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Multidrug Resistance Protein (MRP)-mediated Transport of Leukotriene C_4 and Chemotherapeutic Agents in Membrane Vesicles

DEMONSTRATION OF GLUTATHIONE-DEPENDENT VINCRISTINE TRANSPORT*

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Douglas W. Loe‡, Kurt C. Almquist‡§¶, Roger G. Deeley‡§‖, and Susan P. C. Cole‡§**

From the ‡Cancer Research Laboratories and §Department of Pathology, Queen's University, Kingston, Ontario, K7L 3N6 Canada

The 190-kDa multidrug resistance protein (MRP) has recently been associated with the transport of cysteinyl leukotrienes and several glutathione (GSH) *S*-conjugates. In the present study, we have examined the transport of leukotriene C_4 (LTC_4) in membrane vesicles from MRP-transfected HeLa cells (T14), as well as drug-selected H69AR lung cancer cells which express high levels of MRP. V_{max} and K_m values for LTC_4 transport by membrane vesicles from T14 cells were 529 ± 176 pmol mg^{-1} min^{-1} and 105 ± 31 nM, respectively. At 50 nM LTC_4, the K_m (ATP) was 70 μM. Transport in T14 vesicles was osmotically-sensitive and was supported by various nucleoside triphosphates but not by non- or slowly-hydrolyzable ATP analogs. LTC_4 transport rates in membrane vesicles derived from H69AR cells and their parental and revertant variants were consistent with their relative levels of MRP expression. A 190-kDa protein in T14 membrane vesicles was photolabeled by [^{3}H]LTC_4 and immunoprecipitation with MRP-specific monoclonal antibodies (mAbs) confirmed that this protein was MRP. LTC_4 transport was inhibited by an MRP-specific mAb (QCRL-3) directed against an intracellular conformational epitope of MRP, but not by a mAb (QCRL-1) which recognizes a linear epitope. Photolabeling with [^{3}H]LTC_4 was also inhibitable by mAb QCRL-3 but not mAb QCRL-1. GSH did not inhibit LTC_4 transport. However, the ability of alkylated GSH derivatives to inhibit transport increased markedly with the length of the alkyl group. *S*-Decylglutathione was a potent competitive inhibitor of [^{3}H]LTC_4 transport ($K_{i(app)}$ 116 nM), suggesting that the two compounds bind to the same, or closely related, site(s) on MRP. Chemotherapeutic agents including colchicine, doxorubicin, and daunorubicin were poor inhibitors of [^{3}H]LTC_4 transport. Taxol, VP-16, vincristine, and vinblastine were also poor inhibitors of LTC_4 transport but inhibition by these compounds was enhanced by GSH. Uptake of [^{3}H]vincristine into T14 membrane vesicles in the absence of GSH was low and not dependent on ATP. However, in the presence of GSH, ATP-dependent vincristine transport was observed. Levels of transport increased with concentrations of GSH up to 5 mM. The identification of an MRP-specific mAb that inhibits LTC_4 transport and prevents photolabeling of MRP by LTC_4, provides conclusive evidence of the ability of MRP to transport cysteinyl leukotrienes. Our studies also demonstrate that MRP is capable of mediating ATP-dependent transport of vincristine and that transport is GSH-dependent.

Multidrug resistance can be conferred by overexpression of either the multidrug resistance protein, MRP[1] (1), or by P-glycoprotein (2). Both MRP and P-glycoprotein belong to the ATP-binding cassette transporter superfamily but share only approximately 15% amino acid identity (3). Considerable evidence suggests that P-glycoprotein reduces cellular drug accumulation by acting as an ATP-dependent drug efflux pump (2, 4), but the mechanism of action of MRP is much less certain. The ability to transport drugs directly into plasma membrane vesicles has been firmly established for P-glycoprotein (5, 6), but not for MRP. There is also no evidence that xenobiotics bind directly to MRP (7, 8), as has been demonstrated for P-glycoprotein by cross-linking studies with photoaffinity analogs of chemotherapeutic agents (9). These observations suggest that these two ATP-binding cassette proteins may confer multidrug resistance by different mechanisms.

Some insight into the normal physiological role of MRP has been obtained by the demonstration that membrane vesicles from MRP-overexpressing drug-selected and transfected cells support ATP-dependent transport of cysteinyl leukotrienes (*e.g.* LTC_4) and certain other GSH *S*-conjugates (10–12). Further evidence of a role for MRP in LTC_4 transport was suggested by the observation that photolabeling of a 190-kDa protein with LTC_4 in MRP-expressing cells is inhibited by MK571 (an inhibitor of LTC_4 transport) (10). The cysteinyl leukotrienes are potent mediators of inflammation that increase vascular permeability and smooth muscle contraction (13). LTC_4 is synthesized from GSH and LTA_4 by LTC_4 synthase (14). It is then exported from the cell by an ATP-dependent transport mechanism (15). LTC_4 synthase and the LTC_4 transporter protein are expressed in eosinophils and mast cells (16), as well as endothelial cells (13). LTC_4 is also transported in the liver by at least two routes: uptake from the blood circulation into hepatocytes, and excretion from hepatocytes across canalicular membranes into the bile (13).

Although current evidence strongly suggests that MRP mediates an active transport process, the mechanistic relationship between the involvement of MRP in GSH *S*-conjugate transport and its role in multidrug resistance is unclear. GSH conjuga-

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¶ Supported by an MRCC studentship.
‖ Stauffer Research Professor of Queen's University.
** Career Scientist of the Ontario Cancer Foundation. To whom correspondence and reprint requests should be addressed: Cancer Research Laboratories, Rm. 328, Botterell Hall, Queen's University, Kingston, Ontario, Canada, K7L 3N6. Tel.: 613-545-6507; Fax: 613-545-6830.

[1] The abbreviations used are: MRP, multidrug resistance protein; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); LTC_4, leukotriene C_4; LTB_4, leukotriene B_4; mAb, monoclonal antibody; VCR, vincristine; PAGE, polyacrylamide gel electrophoresis.

tion is not known to be an important pathway for the biotransformation of chemotherapeutic agents to which MRP confers resistance and there is no evidence that this reaction occurs to any significant extent in tumor cells or drug-resistant tumor cell lines (17).

In the present study, the mechanism by which MRP confers resistance to multiple drugs was investigated in a plasma membrane vesicle model system. For these studies, we used membrane vesicles from a population of HeLa cells, termed T14, which had been transfected with a MRP expression vector (8). By using transfected cells as the source of membrane vesicles, those aspects of LTC_4 and drug transport kinetics that are solely attributable to MRP overexpression can be identified (8, 18). Our initial objective was to characterize MRP-mediated LTC_4 transport in membrane vesicles from this new population of transfected HeLa cells so that subsequent studies of drug, or drug conjugate, transport could be evaluated within this context. We provide evidence that [^{3}H]LTC_4 binds specifically to MRP and demonstrate that a MRP-specific mAb inhibits both [^{3}H]LTC_4 transport and binding. We have also characterized the ability of alkylated GSH derivatives and hydrophobic chemotherapeutic agents to inhibit [^{3}H]LTC_4 transport. Finally, we show that GSH not only enhances the ability of vincristine to inhibit LTC_4 transport, but also results in ATP-dependent transport of the drug itself.

