Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells

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P-Glycoprotein (Pgp) was isolated from CH^RC5 membranes by selective detergent extraction and further purified by lentil lectin affinity chromatography. The purified product displayed a very high basal ATPase activity (1.65 μmol/min per mg protein in the absence of added drugs or lipids) with an apparent K_m for ATP of 0.4 mM. There was no evidence of cooperativity, suggesting that the two ATP sites operate independently of each other. Pgp ATPase activity was stimulated by verapamil, trifluoperazine and colchicine, and inhibited by daunomycin and vinblastine. All drugs and chemosensitizers acted as mixed activators or inhibitors, producing changes in both the $V_{max.}$ of the ATPase and the K_m for ATP. ADP competitively inhibited Pgp ATPase, with a K_i of 0.2 mM. The macrolide antibiotics bafilomycin A_1, concanamycin A and concanamycin B, inhibited Pgp ATPase at concentrations of 0.1–10 μM, and at an inhibitor:protein stoichiometry of 0.65–1.0 μmol/mg protein, which is at the low end of the range characteristic of P-type ATPases. Pgp ATPase was relatively selective for adenine nucleotides. Several phospholipids stimulated Pgp ATPase activity in a dose-dependent manner, whereas others produced inhibition. Metabolic labelling showed that the endogenous phospholipids associated with purified Pgp consisted largely of phosphatidylethanolamine and phosphatidylserine, with only a small amount of phosphatidylcholine. ^{32}P-Labelling studies indicated that purified Pgp was partially phosphorylated. It can be concluded that Pgp is a constitutively active, adenine nucleotide-specific ATPase whose catalytic activity can be modulated by both drugs and phospholipids.

INTRODUCTION

The existence of multidrug resistance (MDR) is a major factor in the failure of chemotherapeutic treatment of human cancer. This phenomenon, which may pre-exist in a tumour, or develop after one or more rounds of chemotherapy treatment, is associated with specific drugs, especially the *Vinca* alkaloids and anthracyclines. The molecular basis for one major type of MDR is the overexpression of a 170–180 kDa plasma membrane glycoprotein, known as the P-glycoprotein (Pgp), which is proposed to transport certain classes of hydrophobic drugs out of the cell (for reviews, see [1,2]). Transfection of the cDNA for both class I and III Pgp isoforms has been shown to confer drug resistance on a drug-sensitive recipient cell.

The multidrug transporter is a member of a large group of membrane proteins known as the ABC (ATP-binding cassette) superfamily [3] or the 'traffic ATPases' [4]. The proteins in this superfamily consist of two homologous halves, each made up of six putative transmembrane segments, and a consensus sequence for a nucleotide-binding fold. Other proteins in this family include components of several bacterial transport proteins [5], the yeast *ste6* mating factor *a* exporter [6] and the cystic fibrosis gene product, CFTR [7]. Since Pgp binds photoaffinity analogues of drugs and chemosensitizers [8–11] and ATP [12], it has been proposed to act as an ATP-dependent drug efflux pump. Biochemical evidence that this is indeed the case has accumulated over the past few years. Several studies using plasma-membrane vesicles from cells and tissues expressing Pgp have demonstrated both drug binding and ATP-dependent drug transport [13–17]. Hamada and Tsuruo first reported that affinity-purified Pgp displayed very low ATPase activity [18,19]. Sarkadi et al. found that Sf9 insect cells transfected with human *mdr1* exhibited a high ATPase activity in their plasma membrane, which was stimulated by drugs and chemosensitizers [20], and Al-Shawi and Senior demonstrated similar drug-stimulated ATPase activity in the plasma membrane of a Chinese hamster ovary (CHO) cell line selected for high levels of Pgp expression [21]. Shimabuku et al. constructed a Pgp-β-galactosidase fusion protein, which on purification proved to have ATPase activity [22]. Ambudkar et al. isolated a partially purified preparation of Pgp in octylglucoside which had a high level of ATPase activity [23] and Shapiro and Ling used Zwittergent 3-12 to isolate 90% pure Pgp with substantial levels of ATPase activity [24]. More recently, Urbatsh et al. reported using octylglucoside to isolate purified Pgp with high ATPase activity [25].

We previously isolated and characterized a partially purified Pgp extract with high ATPase activity (> 0.5 μmol/min per mg), from MDR CHO cells [26]. This Pgp preparation showed both ATP-dependent drug transport and ATPase activity following reconstitution into proteoliposomes [27]. In the present work, we have further purified Pgp using lectin affinity chromatography. The resulting product, which displays one of the highest levels of constitutive ATPase activity reported to date for a detergent-solubilized Pgp preparation, has been characterized with respect to kinetics and nucleotide specificity. The kinetic basis for the modulation of Pgp ATPase activity by drugs, chemosensitizers and membrane lipids was examined. In addition, the Pgp ATPase was shown to be inhibited by the macrolide antibiotics bafilo-

Abbreviations used: CHO, Chinese hamster ovary; 2D-HPTLC, two-dimensional high-performance thin-layer chromatography; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; MDR, multidrug-resistance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pgp, P-glycoprotein; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; DMSO, dimethyl sulphoxide.

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mycin A_1 and concanamycins A and B. Finally, the endogenous phospholipids associated with Pgp were identified and the phosphorylation status of the purified protein was explored.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylethanolamine (DPPE), egg phosphatidylethanolamine (PE), and yeast phosphatidylinositol (PI) were obtained from Serdary Research Laboratories (London, Ontario, Canada). CHAPS, disodium-ATP and Mg-ATP, staurosporine, egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), bovine erythrocyte sphingomyelin (SM) and all other lipids were from Sigma Chemical Co. (St. Louis. MO, U.S.A.). Asolectin (30% PC, 31% PI and 30% PE) was supplied by Fluka (Ronkonkoma, NY, U.S.A.). Cell culture supplies were purchased from Gibco Canada (Burlington, Ontario, Canada). Protein was quantified as described by Peterson [28] or Bradford [29], using BSA (crystallized and lyophilized, Sigma) as a standard.

Cell culture and plasma-membrane preparation

The MDR CHO cell line CH^RC5 [30] was grown as described previously [13,26,31]. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in α-minimal essential medium supplemented with 10% heat-inactivated iron-supplemented/defined bovine calf serum (Hyclone Laboratories, Logan, UT, U.S.A.), penicillin (1000 i.u./ml), streptomycin (1 mg/ml) and 2 mM L-glutamine. Plasma-membrane vesicles were isolated from CH^RC5 cells using a method involving cell disruption by nitrogen cavitation, followed by ultracentrifugation on a 35% (w/w) sucrose cushion [31]. Membrane vesicles were stored at −70 °C for no more than 3 months before use.

