Structure and *in Vitro* Substrate Specificity of the Murine Multidrug Resistance-Associated Protein†

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ABSTRACT: MRP is a recently described ATP-binding cassette transporter that confers cellular resistance to natural product cytotoxic drugs. To examine the biochemical activity and cellular physiology of this transporter, we isolated the murine MRP homologue and analyzed its *in vitro* substrate specificity. Murine MRP transcript is widely expressed in tissues and encodes a protein of 1528 amino acids that is 88% identical to its human homologue. Hydropathy analysis indicated that murine and human MRP, the yeast cadmium resistance transporter and the sulfonylurea receptor share a conserved topology distinguished from P-glycoprotein and the cystic fibrosis conductance regulator by an N-terminal hydrophobic region that contains several potential transmembrane domains. Drug uptake assays performed with membrane vesicles prepared from NIH3T3 cells transfected with a murine MRP expression vector revealed ATP-dependent transport for the natural product cytotoxic drugs daunorubicin and vincristine, as well as for the glutathione *S*-conjugates leukotriene C_4 and azidophenacyl-*S*-glutathione. Drug transport was osmotically sensitive and saturable with regard to drug and ATP concentrations, with K_m values of 19 μM, 19 μM, 26 nM, 17 μM, and 77 μM for daunorubicin, vincristine, leukotriene C_4, APA-SG, and ATP, respectively. Consistent with broad substrate specificity, the drug glutathione conjugate APA-SG, oxidized glutathione, the LTD_4 antagonist MK571, arsenate, and genistein were competitive inhibitors of daunorubicin transport, with K_i values of 32 μM, 25 μM, 1.9 μM, 108 μM, and 23 μM, respectively. This study demonstrates that the substrate specificity of murine MRP is quite broad and includes both the neutral or mildly cationic natural product cytotoxic drugs and the anionic products of glutathione conjugation. The widespread expression pattern of murine MRP in tissues, combined with its ability to transport both lipophilic xenobiotics and the products of phase II detoxification, indicates that it represents a widespread and versatile cellular defense mechanism.

Cellular resistance to cytotoxic drugs is a major obstacle to the treatment of disseminated cancers (Young, 1990). Cell lines selected *in vitro* with natural product cytotoxic agents can display a multidrug resistant phenotype that involves simultaneous resistance to a spectrum of structurally and functionally distinct agents, including anthracyclines, vinca alkaloids, and epipodophyllotoxins. One form of multidrug resistance results from expression of P-glycoprotein, the 170-kDa product of the MDR1 gene. P-glycoprotein, an ATP-binding cassette (ABC) family transporter, functions as an ATP-dependent efflux pump that reduces the intracellular concentrations of lipophilic cytotoxic agents by transporting them across the plasma membrane (Endicott & Ling, 1989; Gottesman & Pastan, 1993). Recently MRP,[1] another ABC transporter, was isolated from a doxorubicin-selected cell line that does not overexpress P-glycoprotein (Cole et al., 1992). We and others have reported transfection studies that have established that expression of MRP confers resistance to natural product cytotoxic agents (Grant et al., 1994; Kruh et al., 1994). Several previously characterized non-P-glycoprotein multidrug resistant cell lines have also been shown to overexpress MRP (Barrand et al., 1994; Krishnamachary & Center, 1993; Kruh et al., 1994; Slovak et al., 1993; Zaman et al., 1993). MRP has been reported to be overexpressed in drug-selected murine cell lines (Lorico et al., 1995; Slapak et al., 1994), suggesting that the murine MRP homologue also confers resistance to natural product drugs.

Although the drug resistance phenotype of a variety of MRP-overexpressing cell lines has been described, studies describing the biochemical mechanism by which MRP confers drug resistance have not been reported. The detection of increased drug efflux in cells transfected with the human MRP cDNA (Breuninger et al., 1995; Cole et al., 1994; Zaman et al., 1994) and in human (Coley et al., 1991; McGrath & Center, 1988; Slovak et al., 1988; Versantvoort et al., 1992) and murine (Lorico et al., 1995) drug-selected cell lines that overexpress MRP suggests that MRP may function as a pump for natural product drugs, similar to P-glycoprotein. However, the possibility that MRP confers resistance by transporting either the glutathione *S*-conjugates or other anionic metabolites of lipophilic drugs, as opposed to unaltered drugs, has been suggested based upon reports

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[1] Abbreviations: APA-SG, azidophenacyl-*S*-glutathione; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; LTR, long terminal repeat; MRP, multidrug resistance-associated protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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demonstrating that MRP in membrane vesicle preparations transports glutathione conjugates such as DNP-SG, APA-SG, and the endogenous glutathione-conjugated leukotriene LTC_4 (Jedlitschky et al., 1994; Leier et al., 1994a; Muller et al., 1994; Shen et al., 1996).

In the present study, we examined the biochemical substrate specificity of murine MRP. To accomplish this, we isolated the murine MRP cDNA and analyzed drug transport into inside-out membrane vesicles prepared from transfected NIH3T3 cells. We demonstrate that murine MRP transports natural product cytotoxic drugs such as daunorubicin and vincristine, as well as glutathione *S*-conjugates such as the cysteinyl leukotriene LTC_4. These transport studies indicate that the substrate specificity of murine MRP is quite broad and support the idea that the direct transport of unaltered natural product drugs is an important component of MRP-conferred multidrug resistance.

MATERIALS AND METHODS

Materials and Cell Lines. Antipain, creatine phosphate, ATP, 5′-AMP, pepstatin A, benzamidine, RNase A, DNase I, oxidized glutathione, and genistein were purchased from Sigma. Creatine kinase, leupeptin A, and aprotinin were purchased from Boehringer Mannheim. [^{3}H(G)]Daunomycin (3.7 Ci/mmol) and [^{3}H]leukotriene C_4 (140 Ci/mmol) were purchased from DuPont-NEN; [^{3}H]etoposide (900 mCi/mmol), [^{3}H]vinblastine sulfate (9 Ci/mmol), and [^{3}H]taxol (7.4 Ci/mmol) were purchased from Moravek Biochemicals; and [^{3}H]vincristine sulfate (10.6 Ci/mmol) was purchased from Amersham. Arsenate was purchased from J. T. Baker. Cyclosporin A and PSC833 were generously provided by Sandoz Pharmaceuticals. MK571 was a generous gift of A. W. Ford-Hutchinson (Merck-Frost Center for Therapeutic Research, Quebec).

pSRα-36-8-32, a G418-selected NIH3T3 cell line transfected with a human MRP expression vector, and pSRα-36-7M, a G418-selected NIH3T3 cell line transfected with control plasmid pSRαMSVtkneo, have been previously described (Breuninger et al., 1995). NIH3T3 cells used for transfection were kindly provided by Dr. Stuart Aaronson (Mount Sinai Cancer Center, New York).

