Functional Reconstitution of P-glycoprotein Reveals an Apparent Near Stoichiometric Drug Transport to ATP Hydrolysis*

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Gera D. Eytan, Ronit Regev, and Yehuda G. Assaraf‡

From the Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

We have recently described an ATP-driven, valinomycin-dependent $^{86}$Rb$^+$ uptake into proteoliposomes reconstituted with mammalian P-glycoprotein (Eytan, G. D., Borgnia, M. J., Regev, R., and Assaraf, Y. G. (1994) J. Biol. Chem. 269, 26058–26065). P-glycoprotein mediated the ATP-dependent uptake of $^{86}$Rb$^+$-ionophore complex into the proteoliposomes, where the radioactive cation was accumulated, thus, circumventing the obstacle posed by the hydrophobicity of P-glycoprotein substrates in transport studies. Taking advantage of this assay and of the high levels of P-glycoprotein expression in multi-drug-resistant Chinese hamster ovary cells, we measured simultaneously both the ATPase and transport activities of P-glycoprotein under identical conditions and observed 0.5–0.8 ionophore molecules transported/ATP molecule hydrolyzed. The amount of $^{86}$Rb$^+$ ions transported within 1 min via the ATP- and valinomycin-dependent P-glycoprotein was equivalent to an intravesicular cation concentration of 8 mM. Thus, this stoichiometry and transport capacity of P-glycoprotein resemble various ion-translocating ATPases, that handle millimolar substrate concentrations. This constitutes the first demonstration of comparable rates of P-glycoprotein-catalyzed substrate transport and ATP hydrolysis.

Inherent as well as acquired resistance to antineoplastic agents pose a major obstacle toward curative cancer chemotherapy (1, 2). Multidrug resistance (MDR) is characterized by the development of tumor cell resistance to diverse anticancer drugs. Mammalian cells with the typical MDR phenotype express increased levels of P-glycoprotein (Pgp), an integral component of the plasma membrane (3). Consequently, these cells display resistance to multiple cytotoxic hydrophobic agents, mostly of natural origin, including anthracyclines, Vinca alkaloids, epipodophyllotoxins, actinomycin D, taxoids, and dolastatin 10. Pgp, which possesses an ATPase activity, functions as an energy-dependent extrusion pump that expels these hydrophobic cytotoxic agents out of MDR cells (4).

Sharon et al. (5) have extracted most of the proteins from plasma membranes of MDR cells and have reconstituted the Pgp that remained in the membranes. The reconstituted proteoliposomes displayed an ATP-dependent transport of colchicine, an established substrate of Pgp. Shapiro and Ling (7) have purified Pgp from Pgp-rich cells by a combination of anion exchange and immunoaffinity chromatography. The Pgp preparation was 90% pure and after reconstitution exhibited an ATPase activity that was highly stimulatable by several MDR type drugs and chemosensitizers. Urbatsch et al. (8) have purified Pgp to apparent homogeneity from an extremely Pgp-rich Chinese hamster ovary cell line, reconstituted it, and characterized its drug-stimulatable ATPase activity.

Sharon et al. (5) have shown that colchicine was taken up actively into proteoliposomes with Pgp from CHO cells, against a 5.6-fold concentration gradient. However, the rate of colchicine uptake was about 15 pmol/mg/min with an ATPase activity of ~0.5 μmol/mg/min. This extremely low stoichiometry of drug transport to ATP hydrolysis is probably due to the hydrophobicity and membrane permeability of Pgp substrates. Recently, Shapiro and Ling (9) have described the ATP-dependent uptake of the fluorescent Pgp substrate, Hoechst 33342, into proteoliposomes reconstituted with purified Pgp from highly MDR CHO cells. They reported a stoichiometry of 1 substrate molecule transported/50 ATP molecules hydrolyzed and attributed this low efficiency to fast rebinding of the dye to the vesicles.

