SUMMARY

Cytochrome P450 enzymes are heme-dependent monooxygenases that play a central role in human physiology. Despite the numerous physiological processes that P450 enzymes impact, the electron donors P450 oxidoreductase and cytochrome b5 are the only proteins known to interact with and modulate the activity of ER microsomal P450s. Here, we report that Dap1/PGRMC1 is required for ER P450 function in yeast and humans. We show that S. pombe Dap1 is a hemoprotein that binds and positively regulates Cyp51A1 and Cyp61A1, two P450s required for sterol biosynthesis. Similarly, loss of human PGRMC1 reduces activity of Cyp51A1, blocking cholesterol synthesis and increasing production of toxic sterol intermediates. PGRMC1 stably binds Cyp51A1 and human P450s from three additional families including Cyp3A4, which metabolizes pharmaceutical compounds. These findings demonstrate that PGRMC1 is required for P450 activity and suggest that interindividual variation in PGRMC1 function may impact multiple biochemical pathways and drug metabolism.

INTRODUCTION

Cytochrome P450 enzymes are heme-dependent monooxygenases that participate in the biosynthesis of cholesterol, steroids, bile acids, vitamin D3, and eicosanoids; detoxification of xenobiotics; and metabolism of pharmaceutical drugs (Nebert and Russell, 2002). The human genome codes for 57 cytochrome P450 enzymes, and reduced P450 activity can lead to disease, including congenital adrenal hyperplasia and hypercholesterolemia. Despite the vast array of reactions catalyzed by these enzymes in organisms ranging from bacteria to mammals, the P450 catalytic cycle is remarkably well conserved, requiring one molecule of dioxygen and two electrons from NADPH for the conversion of each substrate molecule to product (Ortiz de Montellano, 2005). Electron donation to eukaryotic ER microsomal P450s occurs primarily through transient interactions with NADPH cytochrome P450 oxidoreductase and, to a lesser extent, cytochrome b5 (Guengerich, 1991; Vermilion et al., 1981; Schenkman and Jansson, 2003). Mammalian mitochondrial P450s interact with the electron-donor proteins adrenodoxin and adrenodoxin reductase (Ortiz de Montellano, 2005). To date, these are the only proteins known to interact with and affect the activity of cytochrome P450s.

The number of P450 enzymes varies widely among species. In contrast to humans and Arabidopsis thaliana, which contain 57 and 246 P450 enzymes, respectively, the fission yeast Schizosaccharomyces pombe has only two P450 enzymes, Cyp51A1/Erg11 and Cyp61A1/Erg5 (Nelson et al., 2004a, 2004b). These ER-localized enzymes from distinct P450 families are both required for ergosterol biosynthesis: Erg11 catalyzes the demethylation of lanosterol, and Erg5 carries out the reduction of ergosta-5,7,24(28)-trienol (see Figure S1 in the Supplemental Data available with this article online) (Lees et al., 1999). Our recent studies revealed that both erg11+ and erg5+ are transcriptionally regulated by the fission yeast sterol regulatory element-binding protein (SREBP), called Sre1, in response to a decrease in oxygen concentration (Hughes et al., 2005; Todd et al., 2006). SREBP is a membrane-bound transcription factor that regulates expression of cholesterol biosynthetic genes and controls lipid homeostasis in mammalian cells (Espenshade, 2006; Goldstein et al., 2006). Importantly, fission yeast Sre1 also controls transcription of cytochrome b5 and cytochrome b5 reductase, raising the possibility that uncharacterized Sre1 target genes may be required for P450 function.

