Cellular and *in Vitro* Transport of Glutathione Conjugates by MRP†

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ABSTRACT: MRP is a recently identified ATP-binding cassette transporter. We previously established that MRP confers resistance to a spectrum of natural product cytotoxic drugs [Kruh, G. D., (1994) *Cancer Res. 54*, 1649−1652], that expression of MRP is associated with enhanced drug efflux [Breuninger, L. M., (1995) *Cancer Res. 55*, 5342−5347], and that MRP transcript is widely expressed in human tissues and solid tumor cell lines [Kruh, G. D., (1995) *J. Natl. Cancer Inst. 87*, 1256−1258]. In the present study the relationship between MRP and drug glutathione S-conjugates was examined. We observed that MRP was labeled by azidophenacylglutathione (APA-SG), a photoaffinity analog of glutathione, and that inside-out membrane vesicles prepared from MRP-overexpressing HL60/ADR cells transported this compound. Transport into membrane vesicles was ATP-dependent, sensitive to osmolarity, and saturable with regard to APA-SG and ATP concentrations, with K_m values of 15 and 61 μM, respectively. APA-SG transport was competitively inhibited by the natural product cytotoxic drugs daunorubicin, vincristine, and etoposide, with K_i values of 4.8, 3.8, and 5.5 μM, respectively. Oxidized glutathione, the drug−glutathione S-conjugate DNP-SG, the LTD_4 antagonist MK571 and arsenate were also competitive inhibitors, with K_i values of 9.0, 23.4, 1.1, and 15.0 μM, respectively. Analysis of the fate of monochlorobimane in MRP transfectants revealed reduced intracellular concentrations of drug-glutathione S-conjugates associated with enhanced efflux and altered intracellular distribution. These results indicate that MRP can transport glutathione conjugates *in vitro* and in living cells and suggest the possibility that the transporter may represent a link between cellular resistance to some classes of cytotoxic drugs and glutathione-mediated mechanisms of resistance. In addition, the observation that both mildly cationic or neutral natural product cytotoxic drugs and anionic compounds such as DNP-SG, MK571, and arsenate are competitive inhibitors of MRP action suggests that the substrate specificity of the transporter is quite broad.

Enzyme systems involved in the detoxification of drugs and xenobiotics can be classified into two major categories. Phase I enzymes, exemplified by the cytochrome P-450 family, typically catalyze oxidoreductase reactions and can often produce active sites on substrates for subsequent phase II metabolism. Phase II enzymes, such as the glutathione S-transferase (GST)[1] family, are typically involved in the addition of more bulky molecules onto drugs. The resulting conjugates are usually less toxic water soluble molecules that are more readily excreted. Although these enzyme systems evolved to protect organisms from environmental toxins, they are also involved in cellular resistance to cytotoxic agents. Of Phase I and phase II enzymes, the most prominent link to cytotoxic drug resistance exists for GSTs (Tew, 1994). Transfection of various GST family cDNAs has been reported to confer resistance to the classical electrophilic alkylating agents chlorambucil, melphalan, and nitrogen mustard (Schecter et al., 1993). Increased resistance to cisplatin and bleomycin has also been observed in GST transfectants (Giaccia et al., 1991; Miyazaki et al., 1990). A potential association between GSTs and resistance to natural product cytotoxic agents has been suggested (Wang & Tew, 1985), based primarily on the observation that many *in vitro* selected multidrug resistant cell lines have increased levels of GST. However, the importance of this mechanism of resistance for natural product drugs is not firmly established (Tew, 1994).

Broad spectrum resistance to natural product drugs, such as anthracyclines, vinca alkaloids, and epipodophyllotoxins is conferred by two members of the ATP cassette superfamily of transporters. P-glycoprotein, the product of the MDR1 gene, decreases intracellular drug concentrations by functioning as an energy dependent efflux pump that extrudes drug from the cell (Endicott & Ling, 1989; Gottesman & Pastan, 1993). Recently, MRP, another ATP cassette superfamily transporter, was isolated from a multidrug resistant cell line (Cole et al., 1992). We and others have reported transfection studies that have established that expression of MRP confers a multidrug resistance phenotype, the drug specificity of which is similar, but not identical, to that of P-glycoprotein (Breuninger et al., 1995; Grant et al., 1994; Kruh et al., 1994; Zaman et al., 1994). In addition, several multidrug resistant

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[1] Abbreviations: APA-SG, azidophenacylglutathione; DNP-SG, dinitrophenacylglutathione; GST, glutathione S-transferase; MCB, monochlorobimane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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cell lines that do not overexpress P-glycoprotein have been shown to overexpress MRP (Barrand et al., 1994; Krishnamachary & Center, 1993; Slovak et al., 1993; Zaman et al., 1993). The detection of altered drug accumulation and/or efflux in MRP transfectants (Breuninger et al., 1995; Grant et al., 1994; Zaman et al., 1994) and MRP-overexpressing drug-selected cell lines (Coley et al., 1991; Marsh et al., 1986; Slovak et al., 1988; Versantvoort et al., 1992) suggests that MRP may decrease intracellular drug concentrations by functioning as an energy dependent efflux pump.

