Characterization of the ATPase Activity of the M_r 170,000 to 180,000 Membrane Glycoprotein (P-Glycoprotein) Associated with Multidrug Resistance in K562/ADM Cells[1]

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ABSTRACT

The M_r 170,000 to 180,000 membrane glycoprotein associated with multidrug resistance (P-glycoprotein) is involved in drug transport mechanisms across the plasma membrane of multidrug-resistant cells. We have recently reported the purification of P-glycoprotein. The purified P-glycoprotein was found to have an ATPase activity, which might be coupled with the active efflux of anticancer drugs. In the present study, we have further studied the properties of the P-glycoprotein ATPase activity by an immobilized enzyme assay procedure using a P-glycoprotein-antibody-Protein A-Sepharose complex. GTP was also hydrolyzed by the P-glycoprotein, although less efficiently than ATP. The ATPase activity of P-glycoprotein had an optimal pH range around neutrality (pH 6.5–7.4). The detergent concentration of 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate used for protein solubilization was essential for enzyme recovery. Maximum activity was obtained when 0.1–0.2% 3-[(3-cholamidopropyl)dimethyl-ammonio]-propane sulfonate was used, while higher concentrations markedly inhibited the ATPase activity. The ATPase activity was dependent on Mg^{2+}; maximum activity was obtained at 2–10 mM. Manganese and cobalt could substitute for magnesium as ionic cofactors. Divalent cations such as Ca^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, and Cu^{2+} inhibited the Mg^{2+}-catalyzed ATP hydrolysis. N-Ethylmaleimide and vanadate inhibited the ATPase activity, while sodium azide or ouabain had no effect. Anticancer agents such as vincristine and Adriamycin did not affect the enzyme activity. In contrast, verapamil and trifluoperazine, agents which inhibit active drug efflux and restore drug sensitivity in resistant cells, caused an increase in the P-glycoprotein ATPase activity suggesting that P-glycoprotein might be the target molecule of these agents.

INTRODUCTION

When tumor cells acquire resistance to naturally occurring anticancer drugs such as *Vinca* alkaloids or anthracyclines, they generally show cross-resistance to other antitumor agents (1). Reduced intracellular drug accumulation resulting from increased active drug efflux across plasma membranes is an important factor in the mechanism of this pleiotropic drug resistance (2–5). However, the molecular mechanisms of this active efflux pump are not fully understood.

Multidrug-resistant cells show characteristic changes in surface glycoproteins. An overexpression of a plasma membrane glycoprotein of relative molecular mass (M_r 170,000–180,000) (P-glycoprotein) is consistently found in different multidrug-resistant human and animal cell lines (6–15). Full-length cDNAs for mouse and human P-glycoprotein genes inserted into viral expression systems and transfected into drug-sensitive cells have been shown to confer multidrug resistance (16–18). Sequence analysis of cDNAs showed that P-glycoprotein has homology with some bacterial transport proteins and has two binding sites for nucleoside triphosphates (19–21). Furthermore, P-glycoprotein is a binding protein for chemotherapeutic agents such as vinblastine (22–25). These results indicate that P-glycoprotein is directly involved in drug transport mechanisms in multidrug-resistant cells (for reviews see Ref. 26). In spite of these findings, the mechanism of its molecular action has remained obscure. Biochemical analysis of the enzymatic activity associated with P-glycoprotein has not been reported.

Recently, we have purified the P-glycoprotein of the human myelogenous leukemia, K562, resistant to Adriamycin (K562/ADM) (13) by means of immunoaffinity chromatography (27). The purified P-glycoprotein was found to have ATPase activity, which might be coupled with the active efflux of anticancer drugs. In this communication, we have studied the properties of the P-glycoprotein ATPase activity by an immobilized enzyme assay procedure (28–31). The activity was characterized and was compared with that of other well-known ATPases such as Na,K-ATPase[3] and Ca^{2+}-ATPase. As calcium antagonists (*e.g.*, verapamil) and calmodulin inhibitors (*e.g.*, trifluoperazine) inhibited the active drug efflux and restored drug-sensitivity in resistant cells (4, 5), we also tested the effects of these drugs on the ATPase activity of P-glycoprotein.

MATERIALS AND METHODS

Reagents and Buffers. ATP, ADP, GTP, and GDP, were obtained as sodium salts from Sigma. [α-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]GTP (3000 Ci/mmol) were purchased from Amersham or ICN. CHAPS was from Dojin Chemicals, Kumamoto, Japan. Buffers were prepared in distilled water and the pH of the reaction buffers was adjusted at the working temperature. When calcium concentration was fixed to 1 μM or less, calcium/EGTA buffer was employed (32). Verapamil was dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide never exceeded 0.01%. Protein A-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. One g of dry material swells in 50 mM Tris-HCl buffer, pH 8.0, to yield 3.5 ml of gel containing 2 mg of protein A per ml.

Cells and Culture Conditions. The highly Adriamycin-resistant tumor cell line, K562/ADM, was established in our laboratory (13) from the human myelogenous leukemia K562 cell line. K562/ADM cells show cross-resistance to various other chemotherapeutic agents including vincristine (15). The cells were prepared and maintained as described previously (33).

