<td>82</td>
</tr>
<tr>
<td>Cynomolgus monkey</td>
<td>4F2</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>4F3B</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>4F11</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>4F12</td>
<td>81</td>
</tr>
<tr>
<td>Marmoset</td>
<td>4F2</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>4F3B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4F11</td>
<td></td>
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</table>
Table 2

Leukotrien B₄ 20-hydroxylation activities by recombinant marmoset P450 4F enzymes

<table>
<thead>
<tr>
<th>P450 forms</th>
<th>Catalytic activity pmol/min/nmol P450</th>
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<tr>
<td>4F2</td>
<td>215 ± 24</td>
</tr>
<tr>
<td>4F3B</td>
<td>304 ± 33</td>
</tr>
<tr>
<td>4F11</td>
<td>924 ± 14</td>
</tr>
<tr>
<td>4F12</td>
<td>116 ± 29</td>
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</table>

Data represent mean values ± standard deviation from triplicate measurements. Recombinant marmoset P450 4F proteins were incubated with 30 μM leukotriene B₄ for 20 minutes.
Table 3

Ebastine hydroxylation and carebastine formation activities by recombinant P450 4F enzymes and liver and intestine microsomes from marmosets, cynomolgus monkeys, and humans

<table>
<thead>
<tr>
<th>P450 forms</th>
<th>Ebastine hydroxylation</th>
<th>Carebastine formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mbol/min/nmol P450</td>
<td>pmol/min/mg P450</td>
</tr>
<tr>
<td>Marmoset P450 4F2</td>
<td>0.013 ± 0.001</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>Marmoset P450 4F3B</td>
<td>0.026 ± 0.003</td>
<td>0.017 ± 0.001</td>
</tr>
<tr>
<td>Marmoset P450 4F11</td>
<td>0.38 ± 0.04</td>
<td>0.031 ± 0.004</td>
</tr>
<tr>
<td>Marmoset P450 4F12</td>
<td>6.6 ± 0.4</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Cynomolgus monkey P450 4F2</td>
<td>&lt;0.010</td>
<td>&lt;0.010</td>
</tr>
<tr>
<td>Cynomolgus monkey P450 4F3B</td>
<td>0.013 ± 0.002</td>
<td>&lt;0.010</td>
</tr>
<tr>
<td>Cynomolgus monkey P450 4F11</td>
<td>0.94 ± 0.19</td>
<td>0.060 ± 0.008</td>
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<tr>
<td>Cynomolgus monkey P450 4F12</td>
<td>2.8 ± 0.1</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>Human P450 4F2</td>
<td>0.081 ± 0.005</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>Human P450 4F3B</td>
<td>0.043 ± 0.001</td>
<td>0.016 ± 0.001</td>
</tr>
<tr>
<td>Human P450 4F11</td>
<td>1.1 ± 0.1</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>Human P450 4F12</td>
<td>2.5 ± 0.1</td>
<td>0.013 ± 0.001</td>
</tr>
<tr>
<td>Marmoset liver microsomes</td>
<td>1460 ± 100</td>
<td>47 ± 8</td>
</tr>
<tr>
<td>Marmoset small intestine microsomes</td>
<td>602 ± 52</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Cynomolgus monkey liver microsomes</td>
<td>1170 ± 184</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Cynomolgus monkey small intestine microsomes</td>
<td>967 ± 90</td>
<td>104 ± 10</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>405 ± 70</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Human small intestine microsomes</td>
<td>112 ± 2</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

Catalytic activities were measured at the substrate concentration of 100 μM for ebastine hydroxylation. All data represent the mean value ± standard deviation of triplicate determination.
Table 4.

Kinetic parameters of ebastine hydroxylation by recombinant P450 4F enzymes and liver microsomes from marmosets, cynomolgus monkeys, and humans

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>$V_{max}$ (μL/min/mg)</th>
<th>$V_{max}/K_m$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marmoset P450 4F12</td>
<td>40 ± 8</td>
<td>7.6 ± 1</td>
<td></td>
<td>0.19</td>
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<tr>
<td>Cynomolgus monkey P450 4F12</td>
<td>5.1 ± 0.6</td>
<td>2.3 ± 0.1</td>
<td></td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Human P450 4F12</td>
<td>5.9 ± 0.8</td>
<td>2.4 ± 0.1</td>
<td></td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Marmoset intestine microsomes</td>
<td>18 ± 4</td>
<td>797 ± 47</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cynomolgus monkey intestine microsomes</td>
<td>54 ± 14</td>
<td>1230 ± 103</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human intestine microsomes</td>
<td>6.4 ± 0.6</td>
<td>124 ± 2</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Kinetic parameters were determined by non-linear regression analysis (mean ± standard error, n = 16 points of substrate concentrations of 0.4–500 μM). Values are based on duplicate determinations. Recombinant P450 protein and intestinal microsomes were incubated with ebastine of optional concentrations for 30 minutes at 37°C in the presence of an NADPH-generating system.
Fig. 4

Relative P450 4F mRNA abundance (Arbitrary units)

- CYP4F2
- CYP4F3B
- CYP4F11
- CYP4F12

Brain, Lung, Liver, Kidney, Small Intestine