One Sre1 target gene that emerged as potentially having a role in P450 function was the S. pombe homolog of mammalian PGRMC1 and S. cerevisiae DAP1 (Falkenstein et al., 1996; Hand et al., 2003). Human PGRMC1 codes for a 22 kDa ER-membrane protein that contains a N-terminal transmembrane segment and a C-terminal cytochrome b5-like domain that binds pentacoordinate heme (Mifsud and Bateman, 2002; Min et al., 2005; Ghosh...
et al., 2005). Previous studies demonstrated that overexpression of PGRMC1 in cultured cells increases hydroxyl-
ation of progesterone by the cytochrome P450 Cyp21, and monoclonal antibody to PGRMC1 inhibits this activity in rat adrenal microsomes (Laird et al., 1988; Min et al., 2005). In addition, S. cerevisiae DAP1 is required for normal Erg11 protein levels and ergosterol synthesis in yeast (Hand et al., 2003; Mallory et al., 2005). These data suggest a role for this family of heme-binding proteins in regulation of cytochrome P450 enzymes in eukaryotes.

Here, we present evidence demonstrating a direct role for Dap1/PGRMC1 in cytochrome P450 function. Fission yeast Dap1 is a hemoprotein that binds and positively regulates Erg11/Cyp51A1 and Erg5/Cyp61A1, two P450s required for sterol biosynthesis. Importantly, Dap1 function is conserved in humans. RNAi-mediated knockdown of human PGRMC1 reduces activity of Cyp51A1, blocking cholesterol synthesis and increasing production of toxic sterol intermediates. PGRMC1 stably binds Cyp51A1 and human P450s from three additional families, suggesting that Dap1/PGRMC1 may function as a general regulator of cytochrome P450s. The presence of PGRMC1 homologs in plants, flies, and worms suggests that this regulation of cytochrome P450 enzymes may be broadly conserved among eukaryotes.

RESULTS AND DISCUSSION

Our previous microarray analysis revealed that fission yeast dap1Δ (SPAC25B8.01) was coordinately regulated with P450 enzymes by Sre1 in response to low oxygen (Todd et al., 2006). To confirm these results, we examined expression of dap1Δ in wild-type and sre1Δ cells grown in the presence or absence of oxygen. Expression of dap1Δ mRNA was induced in the absence of oxygen in a Sre1-dependent manner (Figure 1A), consistent with the regulation of other Sre1 target genes such as sre1+. HMG-CoA synthase (hcs1+) served as a loading control.

To examine whether dap1Δ is required for ergosterol synthesis in fission yeast, we generated a strain lacking dap1Δ by homologous recombination and analyzed sterols from wild-type and dap1Δ cells using gas chromatography and GC-MS. As observed in S. cerevisiae, dap1Δ cells showed defects in sterol synthesis (Mallory et al., 2005). dap1Δ cells were viable under normal growth conditions but contained a reduced amount of ergosterol and elevated amounts of the ergosterol biosynthetic intermediates 24-methylene lanosterol, ergosta-5,7,24(28)-trienol, and ergosta-5,7-dienol, consistent with defects at the Erg11 and Erg5 enzymatic steps (Figure 1B). Ergosta-5,7-dienol is not a normal pathway intermediate, but...
forms when Erg5 is inhibited (Figure S1) (Skaggs et al., 1996). To determine whether the metabolic flux through these enzymatic steps is reduced as a direct result of Dap1 absence, we generated nmt-dap1+, a yeast strain expressing dap1+ under control of the thiamine-repressible nmt1+ promoter. Addition of thiamine to nmt-dap1+ cells resulted in a time-dependent loss of Dap1 protein (Figure 1C, lower panel). Sterol analysis at different times after thiamine addition revealed that, upon loss of Dap1, cells accumulated 24-methylene lanosterol, ergosta-5,7,24(28)-trienol, and ergosta-5,7-dienol, substrates for Erg11 and Erg5. Consistent with this role in sterol synthesis, dap1D cells are sensitive to the inhibitors of sterol synthesis itraconazole and CoCl2 (Figure 1D). These data demonstrate that Dap1 is required in vivo for the activity of Erg11 and Erg5, the entire complement of cytochrome P450 enzymes in fission yeast. The low sequence identity (23%) between these two enzymes from different P450 families suggests that Dap1 may be a general factor required for P450 enzymes.

