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P-GLYCOPROTEIN IS NOT PRESENT IN MITOCHONDRIAL MEMBRANES

Jill K. Paterson and Michael M. Gottesman*

Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, DHHS, Bethesda, MD

Abstract

Recent reports have indicated the presence of P-glycoprotein in crude mitochondrial membrane fractions, leading to the assumption that P-glycoprotein is present in mitochondrial membranes, and may be involved in transport across these membranes. To determine the validity of this claim, two cell lines overexpressing endogenous P-glycoprotein were investigated. Using various centrifugation steps, mitochondria were purified from these cells and analyzed by Western blot reaction with the anti-P-glycoprotein antibody C219 and organelle-specific antibodies. While P-glycoprotein is present in crude mitochondrial fractions, these fractions are contaminated with plasma membranes. Further purification of the mitochondria to remove plasma membranes revealed that P-glycoprotein is not expressed in mitochondria of the KB-V1 (vinblastine resistant KB-3-1 cells) or MCF-7^{ADR} (adriamycin resistant MCF7 cells) cell lines. To further substantiate these findings, we used confocal microscopy and the anti-P-glycoprotein antibody 17F9. This demonstrated that in intact cells, P-glycoprotein is not present in mitochondria and is primarily localized to the plasma membrane. These findings are consistent with the role of P-glycoprotein in conferring multidrug resistance by decreasing cellular drug accumulation. Therefore, contrary to previous speculation, P-glycoprotein does not confer cellular protection by residing in mitochondrial membranes.

Keywords

P-glycoprotein; P-gp; Multidrug resistance; Mitochondria; Cellular localization

Introduction

The simultaneous development of resistance to chemically and structurally distinct drugs, known as multidrug resistance (MDR), contributes to the low success rate of cancer chemotherapy treatments. In part, MDR is conferred by the continued or up-regulation of plasma membrane bound proteins belonging to the ATP binding cassette (ABC) family [1]. To date, 13 transporters have been experimentally shown to confer MDR *in vitro* [2], with additional transporters implicated in MDR through correlative studies [3,4]. These transporters confer resistance by actively transporting drugs across the plasma membrane, decreasing intracellular drug accumulation, and thus decreasing the efficacy of chemotherapy.

Of the 13 transporters implicated in MDR, P-glycoprotein (P-gp, MDR1, ABCB1) is the most widely studied and most prominent form of ABC transporter-mediated MDR [5]. A wide range

*To whom correspondence should be addressed: Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, DHHS, Bethesda, MD, 20892-4256. Email: MGottesman@nih.gov, telephone: 301-496-1530, fax: 301-402-0450

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of drugs, including chemotherapeutic drugs, Vinca alkaloids, anthracyclines, epipodophyllotoxins, antibiotics, cardiac drugs, and HIV protease inhibitors are actively extruded from the cell by P-gp [2].

Several reports have indicated that in addition to localization in the plasma membrane, P-gp may also reside in intracellular membranes. P-gp has been identified in Golgi vesicles and the endoplasmic reticulum [6-11], probably indicating intermediate locations as it is trafficked to the plasma membrane. It has been reported by one group to be present in nuclei [12,13]. More recently, P-gp was observed in crude preparations of mitochondria [14,15], leading to the conclusion that P-gp is present in mitochondria, where it was proposed to either pump drugs out of mitochondria to protect these organelles [15], or pump drugs into mitochondria, leading to the sequestering of drug and protection of the cell [14].

In order to substantiate these findings, we undertook the detailed characterization of cellular compartments using differential centrifugation and Western blotting. We present data to show that P-gp is not localized to mitochondria. Using either KB-V1 cells (KB-3-1 cells selected for resistance to vinblastine [16]) or MCF-7^{ADR} cells (MCF-7 cells selected for resistance to adriamycin [17]), both of which express high levels of endogenous P-gp, we demonstrate that P-gp is present in the crude mitochondrial fraction. However, further purification of the mitochondria reveals that P-gp is not present in mitochondrial membranes, and its presence in the crude mitochondrial fraction is due to plasma membrane contamination. Thus, while MDR is multifaceted, P-gp does not appear able to confer MDR to cells by moving drug substrates across mitochondrial membranes.

