**Progesterone Receptor Membrane Component 1 Modulates Human Cytochrome P450 Activities in an Isoform-Dependent Manner**

Shingo Oda, Miki Nakajima, Yasuyuki Toyoda, Tatsuki Fukami, and Tsuyoshi Yokoi

*Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa, University, Kakuma-machi, Kanazawa, Japan*

Received May 26, 2011; accepted August 8, 2011

**ABSTRACT:**

Cytochromes P450 (P450s) catalyze the metabolism of a wide spectrum of compounds. Recently, progesterone receptor membrane component 1 (PGRMC1), which shares a key structural motif with cytochrome \( b_5 \), has been reported to bind to sterol- or steroid-synthesizing P450s, enhancing their activities. In this study, we investigated whether PGRMC1 affects human drug-metabolizing P450 activities. Using coexpression systems for PGRMC1 and P450s (CYP3A4, CYP2C9, or CYP2E1) in HepG2 cells, we found that PGRMC1 decreased the \( V_{\text{max}} \) values and increased the \( K_m \) values of the CYP3A4A activities, and it decreased the \( V_{\text{max}} \) values but did not affect the \( K_m \) values of the CYP2C9 activities. In contrast, PGRMC1 hardly affected the CYP2E1 activities. These results suggest that PGRMC1 negatively modulates the drug-metabolizing activities of P450, although it was isomeric but not substrate dependent. It is worth noting that communoprecipitation analysis using coexpression systems for FLAG-PGRMC1 and Myc-P450s in human embryonic kidney 293 cells revealed that PGRMC1 interacts with all three P450s, although the affinity seemed to vary. In 29 human liver microsomes (HLMs), there was a 5-fold variability in the PGRMC1 protein levels. By the correlation analyses using the P450 activities and the PGRMC1 levels, we could neither observe the contribution of PGRMC1 to the P450 activities in HLMs nor that of the NADPH-cytochrome P450 reductase or cytochrome \( b_5 \). In conclusion, in contrast to sterol- or steroid-synthesizing P450s, we found that PGRMC1 negatively modulates the human drug-metabolizing activities of P450 through direct interaction. Further studies are needed to determine the clinical significance of PGRMC1 in the pharmacokinetics of drugs.

**Introduction**

Cytochrome P450 (P450) enzymes are heme-containing proteins that catalyze the metabolism of a wide variety of structurally diverse compounds (Nebert and Russell, 2002; Nelson et al., 2004). There are as many as 57 functional P450 genes and 58 pseudogenes in humans (http://drnelson.utmem.edu/CytochromeP450.html). Among them, three families, CYP1, CYP2, and CYP3, contribute to the oxidative metabolism of more than 70% of clinical drugs. Other P450 families (e.g., CYP4, CYP7, CYP11, CYP17, CYP19, CYP21, and CYP51) are involved in the metabolism of endogenous molecules such as steroids, bile acids, leukotrienes, and eicosanoids. P450 enzymes can exert their function by receiving electrons from NADPH-cytochrome P450 reductase (CPR) or cytochrome \( b_5 \) (Guengerich, 2002). CPR is indispensable for the P450 activities, whereas cytochrome \( b_5 \) has a significant role in the activities of some P450s (Shimada et al., 1994; Locuson et al., 2006).

Articulate, publication date, and citation information can be found at [http://dmd.aspetjournals.org](http://dmd.aspetjournals.org).

doi:10.1124/dmd.111.040907.

**ABBREVIATIONS:** P450, cytochrome P450; Ad, adenovirus; CPR, NADPH-cytochrome P450 reductase; GFP, green fluorescent protein; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; MOI, multiplicity of infection; PGRMC1, progesterone receptor membrane component 1; HEK, human embryonic kidney; PCR, polymerase chain reaction; HCM, hepatocyte culture medium; PAGE, polyacrylamide gel electrophoresis; siRNA, small interfering RNA.
CYP61A1, which catalyze sterol biosynthesis, and knockdown of endogenous PGRMC1 in human embryonic kidney (HEK) 293 cells resulted in the decreased cholesterol synthesis catalyzed by human CYP51A1. Thus, it has been demonstrated that PGRMC1 positively regulates P450-mediated sterol or steroid syntheses, making it “a helping hand for P450 proteins” (Debose-Boyd, 2007).

In contrast to endobiotic-metabolizing P450s, there is limited information on the effects of PGRMC1 on xenobiotic-metabolizing P450s. Although it was shown, by coimmunoprecipitation using a coexpression system in HEK293 cells, that PGRMC1 bound to CYP3A4 (Hughes et al., 2007), the functional significance remains to be clarified. In this study, we sought to investigate whether PGRMC1 might be a regulator of human drug-metabolizing P450 activities, focusing on CYP3A4, CYP2C9, and CYP2E1.

