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A Novel Role for Progesterone Receptor Membrane Component 1 (PGRMC1): A Partner and Regulator of Ferrochelatase

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Abstract

Heme is an iron containing cofactor essential for multiple cellular processes and fundamental activities such as oxygen transport. To better understand the means by which heme synthesis is regulated during erythropoiesis, affinity purification coupled with mass spectrometry (MS) was carried out to identify putative protein partners interacting with ferrochelatase (FECH), the terminal enzyme in the heme biosynthetic pathway. Both Progesterone Receptor Membrane Component 1 (PGRMC1) and Progesterone Receptor Membrane Component 2 (PGRMC2) were identified in these experiments. These interactions were validated by reciprocal affinity purification followed by MS analysis and immunoblotting. The interaction between PGRMC1 and FECH was confirmed in vitro and in HEK293T cells, a non-erythroid cell line. When cells that are recognized models for erythroid differentiation were treated with a small molecule inhibitor of PGRMC1, AG-205, there was an observed decrease in hemoglobinization relative to untreated cells. In vitro heme transfer experiments showed that purified PGRMC1 was able to donate heme to apo-cytochrome b_5. In the presence of PGRMC1 in vitro measured FECH activity decreased in a dose dependent manner. Interactions between FECH and PGRMC1 were strongest for the conformation of FECH associated with product release suggesting that PGRMC1 may regulate FECH activity by controlling heme release. Overall, the data illustrate a role for PGRMC1 in regulating heme synthesis via interactions with FECH and suggest that PGRMC1 may be a heme chaperone or sensor.

Graphical Abstract

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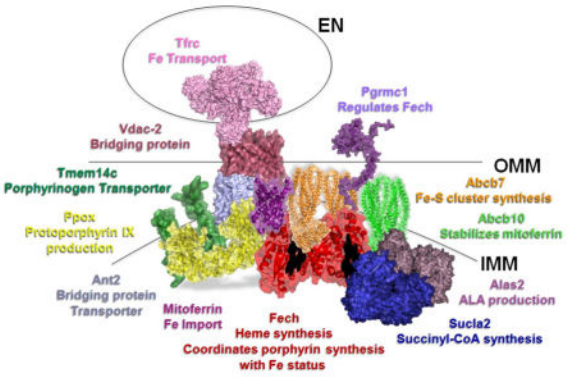
Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. Additional figures and table including additional mitochondrial heme synthesis complex proteins recovered by affinity purification experiments with PGRMC1 and PGRMC2 (Table S1), high stringency wash affinity purification results (Table S2), confocal microscopy experiment images (Figure S1), AG-205 treatment of K562 cells (Figure S2), and heme transfer replicate gels (Figures S3). This material is available free of charge via the Internet at http://pubs.acs.org.



Keywords

Heme; iron; iron metabolism; heme biosynthesis; ferrochelatase; erythropoiesis; porphyrins; protein-protein interaction; progesterone receptor membrane component 1; progesterone membrane component receptor 2

Heme is an essential cofactor in metazoa for many important cellular processes. The ability of heme to bind to small molecules such as gases (O_2 and CO) and to participate in redox reactions are some of its more commonly known roles. However, in the past decade additional essential roles for heme as a regulator of processes including the circadian rhythm[1], microRNA processing[2], protein degradation[3], the cell cycle[4], and ion transport[5–7] have been identified. Hemoproteins are involved in a variety of cellular reactions and are distributed throughout the cell in multiple cellular compartments. For example, the respiratory cytochromes are found in the inner mitochondrial membrane, some cytochrome P_{450}s and cytochrome b_5 are associated with the endoplasmic reticulum (ER) membrane, catalases are localized to the peroxisome and peroxidases to the lysosomes, and heme-binding transcription factors are found in the nucleus[8]. However, despite the biological necessity for heme as a cofactor, heme in its free state is cytotoxic since it can generate harmful free radicals in the presence of oxygen[9, 10].

Heme is synthesized in metazoa by a pathway composed of eight enzymes which starts and ends in the mitochondrial matrix. In erythroid cells, studies show that several components of the heme synthesis pathway exist in a complex to provide feedback regulation on the process of heme synthesis[11]. These components include aminolevulinic acid synthase-2 (ALAS-2), protoporphyrinogen, oxidase (PPOX) and ferrochelatase (FECH). This mitochondrial heme metabolon physically links the first step catalyzed by ALAS-2 with the terminal step catalyzed by FECH, supporting previous studies that showed both enzymes to be important regulatory points in heme synthesis[12–14]. Both ALAS-2 and FECH are regulated at different levels by cellular iron[15–17]. In addition, other proteins involved in mitochondrial cellular iron metabolism were also found in the heme metabolon suggesting an additional level of coordination for heme and iron availability for optimal heme production during erythropoiesis[11].

The convergence of iron and porphyrin metabolism occurs at the terminal step of heme synthesis, which is catalyzed by FECH. FECH inserts ferrous iron into protoporphyrin IX to form protoheme and two protons. Structural studies of human FECH have shown it to be a conformationally dynamic enzyme that undergoes several changes in its structure over the course of the catalytic cycle[18–20]. These conformational changes have been proposed to allow interaction with different protein partners facilitating substrate delivery and product release. Besides its use of iron as a substrate, FECH from animals and some lower organisms possesses a [2Fe-2S] cluster that has been shown to be required for enzyme activity[16] and important in regulating enzyme activity[21]. Release of heme from FECH is the rate-limiting step in the reaction[22] and likely controlled in vivo by interactions with transporters or chaperones.

The protein Progesterone Receptor Membrane Component 1 (PGRMC1) has been proposed to be a heme chaperone[23,24]. PGRMC1 is a 25 kDa protein which belongs to the Membrane-Associated Progesterone Receptor (MAPR) family. The MAPR family is a subset of the cytochrome b_5 family consisting in mice and humans of PGRMC1 and Progesterone Receptor Membrane Component 2 (PGRMC2), as well as the more recently discovered proteins Neudesin and Neuferrin[25]. Of the MAPR proteins, PGRMC1 and PGRMC2 show the closest homology. Neudesin and Neuferrin are secreted proteins that bind heme and are thought to possess neurotrophic and neurogenic activity[26, 27]. Yeast have only a single MAPR family member, DAP1, which has been studied in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The precise cellular roles of PGRMC1 and DAP1 are not clear although it has been implicated in a variety of cellular functions including stimulation of P_{450}s[28, 29] and cholesterol metabolism[30, 31], autophagy[32], endocytosis[33], intracellular signal transduction[34], and iron metabolism[35, 36]. Additionally, PGRMC1 has been reported to play a role in DNA damage protection and response to oxidative stress[33], likely through an iron dependent process[37]. While PGRMC1 has been shown to bind progesterone[24], its involvement in progesterone signaling remains uncertain. However, it has clearly been demonstrated that PGRMC1 binds heme[23, 24, 38] though not with the avidity of a typical stable hemoprotein such as myoglobin or cytochrome b_5.

The PGRMC1 protein consists of an N-terminal region that is proposed to be a transmembrane domain and C-terminal region that contains a cytochrome b_5-like motif heme binding domain[33, 39]. In most cytochrome b_5-like proteins the heme cofactor is coordinated via two histidine residues[40, 41]. However, spectroscopic and mutagenesis studies are consistent with PGRMC1 binding heme via tyrosine ligands rather than histidine[23, 24, 42]. This coordination motif is a feature shared with purported heme transporters such as ShuT[43] and PhuT[44] as well as the heme binding protein HasA[45, 46].