Monoclonal Antibody. The hybridoma cell line producing the anti-P-glycoprotein monoclonal antibody (MRK16) (14) was amplified in the ascites fluid of pristane-primed BALB/c mice and the IgG was purified by 50% ammonium sulfate precipitation, followed by DEAE-Sephacel (Pharmacia) chromatography as described (34). The purified monoclonal antibody was dialyzed against 10 mM Tris-HCl, pH 7.4, 150 mM NH₄Cl.

[3] The abbreviations used are: Na,K-ATPase, sodium- and potassium-activated, magnesium-dependent adenosine triphosphatase; Ca^{2+}-ATPase, Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; S buffer, 50 mM Tris-HCl (pH 8.0), 150 mM NH₄Cl, 2 mM MgCl₂, 1% CHAPS.

Preparation of Crude Membrane Fraction. Cells were washed with phosphate-buffered saline (0.15 M NaCl-20 mM sodium phosphate, pH 7.4) and suspended at 3×10^7 cells/ml in the hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl$_2$, 1 mM dithiothreitol) and incubated for 15 min in an ice bath. The swollen cells were disrupted with 15 to 20 strokes in a tightly fitting Dounce homogenizer. The nuclei were removed by centrifugation at $400 \times g$ for 10 min. Enucleated cell homogenate was spun out at $22,000 \times g$ for 30 min and the pellet was used as a crude membrane fraction. The membrane preparation was suspended in the lysis buffer containing 50% (w/v) glycerol and stored at $-80°C$ until use.

Purification of P-Glycoprotein. Membrane preparations from K562/ADM cells enriched in P-glycoprotein (15) were used for the purification of the protein. The detailed procedures are described elsewhere (27). Briefly, crude membrane fractions from K562/ADM cells were suspended in S buffer (usually membrane fraction from 3×10^7 cells was suspended in 1 ml of S buffer) and incubated for 30 min in an ice bath. After centrifugation at $22,000 \times g$ for 30 min, the resultant supernatant was applied to a column of MRK16 coupled with Affi-Gel 10 (Bio-Rad). The column was washed with S buffer and P-glycoprotein was eluted from the column with 0.1 M sodium citrate buffer, pH 3.5, containing 1% CHAPS. Fractions containing P-glycoprotein were pooled, dialyzed against 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NH$_4$Cl, 2 mM MgCl$_2$, and 0.1% CHAPS. P-Glycoprotein was detected by SDS-PAGE analysis (35) with Coomassie brilliant blue staining. Samples of purified P-glycoprotein were kept frozen in small aliquots (2 µg) and thawed immediately before use.

Protein Determination. Protein was determined by the method of Smith *et al.* (36) with bovine serum albumin as a standard.

Preparation of P-Glycoprotein-Antibody-Protein A-Sepharose Complexes. Membrane preparations from K562/ADM cells were solubilized in S buffer as described above. Immunoprecipitation was carried out by incubating the membrane lysates (0.5 mg protein/ml) with 20 µg/ml of monoclonal antibody MRK16 for 2 h at 4°C. Then 100 µl of Protein A-Sepharose CL-4B suspension (20% by volume, in S buffer) was added to 1 ml of membrane lysates. After incubation for 30 min, the precipitates were washed five times with the reaction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NH$_4$Cl, and 0.1% CHAPS) unless otherwise indicated. The resultant P-glycoprotein-antibody-Protein A-Sepharose complex was used for the ATPase assay. The amount of the protein immobilized on the carrier was estimated by SDS-PAGE analysis of the complex followed by Coomassie blue staining. Antigen-antibody-Protein A-Sepharose complexes were newly prepared from stock membrane preparations in each experiment.

NTPase Assay. The hydrolysis of NTP by P-glycoprotein was assayed by measuring the formation of NDP from [α-^{32}P]NTP as described previously (27) with some modifications. Standard reaction mixtures (100 µl) contained 50 mM Tris-HCl (pH 7.4), 150 mM NH$_4$Cl, 2 mM MgCl$_2$, 0.1% CHAPS, [α-^{32}P]NTP at 300 µM, and P-glycoprotein-antibody-Protein A-Sepharose complex (20 µl of wet gel volume). The preparation was mixed for about 5 s then left at 30°C for 10 min unless otherwise indicated. The reaction was stopped by adding 25 µl of 50 mM EDTA, 2.5% SDS, 25 mM NTP, and 25 mM NDP. The immobilized enzyme was centrifuged off at $10,000 \times g$ for 10 s. Samples were spotted on polyethyleneimine cellulose thin layer plates (20 x 20 cm, Macherey-Nagel) and developed in 0.5 M potassium phosphate buffer, pH 3.4. The ADP-containing spots were visualized by UV light (253.6 nm) and cut out, and the amount of radioactivity (Cerenkov) was determined in a liquid scintillation counter. The ATPase activity was expressed either as the amount of specifically hydrolyzed NTP or as relative activity compared with that obtained in the standard condition. Experimental details are described in the figure legends.