Materials and Methods
Chemicals and Reagents. Testosterone was purchased from Wako Pure Chemical Industries (Osaka, Japan). 5α-Androstan-3β-ol-17β and 17α-hydroxyprogesterone were kindly provided by AstellasPharma (Tokyo, Japan). Clonazepam was kindly provided by Roche (Tokyo, Japan). Chlorzoxazone, 6-hydroxychlorzoxazone, diclofenac, and 7-Ethoxycoumarin, 7-hydroxycoumarin, Midazolam and 1/H11032-hydroxymidazolam were kindly provided by AstellasPharma (Osaka, Japan). Rabbit anti-human/rat CPR antibody was from Millipore (Billerica, MA). Mouse anti-FLAG monoclonal antibody (M2) were from Sigma-Aldrich. 3-Hydroxy-3-methylglutaryl coenzyme A reductase was kindly provided by Sigma-Aldrich (St. Louis, MO), Steraloids (Newport, RI), and SAFC (St. Louis, MO), respectively.

Results
PGRMC1 might be a regulator of human drug-metabolizing P450 activities, focusing on CYP3A4, CYP2C9, and CYP2E1.

TABLE 1
Sequence of oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>5’ to 3’ Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>For construction of cosmid DNA</td>
<td></td>
</tr>
<tr>
<td>S-PGRMC1</td>
<td>GAGTTCGGATCCTCTGCCG&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AS-PGRMC1</td>
<td>ATACCTCCGAGAGTATACCTCCACTG&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>S-CYP2E1-1</td>
<td>ATGTCTGCCCTCAGGATCACC</td>
</tr>
<tr>
<td>AS-CYP2E1-1</td>
<td>CTATGAGGCCGGGAAGTACGA</td>
</tr>
<tr>
<td>For construction of FLAG-tagged PGRMC1 plasmid</td>
<td></td>
</tr>
<tr>
<td>S-PGRMC1</td>
<td></td>
</tr>
<tr>
<td>AS-FLAG PGRMC1</td>
<td></td>
</tr>
<tr>
<td>For construction of FLAG-tagged PGRMC1 plasmid</td>
<td></td>
</tr>
<tr>
<td>S-CYP3A4</td>
<td></td>
</tr>
<tr>
<td>AS-CYP3A4</td>
<td></td>
</tr>
<tr>
<td>S-CYP2C9</td>
<td></td>
</tr>
<tr>
<td>AS-CYP2C9</td>
<td></td>
</tr>
<tr>
<td>S-CYP2E1-2</td>
<td></td>
</tr>
<tr>
<td>AS-CYP2E1-2</td>
<td></td>
</tr>
<tr>
<td>S-3’Xyc</td>
<td></td>
</tr>
<tr>
<td>AS-3’Xyc</td>
<td></td>
</tr>
</tbody>
</table>

<sup>S</sup>, sense; <sup>A</sup>, antisense.
<sup>c</sup> The BamHI site is underlined.
<sup>g</sup> The XhoI site is underlined.
<sup>l</sup> The start codon is in bold.
<sup>k</sup> The XhoI site is underlined. Complementary sequences of FLAG tag are in bold. Complementary sequences of stop codon (TAG) are italicized.
<sup>l</sup> The XhoI site is underlined. Complementary sequences of FLAG tag are in bold. Complementary sequences of stop codon (TAG) are italicized.
<sup>j</sup> The KpnI site is underlined. The deleted stop codon is shown with the inverted caret.
<sup>i</sup> The KpnI site is underlined. The deleted stop codon is shown with the inverted caret.
Dium (Nissui, Tokyo, Japan) supplemented with 0.1 mM nonessential amino acid (Invitrogen) and 10% fetal bovine serum (Invitrogen). For the construction of the coexpression systems for FLAG-PGRMC1 and MUC-P450, the HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, 10 mM HEPES, and 10% fetal bovine serum. Human cryopreserved hepatocytes, lot H704 (Caucasian, female, 49 years old), were purchased from Xenotech, LLC (Lenexa, KS). The hepatocytes were cultured in hepatocyte culture medium (HCMI) (Cambrex, East Rutherford, NJ) on a plate coated with Cell Matrix Type I-C (Nitta Gelatin, Tokyo, Japan). These cells were maintained at 37°C under an atmosphere of 5% CO2, 95% air.

Infection of Recombinant Adenoviruses to HepG2 Cells or Cryopreserved Human Hepatocytes. HepG2 cells were infected at 7.5 × 10^5 cells/well in a six-well-plate and allowed to grow confluent. The cells were infected with a constant MOI of AdCYP (AdCYP3A4, 5; AdCYP2C9, 20; AdCYP2E1, 25; represented as × 1 in Fig. 1) and varied MOI of AdPGRMC1 (0, 2.5, 5, and 10 represented as 0, × 1, × 2, and × 4, respectively). To make the MOI the same value in four different experimental conditions, AdGFP was infected. After 24 h, the cultured medium was replaced with fresh medium without adenovirus. After 48 h, total cell homogenates were prepared by homogenization with TGE buffer (10 mM Tris-HCl, pH 7.4, 20% glycerol, and 0.1 mM EDTA). The protein concentration was determined using Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA) with γ-globulin as a standard.