GST-mediated resistance can involve multiple cellular components (Tew, 1994), including appropriate cellular glutathione concentrations maintained by *de novo* synthetic and salvage enzymes, expression of various GST family members, and possibly the action of a plasma membrane transporter(s) that extrudes conjugates from the cell (Ishikawa, 1992). Our interest in the relationship between glutathione conjugation and cytotoxic drug resistance led us to search for potential transporters of glutathione conjugates using APA-SG, a photoaffinity analog of a glutathione conjugate. In the present study we show that MRP is labeled by APA-SG and that enhanced transport of this agent occurs in both MRP transfectants and in membrane vesicles prepared from MRP-overexpressing cells. These studies thus demonstrate the *in vitro* and cellular transport of glutathione S-conjugates by MRP and suggest that a physiological function of the transporter may be to export the phase II metabolic products of drugs and xenobiotics.

EXPERIMENTAL PROCEDURES

Synthesis of Drug−Glutathione Conjugates. [^{35}S]azidophenacylglutathione ([^{35}S]APA-SG) and [^{3}H]APA-SG were prepared as described (Kunst et al., 1989), with modifications. Direct exposure to light was avoided during operations, and steps prior to thin layer chromatography were conducted under nitrogen or with nitrogen-saturated solutions. [^{35}S]GSH (250 μCi) (517.3 Ci/mmol, NEN, Boston, MA) or [^{3}H]GSH (250 μCi) (43.8 Ci/mmol, NEN) was freed of dithiothreitol by ethyl acetate extraction and added to a reaction mixture containing potassium phosphate buffer (50 mM, pH 7.4), 4-azidophenacylbromide (10 mM), glutathione reductase (120 milliunits) and NADPH (1 mM). The reaction was allowed to proceed at room temperature for 1 h, and the products were separated by Silica G thin layer chromatography using 1-propanol/water (7:3, v/v) as developer. Radiolabeled APA-SG was located by autoradiography, scraped off the plate, and extracted with water. After filtration through a 0.2 μm Gelman filter, the extract was concentrated under nitrogen. Dinitrophenacylglutathione was prepared as previously described (Ishikawa, 1989).

Labeling of Membrane Proteins with [^{35}S]APA-SG. Cells were harvested by centrifugation at 1200*g* for 10 min and washed twice in ice-cold phosphate buffered saline. The cell pellet was diluted 10-fold with hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$), supplemented with protease inhibitors (0.1 mM PMSF, 1 mM leupeptin, 0.3 mM aprotinin), and gently stirred on ice for 10 min. The suspension was then homogenized in a chilled Potter-Elvehjem homogenizer using 30 strokes of the pestle. The homogenate was centrifuged at 4000*g* at 4 °C for 10 min to remove nuclei and nonlysed cells, and the resulting supernatant was centrifuged at 100 000*g* for 1 h at 4 °C. The pellet was then resuspended in 10 mM Tris-HCl (pH 7.6)/250 mM sucrose containing protease inhibitors, aliquotted, and stored at −80 °C.

Membranes (100 μg of protein) were incubated in 100 μM AT-125/10 mM Tris-HCl (pH 7.4)/250 mM sucrose for 30 min at 4 °C. For competition studies, membranes were incubated for an additional 30 min at 4 °C after the addition of various concentrations of competitors. The labeling reaction was conducted in a final volume of 100 μL, and initiated by the addition of [^{35}S]APA-SG (3 μCi). After 30 min of incubation at 4 °C in the dark, the samples were quickly photolyzed in a Stratagene UV Stratalinker (200 000 μJ). The labeled proteins were then analyzed by 7% SDS−PAGE (Laemmli, 1970) and autoradiography. MRP monoclonal antibody MRPmAb-1 (S.P. et al., manuscript submitted) was used for immunoblotting.

