

Arsenic Trigluthathione [As(GS)₃] Transport by Multidrug Resistance Protein 1 (MRP1/ABCC1) Is Selectively Modified by Phosphorylation of Tyr920/Ser921 and Glycosylation of Asn19/Asn23[□]

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ABSTRACT

The ATP-binding cassette (ABC) transporter multidrug resistance protein 1 (MRP1/ABCC1) is responsible for the cellular export of a chemically diverse array of xenobiotics and endogenous compounds. Arsenic, a human carcinogen, is a high-affinity MRP1 substrate as arsenic trigluthathione [As(GS)₃]. In this study, marked differences in As(GS)₃ transport kinetics were observed between MRP1-enriched membrane vesicles prepared from human embryonic kidney 293 (HEK) (K_m 3.8 μ M and V_{max} 307 pmol/mg per minute) and HeLa (K_m 0.32 μ M and V_{max} 42 pmol/mg per minute) cells. Mutant MRP1 lacking *N*-linked glycosylation [Asn19/23/1006Gln; sugar-free (SF)-MRP1] expressed in either HEK293 or HeLa cells had low K_m and V_{max} values for As(GS)₃, similar to HeLa wild-type (WT) MRP1. When prepared in the presence of phosphatase inhibitors, both WT- and SF-MRP1-enriched membrane vesicles had a high K_m value for As(GS)₃ (3–6 μ M), regardless of the cell line. Kinetic parameters of As(GS)₃ for HEK-Asn19/23Gln-MRP1 were similar to those of HeLa/HEK-SF-MRP1

and HeLa-WT-MRP1, whereas those of single glycosylation mutants were like those of HEK-WT-MRP1. Mutation of 19 potential MRP1 phosphorylation sites revealed that HEK-Tyr920Phe/Ser921Ala-MRP1 transported As(GS)₃ like HeLa-WT-MRP1, whereas individual HEK-Tyr920Phe- and -Ser921Ala-MRP1 mutants were similar to HEK-WT-MRP1. Together, these results suggest that Asn19/Asn23 glycosylation and Tyr920/Ser921 phosphorylation are responsible for altering the kinetics of MRP1-mediated As(GS)₃ transport. The kinetics of As(GS)₃ transport by HEK-Asn19/23Gln/Tyr920Glu/Ser921Glu were similar to HEK-WT-MRP1, indicating that the phosphorylation-mimicking substitutions abrogated the influence of Asn19/23Gln glycosylation. Overall, these data suggest that cross-talk between MRP1 glycosylation and phosphorylation occurs and that phosphorylation of Tyr920 and Ser921 can switch MRP1 to a lower-affinity, higher-capacity As(GS)₃ transporter, allowing arsenic detoxification over a broad concentration range.

Introduction

Arsenic, a group 1 (proven) human carcinogen, is an element found throughout Earth's crust, resulting in human exposure through groundwater (Garelick et al., 2008). Chronic exposure to arsenic is known to cause cancers of the lung, skin, and

bladder and is associated with increased incidences of liver and kidney tumors (International Agency for Research on Cancer, 2012). In addition to cancer, arsenic exposure is associated with other adverse health effects, such as diabetes and hypertension (Platanias, 2009). Somewhat paradoxically, arsenic trioxide (As₂O₃) is highly effective in the treatment of acute promyelocytic leukemia and more recently is in clinical trials as a therapy for other hematologic and solid tumors (Kritharis et al., 2013).

The structural resemblance of arsenate (As^V) to phosphate allows its cellular uptake through phosphate carriers such as the Na⁺/phosphate co-transporter type IIb (*SLC34A2*) (Villa-Bellosta and Sorribas, 2010). In aqueous solution at physiologic pH, arsenite (As^{III}) and As₂O₃ exist as the neutral As(OH)₃ and can enter cells passively through aquaglyceroporin channels, as well as the glucose transporter 1 (*SLC2A1*) (Ramirez-Solis et al., 2004; Mukhopadhyay et al., 2014). Once

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ABBREVIATIONS: ABC, ATP-binding cassette; As^V, arsenate; As^{III}, arsenite; As(GS)₃, arsenic trigluthathione; CIP, calf intestinal alkaline phosphatase; CL3, cytoplasmic loop 3; E₂17 β G, 17 β -estradiol 17-(β -D-glucuronide); GSH, glutathione; HEK, human embryonic kidney 293; LTC₄, leukotriene C₄; mAb, monoclonal antibody; MMA^{III}, monomethylarsonous acid; MRP, multidrug resistance protein; MSD, membrane-spanning domain; NBD, nucleotide binding domain; pAb, polyclonal antibody; PIC, phosphatase inhibitor cocktail; PNGase F, peptide, *N*-glycosidase F; SF, sugar free; TM, transmembrane helix; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; WT, wild type.