EXPERIMENTAL PROCEDURES

Materials—[14,15,19,20-^{3}H]-LTC_4 (128 Ci mmol^{-1}) and [^{35}S]methionine (710 Ci mmol^{-1}) were purchased from DuPont NEN (Mississauga, Ontario, Canada), and [^{3}H]vincristine (VCR) (6.9 Ci mmol^{-1}) was from Amersham (Oakville, Ontario, Canada). LTB_4, LTC_4, VP-16, AMP, AMP-PNP, AMP-PCP, ATPγS, GTP, CTP, UTP, colchicine, GSH, GSSG, and *S*-alkyl GSH derivatives were from Sigma. ATP, daunorubicin, doxorubicin, vinblastine, and VCR were purchased from ICN Biochemicals (St. Laurent, PQ, Canada) and taxol was from Omicron BioChemicals (San Antonio, TX). Creatine phosphate and creatine kinase were from Boehringer Mannheim (Dorval, PQ, Canada). QCRL-3 is a IgG_{2a} murine mAb that recognizes a conformation dependent epitope of MRP and has been described previously (19).

Cell Culture—The small cell lung cancer cell line H69, its doxorubicin-selected multidrug resistant H69AR, and the drug-sensitive revertant cell line, H69PR, have been described previously (3, 20). The HeLa cells transfected with the pCEBV7 vector (C6) or the vector containing the MRP coding sequence pCEBV7-MRP1 (T14) have also been described (8). The 8226/Dox40 cell line overexpresses P-glycoprotein (21) and was provided by Dr. W. Dalton. All cell lines were cultured in RPMI 1640 medium with 5% defined bovine calf serum (HyClone Laboratories, Logan, UT) in the absence of antibiotics, except for the transfected HeLa cells which were maintained in 100 μg ml^{-1} hygromycin B. In some experiments, cells were metabolically labeled with [^{35}S]methionine (300 μCi ml^{-1}) as described (18).

Membrane Vesicle Preparation—Plasma membrane vesicles were prepared as described (5, 22) with modifications (18). Briefly, cells were homogenized in buffer containing 10 mM Tris-HCl, 250 mM sucrose, 3 mM KCl, 0.25 mM $MgCl_2$, pH 7.5, and protease inhibitors (18). Cell pellets were frozen at −70 °C for at least 1 h, thawed, and then disrupted by N_2 cavitation (10 min equilibration at 175 p.s.i.). EDTA was added to 1 mM and after centrifugation at 500 × *g* for 15 min, the supernatant was layered over 35% (w/w) sucrose in 10 mM Tris-HCl, 1 mM EDTA (specific gravity, ~1.15 g cm^{-3}) and centrifuged at 100,000 × *g* for 2 h. The interface was collected and washed twice by centrifugation. The membrane pellet was resuspended in transport buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.5) and passed 20 times through a 27-gauge needle for vesicle formation. The resulting vesicles were enriched 4–8-fold in plasma membranes relative to the post-nuclear supernatant, as assessed by Na^+,K^+-ATPase, alkaline phosphatase, and 5′-nucleotidase activities (Ref. 18 and references cited therein). Some contamination from endoplasmic reticulum was observed, as indicated by 2.3-fold enrichment of NADPH cytochrome *c* reductase.

The sidedness of the membrane vesicles was assessed by determining the activities of two plasma membrane-associated ectoenzymes (alkaline phosphatase and 5′-nucleotidase) in the presence or absence of 0.2% Nonidet P-40 (18, 23) and it was determined that 30–34% of T14 PM vesicles were inside-out. T14 vesicles were treated with concanavalin A (1:1 (w/w) ratio) overnight at 4 °C to agglutinate outside-out vesicles (24). Following centrifugation to remove agglutinated material, the supernatant displayed a 2-fold increase in the rate of [^{3}H]LTC_4 uptake (see below), consistent with ATP-dependent transport being attributable to inside-out vesicles.

Vesicle Transport of LTC_4 and VCR—ATP-dependent transport of LTC_4 into membrane vesicles was measured by rapid filtration as described (16), with modifications. Thawed membrane vesicles were diluted in transport buffer and passed 5 times through a 27-gauge needle. Standard transport assays were carried out at 23 °C in a 120-μl volume containing 10 μg of vesicle protein, 4 mM ATP, 10 mM $MgCl_2$, an ATP regenerating system consisting of 10 mM creatine phosphate and 100 μg ml^{-1} creatine kinase, and [^{3}H]LTC_4 (50 nM; 100 nCi) in transport buffer. At the indicated times, 20-μl aliquots were removed and added to 1 ml of ice-cold transport buffer, which was then filtered through nitrocellulose filters (0.22 μm) on a Hoeffer filtration manifold under vacuum. Filters were immediately washed twice with 5 ml of cold transport buffer, solubilized, and radioactivity determined. [^{3}H]LTC_4 uptake was expressed relative to the protein concentration of the membrane vesicles (25). All data were corrected for the amount of [^{3}H]LTC_4 that remained bound to the filter in the absence of vesicle protein, which was usually 5–10% of the total radioactivity.

ATP-dependent uptake of [^{3}H]VCR (200 nM; 70 nCi) was measured as described for [^{3}H]LTC_4 except that 100–120 μg of vesicle protein was used and the incubations were carried out at 37 °C. In some experiments, GSH (1–5 mM) was added to the transport buffer. Uptake of [^{3}H]VCR was stopped by rapid dilution in transport buffer and immediate filtration through glass fiber (Type A/E) filters (Gelman Sciences, Dorval, PQ), which had been presoaked overnight at 37 °C in 10% (w/v) bovine serum albumin (5).

Photolabeling of Membrane Proteins with [^{3}H]LTC_4 and Immunoprecipitation of MRP—Membrane proteins were photolabeled with [^{3}H]LTC_4 essentially as described (16). Briefly, vesicles prepared from different cell types (150 μg of membrane protein in 50 μl) were incubated with [^{3}H]LTC_4 (0.5 μCi, 78 nM) at room temperature for 10 min and frozen in liquid N_2. Samples were alternately irradiated for 30 s at 312 nm in a Stratalinker, followed by snap-freezing in liquid N_2, for a total of 10 min. A portion of the radiolabeled proteins was immunoprecipitated with a mixture of MRP-specific mAbs QCRL-1, QCRL-2, and QCRL-3 (19). Immunoprecipitates were solubilized in Laemmli's buffer and radiolabeled proteins (100 μg) were resolved by SDS-PAGE and gels were subjected to fluorography. To determine whether unlabeled LTC_4, LTB_4, mAb QCRL-3, or mouse IgG_{2a} (isotype control) could inhibit [^{3}H]LTC_4 labeling, T14 membrane vesicles were coincubated with these reagents and [^{3}H]LTC_4 for 10 min at room temperature prior to irradiation and SDS-PAGE.