Preparation of Membrane Vesicles. Membrane vesicles were prepared as described (Ishikawa et al., 1990; Leier et al., 1994b). Briefly, cells were pelleted by centrifugation at 1200*g* for 10 min and washed twice in ice-cold phosphate buffered saline. The cell pellet was diluted 40-fold with hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM EGTA) supplemented with protease inhibitors (0.1 mM PMSF, 1 mM leupeptin, 0.3 mM aprotinin) and gently stirred on ice for 2 h. The lysate was centrifuged at 100 000*g* for 40 min, and the pellet was resuspended in 20 mL of hypotonic buffer and homogenized with a Potter-Elvehjem homogenizer. The resulting homogenate was diluted with incubation buffer (250 mM sucrose/10 mM Tris-HCl, pH 7.4) and centrifuged at 12 000*g* for 10 min. The postnuclear supernatant was stored on ice, and the pellet was resuspended in 20 mL of incubation buffer supplemented with protease inhibitors and homogenized and centrifuged as described above. Both post nuclear supernatants were combined and centrifuged at 100 000*g* for 40 min. The pellets were resuspended in 20 mL of incubation buffer and manually homogenized with a tight fitting Dounce homogenizer. After dilution with 10 mL of incubation buffer, the suspension was layered onto 38% sucrose in 5 mM Hepes/KOH, pH 7.4, and centrifuged at 280 000*g* for 2 h in a Beckman SW41 swinging bucket rotor. The interphases were collected, diluted in 20 mL of incubation buffer, and homogenized with a Dounce homogenizer. The resulting suspension was centrifuged at 100 000*g* for 40 min, and the pellet was resuspended in 1 mL of incubation buffer. All operations were carried out at 4 °C.

Membrane vesicles were formed by passing the final membrane suspension through a 27 gauge needle 20 times. Ten milliliters of the vesicle suspension was then mixed with 2.5 mL of Con-A Sepharose equilibrated with 160 mM Tris-HCl (pH 7.4) and incubated at 4 °C for 30 min with gentle shaking. The Con-A Sepharose was removed by centrifugation at 1400*g* for 5 min, and inside-out membrane vesicles in the supernatant were collected by centrifugation at 100 000*g* for 30 min. The pellet was suspended in incubation buffer and stored at −80 °C. Contamination by mitochondria and endoplasmic reticulum was determined by analysis of marker enzyme activities as described (Leier et al., 1994b).

Analysis of APA-SG Uptake by Membrane Vesicles. Uptake of radiolabeled APA-SG by membrane vesicles was measured by the rapid filtration technique (Leier et al., 1994b). Inverted membrane vesicles were quickly thawed at 37 °C shortly before use and kept on ice. Radiolabeled

APA-SG was mixed with ATP reaction buffer (10 mM Tris-Cl, pH 7.5/4 mM ATP/10 mM $MgCl_2$/10 mM creatine phosphate/0.25 M sucrose/100 μg/mL Creatine kinase), and membrane vesicles (20 μg protein) were added. The suspension was incubated at 37°C and 20 μl aliquots were removed at various time points and diluted in 1.0 mL of ice-cold reaction buffer. The suspensions were applied to 0.45 μm Millipore nitrocellulose filters (presoaked in ATP reaction buffer) under suction and washed twice with 5.0 mL of ice-cold ATP incubation buffer. The filters were dissolved in scintillation fluid, and the radioactivity was assayed in a liquid scintillation counter. In parallel control experiments ATP was replaced with 4 mM 5′-AMP, and ATP-dependent transport was calculated by subtracting the uptake values obtained with 5′-AMP from those obtained with ATP. For Lineweaver−Burk analyses and sucrose inhibition experiments, initial reaction rates were measured at 1 min. All experiments were performed in triplicate and in the absence of photolysis procedures required in the membrane protein labeling experiments.

Analysis of MCB Fluorescence in MRP Transfectants. MRP transfectant pSRα-MRP-16 and control transfectant pSRα (Breuninger et al., 1995) were grown in DMEM on sterile glass coverslips until they were approximately 40% confluent. Cells were incubated for 15 min at room temperature with 70 μM monochlorobimane (Molecular Probes, Eugene, OR) and then rinsed three times with PBS. Fresh media was added, and the coverslips were placed in a 37 °C incubator. For quantitative efflux experiments, coverslips were removed from the incubator at 4 min intervals, rinsed in PBS, and imaged using a Hamamatsu C2400 Newvicon camera attached to a Nikon Optiphot-2 fluorescence microscope. The light source intensity and camera settings were kept constant for all experiments. Cells (25−30) were imaged per time point per experiment. The average pixel intensity of the cells was measured using the "Histogram" function of CoMOS 6.05 (Bio-Rad).

For time course analysis of the intracellular distribution of MCB fluorescence, cells were grown on coverslips and incubated with 60 μM MCB as described above. After being rinsed with PBS, the coverslips were placed cell-side-down on spacers fastened to a glass slide. Hepes-buffered DMEM was injected under the coverslip with a gel-loading tip, and the coverslip edges were sealed with vacuum grease. The cells were maintained at 37 °C using an air blanket (Laboratory Products, Boston, MA), and representative images were rendered at 15 min intervals using Voxel View Ultra 2.01 (Vital Images).

