Human ABCB6 Localizes to Both the Outer Mitochondrial Membrane and the Plasma Membrane†

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ABSTRACT: Expression of the ATP-binding cassette transporter ABCB6 has been associated with multiple cellular functions, including resistance to several cytotoxic agents, iron homeostasis, and porphyrin transport. To further elucidate its physiological function and/or role in drug resistance, we determined the subcellular location of ABCB6. Using three novel ABCB6-specific antibodies, Western blot analysis of cells expressing cDNA-derived or endogenous ABCB6 revealed two distinct molecular weight forms. Confocal microscopy indicates that the protein localizes to both mitochondria and the plasma membrane. Differential centrifugation revealed that the lower molecular weight form predominantly resides in the mitochondria, while the larger protein form is more abundant in the plasma membrane. Preliminary studies indicate that ABCB6 is functionally relevant in the plasma membrane, where its expression prevents the accumulation of specific porphyrins in the cell. Digitonin solubilization of mitochondria demonstrated that ABCB6 is present in the outer mitochondrial membrane, while back-titration assays with the ABCB6-specific antibodies reveal that the nucleotide binding domain of ABCB6 is cytoplasmic. These studies are the first to demonstrate that ABCB6 exists in two molecular weight forms, is localized to both the outer mitochondrial membrane and the plasma membrane, and plays a functional role in the plasma membrane.

The ATP binding cassette (ABC[1]) transporters are one of the largest protein families (*1*). These enzymes are named for the conserved sequences required for ATP binding (Walker A motif, Walker B motif, and the conserved linker region) to create the nucleotide binding domain (NBD). ABC proteins are present in various cellular membranes, transporting a variety of endogenous and xenobiotic substrates, including fatty acids, leukotrienes, phospholipids, inorganic ions, sugars, peptides, glutathione conjugates, polysaccharides, antibiotics, and chemotherapeutic agents (*1*).

The human genome encodes 48 ABC transporters (*2*). Their medical importance stems from genetic disorders linked to mutations in ABC genes (such as cystic fibrosis, Stargardt disease, Tangier disease, and Pseudoxanthoma elasticum (*3*)), their substantial role in pharmacology as gatekeepers at biological barriers (i.e., blood−brain barrier (*4*)), and as defenders of stem cells (*5*). Several ABC transporters, including P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance protein 1 (MRP1/ABCC1), and ABCG2 (MXR/BCRP), are found at high levels in the plasma membrane of cancer cells and have been implicated in multidrug resistance (MDR) (*4*). An additional nine transporters (ABCA2, ABCB11, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11) have been shown to transport specific drugs (*4*), and correlative studies (*6*, *7*) suggest that many of the remaining 36 ABC transporters may be drug transporters.

We became interested in the ABC protein ABCB6 while determining the contribution of the remaining 36 ABC transporters to anticancer drug resistance. Genetic profiling of several cellular models of drug resistance (*6*, *7*) indicated that increased ABCB6 expression correlates with increased resistance to various drugs in 60 diverse cancer cell lines (NCI-60) (*6*). In unrelated experiments, we found that various human and mouse cell lines selected for resistance to arsenite[2] or cisplatin possess elevated levels of ABCB6 mRNA and protein. In other studies, increased ABCB6 mRNA expression was reported in camptothecin-resistant (*8*) or paclitaxel/ FEC (5-fluorouracil, epirubicin, and cyclo-

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[1] Abbreviations: ABC, ATP-binding cassette; NBD, nucleotide binding domain; P-gp, P-glycoprotein; MDR, multidrug resistance; HB, homogenization buffer; RB, resuspension buffer; EDB, enzyme dilution buffer; IAAP, [^{125}I]Iodoarylazidoprazosin; CPIII, coproporphyrin III; PhA, pheophorbide A.

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phosphamide)-treated (*9*) cells. ABCB6-mediated MDR may be clinically relevant since increased expression of ABCB6 in hepatocellular cancer compared to healthy liver tissue (*10*) and breast cancer cells following chemotherapy (*9*) has been reported.

However, this correlation between ABCB6 and drug resistance is surprising because in contrast to other MDR-mediating ABC transporters that reside in the plasma membrane, ABCB6 expression had only been reported in mitochondria (*11*), and most ABC transporters confer MDR by pumping drugs out of the cell. Thus, the question arose as to whether the cellular localization of ABCB6 was restricted to mitochondria.

Beyond cellular protection from chemotherapies, additional functions have been proposed for ABCB6. In yeast, knocking out the orthologous gene Atm1p leads to a decrease in holocytochromes (*12*, *13*), an increase in free Fe^{2+} levels (*14*), and lack of growth in the absence of Leu (*14*). These phenotypes can be reversed by expression of human ABCB6 in the knockout yeast cells (*11*), indicating that ABCB6 is a functional orthologue of Atm1p. A recent report indicates that the addition of hemin to MEL cells leads to an upregulation of ABCB6 at both the mRNA and protein levels and that FLAG-tagged ABCB6 expressed in Saos-2 cells binds heme and other tetrapyrrole carboxylates, indicating that ABCB6 may be responsible for the transport of porphyrins across mitochondrial membranes (*15*).

Since ABC transporters serve very diverse functions in the cell, understanding the cellular localization(s) of each transporter is necessary to properly understand and investigate their cellular role. We determined the intracellular localization of endogenous as well as FLAG-tagged cDNA derived ABCB6. In both cases, we confirmed that ABCB6 is localized to mitochondria, and further characterized its location to the outer mitochondrial membrane with the NBD domain facing the cytoplasm. In addition, we discovered that ABCB6 localization is not restricted to mitochondria, as ABCB6 is also present in plasma membranes. The decreased cellular accumulation of hemin and pheophorbide A (PhA), previously reported substrates of mitochondrial ABCB6, suggest that the plasma membrane form of ABCB6 is also functional. Finally, using a whole cell assay, we have identified a new substrate of ABCB6. We show that iodoarylazidoprazosin (IAAP) has decreased cellular accumulation in cells expressing ABCB6.