Selective detergent-extraction of Pgp

Pgp was partially purified using a two-step CHAPS extraction as described previously [26]. Briefly, plasma-membrane vesicles from CH^RC5 were thawed and sedimented at 164000 *g* for 30 min at 4 °C. The pellet was resuspended in 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) to 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 *g* for 15 min at 4 °C. The pellet was resuspended in 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) to 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 *g* for 15 min at 4 °C. The resulting supernatant (S_2 fraction) contained partially purified Pgp.

Lentil lectin affinity chromatography

The S_2 fraction was further purified by affinity chromatography on lentil lectin-Sepharose 4B (Pharmacia, Dorval, Quebec, Canada), as previously described for detergent-solubilized plasma membrane [31]. In a typical purification, approximately 3.6 ml of the CHAPS extract, containing 0.9 mg of protein, was loaded onto a lentil lectin–Sepharose 4B column equilibrated with buffer B (4 ml bed volume) at 4 °C at a flow rate of 0.2 ml/min. After washing with five column volumes of buffer B, bound proteins were eluted with 0.5 M α-methylmannoside in buffer B. Fractions were monitored for both absorbance at 280 nm and Mg^{2+}-ATPase activity. Since all of the ATPase activity was localized to the run-through, these fractions were pooled (~ 4.2 ml) and applied directly to a second lentil lectin–Sepharose 4B column, using the same conditions as above. The final product was obtained by again pooling the run-through fractions (final volume 4.8 ml).

Electrophoresis and Western blotting

Protein samples from various stages of the purification procedure were first dialysed against 50 mM ammonium bicarbonate and then freeze dried. SDS/PAGE was carried out according to Laemmli [32], 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, Canada) and stained with Coomassie Blue (Bio-Rad). Western blotting was performed as described previously [26,31]. Proteins were transferred to Zetaprobe sheets (Bio-Rad) using a Bio-Rad electroblotting apparatus. Blots were blocked in 5% (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 Pgp-specific monoclonal antibody C219 (ID Laboratories, London, Ontario, Canada) 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, Quebec, Canada) in PBS with 3% BSA, followed by several washes as described above. Dried blots were autoradiographed at −70 °C using DuPont Cronex-4 film and an intensifying screen.

Measurement of Mg^{2+}-ATPase activity

The Mg^{2+}-ATPase activity of Pgp was determined by measuring the release of inorganic phosphate from ATP, using a colorimetric method [26,33]. Samples of solubilized, purified Pgp (0.25 μg) were diluted with assay buffer (50 mM Tris-HCl/0.15 M NH_4Cl/5 mM $MgCl_2$/0.02% NaN_3, pH 7.5) in a 96-well microtitre plate (final volume 90 μl) at 23 °C. To initiate the reaction, 10 μl of ATP solution in assay buffer was added, giving final concentrations of 0–4 mM ATP and 0.4 mM CHAPS. CHAPS concentrations in the range 0.4–0.8 mM produce maximal Pgp ATPase activity [26]. 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 did not exceed 5% of the total activity for pure Pgp and 14% for CH^RC5 plasma membrane. Kinetic data were fitted to the Michaelis–Menten and Hill equations by non-linear regression using the program Enzfitter (Biosoft). Hydrolysis of other nucleoside triphosphates (all from Sigma) was measured using the same procedure.

Stock solutions of colchicine, vinblastine, daunomycin, verapamil and trifluoperazine (all from Sigma) were prepared in assay buffer containing 10% dimethyl sulphoxide (DMSO). Controls contained the appropriate amount of DMSO in buffer alone. Final DMSO concentrations did not exceed 1% (v/v), which had no effect on Pgp ATPase activity. Bafilomycin A_1 from *Streptomyces* sp. [34] was supplied by Dr. Karlheinz Altendorf (Universität Osnabrück, Germany). Concanamycins A and B were provided by Dr. Morrie Manolson (Hospital for Sick Children, Toronto, Canada). Stock solutions of the antibiotics were prepared in DMSO.

Preparation of phospholipids

Stock solutions of phospholipids were made up in chloroform/methanol and stored at −20 °C. Lipids were dispensed into glass tubes, dried under N_2 and pumped under vacuum for 45 min. Dried lipids were then suspended in assay buffer and mixed with small-diameter glass beads using a vortex mixer until homogenous. Lipids were freshly prepared before each experiment.

Metabolic labelling of Pgp and phospholipids with ^{32}P

CH^RC5 cells (1×10^8/ml) were incubated in phosphate-free Hepes-buffered saline solution (20 mM Hepes/140 mM NaCl/2 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/10 mM glucose, pH 7.2) containing 0.15 mCi/ml carrier-free [^{32}P]orthophosphate (ICN Biomedicals). After incubation for 1 h at 37 °C, cells were washed five times to remove unincorporated $^{32}P_i$. Plasma-membrane vesicles and the S_2 fraction were prepared as described above and Pgp was purified using lentil lectin chromatography. ^{32}P-Labelling of Pgp was assessed at various stages of purification by SDS/PAGE and autoradiography. The labelling level of the final product was determined following SDS/PAGE at four different loading levels by cutting out the Pgp bands and counting for ^{32}P. The protein content of the Pgp bands was quantified following Coomassie Blue staining by comparison with BSA standards run on the same gel.

Identification and quantification of phospholipids associated with purified Pgp

Endogenous phospholipids associated with purified Pgp were isolated following metabolic labelling using organic solvent extraction [35] and analysed by two-dimensional high-performance thin-layer chromatography (2D-HPTLC) [36] and autoradiography. Briefly, Pgp (100 μg) purified from [^{32}P]P_i-labelled CH^RC5 cells, together with carrier lipids (20 μg each of PC, PE, PI, PS and SM) were treated with 5 ml of hexane:propan-2-ol (3:2 v/v). The sample was vortexed and then centrifuged at 4000 *g* for 15 min. The organic phase was collected and dried under a stream of N_2. The lipid extract was then redissolved in 20 μl of chloroform/methanol (4:1 v/v) and analysed by 2D-HPTLC on heat-activated high-performance silica gel 60 plates (10 × 10 cm, aluminum-backed, Merck, Darmstadt, Germany). Plates were developed in paper-lined glass chambers in the first dimension in chloroform/methanol/ammonia (65:25:5 v/v), air-dried, and run in the second dimension in chloroform/acetone/methanol/glacial acetic acid/water (6:8:2:2:1 v/v). Lipid spots were located with iodine vapour and identified by comparison with standards, and ^{32}P-labelled species were visualized by autoradiography.

Pgp-associated phospholipids were quantified according to the protocol of Bartlett [37]. Purified Pgp (100 μg) was extracted with organic solvent as described above. To the dried lipid sample in a Pyrex test tube, 30 μl of 10% magnesium nitrate in 95% ethanol was added. The sample was heated in a flame until the brown fumes disappeared, and resuspended in 1 ml of 5 M H_2SO_4. The sample was then assayed for phosphate using KH_2PO_4 as a standard.