The low transport efficiency reported for Pgp, either in membrane vesicles or in reconstituted proteoliposomes, led to proposals that other mechanisms were responsible for the relatively low levels of drugs observed in MDR cells (10). Thus, demonstration of Pgp-mediated transport similar in rate to the Pgp ATPase will prove that Pgp is indeed a drug-efflux pump and could function as such also in vivo.

We have recently described an ATP-driven, valinomycin-dependent $^{86}$Rb$^+$ uptake into proteoliposomes reconstituted with mammalian P-glycoprotein (6). Under these conditions mammalian Pgp transported a cation-ionophore complex and the cation, $^{86}$Rb$^+$ in this case, accumulated in the intravesicular space. The apparent advantage of this assay is that although the actively-transported substrate, ionophore-cation complex, is hydrophobic, the accumulated species is the hydrophilic cation. In the present study, we took advantage of the methodology developed for assay of ionophore- and ATP-dependent Pgp-mediated $^{86}$Rb$^+$ uptake to measure simultaneously both the ATPase and transport functions of reconstituted Pgp. The valinomycin- and ATP-dependent $^{86}$Rb$^+$ uptake into proteoliposomes reconstituted with Pgp was close to the ATPase rate exhibited by these proteoliposomes under identical experimental conditions and thus constitutes the first direct demonstration that isolated Pgp could function as an efficient drug-efflux pump.

**EXPERIMENTAL PROCEDURES**

*Materials*

Gramicidin D, valinomycin, bovine serum albumin (essentially fatty-acid free), poly-L-tryptophan, and soybean phospholipids were purchased from Sigma. $^{86}$Rb$^+$ was purchased from DuPont NEN. Chole-
terol and phosphatidylserine were products of Avanti Polar Lipids.

In transport studies the effect of Na\(^+\)-K\(^+\)-ATPase was abolished by careful depletion of Na\(^+\) from all reagents used in the transport assay. The triphosphate nucleotides were treated with Dowex 50WX8. The pH of the solutions was monitored and resin was added, until no further acidification occurred. The solutions were passed through a 0.22-μm filter and tittered to pH 7.4 with solid Tris base.

Methods

Cell Cultures—A clonal derivative (CL1) of parental CHO AA8 cells and their emetine-resistant sublines were maintained under monolayer conditions in α-minimal essential medium (Biological Industries, Beit Haemek, Israel) containing 5% fetal calf serum (Beth Haemek), 2 mM glutamine, 100 units/ml penicillin G (Sigma), and 100 μg/ml streptomycin sulfate (Sigma). Exponentially growing cells were passaged twice a week using a standard trypsinization procedure. For preparation of membrane fractions, cells were seeded (10⁴ cells/ml) in 25-cm² tissue culture flask (Nunc) in growth medium (5 ml) containing 0.15 mM emetine (−3 times the LD₅₀ for parental AA8 cells). Following growth to midconfluence, emetine-selected cells were detached by trypsinization, counted and replated as above in the presence of a 50–100% increment in the emetine concentration. The gradual increase in emetine concentrations was terminated at 1 μM (−20-fold LD₅₀).

Isolation, Reconstitution of Pgp, and ATPase Assays—Cells (10⁶) grown under suspension culture conditions were hused with leupeptin at 3,000 rpm in a Sorvall GSA rotor, washed with phosphate-buffered saline, and resuspended in 10 ml of lysis buffer containing: 10 mM Hepes-Tris (pH 7.4), 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol (DTT), as well as the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 2 mM), aprotinin (1 mM), pepstatin (10 μM), and leupeptin (10 μg/ml). Following 5 min incubation on ice, cells were lysed using a Teflon-glass homogenizer and diluted 3-fold in lysis buffer. Nuclei and mitochondria were removed by consecutive 10-min centrifugations at 300 and 4,000 × g, respectively, and the microsomal fraction was recovered by a 30-min centrifugation at 100,000 × g and finally resuspended in 1 ml of lysis buffer. The membrane fractions were frozen in liquid nitrogen and stored at −75°C until analysis.

