Functional and Structural Consequences of Cysteine Substitutions in the NH_2 Proximal Region of the Human Multidrug Resistance Protein 1 (MRP1/ABCC1)†

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ABSTRACT: The 190 kDa multidrug resistance protein 1 (MRP1; ABCC1) is comprised of three membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs) configured MSD1-MSD2-NBD1-MSD3-NBD2. MRP1 overexpression in tumor cells results in an ATP-dependent efflux of many oncolytic agents and arsenic and antimony oxyanions. MRP1 also transports GSSG and GSH as well as conjugated organic anions, including leukotriene C_4 and 17β-estradiol 17-(β-D-glucuronide) and certain xenobiotics in association with GSH. Previous studies have shown that portions of MSD1 and the cytoplasmic loop (CL3) connecting it to MSD2 are important for MRP1 transport function. In the present study, Cys residues at positions 43, 49, 85, 148, and 190 in MSD1 and positions 208 and 265 in CL3 were mutated to Ala and Ser, and the effects on protein expression, plasma membrane localization, trypsin sensitivity, organic anion transport, and drug resistance properties were investigated. Confocal microscopy showed that 11 of 14 mutants displayed significant levels of nonplasma membrane-associated MRP1. Most mutant proteins were also more resistant to trypsin proteolysis than wild-type MRP1. All Cys mutants transported organic anions (0.5−1.5-fold wild-type MRP1 activity), and cells expressing Ser-substituted but not Ala-substituted Cys43 and Cys265 MRP1 mutants exhibited a 2.5-fold decrease and a 3-fold increase in arsenite resistance, respectively; Cys43Ser MRP1 also conferred lower levels of vincristine resistance. These results indicate that certain Cys residues in the NH_2 proximal region of MRP1 can be important for its structure and selected transport activities.

Resistance to multiple anticancer agents is a major impediment to the successful treatment of many malignant diseases. In tumor cells, multidrug resistance has most often been associated with overexpression of one of two plasma membrane ATP-binding cassette (ABC) drug transporters: the 170 kDa P-glycoprotein (ABCB1) (*1*, *2*) or the 190 kDa MRP1 (ABCC1)[1] (*3*, *4*). At least two additional proteins closely related to MRP1, MRP2 (ABCC2) and MRP3 (ABCC3) (*5*), and a third more distantly related ABC protein, breast cancer resistance protein (BCRP/ABCG2), can also confer resistance to a variety of natural product drugs (*6*). The overexpression of these polytopic integral membrane proteins results in an ATP-dependent decrease in cellular drug accumulation. Increased expression of MRP1, P-glycoprotein, and BCRP have also been reported in a variety of hematological and solid tumors, suggesting an important role for these transport proteins in clinical drug resistance (*4*, *6*, *7*).

MRP1 and most other members of ABC subfamily C have a five domain structure that includes a third NH_2 proximal MSD with five TM segments and an extracytosolic NH_2 terminus (MSD1-MSD2-NBD1-MSD3-NBD2) (*3*, *8*−*10*). In addition to its role in drug resistance, MRP1 is a primary active transporter of GSH and GSSG as well as GSH, glucuronate, and sulfate-conjugated organic anions. Conjugated substrates of MRP1 that are of potential physiological relevance include the mediator of inflammation, LTC_4, and the cholestatic $E_2$17βG (*10*). In addition, overexpression of MRP1 confers resistance to trivalent, and to a lesser extent, pentavalent arsenic- and antimony-centered oxyanions (*11*).

The transport mechanisms of MRP1 appear varied and complex. For example, many of the anticancer agents to which MRP1 confers resistance are not conjugated to any extent in vivo and there is strong evidence indicating that they are transported in noncovalent association with GSH (*12*−*16*). In addition, transport of certain conjugated substrates of MRP1, which were expected to be transported in a GSH-independent manner, is significantly enhanced by or is completely dependent on the presence of this tripeptide (*17*−*19*). Furthermore, it has been recently demonstrated that the tricyclic isoxazole derivative LY475776 and the azido derivative of the polyhydroxylated sterol acetate agosterol A bind to and inhibit transport mediated by MRP1 in a GSH-

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[1] Abbreviations: DTT, dithiothreitol; TM, transmembrane; MRP1, multidrug resistance protein 1; MSD, membrane spanning domain; NBD, nucleotide binding domain; GSH, reduced glutathione; GSSG, oxidized glutathione; LTC_4, leukotriene C_4; $E_2$17βG, 17β-estradiol 17-(β-D-glucuronide); CL3, cytoplasmic loop 3; MAb, monoclonal antibody.

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dependent manner (*20*−*22*). Transport of GSH by MRP1 can be stimulated by certain compounds such as verapamil that appear not to be transported themselves but nevertheless significantly enhance the affinity (K_m) of MRP1 for this tripeptide (*23*). Overall, the interactions between GSH and MRP1 are clearly critical for MRP1 function but are complex and poorly understood.

The precise function of the extra NH_2-terminal 320 amino acids comprising MSD1 and the CL3 of MRP1 connecting it to MSD2 is yet to be fully elucidated (*8*, *9*). Computer-based secondary structure analyses predict that CL3 contains two α-helical regions. Bakos et al. (*24*) have shown that CL3 is closely associated with the membrane and further that deletion of one amphipathic α-helix (amino acids 221−233) prevents this association. In previous studies, we have shown that an NH_2-truncated MRP1 lacking the first 67 amino acids ($MRP1_{67-1531}$) does not transport LTC_4, which indicates that the region containing the extracellular NH_2 terminus and the first TM helix is required for at least some aspects of MRP1 transport function (*25*). Recently, the Cys residue at position 7 has been shown to be critical for MRP1 structure and function providing further evidence that this region is important (*26*). On the other hand, studies of other truncated mutants have shown that MRP1 lacking the first five TM helices ($MRP1_{204-1531}$) binds and transports LTC_4 at levels comparable to the full length protein while MRP1 polypeptides with further deletions into CL3 (as in $MRP1_{229-1531}$) do not (*25*, *27*, *28*). These findings suggest that CL3 also plays an important role in MRP1 substrate and inhibitor recognition and transport (*24*, *25*, *27*). Interestingly, three nonsynonymous single nucleotide polymorphisms within MSD1 and CL3 of MRP1 have recently been described; however, the functional significance of these amino acid substitutions is not yet known (*29*, *30*).

In the present study, we have examined the phenotypic consequences of substituting Cys residues within these two functionally important regions of MRP1. Five Cys residues at positions 43, 49, 85, 148, and 190 in the predicted TM segments of MSD1 and two Cys residues at positions 208 and 265 in CL3 were substituted with Ala and Ser using site-directed mutagenesis. The Cys mutant proteins were stably expressed in HeLa cells and clonal cell lines tested for various aspects of MRP1 function.

MATERIALS AND METHODS

Materials. [14,15(n)-^{3}H]LTC_4 (38 Ci mmol^{-1}) was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, U.K.). [6,7-^{3}H]$E_2$17βG (55 Ci/mmol) and [^{3}H]GSH (50 Ci/mmol) were from Perkin-Elmer Life Science Research Products (Guelph, ON, Canada). LTC_4 was purchased from CalBiochem (La Jolla, CA) and $E_2$17βG, GSH, nucleotides, $MgCl_2$, 2-mercaptoethanol, DTT, acivicin, doxorubicin HCl, vincristine sulfate, and apigenin were obtained from Sigma Chemical Co. (St. Louis, MO). Heavy metal oxyanions used in chemosensitivity assays were obtained as described previously (*11*). Creatine kinase, creatine phosphate, and protease inhibitor cocktail tablets were from Roche Molecular Biochemicals (Laval, PQ, Canada). Diphenylcarbamyl chloride-treated trypsin was purchased from ICN Biomedicals (St. Laurent, PQ, Canada).