Herein we identify and characterize an interaction between FECH and PGRMC1 as well as PGRMC2. We identify the cellular and subcellular localization of PGRMC1 in murine erythroleukemia cells (MEL) and show that the small molecule inhibitor of PGRMC1, AG-205, decreases heme synthesis in differentiated MEL cells in a dose dependent manner. We show that PGRMC1 interaction with FECH is dependent on the molecular conformation of FECH and that PGRMC1 decreases FECH activity in in vitro assays. These findings along with previous reports suggest PGRMC1 may play a role in regulating heme synthesis.

MATERIALS AND METHODS

Vectors, Cell Lines and Reagents

Human *PGRMC1* and *PGRMC2* were cloned from bacterial expression vectors (gift of Dr. Peter Espenshade) into pEF1alpha FLAG biotag vector (gift of Alan Cantor)[47]. To produce N-terminal FLAG tagged proteins, cloned cDNA encoding full length *PGRMC1* and *PGRMC2* were amplified and cloned into pEF1alpha using the XmaI and BamHI and XmaI and XbaI sites, respectively. An N-terminal FLAG tagged human *FECH* expression vector was produced as previously described[11].

Cell lines utilized for tissue culture experiments were DS19 murine erythroleukemia (MEL) cells[48, 49], human embryonic kidney 293T (HEK 293T) cells (ATCC – CRL3216) and K-562 human myelogenous leukemia (K562) cells (ATCC – CCL243)[50, 51]. To create DS19 MEL and HEK 293T cell lines expressing human FLAG tagged *FECH, PGRMC1* and *PGRMC2*, cells were transfected with expression vectors by electroporation and stably expressing cell lines were selected for puromycin resistance. 5 μg/mL of puromycin (Cellgro, Manassas, VA) was included in media for the selection step. Expression of tagged proteins was confirmed by immunoblot analysis using anti-FLAG antibody (Sigma, St. Louis, MO). All mammalian cells were cultured in DMEM with 25 mM glucose, 1 mM sodium pyruvate and 4 mM glutamine (Cellgro) plus 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Cellgro). For induction of MEL cells 1.5% dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) was included in the growth media and cells were grown for 72 hours for maximal expression of heme biosynthetic enzymes[52]. For addition to MEL cells, hemin (Sigma) was prepared in DMSO and added at a final concentration of 12.5 μM. For induction of K562 cells 1 mM sodium butyrate was included in growth media and cells were grown 6 days[53, 54].

For recombinant production in *Escherichia coli*, wild-type and variant *FECH* were expressed and purified as previously described[55]. Full length human *PGRMC1* was cloned into pTrcHisA (Life Technologies, Grand Island, NY) using the NheI and HindIII sites for production of N-terminal his-tagged proteins. For the non-tagged form *PGRMC1* was cloned with NcoI and HindIII. Wild-type *PGRMC1* was expressed and purified as previously described for human FECH[55].

The PGRMC1 inhibitor AG-205[56, 57](Sigma) was prepared as a 1 mM stock in DMSO.

Affinity Purification and Mass Spectrometry

Affinity purification and MS experiments were carried out in MEL cell lines stably expressing FLAG tagged *FECH, PGRMC1*, and *PGRMC2*. In addition, affinity purification of HEK 293T cells expressing FLAG tagged *FECH* followed by immunoblots was carried out as previously described[11, 58]. For PGRMC1 and PGRMC2, which both form homodimers or homomultimers, we found an equivalent amount of tagged exogenous and endogenous protein with the average ratio being 2.1+0.1 and 1.1+0.1, respectively. This suggests that in the differentiated state there were comparable amounts of the tagged exogenous and the endogenous protein orthologues. From all experiments an average of ~300 proteins were observed with normalized spectral abundance factor values over that of

the control experiments. Pull downs using FLAG tagged PGRMC1 and PGRMC2 resulted in a large number of identified mitochondrial proteins in the recovered pool. From the MS results the criteria used to confirm interactions were based on the number of spectral counts, the number of unique peptides recovered and the percent sequence coverage which occurred over that of the background in two biological replicates and in reciprocal pull down experiments in two biological replicates. The protein interactions presented here have been submitted to the IMEx (http://www.imexconsortium.org) consortium through IntAct[59] and assigned the identifier IM-25485.

Additional experiments with higher stringency washes, specifically 1% Nonidet P-40 in the wash buffer, and harsher elution from the agarose using 6M urea were carried out. Eluted protein samples were then subjected to tryptic digestion and shotgun proteomics performed on a Thermo Fisher Orbitrap XL (Thermo Fisher Scientific, Grand Island, NY) according to a previously described protocol[60]. Data were searched in Proteome Discoverer 1.4 using Sequest HT (Thermo Fisher Scientific) with the percolator node set at a 1% peptide false-discovery rate.

In vitro Interaction Experiments

Expression of his-tagged wild-type FECH, FECH variants, and non-tagged PGRMC1 in *E. coli* was carried out by growth in Circlegrow media (MP Biomedicals, Santa Ana, CA) for 18–20 hours at 30°C. Cells were harvested by centrifugation at 5,000 x g for 10 min, resuspended in solubilization buffer (50 mM Tris-MOPS, pH 8.0, 100 mM KCl, 1% sodium cholate) and sonicated three times on ice for 30 seconds. The resulting lysate was then centrifuged at 100,000xg for 20 minutes and the supernatant reserved. Supernatant from non-tagged PGRMC1 was then mixed with the his-tagged wild-type and variant FECH supernatant and loaded onto HisPur Cobalt Resin (Thermo Fisher Scientific). The column was then washed with wash buffer (50 mM Tris-MOPS pH 8.1, 100 mM KCl, 1% sodium cholate, 15 mM imidazole) and subsequently eluted using elution buffer (50 mM Tris-MOPS pH 8.1, 100 mM KCl, 1% sodium cholate, 250 mM imidazole). Presence of PGRMC1 and relative amounts of PGRMC1 and FECH, both wild-type and variants, were analyzed by SDS-PAGE and immunoblots.

Transcript analysis from *In vitro* erythroid expansion of peripheral blood CD34$^+$ mononuclear cells

In vitro erythroid expansion of peripheral blood CD34$^+$ mononuclear cells and gene expression analysis at multiple days of differentiation was carried as previously described[61]. Probes used were Hs00998344_m1 for *PGRMC1* and Hs01128672_m1 for *PGRMC2*, (Applied Biosystems, Foster City, CA). Levels of mRNA were normalized to an endogenous control human GUSB (beta glucuronidase) (Applied Biosystems) and gene expression is expressed in arbitrary units.

Immunoblots

For immunoblots, eluted protein from affinity purification, column chromatography and cellular lysates was separated on Mini-PROTEAN TGX Stain-Free gels (BioRad, Hercules, CA) and then transferred by Transblot semi-dry blotting (BioRad). Antibodies used included

Anti-FECH (generated in house by H.A.D. at U.G.A.) at a dilution of 1:50,000–100,000, Anti-PGRMC1 (Sigma) at a dilution of 1:2,000, Anti-PGRMC2 (Sigma) at a dilution of 1:500, Anti-cytochrome c (BD Biosciences, San Jose, CA) at a dilution of 1:50,000, Anti-Mitofilin (Gene-Tex, Irvine, CA) at a dilution of 1:500, Anti-SUCLA2 (Gene-Tex) at a dilution of 1:2,500, Anti-ABCB10 (Gene-Tex) at a dilution of 1:1,000, Anti-ABCB7 (Gene-Tex) at a dilution of 1:500, and Anti-α-tubulin (Gene-Tex) at a dilution of 1:1000. Secondary antibodies used included Anti-Rabbit IgG (H+L) HRP conjugate (Promega, Madison, WI) and Anti-mouse IgG (H+L) HRP conjugate (Promega) at dilutions of 1:30,000–60,000. For detection, SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) and X-ray film or ChemiDoc imaging system (BioRad) were used.