The human hepatocytes were seeded at 1.5 × 10^5 cells/well into a 6-well plate. After 3 h, the medium was changed to HCM (albumin and antibiotics free) containing AdPGRMC1 or AdGFP at MOI 30. After 1 h, the medium was replaced with fresh HCM. After 48 h, the total cell homogenates were prepared as described above.

Human Liver Microsomes. Pooled human liver microsomes (HLMs) (n = 50) and individual HLMs (20 donors) were purchased from BD Gentest. Human liver samples from nine donors were obtained from the Human and Animal Bridging Research Organization (Chiba, Japan), which is in partnership with the National Disease Research Interchange (Philadelphia, PA). Microsomes were prepared according to the method described previously (Tabata et al., 2004).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. Total cell homogenates or HLMs (20 µg) were separated with 10% SDS-polyacrylamide gel electrophoresis (PAGE) for the detection of P450s and with 15% SDS-PAGE for the detection of PGRMC1. The separated proteins were electrotransferred onto the polyvinylidene difluoride membrane Immobilon-P (Millipore). The membranes were probed with rabbit anti-human CYP3A4, goat anti-rat CYP2E1, rabbit anti-human CYP2C9, rabbit anti-human PGRMC1, anti-human/rat CPR, or anti-cytochrome b5 antibodies and the corresponding fluorescent dye-conjugated secondary antibodies. The band densities were quantified with the Odyssey Infrared Imaging system (LI-COR Biosciences). The expression levels of P450 proteins were defined on the basis of a standard curve using P450 Supersomes (BD Gentest).

Enzyme Assays. A typical incubation mixture (final volume, 0.2 ml) contained 0.4 mg/ml total cell homogenate or 0.2 mg/ml HLM, 100 mM potassium phosphate buffer, pH 7.4, an NADPH-generating system (0.5 mM NADPH, 5 mM glucose 6-phosphate, 5 mM MgCl2, and 1 unit/ml glucose 6-phosphate dehydrogenase), and each substrate. The reaction mixture was preincubated at 37°C for 2 min, and the reaction was started by adding the NADPH-generating system.

The testosterone 6β-hydroxylase activity was determined as described previously (Nakajima et al., 1999) with a 20-min reaction time. The product formation was determined using HPLC with a LachromUltra C18 column (4.6 × 100 mm; 5 µm; Hitachi, Tokyo, Japan) and monitored at 240 nm. The midazolam 1′-hydroxylase activity was determined as described previously (Kronbach et al., 1989) with slight modifications. The reaction mixture was incubated at 37°C for 15 min, and the reaction was terminated by adding 100 µl of ice-cold methanol. Clonazepam (20 ng) was added as an internal standard. After the removal of the product by centrifugation at 10,000 g for 5 min, a 20-µl portion of the sample was subjected to a liquid chromatography-tandem mass spectrometry system with an HP 1100 system including a binary pump, an automatic sampler, and a column oven (AB Sciex, Tokyo, Japan), which was equipped with a ZORBAX SB-C18 column (2.1 × 50 mm; 3.5 µm; Agilent Technologies). The column temperature was 20°C, and the flow rate was 0.2 ml/min. The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A linear gradient was used from 20% B to 90% B over 2 to 3 min followed by 90% B for 7 min, and then the column was allowed to re-equilibrate at the initial conditions for 4 min. The liquid chromatography was connected to a PE Sciex API 2000 tandem mass spectrometer (AB Sciex) operated in the positive electrospray ionization mode. The turbo gas was maintained at 550°C. Nitrogen was used as the nebulizing gas, turbo gas, and curtain gas at 40, 90, and 40 psi, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using nitrogen as the collision gas. The collision energy was 37 V. The mass/charge (m/z) ion transitions were recorded in the multiple reaction monitoring mode: m/z 342.0 and 203.0 for 1′-hydroxymidazolam; m/z 315.9 and 270.1 for clonazepam.

The 5-warfarin 7-hydroxylase activity was determined as described previously (Yamazaki et al., 1999) with a 20-min incubation time. The product formation was determined using HPLC with a Mightysil RP-18 column (4.6 × 150 mm; 5 µm; Kanto Chemical, Tokyo, Japan) monitored at 280 nm.

The chlorozoxazone 6-hydroxylase activity was determined as described previously (Moiri et al., 2010) with a 30-min incubation time. The product formation was determined using HPLC with a LachromUltra C18 column (4.6 × 100 mm; 3 µm; TOSOH, Tokyo, Japan) monitored at 320 nm.

The diclofenac 4′-hydroxylase activity was determined by the method used by Katoh et al. (2004) with a 30-min incubation time. The product formation was determined using HPLC with a TSK-GEL ODS-80TM column (4.6 × 250 mm; 5 µm; TOSOH, Tokyo, Japan) and monitored at 280 nm.

The 7-ethoxycoumarin O-deethylation activity was determined as described previously (Yamazaki et al., 1999) with a 30-min incubation time. The product formation was determined using HPLC with a Mightysil RP-18 column (4.6 × 150 mm; 5 µm) and monitored with the excitation wavelength set at 338 nm and the emission set at 458 nm.