RESULTS

LTC_4 Uptake in Membrane Vesicles—The time course and ATP dependence of [^{3}H]LTC_4 accumulation by vesicles prepared from HeLa T14 cells is shown in Fig. 1*A*. Accumulation was measured at room temperature with an initial concentration of 50 nM LTC_4 in the presence of 4 mM ATP or AMP. No cations (Na^+, K^+, Ca^{2+}) were present and the membrane potential was not experimentally manipulated. ATP-dependent uptake was rapid, linear up to 30 s and approached steady-state after 90 s (not shown). During the linear phase, the rate of ATP-dependent uptake was approximately 150 pmol mg^{-1} min^{-1}. Increasing the amount of vesicle protein resulted in rates of uptake which were difficult to quantify because of the short duration of linearity. Similarly, quantification was difficult if the experiments were carried out at 37 °C rather than room temperature because of high initial rates of transport and rapid depletion of extravesicular LTC_4. The ATP-dependent rate of LTC_4 uptake in T14 vesicles was at least 100-fold higher than in vesicles from control C6 cells (Fig. 1*A*). Furthermore, the low levels of uptake in T14 vesicles in the presence of AMP were similar to those observed for C6 membrane vesicles with or without ATP. The correlation between LTC_4 transport and levels of MRP expression was also examined by measuring rates of LTC_4 uptake in vesicles isolated from drug-sensitive H69, drug-resistant H69AR, and revertant H69PR cells. As shown in Fig. 1*B*, the rate of ATP-dependent [^{3}H]LTC_4 uptake

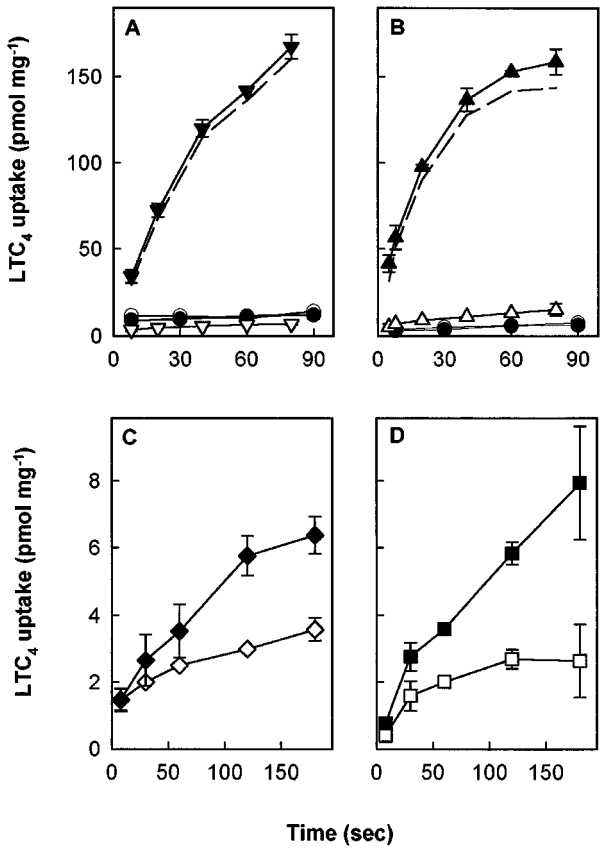

FIG. 1. **Time course of [^{3}H]LTC_4 uptake by membrane vesicles from MRP and control transfected HeLa cells, parental and drug-selected H69 cells, and 8226/Dox40 cells.** Membrane vesicles were incubated with 50 nM [^{3}H]LTC_4 in transport buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.5) for the times indicated. *Closed symbols* represent uptake in the presence of 4 mM AMP; *open symbols* represent uptake in the presence of 4 mM ATP. Vesicles were derived as described under "Experimental Procedures" from the following cell lines: *Panel A*, HeLa C6 (○, ●) and T14 (▽, ▼); *Panel B*, H69 (○, ●) and H69AR (△, ▲); *Panel C*, H69PR (◇, ♦); *Panel D*, 8226/Dox40 (□, ■). The *dotted curve* in *Panel A* (T14) and *Panel B* (H69AR) indicate ATP-dependent uptake, which was calculated by subtracting ATP-independent transport from transport in the presence of ATP. Data points are means of triplicate determinations (±S.E.) in a typical experiment.

in H69AR vesicles was approximately 2-fold faster than for T14 vesicles and was below the limits of detection in vesicles from H69 cells. Low levels of ATP-dependent [^{3}H]LTC_4 uptake could be detected in H69PR vesicles (approximately 1.5 pmol mg^{-1} min^{-1} at room temperature) (Fig. 1*C*) where the levels of MRP are 3–5-fold higher than in H69 cells (3, 19). Vesicles from human 8226/Dox40 cells also exhibited levels of [^{3}H]LTC_4 transport comparable to those of H69PR-derived vesicles (approximately 2 pmol mg^{-1} min^{-1}) (Fig. 1*D*). These cells overexpress P-glycoprotein and contain low levels of MRP similar to those found in H69PR cells (data not shown).

Osmotic Sensitivity and Nucleotide and Cation Specificity of [^{3}H]LTC_4 Transport by T14 Membrane Vesicles—To confirm that LTC_4 accumulation reflected transport of substrate into the vesicle lumen, rather than surface or intramembrane binding, the effect of extravesicular osmolarity on [^{3}H]LTC_4 uptake was measured. As shown in Fig. 2*A*, the rate of [^{3}H]LTC_4 uptake in T14 vesicles was clearly osmotically-sensitive. To establish the nucleotide dependence of LTC_4 transport and to determine whether ATP hydrolysis was required, [^{3}H]LTC_4 uptake was measured in the presence of various nucleoside triphosphates and non-hydrolyzable ATP analogs. As shown in Fig. 2*B*, AMP-PNP, AMP-PCP, and ATPγS (4 mM) were unable to support LTC_4 uptake, suggesting that ATP hydrolysis is necessary for transport. Of the four nucleoside triphosphates tested, LTC_4 uptake was most efficient in the presence of ATP (Fig. 2*B*).

Since the activity of many ATPases is often maintained when other divalent cations are substituted for Mg^{2+}, we investigated the level of ATP-dependent [^{3}H]LTC_4 transport in T14 vesicles in the presence of Mn^{2+}, Ca^{2+}, Co^{2+}, Cd^{2+}, Ba^{2+}, and Zn^{2+} (10 mM). The relative ability of these cations to support LTC_4 transport correlated well with their abilities to support ATP hydrolysis by known ATPases. Thus, when Mn^{2+}, Ca^{2+}, or Co^{2+} were substituted for Mg^{2+}, the [^{3}H]LTC_4 transport rates were measureable but reduced by 25, 50, and 65%, respectively, whereas Cd^{2+}, Ba^{2+}, and Zn^{2+} did not support transport (Fig. 2*C*).

Inhibition of [^{3}H]LTC_4 Transport by ATPase Inhibitors—The inability of non-hydrolyzable ATP analogs to support MRP-mediated LTC_4 uptake and the effect of substituting various cations for Mg^{2+} suggests that ATP hydrolysis, rather than just ATP-binding, is necessary for transport. Consequently, transport activity might be expected to be sensitive to inhibitors of certain classes of ATPases. For this reason, we examined the effects of the P-type ATPase inhibitor sodium vanadate, the Na^+,K^+-ATPase inhibitor ouabain, and the sulfhydryl reagent *N*-ethylmaleimide. [^{3}H]LTC_4 uptake was insensitive to ouabain and vanadate at concentrations up to 1 mM. Preincubation of membrane vesicles with 1 mM *N*-ethylmaleimide at 23 °C had no effect, but at 37 °C, [^{3}H]LTC_4 uptake was inhibited by 70%. This observation suggests that at least one cysteine residue may be important for transport, as has been shown for other transport ATPases (26).