Molecular Cloning of Murine MRP. A radiolabeled human MRP probe was used to screen a murine testes cDNA library (a gift of Dr. Toru Miki, NCI, Bethesda, MD) by plaque hybridization. Approximately 7.5×10^5 phage were screened, and 7 positive clones were isolated. Restriction digestion analysis indicated that the inserts were overlapping cDNAs. Nucleotide sequence analysis was performed on both strands of a single 6 kb clone using an Applied Biosystems automated sequencer.

RNA Blot Analysis. A filter containing 2 μg samples of murine poly(A^+) RNAs (Clontech, Palo Alto, CA) was hybridized with a ^{32}P-labeled murine MRP probe or an actin control probe. Hybridization was performed according to the directions of the manufacturer, and MRP was detected by autoradiography.

Expression of Murine MRP in NIH3T3 Cells. The murine MRP cDNA was inserted into retroviral pSRαMSVtkneo, an LTR-driven expression vector that contains a neomycin selection marker (Muller et al., 1991), to create pSRα-muMRP. NIH3T3 cells were transfected using the $CaCl_2$ precipitation method as described (Wigler et al., 1977) with pSRα-muMRP or pSRαMSVtkneo control DNA. Individual G418 resistant colonies were isolated by the cloning cylinder technique and expanded. Cell lines were not exposed to cytotoxic chemotherapeutic agents.

Generation of MRP Monoclonal Antibody. A segment of the murine MRP cDNA encoding amino acids 1364−1528 was generated by PCR and inserted into the bacterial expression vector pD10 (a gift of Frank Raucher, Wistar Institute). The recombinant histidine fusion protein was expressed in bacteria and purified using a nickel column. Six female BALB/c mice were immunized with 50 μg aliquots of the fusion protein mixed 1:1 with Freund's complete adjuvant. The mice were reinjected 1 month later using Freund's complete adjuvant. One month later, serum was tested by enzyme-linked immunosorbent assay (ELISA) as described (Bizub-Bender et al., 1994) using 96-well microtiter plates coated with the recombinant fusion protein. The mouse with the highest titer was immunized 3 months later by intravenous injection with 50 μg of the antigen, and 3 days later the spleen was removed and its cells were fused with SP2/0 cells using poly(ethylene glycol) as described (Harlow & Lane, 1988; Shulman et al., 1978). The cell mixture was seeded into 24 96-well microtiter plates at a concentration of 2.3×10^4 cells/mL in Opti-MEM medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 5% J774A.1 supernatant, and containing hypoxanthine−aminopterin−thymidine (1 × HAT), 55 mM 2-mercaptoethanol, 50 μg/mL gentamicin, 1.13 nM oxaloacetate, 50 ng/mL sodium pyruvate, and 0.2 unit/mL bovine zinc insulin. Beginning 12 days after fusion, culture medium was tested for the presence of antibodies to the recombinant fusion protein by ELISA. Several positive cultures were tested for reactivity to MRP in immunoblots containing lysates of MRP-overexpressing cell lines. One supernatant detected murine and human MRP in immunoblots and was designated muMRPmAb-1.

Immunoblot Analysis. Fifty microgram protein samples of cell lysates or membrane vesicles were separated by 6% SDS−PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Filters were blocked in PBS containing 0.1% Tween-20 (PBST) and 5% bovine serum albumin, and MRP was detected using muMRPmAb-1, and a secondary antimouse antibody. Blots were washed several times with PBST before and after the secondary antibody treatment and 3 times in PBS alone before development with an Amersham ECL chemiluminescence kit.

Preparation of Membrane Vesicles. Membrane vesicles were prepared as described (Ishikawa et al., 1990; Leier et al., 1994b; Shen et al., 1996). Briefly, $\sim 5 \times 10^9$ control-transfected (pSRα-66-3M) or murine MRP-transfected (pSRα-muMRP-66-16-6) NIH3T3 cells were pelleted by centrifugation at 1200*g* for 10 min, and washed twice in ice-cold phosphate-buffered saline (PBS). The cell pellet was diluted 40-fold with hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM EGTA) supplemented with protease inhibitors (2 mM PMSF, 0.5 μg/mL leupeptin, 2 μg/mL aprotinin, 200 μg/mL benzamidine, 1 μg/mL pepstatin A, 50 μg/mL antipain), 25 μg/mL RNase A, and 50 μg/mL DNase I and gently stirred on ice for 2 h. The cell lysate was centrifuged at 100000*g* for 40 min, and the pellet was resuspended in 20 mL of hypotonic buffer and homogenized with a Potter−Elvehjem homogenizer. The resulting homogenate was diluted with incubation buffer (250 mM sucrose/10 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors and centrifuged at 12000*g* for 10 min. The postnuclear super-