Hemoglobin Measurements

Hemoglobin (Hb) content of intact MEL cells was determined using an Olis CLARiTY Spectrophotometer (Olis, Bogart, GA) as previously described[62]. Cell counts were taken using Scepter handheld automated cell counter (Millipore, Billerica, MA) using the 40 μm tip. % WT hemoglobin/cell was reported to normalize for variation in differentiation as cultures are passaged.

Cellular Fractionation

MEL cells were fractionated using the Mitochondria Isolation kit (Thermo Fisher Scientific). Mitoplasts were prepared from the mitochondrial fraction and protease protection was carried out as previously described[11, 63, 64].

Immunocytochemistry

Preparation of cells for immunofluorescence was carried as previously described[65] with several modifications. Briefly, MEL cells were attached to poly-L-lysine coated coverslips (BD Bioscience) by incubating undifferentiated cells on coverslips for 24 hours at 37°C in 5% CO_2. Cells were fixed in 4% paraformaldehyde for 20 minutes, permeabilized with 0.1% Triton X-100 for 20 minutes and blocked with 5% bovine serum albumin for 1 hour. Cells were incubated with rabbit anti-PGRMC1 (Sigma) diluted 1:100 and mouse anti-TIM23 (BD Biosciences) at a dilution of 1:200 overnight. Incubation with AlexaFluor 488 goat anti-rabbit IgG (H+L) (Life Technologies, Carlsbad, CA) and AlexaFluor 633 goat anti-mouse IgG (H+L) (Life Technologies) was carried out for 1 hour. Finally cells were counterstained with 300 mM DAPI nucleic acid stain (Thermo Fisher Scientific) for 5 minutes prior to mounting with ProLong Gold antifade (Life Technologies). Cells were washed with phosphate buffered saline (PBS) three times between each step in the process, reagents were diluted in PBS and all incubations were carried out at room temperature. Cells were imaged using a Zeiss LSM 710 Inverted Confocal microscope (Zeiss, Thornwood, NY) using a 100X oil immersion objective and images processed using Zen (Zeiss) software.

Structural Model

Interactive modeling was carried out using the full length amino acid sequence of human PGRMC1 via the Protein Model Portal (http://www.proteinmodelportal.org/)[66]. In silico

tools employed to produce predictive structures for the full length protein were RaptorX[67], IntFOLD2[68, 69], Phyre2[70] and ITASSER[71–74]. Images of structures were generated using PyMol[75].

Heme and FECH Activity Measurements

The heme content of purified PGRMC1 was measured as its pyridine hemochromogen as previously described[76]. FECH activity alone and in the presence of PGRMC1 was assayed with mesoporphyrin IX (Frontier Scientific, Logan, UT) and ferrous ammonium sulfate using the continuous direct spectroscopic method[77]. Assays were performed in triplicate on at least two independent protein preparations.

Heme Transfer and Gel Staining

Interprotein transfer of heme was determined by mixing an equivalent amount of PGRMC1, FECH or HasA with apo-cytochrome b_5 protein and incubating 15 minutes at 4°C. Apo-cytochrome b_5 was prepared as previously described[78] except the extraction was carried out at room temperature for 6 hours. For native-PAGE, loading buffer minus SDS and without reductant was added and running buffer without SDS was used. Gels were stained as previously described[79] excluding the trichloroacetic acid wash.

Statistical Analysis

Statistical analysis was carried out using one-way ANOVA followed by Tukey HSD test. A P value of 0.05 was set as the cutoff for statistical significance.

RESULTS

Identification of protein partners of *PGRMC1* and *PGRMC2*

To identify novel protein partners for FECH an affinity purification of FLAG tagged human FECH in induced MEL cells was conducted as previously described[11]. Two of the proteins identified with the largest number of spectral counts, unique peptides and % coverage were PGRMC1 and PGRMC2 (Fig. 1 and Table 1). To validate these interactions we performed the reciprocal pull down experiments using N-terminal FLAG tagged human PGRMC1 and PGRMC2 and recovered murine FECH at levels above that of non-specific interactions (Fig. 1 and Table 1). Results from tagged human forms of PGRMC1 and PGRMC2 showed that these proteins formed multimers with their mouse counterparts (Fig. 1 and Table 1). This is consistent with previous demonstrations showing that PGRMC1 forms multimers[23, 42]. The interactions with the endogenous mouse protein serve as a control for protein folding of the exogenous tagged protein as well as quality control for affinity purification and MS. Additionally, affinity purification of the tagged PGRMC1 resulted in the recovery of murine PGRMC2, and likewise PGRMC2 in the recovery of murine PGRMC1.

A number of additional putative protein partners for PGRMC1 and PGRMC2 were identified in the affinity purification experiments. Of note, many of the protein partners for FECH were recovered in the PGRMC1 and PGRMC2 affinity purification experiments (Table S1). Several of these interactions were confirmed for PGRMC1 by immunoblot (Fig. 2A). Many of these proteins are involved in iron metabolism, including iron transport from

the cell surface[80, 81], iron trafficking[64, 82] and iron-sulfur cluster biogenesis[83], or are putative heme or porphyrin transporters[84, 85].

An additional novel protein partner found in common with FECH, PGRMC1 and PGRMC2 was IMMT (Fig. 1 and Table 1). This interaction was confirmed via immunoblot analysis (Fig. 2A). IMMT, also known as Mitofilin or Mic60, is a protein found at junction points between the inner and outer mitochondrial membranes and is thought to stabilize mitochondrial structure[86]. In addition to IMMT, several other protein components of the mitochondrial inner membrane organizing system (MINOS) were also identified and include OPA1[87, 88] and APOOL (i.e MIC27)[89] (Table 1).

Further characterization of interactions

To confirm that the interactions between PGRMC1 or PGRMC2 and FECH were not an artifact of the cell line utilized or the purification process, we performed several additional experiments. First, we utilized a non erythroid human cell line, human embryonic kidney (HEK) 293T cells, to validate the in vivo interaction. HEK293T cells lines stably expressing FLAG-FECH were created and used for affinity purification and western blot analysis to detect the interaction. Immunoprecipitation of FLAG-FECH from HEK293T cells resulted in the recovery of endogenous PGRMC1 (Fig. 2*B*). This interaction was further confirmed independently in HEK293T cells by high throughput affinity purification studies which used C-terminus HA-tagged FECH as bait and recovered PGRMC1[90]. These data which are available via the Biological General Repository for Interaction Datasets (BioGRID - http://thebiogrid.org/) confirm the FECH/PGRMC1 interaction in a distinct cell line.

Second, we performed a higher stringency purification of FLAG-FECH and associated proteins from induced MEL cells by increasing the detergent concentration in the wash buffer during the affinity purification procedure. MS of the affinity purified tagged proteins resulted in the recovery of PGRMC1 and PGRMC2 with FLAG-FECH and FECH with FLAG-PGRMC1 and FLAG-PGRMC2 (Table S2).