Site-Directed Mutagenesis. Cys mutations were generated using the Transformer site-directed mutagenesis kit (CLONTECH Laboratories, Inc., Palo Alto, CA) or the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The template for mutagenesis was prepared by subcloning the 860 bp *Xba*I/*Bam*HI fragment (first 840 nucleotides of MRP1 coding sequence) of pcDNA3.1(−)$MRP1_K$ into pGEM-3Z (Promega, Madison, WI) (*31*). Mutagenesis was performed according to the manufacturer's instructions with the following sense mutagenic primers (substituted nucleotides are underlined and introduced or lost restriction sites are in italics) as follows: Cys43Ala (5′-G TGG GTG CCT GCT TTT TAC CTC TGG GCC-3′), Cys43Ser (5′-G TGG GTG CCT TCT TTT TAC CTC-3′), Cys49Ala (5′-C CTC TGG GCC *GCA TTC* CCC TTC TAC-3′) (*BsmI*), Cys49Ser (5′-C CTC TGG GCC TCT TTC CCC TTC-3′), Cys85Ala (5′-G TGG ATC GT*C GCG* TGG GCA GAC C-3′) (*BstUI*), Cys85Ser (5′-G TGG ATC GTC AGC TGG GCA GAC C-3′), Cys148Ala (5′-GTA *GCC CTA GTG GC*T GCC CTA GCC-3′) (*BglI*), Cys148Ser (5′-GTA GCC CTA GTG TCT GCC CTA GCC-3′), Cys190Ala (5′-C GTC TTG TCC *GCA TTC* TCA GAT CGC-3′) (*BsmI*), Cys190Ser (5′-C GTC TTG TCC TCT TTC TCA GAT CG-3′), Cys208Ala (5′-C CCT AAT CC*C GCG* CCA GAG TCC AG-3′) (*BstUI*), Cys208Ser (5′-C CCT AAT CCC AGC CCA GAG TCC-3′), Cys265Ala (5′-GTA AAG AAC TGG AAG AAG GAA GCC GC*G AAG ACT A*GG AAG CAG-3′) (*BpiI*), and Cys265Ser (5′-GG AAG AAG GAA TCC GCC AAG ACT AG-3′) (*BsmI*). The 860 bp *Xba*I/*Bam*HI fragment was subcloned back into pcDNA3.1(−)-$MRP1_K$, and the fragments in the full length constructs were sequenced completely (Cortec DNA Service Laboratory Inc., Kingston, ON, Canada).

Stable Transfection of MRP1 Expression Vectors in HeLa Cells. For cell lines stably expressing wild-type and mutant MRP1, approximately 1.5×10^5 HeLa cells were seeded in each well of a six well plate, and 24 h later, DNA (1μg) was transfected using FuGENE6 (3 μL) (Roche Diagnostics, Laval, PQ, Canada). After 48 h, the cells were subcultured 1:12 and the medium was replaced with fresh medium containing G418 (Geneticin) (GIBCO BRL, Burlington, ON, Canada) (1000 μg mL^{-1}). After approximately 17 days, cell colonies were removed individually by scraping and aspirating with a pipet tip as described (*32*) and subcultured in the presence of 600 μg mL^{-1} G418. Levels of MRP1 protein in G418 resistant cell populations were then determined by immunodot blotting with the MRP1 specific MAb QCRL-1 as described previously (*33*, *34*). The MRP1 positive clones were tested for the proportion of cells expressing MRP1 by flow cytometry (Beckman Coulter ALTRA Flow Cytometer) using the MRP1 specific MAb QCRL-3 as described previously (*35*). Populations of less than 80% were further cloned by limiting dilution to obtain populations of 80−100% expressing MRP1. Prior to testing for transport activity and drug resistance levels, MRP1 expression levels were determined by immunoblot and flow cytometry as described above. A second, completely independently generated clonal cell line was established for each Cys mutant and fully characterized when the phenotype of the first mutant clone differed from that of wild-type MRP1 (WT-MRP1).

Confocal Microscopy. Dual staining experiments for nuclei and MRP1 expression were carried out using a Leica TCS SP2 MS multiphoton system confocal microscope (Leica Microsystem, Heidelberg, Germany). Stably transfected HeLa cells expressing wild-type and mutant MRP1 were

seeded at 8×10^5 cells/well in a six well plate on gelatinized coverslips. Thirty-six hours later, the cells were fixed with 4% paraformaldehyde for 10 min and then incubated in permeabilization buffer (0.2% Triton X-100 in PBS) for 5 min, washed three times in blocking solution (0.1% Triton X-100/1% bovine serum albumin in PBS) over 15 min, and then incubated with MAb QCRL-3 (1:2500 dilution in blocking solution) for 1 h. After they were washed with blocking solution three times over 1 h, Alexa Fluor 488 conjugated goat antimouse IgG (H + L), $(Fab')_2$ fragment (Molecular Probes, Eugene, OR) was added (1:500 dilution) with RNase A (10 μg/mL) and incubated in the dark for 30 min. Nuclei were then stained with propidium iodide (2 μg/mL) for 20−45 min, and coverslips were placed on slides with antifade solution (Molecular Probes). Cells were observed at 488 nm excitation and 519 nm emission wavelengths at 1000× magnification. Negative control experiments with IgG_{2a} as the primary antibody and Alexa-488 goat antimouse IgG $(Fab')_2$ as secondary antibody and with the secondary antibody alone were also performed.

Measurement of MRP1 Levels in Cell Lines. For transport experiments, plasma membrane vesicles were prepared from MRP1 wild-type and mutant stably transfected HeLa cells by nitrogen cavitation followed by sucrose gradient centrifugation, as described (*13*). For chemosensitivity testing, crude membranes were prepared as described previously (*35*). Proteins were resolved on a 6−7% polyacrylamide gel and electrotransferred to a nylon membrane. Immunoblotting was performed with MAb QCRL-1 to determine relative levels of MRP1 expression (*33*). Each blot was stained with amido black after chemiluminescence detection to ensure consistent protein loading in each lane. Relative levels of MRP1 expression were estimated by densitometry of multiple film exposures with a ChemiImagerJ 4000 (Alpha Innotech, San Leandro, CA).

Proteolytic Analysis of Membrane Proteins. For trypsinolysis experiments, membrane vesicles were diluted to 0.5 μg/μL in Tris sucrose buffer (250 mM sucrose, 50 mM Tris, pH 7.5) and incubated at 37 °C with diphenylcarbamyl chloride-treated trypsin at trypsin:protein ratios ranging from 1:10 000 to 2.5:1 (w:w) for 15 min. Proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride (10 mM) and leupeptin (16.7 μg/mL) in Laemmli buffer. Samples were then loaded onto a 4−15% Ready Gel (Bio-Rad Laboratories, Hercules, CA) at 2 μg protein per lane. Immunoblotting was performed as described previously with rat MAb MRPr1 (diluted 1:2500), which detects a linear epitope in CL3 (amino acids 238 to 247) (*36*).

Membrane Vesicle Transport Studies. ATP-dependent transport of [^{3}H]LTC_4, [^{3}H]$E_2$17βG, and [^{3}H]GSH into membrane vesicles was determined using the rapid filtration method as described (*37*) with minor modifications. Transport in the presence of AMP was subtracted from transport in the presence of ATP and reported as ATP-dependent uptake for each substrate. LTC_4 uptake was measured for 1 min at 23 °C in a 50 μL volume containing vesicle protein (2 μg), ATP or AMP (4 mM), $MgCl_2$ (10 mM), creatine phosphate (10 mM), creatine kinase (100 μg/mL), and [^{3}H]-LTC_4 (50 nM; 40 nCi). Uptake was terminated by adding the entire reaction mix to 800 μL of ice-cold Tris sucrose buffer. $E_2$17βG uptake was measured as described for LTC_4 except uptake assays were carried out at 37 °C with 5 μg of vesicle protein and [^{3}H]$E_2$17βG (400 nM; 40 nCi). To measure GSH uptake, membrane vesicles (20 μg protein) were preincubated with 500 μM acivicin (an inhibitor of γ-glutamyl transpeptidase) at 37 °C for 10 min. Transport activity was then measured by further incubation at 37 °C for 20 min in a total reaction volume of 60 μL containing [^{3}H]GSH (100 μM, 120 nCi), DTT (10 mM), apigenin (30 μM), and components as described above for [^{3}H]LTC_4 uptake assays.