Kinetic parameters were estimated from the fitted curve using a computer program (KaleidaGraph; Synergy Software, Reading, PA) designed for non-linear regression analysis. The following equations were used: Michaelis-Menten equation, \( V = V_{\text{max}} \times \frac{[S]}{[K_m] + [S]} \); and substrate inhibition equation, \( V = V_{\text{max}} \times \frac{[S]}{[K_m] + [S] + K_i} \), where \( V \) is the velocity of the reaction.
reaction, [S] is the substrate concentration, \( K_m \) is the Michaelis-Menten constant, \( V_{max} \) is the maximum velocity, and \( K_i \) is the substrate inhibition constant. Data are expressed as the means ± S.D. of three independent determinations.

**Coimmunoprecipitation Assay.** The FLAG-PGRMC1 and each Myc-P450 expression plasmid were transiently cotransfected into HEK293 cells. In brief, the day before transfection, the cells were seeded into a six-well plate coated with Cell Matrix Type I-C. After 24 h, each 2 μg of FLAG-PGRMC1 and Myc-P450 plasmids was transfected using Lipofectamine 2000. After 48 h, the cells were collected, and total cell homogenates were prepared by homogenization with TGE buffer. Five hundred micrograms of protein were suspended in either buffer A or buffer B in a final volume of 0.5 ml and rotated at 4 °C for 2 h. Buffer A consisted of 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM (p-amidino-phenyl)-methanesulfon fluoride hydrochloride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. The detergents in buffer A were replaced with 0.1% digitonin in buffer B. The lysates were centrifuged at 100,000g for 30 min. To the supernatants, the anti-FLAG antibody was added and incubated for 8 h followed by precipitation with protein G-Sepharose beads. The beads were washed with buffer A or B and suspended with Laemmli sample buffer. The eluent was subjected to Western blot analyses with anti-FLAG antibody or anti-c-Myc antibody.

**Statistical Analyses.** Statistical significance was determined by using an unpaired, two-tailed Student’s t test for paired data between two groups or the Shirley-Williams’ test when more than three groups were compared. Correlation analyses were performed by Pearson’s product-moment method. When the \( P \) value was less than 0.05, the differences were considered statistically significant.

**Results**

**Establishment of Coexpression Systems for PGRMC1 and P450s in HepG2 Cells.** To establish coexpression systems for PGRMC1 and P450s, AdPGRMC1 was cotransfected with AdCYP3A4, AdCYP2C9, or AdCYP2E1 into the HepG2 cells. Preliminarily, we optimized the MOIs to obtain the expression levels of PGRMC1 and P450s that are close to those in pooled human liver microsomes. When AdPGRMC1 was infected at MOI 3 to 4, the expressed PGRMC1 levels were close to those in the pooled HLMs. Accordingly, we set the MOIs for AdPGRMC1 at 2.5, 5, and 10, which were represented as \( x1 \), \( x2 \), and \( x4 \), respectively (Fig. 1). The MOIs for AdCYP2C9 and AdCYP2E1 were set at 20 and 25, respectively. In the case of AdCYP3A4, MOIs over 10 showed cellular toxicity. Therefore, we set the MOIs for AdCYP3A4 at 5, although the level of CYP3A4 protein produced was lower than that in the pooled HLMs (Fig. 1) but was in the range of those in individual HLMs, as described below. Thus, we obtained four lines for each P450 with various levels of PGRMC1. Using these systems, we investigated the effects of PGRMC1 on the P450 activities.

**Effects of Coexpression of PGRMC1 on CYP3A4 Activities.** To investigate the effects of PGRMC1 on the CYP3A4 activity, kinetic analyses of testosterone 6β-hydroxylation and midazolam 1’-hydroxylation were performed using the total cell homogenates from the HepG2 coexpression system. The kinetics of testosterone 6β-hydroxylation followed the Michaelis-Menten equation (Fig. 2A). The \( K_{m}, V_{max} \) and \( V_{max}/K_{m} \) values of the homogenates from the cells with no exogenous PGRMC1 were 56.9 ± 9.1 μM, 54.3 ± 4.0 pmol · min⁻¹ · pmol P450⁻¹, and 0.96 ± 0.08 μl · min⁻¹ · pmol P450⁻¹, respectively (Table 2). The coexpression of PGRMC1 significantly decreased the \( V_{max} \) values and increased the \( K_{m} \) values, resulting in a decrease in the \( V_{max}/K_{m} \) values in a PGRMC1 concentration-dependent manner. The kinetics of midazolam 1’-hydroxylation followed the substrate-inhibition equation (Fig. 2B). The \( K_{m}, V_{max} \), \( K_{i} \), and \( V_{max}/K_{m} \) values by the homogenates from the cells with no exogenous PGRMC1 were 5.7 ± 0.9 μM, 9.8 ± 1.5 pmol · min⁻¹ · pmol P450⁻¹, 15.9 ± 4.1 μM, and 1.72 ± 0.03 μl · min⁻¹ · pmol⁻¹ P450⁻¹, respectively (Table 2). The coexpression of PGRMC1 significantly decreased the \( V_{max} \) values and increased the \( K_{m} \) values, resulting in a decrease in the \( V_{max}/K_{m} \) values in a PGRMC1 concentration-dependent manner. The \( K_{i} \) value was not affected by the coexpression of PGRMC1. Thus, it was demonstrated that PGRMC1 has the ability to attenuate the CYP3A4 activity independently of the substrate.

