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ATPase activity of purified and reconstituted multidrug resistance protein MRP1 from drug-selected H69AR cells

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Abstract

The ATP-binding cassette transporter protein, multidrug resistance protein MRP1, was purified from doxorubicin-selected H69AR lung tumor cells which express high levels of this protein. A purification procedure comprised of a differential two-step solubilization of MRP1 from plasma membranes with 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate followed by immunoaffinity chromatography using the MRP1-specific monoclonal antibody QCRL-1 was developed. Approximately 300 μg of MRP1 was obtained from 6 mg of plasma membranes at 80–90% purity, as indicated by silver staining of protein gels. After reconstitution of purified MRP1 into proteoliposomes, kinetic analyses indicated that its K_m for ATP hydrolysis was 104 ± 22 μM with maximal activity of 5–10 nmol min^{-1} mg^{-1} MRP1. MRP1 ATPase activity was further characterized with various inhibitors and exhibited an inhibition profile that distinguishes it from P-glycoprotein and other ATPases. The ATPase activity of reconstituted MRP1 was stimulated by the conjugated organic anion substrates leukotriene C_4 (LTC_4) and 17β-estradiol 17-(β-D-glucuronide) with 50% maximal stimulation achieved at concentrations of 150 nM and 1.6 μM, respectively. MRP1 ATPase was also stimulated by glutathione disulfide but not by reduced glutathione or unconjugated chemotherapeutic agents. This purification and reconstitution procedure is the first to be described in which the ATPase activity of the reconstituted MRP1 retains kinetic characteristics with respect to ATP-dependence and substrate stimulation that are very similar to those deduced from transport studies using MRP1-enriched plasma membrane vesicles.

Keywords: Multidrug resistance; ATPase; MRP1; ATP-binding cassette; Purification

1. Introduction

Multidrug resistance protein MRP1 is a 190-kDa plasma membrane protein that confers multidrug resistance on cells in which it is overexpressed [1–3]. Since its cDNA was cloned from the doxorubicin-selected human small cell lung cancer cell line, H69AR [4], MRP1 expression has been detected in many different drug resistant cell lines and in a range of both hematologic and solid tumors [5–8]. In some cases, MRP1 expression has been correlated with clinical outcome [6,7]. MRP1, like the well characterized multidrug resistance protein P-glycoprotein, belongs to the large and ancient ATP-binding cassette (ABC) or traffic-ATPase superfamily of membrane transport proteins [9]. However, MRP1 and P-glycoprotein share rather limited sequence similarity

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(< 20%), which is largely confined to the generally conserved nucleotide binding domains (NBDs) common to all ABC proteins [3,4]. In addition, MRP1 and several of its most closely related proteins are atypical ABC proteins in that they contain an extra, third membrane spanning domain with an extracytosolic NH_2-terminus [2,3,10]. Conserved features of protein structure and gene organization suggest that, unlike P-glycoprotein, MRP1 and related proteins share a common ancestry with the cystic fibrosis transmembrane conductance regulator (CFTR) [2–4].

The function of MRP1 as an ATP-dependent transporter has been studied extensively using membrane vesicles prepared from drug-selected and transfected cells which overexpress the protein. These investigations have shown that vesicles enriched in MRP1 display ATP-dependent, high affinity transport of the cysteinyl leukotriene LTC_4 and other glutathione (GSH) conjugated organic anions as well as oxidized glutathione (GSSG) [11–17]. In addition, an estradiol metabolite formed in the liver, 17β-estradiol 17-(β-D-glucuronide) ($E_2$17βG), has been shown to be a substrate of MRP1 [18,19]. Thus, MRP1 has been demonstrated to be a primary active transporter of structurally diverse conjugated organic anions. In contrast, it has not been possible to detect direct active transport of chemotherapeutic agents by MRP1-enriched membrane vesicles under similar conditions [14,16,20]. However, MRP1-dependent active transport of certain unconjugated xenobiotics such as vincristine, daunorubicin and aflatoxin B_1 is observed if vesicle preparations are supplemented with physiological concentrations of GSH [14,15,21]. More recent studies suggest that MRP1 actively co-transports some unmodified substrates together with GSH [20,22].

The mechanism by which MRP1 transports conjugated organic anions and unmodified xenobiotics has yet to be elucidated. However, it is presumed that, like P-glycoprotein, MRP1 functions as an energy-dependent efflux pump driven by the hydrolysis of ATP. High levels of drug-stimulated ATPase activity have been reported for plasma membrane vesicles enriched in P-glycoprotein [23,24], as well as the purified and reconstituted protein [25–28]. In contrast, studies with MRP1-enriched plasma membrane vesicles have suggested that the constitutive and substrate-stimulated ATPase activity of MRP1 is considerably lower than that observed for P-glycoprotein [29] (unpublished observations). However, characterization of the ATPase activity of MRP1 in such preparations has been complicated by other endogenous ATPases which are often present at very high levels [29] (unpublished observations). Consequently, to investigate the relationship between drug transport, ATP binding and hydrolysis, purification of functional MRP1 is essential. In the present study, we describe a simple immunoaffinity purification procedure for native MRP1 from the membranes of multidrug resistant H69AR cells. We also show that when reconstituted into liposomes, MRP1 has low level constitutive ATPase activity that has broad nucleotide specificity and can be stimulated by conjugated organic anions. Inhibitor studies with various inhibitors indicate that the ATPase activity of MRP1 is distinct from that of typical P-, F-, and V-type ATPases as well as P-glycoprotein.

2. Materials and methods

2.1. Materials

$E_2$17βG, 17β-estradiol 3-(β-D-glucuronide), GSH, GSSG, *S*-decyl GSH, ATP, AMP, sodium orthovanadate, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-C1), EGTA, bafilomycin A_1, oligomycin and all nucleotides were purchased from Sigma (St. Louis, MO, USA). LTC_4 was from Calbiochem (La Jolla, CA, USA). Dithiothreitol (DTT) and 2-mercaptoethanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). NaF and ouabain were from ICN (Aurora, OH, USA). NaN_3 was from BDH Chemicals (Toronto, Ont., USA). *N*-Ethylmaleimide (NEM) was from Aldrich (Milwaukee, WI, USA). All of the above compounds were dissolved in distilled water and the solutions were neutralized to pH 7.5 with 1 M Tris base. The vanadate solution was boiled 10 min and then chilled on ice before use. CHAPS and *n*-octyl-β-D-glucopyranoside were from ICN. The human MRP1-specific murine IgG_1 mAb QCRL-1 has been characterized previously [30,31]. The hexapeptide SSYSGDI corresponding to the QCRL-1 epitope was synthesized by Research Genetics (Huntsville, AL, USA).

2.2. Cell culture and plasma membrane preparation

The small cell lung cancer cell line H69 and its doxorubicin-selected multidrug resistant variant, H69AR, have been described previously [4,32]. Cells were grown as suspension cultures in 500 ml glass bottles in RPMI 1640 medium containing 5% defined bovine calf serum (HyClone Laboratories, Logan, UT, USA) in the absence of antibiotics.

Plasma membranes were prepared essentially as described [14]. Briefly, 1 g of wet cells (approximately 10^8 H69AR cells) stored at −80°C was thawed in 30 ml homogenization buffer containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 3 mM KCl, 0.25 mM $MgCl_2$ and protease inhibitors. The cells were broken by N_2 cavitation (10 min equilibrium at 175 psi) and EDTA was added to 1 mM. Cell debris was removed by low speed centrifugation ($500\times g$, 15 min) and the pellet was washed once with 10 ml of homogenization buffer. Pooled supernatants were layered over 35% sucrose (10 ml) in buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and centrifuged at $100\,000\times g$ for 1.5 h at 4°C. Material at the interface was recovered, pelleted by centrifugation ($100\,000\times g$, 1 h at 4°C) and then resuspended in storage buffer containing 50 mM Tris-HCl, pH 7.5 and 250 mM sucrose. The membrane suspension was passed five times through a 27.5-gauge needle and then stored at −80°C until use. The protein concentration of the plasma membrane preparation was typically 2–4 mg ml^{-1}.