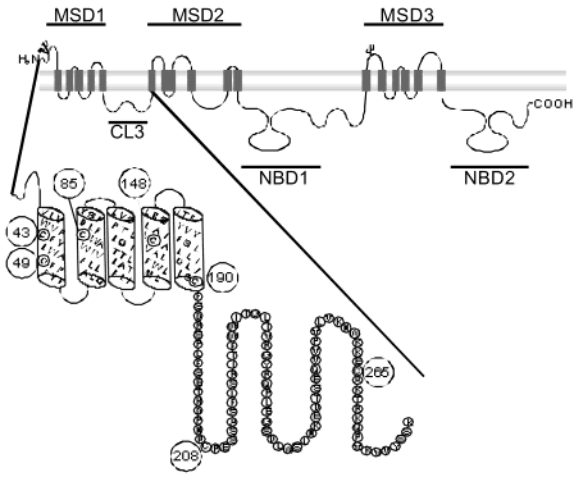

FIGURE 1: Schematic diagram of a predicted secondary structure of MRP1 showing the positions of the Cys residues in MSD1 and CL3 that were mutated in this study.

Chemosensitivity Testing. Drug and heavy metal oxyanion resistance profiles of stably transfected cell lines were analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide microtiter plate assay as described previously (*11*). Within each experiment, determinations were carried out in quadruplicate and a minimum of three independent experiments were performed. Relative resistance factors were calculated as the ratio of the IC_{50} value of cells expressing WT-MRP1 or Cys mutant MRP1 to the IC_{50} value of cells expressing the empty pcDNA3.1(−) vector alone. An immunoblot of crude membrane preparations was performed in parallel with each assay to determine relative MRP1 levels so that resistance levels could be normalized to take into account differences in expression, as required.

RESULTS

Expression of Cys Mutant MRP1 Proteins in Stably Transfected HeLa Cells. To explore the possibility that Cys residues within MSD1 and CL3 of MRP1 may play a role in transport function and/or structure, seven Cys residues at amino acid positions 43, 49, 85, 148, 190 (MSD1), and 208 and 265 (CL3) were mutated to Ser and Ala. Conservative Ser substitutions were created to retain the hydrogen-bonding capability and steric bulk of the Cys side chain, whereas Ala substitutions were generated as a nonconservative replacement (Figure 1). The integrating mammalian expression vector pcDNA3.1(−) containing mutated forms of MRP1 cDNAs was used to generate stably transfected HeLa cells, and clonal cell lines were established by limiting serial dilution. Immunoblotting and flow cytometry showed that the Cys mutants were expressed at levels that ranged from 0.5- to 2.3-fold those of WT-MRP1 (Figure 2). Endogenous

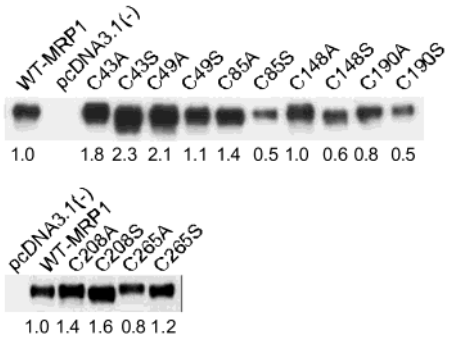

FIGURE 2: Expression levels of wild-type and Cys-substituted MRP1 in stably transfected HeLa cells. The membrane vesicle protein (2 μg) prepared from each transfected clonal cell line was resolved on a 7% polyacrylamide gel, transferred to a poly(vinylidene difluoride) membrane, and probed with MAb QCRL-1 as described previously (*33*). Relative expression levels of the Cys mutant proteins as compared with WT-MRP1 were determined by densitometric analysis of films from multiple exposure times and are shown under each lane. Consistent protein loading of gels was verified by amido black staining of the blot after chemiluminescent detection of MAb binding. Expression levels of WT-MRP1 and the Cys MRP1 mutants were determined for each vesicle preparation and prior to use of cells in chemosensitivity assays. Results shown are from a typical experiment and represent results from $\geq$12 immunoblots.

MRP1 in empty pcDNA3.1(−) vector transfected HeLa cells was not detectable under the conditions used.

Eleven of Fourteen MRP1 Cys Mutants are Not Fully Routed to the Plasma Membrane. To determine whether substitution of Cys residues in MSD1 and CL3 of MRP1 might influence the trafficking of MRP1 to the plasma membrane, the stably transfected HeLa cell lines were examined by indirect immunofluorescent confocal microscopy. MRP1 was not detectable in cells transfected with empty vector (Figure 3A) while cells transfected with pcDNA3.1(−)-$MRP1_K$ (WT-MRP1) showed strong plasma membrane staining as expected (Figure 3B). Plasma membrane staining was also observed in all 14 MRP1-Cys mutant cell lines (Figure 3C-P); however, 11 of them had additional, nonplasma membrane-associated MRP1. Thus, the cell lines expressing the TM1 mutant Cys43Ser-MRP1 (Figure 3D) and the CL3 mutants Cys265Ala-MRP1 (Figure 3O) and Cys265Ser-MRP1 (Figure 3P) exhibited severely disrupted plasma membrane trafficking. Those exhibiting minor disruption of plasma membrane trafficking included cell lines expressing four Cys→Ala mutants [Cys43Ala (Figure 3C), Cys85Ala (Figure 3G), Cys190Ala (Figure 3K), and Cys208Ala (Figure 3M)] and four Cys→Ser mutants [Cys49Ser (Figure 3F), Cys85Ser (Figure 3H), Cys190Ser (Figure 3L), and Cys208Ser (Figure 3N)]. Three of the mutant cell lines with impaired plasma membrane trafficking of MRP1 exhibited filament-like staining (Cys43Ala, Cys43Ser, and Cys49Ser) (Figure 3C,D,F) while the cell lines expressing Cys265Ala and Cys265Ser MRP1 showed a stippled-like staining pattern. Finally, Cys265Ser expressing cells had a unique staining pattern, which was stippled and resembled vacuolar staining.

Several Cys-Substituted MRP1 Proteins Have Increased Resistance to Limited Proteolysis. Previous studies of P-glycoprotein and other membrane proteins have shown that alterations in protein conformation can often be detected by changes in exposure of trypsin cleavage sites (*38*, *39*).

Table 1: Detection of Tryptic Fragments N1 and N2 of MRP1 in Membranes Prepared from HeLa Cells Stably Expressing Cys→Ala and Cys→Ser MRP1 Mutants[a]

transfected HeLa cell line	trypsin:protein ratio (w:w)	
	N1 detected	N2 detected
WT-MRP1	1:10 000	1:1000
C43A-MRP1	1:100	1:100
C43S-MRP1	1:10 000	1:1000
C49A-MRP1	1:250	1:100
C49S-MRP1	1:10 000	1:500
C85A-MRP1	1:10 000	1:1000
C85S-MRP1	1:1000	1:250
C148A-MRP1	1:250	1:250
C148S-MRP1	1:1000	1:500
C190A-MRP1	1:1000	1:1000
C190S-MRP1	1:1000	1:250
C208A-MRP1	1:10 000	1:250
C208S-MRP1	1:10 000	1:500
C265A-MRP1	1:250	1:10
C265S-MRP1	1:1000	1:250

[a] The data shown represent a summary of the limited trypsin digests shown in Figures 4 and 5. The designations N1 and N2 refer to discrete NH_2 proximal tryptic fragments of MRP1 of ~120 and ~40−60 kDa, respectively, described previously in detail (*8*) and shown schematically in the diagram at the top of Figure 4.