Materials and Methods

Reagents

The anti-complex III antibody and Mitotracker Deep Red 633 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-integrin α 2/VLA-2 α , anti-Lamp-1, anti-nucleoporin p62, and P-glycoprotein FITC-conjugated (clone 17F9) antibodies were purchased from BD Biosciences (San Diego, CA). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL). Optiprep iodixanol was purchased from Axis-Shield (Oslo, Norway), Vectashield mounting medium with DAPI was purchased from Vector Laboratories, Inc (Burlingame, CA), and the anti-mouse secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were reagent grade.

Maintenance of cell lines

The KB-V1 and MCF-7^{ADR} cell lines were grown in monolayers at 37°C in 5% CO₂ with DMEM medium (4.5 g of glucose/L) supplemented with 2 mM L-glutamine, 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Purification of mitochondria and plasma membranes

Purified membranes were prepared by modifications of several previously reported methods [18-20]. Cells were washed (PBS, 3×), resuspended in homogenization buffer (HB; 150 mM MgCl₂, 10 mM KCl, 10 mM Tris, pH 6.7, protease inhibitors; 1 mL/ 1 × 10⁷ cells), incubated on ice (15 min), and Dounce homogenized (35 strokes). HB with sucrose (34.2%, 1/3 vol) was added (corresponds to whole cell sample, Fig 1a & 1b, lane 1), and centrifuged ("low" speed, 1000 × g, 5 min, 4°C) to remove nuclei and unlysed cells (resulting pellet corresponds to Fig 1a & 1b, lane 2). The supernatant was centrifuged ("medium" speed, 5000 × g, 10 min, 4°C), the pellet ("crude mitochondria"; Fig 1a & 1b, lane 3) was resuspended in HB + sucrose (20 mL), divided into two samples, and centrifuged (5000 × g, 10 min, 4°C). One resultant pellet

was resuspended in Solution A (3 mL; 20 mM Hepes, 1 mM EDTA, 250 mM sucrose, pH 7.4, Fig 2, lane 3). Iodixanol solution (50% iodixanol, 120 mM Hepes, 6 mM EDTA, 250 mM sucrose, pH 7.4) was added (final concentration of 36%), placed in a centrifuge tube, overlaid with Solution B (10 mL, 30% iodixanol, 80 mM Hepes, 4 mM EDTA, 250 mM sucrose, pH 7.4), then Solution C (to top, 10% iodixanol, 80 mM Hepes, 4 mM EDTA, 250 mM sucrose, pH 7.4), and centrifuged ($50,000 \times g$, 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 ($30,000 \times g$, 10 min, 4°C). The resulting pellet was resuspended in mitochondrial suspension buffer (250 mM sucrose, 10 mM Tris, pH 7.0, Fig 1a & 1b, lane 4) with protease inhibitors.

The second crude mitochondrial pellet was resuspended (resuspension buffer (RB); 3 mL; 10 mM Tris, 0.5 mM EDTA, 10% glycerol, pH 7.5, lane 5) and applied to the top of a sucrose gradient (53.5% sucrose/43.5% sucrose), centrifuged (5 h, $100,000 \times g$, 4°C), and the resulting fraction at the 53.5/43.5% sucrose interface was collected, diluted (RB, 1 volume) and centrifuged ($120,000 \times g$, 1 h, 4°C). The pellets (Fig 1a & 1b, lane 5) were resuspended in TSNa buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 250 mM sucrose) with protease inhibitors.

