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ATP-dependent Transport of Glutathione *S*-Conjugates by the Multidrug Resistance-associated Protein[1]

Gabriele Jedlitschky,[2] Inka Leier, Ulrike Buchholz, Melvin Center, and Dietrich Keppler

Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany [G. J., I. L., U. B., D. K.], and Division of Biology, Kansas State University, Manhattan, Kansas 66506 [M. C.]

Abstract

The ATP-dependent transport of the endogenous glutathione conjugate leukotriene C_4 (LTC_4) was more than 25-fold higher in membrane vesicles prepared from human leukemia cells (HL60/ADR) overexpressing the multidrug resistance-associated protein than from drug-sensitive parental HL60 cells or revertant cells. Similar results were obtained with *S*-(2,4-dinitrophenyl)glutathione as substrate. Photoaffinity labeling detected preferentially in the HL60/ADR membranes a 190-kilodalton protein binding [^{3}H]LTC_4 and 8-azido[α-^{32}P]ATP. The [^{3}H]LTC_4-labeled 190-kilodalton protein was immunoprecipitated by an antiserum against the COOH-terminal sequence of multidrug resistance-associated protein. Our results indicate that multidrug resistance-associated protein mediates the ATP-dependent transport of LTC_4 and structurally related anionic amphiphilic conjugates.

Introduction

Using isolated membrane vesicles from murine mastocytoma cells, we have previously characterized the ATP-dependent export pump for the glutathione conjugate LTC_4[3] in leukotriene-generating cells (1, 2). Photoaffinity labeling of a 190-kilodalton membrane glycoprotein by [^{3}H]LTC_4, which was competed for by a potent inhibitor of the ATP-dependent LTC_4 transport, indicated the involvement of this glycoprotein in the ATP-dependent transport of LTC_4 and structurally related conjugates (2). ATP-dependent transport of glutathione *S*-conjugates was originally described in inside-out-oriented plasma membrane vesicles from human erythrocytes (3) and subsequently established by kinetic measurements in various other tissues (1–6). LTC_4 and its metabolites are the endogenous substrates with the highest known affinities for this ATP-dependent export system (1, 5).

HL60 cells selected for resistance to doxorubicin (HL60/ADR) are defective in the cellular accumulation of the drug. These cells overexpress a 190-kilodalton glycoprotein which is associated with non-P-glycoprotein-mediated multidrug resistance (7, 8) and is encoded by the *MRP* gene (9). The complementary DNA encoding the MRP has been cloned from non-P-glycoprotein multidrug-resistant small cell lung cancer cells (H69/AR) and MRP was identified as a member of the superfamily of ATP-binding cassette transporters (10). Overexpression of the 190-kilodalton MRP in HeLa cells transfected with MRP expression vectors increased multidrug resistance (11). The identification of substrates transported directly by MRP should provide considerable insight into the possible functions of the *MRP*-encoded protein P190 in normal cells and also in those cells isolated for resistance to anticancer drugs. In the present study we identified MRP as a LTC_4-binding protein by photoaffinity labeling, followed by immunoprecipitation by a MRP-specific antiserum. ATP-dependent LTC_4 and *S*-(2,4-dinitrophenyl)glutathione transport were closely correlated with the expression of MRP.

Materials and Methods

Materials. [14,15,19,20-$^3H_4$]LTC_4 (4.7 TBq/mmol) and unlabeled LTC_4 were obtained from DuPont New England Nuclear (Boston, MA) and Amersham-Buchler (Braunschweig, Germany), respectively. 8-Azido[α-^{32}P]ATP (320 GBq/mmol) was obtained from ICN Biochemicals (Irvine, CA). The LTD_4 receptor antagonist MK 571 (12) was kindly provided by Dr. A. W. Ford-Hutchinson (Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). *S*-(2,4-Dinitrophenyl)glutathione was prepared from GSH and 1-chloro-2,4-dinitrobenzene using basic glutathione *S*-transferases from rat liver (Sigma Chemical Co., St. Louis, MO) (13). The protein standard mixture (M_r 43,000–200,000) for SDS-PAGE was from Merck (Darmstadt, Germany) and nitrocellulose filters (pore size 0.2 μm) were from Schleicher & Schuell (Dassel, Germany).