Consequently, to determine whether mutation of Cys residues in MSD1 and CL3 resulted in conformational changes in MRP1, cell membranes were subjected to limited trypsinolysis. The pattern of protein fragments obtained after controlled exposure of MRP1-enriched membranes to trypsin and their detection with several regionally directed, MRP1 specific antibodies has been previously well-characterized (*8*, *21*) (see cartoon at top of Figure 4). Thus, during trypsinolysis, WT-MRP1 is initially cleaved into two polypeptides, one of which is a glycosylated fragment of approximately 120 kDa that is detected by MAb MRPr1 and corresponds to the NH_2 proximal half of MRP1 consisting of MSD1-MSD2-NBD1 (labeled N1 in Figure 4). The other tryptic fragment is 75−80 kDa and is also glycosylated; this smaller fragment corresponds to the COOH proximal half of MRP1 (MSD3-NBD2) and thus is not detected by MAb MRPr1 (labeled C1 in Figure 4).

More extensive digestion of MRP1 with trypsin results in cleavage of the N1 polypeptide into two smaller fragments, designated N2 and N3, of approximately 40−60 and 60 kDa, respectively (Figure 4). The epitope for MAb MRPr1 is located in the NH_2 proximal side of the trypsin cleavage site between the N2 and the N3 fragments and therefore detects only the N2 fragment, which migrates as a broad band because it is N-glycosylated at Asn^{19} and Asn^{23} (*8*, *36*).

Membrane vesicles from the HeLa cells expressing WT-MRP1 and the various Cys → Ala and Cys → Ser mutant MRP1 proteins were treated with trypsin at 12 different trypsin:protein ratios (1:10 000 to 2.5:1) and then immunoblotted. The results are shown in Figures 4 and 5 and are summarized in Table 1. For WT-MRP1, the N1 tryptic fragment (MSD1-MSD2-NBD2) appeared at a trypsin:protein ratio of 1:10 000 and the N2 tryptic fragment (MSD1) appeared when the trypsin:protein ratio was 1:1000. The tryptic digest patterns of Cys43Ser and Cys85Ala-MRP1 were almost indistinguishable from that of WT-MRP1, with the appearance of the N1 fragment and faint N2 bands at trypsin:protein ratios of 1:10 000 and 1:1000, respectively. The tryptic digest patterns for vesicles prepared from mutants

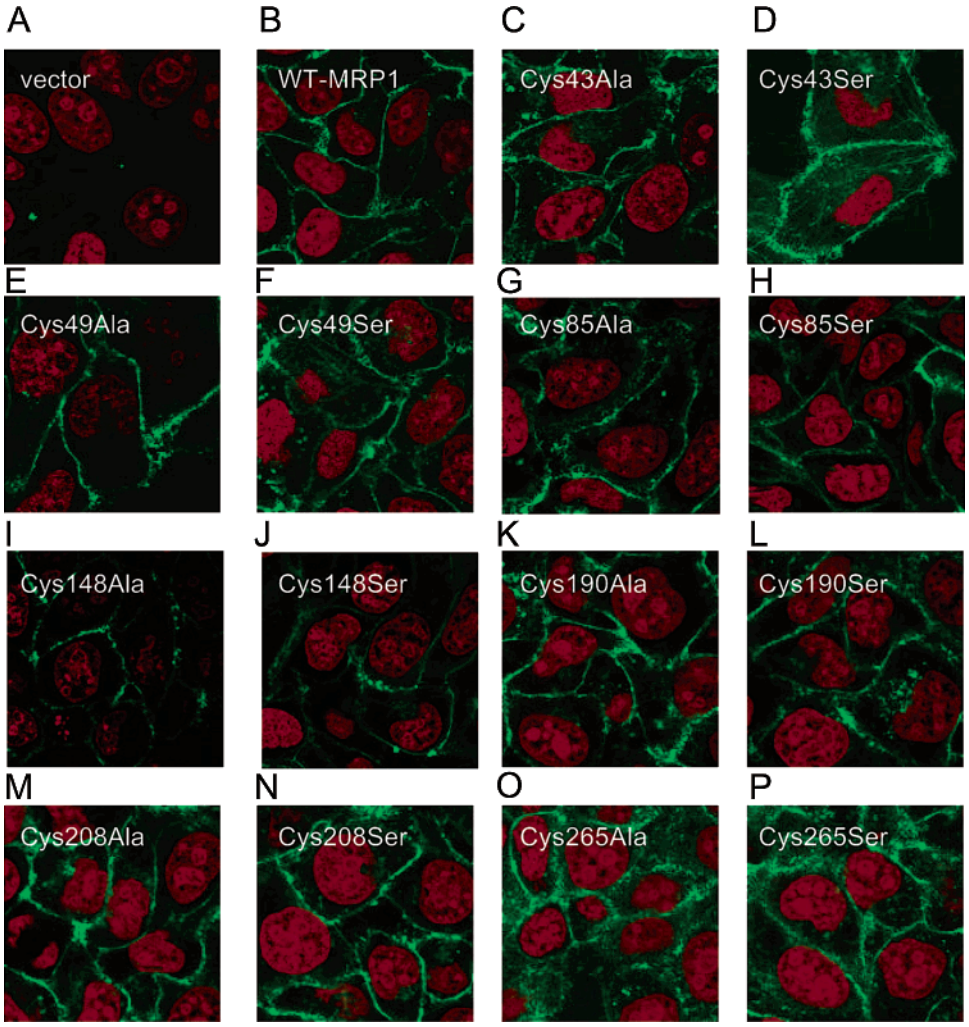

FIGURE 3: Confocal microscopy of HeLa cells expressing WT-MRP1 and single Cys mutants. MRP1 was detected using the MAb QCRL-3, visualized with Alexa 488-tagged secondary antibody and is shown in green. Nuclei were stained with propidium iodide and are shown in red. No green fluorescence was detected in the HeLa cell line transfected with empty pcDNA3.1(−) vector alone (A) or in control experiments using purified murine IgG2a in place of MAb QCRL-3 for WT-MRP1 transfected HeLa cell lines (not shown). Selected fields of HeLa cells transfected with wild-type and Cys mutant MRP1 are shown (B−P) as indicated. Images have been enhanced for maximum contrast between black background and green fluorescence and are not intended to be quantitative.

Cys49Ser, Cys190Ala, Cys208Ala, and Cys208Ser also closely matched those of WT-MRP1 except that the N1 fragment did not appear until a trypsin:protein ratio of 1:1000 for Cys190Ala-MRP1 and the N2 fragments of Cys49Ser-MRP1, Cys208Ala-MRP1, and Cys208Ser-MRP1 did not appear until trypsin:protein ratios of 1:500, 1:250, and 1:500, respectively.

The remaining Cys→Ser MRP1 mutants (Cys85Ser, Cys148Ser, Cys190Ser, and Cys265Ser) were more resistant to trypsinolysis than WT-MRP1 but less resistant than their respective Ala partners. For all four of these Cys→Ser mutants, the N1 tryptic fragment appeared at a trypsin:protein ratio of 1:1000 while the N2 fragment appeared at ratios of 1:500 for Cys148Ser and 1:250 for Cys85Ser, Cys190Ser, and Cys265Ser.

Trypsinolysis of the four remaining Ala-substituted Cys mutants (Cys148Ala, Cys49Ala, Cys43Ala, and Cys265Ala) showed that they were also quite resistant to cleavage by this enzyme. For example, the N1 and N2 fragments of Cys148Ala-MRP1 did not appear until a trypsin:protein ratio of 1:250 while the N1 and N2 fragments of Cys49Ala-MRP1 appeared at ratios of 1:250 and 1:100, respectively. The Cys43Ala-MRP1 mutant was highly resistant to trypsinolysis with the appearance of the N1 band not occurring until the trypsin:protein ratio was 1:100, the same ratio at which the N2 band appeared. The Cys265Ala MRP1 was less resistant than the Cys43Ala mutant with the N1 fragment appearing at a trypsin:protein ratio of 1:250. The cleavage of the N1 fragment into the smaller N2 and N3 fragments was most affected by the substitution of Cys^{265} with Ala since the N2 fragment of this mutant was not detected until a trypsin:protein ratio of 1:10.

