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Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein

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ABSTRACT **Multidrug-resistance-associated protein (MRP) is a plasma membrane glycoprotein that can confer multidrug resistance (MDR) by lowering intracellular drug concentration. Here we demonstrate that depletion of intracellular glutathione by DL-buthionine (*S,R*)-sulfoximine results in a complete reversal of resistance to doxorubicin, daunorubicin, vincristine, and VP-16 in lung carcinoma cells transfected with a *MRP* cDNA expression vector. Glutathione depletion had less effect on MDR in cells transfected with *MDR1* cDNA encoding P-glycoprotein and did not increase the passive uptake of daunorubicin by cells, indicating that the decrease of MRP-mediated MDR was not due to nonspecific membrane damage. Glutathione depletion resulted in a decreased efflux of daunorubicin from *MRP*-transfected cells, but not from *MDR1*-transfected cells, suggesting that glutathione is specifically required for the export of drugs from cells by MRP. We also show that MRP increases the export of glutathione from the cell and this increased export is further elevated in the presence of arsenite. Our results support the hypothesis that MRP functions as a glutathione *S*-conjugate carrier.**

Resistance of cancer cells to natural product drugs is often due to multidrug resistance (MDR). A major form of MDR in human tumors is caused by overexpression of the *MDR1* gene (standard gene symbol, *PGY1*) (1). *MDR1* encodes a large plasma membrane glycoprotein, P-glycoprotein (Pgp) (2), that causes MDR by increased export of drugs from the cell resulting in a decreased intracellular drug concentration (1). Cole *et al.* (3) discovered another membrane transporter gene that can confer MDR (4–6), the MDR-associated protein (MRP) gene. Like Pgp, MRP seems to work as a drug-efflux pump (6). It is mainly present in the plasma membrane of resistant cells (6–8) and is able to decrease cellular drug levels against a concentration gradient (4, 6). However, recent work has also indicated interesting differences between MRP and Pgp. Increased cellular MRP levels are associated with increased reduced glutathione (GSH) *S*-conjugate carrier (GS-X pump) activity in isolated plasma membrane vesicles (9–11). This suggests that MRP is a GS-X pump (12) present in many, if not all, mammalian cells (9–13). These pumps transport substrates containing a large hydrophobic moiety and at least two negative charges (12, 13), as present in drug GSH *S*-conjugates. Moreover, recent studies indicate that GS-X pumps are also involved in the export of cisplatin (14–16) and arsenite (11). Indeed, some cell lines overexpressing *MRP* are moderately resistant to arsenite (4, 11).

These results link MRP to older experiments in which resistance to anthracyclines was found to correlate with increased levels of cellular GSH, GSH synthesis, or GSH *S*-transferases (17–19). This link is supported by the strong decrease in drug resistance in two MDR lung carcinoma cell lines that overexpress *MRP* by DL-buthionine (*S,R*)-sulfoximine (BSO) (20–22), an inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step in GSH synthesis (23). The interpretation of these inhibitor experiments is not unambiguous, however. In both cell lines, other resistance mechanisms (e.g., alterations in topoisomerase II) contribute to resistance, and it is not clear whether the GSH depletion in these cells does not result in membrane damage—e.g., by lipid peroxidation. Damage of the plasma membrane could increase drug influx and, hence, decrease resistance. To test whether GSH is specifically required for MDR caused by MRP but not by Pgp, we have analyzed the effects of BSO treatment on lung cancer cells transfected with an expression vector containing either *MRP* cDNA or *MDR1* cDNA.

MATERIALS AND METHODS

Cell Lines. S1(MRP) was obtained after transfection of non-small cell lung cancer SW-1573/S1 cells with an expression vector containing *MRP* cDNA and a neomycin-resistance marker gene (pRc/RSV-MRP), followed by selection with geneticin (G418) (6). S1(MDR1) was previously named S1(1.1) (24) and was obtained after transfection of S1 cells with the expression vector pJ3Ω (25) containing *MDR1* cDNA, followed by selection with 10 nM vincristine. GLC4/ADR is a *MRP*-overexpressing subline of the non-small cell lung cancer cell line GLC4 and was obtained by selection with doxorubicin (20, 21).