Extraction and Reconstitution of Pgp—Membrane fractions—The extraction and reconstitution of Pgp was performed according to our recently published protocol (6) except for minor modifications. A liposome suspension was prepared from a mixture containing the acetonitrile-insoluble, ethyl-soluble fraction of soybean phospholipids, phosphatidylserine, and cholesterol in a weight ratio of 5:1:1, respectively. The liposomes were dissolved in a stream of nitrogen and exposure to vacuum for 30 min. The lipids were suspended at a concentration of 50 mg/ml in a reconstitution medium containing: 25 mM HEPES-Tris (pH 7.4), 85 mM K₂SO₄, 1 mM MgSO₄, 25 mM Tris-HCl (pH 7.0), 0.5 mM EGTA, and 2 mM ouabain. Aliquots were incubated with the various drugs for 1 h at 37°C in glass test tubes. The ATPase activity was linear for at least 1 h (13). Water-insoluble drugs and peptides were added as ethanol solutions. The total amount of ethanol added was less than 1% and had no effect either on ATPase or on ⁸⁶Rb⁺ uptake assays. At the end of the incubation, the vesicle suspensions were rapidly cooled in ice-cold water bath and, 50-μl portions were distributed into a 96-well microtiter plate. The reaction was not carried out in the plate since it was observed that hydrophobic drugs, such as valinomycin, were adsorbed to polystyrene. The enzymatic reaction was terminated and inorganic phosphate was determined by the addition of a solution consisting of: 0.2% ammonium molybdate, 1.3% sulfuric acid, 0.9% SDS, and freshly prepared 1% ascorbic acid, incubation for 30 min at room temperature, and enzyme-linked immunosorbent assay reading (14). Background values were obtained with samples incubated in parallel on ice and were routinely subtracted from the measurements.

ATPase and Transport Assays—The ATPase activity of Pgp was determined by a colorimetric monitoring of the inorganic phosphate released from ATP (12). Reconstituted vesicles were diluted to a protein concentration of 20 μg/ml in an ice-cold ATPase assay medium, adapted from Urbatsch and Senior (8), which contained: 3 mM ATP, 50 mM KCl, 25 mM MgSO₄, 25 mM Tris-HCl (pH 7.0), 0.5 mM EGTA, and 2 mM ouabain. Aliquots were incubated with the various drugs for 1 h at 37°C in glass test tubes. The ATPase activity was linear for at least 1 h (13). Water-insoluble drugs and peptides were added as ethanol solutions. The total amount of ethanol added was less than 1% and had no effect either on ATPase or on ⁸⁶Rb⁺ uptake assays. At the end of the incubation, the vesicle suspensions were rapidly cooled in ice-cold water bath, and 50-μl portions were distributed into a 96-well microtiter plate. The reaction was not carried out in the plate since it was observed that hydrophobic drugs, such as valinomycin, were adsorbed to polystyrene. The enzymatic reaction was terminated and inorganic phosphate was determined by the addition of a solution consisting of: 0.2% ammonium molybdate, 1.3% sulfuric acid, 0.9% SDS, and freshly prepared 1% ascorbic acid, incubation for 30 min at room temperature, and enzyme-linked immunosorbent assay reading (14). Background values were obtained with samples incubated in parallel on ice and were routinely subtracted from the measurements.

Valinomycin- and ATP-dependent ⁸⁶Rb⁺ uptake was assayed essentially according to the assay strategy we have recently described (6). The transport activity of Pgp was assessed indirectly by measuring uptake of ⁸⁶Rb⁺ ions transported as a ⁸⁶Rb⁺-valinomycin complex into reconstituted proteoliposomes (see Fig. 1 for a scheme describing the methodology). ⁸⁶Rb⁺ serves as a convenient monitor of K⁺-the assay of ⁸⁶Rb⁺ uptake was based on the amplification of the isotopic uptake by an outwardly oriented concentration gradient of K⁺ (15). The principle of the method relies on the trapping of high K⁺ concentrations within the proteoliposomes. Upon dilution of the proteoliposomes into the assay buffer and selective permeation of the proteoliposomes to ⁸⁶Rb⁺ and K⁺ by the ionophore mobile carrier-type valinomycin, a diffusion potential is formed, which maintains the K⁺ gradient. ⁸⁶Rb⁺ is transported into the proteoliposomes until equilibration of its specific radio-