The polyclonal anti-MRP serum was raised in rabbits against the peptide QRGLFYSMAKDAGLV, the deduced COOH-terminal sequence of MRP. Details of the antiserum preparation have been described previously (14).

Cells. The non-P-glycoprotein-expressing doxorubicin-resistant HL60/ADR cells (7–9), as well as the parental and the revertant HL60 cells, were grown in RPMI with 10% fetal calf serum in a humidified incubator (5% CO_2 at 37°C). The resistant cells were cultured in the presence of 200 nM daunorubicin until 3 days before membrane preparation. The revertant cells were isolated as described previously (14).

Preparation of Plasma Membrane Vesicles. Cells were harvested from the cell culture by centrifugation and plasma membrane vesicles were prepared as described (1, 2). In brief, the cells were lysed by incubation in hypotonic buffer (0.5 mM sodium phosphate, pH 7.0-0.1 mM EDTA supplemented with protease inhibitors) for 1.5 h, followed by homogenization with a Potter-Elvehjem homogenizer. After centrifugation of the homogenate at 12,000 × *g* (10 min at 4°C), the postnuclear supernatant was centrifuged at 100,000 × *g* for 45 min at 4°C. The resulting pellet was suspended in incubation buffer (250 mM sucrose-10 mM Tris-HCl, pH 7.4) homogenized with a tight fitting Dounce homogenizer and layered over 38% sucrose in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/KOH, pH 7.4. After centrifugation at 280,000 × *g* for 2 h at 4°C the interphases were collected, washed by centrifugation in the incubation buffer (100,000 × *g*), and passed 20 times through a 27-gauge needle for vesicle formation. The resulting membrane vesicles were enriched in plasma membranes (20–30-fold as compared to the original homogenate) with moderate contaminations from endoplasmic reticulum and Golgi apparatus as calculated from marker enzyme activities (2).

Vesicles Transport Studies. ATP-dependent transport of [^{3}H]LTC_4 and *S*-(2, 4-dinitrophenyl)[^{3}H]glutathione into membrane vesicles was measured by rapid filtration (2, 5). Membrane vesicles (50 μg of protein) were incubated in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and the labeled substrate in an incubation buffer containing 250 mM sucrose and 10 mM Tris-HCl, pH 7.4. The final volume was 110 μl. Aliquots (20 μl) were taken at the times indicated and diluted in 1 ml of ice-cold incubation buffer. The diluted samples were filtered immediately

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: LTC_4, leukotriene C_4; 8-azido[α-^{32}P]ATP, 8-azido-adenosine 5′-[α-^{32}P]triphosphate; GSH, reduced glutathione; HL60/ADR, human leukemia cells selected for resistance to Adriamycin (doxorubicin); MDR, multidrug resistance; MRP, multidrug resistance-associated protein; MK 571, 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio)propanoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

through nitrocellulose filters (0.2 μm pore size), presoaked in incubation buffer by the use of a rapid filtration device, and rinsed twice with 5 ml of incubation buffer. Filters were dissolved and counted in a liquid scintillation counter. In control experiments, ATP was replaced by an equal concentration of 5′-AMP. All rates of ATP-dependent transport were calculated by subtracting values obtained in the presence of 5′-AMP as a blank from those in the presence of ATP (2, 15).