Clonogenic Survival Assay. In six-well dishes, 400 cells per well were seeded and incubated in medium with or without 25 μM BSO for 24 hr prior to incubation with increasing concentrations of drug. After 1 hr, drug was removed, the wells were rinsed with phosphate-buffered saline, and drug-free medium without BSO was added. Seven days after the start of the experiment, the percentage of cells that were able to produce a colony of >50 cells was used as a measure of cell survival. Cellular uptake and efflux of daunorubicin were measured as described (6).

Assay for Glutathione. Cells ($1–2 \times 10^6$) in a 30-mm Petri dish were washed with phosphate-buffered saline and scraped in 10% perchloric acid. Precipitated protein was removed by centrifugation and the supernatant was neutralized by adding 0.4 vol of 0.5 M Mops/5 M KOH. The concentration of total glutathione [GSH and glutathione disulfide (GSSG)] was determined according to the recycling method of Tietze (26). After arsenite exposure, only part of the GSH apparently lost

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Abbreviations: BSO, DL-buthionine (*S,R*)-sulfoximine; GSH, reduced glutathione; GSSG, glutathione disulfide; GS-X pump, GSH *S*-conjugate export carrier; MDR, multidrug resistance; MRP, MDR-associated protein; Pgp, P-glycoprotein.

by the cells was recovered in the medium. Probably part of it was bound to medium components or to arsenic or was metabolized within the cell. To measure GSSG, GSH was derivatized with *N*-ethylmaleimide before the sample was applied to the recycling reaction (27).

Arsenite-Induced Efflux of Thiol Groups. In 25-cm^2 tissue culture flasks 1–2 × 10^6 cells were cultured for 4 hr in cysteine-free RPMI 1640 medium containing 10% fetal calf serum and 37 kBq of [^{35}S]cysteine per ml (>37 TBq/mmol) (Amersham). After rinsing with phosphate-buffered saline, the cells were incubated for 10 min in nonradioactive Dulbecco's modified Eagle's medium (DMEM) to remove free radiolabel. The medium was replaced by 5 ml of DMEM plus serum containing sodium arsenite. At intervals of 10 min, 200-μl samples were taken and radioactivity was determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of the culture medium.

HPLC Analysis of Nonprotein Thiol Groups. Culture medium (500 μl) was spiked with 100 μg of unlabeled cysteine, GSH, and GSSG and extracted with an equal volume of 5% (wt/vol) 5-sulfosalicylic acid. Twenty microliters of the acid-soluble fraction was analyzed by a HPLC system equipped with a ChromSpher C_{18} column (Chrompack, Middelburg, The Netherlands). The mobile phase was delivered by a model 480c ternary gradient pump at a flow rate of 0.4 ml/min. Solvent A was 20 mM phosphate buffer (pH 2.0); solvent B was acetonitrile; and solvent C was 10 mM sodium octyl sulfate. Initial conditions were 60% A and 40% C. After injection, a 10-min linear gradient was started to 40% A and 20% B and 40% C, which was maintained for another 5 min. The system was reequilibrated to initial conditions for 10 min before each new injection. Unlabeled compounds were detected at 215 nm with a UV detector. Column effluent was collected at 1-min intervals and the radioactivity in the samples was determined by liquid scintillation counting.

RESULTS

Role of GSH in MDR Caused by MRP. To test the effect of GSH depletion on drug resistance caused by MRP, we used a human lung carcinoma cell line stably transfected with a *MRP* cDNA construct (6). SW-1573/S1 has a low level of MRP (Fig. 1). This level is 15-fold higher in the *MRP* transfectant S1(MRP). As a control, we used S1 cells transfected with an *MDR1* cDNA construct. S1 cells contain a low amount of *MDR1* mRNA (24), but Pgp is not detectable by antibody; the level in S1(MDR1) is readily visible on the blot in Fig. 1. Transfection of S1 cells with *MRP* cDNA has no effect on *MDR1* gene expression and transfection with *MDR1* cDNA has no effect on MRP (Fig. 1). S1(MRP) is resistant to various anthracyclines (doxorubicin, daunorubicin, idarubicin, epirubicin), *Vinca* alkaloids (vincristine, vinblastine), and VP-16 but not to taxol, bisanthrene, and gramicidin D, to which S1(MDR1) cells are resistant. Unlike some other cell lines that overexpress *MRP* (4, 11), S1(MRP) cells are not resistant to arsenite.