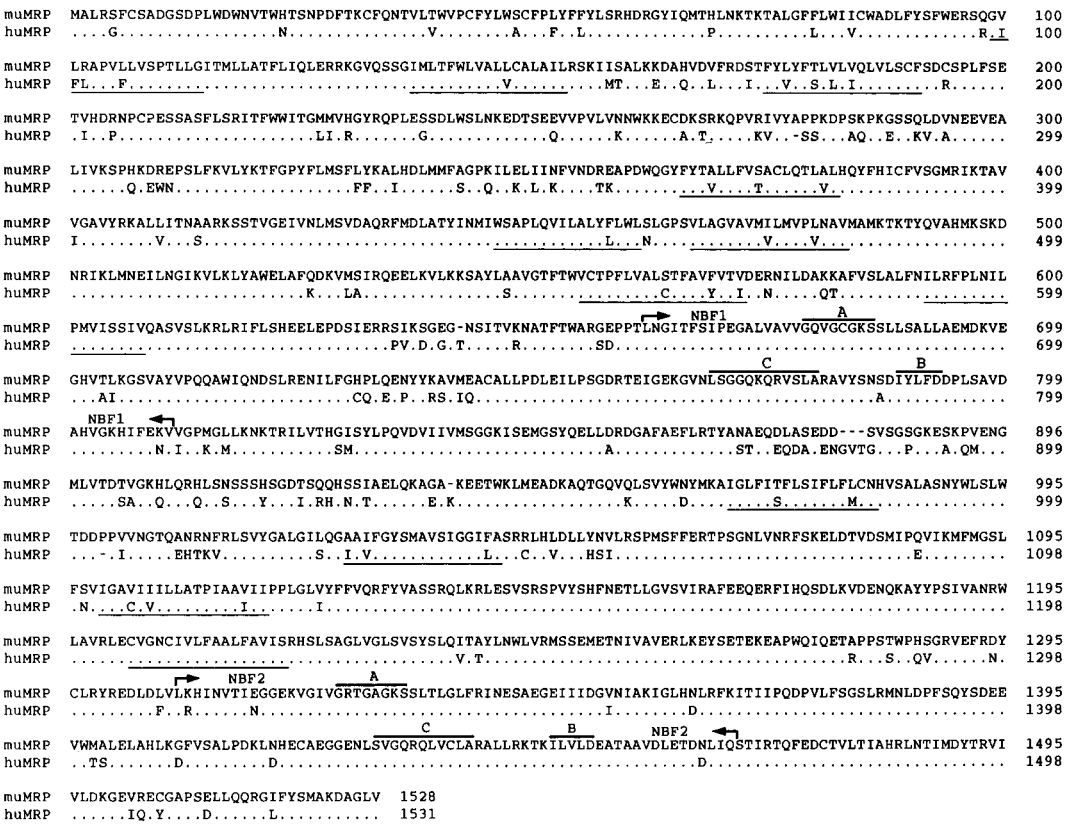

FIGURE 1: Comparison of murine and human MRP. The predicted sequence of murine MRP is shown on top of the human MRP sequence. Identical amino acids are indicated by periods, and gaps are indicated by dashes. The N- and C-terminal nucleotide binding folds (NBF1 and NBF2, respectively) are indicated by arrows. The Walker A, B, and C motifs of the nucleotide binding folds are indicated by overbars, and the suggested transmembrane domains of the human protein (Cole et al., 1992) are underlined.

natant was kept on ice, and the pellet was resuspended in 20 mL of incubation buffer supplemented with protease inhibitors, and homogenized and centrifuged at 12000*g* as described above. The two postnuclear supernatants were combined and centrifuged at 100000*g* for 40 min. The pellets were resuspended in 20 mL of incubation buffer and manually homogenized with a tight-fitting Dounce homogenizer. After dilution with 10 mL of incubation buffer, the suspension was layered onto 38% sucrose/5 mM Hepes−KOH, pH 7.4, and centrifuged in a Beckman SW41 swinging-bucket rotor at 280000*g* for 2 h. The interphase was collected, diluted in 20 mL of incubation buffer, and homogenized with a Dounce homogenizer. The resulting suspension was centrifuged at 100000*g* for 40 min, and the pellet was resuspended in 1 mL of incubation buffer. All operations were carried out at 4 °C.

Membrane vesicles were formed by passing the final membrane suspension through a 27 gauge needle 20 times. Ten milliliters of the vesicle suspension was mixed with 2.5 mL of Con A−Sepharose (Pharmacia) equilibrated with 160 mM Tris-HCl (pH 7.4) and incubated at 4 °C for 30 min with gentle shaking. The Con A−Sepharose was removed by centrifugation at 1400*g* for 5 min, and the inside-out membrane vesicles in the supernatant were collected by centrifugation at 100000*g* for 30 min. The pellet was suspended in incubation buffer and stored at −80 °C. Purity of membrane vesicles was assayed using marker enzyme activities as described (Leier et al., 1994b).

Analysis of Drug Uptake by Membrane Vesicles. Drug uptake by membrane vesicles was measured by the rapid filtration technique (Horio et al., 1988). Inverted membrane vesicles were quickly thawed at 37 °C shortly before use and kept on ice. Radiolabeled drugs were mixed with ATP reaction buffer (10 mM Tris-HCl, pH 7.5/4 mM ATP/10 mM $MgCl_2$/10 mM creatine phosphate/0.25 M sucrose/100 μg/mL creatine kinase), and membrane vesicles (20 μg of protein) were added. The suspension was incubated at 37 °C, and 20 μL aliquots were removed at various time points and diluted in 1.0 mL of ice-cold incubation buffer. The suspensions were immediately applied to 0.45 μm Millipore nitrocellulose filters (presoaked in incubation buffer) under suction and washed twice with 5.0 mL of ice-cold incubation buffer. The filters were dissolved in scintillation fluid, and the radioactivity was measured in a Beckman liquid scintillation counter. In parallel control experiments, ATP was replaced with 4 mM 5′-AMP. ATP-dependent transport was determined by subtracting the uptake values obtained with 5′-AMP from those obtained with ATP. Osmotic sensitivity experiments were performed by incubating the membrane vesicles in incubation buffer containing various concentrations of sucrose for 15 min at room temperature. Drug uptake was then measured in the presence of either ATP or 5′-AMP as described above, except that the sucrose concentration of the ATP reaction buffer was adjusted to that of the incubation buffer.

RESULTS

Molecular Cloning and Predicted Structure of Murine MRP. Overlapping murine MRP cDNA clones were obtained from a testes cDNA library using a probe derived from the human MRP cDNA (Breuninger et al., 1995). Nucleotide sequence analysis of a 6 kb cDNA clone revealed a large open reading frame followed by a stop codon and 1.5 kb of 3′ untranslated sequence. Figure 1 shows a comparison of the predicted murine MRP protein encoded by this open reading frame and the human MRP protein. Overall the

amino acid sequence of the murine protein was quite similar to its human homologue, sharing 88% amino acid identity. The previously suggested transmembrane domains of human MRP (Cole et al., 1992) are well conserved in the murine homologue. Comparison of the murine MRP amino acid sequence revealed that, among the ABC transporters, it is most closely related to the yeast cadmium resistance transporter (YCF1, 43% identity) and the sulfonylurea receptor (SUR, 35% identity), two proteins that have been described since the human MRP sequence was first reported (Cole et al., 1992). Murine MRP was also closely related to the cystic fibrosis transporter (CFTR, 28% identity) and the leishmania P-glycoprotein homologue (PGPA, 32% identity). Figure 2A shows a comparison of the amino (NBF1) and carboxyl (NBF2) nucleotide binding folds of murine MRP with related transporters. A comparison of hydrophobicity plots of murine MRP with other related transporters revealed that murine and human MRP, YCF1, and the sulfonylurea receptor share a series of five hydrophobic segments located at the amino terminus of the protein (Figure 2B). This distinct structural feature was absent in PGPA, CFTR, and MDR1.