Kinetic Parameters of [^{3}H]LTC_4 Transport in T14 Vesicles—Rates of uptake were measured at several LTC_4 concentrations (12.5–1000 nM) to determine K_m and V_{max} for [^{3}H]LTC_4 transport in T14 membrane vesicles. As shown in Fig. 3*A* (*inset*), a Lineweaver-Burk double reciprocal plot yielded an apparent K_m of 77 nM for LTC_4 and a V_{max} of 291 pmol mg^{-1} min^{-1}. The average values (±S.E.) for K_m and V_{max} for six experiments were 105 ± 31 nM and 529 ± 176 pmol min^{-1} mg^{-1}, respectively. This K_m value is in good agreement with that reported previously for vesicles from human MRP-transfected HeLa T5 cells (97 nM) (10), and for [^{3}H]LTC_4 transport in murine mastocytoma membrane vesicles (70 nM) (16). The V_{max} obtained with T14 vesicles is approximately 5-fold higher than the value previously reported for vesicles prepared from HeLa T5 cells transfected with a different MRP expression vector (100 pmol min^{-1} mg^{-1}) (10). The levels of MRP in T14 cells are 2-fold higher than in T5 cells (15) which may explain the differences in V_{max}. However, it is also possible that differences in procedures used to prepare vesicles may contribute to the higher V_{max} values obtained with T14 vesicles.

The apparent K_m for ATP (70 μM) was determined by measuring initial rates of [^{3}H]LTC_4 uptake at 30 s in the presence of different concentrations of the nucleotide (2–4000 μM) (Fig. 3*B*, *inset*).

Photolabeling of Membrane Proteins with [^{3}H]LTC_4 and Inhibition of Transport with MRP-specific MAb QCRL-3—Previous studies have shown that LTC_4 will bind to a 190-kDa protein enriched in membranes from MRP transfected cells (10). To demonstrate conclusively that the labeled 190-kDa protein was MRP, T14 vesicles were incubated with [^{3}H]LTC_4, uv irradiated to cross-link bound LTC_4, and solubilized membranes were immunoprecipitated with MRP-specific mAbs (19). After SDS-PAGE of the immunoprecipitate, a [^{3}H]LTC_4-labeled 190-kDa protein (Fig. 4*A*, *lane 3*) was detected which co-migrated with [^{35}S]methionine-labeled MRP immunoprecipitated with the same mAbs (Fig. 4*A*, *lane 1*). In addition, the

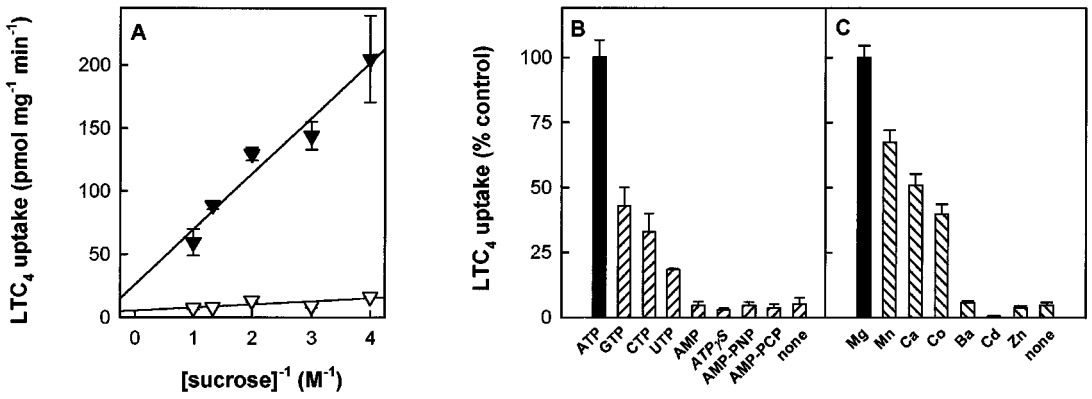

FIG. 2. **Osmotic sensitivity and nucleotide and cation specificity of [^{3}H]LTC_4 transport by T14 membrane vesicles.** *Panel A*, T14 membrane vesicles were preincubated for 10 min in transport buffer containing sucrose (0.25–1 M). Rates of [^{3}H]LTC_4 uptake were measured in the presence of 4 mM ATP (▼) or 4 mM AMP (▼) as described under "Experimental Procedures." *Panel B*, rates of [^{3}H]LTC_4 uptake were measured in the presence of the indicated nucleotides (4 mM). No regenerating system was included in these experiments and the rate of transport in 4 mM ATP was not affected by its omission (*solid bar*). Results obtained with other nucleotides (*hatched bars*) are plotted as a % of values obtained with 4 mM ATP. The data shown are means of triplicate determinations (±S.E.) in a single experiment. *Panel C*, rates of [^{3}H]LTC_4 uptake were measured in the presence of 4 mM ATP and the indicated divalent cations (10 mM) (*hatched bars*). Results are plotted as a % of control LTC_4 uptake values obtained with 10 mM Mg^{2+} at 30 s (*solid bar*). The results shown are means of triplicate determinations (±S.E.) in a single experiment and similar results were obtained in one additional experiment.

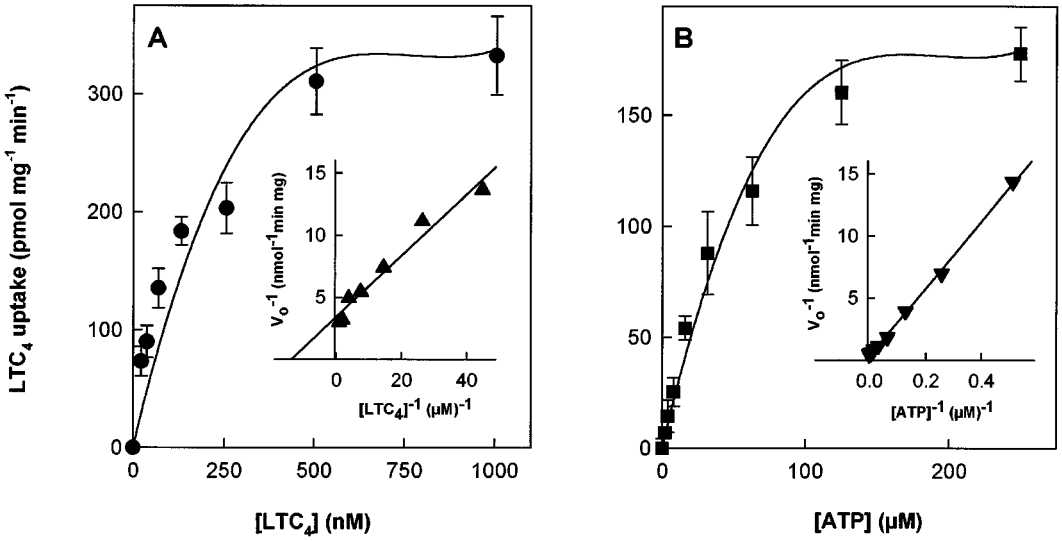

FIG. 3. **Effect of substrate and ATP concentration on [^{3}H]LTC_4 uptake.** *Panel A*, [^{3}H]LTC_4 uptake by T14 membrane vesicles was measured at various LTC_4 concentrations (12.5–1000 nM) for 30 s at 23 °C. Data are plotted as V_o *versus* [S] to confirm that the appropriate concentration range was selected to observe both zero-order and first-order rate kinetics. Kinetic parameters were determined from regression analysis of the Lineweaver-Burk transformation of the data (*inset*). *Panel B*, ATP-dependent uptake of [^{3}H]LTC_4 was measured at various concentrations of ATP (2 μM to 4 mM) in the presence of 50 nM [^{3}H]LTC_4. Uptake at ATP concentrations up to 250 μM are plotted. Kinetic parameters were determined from regression analysis of the Lineweaver-Burk data transformation (*inset*).