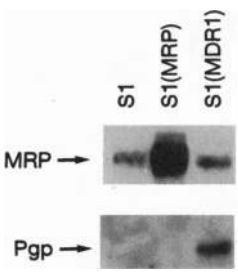

FIG. 1. Immunoblot analysis of MRP and Pgp in cell lysates of drug-sensitive SW-1573/S1, *MRP*-overexpressing S1(MRP), and *MDR1* Pgp-overexpressing S1(MDR1) cells. Total cellular proteins (15 μg per lane) were size fractionated in an SDS/7.5% polyacrylamide gel and transferred to nitrocellulose by electroblotting. MRP was detected with monoclonal antibody MRPr1 (7); Pgp was detected with C219 (Centocor). Binding of antibodies was visualized by enhanced chemiluminescence (Amersham).

Table 1. Intracellular GSH content of lung cancer cell lines at early logarithmic growth phase after exposure to BSO (25 μM; 24 hr) or sodium arsenite (100 μM; 1 hr)

Cell line	GSH, nmol per 10^6 cells		
	Control	BSO	Arsenite
S1	14.9 ± 0.7*	2.3 ± 0.1†	10.9 ± 2.3*
S1(MRP)	14.3 ± 0.8*	1.8 ± 0.6†	3.8 ± 1.8*
S1(MDR1)	15.1 ± 1.1*	2.7 ± 0.4†	10.7 ± 2.2*
GLC4	4.3 ± 0.6†	ND	5.1‡
GLC4/ADR	8.2 ± 1.6†	ND	7.6‡

ND, not determined.
*Mean ± SD of five independent experiments.
†Mean ± SD of three independent experiments.
‡Mean of two experiments.

The GSH contents of the parental drug-sensitive line S1 and the two transfectants were similar and varied from 7 to 15 nmol per 10^6 cells (Table 1), depending on whether the cells were in late or early exponential growth phase. However, S1(MRP) cells secreted 2 times more GSH than S1(MDR1) and S1 cells (Table 2). Exposure to 25 μM BSO for 24 hr reduced the GSH levels in the SW-1573 cell lines by 82–87% (Table 1), in agreement with experiments by Mans *et al.* (28). In clonogenic assays, the fraction of surviving colonies was decreased by 15%, indicating that BSO had little toxic effect. The effect of BSO pretreatment on MDR was determined in clonogenic assays, in which the cells were exposed to drug for 1 hr. BSO decreased the resistance of S1(MRP) against doxorubicin, daunorubicin, VP-16, and vincristine 4- to 7-fold (Fig. 2). BSO also increased the toxicity of doxorubicin, daunorubicin, and VP-16 in S1 and S1(MDR1), but to a lesser extent than in S1(MRP) (i.e., ≈2-fold), and it had no effect at all on vincristine resistance in S1 or S1(MDR1) (Fig. 2) or on taxol resistance in either of the three cell lines (data not shown). These results show that BSO pretreatment is able to completely reverse MDR and that complete reversal is specific for MRP-mediated MDR.

We have previously shown that MRP causes drug resistance by lowering intracellular drug concentration via increased drug efflux (6). Hence, reversal of resistance by GSH depletion might be due either to an increased influx or to a decreased efflux of drug. Experiments that distinguish between these alternatives are presented in Figs. 3 and 4. We examined drug

Table 2. Release of GSH from lung cancer cell lines after incubation for 1 hr with or without sodium arsenite (100 μM) or daunorubicin (10 μM)

Cell line	GSH, nmol per 10^6 cells per hr		
	Control	Arsenite	Daunorubicin
S1	0.14 ± 0.04*	0.81 ± 0.45*	0.16†
S1(MRP)	0.29 ± 0.08*‡	2.91 ± 1.44*§	0.30†
S1(MDR1)	0.10 ± 0.03*	0.86 ± 0.41*	ND
GLC4	0.04 ± 0.00¶	0.09 ± 0.03¶	0.05 ± 0.01¶
GLC4/ADR	0.58 ± 0.16‡¶	2.47 ± 0.25‡¶	0.73 ± 0.21¶

ND, not determined.
*Mean ± SD of five independent experiments.
†Mean of two experiments.
‡Statistically significant difference from value for parental cell line (S1 or GLC4) ($P < 0.01$) according to unpaired Student's *t* test.
§Statistically significant difference from value for parental cell line (S1 or GLC4) ($P < 0.05$) according to unpaired Student's *t* test.
¶Mean ± SD of three independent experiments.