Western blotting

SDS-PAGE was performed as recommended for Novex® Tris-Glycine Gels (Invitrogen, Carlsbad, CA), and samples were transferred to PVDF membranes. The membrane was blocked (20% w/v milk), rinsed (TBST, 3×10 min), incubated with the indicated primary antibody (overnight, RT; anti-P-gp (C219) 1 µg/mL, anti-complex III 0.1 µg/mL, anti-BiP/GRP78 1:250, anti-EEA1 1:2500, anti-GM130 1:250, anti-integrin α 2/VLA-2 α 1:250, anti-Lamp-1 1:250, or anti-nucleoporin p62 1:1000), rinsed (TBST, 4×10 min), incubated with secondary antibody (1:10,000, 1 h, RT), rinsed (TBST, 4×10 min), incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, 5 min), and exposed to Bio-Max MR film (Eastman Kodak Co).

Confocal microscopy using MitoTracker® Deep Red 633 and anti-P-gp (17F9) antibody

To label mitochondria, Mitotracker Deep Red 633 in DMEM medium (30 pg/mL) was added to live cells on a glass chamber slide (30 min, 37°C). Fresh medium was added, and the cells incubated (2×10 min, 37°C). To fix and permeabilize the cells, paraformaldehyde (3.7% in PBS/BSA (0.1%)) was added (20 min, RT). Following washing ($3 \times$ PBS/BSA) and blocking (10 min, RT, PBS/BSA), cells were incubated with the anti-P-gp antibody 17F9 (100 µL/mL, 1 h, RT). Cells were washed ($3 \times$ PBS/BSA), air dried, and Vectashield with Dapi was added. Fluorescent cells were examined using a Zeiss LSM 510 NLO (2-photon) Confocal Microscope with Meta detector (Carl Zeiss Inc, Thornwood, NY, USA) with an Axiovert 200M inverted microscope and Plan-Apochromat 63 $\times$ / 1.4 NA DIC oil immersion objective. With the Zeiss Aim software version 4.0 sp2, images were collected using a multi-track configuration where the FITC, Mitotracker deep red, and DAPI emissions were collected sequentially with a BP 500-550 IR filter, custom BP 641-705 Meta filter, and a BP 435-485 IR filter after sequential excitation with 488 nm, 633 nm and 740nm laser lines, respectively.

Results

P-gp is present in “crude” mitochondrial preparations from KB-V1 cells

To investigate the presence of P-gp in mitochondria, cellular membranes were collected using sequential differential centrifugation and sucrose gradient steps as detailed in the Materials and Methods section. The purity of each fraction was determined by reaction with antibodies selective for proteins present in specific cellular membranes. The whole cell fraction of KB-V1 cells (Fig 1a, lane 1) reacted with all membrane specific antibodies, indicating all cellular

membranes and P-gp are present in the starting lysate, as expected. The lysate was subjected to a low speed centrifugation step and the resulting pellet (Fig 1a, lane 2) contained all cellular membranes and P-gp (Fig 1a, lane 2), most likely indicating the presence of unlysed cells. The remaining supernatant was further centrifuged ($5000 \times g$, 10 min) resulting in a crude mitochondrial fraction (Fig 1a, lane 3). This pellet reacted with anti-complex III antibodies, indicating the presence of mitochondria. However, this fraction also reacted with anti-nucleoporin p62, anti-GM130, anti-BiP/GRP78, anti-LAMP, and anti-integrin α 2/VLA-2 α , indicating the presence of nuclear, Golgi, lysosomal, plasma, and ER membranes, respectively. Positive reaction with C219 [21,22] indicates the presence of P-gp in this crude mitochondrial fraction, but the membrane compartment that contained P-gp could not be determined due to the heterogeneity of this fraction.

P-gp is not present in purified mitochondria preparations of KB-V1 cells

To determine if P-gp is present in the mitochondrial membranes of the crude mitochondrial fraction, we purified the mitochondrial membranes using an iodixanol gradient. Protein residing at the 30%/10% interface was analyzed by Western blot (Fig 1a, lane 4), and reacted with *only* the anti-complex III antibody, indicating that the only cellular membranes present in this fraction are mitochondrial. A lack of reaction with the anti-P-gp antibody C219 indicates that P-gp is not present in mitochondria of KB-V1 cells.