Photoaffinity Labeling. Vesicle suspensions (200 μg protein) were incubated with 74 kBq [^{3}H]LTC_4 (150 nM) at 37°C for 10 min, shock-frozen in liquid nitrogen, and irradiated at 300 nm for 5 min (2, 16). For competition studies, vesicle suspensions were preincubated with 50 μM MK 571 for 30 min at 4°C. Photoaffinity labeling with 370 kBq 8-azido[α-^{32}P]ATP (10 μM) was performed in a similar way, with incubation for 2 min at 4°C and irradiation at 350 nm for 10 min.

Labeled membranes were washed with buffer containing 2% sodium taurocholate to remove proteins which are loosely associated with the membrane and were then subjected to SDS-PAGE (7.5% acrylamide gel containing 5 M urea) as described previously (2). After electrophoresis, gels were cut into 2-mm-thick slices and dissolved in 0.5 ml tissue solubilizer, and the radioactivity was measured in a liquid scintillation counter.

Immunoprecipitation. [^{3}H]LTC_4-labeled HL60/ADR membranes (400 μg) were lysed in 1 ml of solubilization buffer (50 mM Tris-HCl, pH 8.0-140 mM NaCl-0.2 mM phenylmethylsulfonyl fluoride-4 mM EDTA-0.5% sodium deoxycholate) for 40 min at 4°C. The lysates were centrifuged at 10 000 × *g* for 20 min, and the supernatant was incubated with the anti-MRP serum at 75-fold dilution for 18 h at 4°C. Precipitation of the antigen-antibody complex was achieved by the addition of 60-μl 15% Protein A-Sepharose (Sigma) suspended in 10 mM Tris-HCl, pH 8.0, for 1 h, followed by centrifugation. The immunoprecipitate, which was washed 5 times in solubilization buffer, as well as the supernatant, were subjected to SDS-PAGE (7.5% acrylamide-5 M urea), and the distribution of radioactivity in the gels was determined as described above.

Immunoblot. Membrane proteins (50 μg) were separated on a 7.5% acrylamide gel. Immunoblotting was performed essentially according to Towbin (17) using a tank blotting system and an alkaline phosphatase detection system (18).

Statistics. Data are presented as mean ± SD.

Results

ATP-dependent Transport of Glutathione *S*-conjugates by Membrane Vesicles from MRP-overexpressing and Control HL60 Cells. Primary-active ATP-dependent transport of [^{3}H]LTC_4, which proceeds into the fraction of inside-out-oriented vesicles, was studied during a 3-min period (Fig. 1). For a better differentiation between nucleotide-dependent binding and ATP-dependent transport into the membrane vesicles, we calculated the ATP-dependent transport by subtracting the vesicle-associated radioactivity in the presence of 4 mM 5′-AMP from the values obtained in the presence of 4 mM ATP. With vesicles from the parental as well as the revertant cells, which both exhibited a low MRP-expression (Fig. 2, *upper panels*), the rate of ATP-dependent transport of [^{3}H]LTC_4 at the standard concentration of 50 nM was below 1 pmol × mg protein^{-1} × min^{-1}. The HL60/ADR membranes overexpressing MRP (Fig. 2) showed rapid ATP-dependent transport of LTC_4 (50 nM) at an initial transport rate of 25 ± 2 pmol × mg protein^{-1} × min^{-1} (Fig. 1). The amount of [^{3}H]LTC_4 taken up by the vesicles was markedly decreased by increasing the osmolarity of the extravesicular medium. At a concentration of 1 M sucrose (outside the vesicles), the uptake rate was 52 ± 8% (SD; n = 4) of the value obtained under standard conditions with 250 mM sucrose. This indicates that [^{3}H]LTC_4 was transported into the intravesicular space. As recognized earlier (1, 2), the quinoline-based leukotriene D_4 receptor antagonist MK 571 was a potent inhibitor of ATP-dependent LTC_4 transport. The half maximal inhibition of LTC_4 transport by HL60/ADR membranes was observed at 1.3 μM MK 571.