2.3. Solubilization of MRP1

Plasma membranes (5 mg isolated from approximately 1 g of wet H69AR cells) were thawed and centrifuged at $100\,000\times g$ for 15 min at 4°C. The pellet was resuspended in 0.5 ml of buffer A containing 50 mM Tris-HCl, pH 7.4, 150 mM NH_4Cl, 1 mM EDTA and 5 mM CHAPS. The final protein concentration was approximately 10 mg ml^{-1}. The mixture was incubated on ice for 30 min with gentle magnetic stirring and centrifuged at $100\,000\times g$ for 15 min at 4°C. The pellet was washed once with 0.5 ml of buffer B containing 50 mM Tris-HCl, pH 7.4, 10% glycerol (w:v), 0.15 M NaCl and 1 mM EDTA. The washed pellet was resuspended in 5 ml of buffer B containing 10 mM CHAPS and incubated on ice for 30 min with gentle magnetic stirring to solubilize MRP1. The sample was then pelleted at $100\,000\times g$ for 15 min at 4°C. The resulting supernatant was immediately subjected to immunoaffinity chromatography.

2.4. Purification of mAb QCRL-1

GammaBind Plus Sepharose resin (Pharmacia Biotech) (1 ml) was packed in a spin column, then washed five times with 5 ml 0.1 M glycine-HCl, pH 2.7 and equilibrated with 20 mM sodium phosphate buffer, pH 7.0 by a similar washing procedure ($100\times g$, 30 s, 4°C). Mouse ascites fluid containing mAb QCRL-1 was then mixed with the pre-equilibrated resin and gently shaken for 1 h at room temperature. The resin with bound antibody was washed five times with 10 ml of 20 mM sodium phosphate buffer, pH 7.0. mAb QCRL-1 was then eluted by washing six times with 1 ml of 0.1 M glycine-HCl, pH 2.7 and immediately neutralized with 1 M Tris base. The purified mAb was stored at 4°C.

2.5. Preparation of mAb QCRL-1 immunoaffinity column

The glycine buffer in the preparations of purified mAb QCRL-1 was exchanged with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) by dialysis overnight at 4°C. Purified mAb QCRL-1 (5 mg) in coupling buffer was then incubated with 1 g of swollen CNBr-activated Sepharose 4B resin (Pharmacia) that had been pre-washed with 1 mM HCl (200 ml). After incubation with gentle shaking overnight at 4°C in a 15 ml spin column (Bio-Rad), unbound antibody was washed away by brief centrifugation (30 s, $100\times g$) with five times the gel volume of coupling buffer (5×3 ml). Tris-HCl buffer (0.1 M Tris-HCl, pH 8.0) (5 ml) was added to the column and incubation with gentle shaking continued for 2 h at room temperature to block the remaining active groups. The column was then washed with 10 ml of buffer containing 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl, followed by 10 ml of 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl. The column was stored at 4°C in Tris buffer supplemented with 0.02% NaN_3 until use.

2.6. Immunoaffinity chromatography of MRP1

All immunoaffinity purification steps were carried out on ice or at 4°C. The spin column containing 3 ml of Sepharose resin coupled to mAb QCRL-1 was washed four times with 5 ml of glycine buffer (0.1 M glycine, pH 2.7) and then equilibrated by a similar washing procedure with buffer C containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20% glycerol, 0.5 M NaCl, 5 mM CHAPS, all by brief centrifugation (30 s, $100\times g$, each time). Solubilized MRP1 (5 ml) was then mixed with the equilibrated resin and incubated for 3 h with gentle shaking. The resin with bound protein was washed 10 times with 5 ml of buffer C as above. A heptapeptide (10 mg) corresponding to the QCRL-1 epitope (SSYSGDI) [31] was dissolved in buffer C and mixed with the resin and the mixture was then incubated overnight at 4°C with gentle shaking. The final QCRL-1 peptide concentration was approximately 3.3 mg ml^{-1}. MRP1 was eluted by brief centrifugation (30 s, $100\times g$) and the resin washed five times with 2 ml of buffer C without peptide. The eluant and washes were pooled and the MRP1 immediately reconstituted (see below). The immunoaffinity column was regenerated by washing with 25 ml of 0.1 M sodium acetate, pH 4.0, 25 ml of 0.1 M glycine, pH 2.7, 25 ml of buffer C supplemented with 0.02% NaN_3 and stored at 4°C. The column could be re-used up to 10 times.

2.7. Reconstitution of the purified MRP1 into proteoliposomes

Liposome preparations were made as follows. Bovine brain lipid extract containing a minimum of 40% phosphatidylethanolamine (Sigma, brain extract, Type V) and cholesterol were resuspended in a ratio of 80:20 (w:w) in reconstitution buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM DTT, 1 mM $MgCl_2$) at a concentration of 20 mg ml^{-1}. The lipid suspension was sonicated for 30 min at room temperature under argon and then frozen-thawed five times in liquid N_2 followed by sonication for 60 s. The liposomes were stored in aliquots at −70°C. Before use, the liposomes were again frozen-thawed five times followed by sonication for 60 s. For reconstitution, 0.03 mg of purified MRP1 was mixed with 1.2 mg of sonicated liposomes in a 0.75 ml volume of buffer containing 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM DTT, 1 mM $MgCl_2$, 60 mM *n*-octyl-β-D-glucopyranoside and 3.7 mM CHAPS. The mixture was incubated on ice for 1 h and diluted 25-fold into reconstitution buffer. The diluted sample was again incubated for 45 min at 4°C and proteoliposomes were then harvested by centrifugation at $145\,000\times g$ for 1 h at 4°C. The pellet was resuspended in 0.1 ml of storage buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose) and passed five times through a 27.5-gauge needle. The proteoliposomes were aliquoted and stored at −70°C until use. In a typical experiment, recovery of MRP1 in the proteoliposomes was 20–30%.

2.8. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), silver staining and immunoblotting

Gel electrophoresis was carried out using 7.5% SDS-polyacrylamide minigels in a Bio-Rad Mini-Protean II electrophoresis cell. Proteins were stained with alkaline silver [33]. For immunoblotting, proteins were transferred to Immuno-P membranes (Millipore, Bedford, MA, USA) using 25 mM Tris, 192 mM glycine and 20% methanol buffer. Blots were blocked in TBS-T buffer (10 mM Tris-HCl, 0.15 mM NaCl, 0.05% Tween 20, pH 7.5) with 5% skim milk for 1 h at room temperature. The blot was incubated with mAb QCRL-1 ascites (diluted 1:10 000) followed by goat anti-mouse IgG+IgM (H+L) horseradish peroxidase-conjugated $F(ab')_2$ fragment (diluted 1:5000) (Pierce, Rockford, IL, USA). The blots were developed using a chemiluminescence detection kit (Boehringer Mannheim, Laval, Que., Canada). Protein concentrations were determined using a modified Lowry assay [34] and bovine serum albumin as standard.

2.9. Assay of ATPase activity

To measure ATPase activity, purified and reconstituted MRP1 (0.5–1.5 μg) was incubated at 37°C in 0.1 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ for 4 h. The ATP concentration used was 2.5 mM and the pH of the assay buffer was 7.4 unless otherwise indicated. Reactions were stopped

by the addition of 33.3 μl of 18% SDS and the amount of inorganic phosphate released was determined immediately as described [35,36]. In control reactions, 33.3 μl of 18% SDS was included in the assay buffer before MRP1 was added to ensure that MRP1 was inactivated. The added substrates, GSH, inhibitors and drugs did not contribute to the color reaction in the control incubations. To determine the effect of pH on MRP1 ATPase activity, assays were carried out at pH 4.0–9.5 using 50 mM Tris-HC1 buffers to maintain pH 6.5–9.5 and 50 mM sodium acetate buffers to maintain pH 4.0–5.5.

