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ATPase activity of partially purified P-glycoprotein from multidrug-resistant Chinese hamster ovary cells

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In vitro studies of multidrug-resistant cell lines have shown that a membrane protein, the P-glycoprotein, is responsible for resistance to a wide range of structurally and functionally dissimilar anti-cancer drugs. The amino-acid sequence of P-glycoprotein (Pgp) indicates two consensus sequences for ATP binding and the purified protein has been reported to possess a low level of ATPase activity. As part of our goal to further characterize the ATPase activity of P-glycoprotein, we have developed a procedure for rapid partial purification of the protein in a highly active form. Plasma membrane vesicles from multidrug-resistant CH^RC5 Chinese hamster ovary cells were subjected to a two-step procedure involving selective extraction with different concentrations of the zwitterionic detergent CHAPS. The resulting extract was enriched in P-glycoprotein (around 30% pure) and displayed an ATPase activity (specific activity 543 nmol mg^{-1} min^{-1}) that was not found in a similar preparation from drug-sensitive cells. The ATPase specific activity was over 10-fold higher than that previously reported for immunoprecipitated Pgp and 280-fold higher than that of immunoaffinity-purified Pgp. This ATPase activity could be distinguished from that of other ion-motive ATPases and membrane-associated phosphatases and is, thus, proposed to be directly attributable to P-glycoprotein. Optimal P-glycoprotein ATPase activity required Mg^{2+} at an ATP: Mg^{2+} molar ratio of 0.75 : 1 and the apparent K_m for ATP was 0.88 mM. P-Glycoprotein ATPase could be completely inhibited by vanadate and by the sulfhydryl-modifying reagents *N*-ethylmaleimide, $HgCl_2$ and *p*-chloromercuribenzenesulfonate. Certain drugs and chemosensitizers, including colchicine, progesterone, nifedipine, verapamil and trifluoperazine, produced up to 50% activation of P-glycoprotein ATPase activity.

Introduction

The overexpression of P-glycoprotein, a 170–180 kDa membrane protein, is the most commonly observed characteristic of multidrug-resistant cells grown in vitro [1]. The predicted amino acid sequence of Pgp has shown that it shares homology with a superfamily of proteins known as the ABC (ATP-binding cassette) family of proteins [2]. This class of proteins is characterized by the presence of one or two consensus sequences for ATP binding domains and includes such members as bacterial membrane transporters (the *hlyB*, *hisP* and *malK* gene products) [2], the yeast STE6 protein [3,4] and the mammalian cystic fibrosis gene product CFTR [5]. All of these proteins appear to function as ATP-driven importers or exporters in the plasma membrane. By analogy, Pgp is believed to mediate multidrug resistance by acting as an ATP-dependent exporter of drugs to the outside of the cell. Biochemical support for this model comes from the observations that Pgp can be photoaffinity-labelled by both drugs [6–10] and ATP [12] and that ATP-dependent drug binding and transport can be measured in membrane vesicles from MDR cells [13–17].

The mechanism by which P-glycoprotein transports drugs and the role played by ATP in this process are currently unknown. Immunopurified Pgp displayed a very low level of ATPase activity [18,19], which was not affected by the addition of MDR-type drugs. It is our aim to further characterize the ATPase activity of Pgp and eventually elucidate its role in the energization of

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Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; pCMBS, *p*-chloromercuribenzenesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MDR, multidrug-resistant; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; Tris, tris(hydroxymethyl)aminomethane.

drug transport. Towards this goal, we have developed a scheme for rapid partial purification of this integral membrane protein, involving a two-step selective extraction with the zwitterionic detergent CHAPS. The resulting partially purified Pgp preparation retains a high level of ATPase activity. In this report, we present a characterization of this ATPase activity.

Materials and Methods

Materials. CHAPS, colchicine, daunomycin, disodium-ATP, dithioerythritol, mercuric chloride, NEM, pCMBS, Tris-ATP, verapamil hydrochloride and vinblastine sulfate were purchased from Sigma (St. Louis, MO). Magnesium chloride, manganese chloride and cobaltous chloride were all from Fisher Scientific (Don Mills, Ontario). Sodium metavanadate was obtained from Aldrich (Milwaukee, WI). Tissue culture supplies were purchased from Gibco Canada (Burlington, Ontario) and iron-supplemented/defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Protein was quantitated by a microwell adaptation of the Bradford assay [20] using BSA (crystallized and lyophilized, Sigma) as a standard.

Cell culture and plasma membrane preparation. The drug-sensitive parent Chinese hamster ovary cell line (AuxB1) and the MDR cell line selected for colchicine resistance (CH^RC5) have been described previously [21]. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in α-minimal essential medium supplemented with 10% heat-inactivated bovine calf serum, penicillin (1000 U/ml), streptomycin (1 mg/ml), and 2 mM L-glutamine.

Plasma membrane vesicles were isolated from AuxB1 and CH^RC5 as described previously [22]. Briefly, cells harvested using trypsin-citrate-saline were washed with Dulbecco's PBS and disrupted using a Yeda press in homogenization buffer (10 mM Tris-HCl, 0.25 M sucrose, pH 7.3 at 23°C) containing 0.2 mM $CaCl_2$, 0.02% NaN_3 and 1 mg/ml of each of aprotinin, phenylmethylsulfonyl fluoride, pepstatin A and leupeptin (all from Sigma). The homogenate was centrifuged for 10 min at $1000 \times g$ to remove nuclei and unlysed cells and the supernatant was layered onto a 35% (w/w) sucrose cushion and centrifuged for 1 h at $95000 \times g$. The interface layer was removed and washed twice with 10 mM Tris-HCl, 0.25 M sucrose, pH 7.3 at 23°C and collected by ultracentrifugation for 30 min at $100000 \times g$. The final pellet was resuspended in the same buffer using a 25 gauge needle and stored at −70°C for no more than 3 months before use. We have previously shown that these membranes are enriched in plasma membrane marker enzymes relative to the crude homogenate; around 5–10-fold for Na^+/K^+-ATPase and 8–10-fold for 5'-nucleotidase [22].

Selective detergent extraction of partially purified Pgp. Plasma membrane vesicles from CH^RC5 were thawed and sedimented at $164000 \times g$ for 30 min at 4°C. The pellet was resuspended in 100 μl of solubilization buffer A (25 mM CHAPS, 50 mM Tris-HCl, 0.15 M NH_4Cl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 0.02% NaN_3, pH 7.5 at 23°C) to give a final protein concentration of 10 mg/ml and then incubated for 30 min at 4°C. Insoluble material was collected by ultracentrifugation at $164000 \times g$ for 15 min at 4°C and the supernatant (S_1) was saved for analysis. The pellet was resuspended in 1.0 ml of solubilization buffer B (8 mM CHAPS, 50 mM Tris-HCl, 0.15 M NH_4Cl, 5 mM $MgCl_2$, 1 mM dithioerythritol, 0.02% NaN_3, pH 7.5 at 23°C) to give a final protein concentration of 0.4–0.5 mg/ml and incubated for 30 min at 4°C. The sample was then pelleted at $15000 \times g$ for 15 min at 4°C. The resulting supernatant (S_2), which contained partially purified Pgp, was divided into aliquots and stored at −70°C.

Electrophoresis and Western blotting procedures. Protein samples from various stages of the selective detergent extraction procedure were first dialysed against 50 mM ammonium bicarbonate and then lyophilized. SDS-PAGE was carried out as described by Laemmli [23], except that samples were not boiled before electrophoresis. Proteins were separated on a 7.5% polyacrylamide gel using a Bio-Rad Mini-Protean unit (Bio-Rad Laboratories, Mississauga, Ontario) and stained with Coomassie brilliant blue (Bio-Rad). Western blotting was performed essentially according to Towbin et al. [24]. Proteins were transferred to nitrocellulose sheets using a Bio-Rad electroblotting apparatus. Nitrocellulose blots were blocked in 3% (w/v) BSA in PBS (10 mM phosphate, 0.15 M NaCl, pH 7.4) for 3 h at 37°C. Blots were then incubated for at least 5 h with a 0.2 μg/ml solution of the monoclonal antibody C219 in the same buffer at 23°C. After several washes with PBS and PBS containing 0.05% Nonidet P-40, the blot was incubated for 1 h at 23°C with ^{125}I-Protein A (2.5 μCi, ICN Biomedicals, St. Laurent, Québec) in PBS with 3% BSA, followed by several washes as described above. The dried blot was autoradiographed at −70°C using Kodak X OMAT AR 5 film and an intensifier screen.