![Figure 1](image-url)
Valinomycin exhibits a high affinity toward K\(^+\) and 86Rb\(^+\) ions; thus, in the presence of relatively high cation concentrations present in the uptake medium, valinomycin is presented to Pgp predominantly as a cation-ionophore complex. Moreover, most known substrates of Pgp are hydrophobic and cationic in nature, and thus it is likely that the ionophore-cation complex is a preferred substrate for Pgp when compared with the unloaded ionophore. The ATP- and valinomycin-dependent 86Rb\(^+\) uptake required preloading of the proteoliposomes with K\(^+\) ions, and was abolished by dissipation of the K\(^+\) gradient. Thus, Pgp transports the cation-ionophore complex into the intravesicular volume of the vesicles. The 86Rb\(^+\) ions cotransported with valinomycin equilibrate with the cations trapped within the vesicles. Since the amount of 86Rb\(^+\) ions transported is in large excess compared to the total amount of valinomycin present in the medium, valinomycin plays a catalytic role and is recycled. Presumably, the electric potential formed by the K\(^+\)-gradient across the proteoliposome membrane hinders the release of 86Rb\(^+\) together with the accumulated valinomycin, and valinomycin leaks out of the proteoliposomes as the unloaded species. Thus, although the actual Pgp substrate is the hydrophobic 86Rb\(^+\)-valinomycin complex, the accumulated substrate is hydrophilic 86Rb\(^+\) ions.

The transport of 86Rb\(^+\) was measured by rapid removal of extravascular cations with the strong cation exchange resin, Dowex-50WX8, 100-mesh (15), as modified by Garty and Karlish (16). Unless otherwise stated, the transport buffer contained: 25 mM Hepes-Tris (pH 7.4), 0.25 M sucrose, 8 mg/ml bovine serum albumin, 4 mM MgCl\(_2\), 2 mM ATP, various amounts of valinomycin, and either 1 mM orthovanadate. Reconstitution of Pgp by known inhibitors of Pgp including vanadate and oligomycin, and insensitive to ouabain and EGTA. Reconstitution of Pgp by known inhibitors of Pgp including vanadate and oligomycin, and insensitive to ouabain and EGTA.

RESULTS

We undertook this study in order to estimate the stoichiometry of drug transport to ATP hydrolysis catalyzed by Pgp. To this end, both the ATPase and transport functions of Pgp had to be measured simultaneously under identical experimental conditions.

In this respect, we have described an assay of valinomycin uptake into proteoliposomes reconstituted with Pgp from rat liver, the amount of transported valinomycin was assessed as the quantity of 86Rb\(^+\) ions cotransported with the ionophore (6). The amount of Pgp present in canalicular vesicles from rat liver was low, and its presence could be detected only by Western blotting with a monoclonal antibody (6). As a result, its ATPase activity was relatively low and was masked by other ATPases present in the preparation. In contrast, Pgp ATPase activity has been demonstrated with Pgp from multidrug-resistant CHO cells where it is highly overexpressed (5, 8, 9, 13).

Thus, the aim of the present study was to measure simultaneously both ATPase and 86Rb\(^+\) uptake functions assayed under identical conditions as valinomycin-dependent activities of hamster Pgp.

Toward this end, a CHO variant (EmtR\(_1\)) highly-expressing Pgp was established by stepwise selection with the MDR drug, etrione. The Pgp content in the microsomal fraction of this EmtR\(_1\) subline constituted 4.5% of the total protein content. Upon reconstitution, the relative amount of Pgp was increased to 18%, and under the assay conditions used here all the ATPase activity was attributable to Pgp (13). The ATPase activity was stimulated by known substrates of Pgp, inhibited by known inhibitors of Pgp including vanadate and oligomycin, and insensitive to ouabain and EGTA. Reconstitution of Pgp from EmtR\(_1\) plasma membranes, the Pgp content of which was 18%, yielded proteoliposomes with a Pgp content of 40% and a consistently higher ATPase activity (13). However, the yield of these proteoliposomes was low, and since they did not pose a clear advantage over proteoliposomes reconstituted with Pgp from the microsomal fraction, the latter were routinely used.