2.10. MRP1-mediated transport of LTC_4 in membrane vesicles

Inside-out membrane vesicles used for transport assays were isolated from MRP1-transfected HeLa T5 cells and MRP1-mediated ATP-dependent transport of [^{3}H]LTC_4 into the membrane vesicles was measured by rapid filtration essentially as described [14]. To measure the effect of pH on MRP1 transport activity, assays were carried out at pH 5–9 using Tris-HC1 buffers (50 mM) to maintain pH 6.5–9.0 and sodium acetate buffers (50 mM) to maintain pH 5–6.

3. Results

3.1. Purification and reconstitution of MRP1 from H69AR plasma membranes

Approximately 5 mg of plasma membranes were isolated from 1×10^8 H69AR cells by a sucrose density centrifugation procedure. To extract MRP1 from plasma membranes, we used a differential detergent solubilization procedure similar to that used previously to solubilize P-glycoprotein [36]. Treatment of H69AR plasma membranes at a protein concentration of 10 mg ml^{-1} with a buffer containing 5 mM CHAPS solubilized approximately 40% of total protein while more than 90% of the MRP1 remained in an insoluble fraction that could be recovered by centrifugation. However, when the CHAPS concentration was increased to 10 mM, nearly 50% of MRP1 was solubilized. Based on these observations, 5 mM CHAPS and 10 mg ml^{-1} of plasma membranes were routinely used in the first detergent solubilization step. In the second step, the pellet from the first CHAPS extraction was resuspended at a protein concentration of approximately 0.5 mg ml^{-1} in buffers containing various concentrations of CHAPS. At 5 mM CHAPS, approximately one-third of MRP1

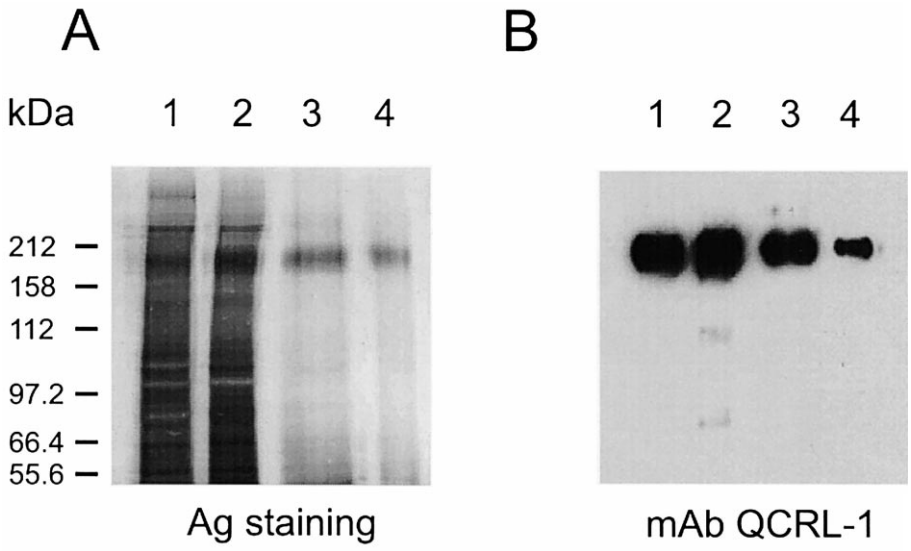

Fig. 1. Analysis of MRP1 at each stage of purification and after reconstitution into proteoliposomes. (A) Proteins were subjected to SDS-PAGE and detected by silver staining. (B) Immunoblot of the protein gel shown in Panel A with mAb QCRL-1. Lane 1, plasma membranes, 10 μg protein; lane 2, membrane proteins solubilized with 10 mM CHAPS, 5 μg protein; lane 3, native MRP1 purified by immunoaffinity chromatography, 1 μg protein; lane 4, reconstituted MRP1 proteoliposomes, 0.4 μg protein.

was solubilized while at 10 mM CHAPS, over 90% of MRP1 and approximately 50% of total protein could be recovered in the supernatant. Thus, we routinely used 10 mM CHAPS and a protein concentration of 0.5 mg ml^{-1} in the second extraction step. Based on densitometry, MRP1 comprised 20–30% of total membrane proteins in the second CHAPS extract (Fig. 1A).

A number of conventional chromatography procedures were investigated for further purification including ion-exchange, gel filtration, hydroxyapatite, Con A affinity Sepharose and isoelectric focusing. However, all were found to be relatively ineffective because of either poor yield or failure to increase purity. As an alternative approach, we investigated the use of immunoaffinity chromatography with the MRP1-specific mAb QCRL-1. The MRP1 CHAPS extract was concentrated to 5 ml and mixed with MAb QCRL-1 Sepharose resin (~3 ml) and over 90% of MRP1 was bound to the resin after incubation at 4°C for 3 h. Incubation of the immunoaffinity resin overnight at 4°C with the synthetic peptide SSYSGDI (which corresponds to the epitope of mAb QCRL-1) [31] resulted in elution of approximately 90% of the bound MRP1. The MRP1 recovered was approximately 80% pure, as judged by densitometry of a silver-stained SDS-PAGE gel (Fig. 1A). With this approach, approximately 300 μg of MRP1 could be purified from 6 mg of H69AR plasma membranes. Based on densitometry of immunoblots using purified MRP1 as a standard, MRP1 accounts for approximately 5–6% of total plasma membrane proteins in the multidrug resistant H69AR cells (not shown). Thus the overall recovery following solubilization and purification was greater than 80%, since 6 mg of plasma membranes would be predicted to contain approximately 300 μg of MRP1.

Proteoliposomes were formed by rapid dilution of the purified MRP1 into a detergent-free buffer. Bovine brain lipids were used in approximately 10 000-fold molar excess to MRP1, assuming an average lipid molecular weight of 700 Da. Because cholesterol has been shown to enhance ATP-dependent substrate transport mediated by solubilized hepatocyte canalicular membranes in a reconstituted system [37], we routinely supplemented the bovine brain lipids with 20% cholesterol. Typically, 20–30% of MRP1 could be recovered in proteoliposome preparations isolated by ultracentrifugation. A silver-stained gel of such MRP1 proteoliposome preparations is shown in Fig. 1A. An immunoblot of the various MRP1 preparations is shown in Fig. 1B. Vesicles containing purified MRP1 were stained with uranyl acetate and determined to have diameters of approximately 200 nm by electron microscopy (not shown).

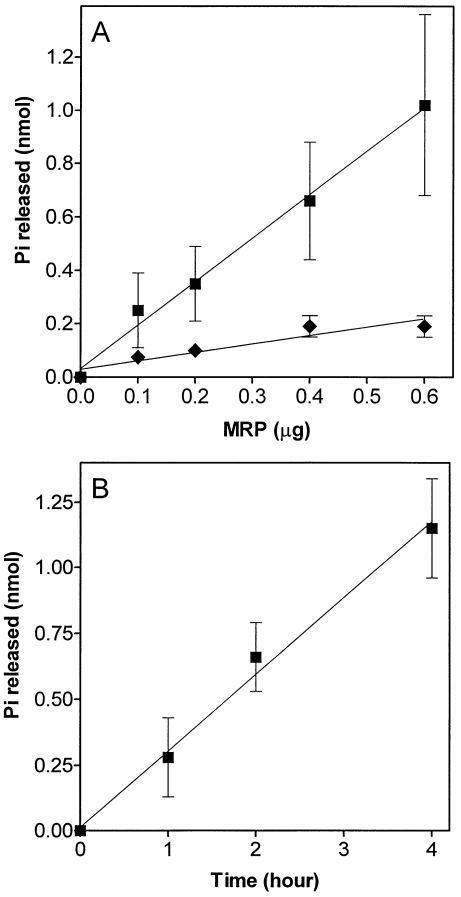

Fig. 2. Basal ATPase activity of purified and reconstituted MRP1. (A) ATP hydrolysis activity exhibited by increasing amounts of purified native MRP1 in assay buffer containing 0.9 mM CHAPS (♦) or purified MRP1 reconstituted in liposomes (■). The ATPase activity was assayed in a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 5 mM ATP, 5 mM $MgCl_2$ and incubated for 4 h at 37°C. (B) The time dependence of ATPase activity exhibited by proteoliposomes containing 0.6 μg of purified MRP1 in a total volume of 10 μl was as described in Panel A. Data points represent mean values (±S.E.) of three independent determinations.