influx by measuring the initial uptake of daunorubicin into untreated cells and cells treated with BSO (Fig. 3). The decrease in fluorescence represents mainly quenching of daunorubicin by its binding to DNA (6). The initial decrease of fluorescence, as determined from the slope of the curve just after addition of drug, was the same for all three cell types and was not affected by BSO. This is indicative of identical passive influx of daunorubicin. At later time points, the accumulation of daunorubicin was lower in S1(MRP) or S1(MDR1) cells than in S1 cells. This reflects the expected accumulation defect due to the presence of an efflux pump (cf. ref. 6), as also shown by the effects of vincristine or digitonin addition. Vincristine is thought to increase daunorubicin accumulation by competing for a common efflux mechanism (29); digitonin permeabilizes the plasma membrane, abolishing the drug gradient set up by active drug efflux (6, 30). BSO pretreatment abolished the daunorubicin accumulation defect in S1(MRP) but not in S1(MDR1). That this is due to a specific effect on drug efflux is illustrated in Fig. 4. The efflux of daunorubicin was faster from S1(MRP) and S1(MDR1) than from S1 cells. BSO abolished the increased efflux of drug from S1(MRP) cells but had no effect on the efflux from S1(MDR1) cells (Fig. 4). We conclude that GSH is required for the export of daunorubicin from cells by MRP but not by Pgp.

In the toxicity experiments presented in Fig. 2, BSO pretreatment also moderately increased the toxicity of anthracyclines and VP-16 in parental S1 cells and in S1(MDR1) cells. This is not due to an increase in drug influx (Fig. 3) or to a decrease in efflux (Fig. 4). It is also probably not due to an effect on the main target of these drugs, topoisomerase II, as BSO did not influence the sensitivity of any of the three cell lines to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (data not shown). The effect remains unexplained at present, but it may be due to a decreased ability of cells with reduced GSH levels to deal with free radicals produced by anthracyclines and VP-16 (31, 32).

Arsenite-Induced Release of Glutathione from Cells Mediated by MRP. The simplest interpretation of the reversal of MRP-mediated drug resistance by BSO pretreatment is that MRP transports GSH *S*-conjugates of drugs. To examine whether stable conjugates of daunorubicin or vincristine exist,

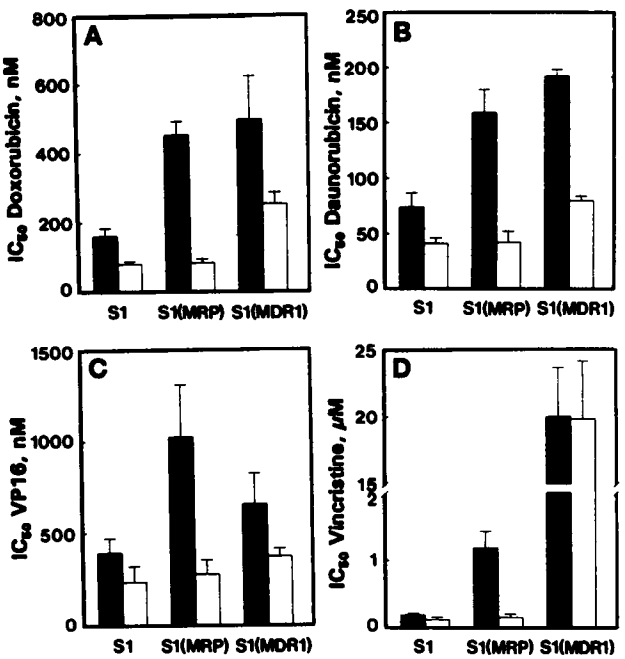

FIG. 2. Effect of BSO on cytotoxicity of doxorubicin (*A*), daunorubicin (*B*), VP-16 (*C*), and vincristine (*D*) in drug-sensitive and MDR SW-1573 cell lines. Solid bars, IC_{50} of untreated cells; open bars, IC_{50} of cells pretreated with BSO.