MRP-specific mAb QCRL-3, which recognizes a conformation-dependent epitope, inhibited [^{3}H]LTC_4 uptake with an IC_{50} of 5 μg ml^{-1} (50 ng of mAb × μg $protein^{-1}$) (Fig. 4*B*). The MRP-specific mAb QCRL-1, which recognizes a linear MRP epitope, had no effect on LTC_4 transport (not shown) nor did isotype control immunoglobulins up to 30 μg ml^{-1} .

To examine the specificity of the interaction between LTC_4 and MRP, the ability of unlabeled LTC_4 or the unconjugated leukotriene, LTB_4, to compete for [^{3}H]LTC_4 labeling of membrane proteins was determined (Fig. 4*C*). In the absence of competitor, photolabeling of MRP was readily detectable in H69AR (Fig. 4*C*, *lane 2*) and T14 (Fig. 4*C*, *lane 4*), but not in C6 membranes (Fig. 4*C*, *lane 3*), as expected. The relative degree of labeling was consistent with the relative level of MRP in these cells. Photolabeling was inhibited by excess unlabeled LTC_4 (Fig. 4*C*, *lane 5*), but not LTB_4 (Fig. 4*C*, *lane 6*). Labeling was also inhibited by mAb QCRL-3 (Fig. 4*C*, *lane 7*) at a concentration which abolishes [^{3}H]LTC_4 transport (100 ng × μg $protein^{-1}$). MAb QCRL-1 did not inhibit photolabeling (not shown), consistent with its inability to inhibit LTC_4 transport. Finally, to exclude the possibility that LTC_4 labeling of MRP was an artifact resulting from nonspecific labeling of an abundant integral membrane protein, labeling experiments were carried out with 8226/Dox40 vesicles known to contain high levels of P-glycoprotein. As shown in Fig. 4*C* (*lane 1*), no labeling with [^{3}H]LTC_4 was observed.

Inhibition of [^{3}H]LTC_4 Uptake by Alkylated GSH Derivatives—It has been reported previously that the transporter(s) responsible for GSH *S*-conjugate in sarcolemmal (27) and hepatocanalicular (28, 29) membranes is inhibited by alkylated GSH derivatives. Consequently, we investigated whether these compounds had a similar effect on MRP-mediated [^{3}H]LTC_4 transport in T14 vesicles. Inhibition of uptake increased with the length of the alkyl chain and hence with the hydrophobicity of the GSH derivative (Fig. 5*A*). For example, *S*-octyl- and *S*-decyl-GSH inhibited LTC_4 uptake by more than 60% at con-

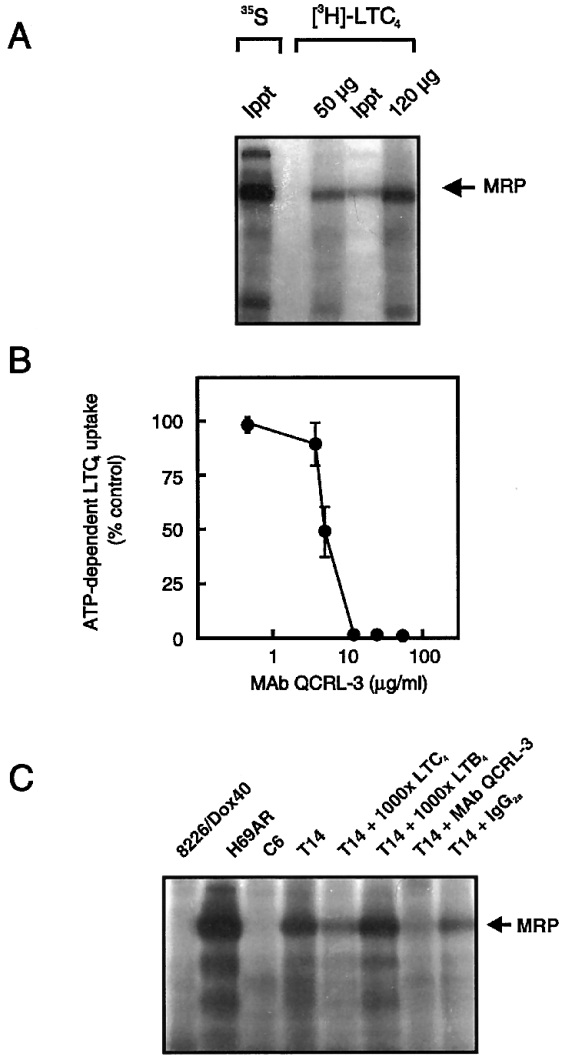

FIG. 4. **Photolabeling of vesicle proteins and inhibition of [^{3}H]LTC_4 uptake and photolabeling by MRP-specific mAb QCRL-3.** *Panel A*, T14 membrane vesicles were photolabeled with [^{3}H]LTC_4 and membrane protein (50 or 120 μg) was analyzed by SDS-PAGE and fluorography. Alternatively, membranes (100 μg of protein) were photolabeled and immunoprecipitated with a mixture of three MRP-specific mAbs ([^{3}H]LTC_4, *middle lane*) (19). For comparison, immunoprecipitations were also performed on membranes prepared from cells labeled in culture with [^{35}S]methionine (^{35}S-*Ippt*, *far left lane*) (18). *Panel B*, [^{3}H]LTC_4 uptake was measured in the presence of MRP-specific mAb QCRL-3. Each point represents the mean of triplicate determinations (±S.E.) in a single experiment. In the experiment shown, the rate of uptake in control incubations was 174 pmol mg^{-1} min^{-1}. *Panel C*, membrane vesicle protein (≈150 μg) from 8226/Dox40, H69AR, C6, or T14 cells was incubated with [^{3}H]LTC_4 (0.5 μCi; 78 nM) in transport buffer, irradiated at 312 nm, and analyzed by SDS-PAGE and fluorography (see "Experimental Procedures"). T14 membrane vesicles were also incubated with [^{3}H]LTC_4 in the presence of excess unlabeled LTC_4, LTB_4, MRP-specific mAb QCRL-3 (10 μg ml^{-1}), and murine IgG_{2a} (70 μg ml^{-1}), prior to irradiation and SDS-PAGE and fluorography. The autoradiograms shown represent a 14-day exposure.

