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PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 (PGRMC1) INHIBITS THE ACTIVITY OF DRUG-METABOLIZING CYTOCHROMES P450 AND BINDS TO CYTOCHROME P450 REDUCTASE

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Running title: PGRMC1 interacts with P450 and P450 reductase

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The abbreviations used are: CYPs, cytochromes P450; CPR, cytochrome P450 reductase; PGRMC1, progesterone receptor membrane component-1; SCAP, SREBP cleavage activating protein; INSIG1, insulin induced gene 1; NP-40, Nonidet P-40; GFP, green fluorescent protein; YFP, yellow fluorescent protein; ER, endoplasmic reticulum; EGFR, epidermal growth factor receptor; siRNA, small inhibitory RNA; shRNA, small hairpin RNA; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamine; WGA, wheat germ agglutinin.

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Abstract

Progesterone receptor membrane component 1 (PGRMC1) has been recently shown to interact with several cytochromes P450 (CYPs) and to activate enzymatic activity of CYPs involved in sterol biosynthesis. We analyzed the interactions of PGRMC1 with the drug-metabolizing CYPs, 2C2, 2C8 and 3A4, in transfected cells. Based on co-immunoprecipitation assays, PGRMC1 bound efficiently to all three CYPs and binding to the catalytic cytoplasmic domain of CYP2C2 was much more efficient than to a chimera containing only the N-terminal transmembrane domain. Downregulation of PGRMC1 expression levels in HEK 293 and HepG2 cell lines stably expressing PGRMC1-specific siRNA, had no effect on the ER localization and expression levels of CYPs, whereas enzymatic activities of CYPs 2C2, 2C8 and 3A4 were slightly higher in PGRMC1-deficient cells. Co-transfection of cells with CYPs and PGRMC1 resulted in PGRMC1 concentration-dependent inhibition of the CYP activities and this inhibition was partially reversed by increased expression of the CYP reductase (CPR). In contrast, CYP51 activity was decreased by downregulation of PGRMC1 and expression of PGRMC1 in the PGRMC1-deficient cells increased CYP51 activity. In cells co-transfected with CPR and PGRMC1, strong binding of CPR to PGRMC1 was observed, however, in the presence of CYP2C2, interaction of PGRMC1 with CPR was significantly reduced suggesting that CYP2C2 competes with CPR for binding to PGRMC1. These data show that in contrast to sterol synthesizing CYPs, PGRMC1 is not required for the activities of several drug-metabolizing CYPs and its overexpression inhibits those CYPs' activities. Further, PGRMC1 binds to CPR which may influence CYP activity.

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Introduction

The cytochrome P450s (CYPs) constitute a superfamily of heme-containing enzymes known to metabolize physiologically important endogenous and xenobiotic compounds. Despite multiple CYPs, a single electron donor, NADPH-dependent cytochrome P450 oxidoreductase (CPR), is required for their enzymatic activities. In most tissues, there is a vast excess of CYPs over CPR, so that rather than forming stable complexes, CYPs enter into transient interactions with CPR. A single CPR molecule may bind to oligomeric complexes of the CYPs, since many CYPs form either homo- or hetero-oligomeric structures (reviewed in(Backes and Kelley, 2003). The role of a second binding partner of CYPs, cytochrome b_5, is less well understood. Cytochrome b_5 is a small heme containing protein also localized in the membranes of the ER that differentially affects the activities of different CYPs (Schenkman and Jansson, 2003a).

Recently, PGRMC1 has emerged as a new binding partner of several CYPs, which unlike CPR or cytochrome b_5, binds to CYP stably and stoichiometrically (Cahill, 2007; Losel, et al., 2008; Rohe, et al., 2009). These results have raised many exciting questions concerning the role of PGRMC1 in the regulation of CYPs from different classes and the mechanism of its effect on CYP function. PGRMC1 is a small 25 kD protein with an N-terminal membrane binding segment and a C-terminal domain with a cytochrome b_5-like structure that binds heme. It is expressed in many tissues, including the liver, kidney and adrenals which have high CYPs activities (Losel, et al., 2008; Meyer, et al., 1996; Min, et al., 2004; Min, et al., 2005b; Raza, et al., 2001). Expression of PGRMC1 is activated by carcinogens and its overexpression has been detected in multiple types of cancer cells (Cahill, 2007; Selmin, et al., 1996; Craven, 2008; Ahmed, et al., 2010). In most cells PGRMC1 is localized in the membranes of the ER (Nolte, et al., 2000; Sakamoto, et al., 2004), but it has also been detected in the plasma membrane, nucleus,

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endosomes, Golgi and cytoplasm (Cahill, 2007; Losel, et al., 2008; Sakamoto, et al., 2004; Peluso, et al., 2006; Craven, et al., 2007). Although its role in the regulation of the CYPs is a recent discovery, PGRMC1 has been shown to affect other cellular functions including suppression of apoptosis, DNA damage repair, and cholesterol and steroid synthesis (Rohe, et al., 2009). An effect of PGRMC1 on CYP-mediated reactions was shown in both yeast and humans (Craven, et al., 2007; Mallory, et al., 2005; Hughes, et al., 2007). The yeast homolog of PGRMC1, Dap1, increased the levels of the sterol synthesizing CYP51 in a heme-dependent manner by stabilization of the protein (Craven, et al., 2007). Recently, CYP51 was shown to bind directly to PGRMC1 in human cells and accumulation of the CYP51 substrate, lanosterol, was detected after down regulation of PGRMC1 (Hughes, et al., 2007). Binding of PGRMC1 to CYP7A1 and CYP21A2, which metabolize cholesterol and progesterone, respectively, was also observed (Hughes, et al., 2007). These data indicate that PGRMC1 positively regulates CYPs involved in sterol biosynthesis. Other classes of CYPs may be also affected by PGRMC1, since strong binding of PGRMC1 to CYP3A4 was observed (Hughes, et al., 2007)), but functional activation of the CYPs may not be not universal. PGRMC1 stimulated the activity of CYP21 in transfected cells, but an inhibition was seen in a reconstituted system and PGRMC1 had no effect on the activity of CYP17 (Min, et al., 2005a). Although recently labeled as a new "helping hand" for CYPs (Debose-Boyd, 2007), the effect of PGRMC1 on CYPs in different classes has not been extensively studied.

It has been suggested that PGRMC1 may play a role in cellular protein trafficking, because it is present in endosomes and contains several YXXΦ motifs (Φ is a large hydrophobic amino acid) that are usually involved in vesicular trafficking (Cahill, 2007; Craven, et al., 2007). PGRMC1 also forms stoichiometric complexes with SCAP and INSIG1, ER proteins involved in

the regulation of ER retention and activities of cholesterol biosynthetic proteins (Suchanek, et al., 2005). Based on these observations, we considered the possibility that PGRMC1 has a role in the retention in the ER of microsomal CYPs, in addition to effects on activity. Our results show that PGRMC1 binds to several CYPs not involved in sterol synthesis, but it has no effect on their ER distribution and retention, and surprisingly, inhibits their activities. Interestingly, we have found that PGRMC1 also binds to CPR and that increased expression of CPR can partially suppress the inhibitory effect of PGRMC1 on CYP activity.

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Materials and Methods

Materials. Cell culture materials were purchased from Invitrogen. Antibodies were from Santa Cruz Biotechnology Inc., except that Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories and anti-PGRMC1 antibody S1 was a kind gift from Dr. Martin Wehling, University of Heidelberg, Mannheim, Germany. The chemiluminescence western blotting detection kit was obtained from Pierce Chemical Co. and the P450-Glo kit for assaying CYP enzymatic activities was from Promega Corp. Sequabrene, pyridine, mevalonolactone, ergosterol, lanosterol and anti-FLAG M2 affinity gel were from Sigma-Aldrich. N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was from Thermo Scientific Inc. (USA).

Plasmid Constructions. Plasmid FLAG/PGRMC1 was obtained from Dr. Peter Espenshade, John Hopkins University School of Medicine, Baltimore, a plasmid encoding myc-tagged human INSIG1 was obtained from Dr. Christoph Thiele, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, and plasmid encoding full length calnexin was obtained from Dr. Michael Brenner, Harvard Medical School, Boston. The plasmid with CYP51A cDNA inserted into pCMV-Sport6 was obtained from Invitrogen. The construction of chimeras CYP2C2/GFP, C1(1-29)/GFP, OEC/GFP, CYP3A4/YFP and CPR/YFP have been described (Szczesna-Skorupa, et al., 1998; Szczesna-Skorupa and Kemper, 2000; Szczesna-Skorupa, et al., 2003; Szczesna-Skorupa and Kemper, 2008b). The plasmid CYP2C8/FLAG/his was constructed by PCR amplification of cDNA coding for CYP2C8 C-terminally tagged with his and FLAG and insertion into the HindIII site of pCMV-5 (Hu, et al., 2010). To construct CYP2C2/FLAG/his, CYP2C2 cDNA from pC2 (Szczesna-Skorupa and Kemper, 1989) was amplified by PCR using forward and reverse primers incorporating Not I and

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Xho I sites, respectively. The PCR product was ligated with 3XFlag and 6XHis sequences containing Xho I/Xba I and Xba I/EcoR V sites, respectively, and the combined sequence was inserted into the Adtrack CMV shuttle vector at the EcoRV and NotI sites to form Adtrack-2C2/Flag/His (B. Li and B. Kemper, unpublished).

Cell Culture and Transfection. Cell culture and transfection with Lipofectamine 2000 reagent of HEK293 and HepG2 cells and selection of HepG2 cells stably expressing CYP2C2/GFP were conducted as described (Szczesna-Skorupa and Kemper, 2001). For co-immunoprecipitation assays, cells were grown in 6-well plates and transfected with 0.5 μg of FLAG/PGRMC1 and 1 μg of either CYP2C2, CYP2C8 or CYP3A4 plasmids. After 24 h, cells were washed with phosphate-buffered saline and lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40 and 1 mM EDTA. Lysates were centrifuged for 15 min at 15K rpm and supernatants were used for western analysis or immunoprecipitation.

Fluorescent Microscopy. Confocal fluorescent microscopy was done as previously described (Szczesna-Skorupa and Kemper, 2006). Briefly, cells were grown on coverslips placed in 6-well plates, and after fixation, were used for the detection of GFP and YFP or were permeabilized for immunostaining with primary and Cy5-conjugated secondary antibodies. Cells were imaged with a Zeiss LSM510 confocal microscope (Szczesna-Skorupa and Kemper, 2006).

Western Analysis. Proteins were separated by SDS-PAGE and for western analysis were blotted to nitrocellulose membranes and detected using a chemiluminescence detection kit (Pierce Chemical Co.).

Preparation of PGRMC1 shRNA Lentivirus. Replication-incompetent lentivirus containing PGRMC1-specific shRNA was produced using the Open Biosystem Trans-Lentiviral GIPZ packaging system following the manufacturer's protocol. Briefly, the PGRMC1 silencing

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clone V2LHS_90636, or the non-silencing control, in pGIPZ transfer vectors was co-transfected with the Trans-Lentiviral packaging mix into subconfluent HEK293 cells grown in 100 mm cell culture dishes. Forty eight h later virus-containing media were collected, centrifuged at 3000 rpm for 30 min, and filtered through sterile 0.45 μm filters. Aliquots were stored at -80^{o} C.