The rate of ATP-dependent transport of *S*-(2,4-dinitrophenyl)-[^{3}H]glutathione at a concentration of 5 μM by HL60/ADR membranes was 50 ± 4 pmol × mg protein^{-1} × min^{-1} (n = 4). This transport was below 5 pmol × mg protein^{-1} × min^{-1} with membranes from the parental or the revertant cells (Fig. 1, *right panel*).

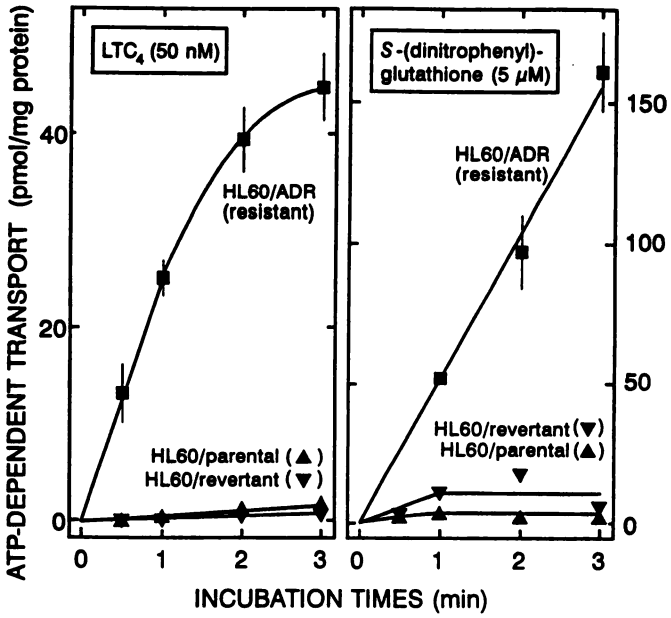

Fig. 1. ATP-dependent transport of [^{3}H]LTC_4 (*left*) and *S*-(2, 4-dinitrophenyl)-[^{3}H]glutathione (*right*) in membrane vesicles of MRP-overexpressing and control HL60 cells. Membrane vesicles (50 μg of protein) from resistant HL60/ADR (■), parental HL60 (▲), and revertant HL60 (▼) cells were incubated with [^{3}H]LTC_4 (50 nM) or *S*-(2,4-dinitrophenyl)[^{3}H]glutathione (5 μM) and the vesicle-associated radioactivity was determined by a rapid filtration technique as described in "Materials and Methods." The rate of net ATP-dependent transport was calculated by subtracting transport in the presence of 4 mM 5′-AMP as a blank from transport in the presence of 4 mM ATP. *Points*, mean; *bars*, SD from 6 experiments with membranes from 3 different preparations for [^{3}H]LTC_4 transport and from 4 experiments for *S*-(2,4-dinitrophenyl)[^{3}H]glutathione transport.

Identification of HL60 Membrane Proteins Binding [^{3}H]LTC_4 and 8-Azido-[α-^{32}P]ATP by Photoaffinity Labeling. LTC_4-binding membrane proteins were detected by direct photoaffinity labeling using [^{3}H]LTC_4 as a photolabile ligand. A marked difference in the [^{3}H]LTC_4 labeling pattern of the membranes from the different cell lines was observed at a molecular mass of approximately 190 kilodaltons (Fig. 2). In the HL60/ADR membranes a predominant 190-kilodalton protein was labeled (Fig. 2*A*), whereas in membranes from the revertant and the parental cells only a slight labeling was detected in this range (Fig. 2, *B* and *C*). The [^{3}H]LTC_4 labeling of the 190-kilodalton protein was competed for by the transport inhibitor MK 571 (Fig. 2*A*). Another significantly labeled protein of about 110 kilodaltons was observed in membranes from all three cell lines, particularly from the HL60 parental cells. In the Coomassie-stained gel no difference was detected in the pattern of proteins from the different cell membranes (not shown), indicating that the differences in the labeling pattern reflect LTC_4 binding to proteins with high affinity rather than nonspecific binding to abundant proteins. The labeling of the 190-kilodalton protein with [^{3}H]LTC_4 (Fig. 2, *lower panels*) was paralleled by the amount of immunoreactive MRP detected with an antiserum directed against the COOH-terminal sequence of this protein (Fig. 2, *upper panels*). Photoaffinity labeling with 8-azido-[α-^{32}P]ATP strongly labeled a 190-kilodalton protein in the membranes from HL60/ADR cells but not in the revertant or the parental cell membranes (Fig. 3). The immunoprecipitate obtained with the anti-MRP serum from [^{3}H]LTC_4-labeled and solubilized HL60/ADR membranes contained a major labeled membrane protein of about 190 kilodaltons, indicating that MRP was the [^{3}H]LTC_4-binding protein (Fig. 4).

