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The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump

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ABSTRACT **The multidrug-resistance associated protein MRP is a 180- to 195-kDa membrane protein associated with resistance of human tumor cells to cytotoxic drugs. We have investigated how MRP confers drug resistance in SW-1573 human lung carcinoma cells by generating a subline stably transfected with an expression vector containing *MRP* cDNA. *MRP*-overexpressing SW-1573 cells are resistant to doxorubicin, daunorubicin, vincristine, VP-16, colchicine, and rhodamine 123, but not to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide or taxol. The intracellular accumulation of drug (daunorubicin, vincristine, and VP-16) is decreased and the efflux of drug (daunorubicin) is increased in the transfectant. The decreased accumulation of daunorubicin is abolished by permeabilization of the plasma membrane with digitonin, showing that MRP can lower the intracellular daunorubicin level against a concentration gradient. Anti-MRP antisera predominantly stain the plasma membrane of *MRP*-overexpressing cells. We conclude that MRP is a plasma membrane drug-efflux pump.**

Cells selected for resistance to a single cytotoxic drug may become crossresistant to a whole range of drugs with different structures and cellular targets. This phenomenon is called multidrug resistance (MDR). The classic form of MDR is due to an increased activity of P-glycoprotein (Pgp), encoded by the human *MDR1* gene (1–4) (standard gene symbol, *PGY1*). This large glycoprotein is located in the plasma membrane and can extrude a range of hydrophobic natural product drugs from the cell against a concentration gradient (1–5). An increase in Pgp activity can result in lowered intracellular drug concentration and, hence, in drug resistance.

Increased Pgp is not the only cause of MDR, however. Several cell lines selected for resistance do not contain increased amounts of Pgp but nevertheless are resistant to a broad range of natural-product drugs (6–9). In one of these non-Pgp MDR lines, the H69AR small-cell lung carcinoma (SCLC) line, Cole *et al.* (10) found amplification and increased expression of a novel gene, the *MRP* (MDR-associated protein) gene. Overexpression of *MRP* has since been observed in several other (11–14), but not all (11), non-Pgp MDR cell lines. Transfection of HeLa cells with an expression vector containing the *MRP* cDNA results in the acquisition of resistance to doxorubicin, vincristine, and VP-16, but not cisplatin (15). We show here that transfection of the *MRP* cDNA into human lung carcinoma cells also results in MDR.

These experiments prove that *MRP* is a drug-resistance gene, but they do not answer the question how MRP acts. As MRP belongs to the ABC superfamily of transporter proteins (10, 16), it could simply act like Pgp, as a plasma membrane pump extruding drugs. Indeed, decreased drug accumulation has been reported for several non-Pgp MDR cell lines that were later found to overexpress *MRP* (7, 11–14). A major exception, however, is the MDR H69AR cell line in which the *MRP* gene was discovered (10). Drug accumulation was reported to be the same as in the parental cell line and this led Cole *et al.* (10, 17, 18) to consider other mechanisms than decreased drug accumulation for MRP action. Moreover, the subcellular location of MRP did not seem to be similar to that of a plasma membrane transporter such as Pgp. A 190-kDa protein detected in non-Pgp MDR cells and thought to be MRP was found mainly in the endoplasmic reticulum, rather than in the plasma membrane (13, 14).

To analyze the mechanism of action of MRP, we examined the effect of *MRP* overexpression on drug resistance and drug accumulation in SW-1573 lung carcinoma cells. In addition, we have raised antibodies against segments of MRP and used these to determine its main cellular location.

MATERIALS AND METHODS

Cell Lines. The drug-sensitive and doxorubicin-selected MDR sublines of the non-SCLC cell line SW-1573 and the SCLC line GLC4 have been described (7–9). The *MDR3* Pgp-expressing cell line V01V01 and the control cell line FVB·c are simian virus 40-immortalized mouse ear fibroblasts obtained from FVB mice transgenic for the human *MDR3* gene and normal FVB mice, respectively (19).