RESULTS

Effect of Monoclonal Antibody MRK16 on the ATPase Activity of P-Glycoprotein. The effect of the purified monoclonal antibody MRK16 on the enzymatic activity of P-glycoprotein was determined. Purified P-glycoprotein (2 µg) was incubated for 1 h at 4°C with an excess amount (20 µg) of the purified

monoclonal antibody MRK16, and the ATPase activity of the incubation mixture was assayed. Treatment with MRK16 did not affect the ATPase activity of the purified P-glycoprotein (Fig. 1). The activity was 1.2 nmol/mg/min when 24 µM ATP was used. The antibody treatment had no effect on the ATPase activity even if various concentrations (3–300 µM) of ATP were used in the assay (data not shown). The result indicates that the ATPase assay using the P-glycoprotein-MRK16 complex is also useful for the characterization of ATPase activity of P-glycoprotein and was used for the remainder of the study.

Time Course of ATP Hydrolysis. As shown in Fig. 2, the amount of ATP hydrolyzed by the P-glycoprotein-MRK16-Protein A-Sepharose complex was linear for the initial 10 min of reaction time. Therefore, we tested the ATPase activity of P-glycoprotein with a 10-min reaction time for the experiments hereafter. When normal mouse IgG (instead of MRK16) was used for the preparation of immune complexes, the ATPase activities of the samples from K562/ADM membrane lysates were negligible, indicating that the P-glycoprotein-antibody complexes obtained by this procedure could be estimated to be practically free from other contaminated ATPase activities (Fig. 2).
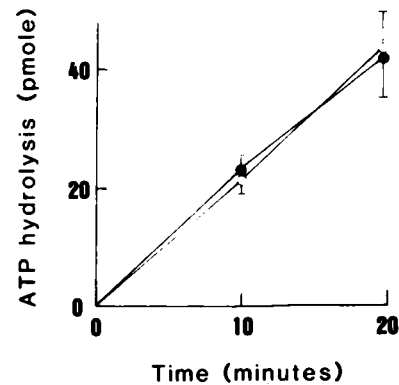

Fig. 1. Effect of monoclonal antibody MRK16 on the ATPase activity of P-glycoprotein. Purified P-glycoprotein (2 µg) was incubated for 1 h at 4°C with 20 µg of purified MRK16 in 80 µl of reaction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NH$_4$Cl, 2 mM MgCl$_2$, and 0.1% CHAPS). Control incubation was done using the same amount of bovine serum albumin instead of MRK16. Then 20 µl of [α-^{32}P]ATP (120 µM, 1.5 ~ 3.0 × 10^6 cpm/20 µl) in the same reaction buffer was added and incubated at 30°C for the indicated time. The reaction was stopped by adding 25 µl of 50 mM EDTA, 2.5% SDS, 25 mM ATP, and 25 mM ADP. Hydrolyzed ATP was measured by thin-layer chromatography. ATPase activity was expressed as the specifically hydrolyzed ATP. ATP hydrolysis obtained by the purified P-glycoprotein incubated with bovine serum albumin (O) and MRK16 (●) in triplicate determinations (mean ± SD) is shown.
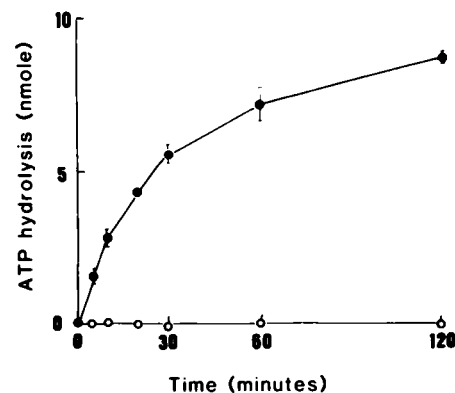

Fig. 2. Time course of ATP hydrolysis by P-glycoprotein-MRK16-Protein A-Sepharose complex. ATPase assay was done as described in "Materials and Methods" for various reaction times. Control experiments were carried out using normal mouse serum in place of MRK16. The time course of ATP hydrolysis by the complex precipitated with MRK16 (●) or with normal mouse serum (O) in triplicate determinations is shown.

Substrate Dependency of Enzymatic Activity of P-Glycoprotein. Fig. 3 shows the effects of substrate concentrations on the enzymatic activity of P-glycoprotein. Half maximum ATPase activity was observed at approximately 150 μM, and maximum activity was obtained when 300–1000 μM ATP was used. GTP was also hydrolyzed by P-glycoprotein, albeit less efficiently than ATP (Fig. 3). The enzyme was not saturated at the highest concentration of GTP used (1 mM). For the characterization of the P-glycoprotein ATPase activity, we used 300 μM ATP in the ATPase assay hereafter. At 300 μM ATP, approximately 90% of maximum ATPase activity was observed (Fig. 3).