All Cys-Substituted MRP1 Mutants Transport [^{3}H]LTC$_4$, [^{3}H]E$_2$17βG, and [^{3}H]GSH. To determine whether MRP1 transport activity was affected by the Cys substitutions, membrane vesicles were prepared from stably transfected HeLa cells and the ATP-dependent uptake of three structurally diverse organic anion substrates ([^{3}H]LTC$_4$, [^{3}H]E$_2$17βG, and [^{3}H]GSH) was determined. As shown in Figure 6, all Cys mutants retained some ability to transport all three of these substrates with activities ranging from 50 to 150% of WT-MRP1 activity.

Four of the seven Cys→Ala MRP1 mutants transported LTC$_4$ at levels very close to those of WT-MRP1 (Figure

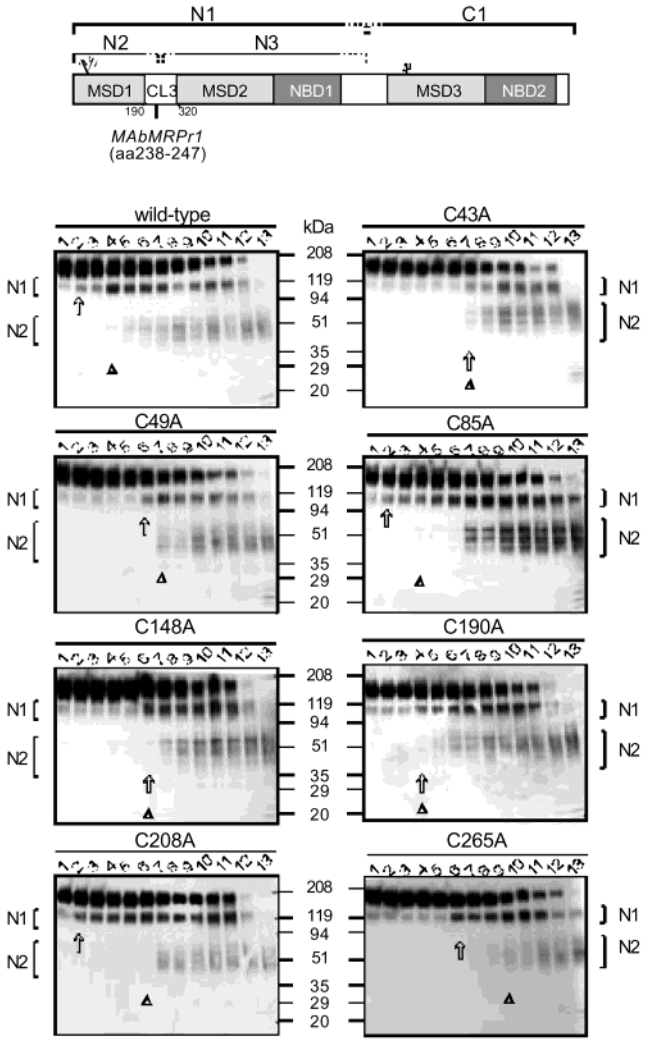

FIGURE 4: Trypsin digestion of MRP1 MSD1 and CL3 Cys → Ala mutants. WT-MRP1 and MRP1 Ala-substituted Cys mutant-enriched membranes (2 μg) from transfected HeLa cells were digested with diphenylcarbamyl chloride trypsin at 37 °C for 15 min at the following trypsin:protein ratios (w:w) (1) control (no trypsin), (2) 1:10000, (3) 1:5000, (4) 1:1000, (5) 1:500, (6) 1:250, (7) 1:100, (8) 1:50, (9) 1:10, (10) 1:5, (11) 1:2.5, (12) 1:1, and (13) 2.5:1. After reactions were stopped, proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis on 4−15% gradient gels and immunoblotted with the MRP1 specific MAb MRPr1. The designations N1 and N2 at the right or left of each blot refer to discrete proteolytic fragments described previously and in the text (*8*); their location is shown at the top of the figure. The arrows on the blots indicate the trypsin:protein ratio at which the larger N1 fragment was detected while the arrowheads indicate the ratio at which the smaller N2 fragment was detected. Molecular mass markers are indicated in the center of the blots.

6A). Ala substitution of Cys^{49} resulted in a 37% decrease in LTC_4 transport activity while replacement of Cys^{208} and Cys^{265} with Ala resulted in an approximate 30% increase in activity. Four of the seven Cys→Ser MRP1 mutants also transported LTC_4 at levels comparable to those of WT-MRP1; however, some differences between the transport activities of the Ala and Ser substituted mutants were noted (Figure 6B). Thus, similar to Cys49Ala-MRP1, Cys49Ser-MRP1 showed a 30% reduction in relative LTC_4 transport activity. However, in contrast to Cys85Ala-MRP1, which transported LTC_4 at levels similar to WT-MRP1, Cys85Ser-MRP1 showed a 38% reduction in LTC_4 uptake. Similar to

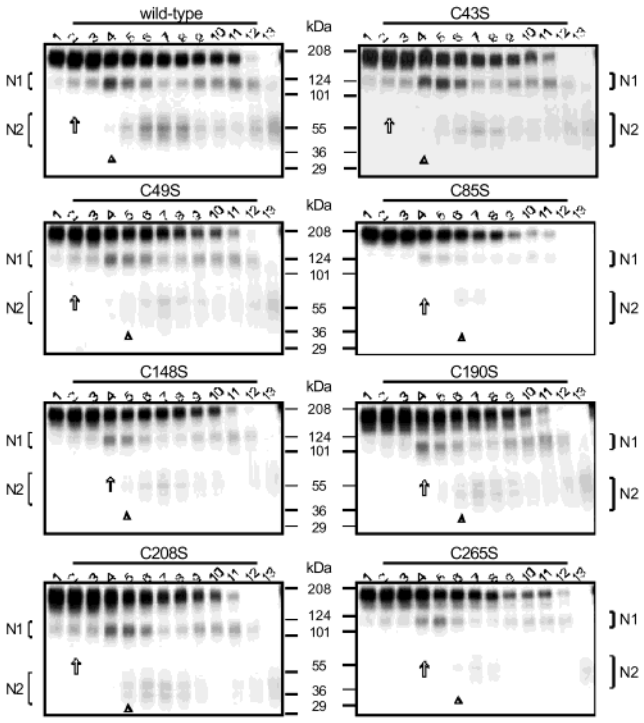

FIGURE 5: Trypsin digestion of MRP1 MSD1 and CL3 Cys → Ser mutants. Trypsin digests were carried out with membranes prepared from Ser-substituted Cys mutants as described in the legend for Figure 4.

Cys208Ala-MRP1, Cys208Ser-MRP1 LTC_4 transport activity was increased 30%. In contrast, unlike Cys265Ala-MRP1, which showed increased activity, Cys265Ser-MRP1 exhibited LTC_4 uptake activity similar to WT-MRP1.