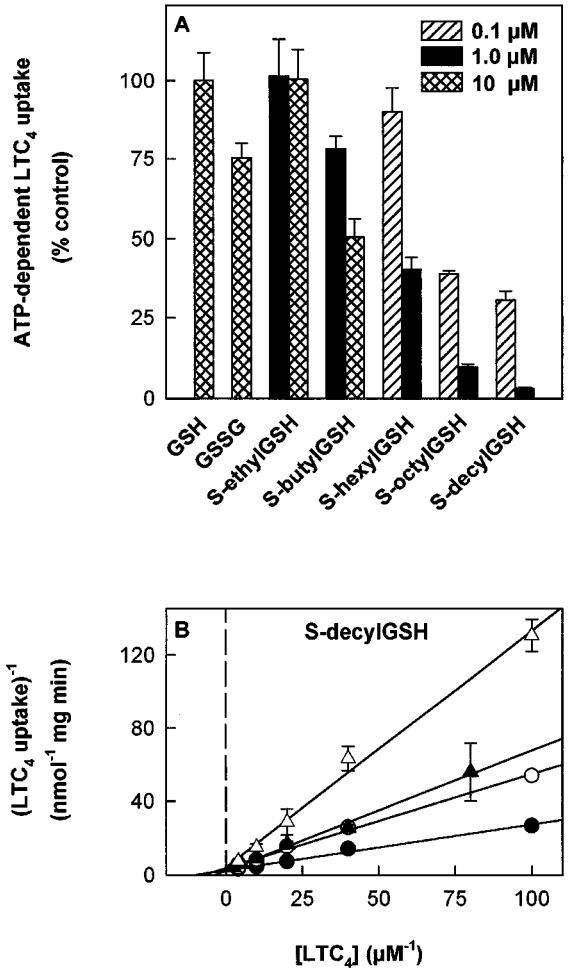

FIG. 5. **Effect of alkylated GSH derivatives on [^{3}H]LTC_4 uptake in MRP-enriched membrane vesicles.** *Panel A*, [^{3}H]LTC_4 uptake into T14 membrane vesicles was measured in the presence of the indicated concentrations of GSH derivatives. Results are plotted as a % of ATP-dependent [^{3}H]LTC_4 uptake in the absence of GSH derivative and each bar represent the mean (± S.E.) of triplicate determinations in a typical experiment. The control uptake rate in this experiment was 190 pmol mg^{-1} min^{-1}. *Panel B*, uptake of [^{3}H]LTC_4 was measured in the presence of various concentrations of *S*-decyl-GSH (control, ●; 50 nM, ○; 100 nM, ▲; 250 nM, △). Double-reciprocal plots were generated and an apparent K_i of 116 nM was calculated from the apparent K_m and V_{max} in the presence of *S*-decyl-GSH.

centrations as low as 100 nM. GSH alone did not inhibit LTC_4 transport even at 5 mM. In contrast, GSSG inhibited transport with an IC_{50} of 100 μM (data not shown) (12). Inhibition of LTC_4 transport by *S*-decyl-GSH was further characterized by determining the kinetic parameters of inhibition. These experiments showed that *S*-decyl-GSH is a competitive inhibitor with an apparent K_i of 116 nM (Fig. 5*B*).

Inhibition of [^{3}H]LTC_4 Transport in Membrane Vesicles by Chemotherapeutic Agents and LTB_4—Several chemotherapeutic agents and the non-cysteinyl leukotriene LTB_4 were tested for their ability to inhibit [^{3}H]LTC_4 transport and the results are shown in Fig. 6. Daunorubicin, doxorubicin, colchicine, and VP-16 at 100 μM and taxol at 40 μM inhibited uptake by only 35–55% (Fig. 6*A*). A modest enhancement of [^{3}H]LTC_4 transport inhibition was observed when the vesicles were coincubated with GSH (1 mM), and colchicine, taxol, or VP-16, but not daunorubicin or doxorubicin (Fig. 6*A*). The small effect seen with colchicine, taxol, and VP-16 may be attributable to the weak inhibition (<15%) of transport exhibited by GSH itself (not shown). In contrast, although VCR and vinblastine alone were also poor inhibitors of [^{3}H]LTC_4 transport (30–50% inhibition at 100 μM), this inhibition was markedly enhanced by coincubation with GSH (1 mM). In the presence of GSH, 50–60 and 90–100% inhibition by vinblastine and VCR was observed at drug concentrations of 10 and 100 μM, respectively (Fig. 6*B*). 2-Mercaptoethanol or dithiothreitol (1 mM) had no effect on the inhibitory potency of vinblastine or VCR (data not shown), indicating that the enhancing effect of GSH was not simply due to its reducing capacity. As expected, LTB_4 was a poor inhibitor of LTC_4 transport, showing little effect at 10 μM, and requiring 100 μM (2000-fold molar excess compared to LTC_4) to inhibit

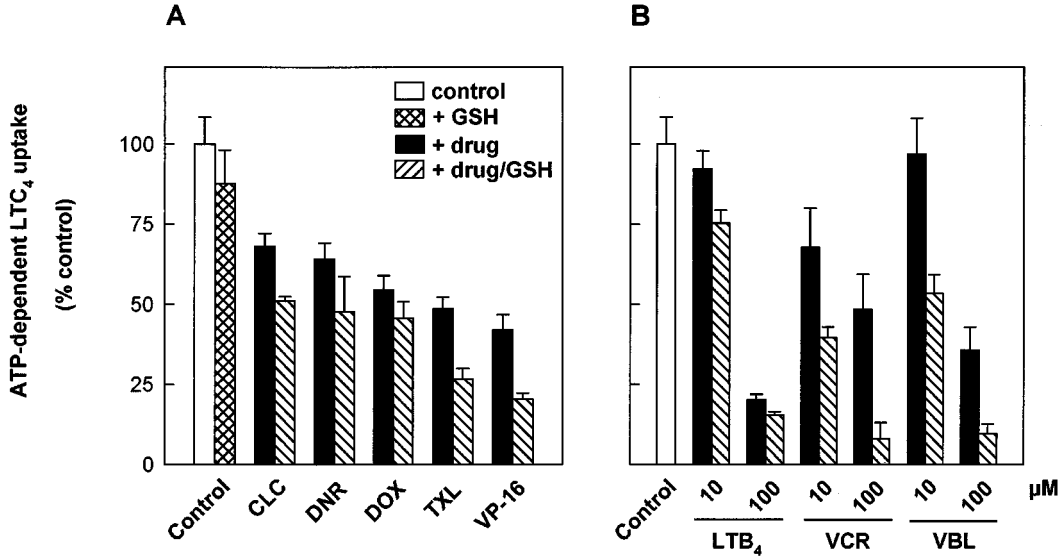

FIG. 6. **Effect of chemotherapeutic agents and LTB_4 on [^{3}H]LTC_4 uptake by T14 vesicles.** The ability of drugs and LTB_4 to inhibit [^{3}H]LTC_4 uptake was measured in the absence or presence of GSH (1 mM) for 30 s at 23 °C. Results were calculated as a % of control values obtained in the absence of both drug and GSH. The *bars* represent the means (±S.E.) of triplicate determinations in a single experiment and similar results were obtained in at least one additional experiment. *Panel A*, the control uptake rate in this experiment was 185 pmol mg^{-1} min^{-1}. *DOX*, doxorubicin; *DNR*, daunorubicin; *CLC*, colchicine; *TXL*, taxol. *Panel B*, the control uptake rate in this experiment was 155 pmol mg^{-1} min^{-1}. *VBL*, vinblastine.

uptake by 80% (Fig. 6*B*). The inhibition by this non-cysteinyl leukotriene was not augmented by GSH.