The basal (i.e. with no substrates added) ATPase activity of proteoliposomes reconstituted with EmtR\(_1\) microsomal fraction was 1.1 ± 0.25 µmol of P/min/mg of protein (Fig. 2A and Ref. 13). A similar basal activity was reported for various Pgp
preparations (5, 7, 8, 17, 18). This basal activity was stimulated by valinomycin and emetine, the selecting drug used to establish EmtR1 cells (Fig. 2). However, a major problem became evident as the minimal valinomycin concentrations required for demonstrating stimulation of ATPase activity were higher than 0.1 μM, whereas appreciable ATP- and valinomycin-dependent 86Rb+ uptake was already evident at a concentration of 0.1 μM valinomycin. Thus, the high basal activity demonstrated by Pgp presumably masked the ATPase activity required for valinomycin-dependent transport. To overcome this obstacle, we looked for a Pgp inhibitor capable of reversibly repressing the basal ATPase activity without exerting a deleterious effect on the proteoliposomes. In this respect we have recently found that various hydrophobic homopolypeptides modulate Pgp ATPase activity. Poly-L-tryptophan met these expectations; at concentrations lower than 100 nM, it inhibited both the basal ATPase and the substrate-stimulatable activities of Pgp (Fig. 2B). These concentrations of poly-L-tryptophan had no deleterious effects on the integrity of the proteoliposomes as revealed by retention of encapsulated 86Rb+ or calcein (data not shown). Most importantly for this study, low concentrations of poly-L-tryptophan repressed the basal ATPase activity and, at concentrations required for mediation of ATP-dependent 86Rb+ uptake (see below), valinomycin reactivated it in a competitive manner (Fig. 3). The Michaelis-Menten type competitive inhibition exerted by poly-L-tryptophan on the stimulatory effect of valinomycin is presented in Fig. 3B as a Lineweaver-Burk plot.

ATP- and Valinomycin-dependent 86Rb+ Uptake into Reconstituted Proteoliposomes—Valinomycin is a cation ionophore with high affinity toward K+ ions, and as expected it allowed uptake of 86Rb+ ions into the proteoliposomes. ATP hydrolysis accelerated both the rate and maximal level of valinomycin-dependent 86Rb+ uptake in K+-loaded Pgp reconstituted proteoliposomes. The ATP-dependent transport, calculated as the difference between the uptake values obtained in the presence of ATP and those obtained in the presence of its non-hydrolyzable analog AMP-PCP, reached a maximum after 2 min (Fig. 4A) and was dissipated after 30 min of incubation (data not shown). The ATP-dependent uptake of 86Rb+ was observed only with K+-loaded proteoliposomes, indicating that the ATP-dependent uptake relies on the dilution of the isotope with trapped K+ ions and that this transport is directed into the intravesicular volume. As expected for Pgp-mediated activity, the ATP-dependent uptake component was restricted to proteoliposomes reconstituted with a Pgp-rich fraction from EmtR1 cells and was absent from proteoliposomes reconstituted with parallel fraction from parental drug-sensitive cells AA8 (Fig. 4B).

In order to discern between the ATP-dependent uptake and the ATP-independent ionophore-mediated equilibration of 86Rb+, reconstituted proteoliposomes were incubated for 3 min in a transport buffer containing valinomycin but lacking ATP. Under these conditions, 86Rb+ was allowed to reach apparent equilibrium with the K+ trapped in the proteoliposomes, and the intravesicular K+ concentration reached a transient constant concentration held by its diffusion potential (Fig. 4). At this point ATP or AMP-PCP was added and 86Rb+ uptake was determined (Fig. 5). The ATP-dependent component of 86Rb+ uptake was not affected by the preincubation. Thus, the ATP-dependent uptake results from an authentic active 86Rb+ uptake and not from effects on the ionophore-mediated equilibration of 86Rb+ across the proteoliposome membrane. The ATP- and valinomycin-dependent 86Rb+ uptake occurred only with K+-preloaded vesicles, indicating that the 86Rb+ ions were transported into the intravesicular milieu. As shown for Pgp from rat liver and MDR cells (6), the ATP- and valinomycin-dependent 86Rb+ uptake mediated by CHO Pgp was specific to ATP and did not occur with UTP, CTP, ADP, and non-hydrolyzable trinucleotides (data not shown).