$E_2 17\beta G$ uptake activity was altered in five of the seven Cys→Ala-substituted MRP1 mutants (Figure 6C). Thus, a moderate (32−42%) decrease in $E_2 17\beta G$ uptake activity was observed for four of the five mutants (Cys49Ala-MRP1, Cys85Ala-MRP1, Cys148Ala-MRP1, and Cys265Ala-MRP1). In contrast, $E_2 17\beta G$ uptake by membrane vesicles prepared from Cys43Ala-MRP1 expressing cells increased 40% relative to uptake by WT-MRP1. Five of the seven Cys→Ser mutants also showed changes in their relative ability to transport $E_2 17\beta G$ (Figure 6D). Consistent with their Ala-substituted counterparts, Cys49Ser-MRP1, Cys148Ser-MRP1, and Cys265Ser-MRP1 showed moderately reduced $E_2 17\beta G$ uptake (34−50%) while uptake by Cys43Ser-MRP1 was increased by 40% relative to WT-MRP1. In contrast to the Cys85Ala mutant, $E_2 17\beta G$ uptake by Cys85Ser-MRP1 was similar to WT-MRP1. In addition, unlike Cys208Ala-MRP1, which transported $E_2 17\beta G$ at a level similar to WT-MRP1, $E_2 17\beta G$ uptake by Cys208Ser-MRP1 was reduced by 35%.

Apigenin-stimulated GSH uptake was similar to WT-MRP1 for all seven of the Cys→Ala-substituted MRP1 molecules (Figure 6E) while uptake of this tripeptide by two of the seven Cys→Ser MRP1 mutants was significantly different (Figure 6F). Thus, membrane vesicles prepared from the Cys43Ser-MRP1 transfected cell line showed a 40% increase in GSH uptake whereas Cys208Ser-MRP1 vesicles showed a 50% decrease in GSH uptake relative to WT-MRP1. In summary, the Cys→Ala or Cys→Ser substitutions in MSD1 or CL3 resulted in no or only moderate (<50%) alterations in LTC_4, $E_2 17\beta G$, or GSH transport activity.

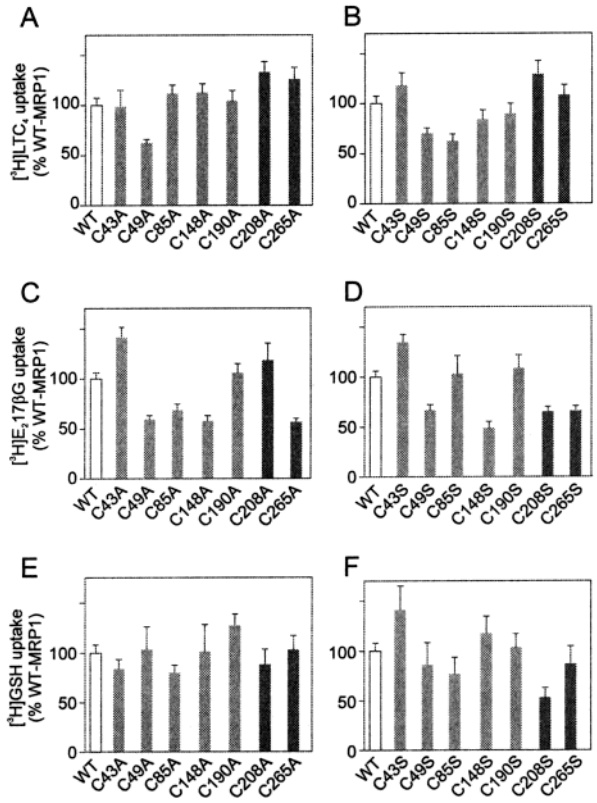

FIGURE 6: Uptake of [^{3}H]LTC$_4$, [^{3}H]E$_2$17βG, and [^{3}H]GSH by inside-out membrane vesicles prepared from wild-type and Cys-substituted MRP1 transfected HeLa cells. The ability of seven Cys → Ala (left panel, A, C, and E) and seven Cys → Ser (right panel, B, D, and F) single substitutions in MSD1 (light gray bars) and CL3 (dark gray bars) of MRP1 to transport [^{3}H]LTC$_4$, [^{3}H]E$_2$17βG, and [^{3}H]GSH was determined, and the activity was expressed relative to WT-MRP1 (unfilled bar). All data have been corrected for differences in expression levels, relative to WT-MRP1. (A,B) ATP-dependent uptake of LTC$_4$ was measured by incubating membrane vesicles (2 μg) with [^{3}H]LTC$_4$ (50 nM, 40 nCi) in transport buffer for 1 min at 23 °C. Each bar represents the mean (±SD) of 3−6 experiments. (C,D) ATP-dependent uptake of E$_2$-17βG was measured by incubating membrane vesicles (5 μg) with [^{3}H]E$_2$17βG (400 nM, 40 nCi) in transport buffer for 1 min at 37 °C. Each bar represents the mean (±SD) of 3−6 experiments. (E,F) ATP-dependent transport of apigenin-stimulated GSH uptake was measured by incubating membrane vesicles (20 μg) with [^{3}H]GSH (100 μM, 120 nCi) and 30 μM of apigenin. Each bar represents the mean (±SD) of triplicate determinations in a single experiment.

Resistance to Heavy Metal-Centered Oxyanions and Chemotherapeutic Agents. We have previously reported that in addition to drugs, both human and murine MRP1/Mrp1 confer low levels of resistance to arsenical and antimonial oxyanions (*11*, *40*). These agents are known to react with thiol groups within proteins. Consequently, we examined the resistance of cells expressing mutant MRP1 molecules harboring substitutions of Cys residues in MSD1 (Cys43Ala, Cys43Ser, Cys49Ala, Cys49Ser, Cys190Ala, and Cys190Ser) and CL3 (Cys208Ala, Cys208Ser, Cys265Ala, and Cys265Ser), to sodium arsenite and potassium antimony tartrate. All 10 mutants tested conferred a 2−4-fold increase in resistance to antimony tartrate, which is similar to or somewhat higher than that conferred by WT-MRP1. However, none of the differences were statistically significant. In contrast, cells expressing two of the mutants showed some differences in levels of sodium arsenite resistance. Thus, the arsenite resistance of cells expressing the TM1 Cys43Ser mutant was 2.5-fold lower than the cell line expressing WT-MRP1 whereas cells expressing Cys265Ser MRP1 were approximately 3-fold more resistant to this heavy metal oxyanion (Table 2). Representative chemosensitivity assays illustrating the sodium arsenite resistance of cell lines expressing the Cys43Ser and Cys265Ser MRP1 mutants are shown in Figure 7.

Table 2: Sensitivity of Stably Transfected HeLa Cells Expressing Wild-Type and Cys-Substituted MRP1 to Sodium Arsenite and Potassium Antimony Tartrate

transfected HeLa cell line	relative resistance[a]	
	Na^+ arsenite	K^+ antimony tartrate
WT-MRP1	3.6 ± 1.3 (1) (n = 6)	2.0 ± 0.5 (1) (n = 7)
C43A-MRP1	4.3 ± 0.7 (1.2) (n = 3)	2.0 ± 0.4 (1) (n = 3)
C43S-MRP1	1.4 ± 0.5 (0.4)[b] (n = 6)	2.8 ± 1.1 (1.4) (n = 4)
C49A-MRP1	4.0 ± 1.6 (1.1) (n = 5)	2.6 ± 0.7 (1.3) (n = 4)
C49S-MRP1	3.0 ± 1.5 (0.8) (n = 4)	3.0 ± 0.6 (1.5) (n = 4)
C190A-MRP1	3.8 ± 0.4 (1) (n = 4)	4.0 ± 1.2 (2) (n = 3)
C190S-MRP1	2.9 ± 0.4 (0.8) (n = 4)	2.7 ± 0.4 (1.4) (n = 3)
C208A-MRP1	3.0 ± 0.6 (0.8) (n = 3)	2.4 ± 0.6 (1.2) (n = 3)
C208S-MRP1	4.2 ± 0.8 (1.2) (n = 3)	3.8 ± 0.7 (1.9) (n = 3)
C265A-MRP1	2.5 ± 0.2 (0.7) (n = 4)	2.3 ± 0.6 (0.9) (n = 3)
C265S-MRP1	10.2 ± 0.4 (2.8)[b] (n = 5)	4.0 ± 1.7 (2) (n = 3)

[a] The resistance of stably transfected HeLa cells was determined using a tetrazolium-based cytotoxicity assay. The relative resistance factors were obtained by dividing the IC_{50} values for wild-type or Cys mutant MRP1 transfected cells by the IC_{50} values for empty vector control transfected cells and were normalized for differences in MRP1 expression levels. The values shown are means (±SD) of relative resistance factors obtained from three or more independent experiments. Numbers in parentheses indicate the relative resistance ratio to WT-MRP1. [b] Statistically different from WT-MRP1 ($P < 0.05$) (ANOVA followed by Newman−Keuls Post-Hoc test).