To determine whether Dap1 interacts directly with P450s or other sterol biosynthetic enzymes, we purified Dap1 fused to the tandem affinity purification tag, Dap1-TAP. Yeast cells expressing dap1-TAP from the endogenous promoter were lysed in detergent, and Dap1-TAP was purified by affinity chromatography. Dap1-TAP protein complexes in the final eluate were subjected to sucrose velocity gradient centrifugation, and gradient fractions were analyzed by SDS-PAGE and silver staining (Figure 2A). Proteins in fractions 12–14 from a parallel gradient were pooled and analyzed by mass spectrometry. Proteins that copurified with Dap1-TAP are indicated. * indicates a truncated form of Erg5. Arrowhead denotes keratin contamination.

(B) Detergent-solubilized extracts from dap1-HA and dap1-HA erg11-Myc yeast were subjected to immunoprecipitation with monoclonal anti-Myc IgG 9E10. Input (I), unbound (U), and bound (B) fractions were subjected to immunoblot analysis with polyclonal anti-Myc IgG, anti-HA-HRP, or anti-Sre1 IgG. Bound fractions are 10-fold overloaded as compared to input and unbound fractions. Sre1 represents the ER-bound precursor form of the protein.

(C) Detergent-solubilized extracts from dap1-HA and dap1-HA erg5-TAP yeast were subjected to purification with IgG-Sepharose. Input (I), unbound (U), and bound (B) fractions were subjected to immunoblot analysis with rabbit IgG, anti-HA-HRP, or anti-Sre1 IgG.

Dap1 homologs from other organisms are pentacoordinate heme-binding proteins predicted to utilize a conserved tyrosine residue in their C terminus for heme binding (Ghosh et al., 2005; Mallory et al., 2005; Min et al., 2005). To test whether fission yeast Dap1 is a heme-binding protein, we purified soluble recombinant ΔTM-Dap1 lacking the N-terminal transmembrane segment. In parallel, we purified ΔTM-Dap1 Y138F that was predicted not to bind heme.
to bind heme. While both proteins expressed well, in-gel peroxidase assays revealed that Dap1, but not Dap1 Y138F, bound heme (Figure 3A). To determine whether heme binding is required for Dap1 function, we expressed HA-dap1 and HA-dap1 Y138F on a plasmid from the constitutive CaMV promoter in dap1Δ cells and examined steady-state sterol levels in these cells. Both wild-type and Y138F mutant Dap1 were expressed at equal levels in yeast (Figure S2). The strain harboring the wild-type HA-dap1 plasmid showed wild-type sterol levels as anticipated. However, similar to dap1Δ, yeast carrying the HA-dap1 Y138F plasmid accumulated 24-methylene lanosterol, ergosta-5,7,24(28)-tri-enol, and ergosta-5,7-dienol, reflecting defects in Erg11 and Erg5 (Figure 3B). These data indicate that dap1 Y138F is a loss-of-function mutation and that Dap1 function requires bound heme.