Detection of P-glycoprotein during detergent extraction by immuno-dot blots. Samples of the supernatants and pellets at each stage of the CHAPS extraction procedure were precipitated with 6% trichloroacetic acid and 0.0125% sodium deoxycholate. The pellets were resolubilized in 20 μl of 0.2 M NaOH/1% SDS and 2.5 μl aliquots were applied to a nitrocellulose strip (0.22 μm, Schleicher and Schuell, Keene, NH). The nitrocellulose was blocked with PBS containing 3% BSA, incubated with the monoclonal antibody C219 as described above for Western blotting and Pgp was

visualized using ^{125}I-Protein A, followed by autoradiography. Amounts of Pgp were estimated semi-quantitatively from the intensity of the spots on autoradiograms by comparison to immuno-dot blots of known quantities of CH^RC5 plasma membrane, with 1 unit (U) of Pgp arbitrarily defined as the amount of Pgp present in 2.5 μg of plasma membrane protein. This assay was linear for Pgp in the region of 2–20 μg of CH^RC5 membrane protein.

Measurement of Mg^{2+}-ATPase activity using a colorimetric assay. Mg^{2+}-ATPase activity was determined by measuring the release of inorganic phosphate from ATP, using a colorimetric method adapted from Chiflet et al. [25]. Samples (1–2 μg) were diluted to 0.89 mM CHAPS with assay buffer (50 mM Tris-HCl, 0.15 M NH_4Cl, 5 mM $MgCl_2$, 0.02% NaN_3, pH 7.5 at 23°C) in a 96-well microtitre plate (final volume 90 μl). To initiate the reaction, 10 μl of ATP solution in assay buffer was added, giving a final concentration of 2 mM ATP and 0.8 mM CHAPS. After 20 min at 37°C, the reaction was stopped by the addition of 100 μl of 6% SDS/3% ascorbate/0.5% ammonium molybdate in 0.5 M HCl. Products were stabilized by the addition of 100 μl of 2% sodium citrate/2% sodium arsenite/2% acetic acid. The absorbance at 750 nm in each well was measured using an ELISA plate reader. Background absorbances were determined by performing the assay with heat-inactivated samples and were subtracted from experimental data. Background values did not exceed 10% of the total activity. Stock solutions of DCCD, oligomycin, NEM, nifedipine, pCMBS, progesterone, quinidine, quinine, trifluoperazine, verapamil and vinblastine (all from Sigma) were prepared in assay buffer containing DMSO. Controls contained the appropriate amount of DMSO in buffer alone. Final DMSO concentrations did not exceed 2.5% (v/v), except for progesterone, where the final concentration was 5% (v/v). DMSO alone, at 2.5% (v/v) and 5% (v/v), stimulated the Mg^{2+}-ATPase activity by 10 and 30%, respectively.

Radiometric assay for Mg^{2+}-ATPase activity. Mg^{2+}-ATPase activity was also determined by measuring the release of [γ-^{32}P]P_i from [γ-^{32}P]ATP according to a method adapted from Shacter [27]. Briefly, samples were incubated at 37°C in 100 μl of assay buffer containing 1 mM ATP, 0.09 μCi [γ-^{32}P]ATP (25 Ci/mmol, ICN Biomedicals) and 0.8 mM CHAPS. After 15 min, the reaction was stopped by adding 200 μl of 5% ammonium molybdate in 2 M H_2SO_4 and 200 μl of 20 mM silicotungstic acid in 4 mM H_2SO_4. Isobutanol/toluene (1:1, v/v, 1.0 ml) was then added and after mixing, the organic layer was separated from the aqueous layer by centrifugation. An aliquot of the organic layer, which contained the released [^{32}P]P_i, was analyzed by liquid scintillation counting.

Measurement of Na^+/K^+-ATPase activity. The ouabain-sensitive Na^+/K^+-ATPase activity was determined by a method adapted from Jones et al. [26]. The final reaction mixture contained 1–2 μg of sample in Na^+/K^+-ATPase assay buffer (50 mM Mops, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 0.02% NaN_3, pH 7.0 at 23°C), 0.8 mM CHAPS and 2 mM ATP, with or without 1 mM ouabain. The release of inorganic phosphate was monitored using the colorimetric method described above.

Results

Partial purification of Pgp from CH^RC5 plasma membrane by selective detergent extraction

During earlier experiments to purify Pgp to homogeneity, we observed that solubilization of this integral membrane protein from the CH^RC5 plasma membrane with the zwitterionic detergent CHAPS required a high detergent-to-protein ratio (30:1, w/w) and even under these conditions, only about 50% of the Pgp was extracted [22]. This observation formed the basis for an alternative approach to Pgp purification, involving differential detergent solubilization. The first step of this procedure removes membrane proteins more readily solubilized at a low detergent/protein ratio, leaving behind a Pgp-enriched pellet. The second step uses a high detergent:protein ratio to extract this pellet, resulting in a solubilized preparation that is highly enriched in Pgp.

To optimize such a selective extraction procedure, CH^RC5 plasma membrane vesicles (10 mg/ml protein) were treated with various concentrations of CHAPS (2, 5, 10 and 25 mM). The relative distribution of Pgp in the pellet and supernatant was determined by semi-quantitative immuno-dot blots using the monoclonal antibody C219 (data not shown). Although over 50% of the total protein was recovered in the supernatant when the membrane vesicles were extracted with 25 mM CHAPS (detergent/protein ratio of 1.5:1, w/w), the bulk of the Pgp remained with the detergent-insoluble pellet. CHAPS concentrations less than 25 mM solubilized lower amounts of total protein, leaving approximately the same amount of Pgp in the insoluble pellet. SDS-PAGE analysis of the 25 mM CHAPS extract (Fig. 1A) showed that a 170–180 kDa band corresponding to Pgp was clearly detected in the insoluble pellet (lane 2), but was virtually absent from the detergent extract (lane 1). This solubilized extract did, however, contain a substantial number of membrane proteins other than Pgp (Fig. 1A, lane 1). These results indicated that 25 mM CHAPS was a suitable detergent concentration to obtain a Pgp-enriched pellet and was therefore routinely used in the first detergent extraction step.

The insoluble pellet from the first CHAPS extraction was then resuspended at a protein concentration of 0.4–0.5 mg/ml in buffers containing different con-

centrations of CHAPS (8, 17 and 33 mM). Protein assays and semi-quantitative immuno-dot blots of the supernatant and pellet showed that in all cases approximately half of the total protein and Pgp were solubilized. Attempts to improve the extraction of Pgp by using an actin-destabilizing buffer [28] in combination with 8 mM CHAPS were unsuccessful. Fig. 1B shows SDS-PAGE analysis of the 8 mM CHAPS extract (detergent/protein ratio of 10:1, w/w). The detergent-soluble supernatant (lane 1) was clearly enriched in Pgp, with densitometric scans of the Coomassie blue-stained gel indicating approx. 30–40% purity. The pellet (lane 2) still contained a substantial amount of this protein. SDS-PAGE analysis of the 17 mM and 33 mM CHAPS extracts indicated no further increase in Pgp content of the supernatant at higher detergent concentrations (data not shown). To avoid the difficulties inherent in handling proteins in high concentrations of detergent (e.g., inhibition of enzyme activity), we routinely used 8 mM CHAPS for the second detergent extraction step.