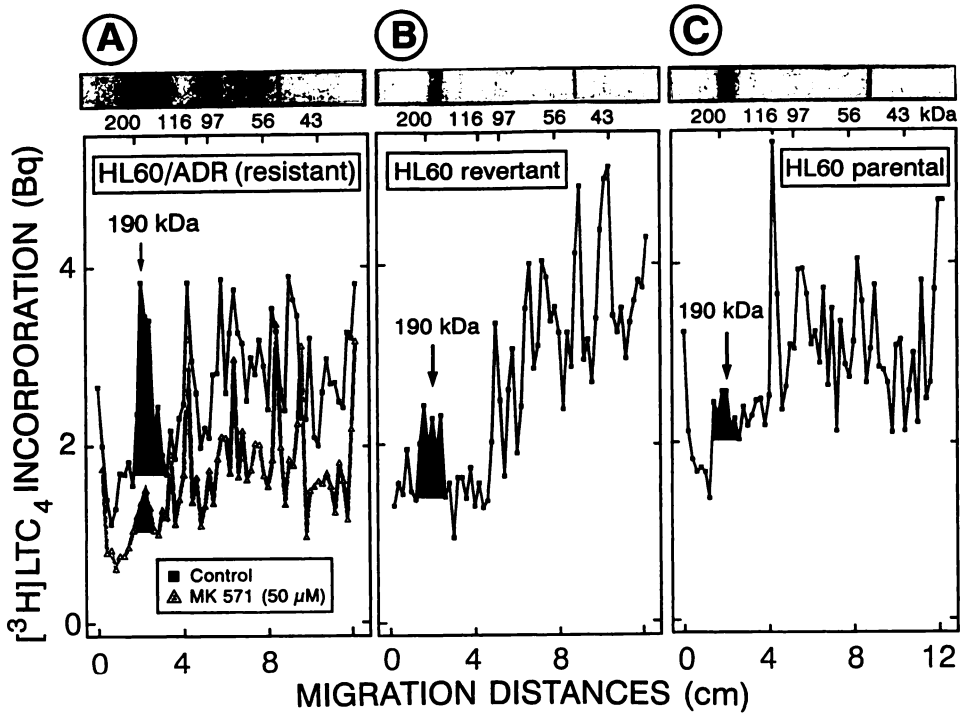

Fig. 2. [^{3}H]LTC$_4$-binding proteins and immunoreactive MRP in membranes of (*A*) HL60/ADR cells, (*B*) revertant cells, and (*C*) parental HL60 cells. Immunoblots with anti-MRP serum are shown in the *upper panels* and distribution of [^{3}H]LTC$_4$-derived radioactivity after SDS-PAGE of photoaffinity-labeled membranes on the *lower panels*. For photoaffinity labeling, membranes (200 μg of protein) were incubated with [^{3}H]LTC$_4$ at 37°C for 10 min, shock frozen, and irradiated at 300 nm. The labeled membranes were treated with 2% sodium taurocholate prior to separation by SDS-PAGE using a 7.5% acrylamide gel containing 5 M urea. In addition, the [^{3}H]LTC$_4$ labeling of HL60/ADR membranes was performed in the presence of 50 μM MK 571 (*A*). Peaks around 190 kilodaltons (*kDa*) are marked.