inside human cells, arsenic can be methylated to four major products: monomethylarsonic acid, monomethylarsonous acid (MMA^{III}), dimethylarsinic acid, and dimethylarsinous acid (Drobna et al., 2010). Although arsenic methylation increases its whole-body clearance, it is considered a bioactivation pathway because MMA^{III} and dimethylarsinous acid are more toxic than inorganic arsenic (Petrick et al., 2000; Mass et al., 2001). Arsenic also undergoes conjugation with glutathione (GSH) (Leslie, 2012). Thus, As^{III} and MMA^{III} as As(GS)₃ and monomethylarsenic diglutathione, respectively, have been isolated from rodent bile and urine (Kala et al., 2000, 2004).

Inorganic, methylated and glutathionylated arsenic species can be actively removed from the cell through ATP-dependent transport proteins, and neutral species can be passively extruded through the aquaglyceroporins (Leslie, 2012; Mukhopadhyay et al., 2014). The multidrug resistance proteins MRP1, MRP2, and MRP4 (gene symbols *ABCC1*, *ABCC2*, and *ABCC4*, respectively) are ATP-binding cassette (ABC) transporter proteins with established roles as arsenic efflux pumps (Kala et al., 2000; Leslie et al., 2004; Carew and Leslie, 2010; Banerjee et al., 2014).

The 190-kDa phosphoglycoprotein MRP1 was originally identified based on its ability to confer resistance to a structurally diverse array of anticancer agents through an ATP-dependent decrease in cellular drug accumulation (Cole, 2014b). In addition to its role in drug resistance, MRP1 is important for the transport of physiologic substrates, including mediators of inflammation such as leukotriene C₄ (LTC₄) and the cholestatic steroid 17 β -estradiol 17-(β -D-glucuronide) (E₂17 β G) (Cole, 2014a). Furthermore, MRP1 transports a variety of other drugs, toxicants, and carcinogens often conjugated to GSH, glucuronate, or sulfate (Jedlitschky et al., 1996; Leslie et al., 2005; Loe et al., 1996a,b). As(GS)₃ was first identified, using membrane vesicles isolated from the doxorubicin-selected human small cell lung cancer cell line H69AR, as a high-affinity, low-capacity substrate of MRP1 with a K_m of 0.32 μ M and V_{max} of 17 pmol mg⁻¹ min⁻¹ (Leslie et al., 2004).

Despite the numerous and important physiologic, pharmacologic, and toxicologic roles MRP1 plays, the influence of post-translational modifications on its functional regulation are not well characterized. MRP1 has three utilized *N*-glycosylation sites at Asn19, Asn23 [in the first membrane-spanning domain (MSD0)], and Asn1006 [in the first extracellular loop of MSD2 connecting transmembrane helix (TM) 12 to TM13] (Hipfner et al., 1997) and is known to be phosphorylated (Almquist et al., 1995; Ma et al., 1995). Although mass spectrometry-based proteomic studies have identified 20 phosphorylation sites on MRP1 to date (Hornbeck et al., 2015), only one site (Thr249) in cytoplasmic loop 3 (CL3), which connects TM5 to TM6, has been functionally characterized (Stolarczyk et al., 2012).

In the current study, MRP1 expressed in HeLa cells was shown to have a 12-fold lower K_m and 7-fold lower V_{max} for As(GS)₃ than observed for MRP1 expressed in human embryonic kidney 293 (HEK) cells. Our findings indicate that MRP1 is differentially *N*-glycosylated and phosphorylated in membrane vesicles prepared from the two cell lines. We also found that the affinity and capacity of MRP1 for As(GS)₃ can be modulated by dual glycosylation at Asn19 and Asn23, as well as dual phosphorylation of MRP1 at Tyr920 and Ser921. Evidence is presented that the phosphorylation of Tyr920/Ser921 results in the markedly lower affinity and

higher capacity of As(GS)₃ transport by MRP1. In addition, the glycosylation status of Asn19/Asn23 influenced the phosphorylation at Tyr920/Ser921, suggesting cross-talk between these two post-translational modifications. Together, our findings suggest that the affinity of MRP1 for As(GS)₃ is influenced by cell type, phosphorylation, and glycosylation, which has important implications for arsenic detoxification by different cell types and tissues over a broad concentration range of this carcinogen.