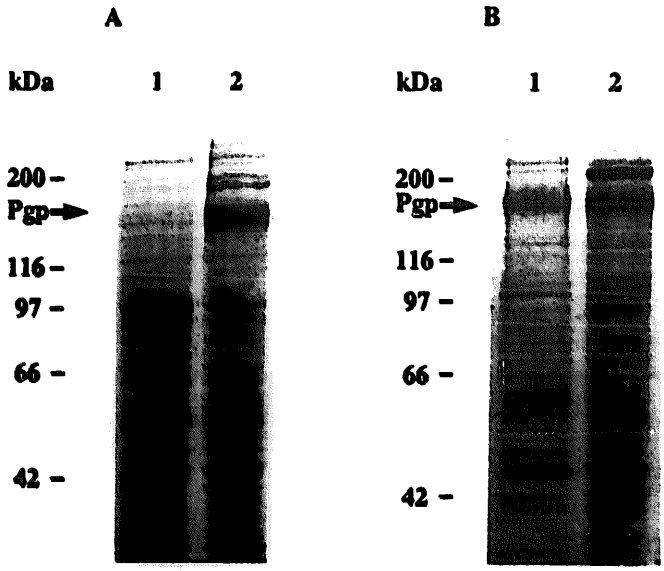

Fig. 1. SDS-PAGE analysis of the selective detergent extraction of Pgp from CH^RC5 plasma membrane. (A) Plasma membrane was extracted with 25 mM CHAPS in buffer A at a protein concentration of 10 mg/ml for 30 min at 4°C. After centrifugation at 164000 × *g* for 15 min, the supernatant and the resuspended pellet were dialyzed against 50 mM ammonium bicarbonate and lyophilized. Samples were analyzed by SDS-PAGE as described in Materials and Methods. Lane 1, soluble supernatant, 18 μg protein; lane 2, membrane pellet, 18 μg protein. (B) The membrane pellet remaining from the first extraction was treated with 8 mM CHAPS in buffer B at a protein concentration of 0.4–0.5 mg/ml for 30 min at 4°C. After centrifugation at 15000 × *g* for 15 min, the supernatant and insoluble pellet were run on SDS-PAGE. Lane 1, soluble supernatant, 10 μg protein; lane 2, insoluble pellet, 20 μg protein. Gels were stained with Coomassie blue. The position of molecular mass markers is indicated on the left and the band corresponding to Pgp is marked by an arrow.

Comparison of the Pgp extraction procedure in AuxB1 and CH^RC5

The rationale for developing a rapid purification procedure for Pgp was to maintain the protein in active form and allow characterization of its ATPase activity. Since the final CHAPS extract from CH^RC5 plasma membrane contained partially purified Pgp, we compared this preparation to a similarly prepared detergent extract from the plasma membrane of the drug-sensitive AuxB1 parent line. SDS-PAGE analysis of the plasma membrane and S_1 and S_2 fractions from both drug-sensitive AuxB1 cells and drug-resistant CH^RC5 cells is shown in Fig. 2A. These results clearly demonstrated that the major difference between the two cell lines was the presence of a 170–180 kDa protein in the plasma membrane and S_2 fractions from CH^RC5, which was absent from the corresponding fractions of AuxB1 (compare lane 1 and 2 and lanes 5 and 6). Western immunoblotting with the monoclonal antibody C219 (Fig. 2B) confirmed the identity of this protein as Pgp. Comparison of the relative intensity of the 170–180 kDa band in each fraction showed that Pgp is enriched 2–3-fold in the CH^RC5 S_2 fraction relative to the plasma membrane starting material and very low amounts are detected in the CH^RC5 S_1 fraction (Fig. 2B).

Time dependence of Mg^{2+}-ATPase activity from the S_2 fractions of AuxB1 and CH^RC5

To test whether elevated ATPase activity was associated with the Pgp-enriched S_2 fraction from CH^RC5, the time course of Mg^{2+}-dependent ATP hydrolysis was measured in the S_2 fractions from both cell lines. ATP hydrolysis by the S_2 fraction from CH^RC5 was linear for at least 30 min (Fig. 3) and was about 10-fold greater than that seen for the S_2 fraction from AuxB1. The assay buffer in this experiment contained 2 mM ATP; ATP hydrolysis measured with 0.1, 0.5 or 1.0 mM ATP was also linear for up to 30 min. In subsequent experiments, single time-point measurements of ATPase activity were carried out at 20 min.

Mg^{2+}-ATPase activity at various stages of the detergent extraction procedure

To further characterize this elevated Mg^{2+}-ATPase activity, ATP hydrolysis was measured in plasma membrane and the S_1 and S_2 fractions from both AuxB1 and CH^RC5. Table I shows the specific and total Mg^{2+}-ATPase activities and the protein recovery at each stage of the extraction procedure. CH^RC5 plasma membrane had a 3.8-fold higher ATPase specific activity than that from AuxB1, whereas the S_2 fraction from the drug-resistant line displayed an ATPase activity 6.4-fold higher than the corresponding S_2 fraction from the drug-sensitive parent. The S_2 fraction from CH^RC5 showed a 2.2-fold increase in Mg^{2+}-ATPase specific

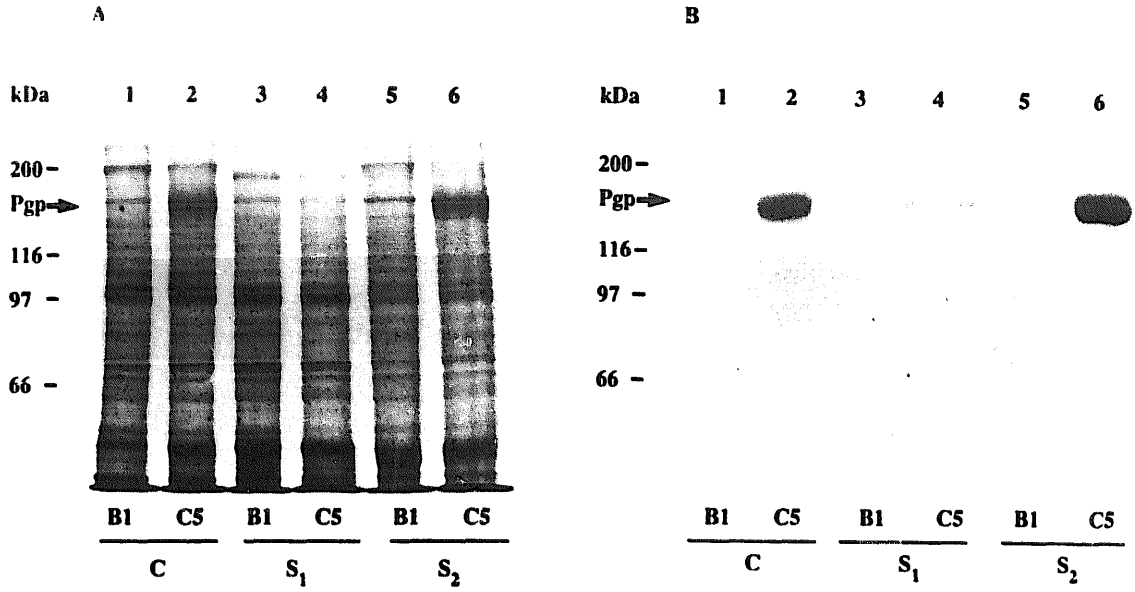

Fig. 2. Comparison of the selective detergent extraction of Pgp from drug-sensitive AuxB1 and drug-resistant CH^RC5 plasma membranes. Detergent extraction was performed as described in Materials and Methods, using plasma membrane from AuxB1 (lanes 1, 3 and 5) and CH^RC5 cells (lanes 2, 4 and 6). Samples were dialyzed into 50 mM ammonium bicarbonate, lyophilized and then analyzed by (A) SDS-PAGE followed by Coomassie blue staining and (B) Western immunoblotting with the Pgp-specific monoclonal antibody C219. Lanes 1 and 2, plasma membrane; lanes 3 and 4, S_1 soluble fraction from the first CHAPS extraction; lanes 5 and 6, S_2 soluble fraction from the second CHAPS extraction. Each lane was loaded with 10 μg of protein. The position of molecular mass markers is indicated on the left and the band corresponding to Pgp is indicated by an arrow.

activity relative to the plasma membrane. This increase in ATPase activity corresponds roughly to the degree of Pgp enrichment shown by SDS-PAGE and Western immunoblotting (Fig. 2A and B). The Mg^{2+}-ATPase activity recovered in the S_2 fraction of CH^RC5 represents about one-third of the total activity present in the plasma membrane starting material. Approximately one-third of the total Mg^{2+}-ATPase activity was also recovered in the CH^RC5 S_1 fraction, although the specific activity was reduced relative to the plasma membrane. Pgp ATPase activity was also measured by monitoring the release of [γ-^{32}P]P_i from [γ-^{32}P]ATP and results quantitatively very similar to those shown in