P-gp is present in purified plasma membrane preparations from KB-V1 cells

In an effort to assign the P-gp present in the crude mitochondria preparation to a specific membrane, we purified a portion of the crude mitochondrial membranes with a sucrose gradient. Analysis of the band at the 53.5%/43.5% interface indicated reaction with *only* the anti-integrin α 2/VLA-2 α antibody (Fig 1a, lane 5), confirming the generation of purified plasma membranes. A positive reaction with C219 indicates the presence of P-gp in the plasma membrane. Thus, the P-gp present in the crude mitochondrial fraction is due to the presence of contaminating plasma membranes in the fraction, and not the presence of mitochondrial membranes.

P-gp is not present in purified mitochondrial preparations from MCF-7^{ADR} cells

Identical localization results were obtained with the MCF-7^{ADR} cell line. Briefly, a positive reaction of the crude mitochondrial membrane fraction (Fig 1b, lane 3) with C219 indicates the presence of P-gp in this fraction. However, positive reaction with anti-nucleoporin, GM130, BiP/GRP78, LAMP, complex III, integrin α 2/VLA-2 α indicates this fraction contains not only mitochondrial, but also nuclear, Golgi, lysosomal, plasma, and ER membranes. Again, we purified the mitochondrial membranes from this crude mitochondrial fraction with an iodixanol gradient. As with the KB-V1 cells, the protein band at the 30%/10% interface contained *only* mitochondrial membranes, as apparent by reaction with only anti-complex III antibodies (Fig 1b, lane 4). The lack of reaction with C219 indicates that P-gp is not present in the mitochondria of MCF-7^{ADR} cells. Plasma membranes were purified from the crude mitochondrial fraction (Fig 1b, lane 5), and reacted *only* with the anti-integrin α 2/VLA-2 α antibody, indicating its purity. P-gp is present in the plasma membrane of these cells, as evident by reaction of C219 with this purified fraction. Again, the presence of P-gp in the crude mitochondrial fraction is not due to its expression in mitochondrial membranes, but due to contamination of the fraction with plasma membranes.

Confocal microscopy confirms the lack of P-gp in KB-V1 and MCF-7^{ADR} mitochondria

To confirm a lack of P-gp in purified mitochondria is indicative of intact cells, we used confocal microscopy. Both KB-V1 (Fig 2a) and MCF-7^{ADR} (Fig 2b) cell lines were labeled with Mitotracker Deep Red (red) to specifically label mitochondria. Following fixation/

permeabilization to ensure antibody accessibility to intracellular epitopes, the cells were reacted with 17F9-FITC to specifically label P-gp (green). DAPI (blue) was also included to specifically label nuclei and ensure permeabilization of the cells. No overlap of mitotracker red with FITC green was observed, indicating P-gp is not present in the mitochondria of these cells.

Discussion

In studying the role of P-gp in drug resistance, pharmacokinetics, or normal cell physiology, it is important to have a correct understanding of the basic cellular biology of P-gp. Following two recent reports that P-gp is present in mitochondria [14,15], we performed detailed studies to determine whether P-gp does, indeed, localize to mitochondria, and whether this location should be considered when studying the role of P-gp in cellular processes or when studying transport across mitochondrial membranes.

We found that purification of mitochondria by differential centrifugation alone is not adequate for mitochondrial-specific studies. The mitochondrial fractions used in the previous studies which assigned P-gp to mitochondrial membranes were contaminated with other cellular membranes [14,15]. Their mitochondrial fractions corresponded to our “crude” mitochondrial fraction; our crude mitochondrial fraction was generated by centrifugation at $5000 \times g$ for 10 min, while the mitochondrial fractions presented in the other reports were collected by centrifugation at $10,000 \times g$ for either 10 [14] or 20 [15] minutes. Thus, any contaminants present in our crude mitochondrial fraction, produced at a lower g -force, should also be present in the mitochondrial fractions used in the cited studies. Our crude mitochondrial fraction contained not only mitochondria, but other cellular membranes, including P-gp-rich plasma membranes.