Phosphorylation of Pgp in CH^RC5 plasma-membrane vesicles using [γ-^{32}P]ATP

CH^RC5 plasma-membrane vesicles (250 μg of protein) were phosphorylated with [γ-^{32}P]ATP (650 Ci/mmol, ICN Biomedicals) in 20 mM Tris-HCl/0.25 M sucrose/5 mM $MgCl_2$/10 mM EGTA, pH 7.5, containing 1 mM sodium orthovanadate and 2 mM ATP. The reaction was allowed to proceed for 5 min at 23 °C and then terminated with an equal volume of 2 × concentrated electrophoresis sample loading buffer. SDS/PAGE was carried out on 7.5% polyacrylamide gels at four different loading levels, followed by Coomassie Blue staining, and Pgp was quantified by comparison with BSA standards run on the same gel. After detection by autoradiography, the ^{32}P-labelled Pgp bands were cut out and counted. The ^{32}P c.p.m., which were linear with protein loading, were used to calculate the specific activity of labelled Pgp.

RESULTS

Purification of ATPase-active Pgp

We previously reported that a two-step selective extraction of the CH^RC5 plasma membrane with the detergent CHAPS generated a partially purified (~ 30%) preparation of Pgp [26] (see Figure 1a, lane 2). The Mg^{2+}-dependent ATPase activity of this S_2 extract under defined assay conditions was confirmed to be due entirely to Pgp, and no contaminating membrane ATPases were detected [26]. When the partially purified Pgp fraction was chromatographed on lentil lectin–Sepharose, over 80% of the ATPase activity was retained in the run-through while over half the total protein was bound by the column (Table 1). SDS/PAGE of the run-through (Figure 1a, lane 3) showed a substantial reduction in contaminating proteins compared with the initial extract. A second chromatography step improved the Pgp purity further, giving a single major band at 180 kDa on SDS/PAGE (Figure 1a, lane 4), which reacted with the Pgp-specific monoclonal antibody C219 on Western blots (Figure 1b). No proteolytic degradation fragments which cross-reacted with the C219 antibody were detected. A large fraction of the protein removed from the preparation during lentil lectin chromatography remained associated with the column and could not be eluted by 0.5 M α-methylmannoside (Table 1), which suggested that it consisted of glycoproteins bound to the column very tightly. Columns could typically be used for up to ten purification runs before their performance decreased. Attempts to regenerate columns using more rigorous washing procedures (high and low pH) succeeded in removing most of the bound protein, but

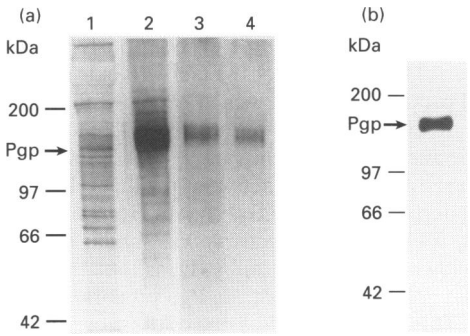

Figure 1 SDS/PAGE analysis of the purification of Pgp by selective CHAPS extraction and lentil lectin affinity chromatography

Samples were dialysed into 50 mM ammonium bicarbonate, freeze dried and then analysed by (**a**) SDS/PAGE followed by silver staining, and (**b**) Western immunoblotting with the monoclonal antibody C219. (**a**) Lane 1, plasma membrane, 1.5 μg of protein; lane 2, CHAPS extract (S_2 fraction), 1.5 μg of protein; lane 3, first lentil lectin run-through, 0.5 μg of protein; lane 4, second lentil lectin run-through, 0.5 μg of protein. (**b**) Western immunoblot of the second lentil lectin run-through, 0.75 μg of protein. Molecular mass marker positions are indicated on the left and the Pgp band is indicated by an arrow.

Table 1 Mg^{2+}-ATPase activity at different stages of Pgp purification

Stage of purification	Mg^{2+}-ATPase Specific activity* (μmol/mg per min)	Total activity (μmol/min)	Protein (mg)
Plasma membrane	0.273 ± 0.009†	0.983	3.60
CHAPS extract	0.570 ± 0.013	0.502	0.88
First lentil lectin run-through	0.966 ± 0.016	0.406	0.42
First lentil lectin eluate	0	0	0.06
Second lentil lectin run-through	1.65 ± 0.04	0.346	0.21

* Samples from various stages of the purification procedure were assayed for Mg^{2+}-ATPase activity as described in Ref. 26. The final reaction mixture contained 2 mM ATP and (except for plasma membrane) 0.4 mM CHAPS, in 100 μl of assay buffer. ATPase activity is expressed as P_i released and is presented as the mean ± S.E.M. ($n = 5$).
† ATPase activity attributable to Pgp is estimated at 0.217 μmol/min per mg (around 80% of the total, see [26]).

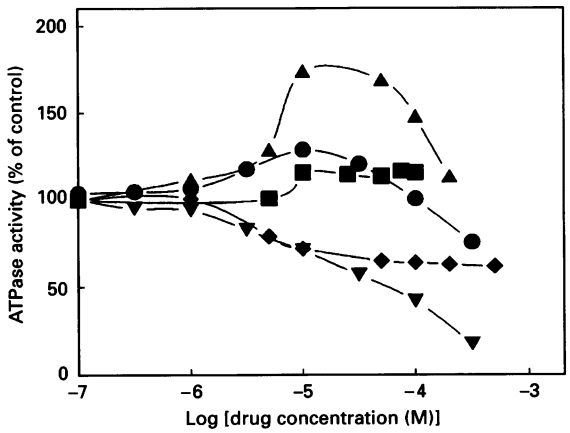

Figure 2 Modulation of Pgp ATPase activity by drugs and chemosensitizers

Purified Pgp (0.2 μg) in 0.4 mM CHAPS was assayed for Mg^{2+}-ATPase activity in the presence of 1 mM ATP and ●, verapamil; ▲, trifluoperazine; ■, colchicine; ◆, daunomycin; and ▼, vinblastine. Data are presented as a percentage of control ATPase activity (means ± S.E.M., $n = 3$), measured without drugs or chemosensitizers.