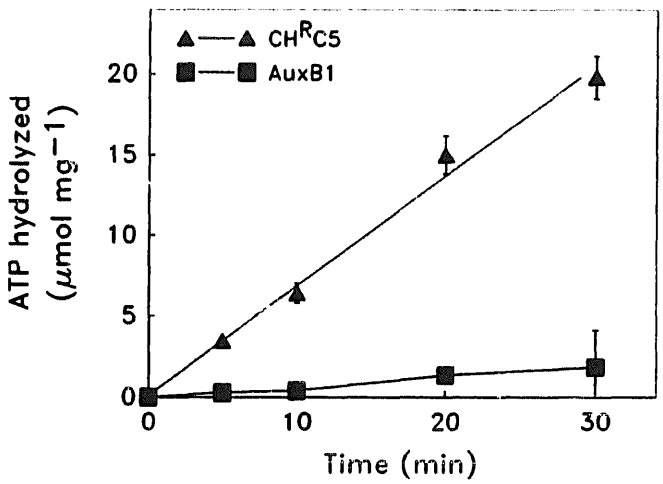

Fig. 3. Time dependence of S_2 fraction Mg^{2+}-ATPase activity from AuxB1 and CH^RC5 plasma membrane. Aliquots containing 1–2 μg of protein from the S_2 fraction of AuxB1 (■) and CH^RC5 (▲) plasma membrane were assayed for Mg^{2+}-ATPase activity at various times as described in Material and Methods. The final reaction mixture contained 2 mM ATP and 0.8 mM CHAPS in 100 μl of assay buffer. ATPase activity is expressed as P_i released and is presented as means ± S.E. (n = 3).

TABLE I

Mg^{2+}-ATPase activity at different stages of the detergent extraction procedure

Aliquots containing 1–2 μg of protein from AuxB1 and CH^RC5 plasma membrane (PM), the soluble fraction from the first extraction step (S_1) and the soluble fraction from the second extraction step (S_2), were assayed for Mg^{2+}-ATPase activity as described in Materials and Methods. The final reaction mixture contained 2 mM ATP and 0.8 mM CHAPS in 100 μl of assay buffer. ATPase activity is expressed as P_i released and is presented as mean ± S.E. (n = 10–15, from six separate extractions of four different membrane preparations from each cell line).

Sample	Mg^{2+}-ATPase		Protein (μg)
	specific activity (nmol mg^{-1} min^{-1})	total activity (μmol min^{-1})	
AuxB1			
PM	55.8 ± 1.6	55.8 ± 2.5	1000 ± 17
S_1	32.4 ± 3.0	14.8 ± 2.0	457 ± 20
S_2	71.8 ± 6.8	9.3 ± 1.9	130 ± 14
CH^RC5			
PM	211 ± 12	211 ± 17	1000 ± 25
S_1	144 ± 13	66.2 ± 9.9	460 ± 27
S_2	458 ± 29	65.0 ± 8.1	142 ± 9

Table I were obtained. This confirmed that the contribution to P_i release from ATP by ADPases or AMPases was negligible. All ATPase measurements presented in this report were thus made using the colorimetric method. The ATPase activity of the CH^RC5 S_2 fraction was stable for several months when stored at −70°C.

Characterization of the Mg^{2+}-ATPase activity in the CH^RC5 S_2 fraction

Since increased Mg^{2+}-ATPase activity in CH^RC5 plasma membrane and S_2 fraction correlated well with elevated levels of Pgp, it seemed likely that Pgp-associated ATPase activity was being measured. To rule out the possibility that phosphatases, or other ATPases, might contribute to ATP hydrolysis by the CH^RC5 S_2 fraction, we tested the effect of different ions and various ATPase/phosphatase inhibitors on ATPase activity. The ion-motive ATP-hydrolyzing enzymes have been divided into three classes, namely the 'P'-type (phosphorylated), 'V'-type (vacuolar) and 'F'-type (F_0F_1) ATPases (for a review, see Ref. 29). These classes of ion-motive ATPases can be distinguished according to their relative sensitivity to inhibitors. Vanadate is a potent inhibitor of P-type ion-motive ATPases (with a K_i in the nM to μM range), as well as a number of other ATP-dependent transporters [30–32]. Fig. 4 shows that low concentrations of vanadate inhibited the Mg^+-ATPase activity of the CH^RC5 S_2 fraction, with an IC_{50} of 1.5 μM. To distinguish this vanadate-sensitive activity from that of other P-type ATPases, such as the Na^+/K^+- and Ca^{2+}-ATPases, it should be noted that the Mg^{2+}-ATPase assay buffer contained no K^+ and the only Na^+ present was that added in the form of NaN_3 and disodium ATP, around 7 mM. In addition, up to 1 mM ouabain (a specific inhibitor of the Na^+/K^+-ATPase) had no effect on the Mg^{2+}-ATPase activity of the CH^RC5 S_2 fraction. Low concentrations of Ca^{2+} (10–500 μM) also had no effect, while higher levels of Ca^{2+} were inhibitory, with a concentration of 25 mM reducing the activity by 72%. EGTA (0.5 mM) had no effect on CH^RC5 S_2 Mg^{2+}-ATPase activity.

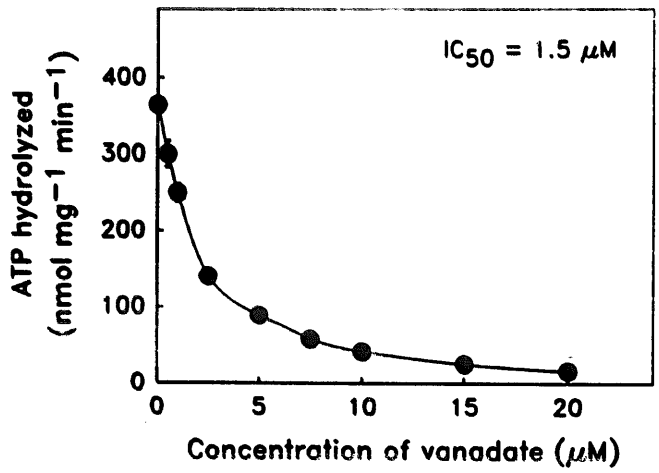

Fig. 4. Inhibition of Pgp ATPase activity by vanadate. ATPase activity was measured in the presence of increasing concentrations of vanadate. Data are expressed as means ± S.E. (n = 3).

TABLE II

Na^+/K^+-ATPase activity at different stages of the detergent extraction procedure

Aliquots containing 1–2 μg of protein from AuxB1 and CH^RC5 plasma membrane (PM), the soluble fraction from the first extraction step (S_1) and the soluble fraction from the second extraction step (S_2) were assayed for ouabain-sensitive Na^+/K^+-ATPase activity as described in Materials and Methods. The final reaction mixture contained 2 mM ATP and 0.8 mM CHAPS in 100 μl of Na^+/K^+-ATPase assay buffer. ATPase activity is expressed as P_i released and is presented as mean ± S.E. (n = 9–12, from four separate extractions of three different membrane preparations from each cell line).

Sample	Na^+/K^+-ATPase		Protein (μg)
	specific activity (nmol mg^{-1} min^{-1})	total activity (μmol min^{-1})	
AuxB1			
PM	96.2 ± 9.4	96.2 ± 11.0	1000 ± 17
S_1	97.5 ± 4.2	41.7 ± 3.9	428 ± 22
S_2	46.2 ± 3.6	5.4 ± 0.7	116 ± 5
CH^RC5			
PM	93.5 ± 8.1	93.5 ± 10.3	1000 ± 23
S_1	92.8 ± 3.9	41.5 ± 4.7	447 ± 32
S_2	37.0 ± 4.2	5.6 ± 0.9	151 ± 8

To further distinguish the Mg^{2+}-ATPase activity from that of the Na^+/K^+-ATPase, the distribution of ouabain-sensitive ATPase activity at various stages of the detergent extraction procedure was measured, using an assay buffer containing 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$ and Mops, pH 7.0 (Table II). Two important points can be noted from these data: first, unlike the Mg^{2+}-ATPase, the relative levels of the ouabain-sensitive Na^+/K^+-ATPase are very similar in both cell lines and second, this activity is depleted in the S_2 fraction of both cell lines relative to the plasma membrane. In the CH^RC5 S_2 fraction, the Mg^{2+}-ATPase activity is more than 10-fold greater than the Na^+/K^+-ATPase activity. Notably, at all stages of the extraction procedure, the ouabain-resistant ATPase activity measured in the Na^+/K^+-ATPase assay buffer was equivalent to the Mg^{2+}-ATPase activity measured in the Mg^{2+}-ATPase assay buffer. These results indicate that the CH^RC5 S_2 fraction contains only very low levels of the Na^+/K^+-ATPase and the buffer conditions chosen for Mg^{2+}-ATPase measurement make any interference essentially negligible. Finally, the Mg^{2+}-ATPase activity attributed to PgP was inhibited by 50% at around 0.1 mM NEM, with inhibition complete at

0.2 mM (see Fig. 8), while the Na^+/K^+-ATPase required a 1 h treatment with 1 mM NEM to reduce its activity by 50% [33].