EXPERIMENTAL PROCEDURES

Reagents. p3×FLAG-CMV-14 expression vector, anti-FLAG M2 antibody, *n*-dodecyl β-D-maltoside, cytochrome *c*, cytochrome *c* oxidase, PGNase F, and *O*-glycosidase were purchased from Sigma (St. Louis, MO). Anti-porin and anti-complex III antibodies, Mitotracker Deep Red 633, Wheat Germ Agglutinin Alexa Fluor 555, Alexa Fluor 488 goat anti-mouse IgG, and Zymed ABTS solution were purchased from Invitrogen (Carlsbad, CA). The protease inhibitor cocktail tablets were from Roche (Indianapolis, IN). The anti-BiP/GRP78, anti-EEA1, anti-GM130, anti-integrinα2/VLA-2α, anti-Lamp-1, and anti-nucleoporin p62 antibodies were purchased from BD Biosciences (San Diego, CA). Optiprep iodixanol was purchased from Axis-Shield (Oslo, Norway), and DAKO mounting media from DakoCytomation (Carpinteria, CA). SNB-19, U251, and HS-578T cells were purchased from the DCTD Tumor/Cell Line Repository (NCI, Frederick, MD). All other chemicals were reagent grade.

Generation and Maintenance of Cell Lines. KB-3-1 cells were stably transfected with human ABCB6 in the p3×FLAG-CMV-14 expression vector (KB-B6) or empty control vector (KB-CV). KB-3-1 (*16*), SNB-19 (human glioblastoma cells of central nervous system origin (*17*)), U251 (human glioblastoma cells of central nervous system origin (*18*)), and HS-578T (human ductal carcinoma (*19*)), KB-B6, and KB-CV cell lines were grown (37 °C, 5% CO_2) in monolayers with DMEM medium (4.5 g of glucose/L) supplemented with L-glutamine (2 mM), FBS (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Protein Assay. Protein content was measured with the micro BCA assay (*20*).

Crude Membrane Preparation. Crude membranes were prepared as described (*21*).

Generation of Monoclonal Anti-ABCB6 Antibodies. In collaboration with BD Biosciences, monoclonal antibodies $ABCB6_{788}$ and $ABCB6_{567}$, both IgG2a kappa, were generated by immunizing mice with the *E. coli*-expressed, purified, and refolded cytoplasmic region of the human ABCB6 protein (amino acids 550 to 842). Bleeds were screened for reaction with ABCB6 via Western blot, and hybridomas were prepared from positive mice.

Generation of Polyclonal Anti-ABCB6 Antibody ($ABCB6_{7474}$). In collaboration with Rockland Immunochemicals, Inc. (Gilbertsville, PA), a peptide of the sequence N-RAMNTQENATRARAVD-C (amino acids 440−455) was synthesized. Rabbits were injected regularly, and bleeds were collected and screened for reaction with ABCB6. Positive sera were affinity purified using the prepared peptide.

Western Blotting. SDS−PAGE and Western blotting were preformed as described (*22*) with anti-FLAG (2.5 μg/mL), anti-porin (1 μg/mL), anti-complex III (0.1 μg/mL), anti-$ABCB6_{567}$ (0.2 μg/mL), anti-$ABCB6_{788}$ (0.2 μg/mL), and anti-$ABCB6_{7474}$ (1:500) and incubations performed overnight.

Deglycosylation Reactions. Deglycosylation reactions of crude membrane fractions were performed in the presence of either PGNase F or *O*-glycosidase with the buffers, temperatures, and time frame recommended by the manufacturer (Sigma, St. Louis, MO).

Purification of Intact, Functional Mitochondria and Plasma Membranes. Purified membranes were prepared by combining several methods (*23*−*25*). Cells were lysed in homogenization buffer (HB; 150 mM $MgCl_2$, 10 mM KCl, 10 mM Tris at pH 6.7, and protease inhibitors; 1 mL/ 1×10^7 cells), incubated on ice (15 min), and Dounce homogenized (35 strokes using only pestle A). HB with sucrose (34.2%, 1/3 vol) was added (corresponds to whole cell sample, Figure 3, lane 1), and low speed centrifugation (1000 × *g*, 5 min, 4 °C) removed nuclei and unlysed cells (corresponds to Figure 3, lane 2). The supernatant was centrifuged at a medium speed (5000 × *g*, 10 min, 4 °C),

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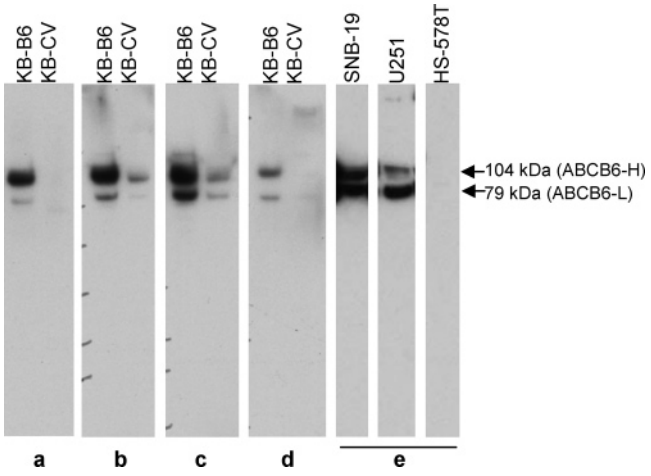

FIGURE 1: Two distinct ABCB6 protein forms exist in cells. Crude membranes from KB-3-1 cells stably transfected with FLAG-tagged ABCB6 (KB-B6) or control vector (KB-CV) were prepared, separated by SDS−PAGE, transferred to a PVDF membrane, and probed with (a) anti-FLAG, (b) anti-ABCB6$_{567}$, (c) anti-ABCB6$_{788}$, or (d) anti-ABCB6$_{7474}$. (e) Crude membranes were prepared from cells that have relatively high endogenous ABCB6 mRNA levels, separated by SDS−PAGE, transferred to a PVDF membrane, and reacted with the anti-ABCB6$_{567}$ antibody. The arrows represent the molecular weight positions of both ABCB6 forms.