Effect of pH and Buffers. A pH profile from 6 to 8 was determined with the following series of buffers at the indicated pH values: MES (pH 6.0, 6.5, and 7.0), HEPES (pH 7.0, 7.4, and 8.0), and Tris-HCl (pH 7.0, 7.4, and 8.0). At this range of pH (6.0 ~ 8.0), the amounts of precipitated P-glycoprotein did not differ significantly, which was reassured by the SDS-PAGE analysis of the immune complexes. The ATPase activity of P-glycoprotein had a rather wide pH optimum range around neutrality (pH 6.5–7.4) (Fig. 4). By using a series of individual buffers, it was important to overlap the working pH with buffers of different pK values to ensure that specific buffer interactions
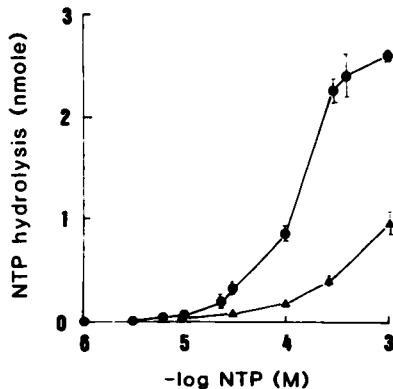
were absent (37). The ATPase activity of P-glycoprotein at overlapping pH values yielded similar results when different buffers at varying concentrations (10 and 50 mM) were used (data not shown), indicating that specific buffer effects were not present.

Effect of Detergent Concentrations. It is well known that enzymatic activities of membrane proteins are often affected by the detergent used for their solubilization (38). We found that the concentration of CHAPS, the detergent used for the solubilization of P-glycoprotein, was critical for enzymatic recovery (Fig. 5). Maximum activity was obtained at a concentration of 0.1–0.2% CHAPS. Higher concentrations of CHAPS markedly inhibited the ATPase activity. The activity mildly decreased with lower concentrations (<0.05%) of CHAPS.

Effects of Metal Ions. When EDTA was added to the ATPase assay mixture, the ATPase activity of P-glycoprotein was completely inhibited, indicating that divalent cation(s) are essential for the ATPase activity. Fig. 6 shows that the ATPase activity of P-glycoprotein was dependent on magnesium. Maximum activity was obtained at 2–10 mM Mg^{2+}. Higher concentrations of Mg^{2+} inhibited the ATPase activity. A low but appreciable level of activity (9% of maximum activity) was observed in the absence of added magnesium (Fig. 6). Mg^{2+} could be replaced by Mn^{2+}, which completely preserved the activity at 3×10^{-5} M (Fig. 7A). Co^{2+} also worked as an efficient substitute for Mg^{2+} at 3×10^{-5} to 3×10^{-4} M, while more than 1 mM of cobalt ions inhibited the ATPase activity (Fig. 7B). Mn^{2+} did not affect the Mg^{2+}-catalyzed hydrolysis of ATP (Fig. 7A), while Co^{2+} weakly inhibited the Mg^{2+}-catalyzed hydrolysis at concentrations higher than 10^{-5} M (Fig. 7B). Calcium worked as a substitute for Mg^{2+}, but it preserved only 20% of the activity (Fig. 7C). Ca^{2+} inhibited the Mg^{2+}-catalyzed hydrolysis at millimolar concentrations (Fig. 7C). Effects of other divalent cations such as Zn, Ni, Cd, and Cu on the ATPase activity of P-glycoprotein were tested (Table 1). In the absence of added Mg^{2+}, these divalent cations (1 mM) preserved 20–30% of the ATPase activity. While in the presence of 2 mM Mg^{2+}, these cations markedly inhibited the Mg^{2+}-catalyzed hydrolysis. In contrast, monovalent cations Na^+ and K^+ did not affect the ATPase activity in the presence or absence of Mg^{2+} (Table 1).

Effect of Various Drugs. The effects of various agents on the

Fig. 3. Effects of substrate concentrations on the enzymatic activity of P-glycoprotein. P-Glycoprotein-MRK16-Protein A-Sepharose complexes (20 μl of wet gel volume) were suspended with 60 μl of the reaction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NH₄Cl, 2 mM MgCl₂, and 0.1% CHAPS). Then 20 μl of the reaction buffer containing various amounts of [α-³²P]ATP or [α-³²P]GTP were added and incubated for 10 min. The nucleoside triphosphatase (NTPase) activity was expressed as the specifically hydrolyzed ATP (●) or GTP (▲).
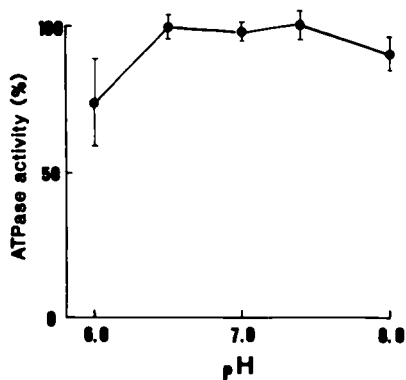