It is also highly unlikely that V- or F-type ATPases contribute to Mg^{2+}-ATPase activity for the following reasons. Neither of these ATPases is sensitive to vanadate [34], which completely inhibited CH^RC5 S_2 ATPase activity. The Mg^{2+}-ATPase assay buffer contained sufficient NaN_3 to inhibit the F_oF_1 ATPase [34,35] and Mg^{2+}-ATPase activity was not affected by 100 ng/ml oligomycin, a concentration sufficient to completely inhibit the mitochondrial ATPases of mouse and rat liver [36,37]. Furthermore, 20 μM DCCD had no effect on the CH^RC5 S_2 ATPase activity, while 100 μM DCCD inhibited the activity by only 16%. By comparison, 1 μM DCCD completely inhibited bovine heart and yeast mitochondrial ATPases [35,38], whereas 100 μM DCCD inhibited liver and yeast lysosomal ATPases [36,38,39].

Phosphatases are also unlikely to contribute to the measured Mg^{2+}-ATPase activity. The pH optimum for the CH^RC5 Mg^{2+}-ATPase activity was around 7.4 (Fig. 5), clearly outside the pH optimum for both acid phosphatases (5.0–6.0) [40] and alkaline phosphatases (8.0–10.5) [41]. In addition, 10 μM sodium molybdate and 10 mM sodium tartrate (acid phosphatase inhibitors [40]) and 25 mM L-phenylalanine (an alkaline phosphatase inhibitor [41]), had no effect on S_2 ATPase activity. Finally, phosphatases hydrolyse ADP and AMP as effectively as ATP [40,41], whereas no ADPase or AMPase activity was detected in the CH^RC5 S_2 fraction.

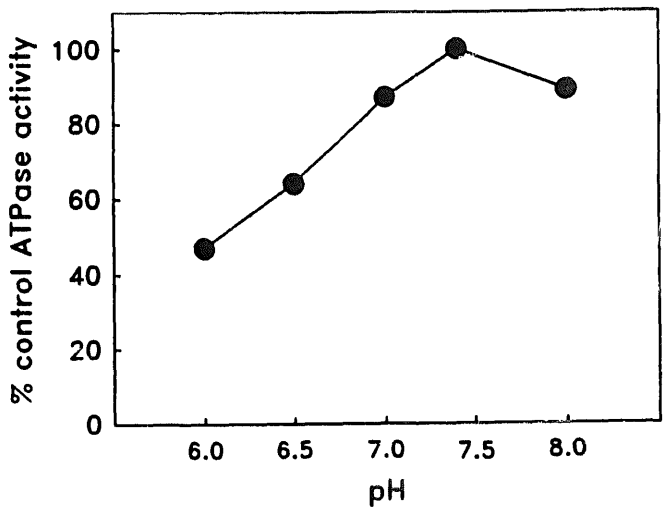

Fig. 5. Effect of pH on the Mg^{2+}-ATPase activity of S_2 fraction from CH^RC5 cells. ATPase activity was measured in Mg^{2+}-ATPase buffer containing either 50 mM Mes (pH 6.0 and 6.5), 50 mM Mops (pH 6.5, 7.0 and 7.4), or 50 mM Tris (pH 7.4 and 8.0). ATP hydrolysis is presented as percent control ATPase activity (404 ± 10 nmol min^{-1} mg^{-1}) measured under standard conditions (i.e., 50 mM Tris, pH 7.4). Data are presented as means ± S.E. ($n = 3$).

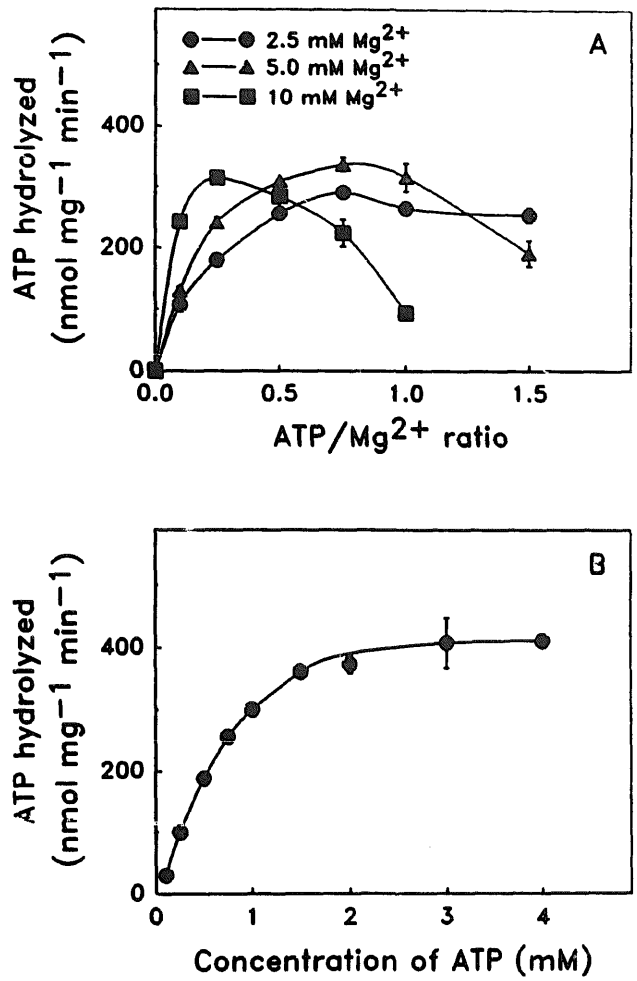

Fig. 6. Dependence of Pgp ATPase activity on ATP and Mg^{2+} concentration. (A) ATPase activity was measured at several different ATP concentrations at fixed Mg^{2+} concentrations of 2.5 mM (●), 5.0 mM (▲) and 10 mM (■). (B) ATPase activity was measured at various ATP concentrations with a fixed Mg^{2+} concentration of 5.0 mM. Data are expressed as means ± S.E. ($n = 3$).

These results indicate that the high Mg^{2+}-ATPase activity in the S_2 fraction from CH^RC5 cells is clearly distinguishable from that of ion-motive ATPases and membrane-associated alkaline and acid phosphatases. These data, along with those demonstrating a close correlation between ATPase activity and Pgp levels, support the proposal that the Mg^{2+}-ATPase activity in the S_2 fraction arises directly from Pgp.

Dependence of Pgp ATPase activity on ATP and Mg^{2+} concentrations

Partially purified Pgp showed optimal ATPase activity at an ATP: Mg^{2+} molar ratio of about 0.75 : 1 at 2.5 and 5.0 mM Mg^{2+} and 0.25 : 1 at 10 mM Mg^{2+} (Fig. 6A). Of the three Mg^{2+} concentrations tested, maximal ATPase activity was achieved with 5.0 mM Mg^{2+} at the optimal ATP : Mg^{2+} ratio, corresponding to an ATP concentration of about 3–4 mM. Fig. 6B shows the ATPase activity as a function of ATP concentra-

tion, at a fixed Mg^{2+} concentration of 5 mM. When these data were fitted to the Michaelis-Menten equation using the software program Enzfitter (BioSoft), the apparent K_m and V_{max} for ATP were determined to be 0.88 mM and 543 nmol mg^{-1} min^{-1}, respectively.