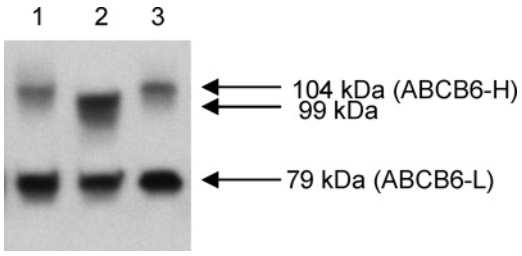

FIGURE 2: ABCB6-H is not a glycosylated form of ABCB6-L. KB-B6 crude membranes were untreated (lane 1) or subjected to *N*-glycosidase (lane 2) or *O*-glycosidase (lane 3) treatment to remove sugar chains. The proteins were then separated by SDS−PAGE and analyzed by Western blot reacted with anti-ABCB6$_{788}$.

the pellet resuspended in HB + sucrose (20 mL), centrifuged (5000 × *g*, 10 min, 4 °C), and resuspended in Solution A (3 mL; 20 mM Hepes, 1 mM EDTA, and 250 mM sucrose at pH 7.4; Figure 3, lane 3). Iodixanol solution (50% iodixanol, 120 mM Hepes, 6 mM EDTA, and 250 mM sucrose at pH 7.4) was added (final concentration of 36%), and the sample was placed in a centrifuge tube, overlaid with Solution B (10 mL, 30% iodixanol, 80 mM Hepes, 4 mM EDTA, and 250 mM sucrose at pH 7.4), Solution C (to top, 10% iodixanol, 80 mM Hepes, 4 mM EDTA, and 250 mM sucrose at pH 7.4), and centrifuged (50,000 × *g*, 4 h, 4 °C, swinging bucket rotor). Protein was collected at the 30%/10% iodixanol interface, an equal volume of solution A (10 mL) was added, followed by centrifugation (30,000 × *g*, 10 min, 4 °C). The resulting pellet was resuspended in mitochondrial suspension buffer (MSB; 250 mM sucrose and 10 mM Tris at pH 7.0; Figure 3, lane 4) with protease inhibitors.

TSNa buffer (10 mM Tris at pH 7.5, 50 mM NaCl, and 250 mM sucrose, with protease inhibitors) was added (1 volume) to the supernatant resulting from the medium speed centrifugation and centrifuged at a high speed (120,000 × *g*, 1 h, 4 °C; microsomes). The pellet was resuspended (resuspension buffer (RB); 3 mL; 10 mM Tris, 0.5 mM EDTA, and 10% glycerol at pH 7.5; lane 5), applied to the

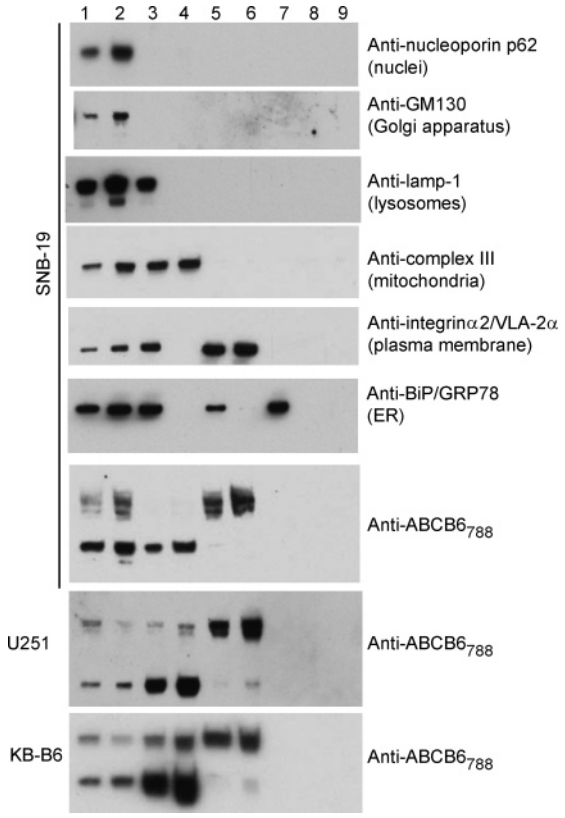

FIGURE 3: ABCB6 resides in both mitochondria and the plasma membrane of overexpressing and native cell lines. Cellular membranes were prepared with various centrifugation steps, as detailed in the text. Organelle location was monitored by separating the resulting proteins with SDS−PAGE, transferring to a PVDF membrane, and reacting with antibodies directed against proteins residing in specific organelle membranes. The antibodies used are indicated with the targeted organelle listed in parentheses. Membrane fractions from SNB-19 cells are shown (similar purity was obtained for U251 and KB-B6 cells). The localization of ABCB6 in membranes from KB-B6, SNB-19, and U251 cells was determined by reaction with the anti-ABCB6$_{788}$ antibody. Lane 1, whole cell lysates; lane 2, low speed pellet; lane 3, medium speed pellet; lane 4, iodixanol band (mitochondria); lane 5, high speed pellet; lane 6, 53.5%/43.5% sucrose interface (plasma membranes); lane 7, lower density proteins; lane 8, higher density proteins; lane 9, soluble proteins.

top of a sucrose gradient (53.5% sucrose/43.5% sucrose), centrifuged (5 h, 100,000 × *g*, 4 °C), and fractions at the 53.5/43.5% sucrose interface, bottom of the tube (higher density proteins), and at the top of the gradient (lower density proteins) were collected, diluted (RB, 1 volume), and centrifuged (120,000 × *g*, 1 h, 4 °C). The pellets (plasma membrane, Figure 3, lane 6; higher density proteins, Figure 3, lane 7; lower density proteins, Figure 3, lane 8) were resuspended in TSNa buffer with protease inhibitors. The supernatants resulting from the high speed spin contained soluble proteins (Figure 3, lane 9).

Generation of Mitochondria with Permeabilized Outer Membranes. Mitochondria with permeabilized outer mitochondrial membranes were generated as described above except following incubation on ice (15 min), the cells were placed at −80 °C overnight, thawed rapidly (RT), incubated on ice (45 min), and then processed though the remaining protocol.

Confocal Microscopy Using MitoTracker Deep Red 633 and Anti-FLAG M2 Antibody. To label mitochondria, Mi-

totracker Deep Red 633 in DMEM media (30 pg/mL) was added to cells (30 min, 37 °C) and then washed (media, 10 min, 37 °C, 2×). To label ABCB6, cells were fixed (4% paraformaldehyde in PBS/BSA (0.1%), 30 min, RT), permeabilized (70% ethanol, 20 min, RT), blocked (PBS/BSA, 2 × 10 min, RT), incubated with anti-FLAG M2 antibody (20 μg/mL, 1 h, RT), incubated with Alexa Fluor 488 goat anti-mouse IgG (1:50, 1 h, RT), dried, and DAKO mounting medium was added. Fluorescent cells were examined with a Zeiss LSM 510 Confocal Microscope (Carl Zeiss Inc, Thornwood, NY) with an Axiovert 100M inverted microscope and 63 × 1.4 NA Zeiss Plan-Apochromat oil immersion objective. Using the Zeiss Aim software, images were collected using a multitrack configuration where the Alexa Fluor 488 and Mitotracker deep red emissions were collected sequentially with a BP 505-530 filter and an LP 650 filter after sequential excitation with 488 and 633 nm laser lines, respectively.