[^{3}H]Vincristine Transport in Membrane Vesicles—The ability of GSH to enhance inhibition of LTC_4 transport by vinblastine and VCR suggested that GSH may also play a role in the transport of these *Vinca* alkaloids. To investigate this possibility directly, we examined the effect of GSH on [^{3}H]VCR uptake. These experiments were performed as described for LTC_4 uptake, except that they were carried out at 37 °C, the concentration of substrate (VCR) was 200 nM, 10–12-fold more vesicle protein was used, and washing conditions were modified to minimize nonspecific binding of VCR to the filters. No ATP-dependent uptake of [^{3}H]VCR uptake could be detected in either C6 or T14 vesicles and steady-state levels of vesicle-associated VCR were very low (approximately 3 and 6 pmol mg^{-1} for C6 and T14 vesicles, respectively, at 20 min) (Fig. 7*A*). In contrast, ATP-dependent [^{3}H]VCR uptake was demonstrable in T14 but not C6 vesicles when GSH was added and this effect was concentration dependent (Fig. 7*B*). Thus, in the presence of 1, 3, and 5 mM GSH, steady state levels of ATP-dependent VCR uptake in T14 vesicles were increased to approximately 15, 22, and 31 pmol mg^{-1}, respectively, at 20 min. 2-Mercaptoethanol, dithiothreitol, or L-cysteine (up to 5 mM) did not increase VCR uptake, indicating that it was not the reducing capacity of GSH that was responsible for the increase in VCR uptake (Fig. 7*B*). [^{3}H]VCR uptake by T14 vesicles was osmotically-sensitive (not shown) and was inhibitable by 500 μM GSSG (23 ± 5% control), and 200 μM *N*-ethylmaleimide (45 ± 5% control), but not by 200 μM vanadate (85 ± 8% control), consistent with the effect of these reagents on LTC_4 transport.

DISCUSSION

Both P-glycoprotein and MRP are capable of causing resistance to a similar spectrum of drugs when overexpressed in mammalian cells (1, 8). Transfection of MRP or P-glycoprotein into drug-sensitive cells has been shown to result in reduced drug accumulation (8, 30). In the case of P-glycoprotein, there is considerable experimental evidence the protein causes resistance by binding and transporting drugs out of the cell or plasma membrane in an ATP-dependent fashion (2, 9, 31). The mechanism by which MRP mediates reduced drug accumulation in resistant cells is less well understood and in contrast to P-glycoprotein, there is no evidence that unmodified chemotherapeutic agents bind directly to, or are transported by, the protein (7, 8).

In the present study, we further characterized the transport properties of MRP using membrane vesicles derived from both drug-selected MRP-overexpressing cells and a population of transfected HeLa cells (8, 18). We found high-affinity rapid transport of LTC_4 in T14 vesicles that was osmotically sensitive, required hydrolyzable nucleotides, and was supported only by those divalent cations that support the activity of other membrane ATPases, thus providing evidence that ATP hydrolysis as well as binding is required for transport. Transport rates were somewhat lower in T14 vesicles than in vesicles from the drug-selected H69AR cells, in keeping with the relative levels of MRP expression in these two cell types (8). The low but detectable transport in revertant H69PR cells was also consistent with the low levels of MRP in these cells (3, 19). The absence of significant LTC_4 transport in vesicles from P-glycoprotein-overexpressing 8226/Dox40 cells confirmed the MRP-specificity of this transport process. These observations confirm and extend previous studies demonstrating that MRP-enriched vesicles are capable of LTC_4 transport (10–12). Binding of LTC_4 to MRP was shown by immunoprecipitation of a 190-kDa [^{3}H]LTC_4 photoaffinity labeled T14 membrane protein with MRP-specific mAbs. MAb QCRL-3 which detects a conformation-dependent epitope of MRP, also strongly inhibited LTC_4 transport and prevented [^{3}H]LTC_4 labeling of MRP. Taken together, these observations provide strong evidence that LTC_4 binds directly to MRP before being transported and further suggest that LTC_4 binds to MRP at a site within or near the epitope detected by mAb QCRL-3.

LTC_4 is the highest affinity substrate identified to date for MRP and while some modifications of the glutathione or arachidonate moieties of the molecule are tolerated, levels of transport are usually diminished by these changes (10). Our present studies demonstrate that LTC_4 transport by MRP is effectively inhibited by alkylated GSH derivatives. The inhibitory potency of alkylated GSH derivatives with respect to LTC_4 transport in hepatocanalicular (28, 29) and sarcolemmal

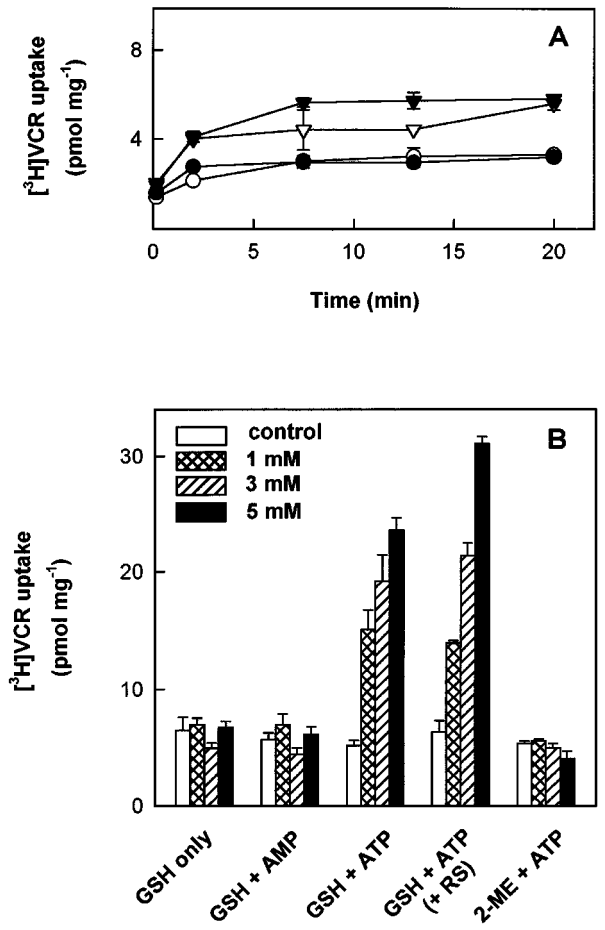

FIG. 7. **Vincristine uptake in membrane vesicles from MRP-transfected HeLa cells.** *Panel A,* membrane vesicles from HeLa C6 (○, ●) and T14 (▽, ▼) cells were incubated with 200 nM [^{3}H]VCR in transport buffer at 37 °C for the times indicated. *Closed symbols* represent uptake in the presence of 4 mM AMP; *open symbols* represent uptake in the presence of 4 mM ATP. *Panel B,* T14 vesicles were incubated for 10 min in 200 nM [^{3}H]VCR in buffer containing 4 mM AMP, ATP, or ATP and an ATP-regenerating system (ATP/RS) as described under "Experimental Procedures." Transport was measured in the absence and presence of the indicated concentrations of GSH and 2-mercaptoethanol (*2-ME*). *Bars* represent the means (±S.E.) of triplicate determinations in a typical experiment and similar results were found in three additional experiments.