Effect of divalent cations on Pgp ATPase activity

Other divalent cations are able to substitute for Mg^{2+} and maintain the function of a variety of different ATPases. Fig. 7 shows the effect of various divalent cations on partially purified Pgp ATPase activity. Maximal ATPase activity was obtained in the presence of 5 mM Mg^{2+}. A lower but significant activity (45% of maximum) was also measured when Mg^{2+} was replaced with Mn^{2+}, with an optimal ion concentration of about 1 mM. When Co^{2+} was substituted for Mg^{2+}, only 22% of the ATPase activity was retained. In this case, the optimal ion concentration was lower than that found for Mg^{2+} or Mn^{2+}, ranging from 50 to 1000 μM. Thus, Pgp ATPase activity can be partially supported by divalent cations other than Mg^{2+}.

Effect of sulfhydryl-reactive agents on Pgp ATPase activity

The sulfhydryl reagents NEM, $HgCl_2$ and pCMBS all inhibited Pgp ATPase activity with an IC_{50} of 80–150 μM (Fig. 8). These results suggest that Pgp contains one or more cysteine residues important for catalysis of ATP hydrolysis. Notably, V-type ATPases are also sensitive to NEM, but the IC_{50} of inactivation is usually less than 10 μM [34].

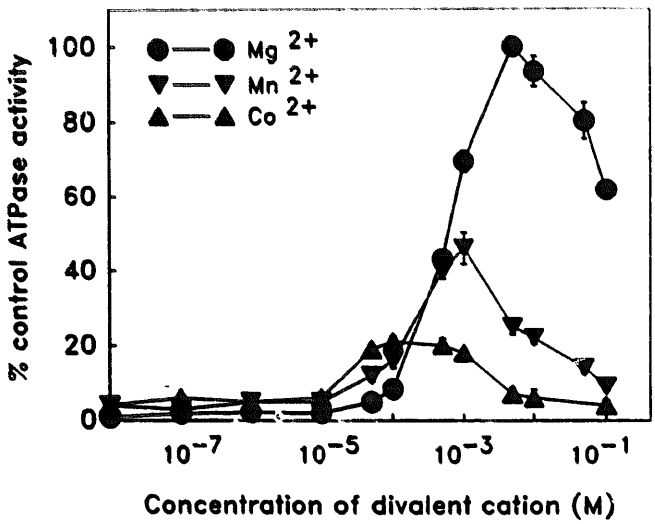

Fig. 7. Effect of divalent cations on Pgp ATPase activity. ATPase activity was determined using 2 mM ATP and varying concentrations of Mg^{2+} (●), Mn^{2+} (▼) or Co^{2+} (▲). ATP hydrolysis is expressed as percent control ATPase activity measured at 5 mM Mg^{2+} (449 ± 10 nmol min^{-1} mg^{-1}). Results are presented as means ± S.E. (n = 3).

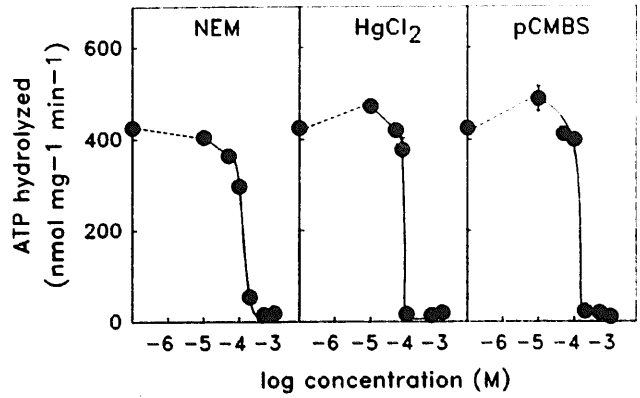

Fig. 8. Inhibition of Pgp ATPase activity by sulfhydryl reagents. ATPase activity was measured in the presence of increasing concentrations of NEM, $HgCl_2$ or pCMBS. Data are expressed as means ± S.E. (n = 3).

Effect of MDR-type drugs and chemosensitizers on Pgp ATPase activity

Since Pgp is believed to be an ATP-dependent drug efflux pump, it was of interest to test the effect on Pgp ATPase activity of various drugs and chemosensitizers known to be part of the MDR spectrum. The response of the ATPase was varied, with several different patterns of change observed. One group of drugs (daunomycin, vinblastine and quinidine) produced inhibition of the ATPase activity over the entire concentration range used (Fig. 9A and B). Except for a small stimulatory effect observed in the 10^{-5}–10^{-4} M concentration range, quinine also fell within this category of inhibitory drugs. The potency of inhibition followed the order, vinblastine > quinidine > quinine = daunomycin. A second group of drugs (trifluoperazine and verapamil) had biphasic effects on Pgp ATPase, producing a large stimulation of activity at < 10 μM, followed by inhibition at higher concentrations (Fig. 9B). Verapamil produced a slightly greater stimulation of the ATPase activity than did trifluoperazine. Finally, a third group of drugs (colchicine, nifedipine and progesterone) produced only a net stimulation of the ATPase activity over the entire concentration range tested (Fig. 9A). Nifedipine is included in this group because the ATPase activity remained greater than the untreated control despite a slight decrease at concentrations greater than 100 μM. At concentrations up to 100 μM, both nifedipine and progesterone showed a similar level of stimulation of Pgp ATPase, which was greater than that observed for colchicine. Higher concentrations of progesterone could not be tested because of its poor solubility in aqueous buffers.

Discussion

Pgp is believed to function as an ATP-dependent drug efflux pump, thereby maintaining intracellular

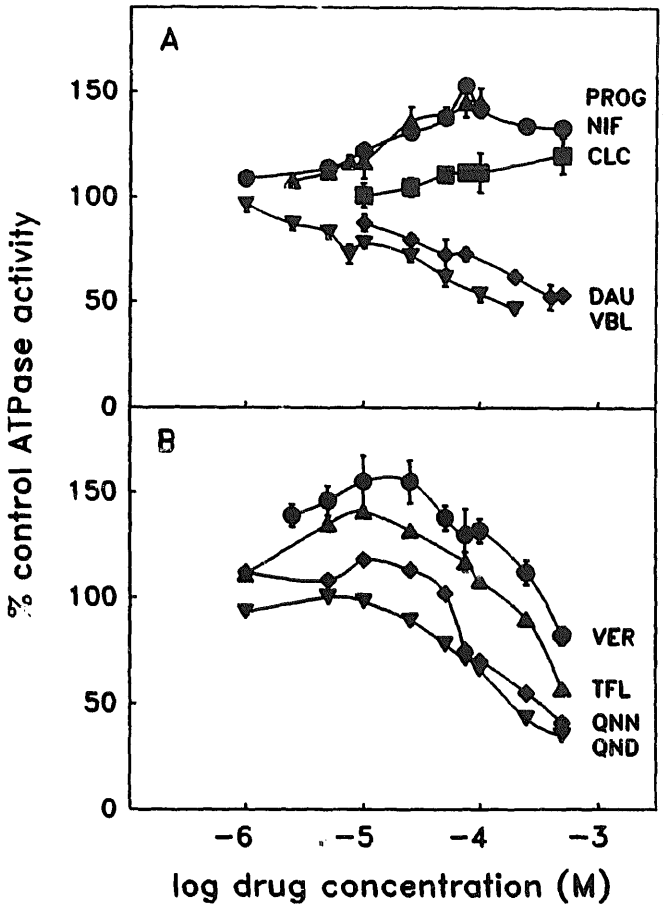

Fig. 9. Effect of drugs and chemosensitizers on Pgp ATPase activity. ATPase activity was measured in the presence of increasing concentrations of (A) progesterone (PROG, ▲), nifedipine (NIF, ●), colchicine (CLC, ■), daunomycin (DAU, ♦) and vinblastine (VBL, ▼) and (B) verapamil (VER, ●), trifluoperazine (TFL, ▲), quinine (QNN, ♦) and quinidine (QND, ▼). Data are expressed as percent control ATPase activity (means ± S.E., $n = 3$) measured in the absence of drug or chemosensitizer, which ranged from 314 ± 9 to 418 ± 7 nmol min^{-1} mg^{-1}.

drug concentrations in MDR cells at subtoxic levels. Many researchers have shown that Pgp can be photoaffinity-labelled by MDR-type drugs [6,8–10] and these same drugs are transported into membrane vesicles containing Pgp in an ATP-dependent fashion [13–15]. In addition, Pgp is photoaffinity-labelled by azido-ATP [11,12]. However, little is known about the ATPase activity of Pgp and how it may be coupled to drug transport. Since a specific inhibitor of Pgp ATPase activity has not yet been identified, Pgp must be separated from the other ATPases present in the plasma membrane of drug-resistant cells, before its ATPase activity can be studied. A few laboratories have reported the purification of Pgp [22,42–44], however, only Hamada and Tsuruo [18,19] have detected ATPase activity in the purified protein. A major limitation to their immunoaffinity purification procedure was that both Pgp recovery and ATPase activity were low. In addition, because of difficulties in the purification of Pgp (aggregation, irreversible binding to ion-exchange columns [19]), further characterization of the ATPase activity was carried out on Pgp-immunoprecipitates rather than immunoaffinity-purified Pgp [18].