Stoichiometry of Pgp-mediated Drug Transport and ATP Hydrolysis—On the one hand, substrate transport mediated by Pgp, was measured indirectly as the ATP-driven and valinomycin-dependent 86Rb+ influx. On the other hand, the ATPase activity of reconstituted Pgp from EmtR1 cells can be readily measured. Assessment of both ATPase activity and drug transport under the same assay conditions should allow estimation of the stoichiometry of drug transport to ATP hydrolysis.

Determination of the stoichiometry of drug transport to ATP hydrolysis relies on a quantitative assay of the valinomycin-dependent ATPase and transport activities of Pgp under identical experimental conditions. As shown in Fig. 6, measurement of Pgp-mediated ATPase and 86Rb+ uptake as a function of increasing valinomycin concentrations, revealed valinomycin-dependent components of both activities. However, as pointed above, Pgp exhibited high basal activity, in

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absence of added substrate, which masked the increase in ATPase activity required to mediate the ATP-driven and valinomycin-dependent $^{86}\text{Rb}^+$ uptake. We have overcome this obstacle by using poly-$L$-tryptophan to suppress the basal activity of Pgp. In the presence of poly-$L$-tryptophan, low valinomycin concentrations mediated an increase in both ATPase activity and $^{86}\text{Rb}^+$ uptake (Fig. 6B). In five independent experiments, it was determined that 0.5–0.8 $^{86}\text{Rb}^+$ ions were transported/ATP molecule hydrolyzed. This apparent stoichiometry of drug transport to ATP hydrolysis was determined as the ratio of the components of $^{86}\text{Rb}^+$ uptake to ATP hydrolysis, measured under identical conditions, which were dependent on both ATP and valinomycin.

An alternative approach to determine the ratio of Pgp-dependent ATP hydrolysis to drug transport was to use inhibitors that suppress both the ATPase and $^{86}\text{Rb}^+$ uptake and thereby determine the apparent stoichiometry as the ratio of parallel reductions in $^{86}\text{Rb}^+$ uptake and ATP hydrolysis. The Pgp ATPase as well as the valinomycin- and ATP-dependent $^{86}\text{Rb}^+$ uptake were both eliminated by established inhibitors of Pgp such as vanadate (Fig. 7A) and oligomycin (Fig. 7B). High concentrations of poly-$L$-tryptophan competitively inhibited both Pgp ATPase activity and the valinomycin-dependent and ATP-driven $^{86}\text{Rb}^+$ uptake (Fig. 7C). The ratio of the drug transport component that was eliminated by these different three inhibitors to the fraction of ATPase activity inhibited by these compounds was again equivalent to 0.5–0.8 mol of $^{86}\text{Rb}^+$ transported/mol of ATP hydrolyzed. Pgp substrates such as doxorubicin inhibited $^{86}\text{Rb}^+$ uptake, with little or no effect on Pgp ATPase activity (Fig. 7D). Presumably, this well known Pgp substrate competed with valinomycin on the Pgp pharmacophore (13).
Thus, the different approaches revealed an apparent near stoichiometry of Pgp-mediated ionophore molecules transported to ATP molecules hydrolyzed.