RESULTS

Photoaffinity Labeling of Membrane Proteins with [^{35}S]-APA-SG. To identify candidate proteins that bind glutathione S-conjugates, we utilized [^{35}S]APA-SG, a photoaffinity analog of glutathione. When membrane proteins were labeled with this reagent, a protein whose size was similar to that of 190 kDa MRP protein was consistently detected. The possibility that MRP was specifically labeled by [^{35}S]-APA-SG was further explored by using membranes prepared from the MRP-overexpressing cell line HL60/ADR (Marsh et al., 1986) or control HL60 cells in photoaffinity-labeling experiments. As shown in Figure 1, a 190 kDa photoaffinity product was readily detected at higher levels in HL60/ADR cells than in control HL60 cells, suggesting that MRP was the labeled protein. When the same filter was immunoblotted with an MRP monoclonal antibody, MRP was detected in HL60/ADR in a similar position as the photoaffinity-labeled protein. (The sensitivity of MRP detection by immunoblotting is substantially reduced by prior photoaffinity labeling.) MRP was confirmed as the labeled protein by detecting MRP in immunoprecipitation experiments of photoaffinity-labeled protein using the MRP antibody (data not shown). The pharmacological specificity of the interaction of MRP with [^{35}S]APA-SG was analyzed by labeling HL60/ADR membranes in the presence of excess molar concentrations of various agents. As shown in Figure 2, labeling of MRP was competed by oxidized glutathione and the glutathione conjugates DNP-SG and S-hexyl-SG. Competition was also observed for unlabeled APA-SG, reduced glutathione (although assay conditions would encourage some oxidation), the glutathione conjugate of ethacrynic acid, and the lipophilic cytotoxic agents etoposide, vinblastine, and doxorubicin (data not shown).

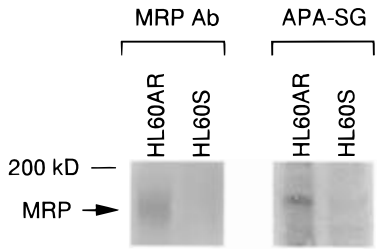

FIGURE 1: Photoaffinity labeling of MRP with [^{35}S]APA-SG. Membranes prepared from HL60/ADR and HL60 cells were photoaffinity labeled with [^{35}S]APA-SG, and the reaction products were separated by SDS−PAGE and transferred to Immobilon-p membranes. The membrane was immunoblotted with MRP monoclonal antibody MRPmAb-1 using ECL detection (left) and subsequently exposed to X-ray film for autoradiographic detection of [^{35}S]APA-SG binding (right).

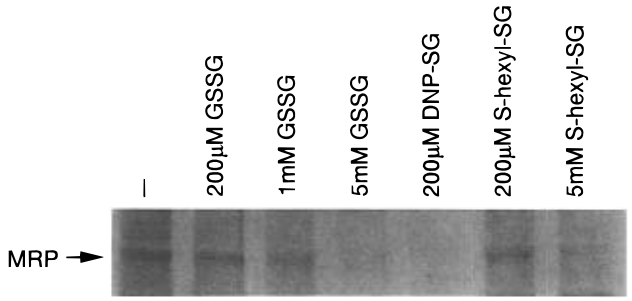

FIGURE 2: Competition of [35S]APA-SG photoaffinity labeling of MRP by drug-glutathione conjugates. Labeling experiments were performed in the presence of 0.2, 1, or 5 mM oxidized glutathione (GSSG), 0.2 mM DNP-SG, and 0.2 or 5 mM S-hexyl-SG, as described in the Experimental Procedures.

ATP-Dependent Uptake of APA-SG by Inside-Out Membrane Vesicles Prepared from HL60/ADR Cells. The labeling studies suggested that MRP might function as a transporter of APA-SG. To examine this possibility directly, APA-SG transport by inside-out membrane vesicles prepared from HL60/ADR and HL60 cells was analyzed. Figure 3A shows a time course of APA-SG uptake over 3 min. To distinguish between ATP-dependent transport of APA-SG into membrane vesicles and nonspecific binding, vesicle associated radioactivity was measured in the presence of AMP or ATP. As has been previously reported for vesicle transport studies (Horio et al., 1988; Kamimoto et al., 1989;