We have also purified Pgp using both lectin affinity chromatography and immunoaffinity chromatography [22]. For various reasons, including heterogeneous glycosylation (in the case of lectin affinity chromatography) and poor column efficiency (in the case of immunoaffinity chromatography), we have found that these methods were unsuitable for large scale purification of Pgp. During these studies, we observed that Pgp was poorly solubilized by CHAPS at a detergent/protein ratio (1.5 : 1) commonly used to extract many other membrane proteins [45]. Only at a much higher detergent : protein ratio were significant quantities of Pgp solubilized from $CH^{R}C5$ plasma membrane. This observation provided the impetus for developing the selective detergent extraction scheme presented in this report. Although this procedure results in only partially purified Pgp, it has the advantage of being rapid, requiring only approx. 2.5 h to complete. In addition, the technique maintains Pgp under mild conditions at all times. The partially purified Pgp obtained by selective CHAPS extraction thus retained a high level of ATPase activity. This procedure may be applicable to the purification of Pgp from other MDR cell lines, although the optimal extraction conditions may vary from one cell line to the other.

The greatest difference between the Pgp ATPase described in this report and that described earlier [18,19], was that our extract displayed a specific activity (V_{max} 543 nmol mg^{-1} min^{-1}) over 10-fold higher than that of immunoprecipitated Pgp (V_{max} 50 nmol mg^{-1} min^{-1}) and 280-fold higher than that of immunoaffinity-purified Pgp. The enhanced specific activity of our partially-purified Pgp relative to the immunoaffinity-purified protein is likely explained by the fact that immunoaffinity chromatography involved prolonged exposure to high detergent concentrations, low pH elution and dialysis, all of which may contribute to partial loss of catalytic activity. However, it is less clear why there was a significant difference in specific activities between our preparation and immunoprecipitated Pgp. Both procedures shared the common advantages of being performed rapidly under mild conditions and the MRK16 monoclonal antibody used for immunoprecipitation did not inhibit Pgp ATPase activity [18]. One possible contributing factor is that our assays were performed at 37°C, whereas the immunoprecipitated Pgp ATPase activity was measured at 30°C.

There are a number of additional differences between the ATPase activity of the partially purified Pgp preparation described in this report and the immunoprecipitated Pgp described earlier. Mn^{2+} and Co^{2+} could only partially substitute for Mg^{2+} in supporting

ATPase activity in the present preparation, whereas activity was completely preserved when the same substitutions were made in immunoprecipitated Pgp. In addition, our preparation had a higher apparent K_m for ATP (880 μM vs. 150 μM) and was approx. 70-fold more susceptible to inhibition by vanadate (IC_{50} of 1.5 μM vs. 100 μM). Immobilization of Pgp by antibody on Protein A-Sepharose may modify the catalytic and kinetic properties of the Pgp ATPase. Alternatively, the presence of other proteins in the partially purified preparation may have similar effects. There are, however, a number of similarities between the ATPase activities of the two preparations. Neither activity was inhibited by up to 1 mM ouabain, nor were they affected by the presence of Na^+, K^+ or EGTA. Furthermore, low concentrations of Ca^{2+} had no effect on activity in both cases, whereas higher concentrations were inhibitory. These characteristics not only illustrate similarities between the two preparations, they also distinguish the ATPase activity of Pgp from that of P-type ion-motive ATPases such as the Na^+/K^+- and Ca^{2+}-ATPases. However, Pgp was strongly inhibited by vanadate, a common inhibitor of these P-type ATPases. There are a number of reports of non-P-type membrane-associated ATPases that are also inhibited by vanadate [30–32,46]. Since vanadate is believed to inhibit ATPase activity by preventing the formation of a phosphoaspartyl intermediate, it is possible that such an intermediate may play a role in the transport function of Pgp, although previous attempts to detect this intermediate have failed [14].

Hamada and Tsuruo reported that NEM inhibited Pgp ATPase activity [18]. In this report, we have confirmed and extended this observation by showing that two other sulfhydryl-reactive reagents, $HgCl_2$ and pCMBS, also inhibited ATPase activity. These results clearly suggest a role for cysteine sulfhydryl residues in ATP hydrolysis. A comparison of the predicted amino acid sequences of various Pgp isoforms showed that six cysteine residues are conserved throughout the three gene classes in hamster and mouse and the two gene classes in humans [47]. Two of these cysteines are located in transmembrane domains, two in the carboxy-terminal tail and one within each of the Walker A motifs of the ATP-binding domains. Further investigation of the role of cysteine residues in ATP hydrolysis and ATP-dependent drug transport may provide some understanding of the mechanism of action of Pgp. We have observed that the same three sulfhydryl reagents also inhibit ATP-dependent colchicine transport into CH^RC5 plasma membrane vesicles [15].

The current model for MDR, which envisions Pgp as an ATP-dependent drug efflux pump, predicts that ATP hydrolysis is directly coupled to drug transport. The ATPase activities of several other transporter ATPases are directly regulated by the transported substrates, e.g., the Na^+/K^+- and Ca^{2+}-ATPases [48], the Cl^--ATPase [30] and the arsenite/antimonite ATPase [49] and also various organic molecule ATP-dependent transporters such as the bacterial histidine [50] and maltose [51] permeases and the erythrocyte glutathione disulfide transporter [52]. In these proteins, significant ATP hydrolysis occurs only when the transported substrate is presented to the enzyme and in the case of the histidine and maltose permeases, only when the substrate is concurrently transported across the membrane. If Pgp behaves similarly, drugs and other molecules that modify transport (e.g., chemosensitizers) might be expected to have a direct effect on Pgp ATPase activity. The effect of MDR drugs on the ATPase activity of partially purified Pgp was inconclusive in this study. Of the transportable drugs tested, only colchicine showed a slight stimulation of ATPase activity, whereas daunomycin and vinblastine showed inhibition at all drug concentrations tested. Although these drugs differ considerably in their structures, photoaffinity labelling experiments [6,8–10] and drug transport studies with membrane vesicles [13–15] show that all three drugs interact directly with Pgp and appear to share overlapping binding sites. It is, therefore, unclear why these three drugs showed different responses and why they did not all show significant stimulation of Pgp ATPase activity. One possibility is that the link between drug binding/transport and ATP hydrolysis is uncoupled when Pgp is solubilized in detergent. However, it should be noted that Pgp appears to be at least partially uncoupled in CH^RC5 plasma membrane vesicles, which, in our hands, show high ATPase activity in the absence of any obvious substrates. Hamada and Tsuruo found neither stimulation nor inhibition of Pgp ATPase activity by 10 μM adriamycin or vincristine [18]. The inhibition of Pgp ATPase activity at high drug concentrations observed in this study may arise from nonspecific effects on Pgp or changes in the micellar structure of the detergent micelles.