Fig. 4. Effect of pH on the ATPase activity of P-glycoprotein. P-Glycoprotein-MRK16-Protein A-Sepharose complexes were prepared as described in "Materials and Methods." The resulting complexes (20 μl of wet gel volume) were washed five times with 1 ml of reaction buffers containing 50 mM MES (pH 6.0 and 6.5) or HEPES (pH 7.0, 7.4, and 8.0), 150 mM NH₄Cl, 2 mM MgCl₂, and 0.1% CHAPS. The pellets were suspended with 60 μl of each buffer. Then 20 μl of 1.5 mM [α-³²P]ATP in appropriate buffers were added and incubated for 10 min. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (50 mM Tris-HCl, pH 7.4, 150 mM NH₄Cl, 2 mM MgCl₂, and 0.1% CHAPS). Data, Means and SD of triplicate determinations.
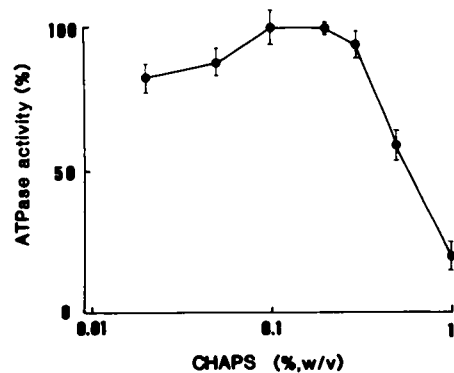

Fig. 5. Effect of CHAPS concentration on the ATPase activity of P-glycoprotein. P-Glycoprotein-MRK16-Protein A-Sepharose complexes were prepared as described in "Materials and Methods." The resulting complexes (20 μl of wet gel volume) were washed five times with 1 ml of reaction buffers containing 50 mM Tris-HCl, pH 7.4, 150 mM NH₄Cl, 2 mM MgCl₂, and various concentrations (0.2–1.0%) of CHAPS. The pellets were resuspended with 60 μl of each buffer. Then 20 μl of 1.5 mM [α-³²P]ATP in the appropriate buffers were added and incubated at 30°C for 10 min. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (50 mM Tris-HCl, pH 7.4, 150 mM NH₄Cl, 2 mM MgCl₂, and 0.1% CHAPS). Data, means and SD of triplicate determinations.
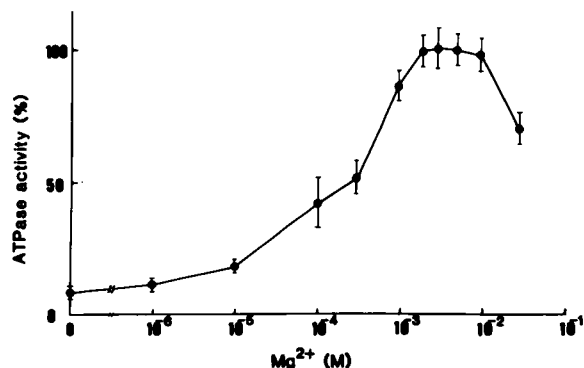
Fig. 6. Effects of Mg^{2+} concentration on the ATPase activity of P-glycoprotein. P-Glycoprotein-MRK16-Protein A-Sepharose complexes were prepared as described in "Materials and Methods." The resulting complexes (20 μl of wet gel volume) were suspended with 40 μl of the reaction buffer (50 mM Tris HCl, pH 7.4, 150 mM NH₄Cl, and 0.1% CHAPS). After adding 20 μl each of the reaction buffer containing various concentrations of $MgCl_2$, 20 μl of 1.5 mM [α-^{32}P]ATP in the reaction buffer was added and incubated at 30°C for 10 min. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (2 mM $MgCl_2$). *Data*, means and SD of triplicate determinations.

ATPase activity of P-glycoprotein were tested (Table 2). The reducing reagent dithiothreitol did not affect the activity in this assay condition, while sulfhydryl reagent *N*-ethylmaleimide inhibited the activity by about 70%. Addition of 1.0 mM ouabain to the assay medium which inhibits the Na,K-ATPase from plasma membranes had no significant effect on the P-glycoprotein enzyme activity. There was no inhibition in the presence of 1 mM sodium azide, which specifically inhibits mitochondrial ATPase. Vanadate, which is known as a blocker of Ca^{2+}-ATPase, myosin-ATPase, and Na,K-ATPase, also inhibited the ATPase activity of P-glycoprotein; 50% inhibition was observed at about 100 μM. Anticancer agents such as vincristine and Adriamycin did not affect the enzyme activity. In contrast, verapamil and trifluoperazine, the agents which inhibit the active drug efflux and restore drug-sensitivity in resistant cells, caused increases in the ATPase activity in dose-response manners (Fig. 8).

DISCUSSION

In this report we have described the initial characterization of the P-glycoprotein ATPase activity. In a first series of experiments, we determined the optimal conditions for the ATPase assay such as substrate concentrations, pH, CHAPS concentration, and Mg^{2+} concentration. Next, the effects of mono- and divalent metal ions and various reagents on the enzyme activity were tested.