Vector Construction and Transfection. A DNA fragment containing the complete predicted *MRP* open reading frame (10, 15) plus 115 nt of 5′ and 800 nt of 3′ noncoding sequence was constructed in the expression vectors pJ3Ω (20) and pRc/RSV (Invitrogen). All cDNA fragments used for the assembly of the *MRP* cDNA were sequenced and the integrity of the *MRP* cDNA fragment in the resulting expression vectors, pJ3Ω-MRP and pRc/RSV-MRP (Fig. 1), was assessed by restriction enzyme mapping and DNA sequence analysis of the cloning junctions. Transfection of SW-1573/S1 cells with pRc/RSV-MRP DNA or African green monkey kidney COS-7 cells with pJ3Ω-MRP DNA followed the standard calcium phosphate precipitation technique (21). Stable transfectants in S1 were selected for 3 weeks in medium with G418 at 800 μg/ml and propagated for further analysis with G418 at 200 μg/ml. COS-7 cells were trypsinized 48 hr after transfection and analyzed by immunocytochemistry.

MRP Fusion Proteins and Immunization. Two fusion genes consisting of the gene for the *Escherichia coli* maltose-binding protein and two different segments of the *MRP* cDNA were constructed in the plasmid vector pMal-c (22).

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Abbreviations: MDR, multidrug resistance (resistant); Pgp, P-glycoprotein; SCLC, small-cell lung cancer; pH_i, intracellular pH; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

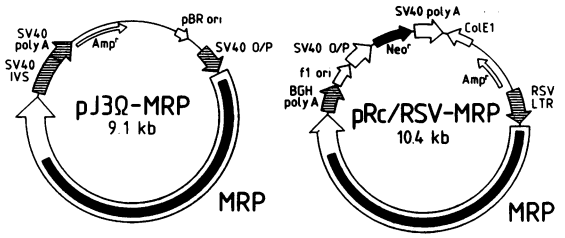

FIG. 1. *MRP* expression vectors. (*Left*) pJ3Ω-MRP, containing the *MRP* cDNA under control of simian virus 40 (SV40) regulatory elements. O/P, SV40 origin and early promoter; IVS, SV40 t-antigen intervening sequence; poly A, SV40 T-antigen polyadenylylation signal; Amp^r, β-lactamase gene; ori, origin of replication. (*Right*) pRc/RSV-MRP, containing the *MRP* cDNA under control of the Rous sarcoma virus long terminal repeat (RSV LTR). BGH poly A, bovine growth hormone gene polyadenylylation signal; Neo^r, neomycin-resistance gene.

The *MRP* sequences in the expression plasmids encoded aa 780–944 (fusion protein II) or 1294–1431 plus 1496–1531 (fusion protein III) of MRP. The fusion proteins were produced in *E. coli* JM101 and purified by amylose resin affinity chromatography (22). Guinea pigs (Sewall–Wright inbred strain) received sub- and intracutaneous injections of 200 μg of purified fusion protein II or III emulsified in Freund's complete adjuvant (Bacto, Detroit, MI). After 2 and 4 weeks booster injections with the same dose of immunogen in Freund's incomplete adjuvant were given intracutaneously, and after an additional 2 weeks the animals were bled.

Immunocytochemistry. Cytocentrifuge preparations of tumor cell lines were air-dried overnight, fixed for 10 min in acetone or 4% (vol/vol) paraformaldehyde in phosphate-buffered saline and incubated for 60 min with diluted antisera. Antibody binding was detected with a biotinylated mouse anti-guinea pig monoclonal antibody (Dako) and streptavidin-conjugated horseradish peroxidase (Zymed). The slides were developed with 3-amino-9-ethylcarbazole, counterstained with hematoxylin, and mounted with Aquamount.