Confocal Microscopy Using Wheat Germ Agglutinin and Anti-FLAG M2 Antibody. ABCB6 was labeled as described above, followed by incubation with wheat germ agglutinin Alexa 533 (1 μg/mL, 1 h, RT). Slides were finished as described above. Alexa 533 was excited with the 543 nm laser line and the emission collected in a PMT with a BP 560-615 filter.

Solubilization with Digitonin. To purified mitochondria (100 μg), increasing amounts of digitonin were added (0, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg digitonin/mg protein), samples were incubated (RT, 30 min) and centrifuged (10,000 × *g*, 10 min, 4 °C), and both supernatants and pellets (resuspended in MSB (50 μL)) were collected.

Cytochrome c Assay. Mitochondria in enzyme dilution buffer (EDB, 5 mM Tris, and 125 mM sucrose at pH 7.0) without (intact) or with (solubilized) *n*-dodecyl β-D-maltoside (2.5 mM) were incubated (10 min, 4 °C) and added (50 μg) to assay buffer (800 μL, 10 mM Tris, and 120 mM KCl at pH 7.0). Reduced cytochrome *c* (50 μL, 2.7 mg/mL cytochrome *c* in 0.5 mM DTT) was added, A_{550} nm was measured (65 s), and the rate of cytochrome *c* oxidation was determined from the slope of the line.

Back-Titration. Modifications of various back-titration techniques (*26*−*28*) were used to determine protein orientation. Antibody (anti-porin (600 ng/mL), anti-complex III (600 ng/mL), anti-FLAG (300 ng/mL), anti-$ABCB6_{567}$ (600 ng/mL), anti-$ABCB6_{788}$ (300 ng/mL), and anti-$ABCB6_{7474}$ (600 ng/mL)) was added to increasing concentrations of either intact or permeabilized mitochondria (0, 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 75, 100, 150, 200 μg/mL; 50 μL total), incubated on a shaker (2 h, RT), centrifuged (5000 × *g*, 20 min, 4 °C), and the supernatant collected.

ELISA. Solubilized mitochondrial protein stocks (0.5 μg) from KB-B6 were loaded in the wells of microtiter ELISA plates (Greiner Labortechnik) and incubated (overnight, RT). The wells were blocked (0.25% BSA in TBST, 30 min, RT), and supernatants from the back-titration were added (2 h, RT). Again, the plates were blocked (10 min, RT), secondary antibody (anti-mouse (125 ng/mL) or anti-rabbit (250 ng/mL)) was added (2 h, RT), followed by ABTS solution (100 μL). After development of color (30−90 min, RT), $A_{405\ nm}$ was read on a microtiter plate reader. Controls were treated with only secondary antibody to generate a background signal, which was subtracted from each well. $A_{405\ nm}$ of the 0 μg/mL sample in the back-titration was set at 100% unbound antibody, and antibody remaining in the supernatant for each condition was determined by dividing this signal by the unbound signal.

[^{125}I]-IAAP Accumulation Assays. [^{125}I]-IAAP (3−5 nM, 2200 Ci/mmol; Perkin-Elmer Life Sciences Wellesley, MA) was added to KB-CV or KB-B6 cells (32 °C, 30 min under subdued light to avoid photocross-linking). After incubation, cells were washed with PBS and lysed (trypsin/EDTA, 37 °C, 30 min). Lysates were transferred to scintillation vials containing Bio-Safe II scintillation fluid (15 mL), and radioactivity was measured. Cells washed with PBS immediately after addition of the [^{125}I]-IAAP were used as the 0 min time point.

Fluorescent Accumulation Assay. KB-CV or KB-B6 cells (3×10^5) in phenol red-free IMDM with 5% FBS were incubated with hemin, coproporphyrin III (CPIII), and PhA (5 μM, 37 °C, 45 min). Cells were pelleted, resuspended in PBS/BSA, and analyzed immediately with a FACSort flow cytometer (Becton-Dickinson Immunocytometry systems, San Jose, CA). For all samples, 10,000 events were counted, and analysis was performed with CellQuest software (Becton-Dickinson Immunocytometry systems, San Jose, CA). The mean fluorescence intensity was calculated using statistics in CellQuest software.

RESULTS

Two Distinct Molecular Weight Forms of ABCB6. ABCB6 cDNA was inserted in a p3×FLAG-CMV-14 expression plasmid, placing a FLAG-tag on the C-terminus. KB-3-1 cells were stably transfected with either the ABCB6-containing vector (KB-B6) or an empty control vector (KB-CV)[2]. Crude membranes were prepared from these cells and analyzed by Western blot using a commercially available anti-FLAG antibody (Figure 1a). As expected, the anti-FLAG antibody only reacted with extracts from the KB-B6 cell line, recognizing a 104 kDa protein, similar to the predicted molecular weight of ABCB6 with the 3× C-terminal FLAG tag (97 kDa). Additionally, another band appeared at 79 kDa.

Using three novel ABCB6-specific antibodies ($ABCB6_{567}$, $ABCB6_{788}$, or $ABCB6_{7474}$), we confirmed that both protein bands were ABCB6. Irrespective of the antibody used, two reactive bands appeared, including light labeling of bands in the KB-CV cell line (Figure 1b−d). This demonstrated that all four antibodies specifically recognize ABCB6, that the reaction with the lower molecular weight band is a specific interaction, and that both the 104 kDa (ABCB6-H) and 79 kDa (ABCB6-L) protein bands are ABCB6.

To determine if the two ABCB6 forms resulted from either overexpression or the presence of the FLAG tag, membranes from cells expressing high levels of endogenous ABCB6 mRNA (*6*) were isolated and analyzed by reaction with the anti-$ABCB6_{567}$ monoclonal antibody (Figure 1e). Intriguingly, HS-578T cells, which have high ABCB6 mRNA levels, express undetectable amounts of ABCB6 protein. SNB-19 and U251 cells express both forms, indicating the FLAG-tagged ABCB6 is processed in a manner similar to that of the endogenous protein. Varying protease inhibitors or sample preparation techniques did not alter these observations. Thus, we conclude that two protein forms of ABCB6 exist in the cell.

ABCB6-H Is Not a Glycosylated Form of ABCB6-L. To determine if ABCB6-H is a glycosylated form of ABCB6-L, KB-B6 crude membranes (Figure 2, lane 1) were incubated with an *N*-glycosidase (PGNase F, Figure 2, lane 2) or *O*-glycosidase (Figure 2, lane 3). Incubation with PGNase F decreased the size of ABCB6-H to 99 kDa but did not affect ABCB6-L. Additionally, no variation in the size of either ABCB6-H or ABCB6-L occurred after incubation with *O*-glycosidase. Thus, ABCB6-H, but not ABCB6-L, is N-glycosylated. However, removal of sugar residues from ABCB6-H did not lead to the generation of ABCB6-L, indicating that ABCB6-H is not a glycosylated version of ABCB6-L.