Third, we investigated the interaction between FECH and PGRMC1 proteins in vitro using affinity chromatography of his-tagged FECH and non-tagged PGRMC1. Purification of his-tagged FECH resulted in the recovery of non-tagged PGRMC1 (Fig. 3). Reciprocal affinity chromatography experiments were not possible due to the non-specific interaction of FECH with anti-his resin. Experiments with PGRMC2 were not carried out due to the low level of PGRMC2 expression. Together the result from MEL cells, HEK293T cells and in vitro experiments demonstrate the interaction between FECH and PGRMC1 or PGRMC2 in a relatively stable protein complex.

Expression of *PGRMC1* and *PGRMC2* during erythropoiesis

In order to understand the roles of PGRMC1 and PGRMC2 in erythropoiesis, we investigated the expression of each during erythroid differentiation. To investigate gene expression of *Pgrmc1* and *Pgrmc2* during murine erythropoiesis, we first queried the ErythronDB database[91, 92]. Transcript levels for *Pgrmc1* decrease throughout adult definitive differentiation, while those for *Pgrmc2* changed less. We further investigated expression levels in peripheral blood $CD34^+$ mononuclear cells in an in vitro erythroid expansion

system[61]. Relative expression levels of *PGRMC1* decreased, while *PGRMC2* expression increased when cells were cultured until day 16 (Fig. 4*A*). To determine if the protein levels correlated to expression data, we differentiated MEL cells for 4 days and performed Western blot analysis on PGRMC1 and FECH. Protein levels of FECH increase as previously described[52]. Levels of PGRMC1 showed significant variation over the time followed (Fig. 4*B*).

Cellular and subcellular localization of *PGRMC1* in MEL cells and *PGRMC1* modeling

Initial affinity purification and MS experiments were conducted using fractionated MEL cells, specifically the mitochondrial cell fraction. The identification of PGRMC1 and PGRMC2 as a partner of FECH in these experiments is consistent with the mitochondrial co-fractionation and possible localization of these proteins. This localization was further investigated using immunohistochemistry and confocal microscopy in both differentiated and undifferentiated MEL cells (Fig. 5*A* and Fig. S1). While fractionation via differential centrifugation may contain contaminates of other membranes including ER and vesicles, data is consistent with PGRMC1 being at least associated with the mitochondrial membrane either directly or via other membrane interactions such as ER-mitochondrial junctions.

In order to further examine submitochondrial localization of PGRMC1, proteinase protection assays were performed on both whole mitochondria and mitoplasts from MEL cells. Results from these studies are consistent with PGRMC1 being associated with the outer face of the outer mitochondrial membrane (Fig. 5*B*). The antibody used to detect PGRMC1 recognizes an antigen on the C-terminal end of the protein. Thus the C-terminus and the identified heme binding domain of PGRMC1 appears to reside on the outside of the mitochondria. This finding is significant since FECH is localized to the inner face of the inner mitochondrial membrane[93–95] and suggests that a portion of the N-terminal end of PGRMC1 interacts with FECH.

To understand how PGRMC1 might interact with FECH yet be associated with the outside of the mitochondria, we carried out modeling studies of full length PGRMC1. Recently, the structure of an N-terminal truncated form of human PGRMC1 (PDB ID 4X8Y) was determined with heme bound[42]. Submission of the human, full length, amino acid sequence of PGRMC1 to the Protein Model Portal[66] produced models that used the structure of the *Arabidopsis thaliana* protein At2g24940 or the truncated PGRMC1 as a backbone. The structure of At2g24940 (PDB ID 1T0G and 1J03) was solved by the NMR spectroscopy by two independent structural genomics groups[96, 97] and shown to possess a cytochrome b_5-like heme binding domain. Amino acid sequence comparison of At2g24940 with PGRMC1 shows these proteins are 25.2% homologous with an identity of 16.1%[98]. From all proposed structures, only those modeled by the four programs, RaptorX[67], ITASSER[71–74], IntFold2[68, 69], and Phyre2[70], were of the full length protein. These models along with the structure of the truncated PGRMC1 with heme bound are shown in Fig. 6. Of note is the long N-terminal, putative helical transmembrane domain which may span the mitochondrial membranes at inner and outer mitochondrial membrane junction points. This would allow FECH and a portion of PGRMC1 to reside in different compartments yet physically interact.

Decreased heme synthesis in the presence of *PGRMC1* inhibitor

A structure-based screen identified several small molecule ligands to the *A. thaliana* protein At2g24940[57]. One of these small molecule ligands AG-205 has a reported K_d of 64 μM for At2g24940[57] and spectroscopic studies suggest that AG-205 alters heme binding to human PGRMC1[56]. In two different cancer cell lines, AG-205 has been shown to increase PGRMC1 protein levels and result in decreased viability in serum free media[56]. To investigate if AG-205 altered the differentiation profile of model erythropoietic cell lines undifferentiated and differentiated MEL and K562 cells grown in serum containing media were treated with AG-205. The inhibitor was added at time 0 at concentrations ranging from 0 to 10 μM and the level of hemoglobin per cell (pg/cell) were measured. While AG-205 had no effect on the undifferentiated cells, it resulted in a dose dependent decrease in the hemoglobin per cell in differentiated MEL cells most pronounced at 72 hours (Fig. 7*A*). This decrease was partially rescued in induced MEL cells by overexpression of PGRMC1 (Fig. 7*A*). K562 cells exhibited the same sensitivity to AG-205 (Fig. S2).

Because heme synthesis during MEL cell differentiation is a biphasic process that requires heme[99], we investigated the effects of AG-205 when added after the onset of differentiation. AG-205 at 5 and 10 μM concentrations was added at time 0, 24 and 48 hours (Fig. 7*B*). The decrease in hemoglobin per cell was less when AG-205 was added at later time points. Additionally, we investigated the ability of hemin to rescue hemoglobinization in AG-205 treated cells. Hemin was able to rescue hemoglobinization at 5 μM concentrations of AG-205 (Figure 7C).

Characterization of *PGRMC1*

To further characterize PGRMC1 and its interaction with FECH, we cloned, expressed and purified a his-tagged version of the full length wild-type human PGRMC1. Heme content of the purified wild-type PGRMC1 was determined by pyridine hemochromogen assay[76]. The extinction coefficient for the Soret band (~410 nm) of heme bound to PGRMC1 was calculated to be 144 mM^{-1} cm^{-1}. This Soret extinction coefficient is similar to that of DAP1 and the truncated mouse PGRMC1 previously described[23]. The average heme content of the as purified wild-type PGRMC1 was determined to be 18.5%+0.6, consistent with reported values[23, 24].

Heme transfer

PGRMC1 has been shown to bind both heme and progesterone and it has been suggested that PGRMC1 may function as a heme chaperone[24]. This function of PGRMC1 was initially proposed from studies with DAP1[23], but later refuted based on the low of affinity of PGRMC1 for heme[38]. In light of the new findings we investigated the ability of PGRMC1 to transfer heme to an apo-hemoprotein. We incubated PGRMC1 with apo-cytochrome b_5 and then separated the protein on native PAGE and stained for heme as well as detected for protein. As controls we also included the bacterial heme-binding protein HasA in both the native and SDS-PAGE and FECH in the SDS-PAGE. Comparison of protein level and heme showed that heme can be transferred from PGRMC1 to apo-cytochrome b_5, while no transfer from HasA or FECH to apo-cytochrome b_5 was observed (Fig. 8 and Fig. S3). Our

findings are consistent with recent data[24] and add support to the function of PGRMC1 as a heme donor for at least some hemoproteins.