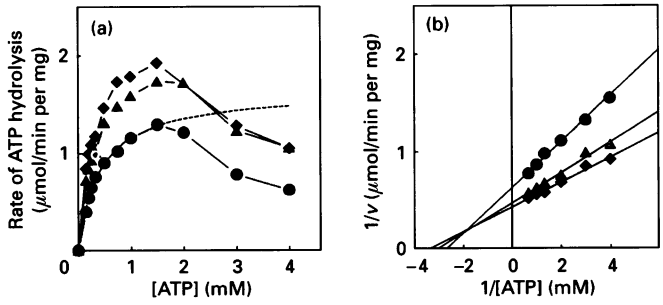

Figure 3 Kinetics of ATP hydrolysis by Pgp in the presence of verapamil

ATPase activity of purified Pgp (0.2 μg) in 0.4 mM CHAPS was measured at various ATP concentrations. Data are presented as means ± S.E.M. ($n = 3$). (**a**) Michaelis–Menten kinetic plots of ATP hydrolysis in the absence of verapamil (●), and in the presence of 5 μM (▲) and 10 μM (◆) verapamil. Kinetic parameters (K_m, V_{max}) were calculated by fitting data for ATP concentrations up to 1.5 mM to the Michaelis–Menten equation using non-linear regression analysis. The calculated curve for these values of K_m and V_{max} is indicated by the broken line. (**b**) Lineweaver–Burk plots of the kinetic data for ATP concentrations in the range 0.25–1.5 mM, in the absence of verapamil (●), and in the presence of 5 μM (▲) and 10 μM (◆) verapamil. Data were fitted by non-linear regression analysis before transformation.

column performance and lifetime were seriously reduced. About 70% of the ATPase activity of the CHAPS extract was retained in the final product (Table 1), which had a 3-fold higher specific activity than the starting material. Several batches of purified Pgp had specific activities in the range 1.2–1.7 μmol/min per mg, mainly as a result of differing activities for the CH^RC5 membrane starting material and CHAPS extract. However, the fold-enrichment in ATPase-specific activity was similar for all detergent extracts following chromatography. Densitometry of Coomassie Blue-stained gels indicated that the Pgp preparation was around 90% pure. This procedure yields one of the most highly ATPase-active purified Pgp preparations reported to date. Purified Pgp in CHAPS appears to exist in the form of protein aggregates of high molecular mass, as assessed by gel-filtration chromatography on Sepharose 4B.

Modulation of the ATPase activity of Pgp by drugs and chemosensitizers

We previously reported that the ATPase activity of partially purified Pgp was modulated by drugs, both in CHAPS solution [26] and in reconstituted proteoliposomes [27]. The effect of various transport substrates and chemosensitizers on purified Pgp ATPase activity was determined at 1 mM ATP (Figure 2). The chemosensitizers trifluoperazine and verapamil increased ATPase activity, with maximal stimulation observed at around 10 μM. The transport substrate colchicine elevated Pgp ATPase activity at concentrations > 75 μM. Daunomycin and vinblastine, both of which are transport substrates, inhibited ATPase activity. In the case of daunomycin, inhibition reached a maximum of ~ 35% at 50 μM, whereas vinblastine progressively inhibited activity to < 20% of the control at 200 μM.

Kinetics of ATP hydrolysis by Pgp

The kinetics of ATP hydrolysis by Pgp were investigated at 5 mM Mg^{2+}. ATPase activity reached a maximum at 1.5 mM ATP and declined at higher concentrations, which is indicative of substrate or product inhibition (Figure 3a). A similar kinetic profile was also observed using 0–4 mM Mg-ATP. Fitting the kinetic data for ATP concentrations up to 1.5 mM to the

Table 2 Kinetic parameters for Pgp ATPase in the presence of drugs and chemosensitizers

Kinetic parameters were determined by fitting experimental data to the Michaelis–Menten and Hill equations using the program Enzfitter. Changes in $V_{max.}$ are expressed in relative terms to account for variations in absolute $V_{max.}$ between different batches of purified Pgp.

Drug	Concentration (μM)	K_m (mM)	Relative $V_{max.}$	Hill coefficient
None		0.40	1.00	1.06
Verapamil	5	0.33	1.34	1.07
Verapamil	10	0.30	1.37	1.14
Trifluoperazine	10	0.54	1.58	0.67
Colchicine	100	0.74	1.71	1.01
Daunomycin	50	0.88	1.26	0.90
Vinblastine	20	1.04	0.80	0.85

Michaelis–Menten equation yielded an apparent K_m of 0.4 mM. The experimental curve showed substantial deviations from the Michaelis–Menten model at higher concentrations, as indicated by both visual inspection (Figure 3a) and analysis of residuals. However, there was no evidence of cooperativity between the two ATP sites at ATP concentrations less than 1.5 mM, and curve fitting gave a value for the Hill coefficient of 1.06.

Effect of drugs and chemosensitizers on the kinetics of Pgp ATPase

The kinetic basis for the stimulatory and inhibitory effects of drugs on Pgp ATPase was examined in more detail, by measuring ATP hydrolysis in the presence of selected concentrations of various compounds. As shown in Figure 3(a) (Michaelis–Menten plots) and Figure 3(b) (results displayed as double reciprocal plots), the apparent K_m of catalysis in the presence of 5 and 10 μM verapamil decreased slightly, while $V_{max.}$ increased. Data at ATP concentrations less than 1.5 mM were used to estimate kinetic parameters in the presence of verapamil (Table 2). $V_{max.}$ increased by 34 and 37%, while K_m was reduced to 0.33 mM and 0.30 mM, at 5 and 10 μM verapamil respectively. The Hill coefficients in the presence of 5 and 10 μM verapamil were close to 1 (Table 2), again indicative of no cooperativity between the two ATPase sites.

Kinetic parameters were determined for Pgp ATPase in the presence of 10 μM trifluoperazine, 100 μM colchicine, 50 μM daunomycin and 20 μM vinblastine. All the drugs tested proved to be either mixed activators (trifluoperazine, colchicine) or inhibitors (daunomycin, vinblastine), with changes observed in both K_m and $V_{max.}$. The kinetic data for these compounds are displayed as double-reciprocal plots in Figure 4. Kinetic data for each drug were fitted to the Michaelis–Menten equation and K_m and $V_{max.}$ parameters were extracted (Table 2). Fitting to the Hill equation allowed estimation of the Hill coefficient for ATP hydrolysis in the presence of each compound. There was no evidence for positively cooperative kinetics of ATP hydrolysis in the presence of any of the compounds tested. All Hill coefficients were close to 1, except that for trifluoperazine, which gave an indication of slight negative cooperativity.

Inhibition of Pgp ATPase by ADP

The deviation from Michaelis–Menten behaviour evident in the kinetic data shown in Figure 3 suggested that product inhibition might be occurring. This was confirmed by determining the kinetics of Pgp ATPase in the presence of increasing concentrations of ADP. ADP acted as a classical competitive inhibitor of ATP hydrolysis by Pgp, with a K_i of 0.2 mM (Figure 5).