ABCB6 Is Localized in Both Mitochondria and Plasma Membranes. ABCB6 is believed to be localized to mitochondria (*11*, *15*). Our ABCB6-specific antibodies provided a new tool to determine the subcellular location of endogenous ABCB6 in various cellular membranes purified from KB-B6, SNB-19, and U251 cells, as detailed in the Experimental Procedures section. The identity and purity of the specific membrane fractions were confirmed with antibodies; anti-nucleoporin p62 identified nuclear membranes, anti-GM130 identified the Golgi apparatus, anti-Lamp-1 monitored lysosomes, anti-complex III identified mitochondrial membranes, anti-integrinα2/VLA-2α ascertained the plasma membrane, anti-BiP/GRP78 identified ER membranes, and anti-ABCB6$_{788}$ detected ABCB6.

As expected, whole cell lysates (Figure 3, lane 1) reacted with the entire panel of antibodies, indicating the presence of all cellular membranes as well as both ABCB6 forms. Analysis of the low speed pellet (Figure 3, lane 2) revealed that all membranes and both ABCB6 forms were present in this fraction, probably because of unlysed cells collected along with the nuclei. Analysis of the medium speed pellet (Figure 3, lane 3) revealed the presence of lysosomes and mitochondria as expected but also nuclei, plasma membranes, and ER. The mitochondria were further purified using an iodixanol gradient, and the fraction at the 30%/10% interface reacted with the anti-complex III antibody but not with other organelle specific antibodies, indicating that only mitochondria remained in the fraction (Figure 3, lane 4). Reaction with the anti-ABCB6$_{788}$ antibody revealed that ABCB6-L is the predominant form in the mitochondria of all three cell lines. Analysis of the high speed pellet (microsomal membranes, Figure 3, lane 5) indicated that only plasma and ER membranes remained in the sample. Additionally, ABCB6-H was highly enriched in this sample. To separate these two membranes, a sucrose gradient was used. The protein band appearing at the 53.5%/43.5% interface was analyzed (Figure 3, lane 6) and found to contain only plasma membranes, as determined by reaction with only anti-integrin α2/VLA-2α. This fraction also reacted with the anti-ABCB6$_{788}$ antibody, indicating the presence of predominantly ABCB6-H in the plasma membrane of all three cell lines. The protein fraction at the bottom of this sucrose gradient contained only ER membranes, as it reacted with only the anti-BiP/GRP78 antibody (Figure 3, lane 7). ABCB6 was not present in the ER membranes, as indicated by a lack of reaction with the anti-ABCB6$_{788}$ antibody. The fraction from the top of the gradient (Figure 3, lane 8) and the soluble proteins (high speed supernatant, Figure 3, lane 9) showed no reaction with the membrane-specific or anti-ABCB6$_{788}$ antibodies (Figure 3, lane 8).

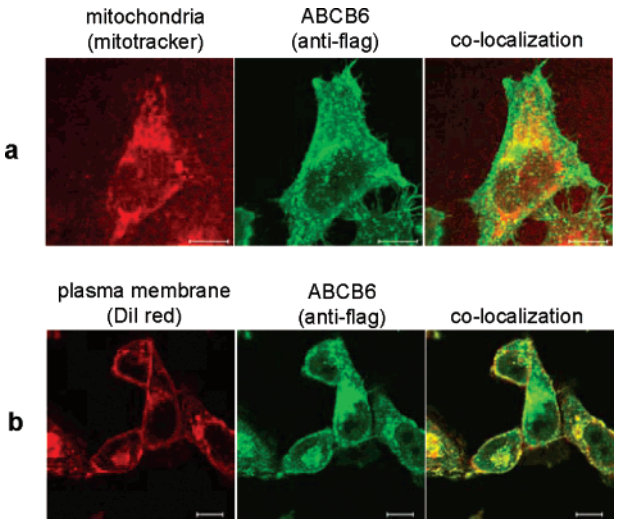

FIGURE 4: Confocal microscopy confirms the localization of ABCB6 in the mitochondria and plasma membranes. (a) Mitochondria of KB-B6 cells were labeled with MitoTracker (red), and ABCB6 was labeled with anti-FLAG M2 antibody (green). An overlay of the MitoTracker and anti-FLAG M2 emissions (yellow) shows co-localization of the two labels. (b) Plasma membranes of KB-B6 cells were labeled with anti-wheat germ agglutinin (red), and ABCB6 was labeled with anti-FLAG M2 (green). Overlaying the two emissions (yellow) demonstrates co-localization of the dyes. The scale bar represents 10 μm.

In summary, ABCB6 was determined to reside in both the mitochondrial and plasma membranes of KB-B6, SNB-19, and U251 cells. While both forms are present in each membrane, ABCB6-L is enriched in mitochondria, whereas ABCB6-H is dominant in the plasma membrane.

Confocal Microscopy Confirms the Presence of ABCB6 in Both Mitochondrial and Plasma Membranes. To verify the dual localization of ABCB6, confocal microscopy was performed. MitoTracker Deep Red 633 (*29*) was used to label mitochondria of KB-B6 cells (Figure 4a, left panel) and anti-FLAG M2 to label ABCB6 (Figure 4a, middle panel). The overlay (yellow) demonstrates that ABCB6 is present in mitochondria (Figure 4a, right panel). In separate cells, wheat germ agglutinin Alexa 555 was used to specifically label the plasma membrane of KB-B6 cells (Figure 4b, left panel) and anti-FLAG M2 (Figure 4b, middle panel) to label ABCB6. Again, the two labels overlapped (yellow), demonstrating co-localization of both labels (Figure 4b, right panel) and confirming ABCB6 localization to the plasma membrane.