In agreement with the previous reports, we identified P-gp in this mitochondrial-enriched crude fraction, but took further steps to determine if the P-gp present in this fraction was located in mitochondrial membranes. Following purification on an iodixanol gradient, the fraction reacted only with anti-complex III, and no other membrane specific antibodies, indicating a successful separation of mitochondrial membranes from other cellular membranes. This fraction did not react with the anti-P-gp antibody C219, indicating that P-gp was not present in the mitochondria of either KB-V1 or MCF-7^{ADR} cells. Therefore, the assignment of P-gp to mitochondrial membranes by the previous reports was incorrect.

To properly assign the P-gp present in the crude mitochondrial fraction to a cellular membrane, we took a fraction of the crude mitochondrial samples, and using a sucrose gradient, purified the plasma membranes from the other cellular membranes, as indicated by reaction of the resulting fraction with only the anti-integrin α 2/VLA-2 α antibody and no other cellular membrane markers. Reaction of this fraction with the anti-P-gp antibody C219 indicated that P-gp was present in the plasma membranes of both KB-V1 and MCF-7^{ADR} cells.

Both previous reports [14,15] used confocal microscopy to confirm the localization of P-gp to mitochondria. While those authors claim P-gp labeling overlaps with mitochondria-specific labels, a lack of P-gp labeling at the plasma membrane, where P-gp is known to reside [5], may indicate technical problems with those labeling studies. Additionally, interpretation of data obtained with the C219 monoclonal antibody using confocal microscopy [15] needs to be performed with caution, as C219 recognizes several different proteins, including MDR3 and myosin [23-25], and when not coupled with correct molecular weight identification, as done with Western blot, can lead to false positive results.

Using confocal microscopy, we confirmed that P-gp is not present in the mitochondria of either KB-V1 or MCF-7^{ADR} cells. Following fixation/permeabilization, reaction of the P-gp specific

antibody 17F9-FITC clearly labels the plasma membrane, as expected, indicating reaction with P-gp. No intracellular labeling is observed with this antibody, but staining with DAPI indicates the cells are permeable to the 17F9 antibody; thus if any intracellular P-gp epitopes are present (including mitochondrial or nuclear), they should be accessible to 17F9-FITC. Additionally, no overlap between the mitochondria-specific label Mitotracker and 17F9-FITC was observed in either KB-V1 or MCF7^{ADR} cells, confirming that P-gp is not present in the mitochondria of these cell lines. These results confirm all of the original immunolocalization studies on P-gp, none of which suggested mitochondrial localization [23,26-28].

While the exact cell lines used in our study and the two previous reports (P5 hepatocellular carcinoma [15] or K562 [14]) differ, in each case the up-regulation of P-gp resulted from selection for doxorubicin (adriamycin) resistance. We also added a cell line selected for resistance to vinblastine. Regardless of the cell line (KB-3-1 or MCF-7) or the drug (doxorubicin or vinblastine) used during selection and up-regulation of P-gp, we did not observe a variation in the cellular localization of P-gp. Furthermore, we believe the assignment of P-gp to mitochondrial membranes in the previous reports was not due to the cell lines used, but due to the fact that purified mitochondria were never obtained.

We believe the P-gp-mediated mitochondrial transport reported in the previous studies [14, 15] is an artifact of the plasma membrane contamination in the mitochondrial fractions. Purified mitochondria were not isolated in the previous studies, thus the functional assays carried out were with mixtures of membranes. Transport data presented in these previous studies very likely represents transport across plasma membrane vesicles present in the crude mitochondrial fraction. Furthermore, upon vesicle formation, there are two possible protein orientations for a membrane protein: right-side out or wrong-side out. This variation in protein orientation may account for the observed inward transport of one study [14], and outward transport [15] of the other.

In conclusion, P-gp is *not* present in mitochondrial membranes. Therefore, the movement of drugs, or other substances, into or out of the mitochondria is not P-gp-dependent, and the numerous effects of P-gp in the cell are not due to a subcellular localization in mitochondria.