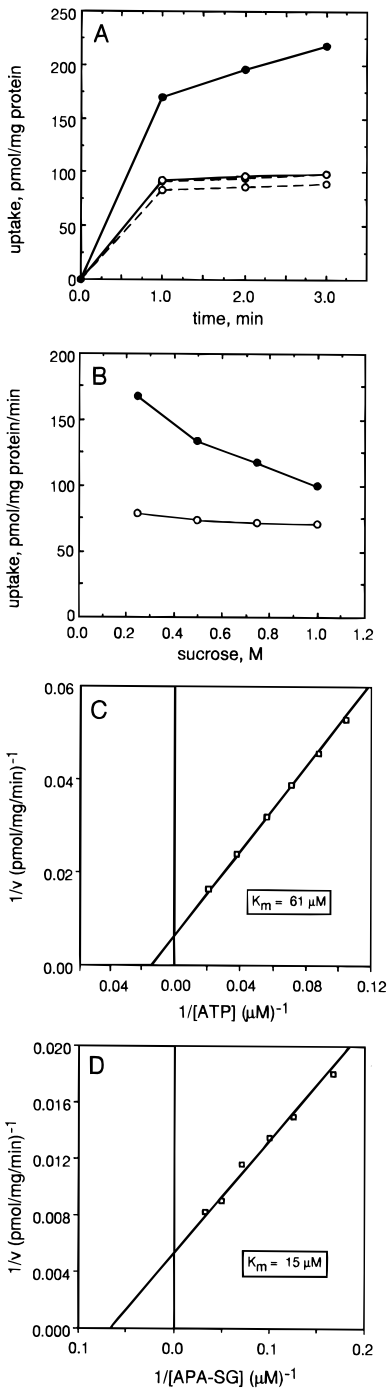

FIGURE 3: ATP-dependent uptake of APA-SG by inside-out HL60/ADR membrane vesicles. (A) Time course of [^{35}S]APA-SG uptake by inside-out membrane vesicles prepared from HL60 (broken lines) or HL60/ADR cells (solid lines). Uptake was measured in the presence of ATP (closed circles) or AMP (open circles). (B) osmotic sensitivity of [^{3}H]APA-SG uptake by HL60/ADR membrane vesicles. Uptake was measured in the presence of ATP (solid circles) or AMP (open circles) (C) Lineweaver−Burk plot of the ATP concentration dependence of [^{3}H]APA-SG uptake by HL60/ADR membrane vesicles. (D) Lineweaver−Burk plot of the [^{3}H]APA-SG concentration dependence of uptake by HL60/ADR membrane vesicles. The concentration of ATP (and AMP controls) was 1.2 mM in A, B, and D, and 9.5−47.5 μM in C. The concentration of [^{35}S]APA-SG was 10 μM in A and B, and the concentration of [^{3}H]APA-SG was 10 μM in C and 6−30 μM in D. The K_m of ATP was 61 μM (C), and the K_m and V_{max} of APA-SG were 15 μM and 180 pmol/(mg·min), respectively (D). Initial reaction rates were measured at 1 min for B−D. Data points represent the average of triplicate determinations.

Lelong et al., 1992), substantial nonspecific binding of APA-SG was detected (AMP controls). Comparable levels of nonspecific binding were detected for the HL60/ADR and control vesicles. However, in the presence of ATP, substantial uptake was observed for the HL60/ADR vesicles but not for the control vesicles. When nonspecific binding was subtracted, the HL60/ADR vesicles displayed ~14-fold greater uptake than the control vesicles at 3 min. To confirm that the observed APA-SG uptake was the result of transport into membrane vesicles, the osmotic sensitivity and ATP concentration dependence of uptake were analyzed. A major biochemical hallmark of transport into membrane vesicles, as opposed to nonspecific binding, is inhibition at high osmotic pressures (Horio et al., 1988; Kamimoto et al., 1989; Lelong et al., 1992). High osmotic pressure reduces the intravesicular space by shrinking the membrane vesicles, the result of which is reduced uptake capacity. Osmotic sensitivity was examined by analyzing ATP-dependent APA-SG uptake by vesicles incubated in various concentrations of sucrose. As expected for transport into membrane vesicles, ATP-dependent uptake decreased as sucrose concentrations were increased above the standard assay conditions of 0.25 M, whereas nonspecific binding in the presence of AMP was unchanged (Figure 3B). At 1.0 M sucrose, vesicle associated APA-SG in the presence of ATP approached the AMP values. Transport into membrane vesicles should increase with increasing concentrations of ATP in the incubation mixture if uptake is ATP-dependent. As shown in Figure 3C, APA-SG uptake was saturable with regard to ATP-concentration, with a K_m of 61 μM. Analysis of the APA-SG concentration dependence of uptake indicated that transport was saturable with respect to APA-SG concentration, with a K_m of 16.3 ± 3.5 μM (average of five independent determinations) (Figure 3D).

Inhibition of APA-SG Transport by Diverse Compounds. The effect of a variety of structurally distinct agents on APA-SG transport was analyzed. As shown in Figure 4, the natural product cytotoxic drugs daunorubicin, vincristine and etoposide inhibited APA-SG uptake. Increases in the apparent K_m of APA-SG with no alteration in V_{max} indicated that these agents were competitive inhibitors, with K_i values of 4.8, 3.8, and 5.5 μM, respectively. Both GSSG and DNP-SG were competitive inhibitors of transport, with K_i values of 9.0 and 23.4 μM, respectively. Competition by these agents was consistent with our observation that they also competed APA-SG labeling of MRP. The LTD_4 receptor antagonist MK571 (Jones et al., 1989) has been reported to modulate MRP-conferred natural product resistance (Gekeler et al., 1995), and MRP has been reported to confer resistance to arsenate (Cole et al., 1994). Both of these compounds were also competitive inhibitors of transport, with K_i values of 1.1 and 15.0 μM, respectively.