The human genome contains two homologs of fission yeast dap1+, called progesterone receptor membrane component 1 and 2 (PGRMC1 and PGRMC2). To date, only PGRMC1 has been characterized (Cradden et al., 2006; Gerdes et al., 1998; Ghosh et al., 2005; Raza et al., 2001; Min et al., 2005). Tissue expression profiles of human PGRMC1 revealed that it was broadly expressed, with high levels present in liver and adrenal gland, two sites of abundant cytochrome P450 activity (Figure S3). To determine whether PGRMC1 is required for cholesterol synthesis in mammals, we used retroviral infection to generate human HEK293 cells that stably expressed RNA hairpins designed to knock down expression of PGRMC1 mRNA. Analysis of mRNA and protein levels revealed that the cell line PGR1-1, but not PGR1-2, had reduced expression of PGRMC1 mRNA and protein (Figure 4A). We performed sterol analysis on HEK293, PGR1-1, and PGR1-2 cells grown in the presence of increasing concentrations of mevalonate to maximize flux through the cholesterol pathway. HEK293 and PGR1-2 cells accumulated the intermediate lanosterol when supplemented with mevalonate (Figure 4B). This level of lanosterol did not affect cell growth after 24 hr (Figure 4B, lower panel). Interestingly, PGR1-1 cells accumulated lanosterol at levels 3-fold higher than control cells. Elevated lanosterol is toxic to cells, and accumulation of lanosterol correlated with the death of PGR1-1 cells at concentrations of mevalonate ≥30 mM. No other significant differences in cellular sterols were observed among the cell lines, suggesting that PGRMC1 function was required specifically for the demethylation of lanosterol by the Erg11 human homolog, Cyp51A1. Unlike ergosterol synthesis, cholesterol synthesis only requires one cytochrome P450, Cyp51A1, and thus no homolog of Erg5 exists in mammals. Microsomes from HEK293, PGR1-1, and PGR1-2 cells treated with 0 or 40 mM mevalonate for 10 hr contained equal amounts of Cyp51A1, suggesting that PGRMC1 affected Cyp51A1 activity and not protein stability as reported for S. cerevisiae Dap1p (Figure S4) (Mallory et al., 2005).

To test whether PGRMC1 binds to Cyp51A1 in mammals as in yeast, we coexpressed FLAG-PGRMC1 and Cyp51-6xMyc by transient transfection in HEK293 cells. PGRMC1 was quantitatively recovered in coimmunoprecipitations from these cells and the majority of Cyp51A1 copurified (Figure 4C). In a parallel experiment, FLAG-PGRMC1 did not bind Site-1 protease, a membrane protein required for activation of mammalian SREBP, demonstrating the specificity of PGRMC1 for Cyp51A1 (Espenshade et al., 1999). Dap1 bound to both P450 enzymes in fission yeast, yet the amino acid sequence identity between Erg11 and Erg5 is no greater than that between Erg11 and other mammalian P450 enzymes. Consequently, we hypothesized that PGRMC1 may bind to a broad spectrum of P450 enzymes. To test this hypothesis, we assayed binding of FLAG-PGRMC1 to three functionally diverse human P450 enzymes: Cyp3A4, required for xenobiotic metabolism and clearance of ~50% of all known drugs (Rendic, 2002); Cyp7A1, the rate-limiting enzyme in bile acid synthesis (Russell, 2003); and Cyp21A2, a progesterone 21-hydroxylase required for production of glucocorticoids and mineralocorticoids (Ortiz de Montellano, 2005). PGRMC1 bound efficiently to all three proteins (Figure 4D). Collectively, these data demonstrate that PGRMC1 is required for Cyp51A1 activity in cholesterol synthesis and that PGRMC1 may function as a common regulator of cytochrome P450 enzymes in mammals.
Our studies demonstrate that Dap1/PGRMC1 is a conserved hemoprotein required for the activity of cytochrome P450 enzymes in eukaryotic sterol synthesis. Several lines of evidence suggest that Dap1/PGRMC1 has a broad function in cytochrome P450 biology: (1) Dap1 is required for the activity of two P450 enzymes from different families, Erg11(Cyp51) and Erg5(Cyp61); (2) Dap1/PGRMC1 binds to P450 enzymes from five different families; and (3) PGRMC1 has been shown to affect Cyp21 activity in vivo and in vitro (Laird et al., 1988; Min et al., 2005).