**Effects of Coexpression of PGRMC1 on CYP2C9 Activities.** To investigate the effects of PGRMC1 on the CYP2C9 activity, kinetic analyses of S-warfarin 7-hydroxylation and diclofenac 4’-hydroxylation were performed. The kinetics of S-warfarin 7-hydroxylation followed the Michaelis-Menten equation (Fig. 3A). The \( K_{m}, V_{max} \), and \( V_{max}/K_{m} \) values by the homogenates from the cells with no exogenous PGRMC1 were 1.6 ± 0.1 μM, 5.4 ± 0.2 pmol · min⁻¹ · pmol P450⁻¹, and 3.3 ± 0.2 μl · min⁻¹ · pmol⁻¹ P450⁻¹, respectively (Table 3). The coexpression of PGRMC1 significantly decreased the \( V_{max} \) values in a PGRMC1 concentration-dependent manner but did not affect the \( K_{m} \) values, resulting in a decrease in the \( V_{max}/K_{m} \) values. The kinetics of diclofenac 4’-hydroxylation followed the Michaelis-Menten equation (Fig. 3B). The \( K_{m}, V_{max} \), and \( V_{max}/K_{m} \) values by the homogenates from the cells with no exogenous PGRMC1 were 7.3 ± 0.3 μM, 91.6 ± 6.8 pmol · min⁻¹ · pmol P450⁻¹, and 12.6 ± 1.5 μl · min⁻¹ · pmol P450⁻¹, respectively (Table 3). The coexpression of PGRMC1
TABLE 2

Kinetic parameters for testosterone 6β-hydroxylation activity and midazolam 1’-hydroxylation activity by recombinant CYP3A4 in single or coexpression systems with PGRMC1

The expression systems were constructed using recombinant adenoviruses as described under Materials and Methods. Kinetic parameters were calculated from curves by nonlinear regression. Data are mean ± S.D. of three independent experiments.

<table>
<thead>
<tr>
<th>AdCYP3A4 + AdPGRMC1</th>
<th>Testosterone 6β-Hydroxylation</th>
<th>Midazolam 1’-Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (pmol/min/μM)</td>
</tr>
<tr>
<td>×1</td>
<td>56.9 ± 9.1</td>
<td>54.3 ± 4.0</td>
</tr>
<tr>
<td>×2</td>
<td>53.1 ± 22.3</td>
<td>51.2 ± 5.9</td>
</tr>
<tr>
<td>×4</td>
<td>71.4 ± 14.2</td>
<td>43.4 ± 4.4*</td>
</tr>
<tr>
<td></td>
<td>91.8 ± 12.2*</td>
<td>37.4 ± 2.7**</td>
</tr>
</tbody>
</table>

* $P < 0.05$ and ** $P < 0.01$ compared with control by Shirley-Williams’ test.

significantly decreased the $V_{max}$ values in a PGRMC1 concentration-dependent manner but did not affect the $K_m$ values, resulting in a decrease in the $V_{max}/K_m$ values. Thus, it was demonstrated that PGRMC1 has the ability to attenuate the CYP2C9 activity independently of the substrate.

Effects of Coexpression of PGRMC1 on CYP2E1 Activities. To investigate the effects of PGRMC1 on the CYP2E1 activity, kinetic analyses of chlorzoxazone 6-hydroxylation and 7-ethoxycoumarin O-deethylation were performed. The kinetics of chlorzoxazone 6-hydroxylation followed the Michaelis-Menten equation (Fig. 4A). The $K_m$, $V_{max}$, and $V_{max}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were 67.1 ± 5.6 μM, 618.3 ± 18.2 pmol/min·μM, and 9.3 ± 0.5 μM/min·μM, respectively (Table 4). The coexpression of PGRMC1 did not affect the kinetic parameters. The kinetics of 7-ethoxycoumarin O-deethylation followed the Michaelis-Menten equation (Fig. 4B). The $K_m$, $V_{max}$, and $V_{max}/K_m$ values by the homogenates from the cells with no exogenous PGRMC1 were 40.2 ± 2.5 μM, 16.0 ± 1.2 pmol/min·μM, and 0.40 ± 0.01 μM/min·μM, respectively (Table 4). The coexpression of PGRMC1 did not affect the $K_m$ and $V_{max}$ values but slightly decreased the $V_{max}/K_m$ values. PGRMC1 likely had a small effect on the CYP2E1 activities in comparison with the CYP3A4 and CYP2C9.