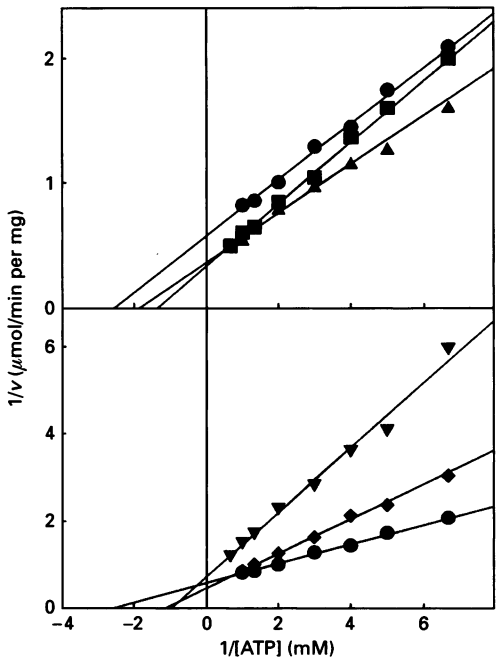

Figure 4 Kinetics of ATP hydrolysis by Pgp in the presence of other drugs and chemosensitizers

ATPase activity of purified Pgp (0.2 μg) in 0.4 mM CHAPS was measured at various ATP concentrations. Kinetic parameters (K_m, $V_{max.}$) were calculated by fitting data to the Michaelis–Menten equation using non-linear regression analysis. Lineweaver–Burk plots of the kinetic data are displayed for ATP concentrations in the range 0.25–1.5 mM, in the absence of drugs (●), and in the presence of 100 μM colchicine (■), 10 μM trifluoperazine (▲), 50 μM daunomycin (◆), and 20 μM vinblastine (▼). Data are presented as means ± S.E.M. ($n = 3$) and were fitted by non-linear regression analysis before transformation.

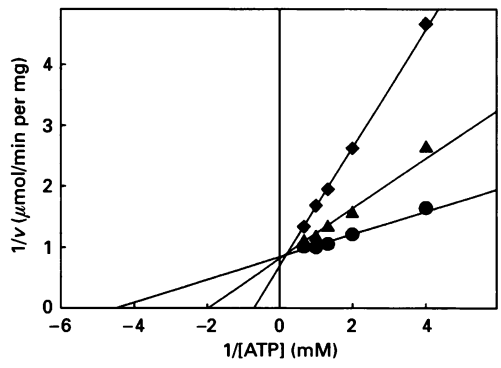

Figure 5 Competitive inhibition of Pgp ATPase by ADP

ATPase activity of purified Pgp (0.2 μg) in 0.4 mM CHAPS was measured at various ATP concentrations. Lineweaver–Burk plots of the kinetic data are presented for ATP concentrations in the range 0.25–1.5 mM, in the absence of ADP (●), and in the presence of 0.25 mM (▲) and 0.50 mM (◆) ADP. Data were fitted by non-linear regression analysis before transformation.

Inhibition of Pgp ATPase by bafilomycin A_1 and concanamycins

Bafilomycins and concanamycins are members of a class of macrolide antibiotics which are known to inhibit a variety of membrane-bound ion-motive ATPases. The concentration range

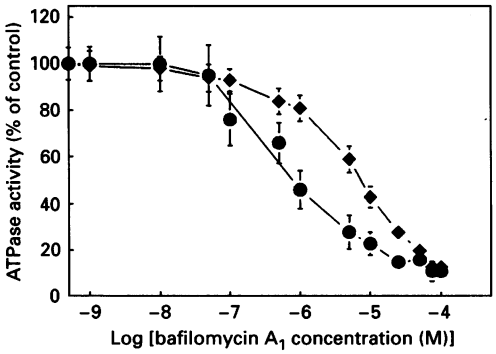

Figure 6 Inhibition of Pgp ATPase by the macrolide antibiotic bafilomycin A_1

ATPase activity of CH^RC5 plasma membrane (◆, 2 μg) and purified Pgp in 0.4 mM CHAPS (●, 0.2 μg) was measured at various concentrations of bafilomycin A_1. Data are presented as a percentage of control ATPase activity (means ± S.E.M., $n = 3$).

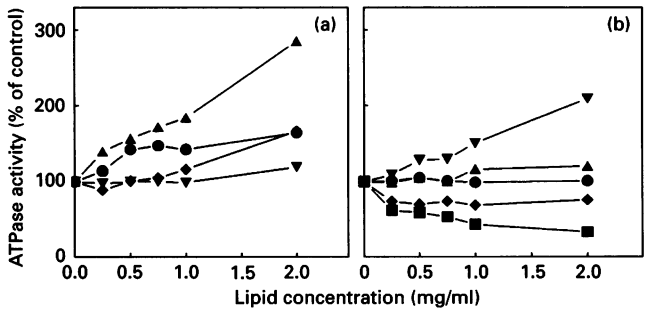

Figure 7 Modulation of the ATPase activity of Pgp by phospholipids

Samples containing purified Pgp (0.25 μg in 0.4 mM CHAPS) were pre-incubated with various concentrations of the following phospholipids: (**a**) DMPE (●), DPPE (▲), egg PE (▼), asolectin (◆); and (**b**) DMPC (●), DPPC (▲), egg PC (▼), PS (◆), PI (■). After 1 h at 4 °C, the ATPase activity was measured at 37 °C. ATP hydrolysis is presented as a percentage of the control ATPase activity assayed without addition of phospholipids (means ± S.E.M., $n = 3$).

Table 3 Ability of Pgp to hydrolyse various nucleoside triphosphates

Purified Pgp (0.25 μg in 0.4 mM CHAPS) was incubated with various ribo- and deoxyribonucleotides at concentrations of 0.5 mM and 1.0 mM and nucleotide hydrolysis was measured as outlined for ATP. Data are presented as means ± S.E.M. ($n = 3$) and are normalized to the values obtained for ATP at the same concentration.

	NTPase activity (% of control)	
	Nucleotide concentration (mM)	
Nucleotide	0.5	1.0
ATP	100	100
CTP	4.4	1.6
GTP	7.1	12.0
UTP	11.4	11.4
dATP	46.8	61.3
dCTP	2.6	8.6
dGTP	4.7	3.6

over which inhibition is observed, as well as the inhibitor:protein stoichiometry, have proved invaluable in distinguishing among the E_1E_2 (P-type), F_oF_1 (F-type) and vacuolar (V-type) ATPases [34,38]. Both the ATPase activity of CH^RC5 plasma membrane (~ 80% of which can be attributed to Pgp) and purified Pgp ATPase were inhibited similarly by bafilomycin A_1 (Figure 6) and the related compounds concanamycin A and B (results not shown). In the case of purified Pgp, inhibition was apparent at 0.1–1 μM for all three antibiotics and was virtually complete at 20 μM. For both CH^RC5 membrane and purified Pgp, the IC_{50} for bafilomycin A_1 inhibition of ATPase activity increased linearly with the amount of protein present in the assay mixture (results not shown). For purified Pgp, ATPase activity was inhibited 50% at 0.9, 1.0 and 0.65 μmol/mg protein for bafilomycin A_1 and concanamycins A and B respectively.