Materials and Methods

Materials. Carrier-free ⁷³As^V (158 Ci/mol) was purchased from Los Alamos Meson Production Facility (Los Alamos, NM), and carrier-free [6', 7'-³H]E₂17 β G (30-60 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Woodbridge, Canada). [3', 5', 7'-³H(N)]-methotrexate (13.4 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). Creatine kinase (rabbit muscle), creatine phosphate, GSH reductase (yeast), complete mini-EDTA-free and EDTA-containing protease inhibitor cocktail tablets, and PhosSTOP phosphatase inhibitor cocktail (PIC) tablets were purchased from Roche Applied Science (Laval, Canada). Calf intestinal alkaline phosphatase (CIP) was purchased from New England Biolabs Canada (Whitby, Canada). Sodium (meta)-arsenite [Na₂AsO₂], sodium metabisulfite [Na₂S₂O₅], sodium thiosulfate [Na₂S₂O₃], E₂17 β G, bovine serum albumin, and GSH were obtained from Sigma-Aldrich (Oakville, Canada). L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was purchased from Thermo Scientific (Rockford, IL). Phenylmethylsulfonyl fluoride was from Bioshop Canada Inc. (Burlington, Canada). Sodium orthovanadate was from Tocris Bioscience (Bristol, UK). MRP1-specific antibodies used in this study include the rat monoclonal antibody (mAb) MRPr1 from Novus Biologicals (Littleton, CO), and the mouse mAb MRPM6 from Millipore (Temecula, CA). The mouse mAb QCRL1 and rabbit polyclonal antibody (pAb) MRP-1 were produced as described (Hipfner et al., 1994; 1996). Rabbit anti-Na⁺/K⁺ATPase (H-300) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Lines and Stable Transfection. HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium with 7.5% fetal bovine serum. HeLa cell lines stably expressing the empty pcDNA3.1(-) vector, pcDNA3.1(-)-MRP1 (HeLa-MRP1) or pcDNA3.1(-)-sugar-free (SF)-MRP1 (HeLa-SF-MRP1) were generated as described previously (Ito et al., 2001; Weigl, 2005) and maintained in RPMI-1640 medium with 5% calf serum and 600 μ g/ml G418 (Geneticin).

Site-Directed Mutagenesis. Mutants of MRP1 were generated using the Agilent QuikChange Lightning site-directed mutagenesis kit (Agilent, Mississauga, Canada). Mutagenesis was performed using pcDNA3.1(-)-MRP1 (Ito et al., 2001) as the polymerase chain reaction template (unless otherwise noted) according to the manufacturer's instructions. Mutagenic primers [Sigma-Aldrich or Integrated DNA Technology (Coralville, IA)] were designed to mutate Asn residues shown previously to undergo glycosylation to Gln (Hipfner et al., 1997), as well as potential phosphorylation sites (Supplemental Table 1). Specifically, eight potential phosphorylation sites in CL3 were mutated individually from Ser, Thr, or Tyr to Ala. In addition, a single construct with all eight potential phosphorylation sites mutated to Ala (Thr249Ala/Ser250Ala/Thr268Ala/Tyr277Ala/Ser278Ala/Ser279Ala/Ser288Ala/Ser289Ala/Ser289Ala) was generated and named 8X_CL3. 8X_CL3 was made using the pcDNA3.1-MRP1-Ser279Ala template with the Thr268Ala primer, followed by the following primers: Thr249Ala/Ser250Ala, Tyr277Phe/Ser278Ala/Ser279Ala, and Ser288Ala/Ser289Ala (Supplemental Table 1). Eleven potential phosphorylation sites in the linker region between nucleotide binding domain (NBD)1 and MSD2 were mutated from Ser or Thr

to Ala and Tyr to Phe. Tyr920 and Ser921 were also mutated to the phosphorylation mimicking Glu.

All mutants were confirmed by sequencing (MacroGenUSA, Rockville, MD, or The Hospital for Sick Children, Toronto, ON). For mutants that exhibited transport activity different than wild type (WT)-MRP1, the entire cDNA was sequenced to confirm that only the desired mutation had been introduced.

Transient Expression of WT and Mutant Forms of MRP1 in HEK293 Cells. HEK293 cells were transiently transfected with WT and mutant pcDNA3.1(-)-MRP1 vectors using a modified version of the calcium-phosphate transfection method (Chen and Okayama, 1988). Briefly, 3×10^6 cells were seeded in 15-cm plates, and 24 hours later, 18 μg of DNA was added using 2 ml of calcium-phosphate transfection solution [125 mM CaCl_2 , 140 mM NaCl, 0.75 mM NaH_2PO_4 , 28 mM HEPES (pH 7.0)]. The media was changed after 24 hours, and cells were harvested after 72 hours. Harvested cells were layered with Tris-sucrose buffer (50 mM Tris, pH 7.4, 250 mM sucrose) containing CaCl_2 (0.25 mM), EDTA-free protease inhibitors, and where indicated, phosSTOP PIC and stored at -80°C .