**DISCUSSION**

Pgp catalyzes the ATP-driven efflux of various cytotoxic xenobiotics out of MDR cells. However, the hydrophobicity of the various Pgp substrates hindered efforts aimed at estimating the stoichiometry of drug transport to ATP hydrolysis. In this respect, using a highly Pgp-rich proteoliposome system, Shapiro and Ling (9) recently reported a stoichiometry of 1 molecule of Hoechst 33342 transported/50 ATP molecules hydrolyzed; this low stoichiometry was attributed to the rapid rebinding of this hydrophobic chromophore to the liposome membrane, thus suggesting that the actual rate of transport is much faster. Recently, we devised an assay that circumvents this obstacle of the hydrophobicity of Pgp substrates; in this assay, Pgp-reconstituted proteoliposomes displayed an ATP-dependent uptake of $^{86}$Rb$^+$-valinomycin complex (6). Thus, Pgp mediated an ATP-driven $^{86}$Rb$^+$ accumulation, whereas the ionophore was recycled. Taking advantage of this assay of hydrophilic cation accumulation, we here combined a simultaneous determination of Pgp ATPase activity and its ability to take up $^{86}$Rb$^+$-valinomycin as reflected in the $^{86}$Rb$^+$ accumulation in the Pgp-reconstituted proteoliposomes. Using this

**FIG. 6.** Pgp ATPase and $^{86}$Rb$^+$ uptake activities as a function of valinomycin concentration. Pgp was extracted and reconstituted for $^{86}$Rb$^+$ uptake as described in Fig. 4. Both the ATPase and $^{86}$Rb$^+$ uptake were measured simultaneously in the same assay medium containing: 0.25 M sucrose, 8 mg/ml bovine serum albumin, 25 mM Hepes-Tris (pH 7.4), 3 mM DTT, 4 mM MgCl$_2$, 2 μl/ml carrier-free $^{86}$Rb$^+$, in the absence (panel A) or presence (panel B) of 2 μM poly-L-tryptophan (5.4 kDa), and either 3 mM ATP, or AMPPCP, in a final volume of 0.125 ml. The buffer was preincubated for 2 min at 37 °C, and the reaction was initiated by the addition of 5 μl of reconstituted proteoliposomes. The $^{86}$Rb$^+$ uptake (circles) and ATPase activity (squares) were assayed for 0.5 and 60 min, respectively. The ATP-dependent $^{86}$Rb$^+$ uptake was calculated by subtracting the values obtained in the presence of AMPPCP. Each point represents the mean value ± S.D., n = 8. The ATPase rates presented were calculated by subtracting the corresponding values obtained in absence of valinomycin.

**FIG. 7.** Inhibition of $^{86}$Rb$^+$ uptake and ATPase activities by vanadate, oligomycin, poly-L-tryptophan, and doxorubicin. The experimental conditions were similar to those described in the legend to Fig. 5, except that the valinomycin concentration here was 0.5 μM and poly-L-tryptophan was omitted except for in panel C. The ATPase rates presented were calculated by subtracting the values obtained in presence of 10 μM orthovanadate.

Thus, the different approaches revealed an apparent near stoichiometry of Pgp-mediated ionophore molecules transported to ATP molecules hydrolyzed.
approach, a stoichiometry of 0.5–0.8 substrate molecules transported/ATP molecule hydrolyzed was estimated. Thus, the high specific activity of Pgp ATPase (12.5 \text{ mmol of Pi/mg of protein/min}) along with its near stoichiometric drug transport to ATP hydrolysis resemble various ion-translocating ATPases including Na\(^{+}\), K\(^{+}\)-ATPase and Ca\(^{2+}\)-ATPase which handle millimolar substrate concentrations. Indeed, the ATP-driven, valinomycin-dependent uptake of \(^{86}\text{Rb}^{+}\) ions was equivalent to an intravesicular concentration of 8 mM.