In vitro activity of *FECH* with *PGRMC1*

To determine the effect of PGRMC1 on the *in vitro* enzyme activity of FECH, we performed assays of wild-type FECH in the presence of PGRMC1. The assay utilized is a direct assay and measures the disappearance of porphyrin substrate to determine heme production[77]. PGRMC1 alone did not bind porphyrin and showed no activity (Table 2). Inclusion of PGRMC1 in assays with FECH showed a dose dependent inhibition of FECH up to equimolar amounts. Beyond equimolar amounts, which resulted in a 40% decrease in FECH activity, no change in inhibition was observed (Fig. 9). In addition to wild-type FECH, we also assayed the F110A FECH variant in the presence of PGRMC1 at equimolar amounts. The activity of the F110A variant showed slightly higher inhibition than the WT enzyme. To rule out non-specific interactions between FECH and PGRMC1, we utilized several other proteins including uroporphyrinogen decarboxylase (UROD)[100] or augmenter of liver regeneration protein (ALR)[101] in the assay in equimolar amounts in the FECH assay. These proteins did not result in any significant loss of activity (Table 2). These data suggest that PGRMC1 and FECH specifically interact and this interaction results in decreased FECH activity in vitro, possibly in a conformationally dependent manner.

Conformation of *FECH* affects *PGRMC1* interaction

The structure of FECH has been well studied and the enzyme has been shown to exist in several distinct conformational states. These conformations are proposed to represent discrete steps in the catalytic cycle of the enzyme and would thus present distinct surfaces for protein-protein interactions[18–20]. Conformational states include the open conformation, the closed conformation with porphyrin bound, and the release conformation with heme bound[18, 20]. In order to determine if PGRMC1 interaction is specific to any one of these conformations, *in vitro* purification and affinity chromatography experiments using his-tagged FECH enzymes, both wild-type and variants, and the non-tagged PGRMC1 protein were carried out. Variants used represent two of the distinct conformation of FECH. The F110A variant is most stable in the product bound or release conformation in which the enzyme has heme bound and a partially unwound π helix[20]. The E343K variant adopts the closed conformation with the porphyrin substrate bound[18]. Results from these experiments showed that PGRMC1 interacts more tightly with the F110A variant of FECH than either the wild-type enzyme or the E343K variant (Figure 3*A*). Quantitation of multiple blots showed over twice as much PGRMC1 was present when the F110A variant was used (Figure 3*B*). This finding is consistent with PGRMC1 interacting with FECH in the release or product bound conformation, suggesting that PGRMC1 may regulate FECH activity by stabilizing a specific conformational state and regulating heme release from FECH.

Discussion

PGRMC1 and PGRMC2 are small hemoproteins whose cellular functions are not well defined. Both proteins are members of the membrane associated progesterone receptor family and are highly homologous[25]. Of the two proteins, PGRMC1 has been studied in

greater detail and reported to be involved in a variety of cellular pathways, including cell proliferation, cholesterol synthesis and autophagy. Several published observations have likely contributed to the unclear function of PGRMC1 in cells, including its upregulation in some cancers, characterization of truncated, tagged and heterogeneous protein, and the lack of functional assays for many of its reported roles. Despite the smaller number of studies carried out on PGRMC2, it is also thought to play a role in cancer[102]. It is not known if PGRMC1 and PGRMC2 have overlapping functions in the cell; however we and others[103] have shown that PGRMC1 and PGRMC2 interact and form heterodimers or multimers.

One property of both PGRMC1 and PGRMC2 which has not been questioned is their ability to bind heme. Heme binding has been characterized through spectroscopic[23, 24, 104] and crystallographic studies[42] for mammalian PGRMC1. Multiple studies also characterized the yeast homolog, DAP1[23, 30, 38] and have shown that several of the proposed functions of PGRMC1 and DAP1 are heme dependent[24, 30, 31, 38, 104]. Spectroscopic studies have been carried out to characterize the heme binding pocket of human PGRMC1 and are consistent with heme binding via a conserved tyrosine residue in a high spin, five coordinate environment[24]. This type of environment is similar to several bacterial heme transport proteins including ShuT and HasA[43, 105]. Recent crystallographic data showed that the dimerization of a truncated form of PGRMC1, which lacks the transmembrane domain, occurs via their heme molecules which interact with each monomer on its surface[42]. This unique and weak binding of heme by PGRMC1 has reinvigorated the proposal that PGRMC1 may function as a heme chaperone in cells[23, 31]. Our data that demonstrate a clear interaction between PGRMC1, PGRMC2, FECH and the mitochondrial heme metabolon add additional evidence to this proposed function.

Consistent with the proposal that PGRMC1, and possibly PGRMC2, function as heme chaperones are the findings that PGRMC1 can be observed in multiple cellular locations; something that would be expected for a heme chaperone that obtains heme from the mitochondrion and transports it to a variety of cellular locations for assembly into holo-hemoproteins. Previous studies have localized PGRMC1 to a variety of cellular compartments including the ER, nucleus, cytoplasm, cell membrane, and the mitochondria[106]. Several of these locations correspond to the proposed functions of PGRMC1. For example, PGRMC1 has been shown to stimulate several cytochrome P_{450}s which are found in the ER membrane[28, 29]. Another role reported for PGRMC1 is vesicle transport of the epidermal growth factor receptor. In these studies, PGRMC1 was cofractionated with EGFR to cytoplasmic vesicles[34]. In our studies, we have shown that PGRMC1 and PGRMC2 cofractionate and colocalize with the mitochondrial heme biosynthesis metabolon[11]. This pattern was observed in both undifferentiated and differentiated MEL cells. Further experiments to characterize the sub-mitochondrial localization were consistent with the majority of PGRMC1 being on the outside of the outer mitochondrial membrane. This localization would make it difficult if not impossible for the heme binding domain of PGRMC1 to interact with FECH to obtain heme and suggests interactions with a transmembrane protein for heme transport, such as ABCB10. Thus FECH and PGRMC1 interaction would take place at or near the N-terminus of PGRMC1 and upon heme binding PGRMC1 would either undergo proteolytic processing or significant conformational rearrangement for heme transport from the mitochondria (Figure 10A). More

detailed studies examining different regions of PGRMC1 are necessary to validate this model of interaction with FECH.

In addition to the possible role of PGRMC1 as a heme chaperone, several equally feasible roles for PGRMC1 in heme production exist that are based on previous data as well as those presented herein. First, as mentioned above, PGRMC1 has been implicated in vesicle transport[34, 107]. Our affinity purification data of PGRMC1 showed that transferrin receptor 1 was recovered from both the PGRMC1 and PGRMC2 experiments similar to what was found with other mitochondrial heme metabolon components. Previous work has proposed a novel delivery system for iron to the mitochondria during erythropoiesis via a kiss and run mechanism[108–110]. Considering the reported vesicle transport function of PGRMC1, it is possible that PGRMC1 could play a role in iron transport to the mitochondria (Figure 10C). If it is shown that PGRMC1 with heme bound is involved in iron transport, heme may serve as an intracellular second messenger to regulate iron homeostasis, which supports the work of Li et al.[36].

Second is the possible function of PGRMC1 as a heme sensor that regulates heme production via either i) stabilizing or destabilizing the mitochondrial heme metabolon (Figure 10D) or ii) directly regulating the activity of FECH (Figure 10B). Our findings clearly support a role for PGRMC1 in regulating heme biosynthesis and/or transport. Inclusion of PGRMC1 in FECH assays resulted in a dose dependent decrease in enzyme activity that saturates at about 60% of maximal FECH activity. We also demonstrated that this interaction is dependent on the conformation of FECH and is strongest when FECH is in the product bound/release conformation. This conformation specific interaction of FECH with a partner was previously hypothesized due to the distinct surfaces of FECH presented during its catalytic cycle[20]. Additionally in an erythroid cell culture model, we were also able to demonstrate that targeting of PGRMC1 with a small molecule inhibitor decreases hemoglobinization in differentiated MEL cells. These data are consistent with PGRMC1 regulating heme synthesis via its interactions with FECH.