Effects of Overexpression of PGRMC1 on Enzyme Activities in Human Hepatocytes. To investigate whether PGRMC1 modulates the activities of endogenous human P450s, we sought to overexpress PGRMC1 in human hepatocytes. When the homogenates from the human hepatocytes were subjected to Western blot analysis, the band density of PGRMC1 protein was similar to that in the pooled HLMs (Fig. 5A). When AdPGRMC1 was infected, the PGRMC1 protein level was significantly (5.6-fold, $P < 0.001$) increased (Fig. 5A). We confirmed that there was no morphological change by the infection with AdPGRMC1. Using the homogenates from these cells, the midazolam 1’-hydroxylase, S-warfarin 7-hydroxylase, and chlorzoxazone 6-hydroxylation activities, at the substrate concentrations of 10, 10, and 500 μM, respectively, were evaluated. Interestingly, we found that the midazolam 1’-hydroxylase and S-warfarin 7-hydroxylase activities in the homogenates from the AdPGRMC1-infected cells were significantly lower than those in control (AdGFP-infected cells). In contrast, the chlorzoxazone 6-hydroxylation activity was not affected by the overexpression of PGRMC1 (Fig. 5B). These results suggest that PGRMC1 modulates the endogenous human P450 activity in an isoform-specific manner, supporting the results from the expression systems.

Coimmunoprecipitation of PGRMC1 and P450s. To investigate whether PGRMC1 directly interacts with P450s, we used a coimmunoprecipitation assay. Because commercially available antibodies against PGRMC1 or P450s are not suitable for immunoprecipitation assays, we constructed FLAG-PGRMC1 and Myc-P450 coexpression systems to perform the immunoprecipitation assay using anti-tag antibodies. We confirmed, by Western blot analyses using anti-FLAG and anti-c-Myc antibodies, that both FLAG-PGRMC1 and Myc-P450s were successfully expressed (Fig. 6). When the lysates using buffer A were assayed, the PGRMC1 in the three expression systems...
was immunoprecipitated to the same extent by using the anti-FLAG antibody, and only Myc-CYP2E1 was coimmunoprecipitated (Fig. 6A). When the lysates using buffer B were assayed, all three P450s were coimmunoprecipitated (Fig. 6B). These results suggest that PGRMC1 binds directly to these P450s, although the degree would be different among the isoforms.

**Relationship between P450 Activities and PGRMC1, CPR, and Cytochrome b<sub>5</sub> Levels in a Panel of 29 Human Liver Microsomes.** The midazolam 1'-hydroxylase, S-warfarin 7-hydroxylase, and chlorzoxazone 6-hydroxylase activities in a panel of 29 human liver microsomes were measured at the substrate concentrations of 10, 5, and 500 μM, and the PGRMC1, CYP3A4, CYP2C9, CYP2E1, CPR, and cytochrome b<sub>5</sub> protein levels were determined by Western blot analysis. The variability of the PGRMC1 protein levels was ~5-fold (Table 5). Although the variability of the CYP3A4 protein levels was large (3–72 pmol/mg, 24-fold), those of the CYP2C9 (5–17 pmol/mg, ~3-fold) and the CYP2E1 (3–16 pmol/mg, ~5-fold) protein levels were relatively small. As shown in Supplemental Fig. 1, A–C, the midazolam 1'-hydroxylase, S-warfarin 7-hydroxylase, and chlorzoxazone 6-hydroxylase activities represented as the metabolite \( \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} \) were significantly correlated with CYP3A4, CYP2C9, and CYP2E1, respectively. To investigate whether the PGRMC1 would be a factor modulating the P450 activities, correlation analyses between the ratio of PGRMC1 to P450 and the midazolam 1'-hydroxylase, S-warfarin 7-hydroxylase, and chlorzoxazone 6-hydroxylase activities represented as the metabolite \( \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1} \) were performed. We expected inverse correlations between PGRMC1/CYP3A4 ratio and midazolam 1'-hydroxylase or PGRMC1/CYP2C9 ratio and S-warfarin 7-hydroxylase activities based on the results of the coexpression systems in HepG2 cells. However, no inverse correlation was observed (Supplemental Fig. 1, D–F). In addition, except for the CPR/CYP2E1 ratio, the CPR/P450 or cytochrome b<sub>5</sub>/P450 ratios did not show a positive correlation with the activities (Supplemental Fig. 1, G–L). Next, we determined the relationship between the PGRMC1 protein level, and each P450 activity corrected with the CPR ratio and cytochrome b<sub>5</sub> protein (Supplemental Fig. 2, A–C) or cytochrome b<sub>5</sub> protein (Supplemental Fig. 2, D–F) levels. However, no inverse correlation was observed in the CYP3A4 or CYP2C9 activities. Because the PGRMC1 protein levels were significantly correlated with the CPR (\( r = 0.50, P < 0.01 \)) and cytochrome b<sub>5</sub> (\( r = 0.76, P < 0.0001 \)) protein levels (Supplemental Fig. 2, G and H), it would be difficult to estimate the contribution of PGRMC1 to the P450 activities in HLM by the correlation analyses.