The expression pattern of murine MRP was examined by analyzing transcript levels in poly(A+) RNA prepared from a variety of tissues. As shown in Figure 3, a 7 kb transcript was readily detected in each of seven tissues examined. Roughly comparable levels of murine MRP transcript were observed in the tissues analyzed.

Expression of Murine MRP in NIH3T3 Cells. To examine the biochemical transport properties of murine MRP, the cDNA was first expressed in NIH3T3 cells. The murine MRP coding sequence was inserted downstream of the LTR promoter of the pSRαMSVtkneo expression vector, to create pSRα-muMRP, and NIH3T3 cells were transfected with either the pSRαMSVtkneo control plasmid or the MRP expression vector. Transfected cells were selected in medium containing G418, resistance to which is conferred by the *neo* resistance gene of the vector. To facilitate protein detection in isolated G418 resistant colonies, a monoclonal antibody was generated against the C-terminal 165 amino acids of murine MRP expressed as a recombinant histidine fusion protein. Since this segment of murine MRP is 93% identical to its human homologue, the monoclonal antibody was initially characterized using our previously reported NIH3T3 cells transfected with the human MRP cDNA (Breuninger et al., 1995; Paul et al., 1996). As shown in Figure 4A, murine MRP monoclonal antibody muMRPmAb-1 readily detected the human MRP protein overexpressed in NIH3T3 cells. Several G418-selected clones were analyzed for protein expression using this antibody, and pSRα-muMRP-66-16-6, the clone with the highest level of murine MRP protein expression, was chosen for further characterization. Figure 4B shows the detection of murine MRP in pSRα-muMRP-66-16-6 and pSRα-66-3M, a G418-selected control cell line obtained by transfection of expression vector lacking cDNA insert. In pSRα-muMRP-66-16-6, the ~175−190 kDa murine MRP protein was expressed at levels approximately ~5−8-fold greater than in the control transfectant. The lower molecular weight bands represent degradation products.

ATP-Dependent Uptake of Daunorubicin by Inside-Out Membrane Vesicles. Drug transport studies using inside-out membrane vesicles prepared from the MRP and control transfectants (Figure 4C) were used to examine the biochemical substrate specificity of the transporter. Figure 5A shows a time course of daunorubicin uptake over 2 min. To distinguish between ATP-dependent transport of drug into membrane vesicles and nonspecific binding, vesicle-associated drug was measured in the presence of AMP or ATP. As has been previously reported for vesicle transport studies of lipophilic cytotoxic drugs (Horio et al., 1988; Kamimoto et al., 1989; Lelong et al., 1992), appreciable nonspecific binding of daunorubicin was detected (AMP controls). Comparable levels of nonspecific binding were detected for the MRP and control vesicles. However, in the presence of ATP, substantial daunorubicin uptake was observed for the pSRα-muMRP-66-16-6 vesicles but not for the control vesicles. When nonspecific binding was subtracted, the MRP transfectant vesicles displayed ~27-fold greater net ATP-dependent uptake than the control vesicles at 2 min. In agreement with a previous analysis of *in vitro* transport by human MRP expressed in NIH3T3 cells (Paul et al., 1996), the activity of the endogenous transporter was not observed, suggesting that its expression level is too low for detection using this assay system.

ATP Dependence and Osmotic Sensitivity of Daunorubicin Transport. Biochemical features of energy-dependent transport into membrane vesicles, as opposed to nonspecific binding, include increasing uptake with increasing ATP concentrations and inhibition of uptake at high osmotic pressure. High osmotic pressure shrinks membrane vesicles, thereby reducing the intravesicular space and decreasing drug uptake (Horio et al., 1988; Kamimoto et al., 1989; Lelong et al., 1992). As shown in Figure 6A, daunorubicin uptake was dependent on ATP concentration. The linear Lineweaver−Burk blot indicated that transport was saturable with respect to ATP concentration, with a K_m of 77 μM for ATP (inset). Osmotic sensitivity was examined by analyzing daunorubicin uptake after the membrane vesicles were incubated in buffer containing various concentrations of sucrose. As expected for transport into membrane vesicles, ATP-dependent uptake of daunorubicin decreased as sucrose concentrations were increased above the standard assay conditions of 0.25 M, whereas nonspecific binding in the presence of AMP was unchanged (Figure 6B). At 1.25 M sucrose, uptake in the presence of ATP was nearly indistinguishable from the AMP values.

Substrate Specificity of Murine MRP. The *in vitro* drug specificity of murine MRP was analyzed by performing membrane vesicle uptake studies with a variety of lipophilic cytotoxic agents. As shown in Figure 5B, in addition to daunorubicin, markedly enhanced ATP-dependent uptake by the MRP transfectant was observed for vincristine. When the nonspecific background was subtracted, the MRP transfectant vesicles exhibited ~23-fold net increased ATP-dependent uptake compared to the control vesicles at 2 min. Similar levels of increased uptake were also observed for doxorubicin (data not shown). In contrast, markedly enhanced uptake was not detected for the natural product drugs etoposide, Taxol, and vinblastine (panels E, F, and G). However, when nonspecific binding was subtracted, slight increases in net ATP-dependent uptake were observed for these three agents (1−2-fold). The ability of murine MRP to transport glutathione conjugates was examined by analyzing the uptake of the cysteinyl leukotriene LTC_4 and the glutathione photoaffinity analogue APA-SG (Kunst et al., 1989). Enhanced uptake was detected for both of these compounds (panels C and D). When the nonspecific