Protein Blots. Total cell lysates were made by lysing harvested cells in 10 mM KCl/1.5 mM $MgCl_2$/10 mM Tris Cl, pH 7.4/0.5% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml). DNA was sheared by sonication and samples containing 25 μg of protein were fractionated by SDS/7.5% PAGE and then transferred onto a nitrocellulose filter by electroblotting. Immunoreactivity of proteins with the antisera against the fusion proteins was visualized with peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (Dako) and 3,3′-diaminobenzidine and 4-chloro-1-naphthol substrate. P-glycoproteins were detected by using the monoclonal antibody C219, rabbit anti-mouse IgG, and ^{125}I-labeled protein A. For glycosylation studies of MRP, membrane-enriched protein fractions were prepared and treated with *N*-glycanase (a mixture of endoglycosidase F and peptide:N-glycosidase F) (Boehringer Mannheim) (21).

Drug Accumulation. Steady-state accumulation of radiolabeled daunorubicin, vincristine, and VP-16 was measured according to Skovsgaard (23), as modified by Broxterman *et al.* (24). Cells in the logarithmic phase of growth (0.2×10^6 cells per ml) were incubated at 37°C with ^{3}H-labeled daunorubicin, vincristine, or VP-16. After 75 min ice-cold phosphate-buffered saline was added and after two cold washes radioactivity was determined by liquid scintillation counting. The cellular influx of daunorubicin was measured by monitoring the fluorescence decrease after addition of cells to daunorubicin with a fluorescence monitor (FluoroMax; Spex Industries, Metuchen, NJ) at excitation and emission wavelengths of 488 and 560 nm, respectively. Intracellular pH (pH_i) was measured with 2′7′-bis(2-carboxyethyl)-5 (and 6)-carboxyfluorescein (BCECF) (25).

RESULTS

Cloning and DNA Sequence Analysis of *MRP* cDNA. *MRP* encodes an mRNA of ≈6.5 kb encompassing a continuous open reading frame of 1531 aa (10, 15). We have isolated a set of overlapping cDNA clones by hybridization screening of a cDNA library of adenovirus-transformed human retinal cells (RCA) and by reverse transcription–PCR of mRNA from the lung cancer cell line GLC4/ADR. Together these clones covered the complete predicted coding region of the *MRP* mRNA plus 137 nt of 5′ and 1137 nt of 3′ noncoding sequence; 916 nt of 3′ noncoding sequence were not published previously and have been deposited in the GenBank database (accession no. X78338). The cDNA clones obtained from the RCA cDNA library differ in a few nucleotides from the sequence published by Cole *et al.* (10): five of these are silent variations (T-1021 → C; T-1258 → C; T-1880 → C; C-1900 → T; A-1907 → C), whereas two lead to amino acid changes [C-546 → T (Thr-117 → Met); T-2250 → C (Leu-685 → Ser)].