**Discussion**

In this study, we investigated the effects of PGRMC1 on the human drug-metabolizing P450 activities, focusing on three major isoforms, CYP3A4, CYP2C9, and CYP2E1. Using coexpression systems for PGRMC1/P450s in HepG2 cells, we found that PGRMC1 increased the \( K_m \) and decreased the \( V_{\text{max}} \) of the CYP3A4 activities and decreased the \( V_{\text{max}} \) of the CYP2C9 activities irrespective of the substrates (Figs. 2 and 3). In contrast to CYP3A4 and CYP2C9, PGRMC1 did not dramatically affect the CYP2E1 activities, indicating the effects of PGRMC1 would be P450 isoform dependent. During the process of preparing this report, an independent study reported that PGRMC1 commonly decreased the CYP3A4, CYP2C8, and rabbit

---

**TABLE 3**

<table>
<thead>
<tr>
<th>AdCYP2C9 + AdPGRMC1</th>
<th>S-Warfarin 7-Hydroxylation</th>
<th>Dichlofenac 4'-Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (μM)</td>
<td>( V_{\text{max}} ) (pmol min(^{-1}) pmol CYP(^{-1}))</td>
</tr>
<tr>
<td>AdCYP2C9 + AdPGRMC1</td>
<td>1.6 ± 0.1</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>x1</td>
<td>1.6 ± 0.1</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>x2</td>
<td>1.7 ± 0.2</td>
<td>2.4 ± 0.2**</td>
</tr>
<tr>
<td>x4</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.1**</td>
</tr>
</tbody>
</table>

\* \( P < 0.05 \) and \** \( P < 0.01 \) compared with control by Shirley-Williams’ test.
CYP2C2 activities using coexpression systems in HEK293 cells (Szeszna-Skorupa and Kemper, 2011). They evaluated the enzyme activities by P450-Glo assay at a substrate concentration. The findings that PGRMC1 decreased the activities of drug-metabolizing P450s were similar between our and recent reports, but our new findings are that the effects of PGRMC1 on the kinetics were different between the P450 isoforms and that there exists a P450(s) not affected by PGRMC1. When we mixed the homogenates from the single expression system for PGRMC1 and the homogenates from the single expression system for P450s, no changes were observed in the kinetics of each P450 activity (data not shown). Therefore, it was suggested that colocalization on the membrane would be critical for PGRMC1 to exert its effect in modulating the P450 activities. It has been reported that PGRMC1 is predominantly located in the endoplasmic reticulum. However, in a human ovarian cancer cell line, Ovarcar-3, PGRMC1 is found in the cytoplasm (Lösel et al., 2008). To analyze the localization of PGRMC1 in human liver, we performed Western blot analysis using cytosol, but PGRMC1 could not be detected. Therefore, the subcellular localization of PGRMC1 appears to be cell-type specific.

To investigate whether PGRMC1 modulates the activities of endogenous P450, we performed experiments using human hepatocytes. First, we sought to investigate the effects of repression of PGRMC1 by siRNA on the P450 activities (data not shown). When siRNA for PGRMC1 (Stealth select RNAi; Invitrogen) was transfected into human hepatocytes, the PGRMC1 mRNA levels were decreased by 70%. However, the PGRMC1 protein level was not decreased (data not shown). Although we used additional siRNA for PGRMC1 from another supplier (Ambion, Austin, TX), favorable results were not obtained. Hence, we sought to investigate the effects of the overexpression of PGRMC1 in human hepatocytes. The overexpression of PGRMC1 resulted in decreases in the CYP3A4 and CYP2C9 activities, but not CYP2E1 activity, which were the same as with the HepG2 coexpression systems (Fig. 5). These results suggest that PGRMC1 modulates the activities of endogenous P450 in an isoform-specific manner.

Using the communoprecipitation assay (Fig. 6), we found that PGRMC1 interacts with P450s (not only CYP3A4 and CYP2C9 but also CYP2E1). When buffer A containing Nonidet P-40 and sodium deoxycholate, which are relatively strong detergents, was used, only CYP2E1 was communoprecipitated (Fig. 6A). However, when buffer B containing digitonin, which is a relatively weaker detergent, was used, all of the three P450 isoforms were communoprecipitated (Fig. 6B). These results suggested that the binding of PGRMC1 to CYP2E1 might be stronger than the binding to CYP3A4 or CYP2C9. Alternatively, the number of CYP2E1 molecules that bound to a PGRMC1 molecule might be larger than that of CYP3A4 or CYP2C9. In other words, a smaller number of PGRMC1 molecules may bind to CYP2E1. Such differences might explain why PGRMC1 did not affect the CYP2E1 activity but decreased the CYP3A4 and CYP2C9 activities, or the effects of PGRMC1 might not be a simple protein-protein interaction.

It was demonstrated that introduction of a mutation in the cytochrome b₅-like domain of PGRMC1, to which heme binds, abolishes the binding to CYP7A1 (Mansouri et al., 2008). Min et al. (2005) also reported that a heme-deficient PGRMC1 mutant could not increase the binding to CYP21A2. These observations suggest the importance of heme binding for PGRMC1 in its function. Cytochrome b₅ has a hexacoordinate heme that is capable of transferring an electron.
cytochrome expression level (data not shown). Alternatively, we measured the cells without the overexpression of CPR, probably because of the low activities. In contrast to their study, the coimmunoprecipitation of CPR and PGRMC1 was observed, they restored by the overexpression of CPR. In addition, because the P450. Therefore, PGRMC1 affects the P450 function without direct transferring of electrons to P450 such as CPR or cytochrome bs. Although these components positively regulate the enzyme activities of CYP3A4, CYP2C9, and CYP2E1, no clear correlation with the P450 activities was observed except in the case of CYP2E1.