In our previous report (27), the effect of ATP concentration on the ATPase activity of the purified P-glycoprotein (free enzyme in solution) was studied. Maximum activity was 1.7 nmol/mg/min when 100 μM ATP was used (27). In contrast, by the immobilized enzyme assay procedure using P-glycoprotein-MRK16-Protein A-Sepharose complex, a higher activity was obtained when a larger amount of ATP was used (Fig. 3). Half maximum activity was observed at approximately 150 μM ATP, and maximum activity was obtained when 300–1000 μM ATP was used (Fig. 3). Since approximately 5 μg of P-glycoprotein was recovered from 0.5 mg of membrane protein of K562/ADM cells (27), the maximum activity of the precipitated ATPase by immobilized enzyme assay procedure was calculated to be 50 nmol/mg/min. This specific activity was much higher than that of the purified P-glycoprotein (1.7 nmol/mg/min).
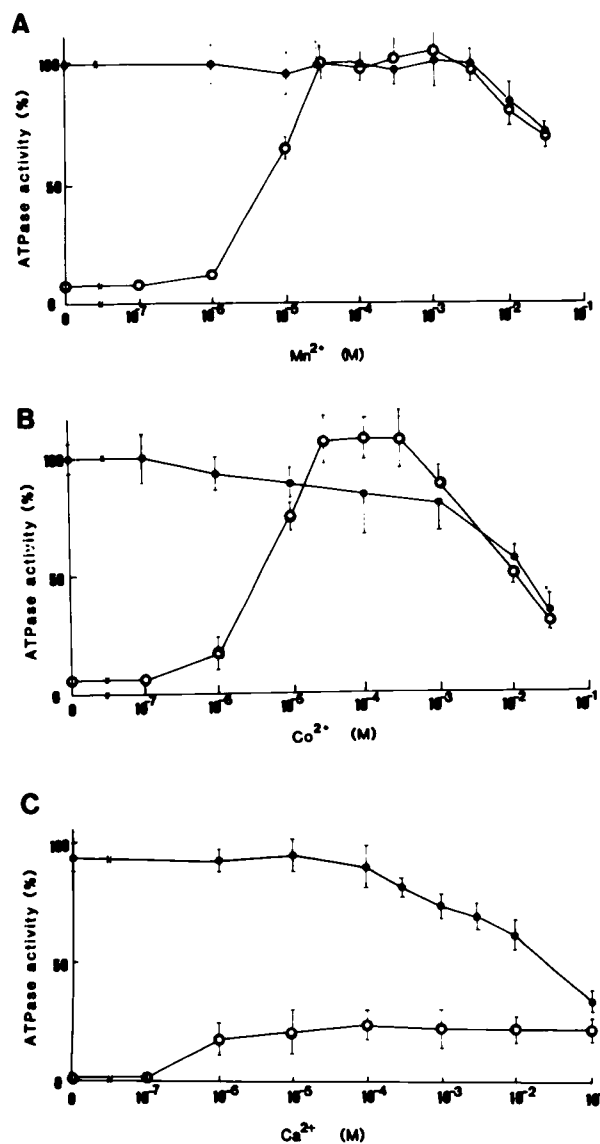

Fig. 7. Effects of Mn^{2+}, Co^{2+}, and Ca^{2+} on the ATPase activity of P-glycoprotein in the presence or absence of 2 mM Mg^{2+}. P-glycoprotein-MRK16-Protein A-Sepharose complexes were prepared as described in "Materials and Methods." The resulting complexes (20 μl of wet gel volume) were suspended with 40 μl of the reaction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NH₄Cl, and 0.1% CHAPS) with or without containing 5 mM $MgCl_2$. After adding 20 μl each of the reaction buffer containing various concentrations of $MnCl_2$, $CoCl_2$, or $CaCl_2$, reaction was started by adding 20 μl of 1.5 mM [α-^{32}P]ATP in the reaction buffer. When calcium concentration was fixed to 1 μM or less, calcium/EGTA buffer was employed (32). The incubation was set at 30°C for 10 min. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (2 mM $MgCl_2$). *Data*, means and SD of triplicate determinations in the presence (●) or absence (○) of 2 mM Mg^{2+}.

The cause of this difference is not clear. One possibility is that denaturation of the enzyme may have occurred during the purification procedure, which caused the changes in the profile of ATP-dependency of the purified enzyme. Another possibility is that the different properties of the purified enzyme and the "immobilized" enzyme may be due to the antibody rather than the state of the process of "immobilization." Comparison of the profile of ATP-dependency of the "immobilized" enzyme (Fig. 3 of this paper) with that of the purified enzyme (Fig. 5 of Ref. 27) shows that "immobilization" increases the values of both V_{max} and K_m. If both of these changes are caused by antibody binding to the enzyme, the antibody may either activate the enzyme (at high ATP), inhibit the enzyme (at low ATP), or have no effect on the enzyme (at "intermediate" ATP

Table 1 *Effects of metal ions on ATPase activity of P-glycoprotein*

ATPase assay was carried out as described in the *legend* for Fig. 6 using various metal ions. The metal ions were used as chlorides with the exception that Zn was used as sulfate. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (2 mM $MgCl_2$). Data, means and SD of triplicate determinations.