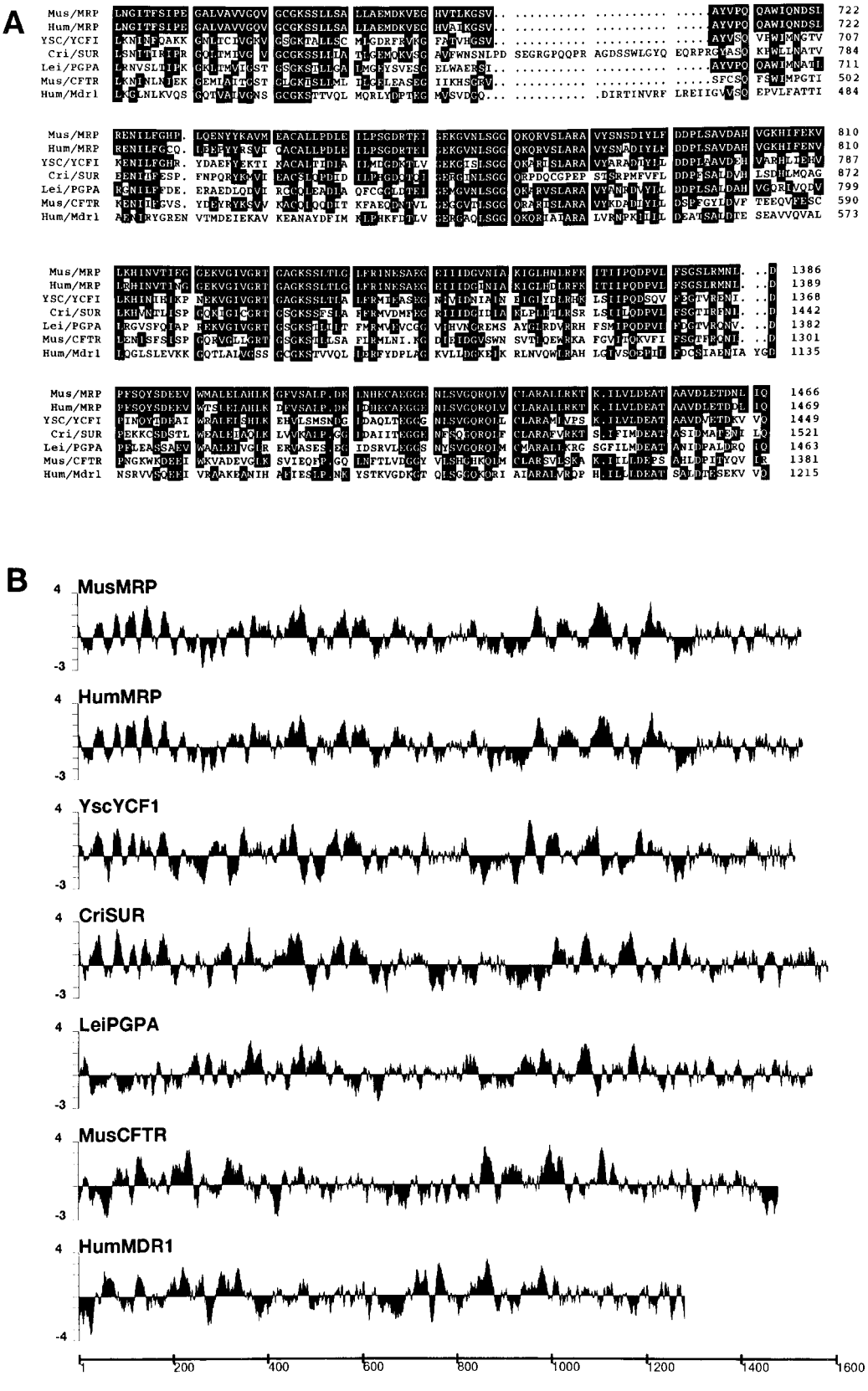

FIGURE 2: Comparison of the nucleotide binding folds and hydropathy profile of murine MRP with those of related ABC transporters. (A) Comparison of murine MRP NBF1 (top two alignments) and NBF2 (bottom two alignments) with the nucleotide binding folds of related transporters. The percent amino acid identity of murine MRP NBF1 was 91, 53, 47, 53, 41, and 35 for Hum/MRP, YSC/YCF1, Cri/SUR, Lei/PGPA, MusCFTR, and Hum/Mdr1, respectively. For murine MRP NBF2, the percent amino acid identities were 94, 63, 58, 47, 40, and 34 for Hum/MRP, YSC/YCF1, Cri/SUR, Lei/PGPA, MusCFTR, and Hum/Mdr1, respectively. Abbreviations and GenBank Accession Numbers: Hum/MRP, human MRP (P33527); YSC/YCF1, yeast metal resistance protein (P39109); Cri/SUR, hamster sulfonylurea receptor (Q09427); Lei/PGPA, leishmania P-glycoprotein (P21441); MusCFTR, murine cystic fibrosis transporter (P26361); Hum/Mdr1, human multidrug resistance transporter (P08183). (B) Comparison of the hydropathy profile of murine MRP with those of related transporters. Hydrophobicity is indicated by positive values, and hydrophilicity is indicated by negative values. Hydropathy was analyzed using the Kyte and Doolittle algorithm (Kyte & Doolittle, 1982).

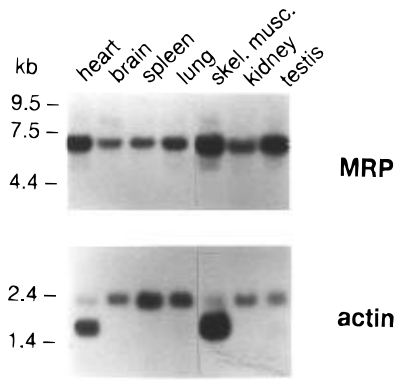

FIGURE 3: Expression of murine MRP transcript in various tissues. Filters that contained 2 μg of poly(A^+) RNA were hybridized with either murine MRP (top) or actin (bottom) probes. Positions of size markers are shown to the left.

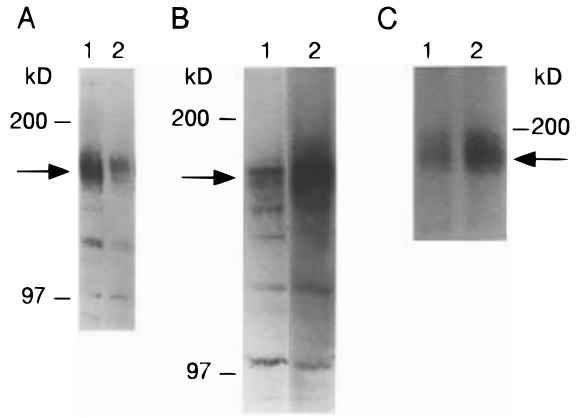

FIGURE 4: Immunoblot analysis of MRP in cell lysates and inside-out membrane vesicles prepared from MRP and control transfectants. (A) Detection of human MRP. Lane 1: human MRP-transfected NIH3T3 cell line pSRα-36-8-32. Lane 2: control vector-transfected NIH3T3 cell line pSRα-36-7M. (B and C) Detection of murine MRP in cell lysates (B) and inside-out membrane vesicles (C) prepared from control transfectant pSRα-muMRP-66-3M (lanes 1) and murine MRP transfectant pSRα-66-16-6 (lanes 2). Protein samples (50 μg) were separated by 6% SDS−PAGE, and MRP was detected by immunoblotting with murine MRP monoclonal antibody muMRPmAb-1. MRP is indicated by arrows, and the positions of molecular mass markers are shown.

background was subtracted, the MRP transfectant vesicles exhibited ~22- and 11-fold increased net ATP-dependent uptake, respectively, compared to the control vesicles.