Analysis of Monochlorobimane Efflux in MRP Transfectants. To determine if MRP confers enhanced cellular efflux of drug-GS conjugates, the fate of monochlorobimane (MCB), an agent for which glutathione conjugation is required for activation of fluorescence, was analyzed in MRP-overexpressing cells. NIH3T3 cells transfected with an MRP expression vector were used for this analysis since cell lines obtained by step-wise selection in drug, such as HL60/ADR, often harbor collateral mechanisms of resistance, and the adherent morphology of fibroblasts facilitates the examination of intracellular fluorescence. MRP transfectant

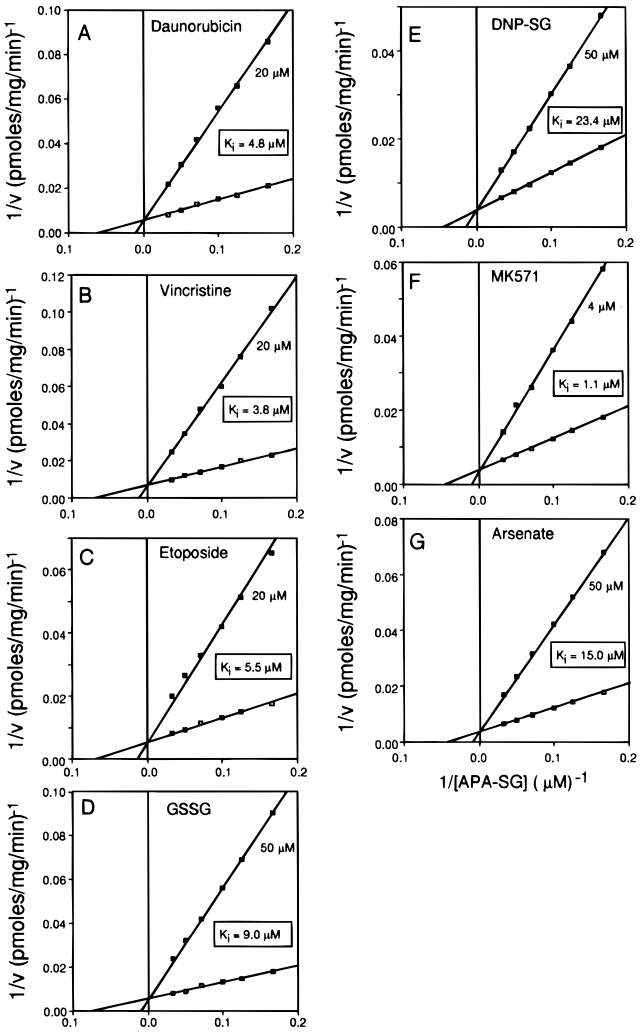

FIGURE 4: Inhibition of APA-SG transport by diverse compounds. Lineweaver−Burk plots of the inhibition of [^{3}H]APA-SG transport by (A) 20 μM daunorubicin, (B) 20 μM vincristine, (C) 20 μM etoposide, (D) 50 μM GSSG, (E) 50 μM DNP-SG, (F) 4 μM MK571, and (G) 50 μM arsenate. Uptake in the absence or presence of inhibitors is indicated by open and closed squares, respectively. The concentrations of ATP was 1.2 mM, and the concentration of [^{3}H]APA-SG was 6 μM−30 μM. Initial reactions rates were measured at 1 min. Data points represent the average of triplicate determinations.

pSRα-MRP-16, which overexpresses the MRP protein (Breuninger et al., 1995), and control transfectant pSRα-7M were grown in the presence of 60 μM MCB for 15 min and examined by fluorescence microscopy at various time points after the cells were washed and the media replaced with drug free media. Figure 5 shows representative images obtained at 15 min intervals. Immediately after incubation with MCB, the MRP and control transfectant appeared to have roughly comparable levels of fluorescence. However, at subsequent time points the MRP transfectant exhibited markedly reduced fluorescence compared to the control cells. In addition, the MRP transfectant exhibited alterations in the intracellular distribution of the conjugate characterized by the development of punctate fluorescence which was initially observed in perinuclear locations and later appeared to be scattered throughout the cytoplasm. The high fluorescence intensities of the control cells were the consequence of the high concentration of MCB required for visualization of intracellular detail in the MRP transfectant. Efflux of the conjugate was quantitated by analyzing fluorescence intensity. As