Discussion

The rates of ATP-dependent transport of the glutathione *S*-conjugates LTC$_4$ and *S*-(2, 4-dinitrophenyl)glutathione by membranes from HL60 cells were directly correlated with the expression of MRP (Figs. 1 and 2). High transport rates were determined with membranes from the MRP-overexpressing HL60/ADR cells, whereas ATP-dependent transport was less than 5%, with the same amounts of membranes from drug-sensitive parental HL60 cells or from revertant cells (Fig. 1). The levels of MRP were low in the revertant as well as in the parental HL60 cells (Fig. 2; Ref. 14). This suggests that MRP rather than another protein, possibly co-overexpressed in the HL60/ADR cells, mediates the ATP-dependent transport of the glutathione *S*-conjugates. We have identified MRP as a membrane protein tightly binding the glutathione *S*-conjugate LTC$_4$ by direct photoaffinity labeling (Fig. 2) and immunoprecipitation with an MRP-specific antiserum (Fig. 4). This antiserum does not recognize

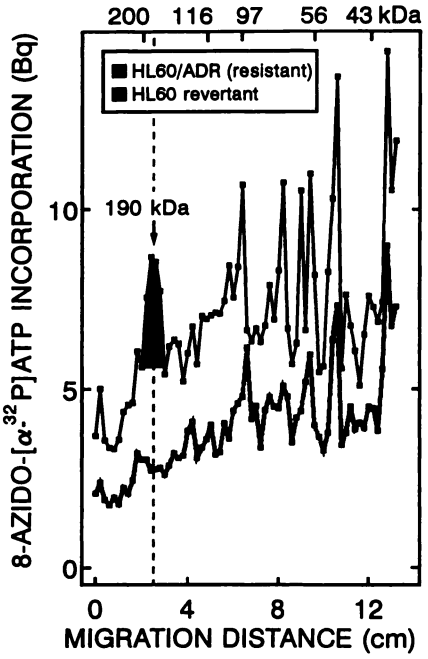

Fig. 3. 8-Azido-[α-^{32}P]ATP-binding proteins in membranes of HL60/ADR cells and revertant cells. For photoaffinity labeling, membranes (200 μg of protein) were incubated with 8-azido-[α-^{32}P]ATP at 4°C for 2 min, shock frozen, and irradiated at 350 nm. The labeled membranes were treated with 2% sodium taurocholate prior to separation by SDS-PAGE using a 7.5% acrylamide gel containing 5 M urea. The labeling pattern of the parental HL60 membranes was similar to that of the revertant cells with no predominant labeling at 190 kilodaltons (*kDa*) (not shown).