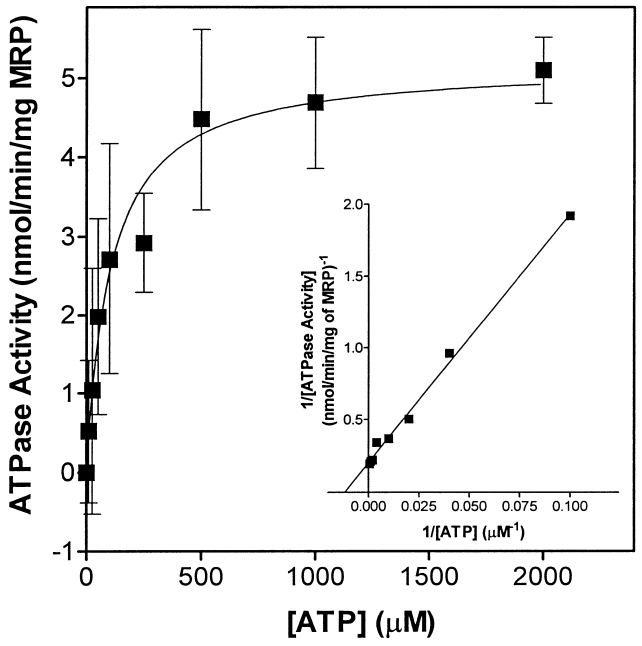

Fig. 3. Effect of ATP concentrations on MRP1 ATPase activity. The ATPase activity of MRP1 proteoliposomes was measured at various concentrations of ATP by incubation for 4 h at 37°C in buffer containing 50 mM Tris-HCl, pH 7.4 and 5 mM $MgCl_2$. The K_m for ATP hydrolysis was 104 ± 22 μM, as determined from regression analysis of the Lineweaver-Burk plot (inset). Data points represent mean values (± S.E.) of three independent determinations.

3.2. ATPase activity of purified and reconstituted MRP1

The ATPase activity of MRP1 solubilized in CHAPS was ~1.5 nmol min^{-1} mg^{-1} which is approximately five-fold lower than that of MRP1 in reconstituted proteoliposomes (Fig. 2A), suggesting that, like many other membrane proteins, MRP1 requires a lipid environment for optimal activity. Reconstituted MRP1 proteoliposomes exhibited ATPase activity proportional to the amount of incorporated purified MRP1 protein (Fig. 2A). The ATPase activity of reconstituted MRP1 was linear with respect to time for up to 4 h at 37°C (Fig. 2B) and exhibited a K_m (ATP) of 104 ± 22 μM, as determined by Lineweaver-Burk plot analysis (Fig. 3). The specific ATPase activity of MRP1 varied somewhat from batch to batch but was typically 5–10 nmol min^{-1} mg^{-1} MRP1. As controls, membranes from drug sensitive H69 and revertant H69PR cells which have levels of MRP1 that are 100- and 25-fold lower than the drug resistant H69AR cells, respectively, were subjected to the same protein purification and reconstitution procedures. No ATPase activity was detected in proteoliposomes prepared from the H69 or H69PR eluates of the QCRL-1 affinity resin (results not shown). Occasionally, the ATPase activity of MRP1 could be stimulated slightly by DTT and 2-mercaptoethanol, suggesting that oxidation during the immunoaffinity purification and reconstitution procedures and/or the relatively long incubation period could be deleterious to the ATPase activity of MRP1. MRP1 contains 25 cysteine residues but which, if any, of

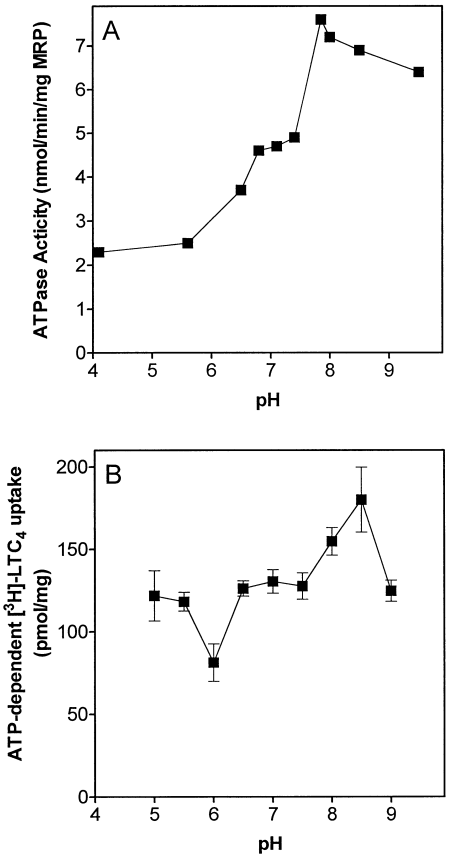

Fig. 4. Effect of pH on MRP1 ATPase activity and LTC_4 transport activity. (A) The ATPase activity of purified and reconstituted MRP1 (1 μg) was measured in buffer containing 2.5 mM ATP and 5 mM $MgCl_2$ at the indicated pH as described in Section 2. Values shown are the means of results obtained in two independent experiments. (B) The ATP-dependent [^{3}H]LTC_4 transport activity by membrane vesicles derived from MRP1-transfected HeLa cells (T5) was measured at the indicated pH values as described in Section 2. Data points represent mean values (± S.D.) of three determinations.

Table 1
Effect of inhibitors on the ATPase activity of purified and reconstituted MRP1

Inhibitor	IC_{50} (μM)
Na_3VO_4	10
NBD-C1	50
NEM	500
NEM +1 mM DTT	1 000
NaN_3	> 6 000
Oligomycin	> 80[a]
EGTA	500
Bafilomycin A_1	> 10
Ouabain	> 6 000
NaF	1 000

MRP1 proteoliposomes were incubated with various concentrations of inhibitors for 30 min at room temperature in buffer containing 50 mM Tris-HCl, pH 7.4 and 5 mM $MgCl_2$ prior to addition of ATP (2.5 mM). ATPase activity was measured and IC_{50} values calculated as described in Section 2.
[a]Concentration of oligomycin is approximate and based on a presumed average molecular weight of 790.

these is important for the function of the protein has not been established [38].

3.3. Effect of inhibitors on MRP1 ATPase activity

The effects of compounds known to inhibit other ATPases were examined and the results are summarized in Table 1. The ATPase activity of MRP1 proteoliposomes was inhibited by orthovanadate, indicating that a coordinate phosphorus complex in the MRP1 molecule likely exists during ATP hydrolysis. The IC_{50} of vanadate was approximately 10 μM, which is comparable to values previously reported for P-glycoprotein [27,28] but is much higher than the values typically reported for P-type ATPases (50–500 nM) [39]. On the other hand, MRP1 ATPase activity was relatively insensitive to NBD-C1 (IC_{50} 50 μM) as are P-type ATPases and P-glycoprotein (IC_{50} 20 μM) [27]. NEM, a potent inhibitor of P-glycoprotein [28] and V-type ATPases, was a relatively weak inhibitor of MRP1 ATPase activity (IC_{50} 500 μM). This inhibition likely involves a reactive sulfhydryl group since the addition of DTT increased the IC_{50} of NEM to 1 mM. These values are comparable to the concentration of NEM that resulted in 70% inhibition of LTC_4 uptake in MRP1-enriched membrane vesicles [14].