Preparation of stable cell lines expressing PGRMC1 siRNA. Selection of HEK293 cells stably expressing either PGRMC1 siRNA or non-silencing control siRNA (Open Biosystems) was performed following the manufacturer's instructions. The PGRMC1 siRNA targets expression of endogenous PGRMC1, but not that of exogenously expressed PGRMC1, which lacks the siRNA target sequence in the 3' untranslated region of the mRNA. About 1x10^{5} cells were plated per each well of a 6-well plate. After 24 h, media were replaced with 1 ml of reduced-serum (0.5%) media without antibiotics and cells were infected with 100 μl of media containing virus per well. Six h later 1 ml of regular medium with serum and antibiotics was added. After 48 h, cells were replated into 25 cm^{2} flasks and grown in medium containing 3 μg/ml of puromycin to select for cells containing the shRNA transfer vector which expresses a puromycin resistance gene. Medium was replaced with fresh selective medium every 2-3 days until only puromycin resistant colonies were obtained and a strong decrease in the expression level of PGRMC1 in pooled cells was confirmed by western analysis. Cells were expanded and maintained in medium containing 3 μg/ml of puromycin. Stable HepG2 cells were obtained following the same protocol except that cells were grown in 60 mm cell culture dishes in 1.5 ml of reduced-serum media supplemented with 4 μg/ml of Sequabrene and were infected with 500 μl of the virus per dish, and selection medium contained 4 μg/ml of puromycin.

CYP activity assays. The enzymatic activities of CYP2C2, CYP2C8 and CYP3A4 were measured using P450-Glo assays (Promega), following the manufacturer's instructions. Briefly,

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subconfluent HEK293 or HepG2 cells grown in 12-well plates were transfected in triplicate with plasmids encoding one of the CYPs with or without CPR and PGRMC1 plasmids. Twenty four h later, cells were washed with PBS and fresh medium containing luminogenic substrate (100 μM Luciferin-ME for CYP2C2 and CYP2C8, or 50 μM Luciferin-PFBE for CYP3A4) was added. Incubation was continued for 3 h after which 100 μl of medium from each well was mixed with 100 μl of Luciferin Detection Reagent, and after 10 min, luminescence was measured using a single-tube Berthold luminometer. Statistical significance was determined by the one-tailed Student's t-test.

Sterol analysis. To analyze lanosterol levels in HEK293 cells stably expressing either control- or PGRMC1-specific siRNA, we followed the procedure of Hughes et al (Hughes, et al., 2007). Subconfluent cells grown in 60 mm plates were either mock-transfected or transfected with FLAG/PGRMC1 expression vector (0.4 μg per plate). Twenty-four h later standard growth medium (DMEM with 10% fetal bovine serum) was replaced with DMEM containing 5% lipoprotein-deficient serum and 40 mM mevalonate. Lipoprotein-deficient serum was used to reduce feedback inhibition of lipoprotein synthesis and, thus, enhance sterol synthesis. After 10 h, cells were collected in PBS and after centrifugation, pelleted cells were resuspended in a mixture of 3 ml methanol and 1.5 ml of 60% KOH. Five μg of ergosterol was added to each sample as a recovery standard. Saponification of sterols was carried out for 2 h at 75^{o}C after which 0.5 ml of water was added and lipids were extracted with 4 ml of hexane. The organic phase was dried down and prior to GC/MS analysis, dried extracts were resuspended in 50 μl pyridine and derivatized with 50 μl MSTFA at 65^{o}C for 40 min. Sample volumes of 1 μl were injected with a split ratio of 5:1. The GC-MS system consisted of a HP5890 gas chromatograph, a HP5973 mass selective detector and HP 7673A (Agilent Inc, Palo Alto, CA, USA)

autosampler. Gas chromatography was performed on a 30 m DB-5 column with 0.32 mm inner diameter and 0.25 μm film thickness (Agilent J&W, USA) with an injection temperature of 250^{o}C, the interface set to 250^{o}C, and the ion source adjusted to 230^{o}C. The helium carrier gas was set at a constant flow rate of 2.5 ml min^{-1}. The temperature program was 2 min isothermal heating at 100^{o}C, followed by an oven temperature increase of 10^{o}C min^{-1} to 320^{o}C and a final 5 min at 320^{o}C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in the m/z 50-800 scanning range. The spectra of all chromatogram peaks were evaluated using the HP Chemstation (Agilent, Palo Alto, CA, USA) program. Identification was performed using the mass spectra obtained from the authentic standards and additionally confirmed with NIST08 and W8N08 libraries (John Wiley & Sons, Inc., USA).

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Results

Binding of PGRMC1 to CYPs 2C2, 2C8 and 3A4. To test whether CYPs bind to PGRMC1, a FLAG-tagged clone of human PGRMC1 was co-transfected with C-terminally GFP-tagged CYP2C2, FLAG/his-tagged CYP2C8, or CYP3A4/YFP in HEK293 cells. After 24 h, cellular lysates were prepared for co-immunoprecipitation assays. A significant fraction (compare bound (B) with unbound (U)) of CYP2C2, detected by western analysis with GFP antisera, co-purified with FLAG/PGRMC1 isolated by binding to M2-agarose while no non-specific binding was observed in the control with agarose (Fig.1A, left panel). In contrast, the ER protein, BAP31 did not co-purify with FLAG/PGRMC1, but as previously shown (Szczesna-Skorupa and Kemper, 2006)), it did co-purify with CYP2C2/GFP (Fig. 1B, right panel). Similar to CYP2C2, a significant fraction of FLAG/PGRMC1was present in CYP2C8 (his) or CYP3A4 immunoprecipitates (Fig.1A, right panel). PGRMC1 is presumed to have a membrane topology similar to that of microsomal CYPs, i.e., an N-terminal membrane-spanning segment and a C-terminal cytosolic domain (Cahill, 2007). To test whether interaction between CYP and PGRMC1 occurs via their respective membrane integrated segments or cytosolic domains, we co-expressed PGRMC1 with GFP chimeras containing either the N-terminal transmembrane domain of CYP2C2 (chimera C1(1-29)/GFP) or its cytosolic (catalytic) domain inserted into the ER membrane by the EGFR transmembrane signal (chimera OEC/GFP) (Szczesna-Skorupa, et al., 1998). Isolation of FLAG/PGRMC1 by binding to M2-agarose followed by western analysis with GFP antisera showed that the chimera OEC/GFP binds much more efficiently to PGRMC1 than C1(1-29)/GFP, consistent with an interaction mediated mainly by the cytosolic domain of CYP2C2 (Fig. 1B,left panel). Minor binding of the chimera C1(1-29)/GFP to PGRMC1 suggests that the membrane spanning region, or the following linker region present in both C1(1-

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29)/GFP and OEC/GFP, may also contribute to the interaction with PGRMC1. These results show that PGRMC1 binds efficiently to three different CYPs that are not involved in sterol metabolism, rabbit CYP2C2 and two human CYPs, 3A4 and 2C8, and that the binding occurs mainly via the interaction with the cytosolic domain of CYP2C2.

Subcellular Localization of PGRMC1 and CYPs. The subcellular distribution of PGRMC1 was analyzed in HepG2 cells stably expressing chimeric CYP2C2/GFP or in HEK293 and HepG2 cells transiently transfected with CYPs 3A4/YFP and 2C2/GFP. Localization of both CYPs was restricted to the ER network and nuclear membranes as has been documented previously (Szczesna-Skorupa and Kemper, 2000; Szczesna-Skorupa and Kemper, 2008b; Ahn, et al., 1993; Szczesna-Skorupa, et al., 1995; Szczesna-Skorupa, et al., 2000; Szczesna-Skorupa and Kemper, 2008a). PGRMC1 was detected in these two compartments, and in a small fraction of HepG2 cells was also present in the plasma membrane whether or not CYP was co-transfected (Fig. 2A-D), which is consistent with earlier observations (Peluso, et al., 2006). Nevertheless, there was considerable colocalization of both proteins in the ER. Similar results were obtained with CYPs 2E1and 2C8 in HepG2, HEK 293 and COS1 cells (not shown).

Since there are conflicting reports about the topology and the orientation of the N-terminus of PGRMC1 in the membrane (Cahill, 2007) we also analyzed non-permeabilized HepG2 cells transfected with a FLAG/PGRMC1, which has the FLAG at the N-terminus. PGRMC1 fluorescence, detected by immunostaining with an anti-FLAG antibody, partially overlapped with a plasma membrane marker, rhodamine conjugated wheat germ agglutinin at the surface of the cells (Fig. 2D). This result indicates that a fraction of PGRMC1 localizes to the plasma membrane and that the N-terminus of PGRMC1 is translocated to the luminal side of the ER

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during synthesis and consequently exposed at the cell surface, in contrast to a previous report (Nolte, et al., 2000).

The Effect of Downregulation of PGRMC1 on CYP Expression Level and Localization. To determine the role of PGRMC1 in the expression and localization of CYPs, PGRMC1 was stably downregulated in cell lines by expressing PGRMC1 siRNA using the lentivirus system. Two kinds of cell lines were prepared that were infected either with control virus that contained a non-silencing siRNA insert or with virus that contained a PGRMC1 siRNA insert. The amount of PGRMC1 detected by western analysis was greatly reduced to almost undetectable levels in the cells expressing the PGRMC1 siRNA compared to cells expressing control siRNA (Fig. 3A). Similar levels of CYP2C2/GFP and exogenously expressed PGRMC1, which is not targeted by PGRMC1 siRNA, were expressed in HEK293 cells that were expressing either control or PGRMC1 siRNA and were transfected with a CYP2C2/GFP expression vector (Fig. 3B, upper panel). The effect of PGRMC1 on the subcellular distribution of CYP2C2 in HEK293 cells was tested by transfecting these cell lines stably expressing PGRMC1 siRNA with the chimera CYP2C2/GFP. CYP2C2/GFP exhibited a typical reticular ER pattern of distribution in cells stably expressing either PGRMC1 or control siRNA (Fig. 3B, lower panel). Similarly, the absence of PGRMC1 had no effect on the ER localization of two other CYPs, CYP2E1 and CYP3A4 (not shown). These results indicate that the expression level and ER localization and retention of CYPs 2C2, 2E1 and 3A4 do not depend on the presence of PGRMC1.

The Effect of PGRMC1 on the Enzymatic Activity of CYPs. To test the effect of PGRMC1 on the enzymatic activity of the CYPs, we either down regulated PGRMC1 by stably expressing PGRMC1 siRNA or overexpressed exogenous PGRMC1 in HEK293 cells. Surprisingly, in PGRMC1 deficient cells, CYP2C2 activity was not decreased, and in several

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independent experiments, activity was slightly higher than in control cells, statistically significantly higher for cells transfected with 25 and 50 ng of PGRMC1 vector (Fig. 4A). When PGRMC1 was exogenously co-expressed with CYP2C2, however, CYP2C2 activity was inhibited in a concentration-dependent manner in cells expressing either control siRNA or PGRMC1 siRNA (Fig. 4A).