Concentration Dependence of Natural Product Drug, LTC_4, and APA-SG Transport. To quantitate the affinity of MRP for its substrates, the concentration dependence of transport was analyzed for daunorubicin, vincristine, LTC_4, and APA-SG. Typical Lineweaver−Burk plots are shown in Figure 7. Transport was saturable with respect to the concentration of each of these compounds with K_m values of 19 μM for daunorubicin and vincristine (panels A and B), and 17 μM for APA-SG (panel D). The K_m of LTC_4 was 26 nM (panel C), substantially lower than the K_m values for the natural product cytotoxic agents, indicating that this compound is a particularly high-affinity substrate.

Inhibition of Daunorubicin Uptake by Diverse Agents. The transport of both neutral or mildly cationic lipophilic cytotoxic drugs and the anionic glutathione conjugates LTC_4 and APA-SG indicated that the substrate specificity of murine MRP is quite broad. To confirm this broad substrate specificity, the inhibition of daunorubicin transport by diverse agents was analyzed. Figure 8 shows that anionic com-

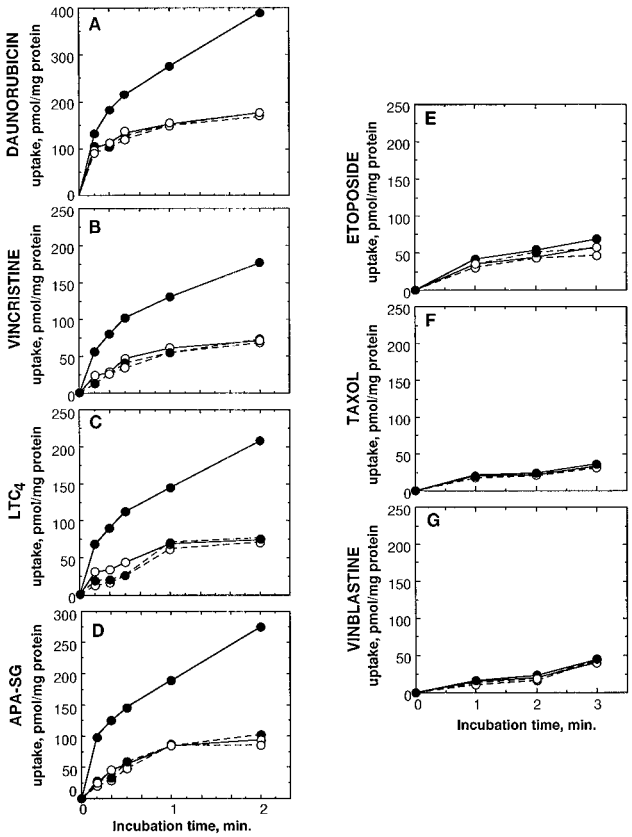

FIGURE 5: ATP-dependent uptake of natural product cytotoxic drugs and glutathione conjugates. ATP-dependent uptake of daunorubicin (A), vincristine (B), LTC_4 (C), APA-SG (D), etoposide (E), Taxol (F), and vinblastine (G) by inside-out membrane vesicles prepared from murine MRP transfectant pSRα-muMRP-66-16-6 (solid lines) or control transfectant pSRα-66-3M (broken lines). Uptake was measured in the presence of 1.2 mM ATP (closed circles) or 1.2 mM 5′-AMP (open circles). The concentrations of cytotoxic drugs, APA-SG, and LTC_4 were 5 μM, 10 μM, and 50 nM, respectively. Each data point represents the average of triplicate determinations. Experiments were performed several times with similar results, and typical experiments are shown.

pounds, including APA-SG, oxidized glutathione, the LTD_4 antagonist MK571 (Jones et al., 1989), and arsenate, a compound for which MRP has been shown to confer resistance (Cole et al., 1994), inhibited daunorubicin uptake with K_i values of 32 μM, 25 μM, 1.9 μM, and 108 μM, respectively. In addition, the lipophilic compound genistein, an isoflavonoid inhibitor of protein tyrosine kinases that has been shown to modulate multidrug resistance in MRP-overexpressing cell lines (Versantvoort et al., 1993), also inhibited uptake ($K_i = 23$ μM). The increases in the apparent K_m of daunorubicin with no alterations in V_{max} indicate that these compounds are competitive inhibitors and suggest the possibility that they share a common or overlapping binding site on the transporter with lipophilic drugs.

DISCUSSION

The observations that transfection of human MRP confers resistance to a spectrum of lipophilic cytotoxic agents (Grant et al., 1994; Kruh et al., 1994) and that overexpression of human and murine MRP occurs in cell lines made drug-resistant by stepwise exposure to natural product cytotoxic agents (Coley et al., 1991; Lorico et al., 1995; McGrath & Center, 1988; Slapak et al., 1994; Slovak et al., 1988; Versantvoort et al., 1992) suggest that expression of both human and murine MRP confers resistance to some members