MRP1 ATPase activity was also relatively insensitive to azide and oligomycin, both potent inhibitors of F_1F_0-ATPase [40,41], exhibiting IC_{50}'s for these compounds greater than 6 mM and 80 μM, respectively. The Ca^{2+}-ATPase inhibitor, EGTA, half-maximally inhibited MRP1 ATPase activity at 0.5 mM but complete inhibition was not observed even at a concentration of 6 mM. Ouabain, a specific inhibitor of Na^+/K^+-ATPase [42] and bafilomycin A, a specific inhibitor of V-type ATPase [43], had no effect on MRP1 ATPase activity up to 6 mM. NaF, which is an inhibitor of gastric H^+,K^+-ATPase [44], Ca^{2+}-ATPase of the sarcoplasmic reticulum [45], and Mg^{2+}-ATPase of human erythrocyte ghosts [46] as well as several classes of phosphatases, half-maximally inhibited MRP1 ATPase activity at 1 mM. Similarly, Al-Shawi and Senior [24] reported NaF inhibited P-glycoprotein ATPase activity with an IC_{50} of 3.3 mM. We also found that NaF inhibited LTC_4 transport by MRP1-enriched membrane vesicles (data not shown).

3.4. pH dependence of MRP1 ATPase activity and LTC_4 transport

The pH profile of the ATPase activity of MRP1 proteoliposomes is shown in Fig. 4A. Maximal ATP-

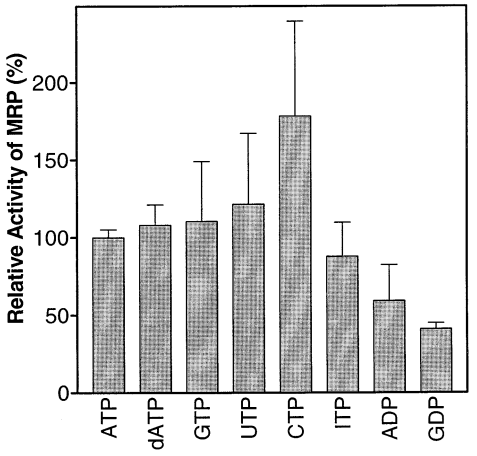

Fig. 5. Nucleotide specificity of MRP1 ATPase activity. The rate of nucleotide hydrolysis by reconstituted MRP1 (1.5 μg) was measured in a reaction mixture containing 50 mM Tris-HC1, pH 7.4, 2.5 mM nucleotides and 5 mM $MgC1_2$ as described in Section 2. In the experiments shown, 100% control activity was 9.2 ± 0.2 nmol min^{-1} mg^{-1} MRP1. Data points represent mean values (± S.E.) of three independent experiments.

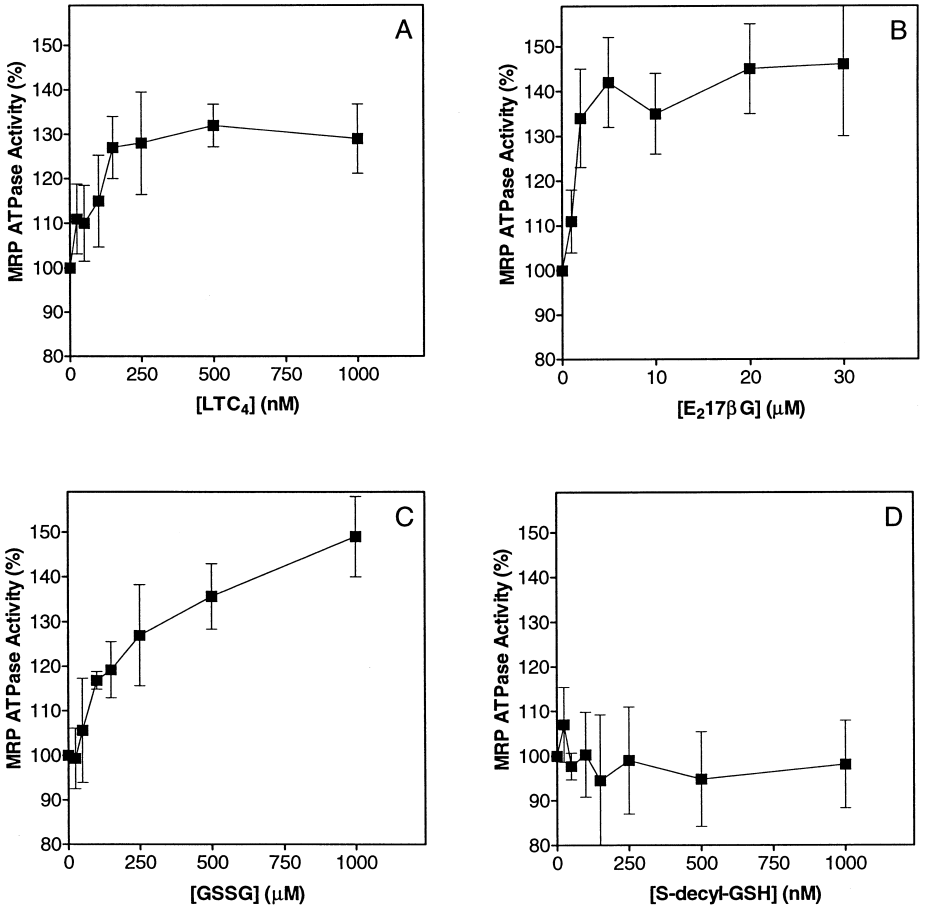

Fig. 6. Effect of organic anions and *S*-decyl-GSH on MRP1 ATPase activity. The ATPase activity of MRP1 proteoliposomes was assayed in a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 2.5 mM ATP, 5 mM $MgCl_2$ and different concentrations of various conjugated organic anions. (A) LTC_4; (B) $E_2$17βG; (C) GSSG; and (D) *S*-decyl-GSH. In the case of $E_2$17βG (B), the assay mixture was supplemented with 17β-estradiol 3-(β-D-glucuronide) (20 μM). In the experiments shown, 100% control activities were 5–10 nmol min^{-1} mg^{-1} MRP1. Data points represent mean values (±S.E.) of three independent experiments.

ase activity was observed at pH 7.5–8.0, which is similar to that of P-glycoprotein [25,27]. For comparison, the pH dependence of LTC_4 transport by T5 membrane vesicles was performed and the results are shown in Fig. 4B. The optimal pH for LTC_4 transport was between 8 and 9, which is slightly higher than the pH value for maximal MRP1 ATPase activity. Thus MRP1 appears to have optimal activities for both ATP hydrolysis and substrate transport in a slightly basic environment.

3.5. Nucleotide specificity of MRP1

To establish the nucleotide dependence of reconstituted native MRP1, various nucleotide triphosphates and diphosphates were substituted for ATP. Fig. 5 shows that purified and reconstituted MRP1 was able to hydrolyze a range of nucleotides. dATP, GTP, UTP and ITP were all good MRP1 substrates with rates of hydrolysis similar to that of ATP. The highest rate of hydrolysis was observed for CTP, which was nearly twice that for ATP. ADP and GDP were also hydrolyzed at a rate of hydrolysis that was 50% or less of that for ATP. Thus, like other ABC transporter proteins [27], MRP1 has a broad nucleotide specificity.

3.6. MRP1 ATPase activity is stimulated by its substrates

Stimulation of P-glycoprotein ATPase activity by many of its substrates, including chemotherapeutic

agents, has been reported by numerous investigators [23–28]. Consequently, it was of interest to determine whether known MRP1 substrates and the drugs to which MRP1 confers resistance affected its ATPase activity in a similar manner. As shown in Fig. 6A, the ATPase activity of purified and reconstituted MRP1 was maximally stimulated 30% by LTC_4, a high affinity substrate of MRP1 [11,13,14]. Half-maximal stimulation by LTC_4 was observed at approximately 150 nM, which is comparable to the K_m (LTC_4) (70–100 nM) obtained in transport studies with MRP1-enriched membrane vesicles [11,13,14].