Nucleotide preferences of Pgp ATPase

We investigated whether purified Pgp was able to utilize nucleoside triphosphates other than ATP. dATP was hydrolysed at slightly less than half the rate observed for ATP at a concentration of 0.5 mM and the rate of hydrolysis increased to around 60% of the rate for ATP at 1.0 mM (Table 3). Other ribo- and deoxyribo-nucleotides were hydrolysed only very slowly (< 10% of the rate for ATP) at both 0.5 and 1.0 mM (Table 3). Thus, Pgp ATPase appears to be relatively specific for adenine nucleotides.

Lipid dependence of Pgp ATPase

To examine the effect of membrane lipids on Pgp ATPase activity, purified Pgp was preincubated for 1 h at 4 °C with a variety of different phospholipids before determination of catalytic activity at 37 °C. The data presented in Figures 7(a) and (b) that several lipid species induced a large, concentration-dependent stimulation of the ATPase activity, while others caused inhibition. DPPE produced the largest stimulation (almost 3-fold at 2 mg/ml), while egg PC increased activity about 2-fold at this concentration. Dimyristoylphosphatidylethanolamine (DMPE) and asolectin had smaller stimulatory effects. Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) produced only small changes in ATPase activity, while PS and PI led to substantial inhibition of 25 and 67% respectively at 2 mg/ml. The Pgp ATPase is thus highly lipid-dependent and specific lipid species, especially DPPE and egg PC, are able to modulate catalytic activity. Analysis of ATP hydrolysis kinetics in the presence of DPPE indicated that this lipid, like verapamil, acted as a mixed activator of the ATPase; $V_{max.}$ increased by 2.5-fold and K_m was reduced to 0.32 mM. There was no evidence of cooperativity between the two ATPase sites in the presence of DPPE (Hill coefficient = 1.2).

Quantification and identification of phospholipids associated with purified Pgp

A sample of purified Pgp was treated with hexane/propan-2-ol to extract associated phospholipids, which were then quantified using the acid hydrolysis/phosphate assay method of Bartlett [37]. Two independent determinations indicated that 53 and 56 phospholipids were associated with each molecule of purified Pgp. To identify the particular phospholipid species present in the Pgp preparation, intact CH^RC5 cells were incubated with [^{32}P]P_i to metabolically label phospholipids, plasma-membrane vesicles were prepared and Pgp was isolated. A sample of purified

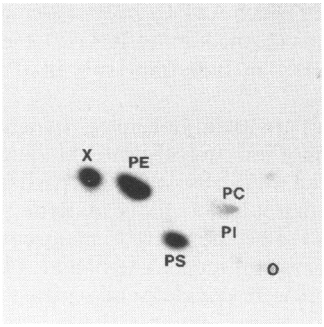

Figure 8 Identification of phospholipids associated with purified Pgp

After metabolic labelling of intact CH^RC5 cells with [^{32}P]P_i, plasma-membrane vesicles were prepared and Pgp was isolated as described. A sample of purified Pgp (100 μg), together with unlabelled carrier lipids, was extracted with hexane–propan-2-ol and the lipid extract was separated by 2D-HPTLC on silica gel 60 plates. Lipid spots were visualized using both autoradiography and staining with I_2 vapour, followed by comparison with lipid standards.

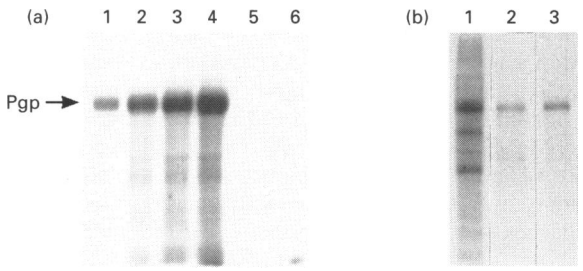

Figure 9 Phosphorylation status of Pgp in plasma membrane vesicles and after purification

(**a**) Phosphorylation of Pgp in CH^RC5 plasma membrane by an endogenous protein kinase. CH^RC5 plasma-membrane vesicles were phosphorylated with 2 mM [γ-^{32}P]ATP at 23 °C for 5 min and SDS/PAGE was carried out on 7.5% acrylamide gels at four different protein loading levels (lane 1, 2 μg; lane 2, 4 μg; lane 3, 6 μg; lane 4, 8 μg), followed by autoradiography. The amount of protein in the Pgp band was quantified by comparison with BSA standards electrophoresed in the same gel after staining with Coomassie Blue. Phosphorylation was also carried out in the presence of a 100-fold excess of unlabelled ATP (200 mM, lane 5), and the protein kinase inhibitor staurosporine (20 μM, lane 6). (**b**) Metabolic labelling of Pgp with ^{32}P. Intact CH^RC5 cells were metabolically labelled with [^{32}P]P_i, followed by plasma-membrane vesicle preparation and Pgp isolation. SDS/PAGE and autoradiography were carried out at various stages of the purification process: CH^RC5 plasma membrane (lane 1, 15 μg), CHAPS extract (S_2 fraction) (lane 2, 0.5 μg) and lentil lectin-purified Pgp (lane 3, 0.75 μg).

Pgp was then treated with hexane/propan-2-ol in the presence of carrier lipids and the lipid extract was separated by 2D-HPTLC. Lipid spots were visualized using both autoradiography and staining with I_2 vapour, followed by comparison with lipid standards. As shown in Figure 8, there were three major ^{32}P-labelled phospholipid species associated with Pgp. The two most prominent lipids were identified as PE and PS. A very small amount of PC was also present, together with barely detectable traces of PI, whereas no SM was observed. 2D-HPTLC analysis and I_2 staining of a total lipid extract prepared from unlabelled CH^RC5 plasma membrane showed that the most abundant phospholipid present was PC, followed by PE, with smaller quantities of PS and SM and trace amounts of PI. Thus, the endogenous phospholipids associated with Pgp are not representative of the composition of the source plasma membrane. In particular, the choline lipids PC and SM are greatly underrepresented in purified Pgp. The third prominently labelled lipid species (marked X in Figure 8) remains unidentified; its migration position on 2D-HPTLC did not correspond to any of the phospholipid standards, including PE, PS, PC, PI, SM, cardiolipin, phosphatidylglycerol and lysophospholipids. The unidentified phospholipid was not present in significant amounts in a total lipid extract prepared from unlabelled CH^RC5 plasma membrane.