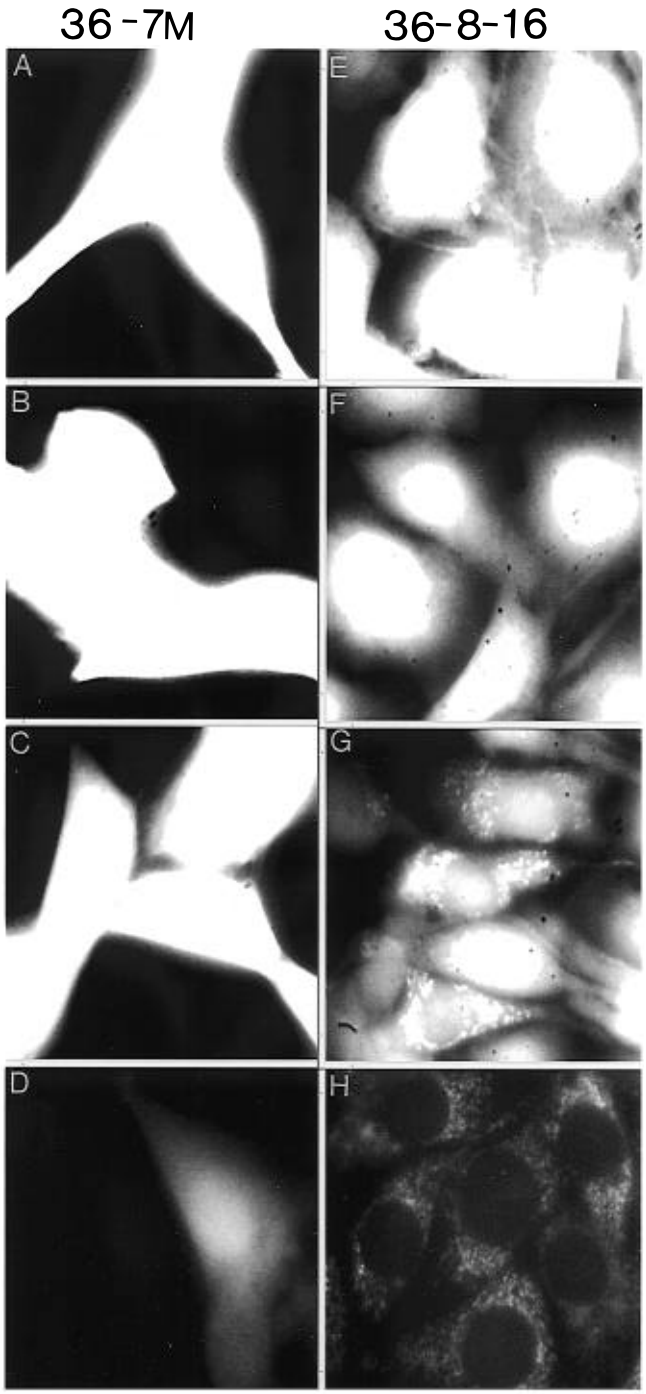

FIGURE 5: Intracellular distribution of bimane−glutathione fluorescence in MRP transfectants. Control NIH3T3 transfectant pSRα-7M (A−D) and MRP transfectant pSRα-MRP-16 (E−H) were grown in the presence of 60 μM monochlorobimane for 15 min and examined by fluorescence microscopy at 0 min (A, E), 15 (B, F), 30 (C, G), and 45 min (D, H) after the medium was replaced with drug-free medium.

shown in Figure 6, increased MCB efflux in the MRP transfectant was evident at 8 min after removal of MCB-containing media. At 16 min the fluorescence intensity of the MRP transfectant was 31% of its initial level, whereas the control cells were unchanged.

DISCUSSION

In the present study we demonstrate that MRP is specifically labeled by the glutathione photoaffinity analog APA-SG, and that this agent is transported in an energy-dependent

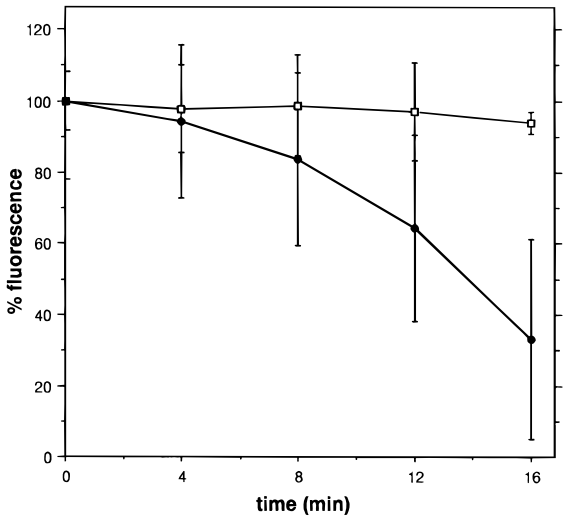

FIGURE 6: Analysis of bimane−glutathione efflux in MRP transfectants. MRP transfectant pSRα-MRP-16 (closed circles) and control transfectant pSRα-7M (open squares) were grown in the presence of 70 μM monochlorobimane for 15 min and examined by fluorescence microscopy at various time points after the medium was replaced with drug-free medium. The fluorescent intensities of 25−30 cells were quantitated per time point.