Since HEK293 cells do not express high levels of endogenous CPR (Fig. 4C), we tested the effect of expressing a human CPR tagged at the C-terminus with YFP (chimera CPR/YFP) in these cells. Co-expression of CPR with CYP2C2 increased the activity of CYP2C2 about 1.5- to 2-fold (Fig. 4B). Interestingly, the percentage inhibition of CYP2C2 activity by PGRMC1 was decreased in the cells expressing CPR/YFP indicating that the exogenously expressed CPR partially reversed the PGRMC1-mediated inhibition (Fig. 4B). Western analysis of lysates prepared from cells used for the activity assay showed that there were no significant changes in CYP2C2 or PGRMC1 levels resulting from co-expression of CPR/YFP (Fig. 4C).

To test whether these unexpected effects of PGRMC1 on CYP2C2 activity are specific for CYP2C2, we examined the effect of PGRMC1 and CPR on the activity of two other CYPs, CYP2C8 and human CYP3A4. For CYP2C8 enzymatic activity was slightly higher in HEK 293 cells stably expressing PGRMC1 siRNA, statistically significantly higher in 3 of the 4 conditions (Fig. 5A). For CYP3A4 the activity was similar in cells expressing either control or PGRMC1 siRNA (Fig. 5B). Similar to the effects on CYP2C2, expression of exogenous PGRMC1 inhibited the activities of CYPs 2C8 and 3A4, although the inhibition by PGRMC1 was greater for these two CYPs compared to CYP2C2. Expression of exogenous CPR increased CYP2C8 (Fig. 5A) and CYP3A4 (Fig. 5B) activities 3- to 4-fold, but in contrast to CYP2C2, exogenous

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CPR led to smaller, but statistically significant, increases in activity of these two CYPs in the presence of PGRMC1 (Fig. 5A, B).

Since HEK293 is not a hepatic cell line, we examined the effects of the PGRMC1 on CYP activities in hepatic-derived HepG2 cells that were stably expressing either control or PGRMC1 siRNA. The activities of both CYP2C2 and CYP2C8 were slightly higher in PGRMC1-deficient cells than in control cells, but the differences were not statistically significant (Fig. 6A, B). Inhibition of the activity of CYP2C8 by exogenously expressed PGRMC1 was similar to that seen in HEK293 cells, however, 5-fold higher amounts of transfected PGRMC1 expression plasmid were required to obtain similar inhibition of CYP2C2 in HepG2 cells compared to HEK 293 cells.

There was little or no effect of exogenous expression of CPR on the CYP2C8 and CYP2C2 activities (Fig. 6A, B), presumably because HepG2 cells express much higher levels of endogenous CPR than HEK293 cells (Fig. 6C). Nevertheless, expression of exogenous CPR modestly, but statistically significantly, reversed the inhibition of the CYP activities by PGRMC1 with greater reversal for CYP2C2 than for CYP2C8 (Fig. 6A, B), similar to the results obtained in HEK293 cells (Fig. 5). The levels of the CYP2C2 were not detectably affected by the expression of PGRMC1 or CPR nor did exogenous expression of CPR substantially increase total CPR levels (Fig. 6C). These results indicate that overexpression of PGRMC1 inhibits the activities of CYPs 2C2, 2C8 and 3A4 in either HEK293 cells or liver HepG2 cells, and the inhibition can be at least partially reversed by increased expression of CPR.

Activity of CYP51 Depends on PGRMC1. The inhibition of the activities of CYPs 2C2, 2C8 and 3A4 by expression of PGRMC1 could possibly result from general nonspecific effects of PGRMC1 overexpression. As a control for that possibility, we examined the activity

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of CYP51 for which positive regulation by PGRMC1 is well documented both in yeast (Mallory, et al., 2005; Hand, et al., 2003) and mammalian cells (Hughes, et al., 2007). In addition, in HEK293 cells, endogenous CYP51 is expressed at high levels, comparable to the expression of CYP2C2 in transiently transfected cells, and the additional expression of exogenous CYP51 only modestly increased total CYP51 protein levels (Fig. 7A).

In contrast to the results with the drug metabolizing CYPs, overexpression of exogenous PGRMC1 in HEK293 cells did not inhibit CYP51 activity since there was no effect on the level of lanosterol (Fig. 7B). Consistent with the studies of yeast and mammalian cells, downregulation of PGRMC1 expression in HEK293 cells resulted in an increased accumulation of the CYP51 substrate, lanosterol, indicating that CYP51 activity was inhibited (Fig.7B). Further, expression of exogenous PGRMC1, which does not contain the siRNA target in its 3' untranslated region, in PGRMC1-deficient cells decreased the levels of lanosterol (Fig. 7B) indicating that CYP51 activity was increased. Thus, in agreement with the data of Hughes et al. (Hughes, et al., 2007) CYP51 activity is increased by PGRMC1 in HEK293 cells. These observations eliminate general non-specific effects of PGRMC1 overexpression on CYP activity as an explanation for the inhibition of the drug metabolizing CYPs and support the conclusion that PGRMC1 has different effects on different classes of CYPs.

PGRMC1 Binds to CPR. Since expression of CPR partially reversed the PGRMC1 inhibition of CYP activity, it is possible that CPR and PGRMC1 interact. To test for an interaction between CPR and PGRMC1 we co-expressed CPR/YFP with either FLAG/PGRMC1 or CYP2C2/FLAG/his in HEK293 cells. CPR and CYP2C2/FLAG were associated with FLAG/PGRMC1 that was isolated by binding to M2 agarose (Fig. 8A). As a negative control for binding specificity, we also tested co-immunoprecipitation of PGRMC1 with another ER

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membrane protein calnexin, which was co-expressed in HEK 293 cells with FLAG/PGRMC1. As shown in Fig. 8A (right panel) calnexin was not detected in the M2 agarose immunoprecipitates indicating that it did not bind to PGRMC1. These results suggest that PGRMC1 may form a specific and stable complex with CPR.

Based on these observations we hypothesized that the partial suppression of the PGRMC1-induced inhibition of CYPs catalytic activity by CPR may result from the competition between CPR and PGRMC1for a shared binding site on the CYP. This would be consistent with the observation that PGRMC1 interacts predominantly with the cytosolic domain of CYP2C2 (Fig. 1C), which is the domain also responsible for binding CPR (Bridges, et al., 1998a). To test this hypothesis, we compared the effect of CPR expression on the binding of PGRMC1 to CYP2C2. In HEK293 cells co-expressing FLAG/PGRMC1 and either CYP2C2/GFP or CPR/YFP, both proteins bound to PGRMC1 at a similar level (Fig. 8B). Surprisingly, in cells co-expressing all three proteins, there was a marked decrease in CPR binding to PGRMC1, whereas binding of PGRMC1 to CYP2C2 was little changed (Fig. 8B). This suggests that binding of PGRMC1 to CYP2C2 is unaffected by CPR but that under these conditions CYP2C2 competes with CPR for binding to PGRMC1 and has a higher affinity for PGRMC1 than CPR does. Alternatively CYP2C2 may induce a conformational change in CPR or PGRMC1 that affects their interaction.

We subsequently determined whether CPR binding to CYP2C2 is affected by PGRMC1. CYP2C2/FLAG/his and CPR/YFP were coexpressed with increasing amounts of FLAG/PGRMC1. After immunoprecipitation of CPR from cell lysates, PGRMC1 and CYP2C2 in the immunoprecipitates were detected by western analysis with PGRMC1 and FLAG antisera, respectively. Expression of PGRMC1 had a minimal effect on CPR binding to CYP2C2 (Fig.

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8C, upper panel) and as expected from the effect of CYP2C2 coexpression, only weak binding of PGRMC1 to CPR was observed (Fig. 8C, third panel) since exogenous CYP2C2 was expressed in these cells. This result suggests that PGRMC1 does not significantly sequester CYP2C2 and reduce its binding to CPR whereas CYP2C2 reduces binding of CPR to PGRMC1.

To test whether PGRMC1 has any effect on the subcellular localization of CPR, we compared the distribution of CPR/YFP and PGRMC1 in HepG2 cells. CPR/YFP was detected in the reticular ER, nuclear membranes and some additional punctuate fluorescence with significant colocalization with transfected FLAG/PGRMC1 (Fig. 9A). A similar pattern of subcellular distribution of CPR/YFP was observed in HEK293 cells expressing either control or PGRMC1 specific siRNA (Fig. 9B) indicating that PGRMC1 is not a determinant of CPR localization in the cell.

INSIG1 Binds to CYP2C2 and Stimulates its Enzymatic Activity. One of the few ER membrane proteins known to form a complex with PGRMC1 is INSIG1 which is involved in the regulation of the activity and localization of the enzymes mediating cholesterol synthesis (Goldstein, et al., 2006). We considered the possibility that INSIG1, as a binding partner of PGRMC1, might mediate the effects of PGRMC1 on CYP activity. We first tested whether INSIG1 binds to CYP2C2 and if so, whether this interaction is dependent on PGRMC1. Analysis of lysates from HEK293 cells co-expressing myc-tagged INSIG1 and CYP2C2/FLAG showed that INSIG1 bound efficiently to CYP2C2 (Fig. 10A). However, similar binding was observed in cells expressing either control or PGRMC1 siRNA (Fig. 10A), indicating that the binding of INSIG1 to CYP2C2 is independent of PGRMC1. In contrast to CYP2C2 and PGRMC1 (Fig. 10A,right panel), binding of INSIG1 to CPR was not detected (Fig. 10B, left panel), although binding to CYP2C2-FLAG, used as a positive control, was observed (Fig. 10B,

right panel). Co-immunoprecipitation of INSIG1 and chimera C1(1-29)/GFP is more efficient than that with the cytoplasmic domain chimera OEC/GFP (Fig. 10C). This result indicates that INSIG1 binds predominantly to the transmembrane domain of CYP2C2, in contrast to PGRMC1, which binds mainly to the cytoplasmic domain of CYP2C2 (Fig. 1B),

Interestingly, INSIG1 increased the activity of CYP2C2 (Fig. 10C) and CYP2C8 (not shown) by 50%, however, unlike CPR, it had no effect on the inhibition of the enzymatic activity of either CYP by PGRMC1. These results show that INSIG1 binds to CYP2C2 and increases CYP activity, but that this interaction is independent of PGRMC1.