Known P450-interacting proteins, NADPH cytochrome P450 oxidoreductase and cytochrome b5, supply electrons to cytochrome P450 through transient interactions and are present in catalytic amounts (Estabrook et al., 1971). In contrast, Dap1/PGRMC1 forms a stable, stoichiometric complex with P450 enzymes. Unlike the hexacoordinate heme protein cytochrome b5, Dap1 function requires binding of pentacoordinate heme. This property suggests that Dap1 does not donate electrons to the P450 enzyme. Previous studies hypothesized that S. cerevisiae Dap1p may act as a heme chaperone and donate heme to newly synthesized Erg11p because deletion of DAP1 reduced Erg11 protein levels (Ghosh et al., 2005; Mallory et al., 2005). However, our data favor a model in which Dap1/PGRMC1 is a stable component of a cytochrome P450 enzyme complex and Dap1/PGRMC1 performs an unrecognized, heme-dependent function in the P450 catalytic cycle. Further studies will be required to define both the mechanism and the structural basis of PGRMC1 function. Mutations in cytochrome P450 enzymes cause multiple human diseases, including congenital adrenal hyperplasia (Cyp21A2) and hypercholesterolemia (Cyp7A1) (Nebert and Russell, 2002). Our findings suggest that mutations in PGRMC1 may lead to more subtle forms of these diseases. In addition, identification of a regulator of P450 enzymes has important implications for drug metabolism research, as polymorphisms in PGRMC1 may contribute to pharmacogenetic variation observed between individuals, and we are actively investigating this possibility (Evans and Relling, 1999).

**EXPERIMENTAL PROCEDURES**

Yeast cell culture, immunoblot analysis, northern analysis, and sterol analysis were performed as previously described with minor modifications (Hughes et al., 2005). A complete description of materials, mammalian cell culture, recombinant protein purification, and other methods is available in the Supplemental Experimental Procedures.

**Dap1-TAP Purification and Sucrose Gradient Fractionation**

The Dap1-TAP purification was conducted as described previously, except that digitonin was substituted for NP-40 in the lysis, wash, and elution buffers (Rigaut et al., 1999). The eluate was subjected to sucrose gradient fractionation (15%–40% sucrose w/v) to separate Dap1-TAP protein complexes from other nonspecific interacting proteins. Fractions from duplicate gradients were TCA precipitated and analyzed by SDS-PAGE or mass spectrometry (Sanders et al., 2002). Proteins identified in Dap1-TAP protein complexes...
were present in the Dap1p-TAP total eluate but absent in a duplicate wild-type, untagged yeast eluate. For communoprecipitations, yeast or transfected mammalian cells were lysed in TAP lysis buffer plus protease inhibitors. Cleared lysates were incubated with antibody and IgG-Sepharose overnight at 4°C and washed with TAP lysis buffer containing 0.1% (w/v) digitonin, and bound protein was subjected to immunoblot analysis.

Retroviral RNAi Production

Retroviruses containing shRNAs homologous to regions in the 3′UTR of human PGRMC1 mRNA (GenBank accession number NM_006667) were produced according to manufacturer’s instructions (Open Biosystems). Briefly, LinX cells seeded at 3 × 10^6 cells per 10 cm dish on day 0 were transfected on day 1 with 10 μg of either pSM2 Expression Arrest clone v2HS_90636 or v2HS_90640. On day 2, cells were refed with 8 ml of fresh HEK293 medium. On day 4, the virus-containing supernatant was harvested by filtering and stored at −80°C until further use.

Generation of PGRMC1 RNAi Knockdown Cell Line

On day 0, HEK293 cells were seeded at 5 × 10^5 cells per 6 cm dish in 2 ml HEK293 medium. On day 1, cells were infected with 100 μl of supernatant containing retrovirus expressing either pGRMC1 shRNA clone v2HS_90636 or v2HS_90640 along with a puromycin-resistance marker. On day 2, cells were refed with HEK293 medium supplemented with 3 μg/ml puromycin for selection of cells stably expressing the indicated shRNA. Cells were refed every 3–4 days until only single clones remained. For each shRNA construct, the clones were pooled and expanded to establish two mixed population cell lines, designated PGR1-2 and PGR1-1. PGR1-1 has reduced PGRMC1 RNA and protein levels and expresses shRNA clone v2HS_90636. PGR1-2, which expresses shRNA clone v2HS_90640, does not have reduced PGRMC1 levels and serves as the negative control RNAi cell line. Both cell lines were maintained in HEK293 medium supplemented with 3 μg/ml puromycin.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/2/143/DC1/.

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