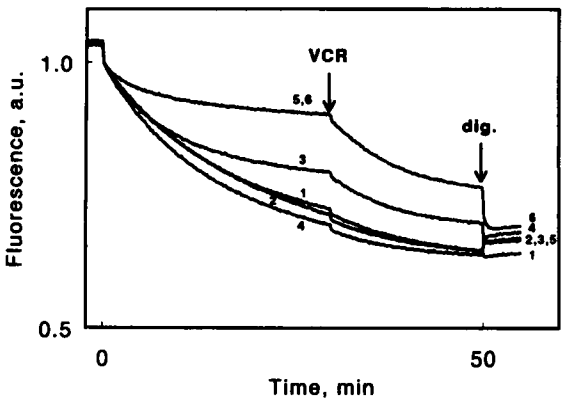

FIG. 3. Time course of daunorubicin uptake by SW-1573 cell lines treated with BSO and untreated cells. One million cells were added to 2.5 ml of medium containing 1 μM daunorubicin. The decrease in fluorescence due to increased influx and binding to DNA was recorded with a fluorescence monitor. After 30 min, vincristine (VCR) (100 μM) was added to the medium; after 50 min, digitonin (dig.) (25 μM) was added. Curves: 1, S1; 2, S1 treated with BSO; 3, S1(MRP); 4, S1(MRP) treated with BSO; 5, S1(MDR1); 6, S1(MDR1) treated with BSO.

we incubated S1(MRP) cells with radiolabeled daunorubicin or vincristine and analyzed the culture medium by HPLC. All added drug was recovered in unmodified form (data not shown), suggesting that if GSH *S*-conjugates of daunorubicin or vincristine exist they are unstable in culture medium. The rate of MRP-mediated drug efflux was of the same order of magnitude as the efflux rate of GSH. Even at high daunorubicin concentration, drug-induced GSH efflux was not found (Table 2; data not shown). An effect on cellular GSH was obtained with arsenite, however, and this was most pronounced in S1(MRP) cells. After exposure to 100 μM sodium arsenite, the glutathione level in S1(MRP) was decreased by 73% and in S1 and S1(MDR1) it was decreased by ≈30% (Table 1). Concomitantly, an increased amount of glutathione was detected in the medium from arsenite-exposed cells (Table 2). In the enzymatic assay used, most of the glutathione detected was in the reduced form (GSH) and <10% was oxidized glutathione (GSSG).

To further analyze the excreted products, we labeled the cells with [^{35}S]cysteine prior to arsenite exposure. Addition of 100 μM arsenite resulted in a 4-fold higher increase of efflux of radioactivity from S1(MRP) cells than from S1 or

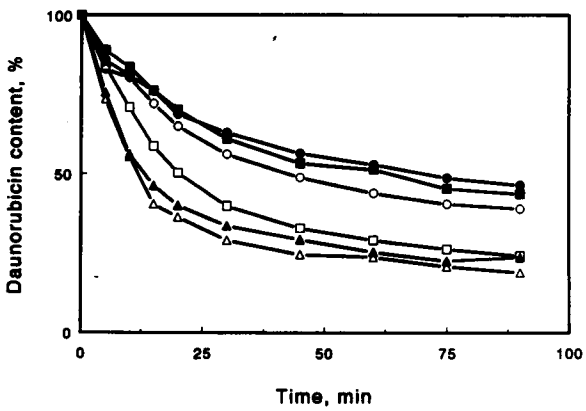

FIG. 4. Normalized cellular efflux of [G-^{3}H]daunorubicin from SW-1573 cells treated with BSO (solid symbols) and from untreated cells (open symbols). S1 (circles), S1(MRP) (squares), and S1(MDR1) (triangles) cells were loaded with [G-^{3}H]daunorubicin (0.5 μM) for 60 min and suspended in daunorubicin-free medium. At intervals thereafter, the amount of cellular daunorubicin was measured.

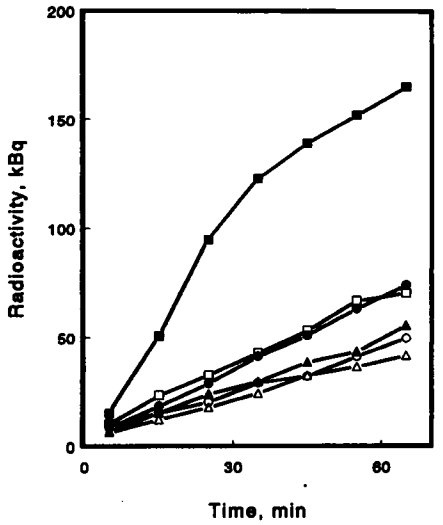

FIG. 5. Radioactivity in medium from [^{35}S]cysteine-labeled SW-1573 cells with (solid symbols) or without (open symbols) exposure to arsenite. One million S1 (circles), S1(MRP) (squares), or S1(MDR1) (triangles) cells were exposed to 100 μM arsenite, and at intervals thereafter the amount of radioactivity in the medium was determined by liquid scintillation counting.