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Discussion

Our studies show that PGRMC1 binds efficiently to CYPs, 2C2, 2C8, and 3A4, and surprisingly, inhibits the activities of these CYP's but is not essential for their localization or retention in the ER. PGRMC1 has been shown to increase the activity of two yeast CYPs, CYP51A1 and CYP61A1, and two mammalian CYPs, CYP51 and CYP21 involved in sterol biosynthesis (Craven, et al., 2007; Mallory, et al., 2005; Hughes, et al., 2007; Min, et al., 2005a). Down regulation of PGRMC1 (Dap1 in yeast) expression resulted in accumulation of CYP51 and CYP61A1 substrates in yeast and accumulation of CYP51 substrates in mammalian cells, and direct binding of Dap1 to yeast CYP51 and CYP61A was shown (Hughes, et al., 2007). In earlier studies, Dap1 was shown to positively regulate CYP51 by stabilizing this protein in a heme-dependent fashion, but direct binding of CYP51 to Dap1 was not detected (Craven, et al., 2007; Mallory, et al., 2005). Our studies on CYP51 are consistent with these previous studies since the CYP51 substrate, lanosterol, accumulated in PGRMC1-deficient cells and the increase was reversed by overexpression of exogenous PGRMC1. In contrast to this sterol synthesizing CYP, rabbit CYP2C2 is a fatty acid hydroxylase and human CYP2C8 and CYP3A4 are responsible for metabolism of the majority of drugs that are metabolized by human CYPs (Danielson, 2002). The inhibition of the activity of these CYPs by PGRMC1 indicates that PGRMC1 may activate or inhibit specific CYPs. This conclusion is consistent with the observations that PGRMC1 did not affect CYP17 activity and had condition-dependent effects on CYP21 for which activity was stimulated by PGRMC1 in cells, but inhibited in reconstituted systems (Min, et al., 2005b).

A possible concern is that PGRMC1 inhibition of exogenously expressed CYPs might be an artifact of over expression. Several considerations indicate that this is not the case. The level

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of CYP proteins present in the stably transfected cells was about 25% that of cells transiently transfected as determined by fluorescence intensity of the CYP-GFP proteins (data not shown) and in both types of cells, CYP2C2 activity was decreased. Further, the levels of expression of CYPs in transiently transfected COS cells are reported to be 5 to 40 pmol/mg microsomal protein (Clark and Waterman, 1991) and range from 10 to over 100 pmoles/mg microsomal protein in stably transfected cells (Gonzalez and Korzekwa, 1995). These values are comparable to those reported for 11 different CYPs in human liver which range from 1.3 to 80.2 pmoles/mg microsomal protein (64 pmoles/mg for CYP3A4) (Kawakami, et al., 2010) so that the levels of exogenously expressed CYPs are similar to the levels in hepatocytes *in vivo*. Finally, the levels of endogenous CYP51 are similar to the levels of the CYP2C2, CYP2C8, and CYP3A4 in transiently transfected cells. These considerations indicate that the inhibition of the CYP2C2/8 and CYP3A4 by PGRMC1 is not an artifact of overexpression of these proteins.

Isozyme-specific, and probably also concentration-dependent, effects of PGRMC1 on different CYPs activities, may be of special importance in view of the fact, that PGRMC1 is induced by carcinogens and overexpressed in cancer cells (Rohe, et al., 2009; Ahmed, et al., 2010; Nie, et al., 2006). Further, PGRMC1 has been shown to contribute to chemotherapy resistance, so it's inhibitory effect on drug-metabolizing CYPs is of particular interest. Interestingly, in lung cancer cells PGRMC1 appears to have no effect on cholesterol biosynthesis, since downregulation of PGRMC1 did not induce accumulation of lanosterol (Ahmed, et al., 2010). Thus, PGRMC1 effects may be also cell specific, which could also explain our observations of lower inhibitory effect of PGRMC1on drug metabolizing CYPs in HepG2 than in HEK293 cells.

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PGRMC1 contains a heme-binding cytochrome b_5-like domain in its cytoplasmic domain so it is tempting to postulate that CYPs may bind to the cytochrome b_5 domain of PGRMC1 since CYPs interact with cytochrome b_5. Consistent with this idea, binding of PGRMC1 to CYP2C2 is predominantly to its cytoplasmic domain, as is binding of cytochrome b_5 to CYPs (Bridges, et al., 1998b), and a mutation in the cytochrome b_5 domain of PGRMC1 eliminated binding to CYP7A1(Mansouri, et al., 2008). In contrast to cytochrome b_5 which can donate electrons to CYPs, transfer of electrons from PGRMC1 to a CYP seems unlikely in view of the pentacoordinate binding of heme to PGRMC1, as opposed to hexacoordinate heme in CYPs. However, cytochrome b_5 apo-protein, without a heme, affects the activity of some CYPs (Yamazaki, et al., 1996), presumably allosterically, which is a possible mechanism for PGRMC1 that does not involve electron transfer. Interestingly, the effects of cytochrome b_5, like PGRMC1, are isozyme-specific for reasons that are not entirely clear (Schenkman and Jansson, 2003b).

A novel observation of our studies is that PGRMC1 binds efficiently to CPR. Cytochrome b_5 can be reduced by CPR and presumably binds CPR although this has not been shown directly (Schenkman and Jansson, 2003b) so that CPR may interact with the cytochrome b_5 domain of PGRMC1. Interestingly, PGRMC1-induced inhibition of CYP activity can be partially reversed by increased expression of CPR. The simplest explanation of these results would be that CPR competes for binding of PGRMC1 to the CYPs which would be consistent with binding to the cytochrome b_5 site that overlaps the CPR binding site. However, studies of the interactions of these proteins by co-immunoprecipitation are not consistent with this explanation. CPR expression had little effect on the interaction of CYP2C2 with PGRMC1. In contrast, expression of CYP2C2 dramatically reduced the interaction of CPR with PGRMC1. Alternatively, PGRMC1 might inhibit CYP activity by competing for CPR binding. Although

increasing levels of PGRMC1 did not affect the binding of CYP2C2 to CPR, the binding was measured in the absence of substrate, and co-immunoprecipitation may not detect effects on transient functional interactions between CYP and CPR. Interestingly, human CPR was reported to exist in two conformational states: a compact structure that is suitable for interflavin electron transfer and an extended form involved in transfer of electrons to a CYP (Ellis, et al., 2009). PGRMC1 may interfere with the transition between the closed and open conformation. The interactions among these proteins and the ultimate effects on activity appear to be complex and not completely explained by the present data.

Two different models to explain the PGRMC1 effect on microsomal CYPs have been proposed: stabilization of CYPs by PGRMC1 in a heme-dependent manner (Mallory, et al., 2005) or stable heme-dependent binding of PGRMC1 to CYPs affecting activity by an unknown mechanism (Hughes, et al., 2007). Our own results which show that PGRMC1 inhibits drug-metabolizing CYPs activity, whereas it activates sterol synthesizing CYP51, are not compatible with increased CYP stability and are more consistent with the second model in which activity could be affected positively or negatively in an isozyme-specific manner. Explanations for a mechanism are largely speculative and may include allosteric effects of PGRMC1 on CYPs; decreased CPR binding to CYP which cannot be detected by the co-immunoprecipitation assays in this study; induction of covalent modification, such as phosphorylation of CYP or CPR; or mediation of the effects by an unidentified binding partner of PGRMC1 that affects the activity of CYP and might also regulate PGRMC1 binding to CPR in the presence of CYP. With regard to the last possibility, the presence of multiple protein motifs in PGRMC1 that are associated with signal transduction strongly suggest that PGRMC1 may be involved in the formation of multiple protein-protein interactions and signal transduction (Cahill, 2007; Losel, et al., 2008).

INSIG1, which binds to both PGRMC1 and CYP, was a potential intermediary protein, but our results suggest that INSIG1 and PGRMC1 act independently on CYP.

Although expression of CPR partially reversed the inhibition of CYP activity in cells, the extent of the reversal was different for the different CYPs. Different CYPs may interact with different residues of the CPR which suggests formation of complexes with different stoichiometry and/or differences in the affinity of a CYP toward CPR (Miller, et al., 2009; Yamazaki, et al., 2002). Two of the CYPs used in our studies, CYP2C8 and CYP3A4, in a reconstituted system required higher amounts of CPR than other CYPs for their optimal activities, and both were additionally stimulated by cytochrome b_5 (Yamazaki, et al., 2002). We observed that compared to CYP2C2, these two CYPs were more sensitive to both activation by exogenous CPR and inhibition by PGRMC1 and the inhibition was only weakly reversed by expression of CPR. The reversal of PGRMC1 inhibition of CYPs, thus, may be influenced by the binding affinity for CPR and whether cytochrome b_5 binds and activates the CYP.

Our results support previous studies showing that PGRMC1 forms stable complexes with CYPs, but in contrast to the earlier studies on CYPs involved in steroid biogenesis, PGRMC1 inhibits, rather than activates, CYPs involved mainly in drug metabolism. PGRMC1 also binds CPR which was not appreciated before and adds a layer of complexity in understanding how PGRMC1 affects the activity of CYPs in an isozyme-specific manner.

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Authorship Contributions

Participated in research design: Szczesna-Skorupa and Kemper.

Conducted experiments: Szczesna-Skorupa.

Contributed new reagents or analytical tools: Szczesna-Skorupa

Performed data analysis: Szczesna-Skorupa.

Wrote or contributed to the writing of the manuscript: Szczesna-Skorupa and Kemper.

Other: Kemper acquired funding for the research

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Footnotes

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Figure Legends

Fig. 1. Binding of FLAG/PGRMC1 to CYPs 2C2, 3A4 and 2C8. A. HEK293 cells were transfected with expression plasmids for FLAG/PGRMC1 and either CYP2C2/GFP, CYP3A4/YFP or CYP2C8/FLAG/his. After 24 h, cellular lysates were prepared and FLAG/PGRMC1 was immunoprecipitated with M2-agarose, CYP3A4 with anti-3A4 or CYP2C8/FLAG/his with anti-his antisera. Western blots were probed with either anti-GFP or anti-FLAG antibodies. Input (I) and bound (B) and unbound (U) fractions were analyzed. In both A and B, the bound fraction was concentrated 10-fold relative to unbound and input. B. HEK293 cells were transfected with FLAG/PGRMC1 and either C1(1-29)/GFP or OEC/GFP and after 24 h cellular lysates were prepared and FLAG/PGRMC1 was immunoprecipitated with M2-agarose. Unbound (U) and bound (B) fractions were analyzed by western blots probed with anti-GFP antibody. C. HEK293 cells were transfected with FLAG/PGRMC1 and CYP2C2/GFP as in A. FLAG/PGRMC1 and CYP2C2/GFP were immunoprecipitated from cellular lysates with M2-agarose and GFP antibodies, respectively, and the amount of BAP31 in the immunoprecipitates was determined by western blotting with an antibody against BAP31

Fig. 2. Subcellular localization of PGRMC1 and CYPs. A. HepG2 cells stably expressing CYP2C2/GFP were transfected with FLAG/PGRMC1 (1 μg) (upper row), HEK293 cells were transiently co-transfected with FLAG/PGRMC1 (0.5 μg) and CYP2C2/GFP (1 μg) (middle row) and HepG2 cells were transiently co-transfected with FLAG/PGRMC1 (1 μg) and CYP3A4/YFP (1 μg) (bottom row). Twenty-four h after transfection, cells were fixed, permeabilized and CYPs were detected by fluorescence and PGRMC1 by immunostaining with

an anti-FLAG antibody followed by Cy5-conjugated secondary antibody. B. A higher magnification image of HEK293 cell transiently co-transfected with CYP2C2/GFP and FLAG/PGRMC1 and processed as in A. C. HepG2 or HEK293 cells were transiently transfected with FLAG/PGRMC1 only and processed as in A. D. HepG2 cells were transfected with FLAG/PGRMC1 (1 μg), and after 24 h, cells were fixed and immunostained with anti-FLAG antibody or with wheat germ agglutinin (a plasma membrane marker) without permeabilization. Cells were analyzed by confocal microscopy. Scale bars=10 μm.