The effect of chemosensitizers on Pgp ATPase activity was inconclusive. Nifedipine, progesterone, trifluoperazine and verapamil all stimulated Pgp ATPase activity to a significant degree, whereas both quinine and quinidine inhibited the Pgp ATPase activity, with a small degree of stimulation by quinine at low concentrations. Hamada and Tsuruo observed a similar stimulation of Pgp ATPase activity by trifluoperazine and verapamil [18]. It is difficult to predict how chemosensitizers might affect Pgp ATPase activity, since the mechanism by which they reverse MDR is not known and may vary depending on the type of chemosensitizer [53]. The six chemosensitizers chosen in this study represent a cross-section of the various classes of molecules known to sensitize MDR cells to chemotherapeutic agents, e.g., calcium channel blockers (nifedi-

pine and verapamil), calmodulin inhibitors (trifluoperazine), steroids (progesterone) and anti-arrhythmics (quinine and quinidine) (for a review, see Ref. 54). All these agents inhibit ATP-dependent drug binding and transport in membrane vesicles from MDR cells [13–17,55] and progesterone and a photoaffinity analogue of verapamil are known to label Pgp directly [7,56]. Of these six chemosensitizers, only verapamil appears to be a substrate for efflux in MDR cells [57–59]. We were unable to observe consistent patterns relating the potency of the chemosensitizers to reverse MDR or inhibit Pgp labelling, to their ability to inhibit or stimulate the Pgp ATPase activity. Further characterization of the effect of MDR drugs and chemosensitizers on Pgp ATPase activity awaits the functional reconstitution of Pgp into defined lipid vesicles.

Acknowledgements

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References

1 Kane, S.E., Pastan, I. and Gottesman, M.M. (1990) J. Bioenerg. Biomembr. 22, 593–618.
2 Higgins, C.F., Hyde, S.C., Mimmack, M.M., Gileadi, U., Gill, D.R. and Gallagher, M.P. (1990) J. Bioenerg. Biomembr. 22, 571–592.
3 Kuchler, K., Sterne, R.E. and Thorner, J. (1989) EMBO J. 8, 3973–3984.
4 McGrath, J.P. and Varshavsky, A. (1989) Nature 340, 400–404.
5 Riordan, J.R., Rommens, J.M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S. and Tsui, L.-C. (1989) Science 245, 1066–1073.
6 Safa, A.R., Glover, C.J., Meyers, M.B., Biedler, J.L. and Felsted, R.L. (1986) J. Biol. Chem. 261, 6137–6140.
7 Safa, A.R. (1988) Proc. Natl. Acad. Sci. USA 85, 7187–7191.
8 Safa, A.R., Mehta, N.D. and Agresti, M. (1989) Biochem. Biophys. Res. Commun. 162, 1402–1408.
9 Cornwell, M.M., Safa, A.R., Felsted, R.L., Gottesman, M.M. and Pastan, I. (1986) Proc. Natl. Acad. Sci. USA 83, 3847–3850.
10 Busche, R., Tümmler, B., Riordan, J.R. and Cano-Gauci, D.F. (1989) Mol. Pharmacol. 35, 414–421.
11 Cornwell, M.M., Tsuruo, T., Gottesman, M.M. and Pastan, I. (1987) FASEB J. 1, 51–54.
12 Georges, E., Zhang, J.-T. and Ling, V. (1991) J. Cell. Physiol. 148, 479–484.
13 Horio, M., Gottesman, M.M. and Pastan, I. (1988) Proc. Natl. Acad. Sci. USA 85, 3580–3584.
14 Kamimoto, Y., Gatmaitan, Z., Hsu, J. and Arias, I.M. (1989) J. Biol. Chem. 264, 11693–11698.
15 Doige, C.A. and Sharom, F.J. (1992) Biochim. Biophys. Acta 1109, 161–171.
16 Naito, M., Hamada, H. and Tsuruo, T. (1988) J. Biol. Chem. 263, 11887–11891.
17 Sehested, M., Bindslev, N., Demant, E.J.F., Skovsgaard, T. and Jensen, P.B. (1989) Biochem. Pharmacol. 38, 3017–3027.
18 Hamada, H. and Tsuruo, T. (1988) Cancer Res. 48, 4926–4932.
19 Hamada, H. and Tsuruo, T. (1988) J. Biol. Chem. 263, 1454–1458.
20 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
21 Kartner, N., Evernden-Porelle, D., Bradley, G. and Ling, V. (1985) Nature 316, 820–823.
22 Doige, C.A. and Sharom, F.J. (1991) Protein Express. Purif. 2, 256–265.
23 Laemmli, U.K. (1970) Nature 227, 680–685.
24 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
25 Chifflet, S., Torriglia, A., Chies: R. and Tolosa, S. (1988) Anal. Biochem. 168, 1–4.
26 Jones, L.R., Maddock, S.W. and Besch, H.R., Jr. (1980) J. Biol. Chem. 255, 9971–9980.
27 Shacter, E. (1984) Anal. Biochem. 138, 416–420.
28 Woda, B.A. and Woodin, B. (1984) J. Immunol. 133, 2767–2772.
29 Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150.
30 Gerencser, G.A. and Lee, S.-H. (1985) Am. J. Physiol. 248, R241–R248.
31 LaBelle, E.F., Singh, S.V., Srivastava, S.K. and Awasthi, Y.C. (1986) Biochem. J. 238, 443–449.
32 Prossnitz, E., Gee, A. and Ames, G.F.-L. (1989) J. Biol. Chem. 264, 5006–5014.
33 Kirkpatrick, D.L. and King, K.M. (1990) Biochem. Pharmacol. 39, 1484–1487.
34 Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700.
35 Stone, D.K., Xie, X.-S. and Racker, E. (1983) J. Biol. Chem. 258, 4059–4062.
36 D'Souza, M.P., Ambudkar, S.V., August, J.T. and Maloney, P.C. (1987) Proc. Natl. Acad. Sci. USA 84, 6980–6984.
37 Glickman, J., Croen, K., Kelly, S. and Al-awqati, Q. (1983) J. Cell Biol. 97, 1303–1308.
38 Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) J. Biol. Chem. 260, 1090–1095.
39 Schneider, D.L. (1983) J. Biol. Chem. 258, 1833–1838.
40 Ketcham, C.M., Baumbach, G.A., Bazer, F.W. and Roberts, R.M. (1985) J. Biol. Chem. 260, 5768–5776.
41 Fernley, H.N. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 4, pp. 417–447, Academic Press, New York.
42 Riordan, J.R. and Ling, V. (1979) J. Biol. Chem. 254, 12701–12705.
43 Roy, S.N. and Horwitz, S.B. (1985) Cancer Res. 45, 3856–3863.
44 Chambers, T.C., McAvoy, E.M., Jacobs, J.W. and Eilon, G. (1990) J. Biol. Chem. 265, 7679–7686.
45 Hjelmeland, L.M. (1990) Methods Enzymol. 182, 253–264.
46 Xie, X.-S., Stone, D.K. and Racker, E. (1989) J. Biol. Chem. 264, 1710–1714.
47 Devine, S.E., Hussain, A., Davide, J.P. and Melera, P.W. (1991) J. Biol. Chem. 266, 4545–4555.
48 Racker, E. (1985) in Reconstitution of Transporters, Receptors and Pathological States, pp. 87–103, Academic Press, Orlando.
49 Hsu, C.-M. and Rosen, B.P. (1989) J. Biol. Chem. 264, 17349–17354.
50 Bishop, L., Agbayani, R., Ambudkar, S.V., Maloney, P.C. and Ames, G.F.-L. (1989) Proc. Natl. Acad. Sci. USA 86, 6953–6957.
51 Dean, D.A., Davidson, A.L. and Nikaido, H. (1989) Proc. Natl. Acad. Sci. USA 86, 9134–9138.
52 Kondo,T., Kawakami, Y., Taniguchi, N. and Beutler, E. (1987) Proc. Natl. Acad. Sci. USA 84, 7373–7377.
53 Georges, E., Sharom, F.J. and Ling, V. (1990) Adv. Pharmacol. 21, 185–220.

54 Ford, J.M. and Hait, W.N. (1990) Pharmacol. Rev. 42, 155–199.
55 Horio, M., Lovelace, E., Pastan, I. and Gottesman, M.M. (1991) Biochim. Biophys. Acta 1061, 106–110.
56 Qian, X.-d. and Beck, W.T. (1990) J. Biol. Chem. 265, 18753–18756.
57 Kessel, D. and Wilberding, G. (1984) Biochem. Pharmacol. 33, 1157–1160.
58 Cano-Gauci, D.F. and Riordan, J.R. (1987) Biochem. Pharmacol. 36, 2115–2123.
59 Yusa, K. and Tsuruo, T. (1989) Cancer Res. 49, 5002–5006.