The models proposed herein for PGRMC1 function in heme synthesis are not exclusive and likely overlap in some fashion. For example, our findings demonstrating the ability of PGRMC1 to transfer heme to an apo-hemoprotein and to interact with the “release conformation” of FECH support a model in which PGRMC1 serves as both a heme chaperone and regulator of FECH. Overall our data are consistent with PGRMC1 being an essential component of the mitochondrial heme metabolon and playing a role in regulating heme synthesis in erythroid differentiation. While the precise roles of PGRMC1 and PGRMC2 need additional clarification, planned studies, including the production of transgenic animals and knock-out cell lines, will define the roles of these proteins in heme biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FECH	ferrochelatase
PGRMC1	progesterone receptor membrane component 1
PGRMC2	progesterone receptor membrane component 2
MAPR	Membrane-Associated Progesterone Receptor
MudPIT	multidimensional protein identification technology
MS	mass spectrometry

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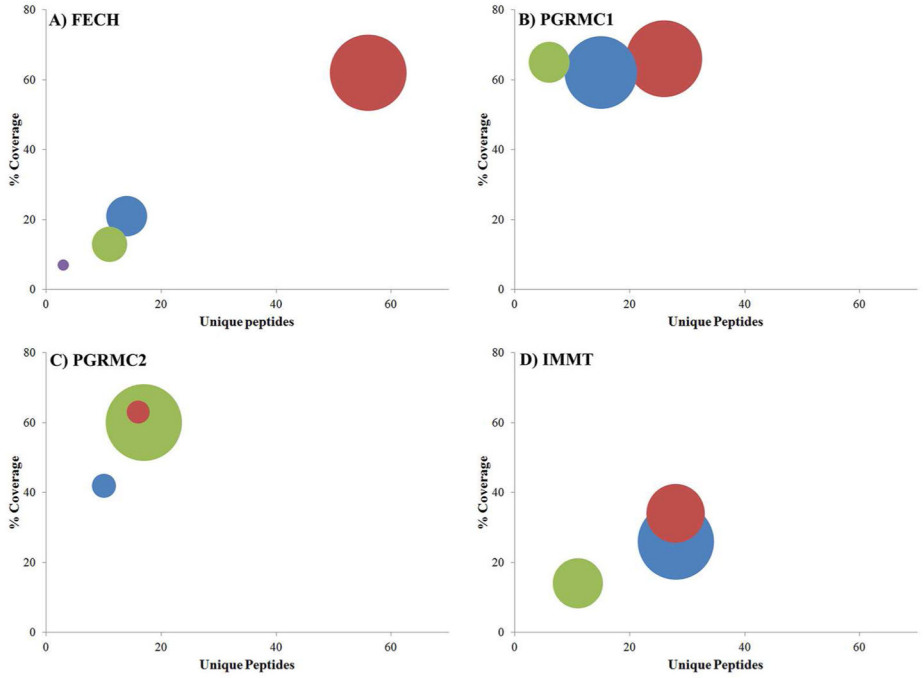

Figure 1.

Graphical representation of affinity purification and MS analysis of FLAG-FECH (red), FLAG-PGRMC1 (blue) and FLAG-PGRMC2 (green) and FLAG-CPOX (purple, negative control). Each panel represents an identified mouse protein recovered with the bait human protein listed in the legend of panel A. Panels are as follows: A - FECH, B - PGRMC1, C - PGRMC2 and D - IMMT. Number of unique peptides (x axis), % coverage (y axis) and spectral counts (bubble size) for each was calculated using the maximal values obtained minus the maximal values observed in the control samples (empty vector). Size of bubbles represents the % of the total spectral counts identified for each mouse protein. The maximal spectral counts of each of the proteins was FECH=417, PGRMC1 = 115, PGRMC2 = 491 and IMMT = 59.

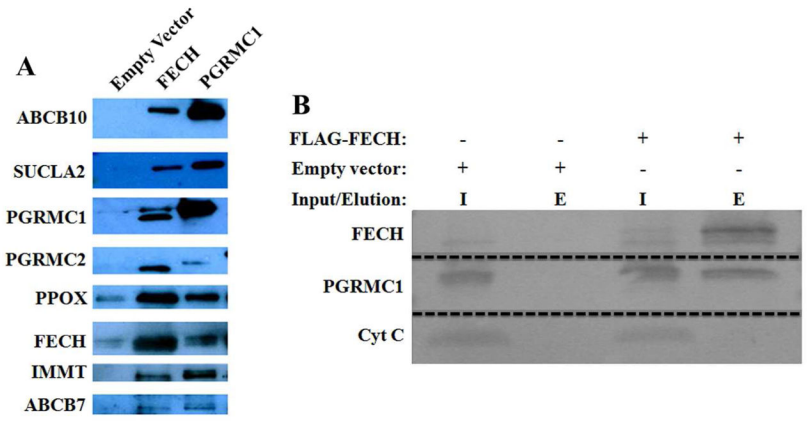

Figure 2.

Immunoblot from affinity purification of FLAG elutions. A, A representative immunoblot of FLAG elutions from an affinity purification experiment of differentiated MEL cells with empty vector (lane 1), FLAG-FECH (lane 2) and FLAG-PGRMC1 (lane 3). Blots were probed for ABCB10, SUCLA2, PGRMC1, PGRMC2, PPOX, FECH, IMMT and ABCB7. B, Affinity purification from HEK293T cells using empty vector (lanes 1 and 2) and FLAG FECH (lanes 3 and 4). Blot was probed for PGRMC1. I is input and E is FLAG elution.

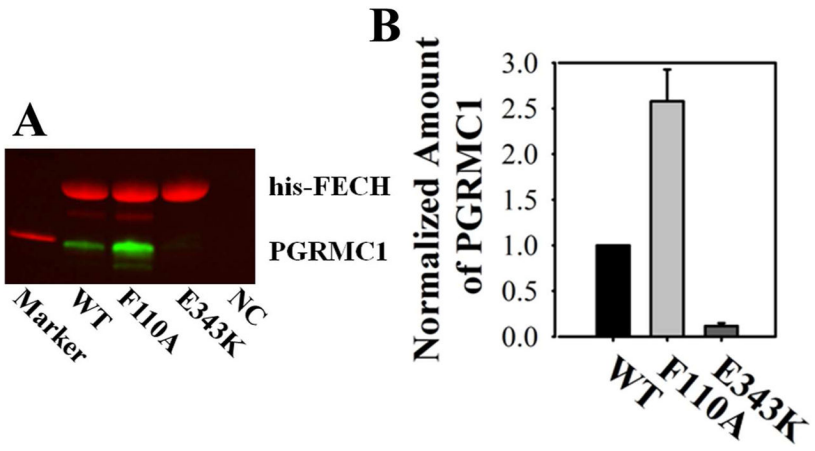

Figure 3.

Analysis of WT FECH and FECH variant interactions with PGRMC1. A, Representative SDS-PAGE stain free gel visualization of FECH (red) and immunoblot analysis of non-tagged PGRMC1 (green) recovered from in vitro experiments. Protein standard is in lane 1, wild-type (WT) FECH is in lane 2, the F110A FECH variant in lane 3, the E343K FECH variant in lane 4 and the negative control (NC), only non-tagged PGRMC1 in lane 5. Experimental conditions are described in *Materials and Methods* section. FECH variants are described in Results section Conformation of *FECH* affects *PGRMC1* interaction. B, Quantitation of PGRMC1 with wild-type (WT), F110A and E343K FECH from four biological replicates. P values for each variant with the wild-type and each other are < 0.0001.