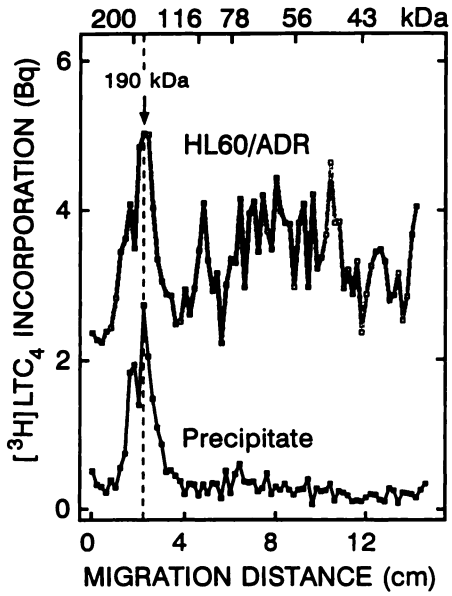

Fig. 4. Immunoprecipitation of MRP after [^{3}H]LTC$_4$ labeling of HL60/ADR membranes. [^{3}H]LTC$_4$-labeled HL60/ADR membranes (400 μg of protein) were lysed and incubated with anti-MRP serum as described in "Materials and Methods." After addition of protein A-Sepharose, the washed immunoprecipitate was subjected to SDS-PAGE (7.5% acrylamide gel containing 5 M urea) and the distribution of [^{3}H]LTC$_4$-derived radioactivity in the gel was determined. Comparison with the [^{3}H]LTC$_4$ labeling pattern in taurocholate-washed HL60/ADR membranes separated on the same gel indicates that the labeled peak of about 190 kilodaltons (*kDa*) consists of [^{3}H]LTC$_4$-labeled MRP. In the supernatant after immunoprecipitation (not shown) the labeled peak at 190 kilodaltons was not detectable.

MDR1 P-glycoprotein (14). The photoaffinity labeling of MRP with [^{3}H]LTC_4 was competed for by a potent competitive inhibitor of ATP-dependent LTC_4 transport, the quinoline-based leukotriene receptor antagonist MK 571 (Fig. 2A). This result corresponds to the inhibition of LTC_4 transport and suppression of the photoaffinity labeling of a 190-kilodalton glycoprotein by MK 571 in membrane vesicles from murine mastocytoma cells (2). The MRP-specific antiserum used in Fig. 4 also served to detect by immunoblotting and immunoprecipitation the 190-kilodalton membrane glycoprotein from murine mastocytoma cells, suggesting the existence of a murine MRP homologue.[4] We have confirmed by photoaffinity labeling with 8-azido-[α-^{32}P]ATP (Fig. 3) that MRP is an ATP-binding membrane glycoprotein (9, 10).

Taken together, our results indicate that MRP mediates the transport of glutathione *S*-conjugates in an ATP-dependent manner. This represents the first direct functional characterization of this member of the ATP-binding cassette superfamily of transmembrane proteins. Furthermore, LTC_4 was identified as an endogenous transport substrate of P190. Previously, kinetic studies on ATP-dependent LTC_4 and glutathione *S*-conjugate transport in various tissues, particularly in liver, indicated that anionic amphiphilic compounds, especially glutathione, glucuronate, and sulfate conjugates, are substrates for this transporter (5, 15, 19). MRP was identified as a component of a non-P-glycoprotein-mediated resistance to anticancer drugs such as anthracyclines, *Vinca* alkaloids, and epipodophyllotoxins in several MDR cell lines including the human leukemia cell line HL60/ADR (7–9) and cell lines derived from both small cell and non-small cell lung tumors (10, 20, 21). Studies with cells transfected with *MRP* complementary DNA demonstrated that MRP overexpression alone is sufficient to confer this multidrug resistance phenotype (11, 22). For some of the above mentioned anticancer drugs, intracellular metabolism and conjugation has been described (23–25). Whether such metabolites are substrates for the 190-kilodalton conjugate export pump is currently under investigation in our laboratories. Oxidation and/or conjugation followed by ATP-dependent export would explain the cross-resistance to compounds of various structures. The pattern of resistance would then be dependent on the pattern of metabolizing and/or conjugating enzymes, as well as on the expression of MRP in different cell types. The mechanism of doxorubicin-induced cytotoxicity appears to be linked at least in part to its enzymatic reductive activation to a semiquinone free radical with subsequent generation of toxic radicals (26). GSH depletion was shown to potentiate the cytotoxicity of doxorubicin in multidrug-resistant human breast tumor cells (27). An increased oxidation of GSH catalyzed by glutathione peroxidase leads to enhanced formation of oxidized glutathione, which is a substrate for the ATP-dependent conjugate export pump (3). This mechanism may explain the resistance but not the decreased accumulation of doxorubicin in MRP-overexpressing cells. Further elucidation of the substrate specificity and the function of MRP should include transport studies with the purified and functionally active reconstituted protein, as well as ATP-dependent transport in membrane vesicles from *MRP*-transfected cells.

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