One perplexing theme that emerges from the present study is that 1) despite the millimolar substrate translocation capability of Pgp, even when consisting 18% (this paper) or 32% (8) of total plasma membrane proteins, and 2) although Pgp can surprisingly consume as much as 12% of total cellular ATP in highly MDR cells (19, 20), Pgp can protect highly MDR cells only against 10\(\times\)10\(^{-6}\) M emetine (this paper) or 30\(\times\)10\(^{-6}\) M colchicine (8). This apparent discrepancy of 3 orders of magnitude between Pgp’s translocation ability combined with its strong ATPase activity versus its low efficiency in protecting cells from cytotoxic agents is highly dependent on the preferred, rapid copartition, and rapid diffusion of these hydrophobic drugs through the membrane. This is best exemplified in the case of the hydrophobic peptide ionophores valinomycin and gramicidin D. Although valinomycin proved an excellent Pgp substrate (i.e. low \(K_m\) and high ATPase \(V_{\text{max}}\)), Pgp was shown to confer upon highly MDR cells only a modest protection against this ionophore (see Refs. 13 and 21), the transmembrane flip-flop of which was found to be on the order of 25 \times 10^4/s (22). In contrast, in spite of the slow gramicidin D translocation and consequent inhibition of Pgp ATPase activity, Pgp proved very efficient in protecting highly MDR cells against this channel-forming ionophore (13, 21). This is not surprising given the relatively slow transmembrane flip-flop (i.e. minutes half-time) gramicidin D monomers must undergo prior to dimerization and channel formation (23). Based on the turnover number of Pgp, which was estimated to be 900 substrate molecules/min (\(K_{\text{cat}} = 15\) s; see Refs. 8 and 13), gramicidin D monomers, but not valinomycin, can be efficiently extracted from the plasma membrane and extruded.

REFERENCES
1. Frei, E., III (1985) Cancer Res. 45, 6523–6537
2. Chabner, B. A., and Collins, J. M. (eds) (1990) Cancer Chemotherapy: Principles and Practice, Lippincott, Philadelphia
3. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
4. Gottesman, M. M., and Pastan, I. (1988) J. Biol. Chem. 263, 12163–12166
5. Sharom, F. J., Yu, X. H., and Doige, C. A. (1993) J. Biol. Chem. 268, 24197–24202
6. Eytan, G. D., Borgnia, M. J., Regev, R., and Assaraf, Y. G. (1994) J. Biol. Chem. 269, 26058–26065
7. Shapiro, A. B., and Ling, V. (1994) J. Biol. Chem. 269, 3745–3754
8. Urbatsch, I. L., Al-Shawi, M. K., and Senior, A. E. (1994) Biochemistry 33, 7069–7076
9. Shapiro, A. B., and Ling, V. (1995) J. Biol. Chem. 270, 16167–16175
10. Simon, S., Roy, D., and Schindler, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1128–1132
11. Essen, A. (1978) Anal. Biochem. 89, 264–273
12. Chifflet, S., Torriglia, A., Chiesa, R., and Toloza, A. (1988) Anal. Biochem. 168, 1–4
13. Borgnia, M. J., Eytan, G. D., and Assaraf, Y. G. (1996) J. Biol. Chem. 271, 3163–3171
14. Doige, C. A., Yu, X., and Sharom, F. J. (1993) Biochim. Biophys. Acta 1146, 65–72
15. Garty, H., and Karlish, S. J. D. (1989) Methods Enzymol. 172, 155–164
16. Gasko, O. D., Knowles, A. F., Shetzer, H. G., Sudilina, E.-M., and Racker, E. (1976) Anal. Biochem. 72, 57–65
17. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8472–8476
18. Al-Shawi, M. K., and Senior, A. (1993) J. Biol. Chem. 268, 4197–4206
19. Broxterman, H. J., Pinedo, H. M., Kuiper, C. M., Kapsein, L. C. M., Schuurhuis, G. J., and Lankelma, J. (1988) FEBS Lett. 227, 2278–2282
20. Broxterman, H. J., Pinedo, H. M., Kuiper, C. M., Schuurhuis, G. J., and Lankelma, J. (1989) FEBS Lett. 247, 405–410
21. Assaraf, Y. G., and Borgnia, M. J. (1994) Eur. J. Biochem. 222, 813–824
22. Benz, R., and Läuger, P. (1976) J. Membr. Biol. 27, 1441–1450
23. O’Connell, A. M., Koepe, R. E., II, and Andersen, O. S. (1990) Science 250, 1256–1259