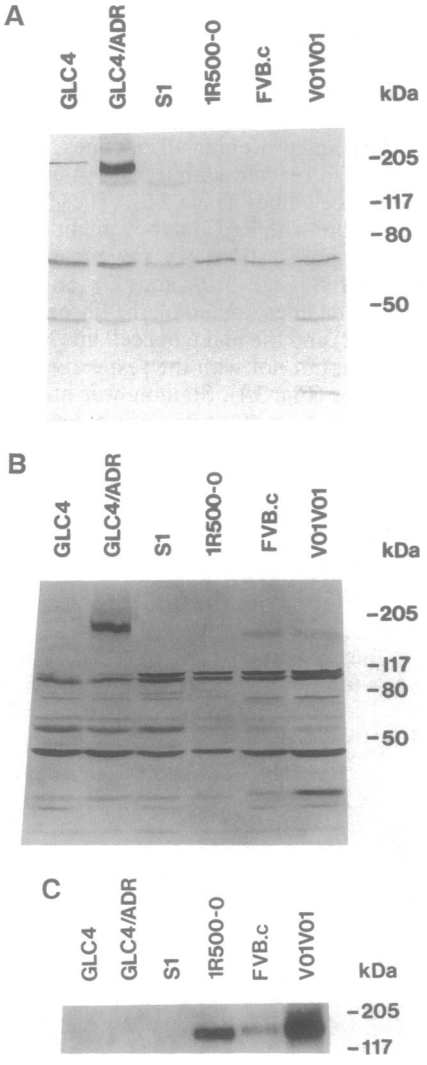

FIG. 2. Immunoblot analysis of total protein of cell lines with increased levels of MRP (GLC4/ADR), *MDR1* Pgp (1R500-0), or *MDR3* Pgp (V01V01) and of the corresponding parental cell lines (GLC4, S1, and FVB·c). Protein blots were incubated with antiserum against MRP fusion protein II (*A*), antiserum against MRP fusion protein III (*B*), or the Pgp-reactive monoclonal antibody C219 (*C*).

As the variation at aa 685 is in the highly conserved Walker A consensus motif in the amino-proximal nucleotide-binding domain (10, 16), we checked whether it was also present in cDNA from GLC4/ADR cells. This was the case. Since the reconstructed cDNA from the RCA cDNA clones encodes a functional MRP (see below), we conclude that the two amino acid changes do not impair the activity of MRP.

Generation of Anti-MRP Polyclonal Antisera. To examine the expression level and the cellular localization of MRP, we prepared polyclonal antisera against two bacterial fusion proteins containing different fragments of MRP. The first fusion protein contained the "linker" domain that connects the two halves of the protein (fusion protein II) (10). The second contained the carboxyl-terminal end and part of the predicted carboxyl-proximal nucleotide binding domain of MRP (fusion protein III) (10). The specificity of the antisera was determined on protein blots (Fig. 2) and on cytocentrifuge preparations of tumor cells and of African green monkey kidney COS-7 cells that transiently overexpress MRP (Fig. 3).

On immunoblots both antisera detected a protein of 190–195 kDa in total protein isolates of the GLC4/ADR cells, but not of the parental drug-sensitive cell line GLC4 (Fig. 2). This is in agreement with the massive increase of *MRP* mRNA and *MRP* gene-copy number in the GLC4/ADR cells, relative to the low basal *MRP* mRNA level in GLC4 (11). The sera did not crossreact with the human *MDR1* or *MDR3* Pgps (Fig. 2) but did crossreact with several small proteins not affected by the level of MRP and present in all cell lines. In addition, one of the antisera also reacted with a band of about 180 kDa in the two mouse cell lines (Fig. 2*B*). These bands did not precisely comigrate with Pgp and they might be mouse MRP.

In cytological preparations we saw strong staining with GLC4/ADR cells (Fig. 3 *B* and *C*) and other *MRP*-overexpressing cell lines, including the fibrosarcoma cell line HT1080/DR4 (12) and the leukemia cell line HL60/ADR (13) (data not shown), but not with the respective parental drug-sensitive cell lines (Fig. 3*A*). Staining was primarily over the plasma membrane. In most of the cells a dense spot near the nucleus was also seen, probably corresponding to the Golgi network. This may represent nonfunctional MRP or protein that is on its way to the plasma membrane. Both antisera showed the same staining pattern and specificity. We also saw strong reaction with COS-7 cells that were transiently transfected with the *MRP* expression vector pJ3Ω-MRP (Figs. 1 and 3*G*), but not with "mock"-transfected cells (Fig. 3*F*).