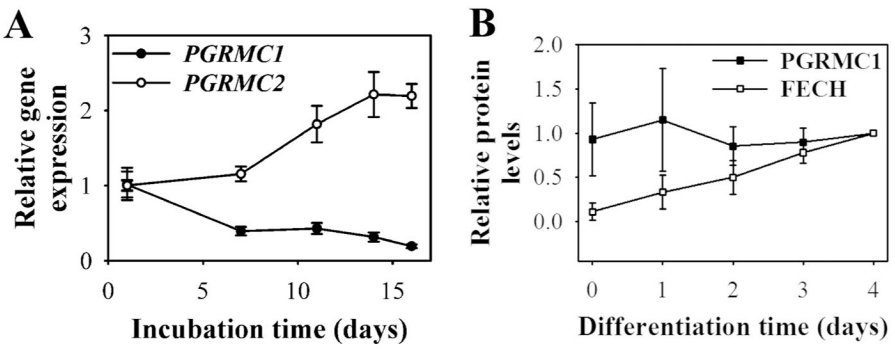

Figure 4.

Gene expression of *Pgrmc1* and *Pgrmc2* and protein levels of PGRMC1 and FECH during differentiation in two model systems. A, Expression levels of PGRMC1 and PGRMC2 in an erythroid expansion model system relative to the control GUSB. B, Protein levels of PGRMC1 and FECH in differentiating MEL cells normalized to day 4 levels.

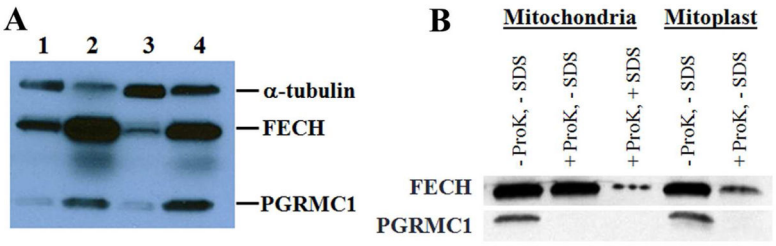

Figure 5.

Cellular and subcellular localization of PGRMC1 and FECH in MEL cells. A, Cellular localization of PGRMC1 and FECH in undifferentiated and differentiated MEL cells. Representative immunoblot of FECH, PGRMC1 and α-tubulin detected from MEL cellular fractions. Lane 1 is the cytosolic fractions and lane 2 the mitochondrial fraction from differentiated MEL cells, while lane 3 is the cytosolic fractions and lane 4 the mitochondrial fraction from undifferentiated MEL cells. 12.5 μg total protein was loaded in each lane. B, Submitochondrial localization of PGRMC1 was determined by mitoplast preparation and protease protection assay. Representative immunoblot of FECH and PGRMC1 from induced fractionated MEL cells.

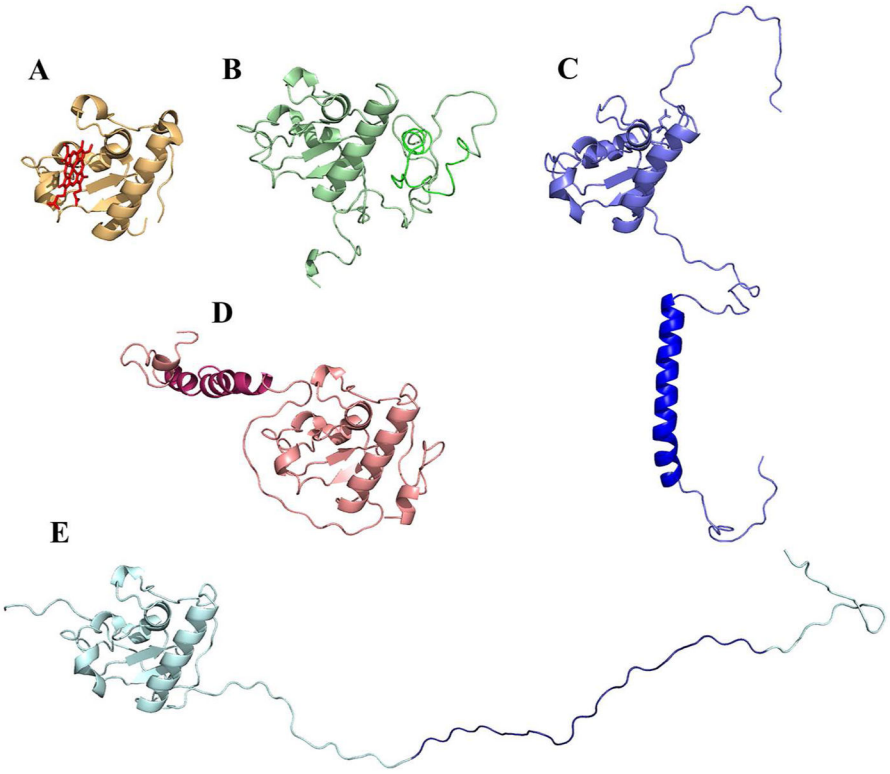

Figure 6.

Structure of truncated PGRMC1 and models of full length PGRMC1. A, PGRMC1 (PDB ID 4X8Y) structure and models of PGRMC1 as generated by B, ITASSER[71–74], C, RaptorX[67], D, Phyre2[70] and E, IntFOLD2[68, 69]. Putative transmembrane domains in B thru E are shown with darker color and heme bound by PGRMC1 is shown as red sticks. All structures rendered using PyMol[75].

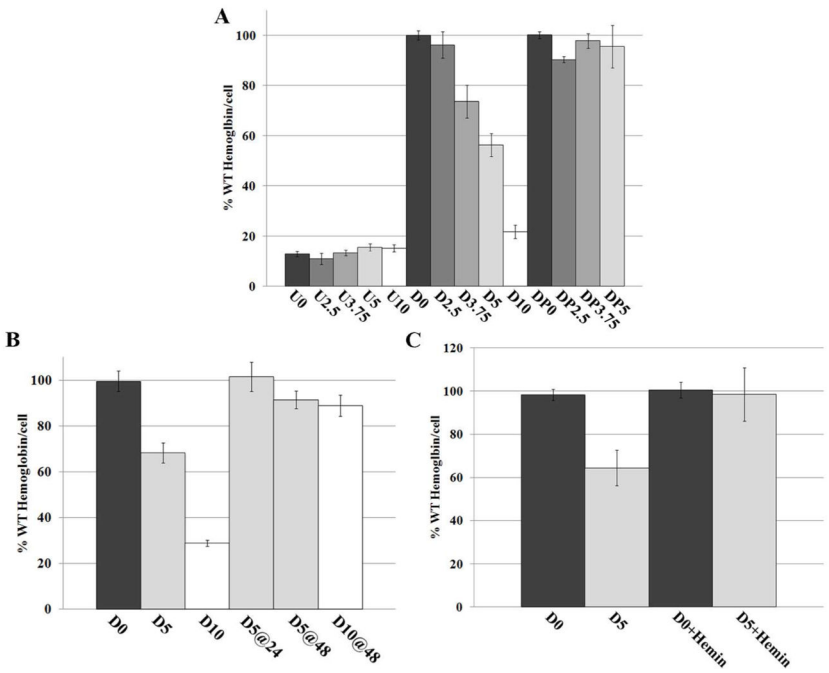

Figure 7.