fashion by membrane vesicles prepared from MRP-overexpressing HL60/ADR cells. These results indicate that MRP is a glutathione S-conjugate transporter and are consistent with reports that MRP can transport *in vitro* the endogenous glutathione conjugated leukotriene LTC_4 and DNP-SG (Jedlitschky et al., 1994; Leier et al., 1994a; Muller et al., 1994). In addition, the analysis of MCB cellular kinetics described in the present study extends the previous *in vitro* analyses by providing the first direct evidence that MRP confers enhanced cellular efflux of glutathione conjugates, thus establishing MRP as a physiological glutathione S-conjugate transporter. Whether the primary physiological function of MRP is in the transport of endogenous glutathione S-conjugates, such as cysteinyl leukotrienes, or in the efflux of exogenous toxins remains to be determined.

In addition to increased cellular efflux of glutathione conjugates, the analysis of the fate of MCB in MRP transfectants revealed an altered pattern of intracellular glutathione conjugate distribution characterized by the initial accumulation of glutathione conjugates in perinuclear structure(s) followed by the development of a scattered punctate pattern, possibly suggestive of a process of vesicle transport. Both increased efflux and altered intracellular distribution are consistent with the subcellular location of MRP, which was detected in both the plasma membrane and cytoplasmic structure(s) by immunofluorescence and subcellular fractionation studies (Flens et al., 1994; Krishnamachary & Center, 1993; Slovak et al., 1993). MRP in the plasma membrane could directly pump glutathione S-conjugates out of the cell, resulting in the reduced intracellular glutathione S-conjugate concentrations we observed, and the cytoplasmic membrane associated MRP could account for the perinuclear accumulation and subsequent scattered pattern. We observed a similar pattern of increased efflux and altered intracellular drug distribution in studies of the fate of daunorubicin in MRP transfectants (Breuninger et al., 1995), and similar observations have been made in drug-selected MRP-overexpressing cell lines (Barrand et al., 1993; Coley et al., 1993; Marquardt & Center, 1992). The possibility that MRP mediated sequestration confers a protective effect, either by shielding cellular targets and/or contributing to drug efflux by a process of vesicle transport is intriguing and warrants further study.

Although this study indicates that the substrate specificity of MRP includes drug−glutathione S-conjugates and that this activity has *in vivo* consequences in terms of reducing intracellular levels of conjugates, the relationship between these findings and cellular resistance to natural product drugs is less clear. Two considerations support the idea that the extrusion of glutathione S-conjugates may not be the predominant mechanism of MRP mediated multidrug resistance: (1) transfection experiments have shown that MRP confers resistance to lipophilic cytotoxic drugs, but increased resistance to alkylating agents has not been observed (Breuninger et al., 1995, Cole et al., 1994); and (2) convincing evidence of GST-mediated catalysis exists for alkylating agents but not for the natural product drugs that constitute the MRP multidrug resistance phenotype (Tew, 1994). These considerations and our observation that daunorubicin, etoposide, and vincristine are competitive inhibitors of *in vitro* transport of APA-SG by MRP suggest that the direct transport of unaltered natural product drugs may be an important component of MRP-conferred multidrug resistance. Consistent with this idea, we have found that membrane vesicles prepared from MRP-overexpressing cells transport natural product cytotoxic drugs.[2] The possibility that anionic oxidative metabolites of natural product drugs are also substrates of MRP cannot be dismissed, although the significance of such metabolites has not been established.

In view of our observation that MRP confers increased cellular efflux of glutathione conjugates, and the substantial evidence that alkylating agents are conjugated to glutathione in the cell (Bolton et al., 1993; Tew, 1994), the absence of increased resistance to alkylating agents in MRP transfectants warrants further consideration. One possibility is that MRP can transport the conjugates of these agents but that expression of MRP in transfectants is not sufficient by itself to confer resistance. A potential explanation for this is that glutathione-mediated resistance requires the participation of a variety of cellular components that generate glutathione and catalyze its conjugation to drugs, in addition to the action of a transporter to eliminate the product from the cell. Thus, overexpression of MRP may not be sufficient to confer increased resistance in the absence of increases in the expression levels of other involved proteins. If this hypothesis is correct, then in the clinical setting, in which simultaneous elevations in the levels of several proteins can occur, increased expression of MRP might indeed contribute to alkylating agent resistance. Expression of MRP might then confer a broad form of cytotoxic drug resistance involving both natural products and alkylating agents, the scope of which could exceed the ordinary definition of "multidrug resistance". Although this possibility is speculative, the clinical implications are indeed formidable, since many chemotherapy regimens are designed to circumvent cellular resistance mechanisms by combining both natural product and alkylating agents.

[2] S.P., L.M.B, K.D.T., H.S., and G.D.K., manuscript submitted.

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