The HeLa cell lines expressing Cys43Ser-MRP1 and Cys265Ser-MRP1 that showed changes in arsenite resistance were also tested for their sensitivity to the anticancer drugs vincristine and doxorubicin. For comparison, cells expressing the Ala-substituted Cys^{43} and Cys^{265} mutants were also tested (Figure 8). The Cys43Ala, Cys43Ser, Cys265Ala, and Cys265Ser MRP1 mutant expressing cell lines were 6.5-, 2.6-, 7.1-, and 5.1-fold resistant to doxorubicin, respectively, levels of resistance that did not differ significantly from the ∼5-fold resistance observed with cells expressing WT-MRP1 (Figure 8A). In contrast, the cell line expressing Cys43Ser-MRP1 was only 5-fold resistant to vincristine while the cell lines expressing Cys43Ala, Cys265Ala, and Cys265Ser-MRP1 were 21-, 19-, and 13-fold resistant, respectively, levels of resistance comparable to those observed in cells expressing WT-MRP1 (15-fold resistant) (Figure 8B).

DISCUSSION

In this study, we examined the effects of conservative (Ser) and nonconservative (Ala) substitutions of five Cys residues in MSD1 and two Cys residues in CL3 on some structural and functional properties of MRP1. All 14 of the Cys MRP1 mutants were expressed at levels that were, for the most part, comparable or only moderately different from the levels of WT-MRP1, suggesting that none of these Cys residues play a critical role in MRP1 protein biosynthesis or stabilization. The lowest expression levels were observed with the MSD1 mutants Cys85Ser, Cys148Ser, and Cys190Ser, which were

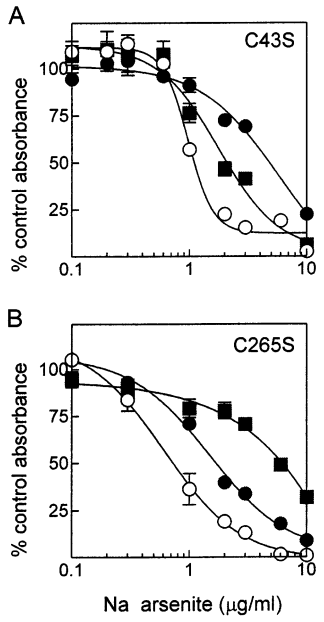

FIGURE 7: Resistance of HeLa cells expressing Cys43Ser and Cys265Ser mutant MRP1 to sodium arsenite. HeLa cell lines stably transfected with the pcDNA3.1(−) vector (○), pcDNA3.1(−)-$MRP1_K$ (●), and (A) pcDNA3.1(−)C43S-$MRP1_K$ (■) or (B) pcDNA3.1(−)-C265S-$MRP1_K$ (■) were exposed to sodium arsenite for 72 h at 37 °C at the concentrations indicated, and then, cell viability was measured using a tetrazolium assay. The results shown are those of a typical experiment in which each data point represents the mean ±SD of quadruplicate determinations. Similar results were obtained in at least three additional independent experiments. Data are not normalized for MRP1 expression; however, Cys265Ser-MRP1 and Cys43Ser-MRP1 were expressed at levels comparable to, and 1.5-fold higher than, WT-MRP1, respectively. Consequently, normalization of the data would not affect the relative resistance of Cys265Ser-MRP1 and would only further emphasize the decreased arsenite resistance of Cys43Ser-MRP1.

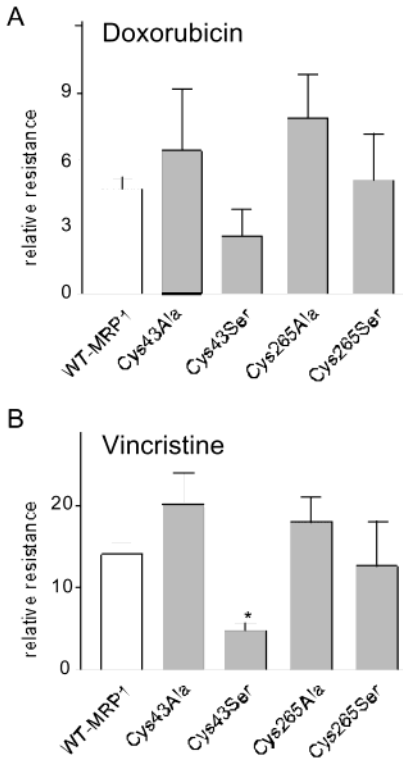

FIGURE 8: Resistance of HeLa cells expressing wild-type, Cys43, and Cys265 mutant MRP1 to anticancer agents. HeLa cell lines stably transfected with pcDNA3.1(−)-$MRP1_K$ (empty bars) and the indicated mutant MRP1 (cDNAs) (shaded bars) were exposed to (A) doxorubicin or (B) vincristine for 72 h at 37 °C at concentrations ranging from 0.1 to 1000 nM or 0.1 to 10 μM, respectively. Cell viability was measured using a tetrazolium-based assay. The relative resistance values were determined by dividing the IC_{50} value for wild-type or mutant MRP1 transfected cells by the IC_{50} value for empty vector control transfected cells and were normalized for MRP1 expression levels as appropriate. The bars represent the mean values ± SEM of 3−6 experiments. * statistically different from WT-MRP1 ($p < 0.05$) (Student's *t*-test).

reduced 40−50% as compared to WT-MRP1. In addition, several Cys mutants did not appear fully routed to the plasma membrane, with the most severely disrupted pattern of subcellular localization being observed with the TM1 mutant Cys43Ser and the CL3 mutants Cys265Ala and Cys265Ser. This suggests that these mutations may have introduced a structural change in the protein, perhaps through a small degree of misfolding, which reduces the efficiency of MRP1 trafficking to the plasma membrane. The disrupted membrane trafficking of the Cys43Ser MRP1 mutant is of particular interest since a single nucleotide polymorphism resulting in this amino acid substitution has recently been reported in a Japanese population (*29*).

Additional evidence for structural changes introduced into MRP1 by the Cys substitutions was indicated by differences in the sensitivity of the Cys mutants to trypsin-mediated limited proteolysis. In the case of the cystic fibrosis transmembrane conductance regulator CFTR (ABCC7) and P-glycoprotein, several single amino acid changes have been described, which cause a marked increase in the proteolytic susceptibility of these related ABC proteins (*41*, *42*). However, rather than an increase, almost all of the MRP1 Cys mutants examined here showed a tendency toward a decrease in trypsin susceptibility. The most significant decreases were observed in membranes from cells expressing the Cys43Ala, Cys49Ala, Cys148Ala, and Cys265Ala MRP1 mutants, suggesting that an alteration in the conformation of these proteins has occurred that decreases the accessibility of specific trypsin cleavage sites in CL3 (appearance of N2 fragment) and, in some cases, in the linker region of the protein between NBD1 and MSD3 (appearance of N1 fragment). Interestingly, the trypsin susceptibility of the Ser-substituted partners of these four Cys mutants is more similar to WT-MRP1. The basis for this difference between these Ser- and Ala-substituted mutants is not known but may become more evident as more detailed structural information on this region of MRP1 becomes available.