ABCB6 Resides in the Outer Mitochondrial Membrane. Mitochondria contain an outer and an inner membrane separated by intermembrane space. To determine which mitochondrial membrane contains ABCB6, we used digitonin solubilization (*30*, *31*); the addition of low levels of digitonin to intact mitochondria will only solubilize the outer mitochondrial membrane, leaving the inner mitochondrial membrane intact. Increasing amounts of digitonin were added to purified mitochondria aliquots (Figure 5, lane 1). Following incubation, the samples were centrifuged, generating supernatants (Figure 5, *s*) containing solubilized membranes and pellets (Figure 5, *p*) containing non-solubilized, intact membranes. Outer mitochondrial membrane solubilization

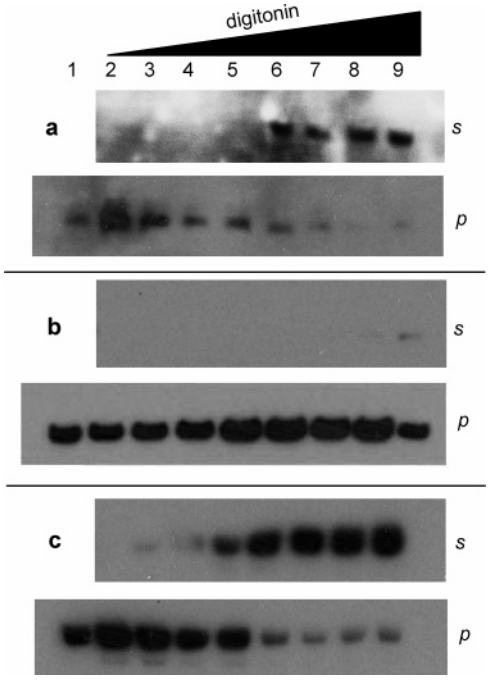

FIGURE 5: ABCB6 resides in the outer mitochondrial membranes. Purified mitochondria from KB-B6 cells were intact (lane 1) or subjected to increasing concentrations of digitonin (lane 2−9) and centrifuged to separate solubilized membranes (supernatants (s)) and intact membranes (pellets (p)). Proteins in the supernatant and pellet were separated by SDS−PAGE, transferred to a PVDF membrane, and reacted with (a) anti-porin, an antibody specific for the outer mitochondrial membrane protein porin, (b) anti-complex III, an antibody specific for the inner mitochondrial membrane−protein complex III, or (c) anti-FLAG, an antibody that recognizes FLAG-tagged ABCB6.

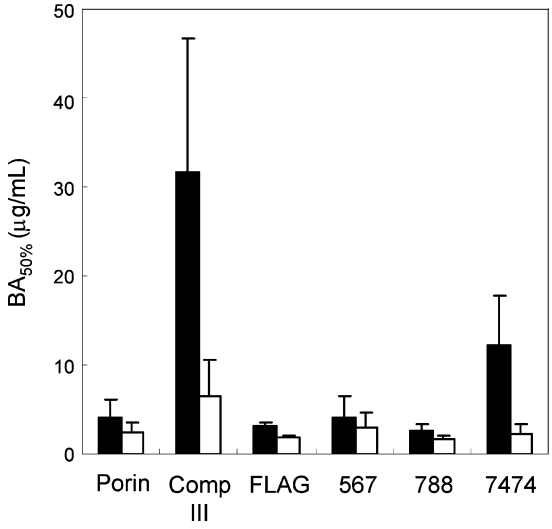

FIGURE 6: NBD of ABCB6 is cytoplasmic. The amount of intact (filled) or permeabilized (open) mitochondrial protein required to bind half ($BA_{50\%}$) of each specified antibody (indicated on the *x*-axis) was determined from the curve fits (Figure S2, Supporting Information; $N = 3$). A difference between intact and permeabilized mitochondria indicates that the epitope is in the intermembrane space, while no difference between the two preparations indicates a cytoplasmic epitope.

was monitored by following the solubilization of porin, an outer mitochondrial membrane protein, with an anti-porin monoclonal antibody (Figure 5a). As expected, increasing digitonin concentration (Figure 5, lanes 2−9) increased the solubilization of porin, indicating solubilization of the outer mitochondrial membrane. Solubilization of the inner mitochondrial membrane was monitored by following the solubilization of complex III, an inner mitochondrial membrane protein, with an anti-complex III monoclonal antibody (Figure 5b). Even at the highest digitonin concentration, very little complex III was present in the supernatant, indicating a lack of inner mitochondrial membrane solubilization.

ABCB6 solubilization was monitored using the anti-FLAG antibody. As digitonin concentration increased, the level of ABCB6 in the supernatant increased (Figure 5c), consistent with porin and outer mitochondrial membrane solubilization but not with complex III and inner mitochondrial membrane solubilization, indicating that ABCB6 is expressed in the outer mitochondrial membrane. Note that only ABCB6-L is observed in this blot, as detection of ABCB6-H requires longer exposure, which is not compatible with precise quantitation. ABCB6-H was solubilized in a similar manner, with complete solubilization achieved at 1.0 mg digitonin/mg protein.

Nucleotide Binding Domain of ABCB6 Faces the Cytoplasm. A back-titration assay was used to determine the NBD orientation of ABCB6 in the outer mitochondrial membrane. To increasing amounts of mitochondria (0−400 μg/mL) prepared with either intact or permeabilized outer mitochondrial membranes, antibodies were added at a single specific concentration. Generation of intact and permeabilized outer mitochondrial membranes was determined by the release and oxidation of cytochrome *c* (Fe(II) → Fe(III)) as monitored by a decrease in A_{550nm} (*23*) (Figure S1, Supporting Information). After incubation, mitochondria (and bound antibody) were removed with centrifugation, supernatants were collected, and the amount of *unbound* antibody was measured with ELISA. The ELISA signal obtained following incubation in the absence of mitochondria was set to 100%, and all other values are presented relative to this reading. Plotting the percentage of unbound antibody as a function of mitochondrial protein leads to typical titration curves, which were fit to a standard first-order equation (Figure S2, Supporting Information), and the protein amount required to bind half the antibody ($BA_{50\%}$) was determined (Figure 6).

If an epitope is cytoplasmic, it is accessible in both intact and permeabilized mitochondrial samples, and $BA_{50\%}$ will be similar under both conditions. If the epitope is in the intermembrane space, it will only be accessible in permeabilized samples; thus, $BA_{50\%}$ will be lower for the permeabilized sample as compared to an intact sample, as antibody binding in the intact sample reflects only nonspecific binding.

Two antibodies were employed in control experiments. The anti-porin antibody recognizes a cytoplasmic epitope (*26*, *28*, *32*, *33*). As expected, similar $BA_{50\%}$ results were obtained for both intact (4.1 ± 2.0 μg/mL) and permeabilized (2.5 ± 1.0 μg/mL) mitochondria. The anti-complex III antibody (clone 16D10) has an intermembrane space epitope, which explains the different $BA_{50\%}$ values for the two mitochondrial preparations (31.8 ± 15 μg/mL for intact and 6.5 ± 4.0 μg/mL for permeabilized).