Overexpression of MRP in SW-1573 Cells. To make stable transfectants in SW-1573 cells we cloned the *MRP* cDNA behind the Rous sarcoma virus promoter in the expression vector pRc/RSV (Fig. 1*B*). This vector also contains the bacterial *neo* gene allowing the selection of transfected cells with the neomycin analogue G418. pRc/RSV-MRP (Fig. 1*B*) was transfected into SW-1573/S1 cells, and after 3 weeks of selection with G418, 40 colonies were picked randomly and analyzed for *MRP* expression. Only 5 clones showed increased *MRP* mRNA, as measured by an RNase protection assay (11), indicating that in the other clones only the *neo* gene was correctly integrated. Of the 5 clones with increased *MRP* mRNA, 1, designated S1(MRP), also showed increased MRP in immunoblot analysis (Fig. 4*A*). In a second transfection experiment 1 of 30 G418-resistant transfectants analyzed overexpressed *MRP* [S1(MRP)-2, Fig. 4*A*]. Both transfectants with raised *MRP* mRNA levels were MDR, whereas G418-resistant clones that did not overexpress *MRP* were not (Table 1).

Glycosylation of MRP. MRPs from the transfectants comigrated with MRP present in S1 cells in SDS/PAGE and their mobility corresponded to ≈180 kDa (Fig. 4). MRPs from the resistant GLC4 and SW-1573 sublines all migrated slower (Fig. 4*B*). After treatment with N-glycanase, the two types of MRP both decreased in apparent mass to ≈170 kDa (Fig. 4*C*), in agreement with the 172 kDa predicted from the primary amino acid sequence of MRP. This suggests that the difference in mobility seen in untreated protein samples is due to a difference in glycosylation.

Location of MRP in the Cell. In cytocentrifuge preparations both anti-MRP antisera mainly stained the plasma membrane of S1(MRP) cells (Fig. 3*E*). There was also cytoplasmic staining, however, mostly homogeneous, but in some cells present as a concentric ring around the nucleus. Whether this ring represents the endoplasmic reticulum or the nuclear membrane remains to be determined. The relative fraction of MRP present in the plasma membrane of S1(MRP) seemed lower than in GLC4/ADR, suggesting that the routing of nascent MRP may differ in the two cell types. Untransfected S1 cells did not stain (Fig. 3*D*), confirming the specificity of the antisera for MRP.

Mechanism of MRP-Mediated MDR. The resistance spectrum of the *MRP*-overexpressing transfectants was compared with that of untransfected SW-1573/S1 cells in clono-