Fig. 3. Down regulation of PGRMC1expression in HEK293 and HepG2 cells. A. Lysates from HEK293 and HepG2 cells stably expressing either non-silencing control siRNA (C) or PGRMC1 specific siRNA (si) were prepared and PGRMC1 or actin was detected by western analysis with an antibodies against PGRMC1 or actin, respectively. B. HEK293 cells stably expressing either control or PGRMC1 specific siRNA were transfected with expression plasmids for CYP2C2/GFP with or without FLAG/PGRMC1, and after 24 h, cellular lysates were prepared and CYP2C2/GFP and PGRMC1 were detected by western analysis using antisera to GFP and PGRMC1, respectively (Upper Panel). Both endogenous (lower band) and transfected FLAG-tagged PGRMC1 (upper band) were detected. M indicates mock-transfected cells. The lower panel shows, HEK293 cells stably expressing either control or PGRMC1 siRNA transfected with CYP2C2/GFP fixed and imaged by fluorescent microscopy.

Fig. 4. PGRMC1 effect on the activity of CYP2C2 in HEK293 cells. A. HEK293 cells stably expressing either control (black bars) or PGRMC1 siRNA (gray bars) were plated into 12-well plates and 24 h later cells were transfected in triplicate either with an expression vector for

CYP2C2/FLAG (0.5 μg per well) alone or additionally with indicated amounts of FLAG/PGRMC1 expression vector. After 24 h, the enzymatic activity of CYP2C2 was measured using the P450-Glo assay as described in Materials and Methods. B. HEK293 cells were transfected with an expression vector for CYP2C2/FLAG (0.5 μg per well) alone or with FLAG/PGRMC1 and 0.1 μg of CPR/YFP expression vectors. The activity of CYP2C2 was assayed 24 h after transfection. Background luminescence from mock-transfected cells was subtracted from all samples. Data were analyzed by the Student's t-test. * indicates a significant different between the PGRMC siRNA sample and the control siRNA sample, $p < 0.05$, $n = 3$. (A,B) The mean value from triplicates and the standard deviation are shown. C. HEK293 cells were transfected with CYP2C2/FLAG (0.5 μg), CPR/YFP (0.1 μg) and FLAG/PGRMC1 (0.2 μg), as indicated, and after 24 h cells were lysed in RIPA buffer and respective proteins detected by western analysis. Both endogenous (lower bands) and transfected CPR/YFP or FLAG/PGRMC1 (upper bands) were detected as indicated.

Fig. 5. PGRMC1 effect on the activities of CYP2C8 and CYP3A4 in HEK293 cells. HEK293 cells stably expressing control (black bars) or PGRMC1 siRNA (gray bars) were transfected as in Fig. 4 either with 0.5 μg per well of CYP2C8/FLAG/his (A) or CYP3A4 (B) expression vectors with or without 0.2 μg of FLAG/PGRMC1 and 0.1 μg of CPR/YFP. Enzymatic activity was assayed after 24 h as described in the legend to Fig. 4. Data were analyzed by the Student's t-test. * indicates a significant difference between the PGRMC1 siRNA sample and the control siRNA sample and ** indicates a significant difference between cells expressing CPR and PGRMC1 compared to cells expressing only PGRMC1, $p < 0.05$, $n = 3$.

Fig. 6. PGRMC1 effect on CYP2C2 and CYP2C8 activity and expression levels in transfected HepG2 cells. A and B. HepG2 cells stably expressing control (black bars) or PGRMC1 siRNA (gray bars) were transfected as in Fig. 4 with 1 μg per well of CYP2C2/FLAG (A) or 1 μg of CYP2C8/FLAG/his (B) (compared to 0.2 μg in Fig. 4) expression vectors with or without 0.1 μg of CPR/YFP and either 1 μg (A) or 0.5 μg (B) of FLAG/PGRMC1. Activities were assayed after 24 h as described in the legend to Fig. 4. C. Transfected HepG2 cells were lysed in RIPA buffer and CPR (upper panel), CYP2C2 (middle panel) and PGRMC1 (lower panel) were detected by western analysis. Both endogenous (lower band) and transfected CPR/YFP (upper band) were detected. ** indicates a significant difference between cells expressing CPR and PGRMC1 compared to cells expressing only PGRMC1, $p < 0.05$, $n = 3$

Fig. 7. PGRMC1 effect on lanosterol levels in HEK293 cells. A. HEK293 cells were transfected with CYP2C2/his (left panel) or CYP51 (right panel) and 24 h later cellular lysates were analyzed by western analysis with an anti-his or anti-CYP51 antibodies. B.HEK293 cells stably expressing control or PGRMC1 siRNA were either mock-transfected (black bars) or transfected with a FLAG/PGRMC1 expression plasmid (grey bars) and 24 h later incubated in DMEM medium containing 5% lipoprotein-deficient serum and 40 mM mevalonate for 10 h. Extracted sterols were analyzed with QC/MS as described in Materials and Methods. The mean values and standard deviations from triplicates are shown. * indicates a significant difference between PGRMC1 siRNA and control siRNA samples and ** indicates a significant difference between cells expressing PGRMC1 with cells not expressing PGRMC1, $p < 0.05$, $n = 3$.

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Fig. 8. Binding of PGRMC1 to CPR. A. HEK293 cells were transfected with expression vectors for CPR/YFP and either FLAG/PGRMC1 or CYP2C2/FLAG (left panel) or FLAG/PGRMC1 and calnexin (right panel) and after 24 h, cellular lysates were subjected to immunoprecipitation with M2-agarose or agarose-conjugated non-immune IgG. CPR in the immunoprecipitates was detected by western analysis using an antibody against CPR and calnexin with an anti-calnexin antibody (upper panels). In the lower panels, controls are shown in which FLAG immunoprecipitates were analyzed by western with FLAG antibody. Bound (B) fractions are concentrated 5-fold compared to input (I) fractions. B. HEK293 cells were transfected with expression vectors for FLAG/PGRMC1 and either CYP2C2/GFP, CPR/YFP or both, as indicated. After 24 h, lysates from transfected cells were analyzed by immunoprecipitation with M2-agarose or agarose-conjugated IgG and CYP2C2/GFP and CPR/YFP were detected in the immunoprecipitates by western analysis with an antibody against GFP, which recognizes both GFP and YFP. C. HEK293 cells were co-transfected with expression vectors for CYP2C2/FLAG/his and CPR/YFP without or with 0.5 μg (+) or 1 μg (++) of FLAG/PGRMC1. After 24 h, CPR was immunoprecipitated from cellular lysates with an antibody against CPR and PGRMC1 was immunoprecipitated with an antibody against PGRMC1. CYP2C2/FLAG/his (upper panel),CPR/YFP (second panel), and FLAG/PGRMC1 (third and fourth panels) in the immunoprecipitates were detected by western analysis with an anti-FLAG, anti-CPR and anti-PGRMC1 antibodies, respectively. (A-C) At least 3 experiments were performed and representative data for each are shown.

Fig. 9. Subcellular localization of CPR in cells expressing control and PGRMC1 siRNA. A. HepG2 cells were transfected with expression vectors for CPR/YFP and FLAG/PGRMC1, and

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after 24 h, fixed cells were permeabilized and subjected to immunostaining with anti-FLAG antibody followed by Cy5- conjugated secondary antibody to detect PGRMC1 (red). B. Control or PGRMC1-deficient HEK293 cells were transfected with expression vectors for CPR/YFP only or with CPR/YFP and FLAG/PGRMC1 (far right panel). Fixed cells were analyzed by confocal microscopy. Scale bars=10 μm.

Fig. 10. INSIG1 binds CYP2C2 and stimulates its activity. A. HEK293 cells expressing control or PGRMC1 siRNA were transfected with expression vectors for INSIG1/myc and either CYP2C2/FLAG/his (left panel) or FLAG/PGRMC1 (right panel). Twenty-four h later, cellular lysates were immunoprecipitated with M2-agarose or agarose-conjugated IgG and INSIG1/myc in the immunoprecipitates was detected by western analysis with anti-myc antibody. B. Lysates of HEK293 cells cotransfected with CPR/YFP and either INSIG/myc or CYP2C2/FLAG, as indicated, were immunoprecipitated with an antibody against CPR and western blots were probed with anti-myc antibody to detect INSIG/myc (left panel), anti-CPR to detect CPR (middle panel) or anti-FLAG to detect CYP2C2/FLAG. C. Lysates of HEK293 cells cotransfected with INSIG/myc and either C1(1-29)/GFP or OEC/GFP, as indicated, were immunoprecipitated with an antibody against GFP or agarose-conjugated IgG and INSIG/myc was detected on western blots with an anti-myc antibody (left panel). The right panel shows identical lysates immunoprecipitated with an anti-myc antibody and probed with an antibody against GFP. For A-C, bound (B) fractions were concentrated 5-fold compared to input (I) fractions. Similar results were obtained in three independent experiments. D. CYP2C2 activity in the presence of INSIG1. HEK293 cells grown in 12-well plates were transfected with 0.5 μg of CYP2C2/FLAG/his with or without addition of CPR/YFP (0.1 μg), FLAG/PGRMC1 (0.2 μg)

and INSIG1/myc (0.2 μg per well). Activities were determined after 24 h as described in the legend to Fig. 4. Background luminescence from mock-transfected cells was subtracted from all samples. The mean value from triplicates and the standard deviation are shown. * indicates significant differences between cells expressing the indicated protein and untransfected cells, $p < 0.05$, $n = 3$.