Phosphorylation status of Pgp

Pgp is known to be phosphorylated, both *in vivo* and *in vitro*. Two different approaches were used to assess the state of phosphorylation of Pgp in the present study. Addition of [γ-^{32}P]ATP to CH^RC5 plasma membrane vesicles resulted in rapid phosphorylation of Pgp by an endogenous protein kinase (Figure 9a). Pgp was the only membrane protein to be ^{32}P-labelled under these conditions, and the fact that phosphorylation takes place so readily *in vitro* suggests that Pgp is partially dephosphorylated in CH^RC5 plasma-membrane vesicles. A similar kinase has been reported to phosphorylate Pgp in the plasma membrane of MDR KB-V1 cells [39]. Phosphorylation of Pgp was completely abrogated by excess unlabelled ATP and the protein kinase inhibitor staurosporine (Figure 9a). To determine the stoichiometry of phosphorylation, CH^RC5 plasma membrane was phosphorylated with [γ-^{32}P]ATP and SDS/PAGE was carried out at different protein loading levels. Determination of the specific activity of the Pgp band revealed that the stoichiometry of labelling was $\sim$ 0.27 phosphates per protein. Thus, Pgp in the plasma membrane starting material is not fully phosphorylated.

In the second approach, intact CH^RC5 cells were metabolically labelled using [^{32}P]P_i, followed by plasma-membrane vesicle preparation and Pgp isolation. SDS/PAGE and autoradiography were performed at various stages of the purification process. Pgp appeared as a prominently phosphorylated band in the CH^RC5 plasma membrane (Figure 9b, lane 1). Pgp was the only ^{32}P-labelled protein band in both the S_2 fraction and the purified final product (Figure 9b, lanes 2 and 3 respectively).

DISCUSSION

In earlier studies, we succeeded in purifying Pgp to homogeneity [31] using various combinations of immunoaffinity and lectin-affinity chromatography. However, we observed that purification steps during which Pgp bound to the column matrix, and was subsequently eluted, usually resulted in a partial or total loss of ATPase activity (depending on the harshness of the eluent). We then developed a procedure for rapid extraction of Pgp with high ATPase activity from the CH^RC5 plasma membrane using CHAPS [26]. Here we show that affinity chromatography on lentil lectin–Sepharose provides a rapid and convenient technique for further purification of the detergent extract; many glycoprotein contaminants were bound selectively and Pgp ATPase activity was retained. The final ATPase activity of 1.65 μmol/min per mg is 5-fold higher than that reported by Shapiro and Ling [24] for 90% pure CH^RC5 Pgp isolated by ion-exchange and immunoaffinity chromatography. In addition, the overall yield of Pgp from the plasma membrane approaches 50% (compared with 10% for the Shapiro and Ling preparation). Since the completion of this study, Urbatsch et al. have reported the isolation and purification of hamster Pgp with comparable ATPase catalytic activity, using octyl glucoside extraction followed by chromatography on Reactive Red 120 [25]. However, the final product is dilute, and contains 50 mM ATP, *E. coli* lipids and 1 M NaCl, so that further experiments required reconstitution of the protein into lipid bilayers. Because the

protein obtained in the present study undergoes little dilution (final concentration ~ 50 μg/ml) it can be used directly without concentration and it is obtained free from interfering substances, such as ATP, lipids, salt, peptides, sugars etc. Finally, the entire membrane solubilization and purification protocol is convenient and rapid; it can be completed in 4–5 h and produces sub-milligram amounts of Pgp with high ATPase activity.

Pure Pgp displays a relatively high K_m for ATP (around 0.4 mM), consistent with other reports that drug efflux from MDR cells is particularly sensitive to ATP depletion (e.g. [40]). An even higher K_m value was recently reported for the ATPase activity of plasma membrane from a cell line selected for Pgp overexpression [21] and for the Pgp isolated from this membrane [25]. The kinetic plots for ATP hydrolysis are diagnostic of product inhibition at ATP concentrations over 1.5 mM, an effect which was previously observed with partially purified Pgp [26], although it was less pronounced. The kinetic properties of proteins often change somewhat during the course of purification. Kinetic experiments carried out in the presence of ADP confirmed that the nucleotide acted as a competitive inhibitor of the ATPase. A similar conclusion was reached in a study of the ATPase activity of Pgp from the plasma membrane from an overexpressing CHO cell line [21,25].

The ATPase activity of purified Pgp was stimulated by trifluoperazine, verapamil and colchicine, whereas the transport substrates daunomycin and vinblastine caused inhibition. Kinetic studies indicated that all of these compounds acted as 'mixed' activators or inhibitors, indicating that ATP is hydrolysed more rapidly, or more slowly, in the Pgp–drug–ATP complex than in the absence of the drug. The activation of solubilized Pgp by verapamil and trifluoperazine is lower relative to CH^RC5 membrane [40a], which suggests that the detergent CHAPS disrupts the interaction between the drug binding site(s) and the ATPase domains. This is not entirely surprising, since the drug-binding sites are likely to be contained within the transmembrane regions of Pgp [41,42]. Ambudkar et al. also reported that detergent-solubilized Pgp was activated only slightly by the substrate vinblastine [23]. Daunomycin and vinblastine cause inhibition of the ATPase activity of both pure Pgp in CHAPS and reconstituted Pgp in proteoliposomes [27], which suggests that ATPase stimulation *per se* is not a necessary correlate of all Pgp transport substrates. Indeed, we have recently reported that the extent of Pgp ATPase stimulation by hydrophobic peptides does not correlate with their affinity as transport substrates [40a]. The coupling between drug binding and ATP hydrolysis is clearly complex, perhaps reflecting the presence of multiple or overlapping substrate-binding sites. The reported ability of various drugs and chemosensitizers to stimulate Pgp ATPase activity varies widely between different Pgp preparations and Pgp-containing membranes, which suggests that the lipid/detergent environment may be a crucial determinant.

The stimulatory effect of verapamil on Pgp ATPase appears to operate in intact MDR cells, since ATP consumption increased markedly after treatment with verapamil [43]. While most membrane ATPases are tightly coupled to substrate binding and transport, Pgp shows high constitutive levels of ATPase activity, accounting for the high energy consumption noted for MDR cells in the absence of exogenous drugs [43]. It seems unlikely that compounds present in the experimental system act as substrates, since we observe high ATPase activity for both purified Pgp in a simple buffer solution (this work) and partially purified Pgp in proteoliposomes [27]. Since the constitutive ATPase activity of Pgp is fully retained during purification, and also after reconstitution [27], it seems likely that it represents an integral molecular property of Pgp. Little is currently known about the means by which ATP hydrolysis is coupled to substrate translocation across the membrane. It is possible that continuous ATP turnover plays an important role in the mechanism of transport by Pgp.