Metal ions	Concentration (mM)	Relative ATPase activity (%)	
		Mg 0 mM	Mg 2 mM
Buffer		9.1 ± 0.5	100 ± 2.1
Na	20.0	9.6 ± 1.6	103.0 ± 3.9
K	20.0	6.6 ± 0.2	97.3 ± 3.3
Mn	1.0	105.4 ± 7.9	99.9 ± 12.5
Co	1.0	87.9 ± 7.2	80.7 ± 12.0
Ca	1.0	21.7 ± 7.7	74.1 ± 4.3
Zn	1.0	19.2 ± 4.6	16.7 ± 5.2
Ni	1.0	34.0 ± 5.2	35.1 ± 6.3
Cd	1.0	27.0 ± 4.5	22.7 ± 1.5
Cu	1.0	25.0 ± 12.0	17.0 ± 8.1

Table 2 *Effects of various drugs on ATPase activity of P-glycoprotein*

ATPase assay was carried out as described in the *legend* for Fig. 6, except that various drugs were added in the presence of 2 mM (final concentration) $MgCl_2$. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (no drug added). Data, means and SD of triplicate determinations.

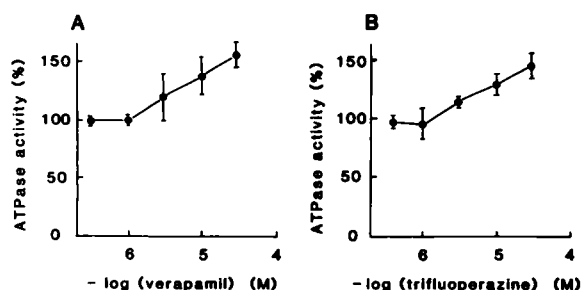| Drugs	Concentration (mM)	Relative ATPase activity (%)
No drug		100 ± 3.3
Dithiothreitol	1.0	102.9 ± 4.3
N-Ethylmaleimide	1.0	29.0 ± 2.8
Sodium azide	1.0	101.2 ± 1.3
Ouabain	1.0	102.2 ± 1.3
Vanadate	0.01	90.4 ± 0.5
	0.1	52.1 ± 2.0
	1.0	28.5 ± 0.5
Vincristine	0.01	105.4 ± 5.1
Adriamycin	0.01	103.2 ± 2.0

Fig. 8. Effects of verapamil and trifluoperazine on the ATPase activity of P-glycoprotein. ATPase assay was carried out as described in the legend for Table 2. The ATPase activity was expressed as relative activity compared with that obtained in the standard condition (no drug added). *Data*, means and SD (*bar*) of triplicate determinations.

levels where the K_m and the V_{max} effects cancel each other out). However, this seems not the case, because the antibody treatment had no effect on the ATPase activity of the purified P-glycoprotein even if various concentrations (3–300 μM) of ATP were used in the ATPase assay. These possibilities, especially the first one, remain to be examined further.

Since the treatment of purified P-glycoprotein with monoclonal antibody MRK16 did not affect the ATPase activity of the protein (Fig. 1), we used the immobilized enzyme assay procedure (31) for the characterization of the P-glycoprotein ATPase activity. The assay procedure using P-glycoprotein-MRK16-Protein A-Sepharose complex had several advantages: (a) the complex could be prepared in a few hours; (b) ATP hydrolysis by contaminated proteins were practically negligible (Fig. 2); (c) P-glycoprotein was not treated with harsh conditions such as low pH, and therefore, native activity of the protein would not be severely damaged; (d) for buffer substitu-

tion, washing the gel with the desired buffer was sufficient, instead of extensive dialysis; (e) P-glycoprotein had high hydrophobic domains (19–21) and was easily lost during purification procedures by apparent irreversible hydrophobic reactions with other hydrophobic substances or by intermolecular self-aggregation (27, 39). Rapid isolation of P-glycoprotein using specific antibody and Protein A-Sepharose may circumvent these protein losses. In spite of these advantages, assay procedures for immobilized enzymes might also have their limitations (28, 30). The kinetic behavior of immobilized enzymes might, in some respects, differ from that of free enzymes in solution. Problems such as diffusional restrictions (29) and enzyme leakage from the matrix might exist. Taking into account of these considerations, details of the assay conditions used are given precisely in the figure legends to permit proper comparison and evaluation.

Cornwell *et al.* have reported the ATP-binding properties of the P-glycoprotein from multidrug-resistant KB cells (40). Both ATP and GTP inhibited labeling of P-glycoprotein by the photoaffinity reagent 8-azido-α-$[^{32}P]$ATP, although it was not determined whether ATP or GTP or both nucleotides function as enzymatic substrate in the cell (40). In this report we have found that GTP is also hydrolyzed to GDP and phosphate by P-glycoprotein in the presence of Mg^{2+} (Fig. 3). As shown in Fig. 3, ATP was the preferred substrate for the hydrolysis reaction *in vitro*. Other nucleotides such as ADP were also hydrolyzed by P-glycoprotein, although less efficiently than ATP (data not shown). As the intracellular concentration of ATP in human cells is estimated to be higher than that of GTP and other NTPs (41) it is likely that ATP is the actual substrate used *in vivo* by P-glycoprotein in the hydrolysis reaction.