S1(MDR1) cells (Fig. 5). Increasing the arsenite concentration to 200 μM did not further increase efflux, whereas at 50 μM the efflux rate was about half that at 100 μM. Nearly all radioactivity in the medium from arsenite-induced cultures as well as from the controls was acid soluble and there was no release of lactate dehydrogenase, indicating that the increased release of radiolabel after exposure of the cell was not caused by permeabilization of the cell membrane. The acid-soluble thiol compounds in the medium were separated by HPLC (Fig. 6). The majority of radioactivity eluted together with GSSG and this peak was 4-fold higher in medium from S1(MRP) cells that had been incubated with arsenite than in medium from the controls (Fig. 6). In the absence of arsenite, S1(MRP) cells lost $\approx$2 times more (i.e., 1.94 $\pm$ 0.65; n = 5) radioactivity than S1 cells (Figs. 5 and 6), confirming the 2-fold higher release of GSH from S1(MRP) as found by enzymatic assay (Table 2). Similar arsenite-induced secretion of GSH was found in the GLC4/ADR cell line (Table 2), containing much higher amounts of MRP than S1(MRP) or its parental drug-sensitive cell line GLC4 (6, 7). We conclude that overexpression of *MRP* is associated with an increased release of GSH from cells after exposure to arsenite.

DISCUSSION

We show here that pretreatment of *MRP*-transfected lung cancer cells with BSO abolishes the drug resistance caused by MRP. BSO is a specific inhibitor of GSH synthesis and its known effects on cells can be attributed to the decrease in cellular GSH caused by the block in GSH synthesis (23). Hence, our results firmly link the action of MRP to the intracellular supply of GSH. This link does not appear to be the result of an indirect effect of GSH depletion on cell vitality and plasma membrane permeability. The cloning efficiency of SW-1573 cells decreased only 15% by incubation with BSO, and drug influx into the cells was not increased. Moreover, the BSO effect was specific for MRP-mediated MDR, as the vincristine resistance of the same lung cancer cells transfected with *MDR1* was not affected at all.

The simplest interpretation of our results is that MRP catalyzes the cotransport of drug and GSH. This interpretation is now supported by several lines of evidence. (*i*) Cells that overexpress *MRP* secreted more GSH into the medium than