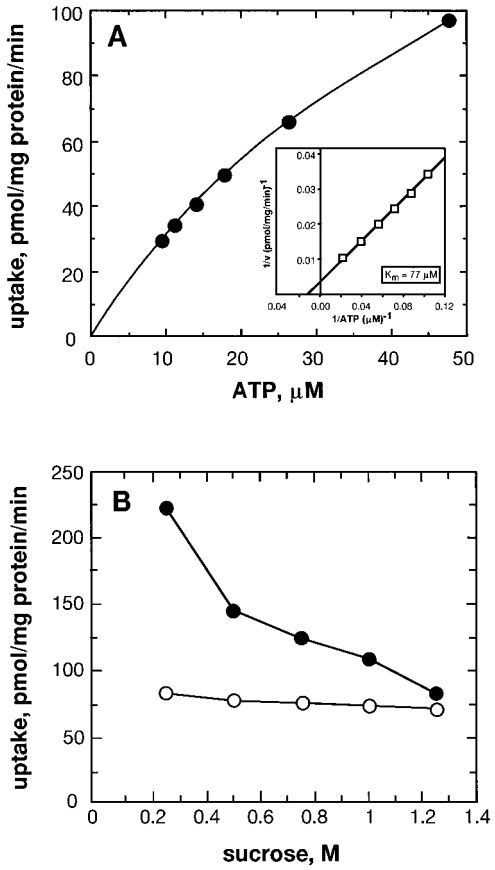

FIGURE 6: ATP dependence and osmotic sensitivity of daunorubicin transport. ATP concentration dependence and osmotic sensitivity of daunorubicin transport by inside-out membrane vesicles prepared from MRP transfectant pSRα-muMRP-66-16-6. (A) ATP concentration dependence of daunorubicin transport. The inset shows a Lineweaver−Burk plot of the data. The K_m and V_{max} of ATP were 77 μM and 286 pmol mg^{-1} min^{-1}, respectively. (B) Osmotic sensitivity of daunorubicin uptake. Open and closed circles indicate AMP and ATP values, respectively. The concentration of ATP was 9.5−47.6 μM in (A) and 1.2 mM in (B). The concentration of daunorubicin in (A) and (B) was 5 μM.

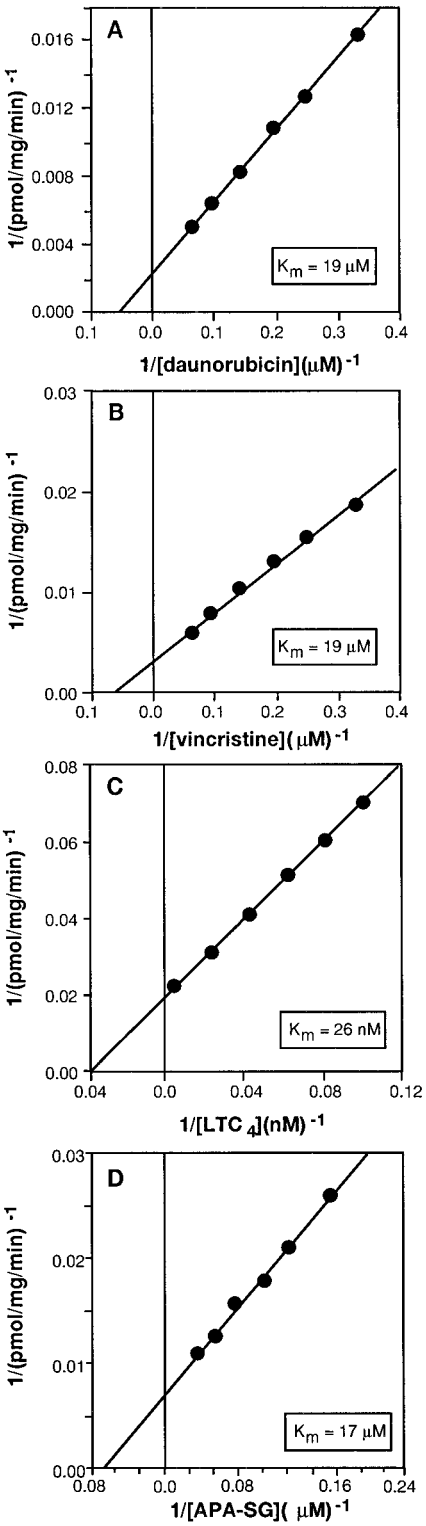

FIGURE 7: Kinetic analysis of drug and glutathione conjugate transport. Lineweaver−Burk analysis of the concentration dependence of daunorubicin (A), vincristine (B), LTC_4 (C), and APA-SG (D) transport by inside-out membrane vesicles prepared from MRP transfectant pSRα-muMRP-66-16-6. The concentrations of daunorubicin and vincristine were 3−15 μM, and the concentrations of LTC_4 and APA-SG were 10−150 nM and 6−30 μM, respectively. The concentration of ATP was 1.2 mM. The K_m values of vincristine, daunorubicin, LTC_4, and APA-SG were 19 μM, 19 μM, 26 nM, and 17 μM, respectively. The V_{max} values were 417, 357, 53, and 147 pmol mg^{-1} min^{-1}, respectively.

of this class of drugs. Previous studies indicating that human MRP in membrane vesicles transports glutathione conjugates (Jedlitschky et al., 1994; Leier et al., 1994a; Muller et al., 1994; Shen et al., 1996) and the observation that it shares only 15% amino acid identity with the natural product efflux pump P-glycoprotein (Cole et al., 1992), have led to speculation that MRP confers resistance to lipophilic cytotoxic drugs by transporting either their glutathione conjugates or their anionic metabolites (Ishikawa et al., 1995). To understand how the biochemical substrate specificity of MRP relates to its cellular drug resistance activity, we isolated the murine MRP homologue and analyzed its *in vitro* activity. Using membrane vesicles prepared from NIH3T3 cells transfected with a murine MRP expression vector, we found that the transporter is an energy-dependent pump of both lipophilic cytotoxic drugs and glutathione conjugates such as APA-SG and the cysteinyl leukotriene LTC_4. Consistent with this substrate specificity, daunorubicin transport was competitively inhibited by a variety of agents, including glutathione conjugates and oxidized glutathione. This substrate specificity is similar to that of human MRP, for which we have also observed transport of lipophilic cytotoxic agents and glutathione conjugates (Paul et al., 1996; Shen et al., 1996), and supports the idea that the transporter confers resistance to natural product cytotoxic agents by functioning as an efflux pump for unaltered chemotherapeutic agents. This idea is consistent with the observation that transfection of human MRP does not confer resistance to alkylating agents (Breuninger et al., 1995), the class of chemothera-