$E_2$17βG is also transported by MRP1 in membrane vesicles [18,19] but unexpectedly, this compound caused moderate inhibition of MRP1 ATPase activity over the concentration range 1–15 μM. At 15 μM $E_2$17βG, MRP1 ATPase activity was decreased by 10–20% and the extent of inhibition did not increase with higher $E_2$17βG concentrations up to 100 μM (not shown). Because of the potential of the lipid environment to affect the ATPase activity of MRP1, we considered the possibility that the inhibition could be the consequence of a non-specific effect of the relatively lipophilic steroid glucuronide on proteoliposome structure. In agreement with this suggestion, we found that the structural isomer 17β-estradiol 3-(β-D-glucuronide) which we have shown previously does not compete for $E_2$17βG transport by MRP1 [19], was also inhibitory (not shown). To investigate the possibility that the non-specific inhibition by estrogen glucuronide had obscured a stimulation of ATPase activity, we repeated the experiments with $E_2$17βG in the presence of 20 μM 17β-estradiol 3-(β-D-glucuronide), a concentration at which non-specific inhibition was maximal. Under these conditions, the addition of 1–5 μM $E_2$17βG stimulated ATPase activity in a concentration dependent manner up to a maximal 40% increase (Fig. 6B). Additional 17β-estradiol 3-(β-D-glucuronide) in the same concentration range had no effect. The concentration of $E_2$17βG that resulted in 50% maximal stimulation was 1.6 μM which is similar to the K_m for this compound determined by transport studies with MRP1-enriched membrane vesicles [18,19].

GSSG, also a substrate of MRP1 [16,17], stimulated ATPase activity 50% at 1 mM (Fig. 6C). In addition, MRP1 ATPase activity was stimulated by GSH (not shown). However, this stimulation by GSH was likely the result of the presence of small amounts of GSSG formed during the relatively long ATPase assay time of 4 h. In support of this interpretation, GSH did not significantly stimulate ATPase activity in the presence of 5 mM DTT (not shown). *S*-decyl-GSH, which has been previously shown to be a potent competitive inhibitor of MRP1-mediated LTC_4 transport activity [13,14], had no effect on MRP1 ATPase activity (Fig. 6D). The chemotherapeutic agents vincristine, VP-16 and daunorubicin were tested at concentrations of 30–100 μM. None of these compounds detectably stimulated MRP1 ATPase activity, either in the presence or absence of 2 mM GSH (data not shown). Verapamil, a potent stimulator of P-glycoprotein ATPase activity and reversing agent of P-glycoprotein-mediated resistance also had no effect on MRP1 ATPase activity (data not shown)

4. Discussion

In the present study, we describe a simple procedure for purification of native MRP1 from multidrug resistant H69AR lung cancer cells. This procedure is comprised of a differential two-step CHAPS solubilization of MRP1 from plasma membranes followed by immunoaffinity chromatography using the human MRP1-specific mAb QCRL-1. MAb QCRL-1 has been well characterized and shown to recognize a human MRP1-specific heptapeptide epitope in the linker region which connects the two halves of the protein [30,31]. Using this method, MRP1 of approximately 80% purity as judged by silver staining was obtained with a total recovery of more than 80%. We estimate that MRP1 accounts for approximately 5% of total proteins in the plasma membranes isolated from H69AR cells, consistent with the high levels of drug resistance in these cells [32].

When purified MRP1 from H69AR cells was reconstituted into proteoliposomes by rapid dilution, relatively low levels of ATPase activity were detectable. Purified recombinant histidine-tagged MRP1 as judged by Coomassie Blue staining was recently reported to possess high constitutive ATPase activity (460 nmol mg^{-1} min^{-1}) comparable to that of P-glycoprotein [47]. However, in our studies, purified

and reconstituted native MRP1 showed a basal ATPase activity of 5–10 nmol min^{-1} mg^{-1}. This activity is 60–100-fold lower than that of P-glycoprotein [23–28]. In this regard, it is interesting to note that the MRP1-related yeast protein Yor1p exhibits ATPase activity that is at least 15-fold lower than that of the P-glycoprotein-related yeast protein Pdr5p [48]. Other reconstituted ABC transporters with relatively low levels of basal ATPase activity include CFTR (50 nmol min^{-1} mg^{-1}) [49,50] and the recently described ABCR (1.3 nmol min^{-1} mg^{-1}), a protein involved in early onset macular degeneration [51].

The possibility that MRP1 may lose some of its activity during the immunoaffinity purification and reconstitution procedures cannot presently be excluded. It is also possible that the reconstitution system used in this study is not optimal for MRP1. Integral membrane proteins, including P-glycoprotein, usually require specific lipids for optimal activity and may be inhibited by other lipid species. Their activities are also sensitive to the lipid bilayer dynamics and physico-chemical state [52–55]. Preliminary experiments indicate that the activity of MRP1 is also modulated significantly by changes in its lipid environment (unpublished observation). Nevertheless, the relatively low level ATPase activity of reconstituted native MRP1 reported here is consistent with the level of ATPase activity attributable to MRP1 in membrane vesicles [29] (unpublished observation). Taken together, these results suggest that MRP1 has a low intrinsic ATPase activity. It is important to note, however, that the dependence of the activity of the reconstituted native MRP1 on ATP concentration [K_m (ATP) 104 μM] is comparable to that obtained from transport studies with MRP1-enriched membrane vesicles [12,13,16,17]. In contrast, the affinity reported for purified histidine-tagged recombinant MRP1 solubilized in *n*-dodecyl-β-D-maltoside [K_m (ATP) 3 mM] is markedly lower, for reasons which are presently not understood [47].

The comparatively low ATPase activity of reconstituted native MRP1 from H69AR cells and its relatively high affinity for this nucleotide may be linked to the structural differences between the two NBDs of MRP1 and its related proteins and those of the P-glycoproteins. The NH_2- and COOH-proximal NBDs of the P-glycoproteins are quite similar, and although cooperativity between the two domains is required for activity, they appear to be functionally quite similar although not necessarily equivalent [56–58]. In contrast, the two NBDs of MRP1 and its related proteins as well as CFTR are considerably less similar to each other [1–4]. In the case of CFTR, there is convincing evidence that the two NBDs are functionally distinct [59,60]. There are 13 amino acids present in the NBDs of the P-glycoproteins that are absent from the comparable location in the first NBD of the MRP1-related proteins and CFTR [3,4]. The absence of these amino acids significantly shortens the distance between the Walker A and B motifs and is known to affect the folding of this domain [61]. Furthermore, the ABC 'active transport family' signature (LSSG-GQX_3RHydXHydA), which is relatively invariant in both NBDs of the P-glycoproteins, is highly conserved in the first NBD, but not in the second NBD of CFTR and the MRP1-related proteins [4]. Thus it would not be surprising if the structural characteristics of the two NBDs common to MRP1-related proteins had important consequences with respect to the mechanism of ATP binding and hydrolysis and possibly for the coupling between ATP hydrolysis and substrate transport as well.

To further characterize the ATPase activity of reconstituted MRP1 and to distinguish it from that of other possible contaminating ATPases, the effects of several inhibitors were examined (Table 1). The presence of contaminating vacuolar, or V-type, ATPases could be excluded based on the observation that the MRP1 ATPase activity was relatively sensitive to vanadate but insensitive to *N*-ethylmaleimide, NBD-C1 and bafilomycin A_1. In contrast, P-glycoprotein ATPase activity is sensitive to *N*-ethylmaleimide and bafilomycin A_1 [28,62]. The concentration of vanadate required for 50% inhibition (10 μM) of MRP1 ATPase activity is much higher than that typically required to inhibit P-type ATPases [39], although it is similar to that required to inhibit P-glycoprotein ATPase activity [28]. Contamination with Na^+/K^+-ATPase, a highly abundant P-type ATPase, could be excluded based on the insensitivity of MRP1 ATPase activity to ouabain. The insensitivity of MRP1 ATPase activity to sodium azide and oligomycin indicates that typical F_1F_0-ATPases may also be excluded as possible contaminants. In contrast, P-glycoprotein ATPase activity, although in-

sensitive to azide, is quite sensitive to oligomycin [24]. Taken together, these results indicate that contributions to the observed MRP1 ATPase activity by contaminating typical P-, F-, and V-type ATPases may be excluded. They also indicate that the MRP1 ATPase activity does not share a common ATPase inhibitor profile with P-glycoprotein.