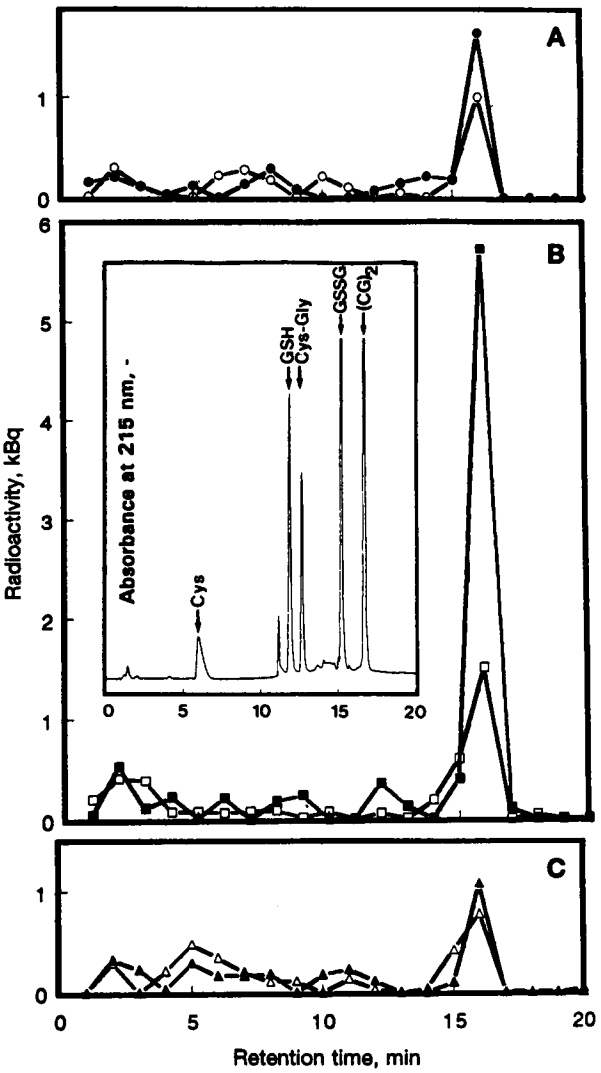

FIG. 6. Chromatographic analysis of acid-soluble ^{35}S-labeled sulfhydryl groups in the culture medium of S1 (*A*), S1(MRP) (*B*), and S1(MDR1) (*C*) cells with (solid symbols) or without (open symbols) exposure to arsenite (100 μM; 1 hr). (*Inset*) Chromatogram of five standards in 10 mM phosphate buffer (pH 2.0). Cys-Gly, cysteinyl glycine; $(CG)_2$, oxidized cysteinyl glycine (Gly-Cys-Cys-Gly).

parental cells, showing that MRP affects GSH transport even in the absence of drug. (*ii*) GSH depletion prevented the extrusion of drug from the cells by MRP but not by Pgp. (*iii*) BSO does not directly inhibit drug transport, as its effect can be abolished by preventing GSH depletion with added GSH ester (33). (*iv*) Experiments with plasma membrane vesicles show that overexpression of *MRP* results in the increased ATP-dependent transport of GSH *S*-conjugates (9–11). (*v*) A 190-kDa protein that reacts with an antibody against MRP can be cross-linked to a GSH *S*-conjugate, leukotriene C_4 (LTC_4) (9). (*vi*) We have found an arsenite-induced efflux of GSH from cells overexpressing *MRP*, presumably due to the transport of an arsenite–GSH complex by MRP. All these results support the interpretation that MRP is a GSH *S*-conjugate transporter. MRP may therefore be identical, or functionally similar, to transporters previously described as the GS-X pump (12), the multispecific organic anion transporter (MOAT) (13), or the LTC_4 transporter (9, 10).

This interpretation rests on circumstantial evidence, however, and even this evidence is still lacunar. Conjugates of anthracyclines and *Vinca* alkaloids with GSH (or other negatively charged compounds) are not known to exist and we have

been unable to find them. It is possible that the drug–GSH complex is unstable or even noncovalent. As GSH *S*-transferases can bind both hydrophobic compounds and GSH (34), such a noncovalent complex might be presented to MRP by a transferase. These are speculations, however, and we cannot yet exclude the possibility that GSH depletion affects MRP-mediated drug transport in an indirect fashion. More experiments with membrane vesicles containing MRP are clearly required to settle the exact substrate specificity and transport mechanism of this transporter.

Why MRP-overexpressing cells secrete so much GSH is unclear. In experiments with isolated plasma membrane vesicles, GSH did not inhibit the ATP-dependent transport of LTC_4 or dinitrophenylglutathione by MRP (11, 12). However, this does not exclude the possibility that GSH is a low-affinity substrate for MRP or that GSH is transported together with an endogenous ligand. In TR^- rats, which lack a functional hepatic GS-X pump, the release of GSH from hepatocytes into bile is impaired (13). Although this was suggested to be a secondary effect of the accumulation of organic anions in the circulation (35), the results presented here suggest that GSH indeed may be a low-affinity substrate for MRP and perhaps for GS-X pumps.

Our results also indicate that MRP can transport a complex of arsenite and GSH. Previous work had already provided a link between arsenite and GSH metabolism (36, 37). Arsenite and GSH can form an $As(SG)_3$ complex, as detected by NMR (38, 39), and this may be the form in which arsenite is excreted. In our HPLC analysis, the main peak of radioactivity comigrated with GSSG, but no GSSG was detected when the medium was directly analyzed by an enzymatic method. Other methods will be required to determine whether the $As(SG)_3$ complex is indeed the form in which arsenite is extruded by MRP.

Resistance to many anticancer drugs has been linked to increased cellular levels of GSH and GSH *S*-transferases (19). Attempts have been made to modify drug sensitivity with BSO, and this compound has even been tested in some humans (40). Our work shows that lowered GSH has only minor effects on MDR associated with overexpression of *MDR1* and specifically affects MRP-mediated MDR. With a more precise dissection of the causes of MDR in patients, it may become possible to identify the forms of MDR that could benefit from GSH depletion.

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