Figure 1

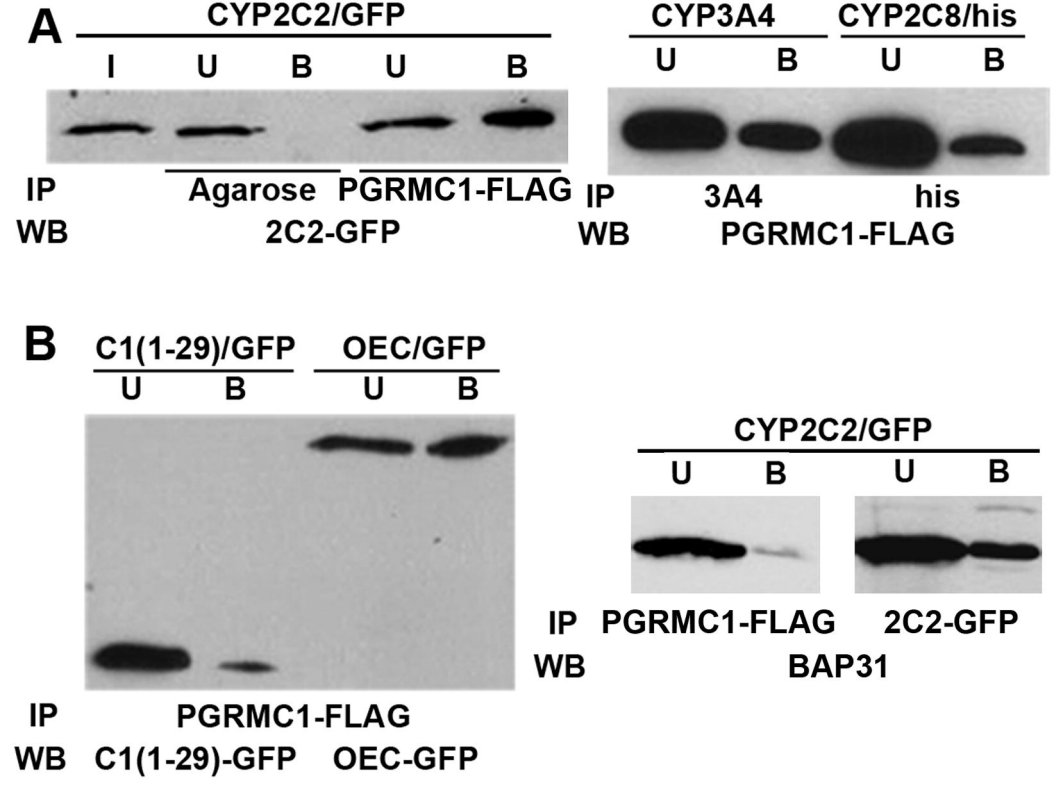

A
CYP2C2/GFP
I
U
B
U
B
IP
Agarose
PGRMC1-FLAG
WB
2C2-GFP
CYP3A4
CYP2C8/his
U
B
U
B
IP
3A4
his
WB
PGRMC1-FLAG
B
C1(1-29)/GFP
OEC/GFP
U
B
U
B
IP
PGRMC1-FLAG
WB
C1(1-29)-GFP
OEC-GFP
CYP2C2/GFP
U
B
U
B
IP
PGRMC1-FLAG
2C2-GFP
WB
BAP31

Figure 2

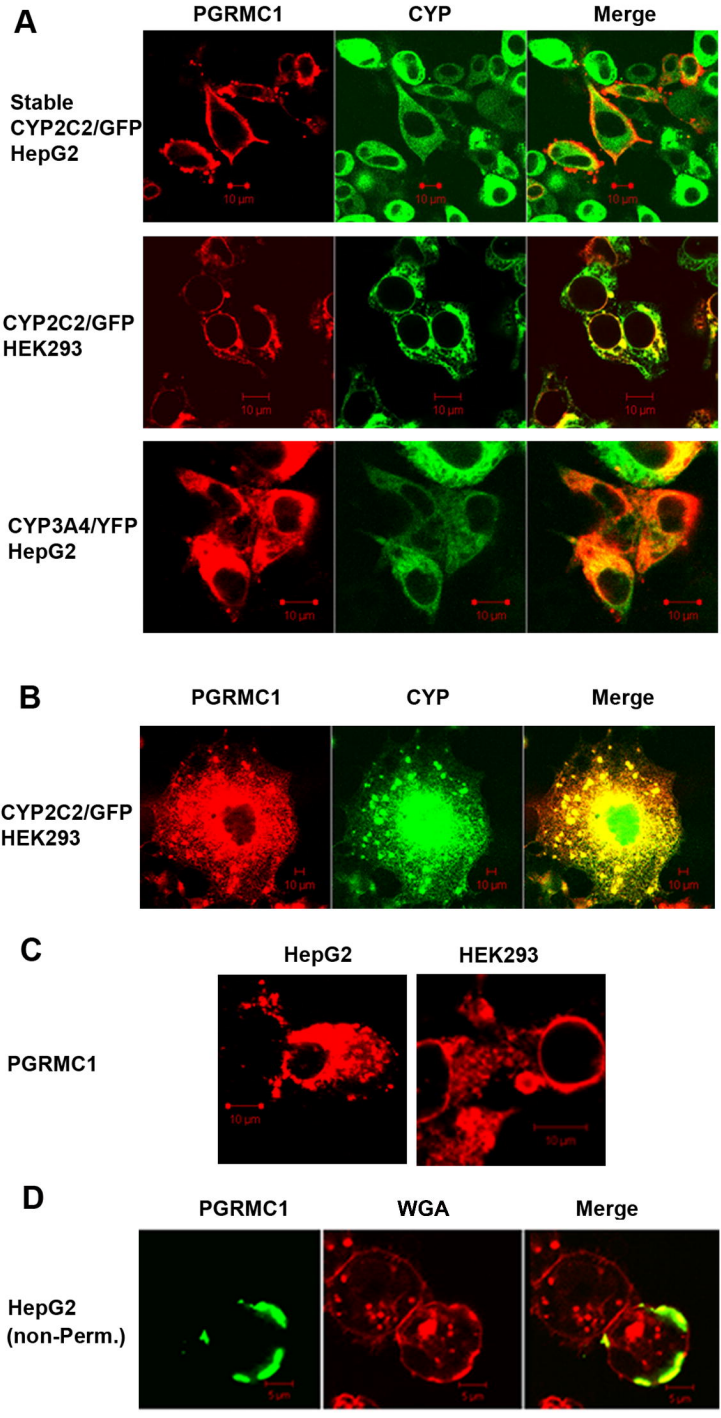

A
PGRMC1
CYP
Merge
Stable CYP2C2/GFP HepG2
CYP2C2/GFP HEK293
CYP3A4/YFP HepG2
10 µm
B
PGRMC1
CYP
Merge
CYP2C2/GFP HEK293
10 µm
C
HepG2
HEK293
PGRMC1
10 µm
D
PGRMC1
WGA
Merge
HepG2 (non-Perm.)
5 µm

Figure 3

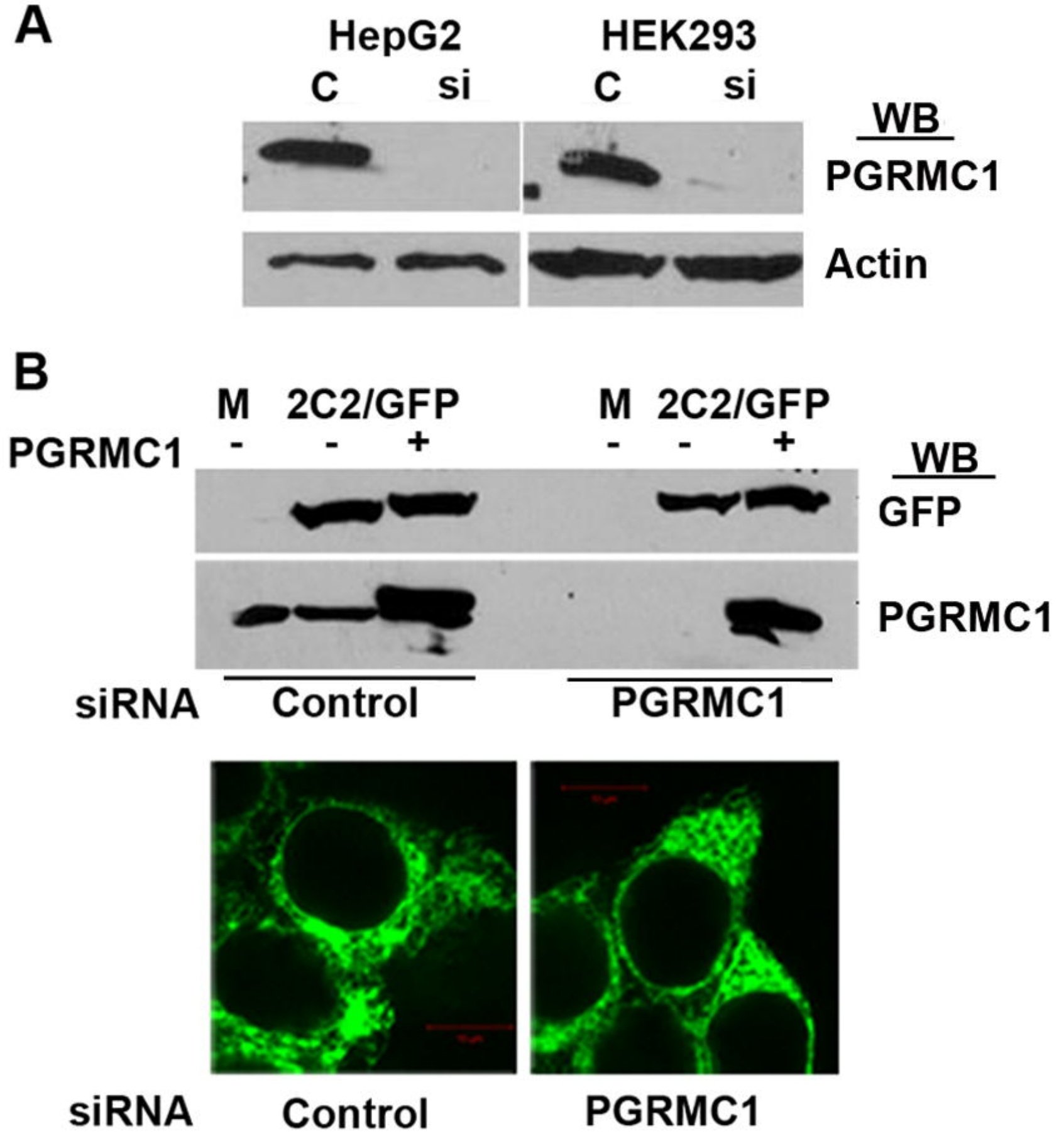

A
HepG2
HEK293
C
si
C
si
WB
PGRMC1
Actin
B
M
2C2/GFP
M
2C2/GFP
PGRMC1
-
-
+
-
-
+
WB
GFP
PGRMC1
siRNA
Control
PGRMC1
siRNA
Control
PGRMC1