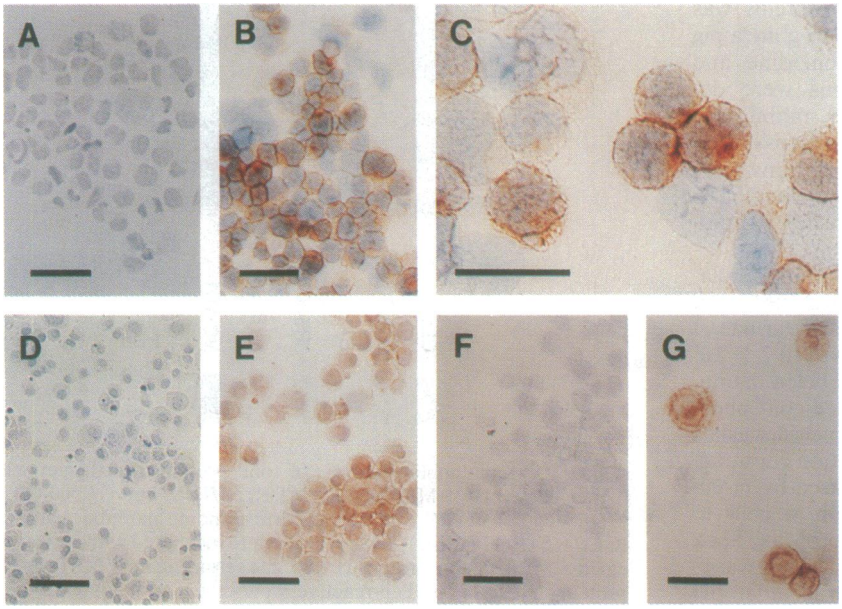

FIG. 3. Immunochemical staining of cytocentrifuge preparations of *MRP*-overexpressing cells. (*A*) Drug-sensitive GLC4 cells. (*B* and *C*) Drug-resistant MRP-overexpressing GLC4/ADR cells (two different magnifications). (*D*) Drug-sensitive SW-1573/S1 cells. (*E*) S1(MRP), an *MRP*-overexpressing transfectant of S1. (*F*) COS-7 cells transfected with plasmid DNA lacking *MRP* cDNA sequences ("mock" transfection). (*G*) COS-7 cells transfected with pJ3Ω-MRP DNA (Fig. 1). The stained cells are the transiently transfected cells that highly overproduce MRP. In the COS-7 cells most MRP is in the cytoplasm. Therefore, the COS-7 expression system may not be suitable for functional studies on MRP. Slides were stained with antiserum against MRP fusion protein III. Staining with antiserum against fusion protein II gave similar results. (Bars = 50 μM.)

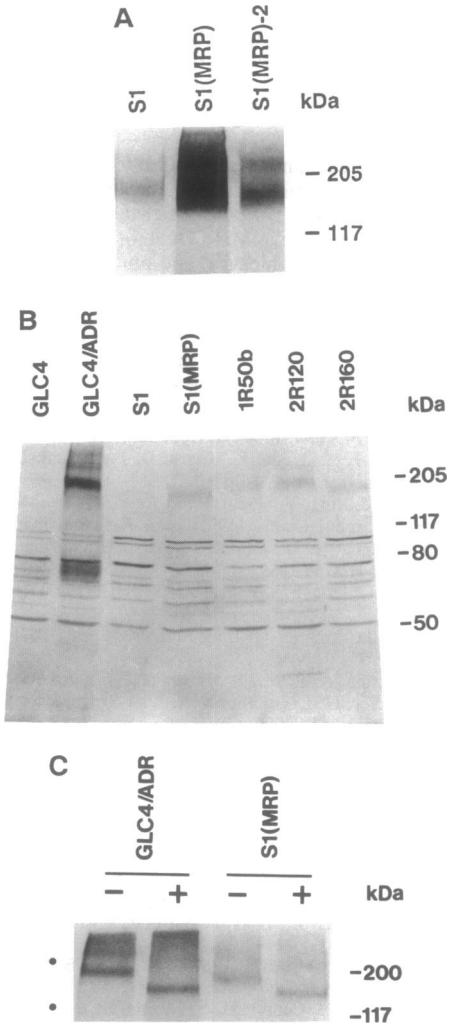

FIG. 4. Immunoblot analysis of MRP in untransfected S1 cells and *MRP*-overexpressing transfectants [S1(MRP) and S1(MRP)-2] (*A*) and drug-sensitive (GLC4 and S1) and drug-resistant lung cancer cell lines [GLC4/ADR, S1(MRP), 1R50b, 2R120, and 2R160] (*B*) and of MRP glycosylation in GLC4/ADR and S1(MRP) cells (*C*). +, Membrane-enriched protein fractions treated with *N*-glycanase; −, untreated control samples. Migration of protein markers of 117 and 200 kDa is indicated with dots at left and with bars at right. Blots were stained with the anti-MRP monoclonal antibody MRPr1 (M.J.F., R.J.S., and G.J.R.Z., unpublished data) (*A*) or with polyclonal antiserum against MRP fusion protein III (*B* and *C*).

genic survival assays. The results for S1(MRP) are summarized in Table 1. Overexpression of *MRP* in SW-1573 cells increases resistance to doxorubicin, vincristine, and VP-16 (Table 1), as also reported by Grant *et al.* (15) for HeLa cells. In addition we found increased resistance to daunorubicin, colchicine, and rhodamine 123, but not to *m*-AMSA and taxol (Table 1). S1(MRP)-2 was about 2-fold resistant to doxorubicin, vincristine, and VP-16, and not to *m*-AMSA, thus confirming the results for S1(MRP).