(27) membranes has been shown to increase proportionately with the length of the alkyl chain. We also found this to be the case in vesicles from MRP transfectants. Indeed, LTC_4 transport by MRP was significantly more sensitive to inhibition by these compounds than reported for the transporters in rat muscle and liver (27, 29). Inhibition of MRP-mediated LTC_4 transport by the most potent GSH derivative, *S*-decyl-GSH, was competitive, suggesting that it binds to a site in MRP that is similar or possibly overlapping the site to which LTC_4 binds. The K_i (116 nM) for *S*-decyl-GSH was similar to the apparent K_m (105 nM) for LTC_4 transport in T14 membrane vesicles, indicating that this GSH derivative is potentially a high affinity substrate for MRP.

In contrast to cysteinyl leukotrienes and alkylated GSH derivatives, we and others have found that chemotherapeutic drugs are poor inhibitors of LTC_4 transport, exerting significant inhibition only at concentrations 200-2000-fold greater than the K_m of LTC_4 (12, 32). However, we observed that inhibition of LTC_4 transport by certain drugs, most notably the *Vinca* alkaloids VCR and vinblastine, could be significantly enhanced by incubation with physiological concentrations of GSH. Why this enhancement is more pronounced with these two drugs than with others is presently unclear. It does not appear to be simply related to the relative degree of MRP-mediated resistance to a particular agent since T14 cells are considerably more resistant to VCR, doxorubicin, and daunorubicin than they are to vinblastine (8). Nevertheless, the ability of both *Vinca* alkaloids to inhibit LTC_4 transport in T14 membrane vesicles is similarly and markedly enhanced by GSH while inhibition by doxorubicin and daunorubicin are unaffected. A similar potentiating effect of GSH is observed for inhibition of 17β-estradiol 17-(β-D-glucuronide) transport by MRP (44). This effect appears specific for GSH since other thiols (2-mercaptoethanol, L-cysteine, and dithiothreitol) or other organic anions such as glucuronic acid could not substitute for GSH.

We also found that the presence of GSH resulted in demonstrable ATP-dependent [^{3}H]VCR transport by MRP-enriched vesicles. ATP-dependent uptake of [^{3}H]VCR was approximately 31 pmol mg^{-1} at steady state in the presence of 200 nM VCR and 5 mM GSH. This is substantially lower than steady state levels of LTC_4 uptake, GSSG uptake (33), and 17β-estradiol 17-(β-D-glucuronide) uptake (44) in MRP-enriched vesicles but is comparable to that reported for vinblastine uptake in vesicles from certain cell lines overexpressing P-glycoprotein (6, 34).

The mechanism by which GSH enables ATP-dependent VCR transport by MRP, and possibly other chemotherapeutic agents as well, is unclear. GSH has also been reported to potentiate binding of the hydrophobic ligand MK 801 to the integral membrane NMDA receptor (35) but whether the mechanism involved is similar to that which potentiates VCR transport is unknown. It is possible that MRP contains a bipartite binding site for hydrophobic and anionic moieties that would allow binding of non-covalent drug-GSH complexes, or the sequential binding of GSH and drug. Occupation of both elements of the site may be necessary before transport can occur. At present, there is no convincing evidence that GSH is actively co-transported with drug. GSH by itself is not a substrate for transport by MRP (33), and neither does it inhibit LTC_4 transport, even at 5 mM, the highest intracellular concentration likely to be encountered *in vivo*. In contrast, GSSG caused 50% inhibition of LTC_4 transport at approximately 100 μM. Finally, it has been reported that transport of daunorubicin by MRP does not increase GSH release by intact cells (36, 37). Thus it appears unlikely that MRP transports drugs in association with reduced GSH. Alternatively, interaction of GSH with MRP may cause a conformational change (37) or an alteration in the exposure of nonpolar residues, that may favor binding of some hydrophobic compounds prior to transport. Further studies are required to elucidate precisely how GSH enhances VCR transport.

In addition to the cysteinyl leukotrienes and vincristine in the presence of GSH, MRP can transport 17β-estradiol 17-(β-D-glucuronide) and possibly certain other cholestatic steroid glucuronides (44). We have determined that LTC_4 can inhibit 17β-estradiol 17-(β-D-glucuronide) transport and vice versa. Consequently, the presence of a cysteinyl residue is not absolutely required for a compound to be a substrate for MRP-mediated transport. A number of organic anions and cyclic peptides which are neither GSH nor glucuronide conjugates such as MK 571 (K_i 0.6 μM) (16, 38) and cyclosporin A (K_i 5 μM) (10) can behave as competitive inhibitors of LTC_4 transport in membrane vesicles from drug-selected cells known to overexpress MRP. Since cyclosporin A is not an effective chemosensitizer in MRP-overexpressing cells (30, 39, 40), the ability of a compound to inhibit LTC_4 transport is clearly not always in-

dicative of its capacity to reverse MRP-associated resistance nor its ability to act as a substrate.

We and others have clearly shown that MRP-overexpressing cells are not resistant to cisplatin (8, 20, 30, 41, 42). The presence of ATP-dependent transport of a glutathione-platinum complex in a platinum-resistant cell line has been demonstrated and it was suggested that the transporter or "GS-X pump" in these cells may be MRP (43). Although this transport activity is pharmacologically similar to MRP in some respects, in that it is inhibitable by LTC_4, GSSG, and *S*-dinitrophenylglutathione, the overexpression of MRP mRNA and protein in these cells has not been shown. Furthermore, we found that cisplatin did not inhibit LTC_4 transport in T14 vesicles either by itself or in combination with GSH (results not shown). Finally, Muller and co-workers (12) were unable to demonstrate LTC_4 transport in a platinum-resistant lung cancer cell line which exhibits reduced drug accumulation. Thus current evidence suggests that MRP is not involved in conferring resistance to platinum-containing drugs although it remains possible that a transporter related to MRP may be responsible, at least in some platinum-resistant cell lines.

To date, there is little known about the mechanism of ATP-dependent MRP-mediated transport of cysteinyl leukotrienes, steroid glucuronides, or drugs in association with GSH. However, it is possible that substrate binding by MRP (in the presence of GSH in the case of chemotherapeutic drugs) facilitates the binding and subsequent hydrolysis of ATP, which in turn induces a conformational change in MRP that allows for the release of the transported molecule(s) into the extracellular space. Experiments aimed at determining whether the ATPase activity of MRP is stimulated by LTC_4, 17β-estradiol 17-(β-D-glucuronide), or chemotherapeutic agents in the absence or presence of GSH are underway. Mapping of the mAb QCRL-3 epitope is also in progress and together with proteolytic mapping studies of [^{3}H]LTC_4-labeled MRP, should allow the LTC_4 binding site on the MRP molecule to be identified.

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