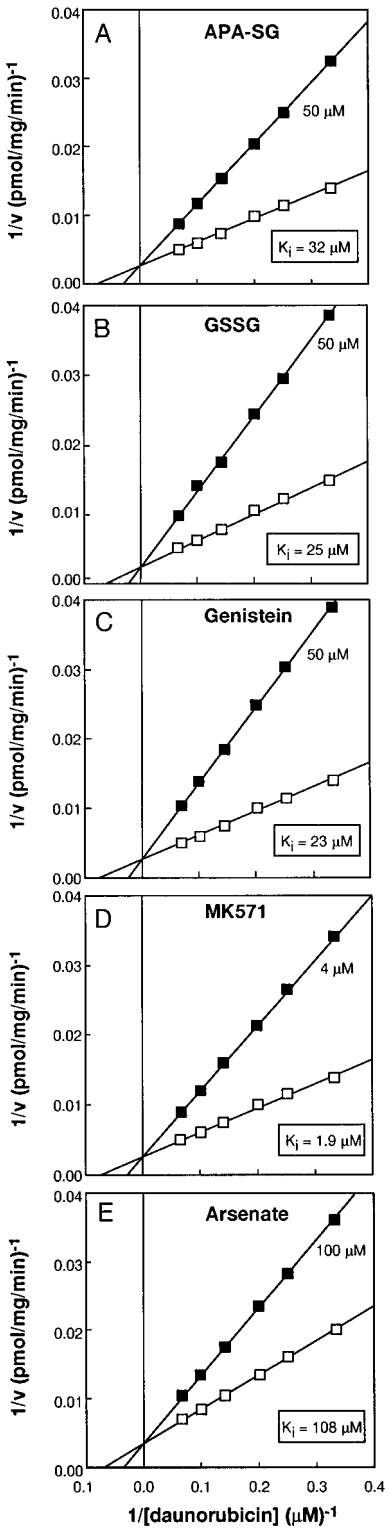

FIGURE 8: Inhibition of daunorubicin transport by various compounds. Lineweaver−Burk analysis of the inhibition of daunorubicin transport into inside-out membrane vesicles prepared from MRP transfectant pSRα-muMRP-66-16-6 by APA-SG (A), oxidized glutathione (B), genistein (C), MK571 (D), and arsenate (E). Open boxes indicate values in the absence of inhibitors, and closed boxes indicate values in the presence of inhibitors. The concentration of ATP was 1.2 mM, and the concentration of daunorubicin was 3−15 μM. The concentrations of inhibitors were 50 μM (APA-SG), 50 μM (GSSG), 50 μM (genistein), 4 μM (MK571), and 100 μM (arsenate). K_i values are indicated in the enclosed boxes.

peutic agents for which glutathione conjugation is firmly established, the absence of evidence indicating that natural product cytotoxic drugs are conjugated to glutathione (Tew, 1994), and the observation that natural product cytotoxic agents are competitive inhibitors of glutathione conjugate transport by human MRP in membrane vesicles (Shen et al., 1996).

Although this analysis indicates that the amino acid sequences of murine and human MRP are 88% identical and that the proteins transport a similar spectrum of compounds, the present study suggests that there may be differences in the biochemical activities of the two transporters. While we found that both proteins pump anthracyclines and vincristine, etoposide transport was detected for the human (Paul et al., 1996) but not the murine protein. In addition, while the K_m values for APA-SG and LTC_4 for the murine (17 μM and 26 nM) and human protein (15 μM and 35 nM) (Paul et al., 1996) are quite similar, the K_m values for daunorubicin and vincristine for the murine protein (19 μM) are significantly higher than the K_m values for the human protein (6.3 μM and 4.2 μM). These differences may be of functional significance in terms of the biological activities of the two proteins and possibly suggest that the murine protein may be less potent than its human counterpart in conferring natural product resistance.

Consistent with the ability of murine MRP to transport lipophilic agents, amplification and overexpression of this gene have been observed in two drug resistant murine leukemia cell lines obtained by stepwise selection in natural product agents. Overexpression of murine MRP has been reported for PC4 cells selected for high levels of resistance to vincristine (Slapak et al., 1994), an agent for which we detected transport activity in membrane vesicles, and for WEHI-3B cells selected for resistance to novobiocin (Lorico et al., 1995), a topoisomerase II targeted natural product antibiotic. However, the drug resistance profiles described for these two cell lines are neither in complete agreement with each other nor with the substrate specificity we describe here. The PC4/VCR cell line is not resistant to doxorubicin but displays etoposide resistance, whereas we detected membrane vesicle transport for doxorubicin but not etoposide. In contrast, the WEHI-3B/NOVO cell line is not resistant to vincristine, but displays increased resistance to etoposide (Lorico et al., 1995). The absence of complete agreement is not surprising since the basis for resistance in cell lines obtained by stepwise selection in drug is multifactorial (de Jong et al., 1990). In addition to potential contributions to resistance by other cytoplasmic and membrane proteins, it is possible that mutations in the MRP protein may occur in response to selective pressure by drugs and these alterations could potentially influence the activity of the transporter. This possibility is consistent with the observations that human MRP cDNAs isolated from different drug resistant cell lines differ in their amino acid sequences (Breuninger et al., 1995; Cole et al., 1992) and that mutations in MDR1 cDNAs isolated from drug selected cell lines influence the biological activities of the encoded proteins (Gottesman & Pastan, 1988). Analysis of murine MRP transfectants should define the drug resistance phenotype conferred by this protein.

Hydropathy analysis of the predicted murine and human MRP proteins revealed strikingly similar profiles. A distinctive feature of this membrane topology is a hydrophobic N-terminal region of ~200 amino acids that contains several potential transmembrane spanning domains. This feature is also present in the yeast cadmium resistance transporter (Wemmie et al., 1994) and the sulfonylurea receptor (Agui-

lar-Bryan et al., 1995), but absent in P-glycoproteins and the cystic fibrosis transporter. Although the significance of this region is currently unknown, its presence in several ABC transporters suggests a conserved function. In the initial report describing the amino acid sequence of human MRP, a unique membrane topology consisting of eight N-terminal and four C-terminal transmembrane helices appended N-terminal to two cytoplasmic ATP-binding domains was suggested (Cole et al., 1992). This membrane topology is distinct from the typical 6 + 6 configuration that has been proposed for ABC transporters [for review, see Higgins (1992)]. Analysis of the murine MRP sequence indicates that the transmembrane domains suggested for the human protein are well conserved. Experiments designed to elucidate the membrane topology of MRP should help to define the significance of the N-terminal hydrophobic segment and establish the precise location of transmembrane domains.

In this report, we establish that murine MRP has broad substrate specificity that includes both the anionic products of glutathione conjugation as well as neutral or weakly cationic natural product cytotoxic drugs. In addition, we found that murine MRP is widely expressed in normal tissues. The widespread expression pattern of murine MRP, combined with its ability to transport both phase II detoxification products as well as lipophilic xenobiotics, indicates that it represents a widespread and remarkably versatile cellular defense against potentially harmful xenobiotics. Additional studies should help to define the physiological functions of this protein.

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