Figure 4

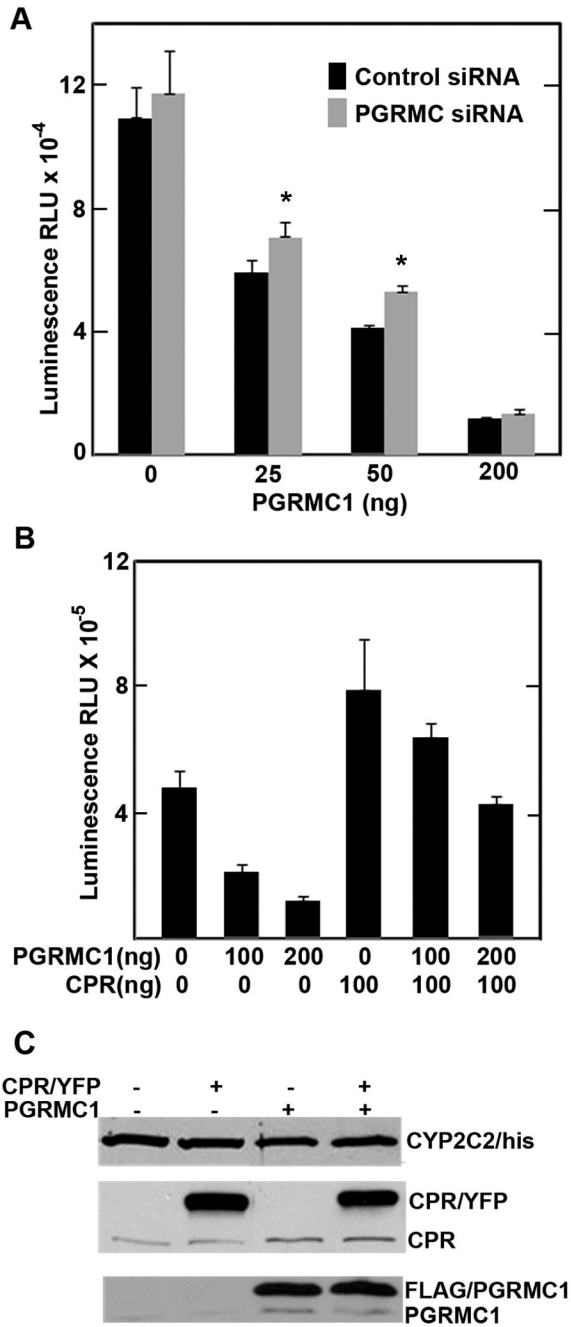

A
Control siRNA
PGRMC siRNA
Luminescence RLU x 10-4
12
8
4
0
*
*
0
25
50
200
PGRMC1 (ng)
B
Luminescence RLU X 10-5
12
8
4
PGRMC1(ng) 0 100 200 0 100 200
CPR(ng) 0 0 0 100 100 100
C
CPR/YFP - + - +
PGRMC1 - - + +
CYP2C2/his
CPR/YFP
CPR
FLAG/PGRMC1
PGRMC1

Figure 5

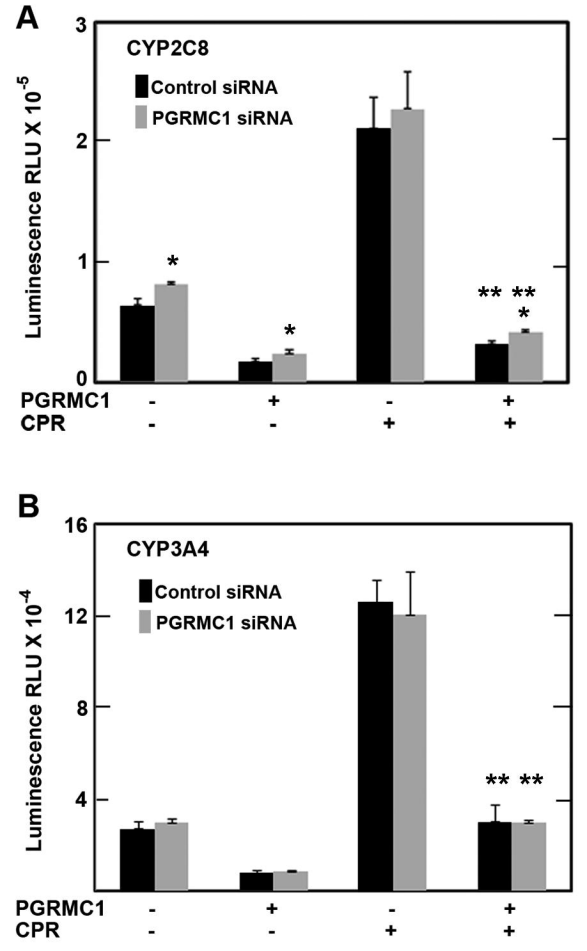

A
CYP2C8
Control siRNA
PGRMC1 siRNA
Luminescence RLU X 10-5
3
2
1
0
*
*
** **
*
PGRMC1 - + - +
CPR - - + +
B
CYP3A4
Control siRNA
PGRMC1 siRNA
Luminescence RLU X 10-4
16
12
8
4
** **
PGRMC1 - + - +
CPR - - + +

Figure 6

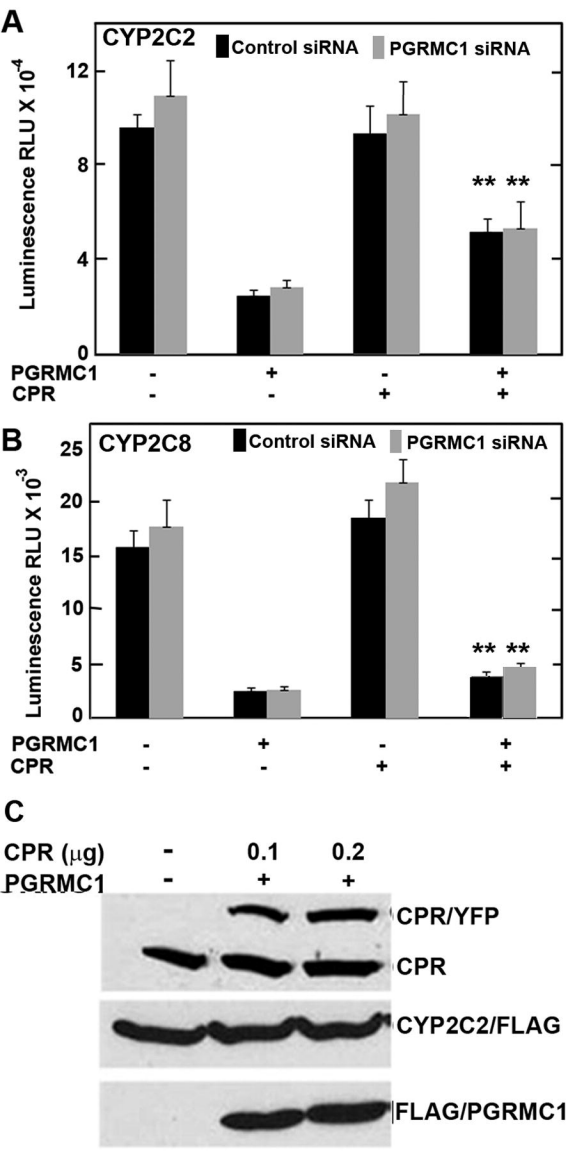

A
CYP2C2
Control siRNA
PGRMC1 siRNA
Luminescence RLU X 10-4
12
8
4
0
** **
PGRMC1 - + - +
CPR - - + +
B
CYP2C8
Control siRNA
PGRMC1 siRNA
Luminescence RLU X 10-3
25
20
15
10
5
0
** **
PGRMC1 - + - +
CPR - - + +
C
CPR (μg) - 0.1 0.2
PGRMC1 - + +
CPR/YFP
CPR
CYP2C2/FLAG
FLAG/PGRMC1

Figure 7

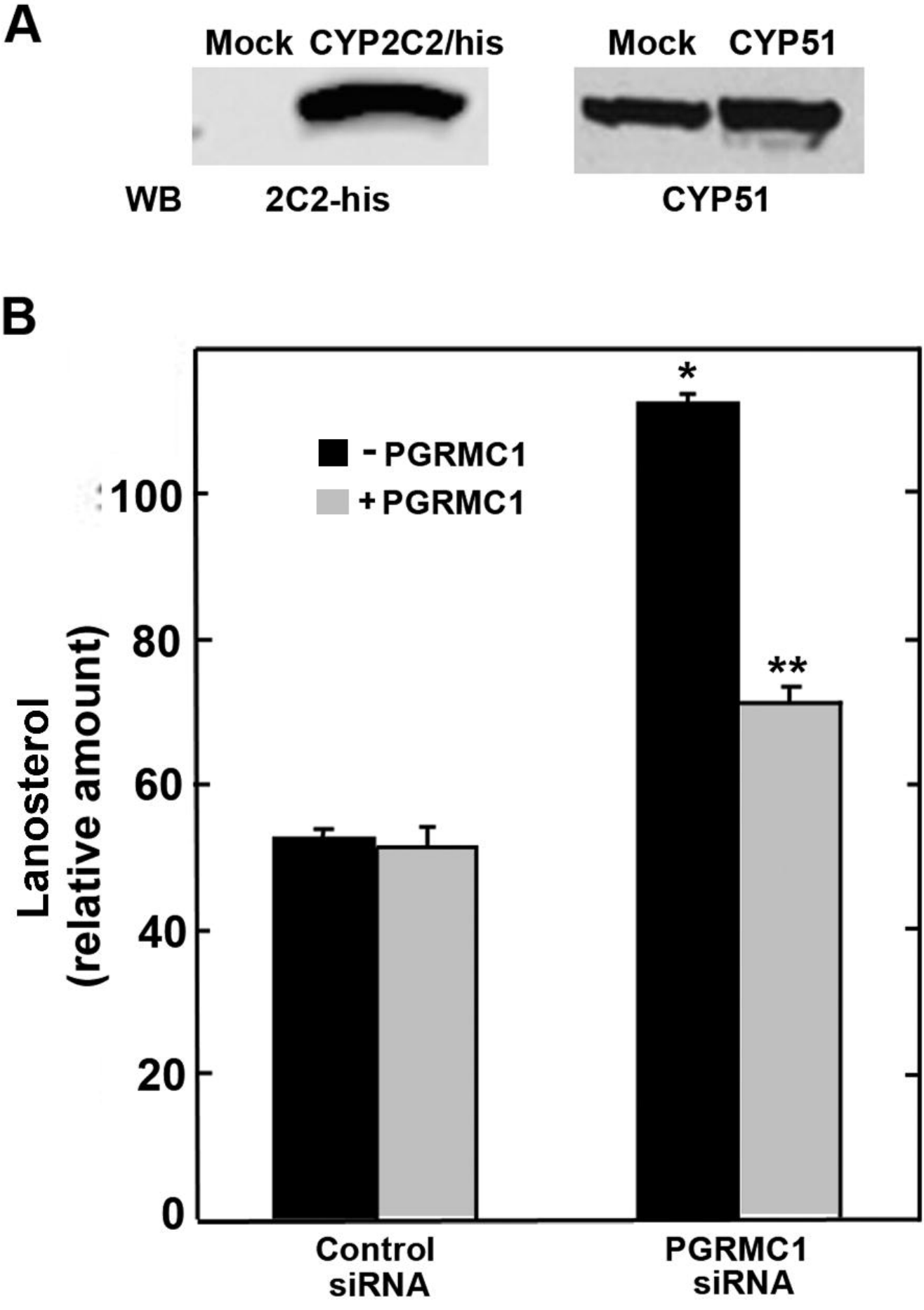

A
Mock
CYP2C2/his
Mock
CYP51
WB
2C2-his
CYP51
B
- PGRMC1
+ PGRMC1
Lanosterol
(relative amount)
100
80
60
40
20
0
*
**
Control
siRNA
PGRMC1
siRNA