We used CHAPS for the solubilization of P-glycoprotein, since it was the detergent selected for the efficient immunoprecipitation of P-glycoprotein and for the preservation of the ATPase activity of the protein (27). CHAPS produced a concentration-dependent effect on the P-glycoprotein ATPase activity (Fig. 5). The activity was slightly decreased when lower concentrations of CHAPS (less than 0.05%) were used in the reaction. This might reflect the aggregation of P-glycoprotein, which has highly hydrophobic domains (19–21). In contrast, the ATPase activity was markedly suppressed in the presence of high concentrations of CHAPS (0.5–1%). Enzymatic activities of membrane ATPases are often affected by the detergent used for their solubilization (38). Detergents disrupt and delipidate membrane-bound enzymes to cause inactivation of lipid-dependent enzyme function. Total delipidation with higher concentrations or longer detergent treatment times, may result in total and irreversible inhibition (42–48). In addition to the effects of solubilization, detergent effects at the regulatory site of enzymes have been reported (49, 50). For example, Huang *et al.* (50) showed the direct nonsolubilizing effects of detergents on the enzymatic activity of Na,K-ATPase. Their findings indicated (a) detergent binding to hydrophobic sites on extramembranous segments of enzyme subunits; (b) that occupation of these sites mimicked the effects of ATP at a low-affinity regulatory site of the enzyme. Thus, in studies on detergent-solubilized preparations of P-glycoprotein, it would be necessary to consider both the effects of solubilization *per se* and detergent effects at the regulatory sites. The denaturation of the purified P-glycoprotein (27) may be due to the longer treatment with detergent during the purification procedure. The optimal concentration of CHAPS for the immobilized enzyme assay procedure was 0.1–0.2% (Fig. 5). Even at this optimal concentration, we can not rule out the possibility that the

detergent might damage the native activity of P-glycoprotein ATPase.

P-Glycoprotein has been postulated as the pump molecule transporting chemotherapeutic agents such as vincristine and Adriamycin (16–21). However, it remains to be determined whether the ATPase activity of the protein is actually coupled with the drug transport. P-Glycoprotein has been proved to be an acceptor protein of the chemotherapeutic agents such as vincristine (22–25). In our preliminary experiments, however, we could not detect [^{3}H]vincristine binding to the purified P-glycoprotein preparations when they were solubilized with CHAPS,[4] suggesting that treatment with the detergent might damage the drug-binding capacity of P-glycoprotein. In our experimental conditions, chemotherapeutic agents such as vincristine and Adriamycin did not affect the ATPase activity of P-glycoprotein (Table 2). The results might be due to the detergent effects at the drug-binding sites of P-glycoprotein. Studies on the P-glycoprotein ATPase activity using the detergent-free purified protein reconstituted into liposomes may be necessary.

Interestingly, verapamil and trifluoperazine, the agents which inhibit the active drug efflux and restore drug-sensitivity in resistant cells, caused increases in the ATPase activity of P-glycoprotein (Fig. 8). The concentrations of these drugs used for the activation of the P-glycoprotein ATPase were similar to those used for the circumvention of resistance in multidrug-resistant cells (4, 5). Recently, it has been demonstrated that P-glycoprotein is an acceptor protein for certain calcium channel blockers such as verapamil, diltiazem, and azidopin (24, 51). Thus, verapamil (or trifluoperazine) might interact directly with P-glycoprotein, resulting in inhibition of drug efflux mechanisms in resistant cells. Apparently it is paradoxical that verapamil and trifluoperazine, the reagents which inhibit the active efflux of chemotherapeutic drugs, enhanced the ATPase activity of the P-glycoprotein which is a candidate molecule for the putative "drug-efflux pump." One explanation is that verapamil (or trifluoperazine) works as a competitive inhibitor to the transport of chemotherapeutic agents via P-glycoprotein. Verapamil (or trifluoperazine) might be more easily transported by P-glycoprotein than chemotherapeutic agents. A second explanation is that verapamil (or trifluoperazine) is not transported by P-glycoprotein, but modifies the pump function, resulting in a decreased efficiency of the transport of chemotherapeutic drugs in spite of enhanced ATP hydrolysis. A third explanation is that verapamil (or trifluoperazine) works in cooperation with other cellular factor(s) regulating the drug-efflux mechanism of P-glycoprotein. In the absence of this regulatory factor, verapamil (or trifluoperazine) could not inhibit the pump function of P-glycoprotein, paradoxically enhancing the ATPase activity of the protein. All these possibilities remain to be examined. In any case, it is likely that P-glycoprotein is a target molecule of verapamil (or trifluoperazine). Modification of the enzymatic activity of the P-glycoprotein ATPase might be the key mechanism in the action of these drugs.

Cellular factors regulating the activity of P-glycoprotein are not known. The P-glycoprotein ATPase activity might be controlled by some cellular stimulators or inhibitors. Previously, we demonstrated that P-glycoprotein was phosphorylated by an array of complex regulation mechanisms (15, 33). The reversible protein phosphorylation/dephosphorylation might be involved in the regulation of the P-glycoprotein ATPase

activity. Studies using a cell-free system reconstituted from purified components would be required.

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