Inhibition of heme synthesis in MEL cells by AG-205. A, AG-205 inhibits heme synthesis in differentiating (D) MEL cells, but not undifferentiated (U) cells. Concentrations of AG-205 were 0, 2.5, 3.75, 5 and 10 μM. Differentiated cells overexpressing PGRMC1 (DP) were treated with AG-205 at 2.5, 3.75, and 5 μM. For undifferentiated cells, $P < 0.01$ for U2.5 vs U5 and U2.5 vs U10. For differentiated cells, $P<0.01$ was found for all comparisons except D0 vs D2.5. For differentiated cells expressing PGRMC1, no statistically significant differences were found. B, AG-205 added after initiation of differentiation at either 24 (@24) or 48 (@48) hours had less of an effect at 5 and 10 μM than when added at the initiation of differentiation. $P < 0.01$ was found for D0 vs D5, D0 vs D10, D5 vs D5@24, D5 vs D5@48, D5 vs D10@48, D5 vs D10, D10 vs D5@24, D10 vs D5@48 and D10 vs D10@48. $P<0.05$ was found for D0 vs D10@48 and D5@24 vs D10@48. C. Addition of 12.5 μM hemin results in rescue of hemoglobinization at 5 μM AG-205. % WT Hemoglobin (pg) per cell for cultures with hemin are normalized to D0 control with hemin added. $P < 0.01$ for D0 vs D5, D5 vs D0+hemin and D5 vs D5+hemin.

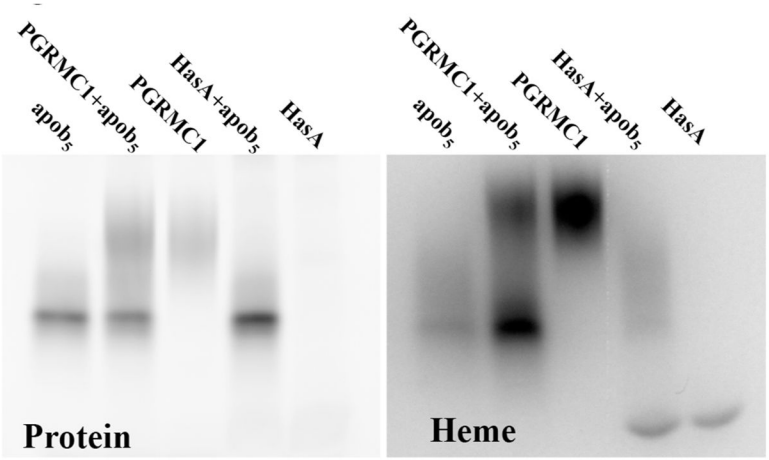

Figure 8.

Heme transfer to apo-cytochrome b_5. A, Native PAGE of proteins alone or in combination of apo-cytochrome b_5 ($apob_5$), PGRMC1 and HasA detected for protein (left) and heme (right).

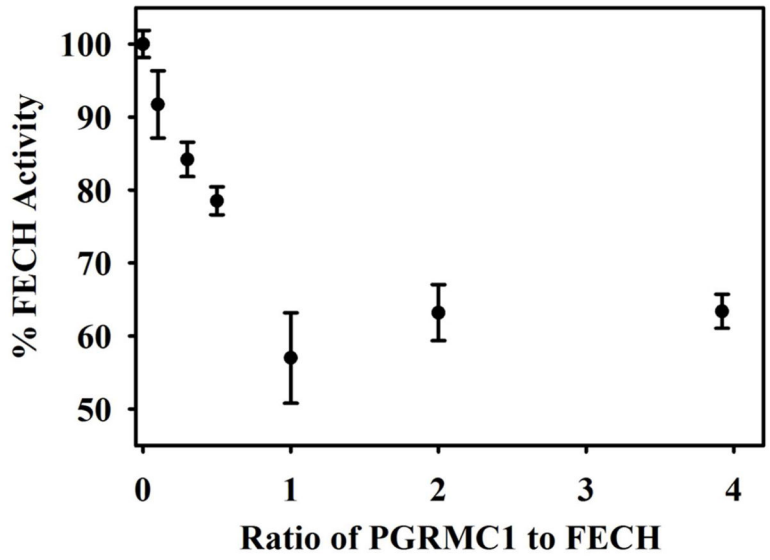

Figure 9.
Effect of PGRMC1 on FECH activity. % FECH activity with different molar amounts of PGRMC1 is shown.

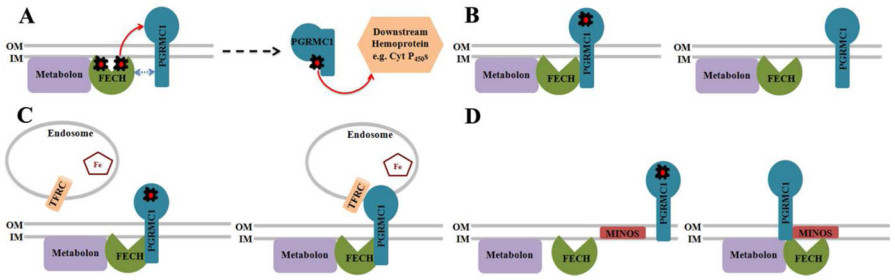

Figure 10.

Models for role of PGRMC1 in heme synthesis. A, PGRMC1 functions as a heme chaperone to deliver newly synthesized heme to hemoproteins in different cellular locations, B, PGRMC1 serves as a heme sensor which directly interacts with FECH and decreases FECH activity, C, PGRMC1 serves as a heme sensor to regulate endosomal trafficking of iron to the mitochondria for heme synthesis, D, PGRMC1 serves as a heme sensor to regulate the localization of the mitochondrial heme biosynthesis complex to inner and outer membrane junction points.

Table 1

Affinity Purification and MS Results of FECH, PGRMC1 and PGRMC2.

Murine Protein	Human Input			
	FECH (n=2)	PGRMC1 (n=2)	PGRMC2 (n=2)	Empty Vector (n=2)
FECH	297–443[a] 26–63 46–79%	81–142 12–21 22–38%	72–115 16–18 26–30%	9–26[a] 4–7 13–17%
PGRMC1	39–115 16–26 52–66%	93–104 11–15 36–62%	57–65 5–6 27–33%	0 0 0%
PGRMC2	22–43 10–16 54–63%	34–49 3–10 16–42%	241–491 15–17 42–61%	0 0 0%
IMMT	12–40 8–34 13–45%	63–65 34 37%	30–31 16–17 21–25%	3–6 2–6 4–11%
APOOL	0–9 0–9 0–43%	13–29 6–7 25–29%	7 5 21–22%	0 0 0%
OPA1	0–28 0–28 0–37%	9–25 6–17 8–21%	3–9 3–6 5–9%	0 0 0%

Data shown from top value to bottom as: spectral counts/unique peptides, percent sequence coverage.

[a]data from FECH-FECH and Empty vector-FECH interactions as previously reported[11].

Table 2

FECH Enzyme Activity. Equimolar amounts of WT and Variant FECH as well as control proteins were used.

Sample	Activity (% + SD)
FECH	99 + 2.4
FECH + PGRMC1	57 + 6.2
F110A FECH	90 + 6.8
F110A FECH + PGRMC1	41 + 3.3
FECH + UROD	89 + 1.9
FECH + ALR	106 + 8.6
PGRMC1	3 + 1.3