Figure 8

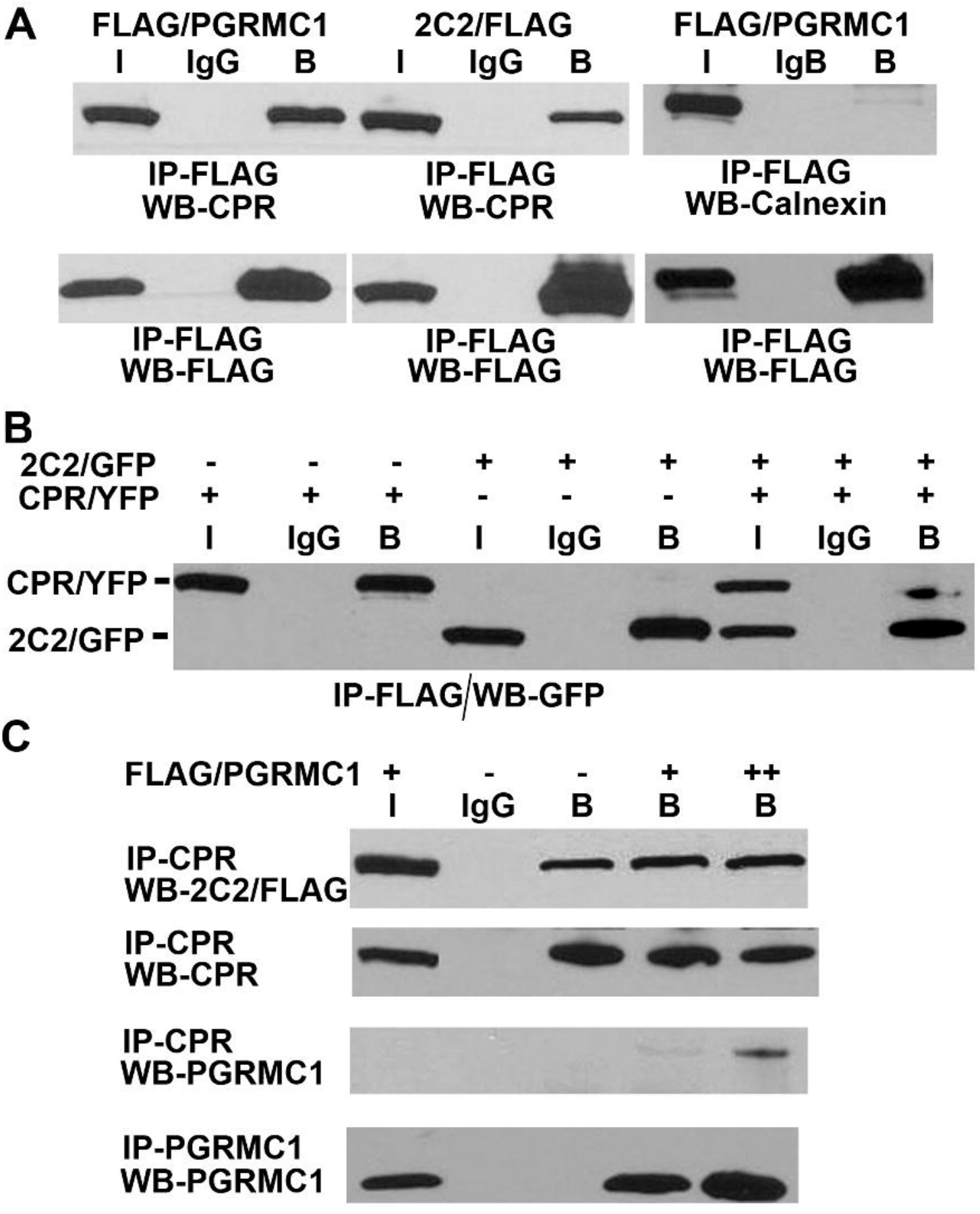

A
FLAG/PGRMC1
I IgG B
2C2/FLAG
I IgG B
FLAG/PGRMC1
I IgB B
IP-FLAG
WB-CPR
IP-FLAG
WB-CPR
IP-FLAG
WB-Calnexin
IP-FLAG
WB-FLAG
IP-FLAG
WB-FLAG
IP-FLAG
WB-FLAG
B
2C2/GFP - - - + + + + + +
CPR/YFP + + + - - - + + +
I IgG B I IgG B I IgG B
CPR/YFP
2C2/GFP
IP-FLAG/WB-GFP
C
FLAG/PGRMC1 + - - + ++
I IgG B B B
IP-CPR
WB-2C2/FLAG
IP-CPR
WB-CPR
IP-CPR
WB-PGRMC1
IP-PGRMC1
WB-PGRMC1

Figure 9

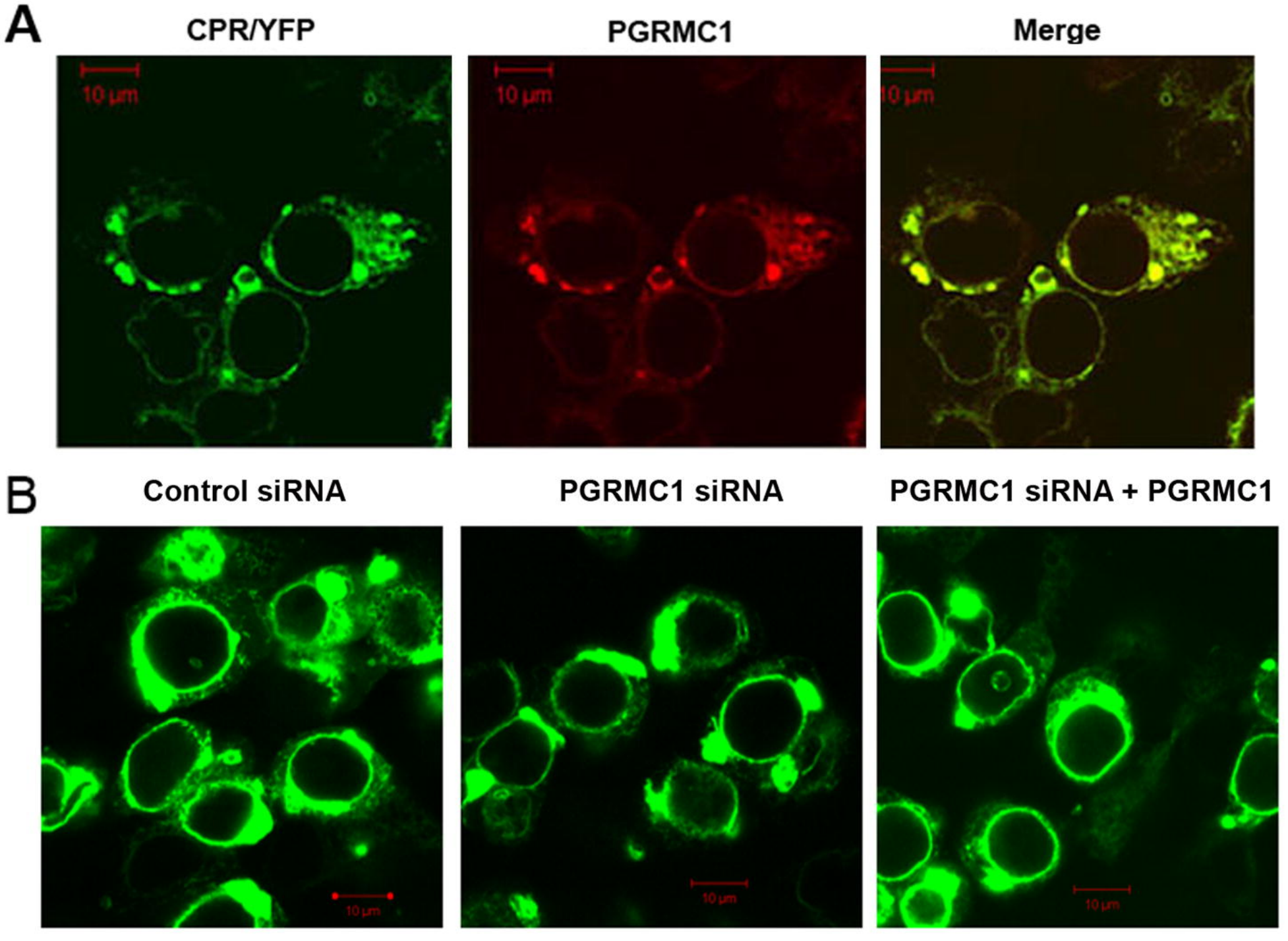
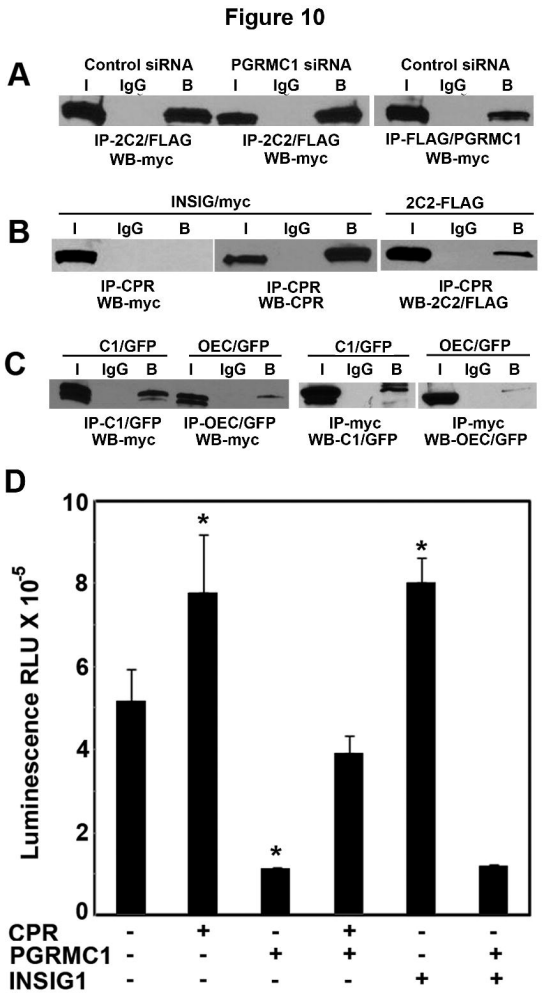

A
CPR/YFP
PGRMC1
Merge
10 µm
B
Control siRNA
PGRMC1 siRNA
PGRMC1 siRNA + PGRMC1
10 µm

Figure 10


A
Control siRNA
I IgG B
IP-2C2/FLAG
WB-myc
PGRMC1 siRNA
I IgG B
IP-2C2/FLAG
WB-myc
Control siRNA
I IgG B
IP-FLAG/PGRMC1
WB-myc
B
INSIG/myc
I IgG B
IP-CPR
WB-myc
I IgG B
IP-CPR
WB-CPR
2C2-FLAG
I IgG B
IP-CPR
WB-2C2/FLAG
C
C1/GFP
I IgG B
IP-C1/GFP
WB-myc
OEC/GFP
I IgG B
IP-OEC/GFP
WB-myc
C1/GFP
I IgG B
IP-myc
WB-C1/GFP
OEC/GFP
I IgG B
IP-myc
WB-OEC/GFP
D
Luminescence RLU X 10-5
0
2
4
6
8
10
*
*
*
CPR - + - + - -
PGRMC1 - - + + - +
INSIG1 - - - - + +