In conclusion, we found that PGRMC1 decreases the activities of drug-metabolizing P450s in HLMs, which is supported in the study by Szczesna-Skorupa and Kemper (2011), although it is possibly isoform dependent. It has been reported that PGRMC1 is highly expressed in breast and ovary tumors and in cancer cell lines from the colon, thyroid, lung, and cervix (Crudzen et al., 2005; Peluso et al., 2008a). Recently, it has been reported that PGRMC1 is highly expressed in human myometrium during pregnancy and may mediate the relaxation effect on myometrium (Wu et al., 2011). The changes of PGRMC1 expression under certain physiological conditions might impact on the metabolism of steroids. Meanwhile, little is known about the factors that affect the PGRMC1 expression level in human liver. Further studies are warranted to clarify the physiological significance of PGRMC1 in the modulation of drug-metabolizing P450s in liver.

In this study, the variability of the PGRMC1 protein levels in HLM was first evaluated. In 29 human liver samples, there was 5-fold variability (Table 5). We sought to estimate the contribution of PGRMC1 to the modulation of the P450 activities by correlation analyses between the P450 activities and PGRMC1 protein levels. In the analysis, we took into account the other components, the transferring of electrons to P450 such as CPR or cytochrome bs. We can determine the absolute expression levels of P450, CPR, and cytochrome bs but not PGRMC1. Understanding the absolute expression level of PGRMC1 in HLMs would be helpful to determine the relative importance of PGRMC1 in the modulation of the P450 activity.

Previous studies reported that PGRMC1 increased the activities of CYP21A2 and CYP51A1 that are responsible for steroid or sterol metabolism (Min et al., 2005; Hughes et al., 2007). In contrast, this study found that PGRMC1 decreased the activity of human drug-metabolizing P450s, which is supported in the study by Szczesna-Skorupa and Kemper (2011), although it is possibly isoform dependent. It has been reported that PGRMC1 is highly expressed in breast and ovary tumors and in cancer cell lines from the colon, thyroid, lung, and cervix (Crudzen et al., 2005; Peluso et al., 2008a). Recently, it has been reported that PGRMC1 is highly expressed in human myometrium during pregnancy and may mediate the relaxation effect on myometrium (Wu et al., 2011). The changes of PGRMC1 expression under certain physiological conditions might impact on the metabolism of steroids. Meanwhile, little is known about the factors that affect the PGRMC1 expression level in human liver. Further studies are warranted to clarify the physiological significance of PGRMC1 in the modulation of drug-metabolizing P450s in liver.

In conclusion, we found that PGRMC1 decreases the activities of drug-metabolizing P450s in an isoform-dependent manner. The action was opposite to that for steroid-metabolizing P450s. Thus, PGRMC1 seems to affect P450s depending on their functions. The present study activities by PGRMC1 might not be due to the decrease in CPR activity, although the possibility that PGRMC1 might influence the electron transferring from CPR to P450, because the Vmax values of CYP3A4 and CYP2C9 were decreased, could not be excluded. In the case of CYP3A4, an increase in the Km values was also observed. Therefore, as another mechanism, it is suggested that PGRMC1 might cause an allosteric change in the CYP3A4 structure that affects the affinity to substrates. Nevertheless, the effects of PGRMC1 on the P450 activity were diverse depending on the P450 isoforms, and further detailed studies are needed to clarify the underlying mechanisms.

### Table 5

Expression levels of PGRMC1, P450s, CPR, and cytochrome bs and enzyme activities in human liver microsomes

<table>
<thead>
<tr>
<th>Relative Expression Level</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGRMC1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MDZ&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>pmol/mg</td>
</tr>
<tr>
<td>Cyt b&lt;sub&gt;s&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>28 ± 20</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>9 ± 4</td>
</tr>
</tbody>
</table>

<sup>a</sup> PGRMC1 protein level was expressed as relative to the pooled HLMs set at 1.0.

<sup>b</sup> CPR and cytochrome b<sub>s</sub> protein levels are indicated as relative to the lowest set at 1.0.
revealed a novel function of PGRMC1 in modulating the drug-metabolizing activity.

Acknowledgments

We acknowledge Brent Bell for reviewing this manuscript.

Authorship Contributions

Participated in research design: Oda, Nakajima, Fukami, and Yokoi.

Conducted experiments: Oda.

Contributed new reagents or analytic tools: Oda, Toyoda, and Fukami.

Performed data analysis: Oda and Nakajima.

Wrote or contributed to the writing of the manuscript: Oda, Nakajima, and Yokoi.

References


Address correspondence to: Dr. Tsuyoshi Yokoi, Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakumamachi, Kanazawa 920-1182, Japan. E-mail: tyokoi@kenroku.kanazawa-u.ac.jp