Inhibitor studies are useful in helping to identify those amino acid residues important in substrate binding and catalysis. Previous studies in our laboratory on partially purified Pgp ATPase revealed that it had a unique inhibition profile [26]. Pgp ATPase was found to be insensitive to many compounds known to inhibit other classes of membrane-bound ATPases. The only identified inhibitors were vanadate and several sulfhydryl agents, which suggested that the catalytic site contains one or more reactive cysteine residues. These observations were later confirmed by Al-Shawi and Senior [21], who also found that fluoroaluminate was a good inhibitor. The bafilomycins and concanamycins, members of a family of macrolide antibiotics with a 16-membered lactone ring, have proved useful in both distinguishing among various classes of ATPases and identifying new ATPases. The antibiotic concentration and the antibiotic:protein stoichiometry at which 50% inhibition occurs (designated as I_{50}, in μmol/mg protein) have been shown to be diagnostic of the ATPase class [34,38]. F_oF_1-ATPases are not inhibited by these compounds at concentrations as high as 1 mM. Vacuolar H^+-pumping ATPases are extremely sensitive to bafilomycin A_1, and even more so to the concanamycins: 50% inhibition occurs at ~ 0.5 nM for bafilomycin A_1 and at ~ 20 pM for concanamycin A [38]. The I_{50} values for inhibition of H^+-ATPases by these antibiotics are in the range 5×10^{-5} (bafilomycin A_1) to 2×10^{-6} (concanamycin A) μmol/mg, which approaches one molecule of antibiotic per molecule of protein. E_1E_2-type ATPases, such as the Na^+K^+- and Ca^{2+}-ATPases, show intermediate sensitivity, with inhibition in the 10–100 μM range and I_{50} values of 3.9 and 0.94 μmol/mg respectively [34,38]. The Pgp ATPase clearly falls at the low end of the range for this latter group, with inhibition occurring between 0.1 and 10 μM, and I_{50} values of 0.65–1.0 μmol/mg for bafilomycin A_1 and concanamycins A and B. Further inhibitor studies may be useful in defining the molecular properties of the ATP-binding folds of Pgp.

The possible role of the two ATP-binding folds of Pgp in the transport process has been the subject of much speculation. Kinetic analysis of the data for purified Pgp showed no evidence for allosteric cooperative interactions between the two ATPase sites, in either the absence or presence of drugs, chemosensitizers or lipids. This suggests that, whatever their role in the transport process, the two ATPase sites operate independently of each other. Similar conclusions were reached by Garrigos et al. using plasma membrane from MDR Chinese hamster lung fibroblasts [44].

We previously determined that ATP, and to a lesser extent dATP, were able to support Pgp-mediated drug transport into CH^RC5 plasma vesicles, while GTP and non-hydrolysable ATP analogues were not [13]. The present study indicates that Pgp is able to hydrolyse other nucleotides only very poorly and thus appears to be relatively selective for adenine nucleotides. This conclusion is supported by the recent report of Urbatsch et al., who concluded that Pgp was relatively specific for adenine nucleotides but was tolerant of modifications [25]. A previous study reported significant levels of GTP hydrolysis by Pgp [21], but since the source used was native plasma membrane, it is likely that other membrane-bound nucleotidases contributed to GTP hydrolysis. It has also been reported that GTP supported vinblastine transport in plasma membrane vesicles from MDR KB cells [45], and it was thus suggested that Pgp may be able to use GTP to energize transport. However, in many cases, drug

transport is very sensitive to ATP depletion, requiring an ATP regenerating system for maximal transport (see for example, [13–15]). Our data suggest that the effect of GTP in KB plasma-membrane vesicles may have been to 'rescue' endogenous ATP from hydrolysis by other nucleotidases, rather than to act as an alternative energy source for Pgp. This proposal is supported by our recent study of reconstituted Pgp in proteoliposomes, where an ATP-generating system was not required for drug transport [27].

Pgp ATPase was modulated by phospholipids: some species greatly stimulated catalytic activity, while others produced inhibition. DPPE both increased the $V_{max.}$ and decreased the K_m for ATP. Saeki et al. recently reported that the ability of a Pgp–β-galactosidase fusion protein to be photolabelled with azidopine could be modulated by various sterols [46]. It is likely that the presence of small amounts of detergent promotes the exchange of exogenous lipids into the boundary lipid region of Pgp. The protein may also be 'reconstituted' into the lipid structures under the conditions of these experiments, in which case the large catalytic activation observed for egg PC and DPPE suggests that these lipid species are good choices for functional reconstitution of Pgp. Indeed, successful reconstitution of drug transport in proteoliposomes of DPPE/egg PC containing partially purified Pgp has been achieved in our laboratory [27].

Extraction of purified Pgp with organic solvents revealed that each molecule of protein is associated with 53–56 phospholipids. This number seems entirely reasonable given the size and hydrophobicity of Pgp and the fact that no attempt was made to delipidate the protein. Indeed, detergent delipidation inactivates Pgp ATPase activity, which can be restored subsequently by the addition of phospholipids [33]. Analysis of phospholipids, following metabolic labelling of intact cells with [^{32}P]P_i, revealed that the major phospholipid associated with Pgp was PE, with lesser amounts of PS. Only very small amounts of PC and no SM were detected, although PC was the major lipid present in the CH^{R}C5 plasma membrane. Thus, Pgp selectively associates with only certain phospholipid species in the source plasma membrane. The amino phospholipids PE and PS, which predominate in the inner leaflet of the plasma membrane of eukaryotic cells, are favoured, whereas the choline lipids PC and SM, which predominate in the outer leaflet, are greatly under-represented. It is interesting that as the major phospholipid associated with Pgp, PE, is also the species that best activates the ATPase activity. Also associated with Pgp was an unidentified phospholipid, which was present below the detection limits in CH^{R}C5 membrane. It seems likely that this species is a metabolic intermediate which turns over rapidly and thus becomes labelled with ^{32}P to a high specific activity.

Variations in the ability of various drugs to stimulate or inhibit ATPase activity have been noted by several research groups. It has been suggested that these disparities may arise from either a different lipid/detergent environment (discussed above) or differences in post-translational modification of Pgp, especially phosphorylation, which is known to modify the level of drug resistance seen in intact cells [47–49]. The sites of phosphorylation of Pgp by protein kinase C have recently been identified in the human [50] and the mouse [51], as multiple serine/threonine residues in the linker region joining the N- and C-terminal halves of the protein. It was not possible to carry out phosphorylation/dephosphorylation of purified Pgp by defined enzymes in the present study because of the presence of high concentrations of the detergent CHAPS. Our studies of the phosphorylation state of Pgp during the solubilization and purification process showed that the final purified product is phosphorylated. However, investigation of the phosphorylation state of the plasma-membrane starting material showed that Pgp can be further labelled with [γ-^{32}P]ATP, indicating that purified Pgp is likely to be phosphorylated at sub-stoichiometric levels.

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