Resistance was associated with a decreased steady-state accumulation of drug (Table 2). The mechanism of decreased drug accumulation in S1(MRP) was examined in detail for daunorubicin. As this is a weak base, its accumulation could be decreased by an increase in pH_i (26). This was not the case, as we found a pH_i of 7.27 ± 0.13 (mean ± SD; $n = 3$) in S1 cells and a pH_i of 7.30 ± 0.10 in S1(MRP) cells. A decrease in drug accumulation could also be caused by a decrease in cellular binding sites. To test this possibility, we

Table 1. Resistance spectrum of the *MRP* transfectant S1(MRP)

Drug	Relative resistance
Doxorubicin	2.7 ± 0.4
Daunorubicin	3.2 ± 0.8
Vincristine	5.3 ± 1.3
VP-16	4.9 ± 1.1
m-AMSA*	1.1 ± 0.1
Colchicine	3.6 ± 0.1
Rhodamine 123	4.1 ± 0.3
Taxol	1.0 ± 0.1

Resistance was determined in clonogenic survival assays (8). Relative resistance = IC_{50} S1(MRP)/IC_{50} S1 (IC_{50}, inhibitory concentration where 50% of the cells survive). The values are the means ± SD of at least three experiments, each performed in duplicate.
*4′-(9-Acridinylamino)methanesulfon-*m*-anisidide.

selectively permeabilized the cellular plasma membrane with digitonin (see ref. 25). Upon addition of digitonin, the accumulation of daunorubicin in S1(MRP) cells became equal to that in S1 cells (Table 2), showing that the accumulation deficit in S1(MRP) was not due to fewer cellular binding sites. This was confirmed by an experiment where the uptake of daunorubicin into S1 and S1(MRP) was followed in time and compared with the uptake into the 2R120 subline (Fig. 5). The fluorescence decrease represents mainly quenching of daunorubicin by binding to the DNA (27). The accumulation of daunorubicin was lower in S1(MRP) and 2R120 cells than in S1 cells, in agreement with Table 2. Upon addition of digitonin, S1(MRP) and 2R120 showed a large increase in DNA binding (Fig. 5). These results demonstrate that the decreased accumulation of daunorubicin in the *MRP*-overexpressing cells was not due to a passive redistribution of drug, but due to an active process. When we therefore tested daunorubicin efflux from transfected and control cells; the efflux of daunorubicin was faster from S1(MRP) than from S1 cells (Fig. 6). We conclude that MRP can mediate the active extrusion of daunorubicin from cells.

DISCUSSION

Our results show that MRP is remarkably similar to the drug-transporting Pgps in its mode of action. (*i*) Like Pgp, MRP can cause resistance to a range of hydrophobic drugs. (*ii*) MRP is predominantly located in the plasma membrane. (*iii*) MRP can decrease drug accumulation in the cell and this decrease is abolished by permeabilization of the plasma membrane. (*iv*) MRP can increase the efflux of drugs from cells. We therefore think that MRP acts as a drug pump, like Pgp, extruding hydrophobic compounds from cells against a concentration gradient. Presumably the two ATP-binding motifs in MRP allow the protein, just like Pgp, to use ATP hydrolysis for active transport.

The drug-resistance spectra associated with *MRP* and *MDR1* overexpression are remarkably similar, given the

Table 2. Accumulation of daunorubicin (0.5 μM, 75 min), vincristine (0.5 μM, 75 min) and VP-16 (5 μM, 75 min) in untransfected S1 cells and the *MRP* transfectant S1(MRP)

	pmol per 10^6 cells			
	Daunorubicin*			
Cell line	− digitonin	+ digitonin†	Vincristine*	VP-16‡
S1	367 ± 30	292 ± 39	28.8 ± 3.6	13.0
S1(MRP)	211 ± 22	295 ± 25	16.1 ± 4.4	5.5

*Mean ± SD of four independent experiments, each performed in quadruplicate.
†Digitonin (20 μM) was added 5 min before the end of the incubation.
‡Mean of two independent experiments, each performed in quadruplicate. Variation was <2 pmol per 10^6 cells.