Identical comparisons were made with the anti-ABCB6 antibodies. In the case of anti-FLAG, there was no difference between intact (3.1 ± 0.3 μg/mL) and permeabilized (1.9 ± 0.2 μg/mL) $BA_{50\%}$ values, indicating that the C-terminal

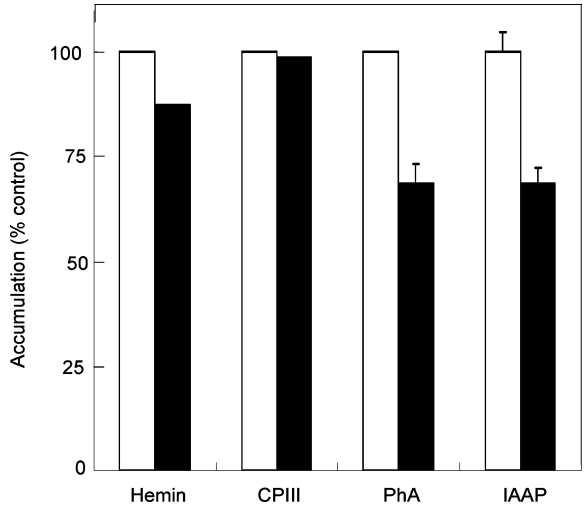

FIGURE 7: Expression of ABCB6 in plasma membranes leads to decreased accumulation of specific porphyrins. KB-CV (open bars) and KB-B6 (filled bars) cells were incubated with the indicated porphyrin, and total accumulation (pmol/10^6 cells) was measured as described in the text. Data is presented as the percent accumulation in KB-CV cells.

FLAG-epitope is cytoplasmic. Similarly, in the case of anti-$ABCB6_{567}$ and anti-$ABCB6_{788}$ antibodies, there was no difference between the intact (4.1 ± 2.4 and 2.7 ± 0.6 μg/mL, respectively) and permeabilized (2.9 ± 1.7 and 1.6 ± 0.4 μg/mL, respectively) samples, indicating that these epitopes are also cytoplasmic. For the anti-$ABCB6_{7474}$ antibody, large differences between the intact (12.3 ± 5.6 μg/mL) and permeabilized (2.2 ± 1.2 μg/mL) samples occurred, indicating an intermembrane space epitope.

Plasma Membrane ABCB6 Is Functionally Active. A recent report has elucidated the function of ABCB6 in mitochondria (*15*). To determine if the plasma membrane form of ABCB6 has a similar function, we measured the cellular accumulation of various porphyrins. We incubated cells with three porphyrin compounds (hemin, CPIII, and PhA) shown by Krishnamurthy et al. (*15*) to accumulate in mitochondria expressing ABCB6. Since PhA is an ABCG2 substrate (*34*), we also screened the ABCG2 substrate IAAP. Accumulation levels in KB-B6 cells were compared to KB-CV after 30 min (Figure 7). ABCB6 expression led to a reduction in PhA (69%) and IAAP (68%) cellular accumulation, a slight reduction in hemin (87%) accumulation, and no difference in CPIII (99%) accumulation. This indicates that ABCB6 expression in the plasma membrane leads to a decreased accumulation of certain porphyrins and that it may be involved in the cellular efflux of porphyrins from the cell, in addition to its previously reported role in mitochondria (*15*).

DISCUSSION

ABCB6 is reported to be a mitochondrial transporter (*11*, *15*) involved in porphyrin transport (*15*), iron homeostasis (*11*), or resistance to cytotoxic agents (*6*, *8*−*10*). Because of its uncertain physiological role, we decided to seek additional information about its function from studies on subcellular localization. This work shows that ABCB6 localizes both to mitochondrial and plasma membranes. Two protein forms were detected, with the higher molecular weight form predominant in plasma membrane and the lower molecular weight form predominant in mitochondria. We determined that the mitochondrial form resides in the outer mitochondrial membrane, with the NBD facing the cytoplasm. Furthermore, we show that plasma membrane bound ABCB6 may be functionally similar to mitochondrial ABCB6.

KB-B6 cell lines were created to stably express high levels of ABCB6. ABCB6 reacted with four different antibodies, one specific for a FLAG-tag, and three developed to specifically recognize ABCB6. Upon reaction with all four antibodies, two protein bands appeared. In addition to the band migrating at 104 kDa (ABCB6-H), near the predicted molecular weight, a second band (79 kDa, ABCB6-L) was observed in samples prepared from cells expressing endogenous or cDNA-derived ABCB6. The presence of lower molecular weight bands could indicate proteolysis, alternative transcripts, or post-translational modification. Since varying protease inhibitors or sample preparation techniques did not prevent the appearance of ABCB6-L, this form most likely represents a post-transcriptionally or post-translationally modified form. Reaction of both bands with the anti-FLAG antibody indicates that both forms are generated from the cDNA construct, suggesting that the lower band results from post-translational, not post-transcriptional, modification. While PNGase F addition indicates that ABCB6-H is *N*-glycosylated, removal of the sugars does not lead to the generation of ABCB6-L, indicating that ABCB6-H is not simply a glycosylated version of ABCB6-L.

The *S. cerevisiae* functional orthologue of ABCB6, Atm1p, has been shown to localize to the inner mitochondrial membrane with the nucleotide binding domain facing the matrix (*13*). Because of its sequence homology to Atm1p (39% identical, 58% similar), ABCB6 was hypothesized, and a FLAG-tagged version was shown to reside in mitochondria (*11*, *15*). Because expression systems may be associated with specific artifacts, such as derailed targeting, we assessed the endogenous location of ABCB6 in cells expressing high ABCB6 mRNA levels. In agreement with the previous reports (*11*, *15*), using both cell fractionation and confocal microscopy, we show that not only FLAG-tagged ABCB6 but also endogenous ABCB6 resides in the mitochondria.

Our results showing outer mitochondrial localization of ABCB6 were unexpected for several reasons: (1) The three *S. cerevisiae* mitochondrial ABC transporters (Atm1p, Mdl1p, Mdl2p (*13*, *35*, *36*)) and the mouse homologue of the human transporter ABCB10 (ABC-me (*37*)) have been shown to localize to the inner mitochondrial membrane. Thus, we expected ABCB6 to reside in the inner mitochondrial membrane. (2) The outer mitochondrial membrane contains numerous aqueous channels, making the membrane permeable to most molecules <6000 Da in size (*38*), the size of a typical ABC substrate. In such a context, an active transporter may be superfluous. (3) ABCB6 was reported to be a functional orthologue of the inner mitochondrial membrane protein Atm1p (*11*). While this article was in preparation, a paper by Krishnamurthy and colleagues appeared reporting similar outer mitochondrial membrane localization in an independent cell line (*15*).