The ATPase activity of MRP1 was stimulated by several compounds known from previous studies to be substrates for this transport protein. LTC_4 has the highest known affinity for MRP1 and this compound stimulated ATPase activity approximately 30% above basal activity. Presuming that substrate stimulation of ATPase activity is the driving force of substrate translocation, then 1.5–3 nmol min^{-1} mg^{-1} MRP1 of ATP hydrolysis would contribute to LTC_4 transport by MRP1. Previous studies of MRP1-enriched membrane vesicles isolated from H69AR cells [14], indicated that the specific LTC_4 transport activity of MRP1 was approximately 100–300 pmol min^{-1} mg^{-1} protein. Since MRP1 comprises approximately 5% of total plasma membrane protein in H69AR cells, LTC_4 transport activity may be estimated to be approximately 2–6 nmol min^{-1} mg^{-1} MRP1. This implies a stoichiometry of molecules LTC_4 transported to molecules ATP hydrolyzed of 1–2, which in turn suggests a mechanism for substrate translocation by MRP1 that is tightly coupled to ATP hydrolysis. Similarly, on the basis of the greater than 10-fold difference in their ATPase activities, it was suggested that the MRP1-related Yor1 yeast protein is a more highly coupled transporter than the P-glycoprotein-related Pdr5p yeast protein [48].

Reconstituted MRP1 ATPase activity from H69AR cells was stimulated by LTC_4 in a linear manner with respect to substrate concentration with maximal stimulation at approximately 250 nM and half-maximal stimulation at approximately 150 nM. These values are consistent with the K_m (LTC_4) (70–100 nM) obtained in transport studies with MRP1-enriched membrane vesicles [11,14]. In contrast, stimulation of the recombinant histidine-tagged MRP1 by LTC_4 showed no concentration dependence over a 1000-fold range (1 to 1000 nM) and maximal stimulation required a concentration of at least 10 μM [47]. The basis for this difference in potency between the two preparations of MRP1 is unclear.

The observation that GSSG but not GSH stimulated MRP1 ATPase activity is consistent with the ability of GSSG to be transported by MRP1 [16,20]. GSH itself is not a substrate but may be co-transported with the *Vinca* alkaloid vincristine [20,21]. The alkyl derivative *S*-decyl-GSH also did not significantly stimulate MRP1 ATPase activity although it is a competitive inhibitor of LTC_4 transport in MRP1-enriched membrane vesicles [14]. These observations suggest that *S*-decyl-GSH may interact with MRP1 at the same site as GSH but is not itself transported and thus it can compete effectively with GSH for transport.

In contrast to our inability to demonstrate stimulation of reconstituted MRP1 ATPase activity by anticancer drugs, Chang et al. [47] reported that daunorubicin, doxorubicin, colchicine, vinblastine and vincristine all moderately stimulated the ATPase activity of histidine-tagged recombinant MRP1. The concentration dependence of this stimulation was comparable for all five drugs from 0.1–500 μM. This finding is somewhat surprising given that these compounds are not transported by MRP1 in membrane vesicles and cells overexpressing MRP1 typically display very low levels of resistance to colchicine and vinblastine compared to vincristine, doxorubicin and daunorubicin [1,14,16,20,21]. The basis for the apparent discrepancy between our findings and those reported previously is unknown. Unlike membrane vesicle studies where MRP1-mediated transport of vincristine and daunorubicin may be stimulated by physiological concentrations of GSH [14,20,21], we found that addition of 2 mM GSH did not enhance the ability of these drugs to stimulate MRP1 ATPase activity. The intrinsically low levels of MRP1 ATPase activity may make detection of stimulation by these drugs difficult, particularly since previous studies have shown that the efficiency with which MRP1 transports or is inhibited by unmodified compounds is considerably lower than for their conjugated derivatives [15,18,63]. It should also be noted that in the case of P-glycoprotein, not all substrates stimulate activity and in some cases, inhibition rather than stimulation has been observed [26,36,62]. Whether this is also true of MRP1 remains to be determined. Alternatively, the lipids used to reconstitute MRP1 in this study might not be optimal for observation of MRP1 activation by

these substrates. Like P-glycoprotein [26,53–55,64], the lipid environment in which MRP1 is reconstituted is likely to markedly affect the interaction between drugs and MRP1 and thus modulate the degree of drug stimulation of ATPase activity. This possibility is the subject of ongoing investigations.

In summary, we have purified native MRP1 and reconstituted it into proteoliposomes. The reconstituted MRP1 showed low level ATPase activity which could be stimulated by its conjugated organic anion substrates LTC_4 and $E_2$17βG at concentrations that correspond well with K_m values reported previously from transport studies with MRP1-enriched membrane vesicles. ATPase inhibitor experiments clearly distinguished MRP1 ATPase activity from that of other ATPases, including P-glycoprotein. A tight coupling mechanism between ATP hydrolysis and translocation of substrate is proposed for MRP1 based on the results obtained. These studies represent the first characterization of the ATPase activity of reconstituted native MRP1. The simple immunoaffinity purification procedure described will facilitate mechanistic studies of ATP dependent MRP1-mediated conjugated organic anion and drug transport.

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References

[1] D. Lautier, Y. Canitrot, R.G. Deeley, S.P.C. Cole, Biochem. Pharmacol. 52 (1996) 967–977.
[2] R.G. Deeley, S.P.C. Cole, Semin. Cancer Biol. 8 (1997) 193–204.
[3] S.P.C. Cole, R.G. Deeley, BioEssays 20 (1998) 931–940.
[4] S.P.C. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M.V. Duncan, R.G. Deeley, Science 258 (1992) 1650–1654.
[5] K. Nooter, A.M. Westerman, M.J. Flens, G.J.R. Zaman, R.J. Scheper, K.E. van Wingerden, H. Burger, R. Oostrum, T. Boersma, P. Sonneveld, J.W. Gratama, T. Kok, A.M.M. Eggermont, F.T. Bosman, G. Stoter, Clin. Cancer Res. 1 (1995) 1301–1310.
[6] H.S.L. Chan, Y. Lu, T.M. Grogan, G. Haddad, D.R. Hipfner, S.P.C. Cole, R.G. Deeley, V. Ling, B.L. Gallie, Cancer Res. 57 (1997) 2325–2330.
[7] M.D. Norris, S.B. Bordow, G.M. Marshall, P.S. Haber, S.L. Cohn, M. Haber, New Engl. J. Med. 334 (1996) 231–238.
[8] J. Kreisholt, M. Sorensen, P.B. Jensen, B.S. Nielsen, C.B. Andersen, M. Sehested, Br. J. Cancer 77 (1998) 1469–1473.
[9] C.F. Higgins, Annu. Rev. Cell Biol. 8 (1992) 67–113.
[10] D.R. Hipfner, K.C. Almquist, E.M. Leslie, J.H. Gerlach, C.E. Grant, R.G. Deeley, S.P.C. Cole, J. Biol. Chem. 272 (1997) 23623–23630.
[11] I. Leier, G. Jedlitschky, U. Buchholz, S.P.C. Cole, R.G. Deeley, D. Keppler, J. Biol. Chem. 269 (1994) 27807–27810.
[12] R. Evers, N.H.P. Cnubben, J. Wijnholds, L. van Deemter, P.J. van Bladeren, P. Borst, FEBS Lett. 419 (1997) 112–116.
[13] M. Muller, C. Meijer, G.J.R. Zaman, P. Borst, R.J. Scheper, N.H. Mulder, E.G.E. de Vries, P.L.M. Jansen, Proc. Natl. Acad. Sci. USA 91 (1994) 13033–13037.
[14] D.W. Loe, K.C. Almquist, R.G. Deeley, S.P.C. Cole, J. Biol. Chem. 271 (1996) 9675–9682.
[15] D.W. Loe, R.K. Stewart, T.E. Massey, R.G. Deeley, S.P.C. Cole, Mol. Pharmacol. 51 (1997) 1034–1041.
[16] I. Leier, G. Jedlitschky, U. Buchholz, M. Center, S.P.C. Cole, R.G. Deeley, D. Keppler, Biochem. J. 314 (1996) 433–437.
[17] M. Heijn, J.H. Hooijberg, G.L. Scheffer, G. Szabo, H.V. Westerhoff, J. Lankelma, Biochim. Biophys. Acta 1326 (1997) 12–22.
[18] G. Jedlitschky, I. Leier, U. Buchholz, K. Barnouin, G. Kurz, D. Keppler, Cancer Res. 56 (1996) 988–994.
[19] D.W. Loe, K.C. Almquist, S.P.C. Cole, R.G. Deeley, J. Biol. Chem. 271 (1996) 9683–9689.
[20] D.W. Loe, R.G. Deeley, S.P.C. Cole, Cancer Res. 58 (1998) 5130–5136.
[21] J. Renes, E.G.E. de Vries, E.F. Nienhuis, P.L.M. Jansen, M. Muller, Br. J. Pharmacol. 126 (1999) 681–688.
[22] G. Rappa, A. Lorico, R.A. Flavell, A.C. Sartorelli, Cancer Res. 57 (1997) 5232–5237.
[23] B. Sarkadi, E.M. Price, R.C. Boucher, U.A. Germann, G.A. Scarborough, J. Biol. Chem. 267 (1992) 4854–4858.
[24] M.K. Al-Shawi, A.E. Senior, J. Biol. Chem. 268 (1993) 4197–4206.
[25] S.V. Ambudkar, I.H. Lelong, J. Zhang, C.O. Cardarelli, M.M. Gottesman, I. Pastan, Proc. Natl. Acad. Sci. USA 89 (1992) 8472–8476.