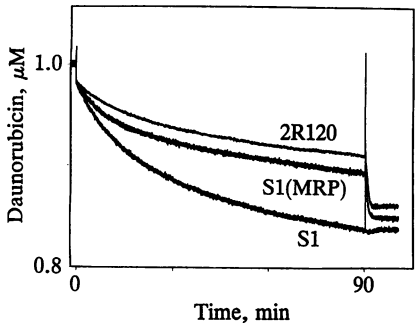

FIG. 5. Time course of daunorubicin uptake by SW-1573 cell lines. One million S1, S1(MRP), or 2R120 cells were added to 2.5 ml of medium containing 1 μM daunorubicin. The decrease in fluorescence due to increased influx of daunorubicin and binding to DNA was recorded. After 90 min of incubation, digitonin (20 μM) was added to the medium.

large difference in sequence between MRP and the *MDR1*-encoded Pgp (10). Nevertheless, there are also important differences between MRP and *MDR1* Pgp in the drugs that they transport or interact with. We found no resistance to taxol in lung carcinoma cells transfected with *MRP* cDNA (Table 1), whereas mouse bone marrow cells transgenic for *MDR1* are highly resistant (28). In a preliminary search for reversal agents of MRP-mediated MDR, we found that the decreased accumulation of daunorubicin in the *MRP* transfectant was not affected by cyclosporin A, an effective reversal agent of Pgp-mediated MDR. In contrast, the isoflavinoid genistein, a drug that does not inhibit *MDR1* Pgp-mediated drug transport (29), slightly reduced the decreased drug accumulation in the *MRP* transfectant. This suggests that genistein is a modulator of MRP-mediated MDR. Since genistein is too toxic for use in patients and even too toxic to use as a convenient reversal agent in drug resistance tests (29), less toxic analogues of genistein that also act on MRP are needed.

MRP in the non-Pgp MDR cell line SW-1573/2R120 is not further increased in the Pgp-overexpressing cell line 2R160 (Fig. 4*B*). As this cell line was derived from 2R120 by further selection on doxorubicin (9), the extent to which *MRP* overexpression is tolerated in SW-1573 cells may be limited to the level reached in 2R120. Consequently, MRP-mediated MDR may play a role only in low-level MDR in SW-1573 cells and possibly in other cells as well.

There is little information on the contribution of increased levels of MRP to drug resistance of human cancers. High levels of *MRP* mRNA were found in leukemic cells of a high

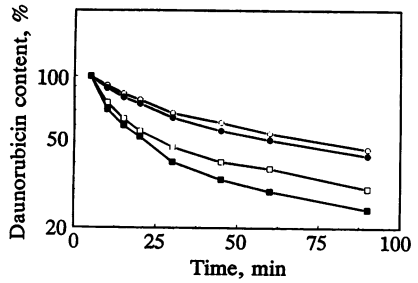

FIG. 6. Normalized cellular efflux of [G-^{3}H]daunorubicin from SW-1573/S1 cells (circles) or S1(MRP) cells (squares). Cells were loaded with daunorubicin (0.5 μM) for 60 min and suspended in daunorubicin-free medium. At intervals thereafter, the amount of cellular daunorubicin was measured. Two separate experiments (closed and open symbols) are shown.

percentage of patients with chronic lymphocytic leukemia, but there was no relation to prior chemotherapy or treatment outcome (30). The phenotype delineated in this paper, the anti-MRP antisera, and the methods used here to demonstrate reduced drug accumulation should help to determine the role of MRP-mediated MDR in clinical drug resistance.

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