Despite the structural changes suggested by the confocal microscopy and limited proteolysis studies, all of the Cys mutants tested retained the ability to transport conjugated organic anions and GSH. In fact, one of the mutants that showed extensive intracellular staining, Cys43Ser-MRP1, had LTC_4 and $E_2 17\beta G$ transport activity that was greater than or equal to that of WT-MRP1. This contrasts with other amino acid substitutions of MRP1 (such as when Lys^{332} is replaced by Asp or Leu) that completely eliminate LTC_4 transport without disrupting plasma membrane trafficking (*43*). Thus, there appears to be no strict correlation between the relative ability of the Cys-MRP1 mutants to efficiently traffic to the plasma membrane and their in vitro organic anion transport activity. All Cys-substituted proteins were present to a certain extent in the plasma membrane of the

transfected cell lines even if intracellular staining was extensive. Therefore, although structural changes could alter the efficiency of plasma membrane localization and decrease trypsin sensitivity, the MRP1 present in the membrane vesicles remained transport competent.

Yang et al. (*26*) recently reported that when Cys^{7} and Cys^{32} in the extracellular NH_2 terminus of MRP1 were replaced with Ala, the Cys7Ala but not the Cys32Ala mutant showed significant structural and functional changes. Thus, as we have now shown for several Cys residues in MSD1 and CL3, mutation of Cys^{7} resulted in structural changes of MRP1 manifested as a change in trypsin sensitivity. However, in contrast to our findings with the seven Cys mutants examined here, LTC_4 transport by Cys7Ala-MRP1 was reduced by more than 90%, providing indirect evidence that the structure of MRP1 was more disrupted in this mutant. On the other hand, the apparent changes in Cys7Ala-MRP1 conformation resulted in little or no impairment of plasma membrane localization unlike several of the Cys-substituted mutants investigated here (*26*).

Little is known about how exactly MRP1 trafficks to the plasma membrane of nonpolarized tumor cells or to the basolateral membrane of polarized epithelial cells. However, several of the Cys mutants exhibited an actin-like staining pattern. Disruption of the actin cytoskeleton alters the function of several ABC proteins including other subfamily C members, such as the cystic fibrosis TM conductance regulator (CFTR/ABCC7), MRP2 (ABCC2), and the K^+ channel regulator SUR2 (ABCC9), as well as the more distantly related P-glycoprotein, and cholesterol and phospholipid transporter ABCA1 (*44*−*48*). Thus, it will be of interest to determine whether MRP1 can also associate with components of the cytoskeleton.

There are 25 Cys residues in MRP1, and our results clearly indicate that conservative and nonconservative substitutions of seven of them in the NH_2 proximal region of MRP1 have no or only a moderate impact on organic anion transport. Similarly, replacing the vicinal Cys^{1205} and Cys^{1209} residues in MSD3 of MRP1 with Ser singly or in combination did not affect transport of LTC_4 or vincristine (*49*). In addition, LTC_4 transport was only moderately reduced in a truncated MRP1 molecule lacking MSD1 ($MRP1_{204-1531}$) in which all 18 Cys residues (including Cys^{208} and Cys^{265}) were replaced with Ala residues (*50*). In contrast, Cys^{7}, which is predicted to be extracellular, was critical for LTC_4 transport (*26*). Transport of other substrates was not examined in this latter study, so whether this mutation causes a substrate selective or more global loss of organic anion transport by MRP1 is not known. Cys residues in several GSH-dependent proteins, including glutaredoxins and the omega class of GSTs, have been shown to be important for their interaction with GSH (*51*, *52*). However, none of the 14 Cys MRP1 mutants examined in the present study showed more than a 50% decrease in GSH transport activity indicating that none of them are critical for binding or transport of this tripeptide substrate.

In addition to their limited effects on organic anion transport function, the mutation of Cys residues in MSD1 and CL3 Cys had no effect on the ability of MRP1 to confer resistance to heavy metal-centered oxyanions with just two exceptions (Cys43Ser-MRP1 and Cys265Ser-MRP1). Similarly, vincristine resistance was decreased only in the Cys43Ser-MRP1 mutant. The reason for the differences in resistance between the Ala- and the Ser-substituted Cys^{43} and Cys^{265} MRP1 mutants and why this difference was not observed for all four cytotoxic agents tested is unknown. Two completely independently derived clonal cell lines expressing these mutants exhibited the same resistance patterns indicating it cannot be explained by clonal variation. However, it is perhaps worth noting that the Ser-substituted mutants exhibited the most disrupted plasma membrane trafficking. On the other hand, the limited proteolysis studies suggest that the differences in chemosensitivity are not related to conformational changes that reduce the accessibility of trypsin cleavage sites in this region.

MRP1-Cys^{43} is predicted to be located in the outer lipid leaflet of TM1; therefore, the reduced ability of the Ser-substituted mutant to confer arsenite resistance was not necessarily expected since Cys residues important for interaction with heavy metals in other transporters are typically located in cytoplasmic domains (*53*). However, the fact that the Ala-substituted Cys^{43} mutant could still confer wild-type levels of arsenite and vincristine resistance indicates that it is likely not the thiol group of Cys per se that is of importance. The hydroxyl group of the Ser side chain occupies less space than the Cys thiol group; furthermore, the Ser side chain has hydrogen-bonding capabilities whereas the Ala side chain does not. These properties of Ser could conceivably introduce changes in the packing of TM1 with other helices of MRP1 that lead to less favorable interactions with arsenite and vincristine. In addition, as noted above, Cys43Ser-MRP1 was not completely routed to the plasma membrane, and although this had no significant effect on organic anion transport, it might still contribute to changes in arsenite and vincristine resistance since previous studies have shown that these properties of MRP1 are not inextricably linked to each other (*31*). Finally, the reduced ability of Cys43Ser-MRP1 to confer resistance to arsenite and vincristine is of potential clinical relevance because as mentioned previously, Ito et al. (*29*) have reported the occurrence of a naturally occurring Cys43Ser polymorphism in healthy Japanese subjects. Thus, our findings raise the possibility that if individuals bearing this polymorphism are exposed to arsenite or treated with vincristine, they may show a reduced response to the toxicity of these xenobiotics.

The location of Cys^{265} in the cytoplasmic loop between MSD1 and MSD2 places this residue in a more favorable position than Cys^{43} to directly interact with a heavy metal oxyanion substrate. The increased ability of the Ser-substituted but not the Ala-substituted Cys^{265} mutant to confer arsenite resistance suggests that Cys^{265} and arsenite do not interact directly with one another unless another Cys residue in close spatial proximity to Cys^{265} can replace this interaction when Ala is present at position 265. The crystal structure of the ATPase subunit of the ArsAB transporter, which confers resistance to arsenite and antimonite in *Escherichia coli*, indicates that Ser residues can be involved in the formation of complexes with antimonite (*53*). Thus, one possible explanation for the increased arsenite resistance of Cys265Ser-MRP1 is that arsenite could be forming a complex with the mutant protein that is not held with as high affinity as WT-MRP1. Consequently, the arsenite could be released more quickly after translocation across the membrane resulting in increased transport efficiency. Another

possible explanation is that substitution of Cys^{265} with Ser but not Ala results in a conformational change within CL3 that indirectly increases the efficiency with which arsenite interacts with the protein. However, if such a change occurs, it is not associated with differences in accessibility of trypsin cleavage site(s) in this region of the two mutant proteins.

A final implication of our findings concerns the technique known as Cys scanning mutagenesis. This technique is a powerful method for determining membrane protein topology, the location of substrate binding regions, and conformational changes as well as for probing interactions between protein domains. However, this technique usually requires that a Cys-less form of the target protein is functionally active and structurally equivalent to the wild-type protein so that the reactivity of various sulfhydryl modifying reagents with Cys residues introduced into specific locations can be interpreted in a biologically meaningful context. Our observations and those of Yang et al. (*26*) that several single Cys substitutions in the NH_2 terminus of MRP1 can significantly affect the structural and functional properties of MRP1 may complicate the application of this technique to the study of this transport protein.

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