Notwithstanding the differences between yeast and human mitochondria, the fact that functional orthologues Atm1p and ABCB6 reside in different mitochondrial membranes is puzzling. However, two additional mitochondrial proteins,

human ABCB7 and yeast Mdl1p, have also been shown to be functional orthologues of Atm1p (*39*−*41*), while maintaining distinct roles. Mutation in ABCB7, but not ABCB6 or Mdl1p, leads to a rare form of X-linked sideroblastic anemia associated with ataxia (*40*, *42*, *43*), and only Mdl1p, not ABCB6 or ABCB7, has been implicated in peptide transport (*36*), indicating that these proteins have independent roles in mammalian cells. Thus, while some functions of the mitochondrial transporters may overlap regardless of membrane location, they may each also have a unique cellular role.

Intriguingly, we find a distinct form of ABCB6 in the plasma membrane of KB-B6, SNB-19, and U251 cells. While a dual localization of this protein might be surprising, this cellular localization is in agreement with reports that indicate that ABCB6 is present in red blood cells (*44*, *45*), cells which possess only a plasma membrane.

The expression of ABCB6 in the plasma membrane results in decreased accumulation of PhA, IAAP, and hemin in the cell, indicating that it may play a similar functional role in the plasma membrane as reported in mitochondria (*15*). While Krishnamurthy and colleagues speculated that CPIII was the most probable substrate for mitochondrial ABCB6 (*15*), we found that plasma membrane ABCB6 expression did not affect CPIII accumulation. Thus, it appears that the two molecular weight forms and two cellular locations may transport similar but also distinctive substrates. Plasma membrane ABCB6 may work to protect cells from the toxic effects of PhA (found naturally in foods (*46*−*49*)), similar to the role proposed for ABCG2 (*50*). Additionally, this finding may explain its appearance in red blood cells, where if porphyrin levels are not well controlled, symptoms similar to those of the genetic disorder erythrohepatic protoporphyria (*51*−*53*) occur.

How ABCB6 is trafficked to different cellular membranes is currently unknown. Many mitochondrial targeted proteins possess targeting peptide signals (*54*, *55*). Two web-based programs (*56*, *57*) assess the presence of a mitochondrial targeting sequence and indicated that the inner mitochondrial membrane proteins Atm1p (probability of 0.935 out of 1.000), ABCB7 (0.867), ABCB8 (0.923), Mdl1p (0.832), Mdl2p (0.950), and ABC-me (0.907) possess N-terminal mitochondrial targeting sequences, and extensive biochemical work confirms the importance of this sequence for mitochondrial targeting (*13*, *37*, *39*, *58*, *59*). Conversely, ABCB6 lacks a mitochondrial localization peptide (probability of 0.021), with a similar value obtained for human ABCB10 (0.116). It is possible that the lack of a targeting signal allows for ABCB6 to be trafficked to different membranes, including the plasma and outer mitochondrial membranes. Targeting of proteins that traverse the outer mitochondrial membrane numerous times is reported to be structurally specific and not due to a specific amino acid sequence (*60*−*62*).

We determined that the NBD domain of ABCB6 is cytoplasmic. This is also the case with other outer mitochondrial membrane proteins where the bulk of the protein extends into the cytoplasm (*60*), regardless of whether they are N-terminally linked (*63*), C-terminally linked (*64*), possess only two transmembrane domains (*65*), or traverse the membrane numerous times (*66*).

Finally, we propose a structural model for ABCB6 (Figure 8). Initial models were generated by hydropathy analyses

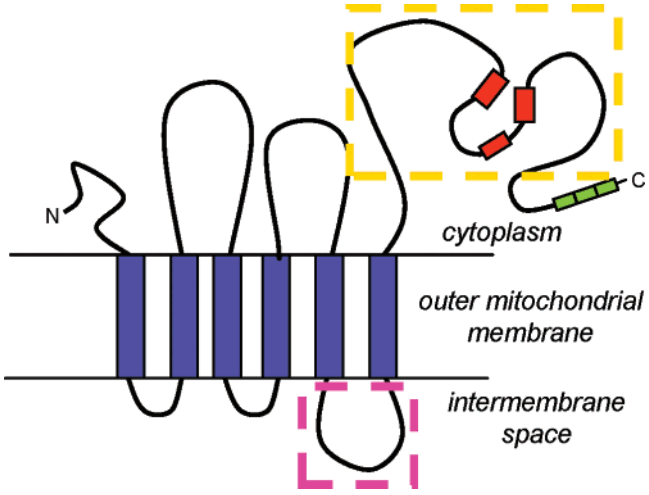

FIGURE 8: Model of ABCB6 orientation in mitochondria. Using various hydropathy calculation programs, comparison modeling to P-gp, and improvement with the back-titration data, ABCB6 is predicted to have six transmembrane segments. Both termini and the NBD are cytoplasmic, while the RAMNTQENATRARAVD sequence is in the intermembrane space. Solid green rectangles denote the FLAG-tag, the dotted yellow square indicates the anti-$ABCB6_{567}$ and anti-$ABCB6_{788}$ epitopes, the solid red rectangles represent the three major conserved components of the NBD, while the dotted purple square indicates the anti-$ABCB6_{7474}$ epitope.

(*67*−*70*), modified on the basis of alignment with a P-gp model based on biochemical and crystallographic data (*71*), and finally modified on the basis of our back-titration data. We predict that the protein has six transmembrane segments, orienting the termini on the same side of the membrane. Back-titration indicated that the C-terminus (FLAG-tag, represented by solid green rectangles in Figure 8) is cytoplasmic; thus, we predict that the N-terminus will also be cytoplasmic, the NBD is cytoplasmic, and the sequence RAMNTQENATRARAVD (denoted with the dotted purple square) is located in the intermembrane space.

To conclude, this work shows that two distinct molecular weight forms of human ABCB6 exist in all investigated mammalian cells expressing ABCB6. Both forms of the protein localize to the outer mitochondrial membrane, with a cytoplasmic NBD, while the plasma membrane is enriched with ABCB6-H. We have shown that ABCB6 expression in the plasma membrane leads to decreased cellular accumulation of IAAP, PhA, and hemin, indicating the functional relevance of the plasma membrane form. The difference between the two molecular weight species, the trafficking of these proteins, and additional functional roles are currently being investigated.

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SUPPORTING INFORMATION AVAILABLE

Control experiments and curves fits used to calculate the data presented in Figure 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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