[26] F.J. Sharom, X. Yu, C.A. Doige, J. Biol. Chem. 268 (1993) 24197–24202.
[27] I.L. Urbatsch, M.K. Al-Shawi, A.E. Senior, Biochemistry 33 (1994) 7069–7076.
[28] A. Shapiro, V. Ling, J. Biol. Chem. 269 (1994) 3745–3754.
[29] J.H. Hooijberg, H.J. Broxterman, M. Heijn, D.L.A. Fles, J. Lankelma, H.M. Pinedo, FEBS Lett. 413 (1997) 344–348.
[30] D.R. Hipfner, S.D. Gauldie, R.G. Deeley, S.P.C. Cole, Cancer Res. 54 (1994) 5788–5792.
[31] D.R. Hipfner, K.C. Almquist, B.D. Stride, R.G. Deeley, S.P.C. Cole, Cancer Res. 56 (1996) 3307–3314.
[32] S.E.L. Mirski, J.H. Gerlach, S.P.C. Cole, Cancer Res. 47 (1987) 2594–2598.
[33] W. Wray, T. Boulikas, V.P. Wray, R. Hancock, Anal. Biochem. 118 (1981) 197–203.
[34] G.L. Peterson, Anal. Biochem. 83 (1977) 346–356.
[35] S. Chifflet, A. Torriglia, R. Chiesa, S. Tolosa, Anal. Biochem. 168 (1988) 1–4.
[36] C.A. Doige, X. Yu, F.J. Sharom, Biochim. Biophys. Acta 1109 (1992) 149–160.
[37] M. Buechler, M. Boehme, H. Ortlepp, D. Keppler, Eur. J. Biochem. 224 (1994) 345–352.
[38] S.L. Olsen, E.M. Leslie, D.W. Loe, A.L.-J. Ooi, R.G. Deeley, S.P.C. Cole, Proc. Am. Assoc. Cancer Res. 39 (1998) 1145.
[39] G. Inesi, Annu. Rev. Physiol. 47 (1985) 573–601.
[40] R.E. Ebel, H.A. Lardy, J. Biol. Chem. 250 (1975) 191–196.
[41] L.M. Amzel, P.L. Pedersen, Annu. Rev. Biochem. 52 (1983) 801–824.
[42] P.L. Pedersen, E. Carafoli, Trends Biochem. Sci. 12 (1987) 146–150.
[43] E.J. Bowman, A. Siebers, K. Altendorf, Proc. Natl. Acad. Sci. USA 85 (1988) 7972–7976.
[44] J.G. Forte, A. Ganser, R. Beesley, T.M. Forte, Gastroenterology 69 (1975) 175–189.
[45] A.J. Murphy, R.J. Coll, J. Biol. Chem. 267 (1992) 5229–5235.
[46] M.B. Morris, G. Monteith, B.D.J. Ronfogalis, Cell. Biochem. 48 (1992) 356–366.
[47] X.-B. Chang, Y.-X. Hou, J.R. Riordan, J. Biol. Chem. 272 (1997) 30962–30968.
[48] A. Decottignies, A.M. Grant, J.W. Nichols, H. de Wet, D.B. McIntosh, A. Goffeau, J. Biol. Chem. 273 (1998) 12612–12622.
[49] C. Li, M. Ramjeesingh, W. Wang, E. Garami, M. Hewryk, D. Lee, J.M. Rommens, K. Galley, C.E. Bear, J. Biol. Chem. 271 (1996) 28463–28468.
[50] C.E. Bear, C. Li, K. Galley, Y. Wang, E. Garami, M. Ramjeesingh, J. Bioenerg. Biomembr. 29 (1997) 465–473.
[51] H. Sun, R.S. Molday, J. Nathans, J. Biol. Chem. 274 (1999) 8269–8281.
[52] G. In 't Veld, A.J.M. Driessen, W.N. Konings, FEMS Microbiol. Rev. 12 (1993) 293–314.
[53] C.A. Doige, X. Yu, F.J. Sharom, Biochim. Biophys. Acta 1146 (1993) 65–72.
[54] Y. Romsicki, F.J. Sharom, Eur. J. Biochem. 256 (1998) 170–178.
[55] I.L. Urbatsch, A.E. Senior, Arch. Biochem. Biophys. 316 (1995) 135–140.
[56] I.L. Urbatsch, B. Sankaran, S. Bhagat, A.E. Senior, J. Biol. Chem. 270 (1995) 26956–26961.
[57] C.A. Hrycyna, M. Ramachandra, S.V. Ambudkar, Y.H. Ko, P.L. Pedersen, I. Pastan, M.M. Gottesman, J. Biol. Chem. 273 (1998) 16631–16634.
[58] Y. Takada, K. Yamada, Y. Taguchi, K. Kino, M. Matsuo, S.J. Tucker, T. Komano, T. Amachi, K. Ueda, Biochim. Biophys. Acta 1373 (1998) 131–136.
[59] M.R. Carson, S.M. Travis, M.J. Welsh, J. Biol. Chem. 270 (1995) 1711–1717.
[60] K. Szabo, G. Szakacs, T. Hegedus, B. Sarkadi, J. Biol. Chem. 274 (1999) 12209–12212.
[61] D.R. Hipfner, Q. Mao, W. Qiu, E.M. Leslie, R.G. Deeley, S.P.C. Cole, J. Biol. Chem. 274 (1999) 15420–15426.
[62] F.J. Sharom, X. Yu, J.W. Chu, C.A. Doige, Biochem. J. 308 (1995) 381–390.
[63] W. Priebe, M. Krawczyk, M.T. Kuo, Y. Yamane, N. Savaraj, T. Ishikawa, Biochem. Biophys. Res. Commun. 247 (1998) 859–863.
[64] F.J. Sharom, Biochem. Soc. Trans. 25 (1997) 1088–1096.