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References

1. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48–58. [PubMed: 11902585]
2. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006;5:219–34. [PubMed: 16518375]
3. Annereau JP, Szakacs G, Tucker CJ, Arciello A, Cardarelli C, Collins J, Grissom S, Zeeberg BR, Reinhold W, Weinstein JN, Pommier Y, Paules RS, Gottesman MM. Analysis of ATP-binding cassette transporter expression in drug-selected cell lines by a microarray dedicated to multidrug resistance. *Mol Pharmacol* 2004;66:1397–405. [PubMed: 15342794]
4. Szakacs G, Annereau JP, Lababidi S, Shankavaram U, Arciello A, Bussey KJ, Reinhold W, Guo Y, Kruh GD, Reimers M, Weinstein JN, Gottesman MM. Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. *Cancer Cell* 2004;6:129–37. [PubMed: 15324696]
5. Ambudkar SV, Kim IW, Sauna ZE. The power of the pump: mechanisms of action of P-glycoprotein (ABCB1). *Eur J Pharm Sci* 2006;27:392–400. [PubMed: 16352426]
6. Fu D, Bebawy M, Kable EP, Roufogalis BD. Dynamic and intracellular trafficking of P-glycoprotein-EGFP fusion protein: Implications in multidrug resistance in cancer. *Int J Cancer* 2004;109:174–81. [PubMed: 14750166]

7. Bour-Dill C, Gramain MP, Merlin JL, Marchal S, Guillemin F. Determination of intracellular organelles implicated in daunorubicin cytoplasmic sequestration in multidrug-resistant MCF-7 cells using fluorescence microscopy image analysis. *Cytometry* 2000;39:16–25. [PubMed: 10655559]
8. Belhoussine R, Morjani H, Millot JM, Sharonov S, Manfait M. Confocal scanning microspectrofluorometry reveals specific anthracycline accumulation in cytoplasmic organelles of multidrug-resistant cancer cells. *J Histochem Cytochem* 1998;46:1369–76. [PubMed: 9815278]
9. Ferrao P, Sincok P, Cole S, Ashman L. Intracellular P-gp contributes to functional drug efflux and resistance in acute myeloid leukaemia. *Leuk Res* 2001;25:395–405. [PubMed: 11301107]
10. Molinari A, Calcabrini A, Meschini S, Stringaro A, Del Bufalo D, Cianfriglia M, Arancia G. Detection of P-glycoprotein in the Golgi apparatus of drug-untreated human melanoma cells. *Int J Cancer* 1998;75:885–93. [PubMed: 9506534]
11. Meschini S, Calcabrini A, Monti E, Del Bufalo D, Stringaro A, Dolfini E, Arancia G. Intracellular P-glycoprotein expression is associated with the intrinsic multidrug resistance phenotype in human colon adenocarcinoma cells. *Int J Cancer* 2000;87:615–28. [PubMed: 10925353]
12. Baldini N, Scotlandi K, Serra M, Shikita T, Zini N, Ognibene A, Santi S, Ferracini R, Maraldi NM. Nuclear immunolocalization of P-glycoprotein in multidrug-resistant cell lines showing similar mechanisms of doxorubicin distribution. *Eur J Cell Biol* 1995;68:226–39. [PubMed: 8603675]
13. Maraldi NM, Zini N, Santi S, Scotlandi K, Serra M, Baldini N. P-glycoprotein subcellular localization and cell morphotype in MDR1 gene-transfected human osteosarcoma cells. *Biol Cell* 1999;91:17–28. [PubMed: 10321019]
14. Munteanu E, Verdier M, Grandjean-Forestier F, Stenger C, Jayat-Vignoles C, Huet S, Robert J, Ratinaud MH. Mitochondrial localization and activity of P-glycoprotein in doxorubicin-resistant K562 cells. *Biochem Pharmacol* 2006;71:1162–74. [PubMed: 16499877]
15. Solazzo M, Fantappie O, Lasagna N, Sassoli C, Nosi D, Mazzanti R. P-gp localization in mitochondria and its functional characterization in multiple drug-resistant cell lines. *Exp Cell Res* 2006;312:4070–8. [PubMed: 17027968]
16. Shen DW, Cardarelli C, Hwang J, Cornwell M, Richert N, Ishii S, Pastan I, Gottesman MM. Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. *J Biol Chem* 1986;261:7762–70. [PubMed: 3711108]
17. Clarke R, Currier S, Kaplan O, Lovelace E, Boulay V, Gottesman MM, Dickson RB. Effect of P-glycoprotein expression on sensitivity to hormones in MCF-7 human breast cancer cells. *J Natl Cancer Inst* 1992;84:1506–12. [PubMed: 1359153]
18. Storrie B, Madden EA. Isolation of subcellular organelles. *Methods Enzymol* 1990;182:203–25. [PubMed: 2156127]
19. Attardi G, Ching E. Biogenesis of mitochondrial proteins in HeLa cells. *Methods Enzymol* 1979;56:66–79. [PubMed: 459888]
20. Shukla S, Saini P, Smriti, Jha S, Ambudkar SV, Prasad R. Functional characterization of *Candida albicans* ABC transporter Cdr1p. *Eukaryot Cell* 2003;2:1361–75. [PubMed: 14665469]
21. Ramachandra M, Ambudkar SV, Gottesman MM, Pastan I, Hrycyna CA. Functional characterization of a glycine 185-to-valine substitution in human P-glycoprotein by using a vaccinia-based transient expression system. *Mol Biol Cell* 1996;7:1485–98. [PubMed: 8898356]
22. Kartner N, Evernden-Porelle D, Bradley G, Ling V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* 1985;316:820–3. [PubMed: 2412130]
23. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. *J Histochem Cytochem* 1989;37:159–64. [PubMed: 2463300]
24. Georges E, Bradley G, Gariepy J, Ling V. Mapping of P-glycoprotein monoclonal antibody epitopes: application for immunohistological staining. *Proc Am Assoc Cancer Res* 1989;30:510.
25. Meyers MB, Rittmann-Grauer L, O'Brien JP, Safa AR. Characterization of monoclonal antibodies recognizing a Mr 180,000 P-glycoprotein: differential expression of the Mr 180,000 and Mr 170,000 P-glycoproteins in multidrug-resistant human tumor cells. *Cancer Res* 1989;49:3209–14. [PubMed: 2566379]

26. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* 1987;84:7735–8. [PubMed: 2444983]
27. Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* 1990;38:1277–87. [PubMed: 1974900]
28. Willingham MC, Richert ND, Cornwell MM, Tsuruo T, Hamada H, Gottesman MM, Pastan IH. Immunocytochemical localization of P170 at the plasma membrane of multidrug-resistant human cells. *J Histochem Cytochem* 1987;35:1451–6. [PubMed: 2890686]

Abbreviations

MDR	multidrug resistance
ABC	ATP-binding cassette
P-gp	P-glycoprotein
HB	homogenization buffer
RB	resuspension buffer
BSA	bovine serum albumin

Figure 1. While P-gp is present in crude mitochondrial fractions, it is not present in purified mitochondria

Whole cell lysates (lane 1), low speed pellets (lane 2), crude mitochondria (lane 3), purified mitochondria (lane 4), and purified plasma membranes (lane 5) were generated from either KB-V1 (a) or MCF-7^{ADR} (b) cells, as described in the text. Following SDS-PAGE separation, the presence of cellular membranes in each fraction was determined by Western blot analysis reaction of antibodies specific for proteins (in parentheses) expressed only in particular membranes. P-gp expression was determined by reaction with C219.

Figure 2. P-gp is not expressed in mitochondria of KB-V1 or MCF-7^{ADR} cells
Mitochondria of KB-V1 (a) or MCF-7^{ADR} (b) were labeled with Mitotracker Deep Red (red), fixed and permeabilized, reacted with the anti-P-gp antibody 17F9-FITC (green), labeled with DAPI (blue), and viewed by confocal microscopy.