Membrane Vesicle Preparation and Immunoblotting. Membrane vesicles were prepared using the nitrogen cavitation method as previously described (Carew and Leslie, 2010). Membrane vesicle proteins were resolved on SDS-PAGE gels (Hipfner et al., 1997) and transferred to a polyvinylidene fluoride membrane and immunoblotted in 4% skim milk to determine the relative expression levels using the MRP1-specific antibodies: MRPr1 (diluted 1:10,000), QCRL1 (diluted 1:10,000), MRP-1 (diluted 1:5000), and/or MRpm6 (diluted 1:10,000). The Na^+/K^+ -ATPase (H-300) (diluted 1:1000) specific antibody was used as a loading control. Relative levels of MRP1 were quantified using ImageJ Software (National Institutes of Health, Bethesda, MD).

As(GS)₃ and [⁷³As](GS)₃ Synthesis. Arsenite [⁷³As^{III}] was reduced from arsenate [⁷³As^V] as previously described (Reay and Asher, 1977). Briefly, NaAsO_2 (6 mM) was added to a metabisulfite-thiosulfate reagent (66 mM $\text{Na}_2\text{S}_2\text{O}_5$, 27 mM $\text{Na}_2\text{S}_2\text{O}_3$ and 82 mM H_2SO_4) and then serially diluted using arsenic-free metabisulfite-thiosulfate reagent. These were then added at a 1:1 ratio with carrier-free ⁷³As^V to form ⁷³As^{III} and incubated for a minimum of 12 hours at room temperature. To form As(GS)₃ and ⁷³As(GS)₃ the reagent mixture was then mixed at a 1:1 ratio with reduced GSH (150 mM) and incubated for at least 30 minutes at room temperature to obtain the appropriate As(GS)₃ concentrations with a GSH concentration of 75 mM.

As(GS)₃ Transport Studies. As(GS)₃ transport assays were performed as previously described with minor modifications (Leslie et al., 2004). Briefly, 20 μg of membrane vesicles were incubated with As(GS)₃ (0.05–30 μM , 30–120 nCi), GSH reductase (0.6 U/ml), NADPH (0.35 mM), GSH (3 mM), AMP or ATP (4 mM), MgCl_2 (10 mM), creatine phosphate (10 mM), and creatine kinase (100 $\mu\text{g}/\text{ml}$) at 37°C for 60 seconds. Transport was then stopped by diluting with 800 μl of ice-cold Tris-sucrose buffer and filtered through glass fiber filters (type GF/B) using a 96-well plate Cell-Harvester (Perkin Elmer, Waltham, MA), washed five times with Tris-sucrose buffer, and radioactivity quantified by liquid scintillation counting. Data were plotted using Prism 5 (Graphpad Software, La Jolla, CA) and K_m and V_{max} values determined using Michaelis-Menten analysis.

E₂17 β G and Methotrexate Transport Studies. E₂17 β G transport assays were performed essentially as described (Carew et al., 2011). Briefly, 5 μg of membrane vesicles was incubated with E₂17 β G (0.1–30 μM , 40–80 nCi), AMP or ATP (4 mM), MgCl_2 (10 mM), creatine phosphate (10 mM), and creatine kinase (100 $\mu\text{g}/\text{ml}$) at 37°C for 90 seconds. Transport was then stopped, and samples were processed and analyzed as described for As(GS)₃. [³H]Methotrexate transport assays were performed by incubating membrane vesicles (10 μg protein) with methotrexate (100 μM , 250 nCi) and components described for E₂17 β G at 37°C for 20 minutes.

Glycolytic and Proteolytic Analysis of MRP1. Membrane vesicles were diluted to 2 $\mu\text{g}/\mu\text{l}$ in 50 mM Tris buffer (pH 7.4) and subjected to limited trypsinolysis of MRP1 as previously described,

with slight modifications (Hipfner et al., 1997). Briefly, MRP1-enriched vesicles were incubated with TPCK-treated trypsin at a trypsin:membrane protein ratio of 1:500 (w/w) for 30 minutes at 37°C . Proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride. Removal of total and core N-glycosylation by peptide: N-glycosidase F (PNGase F) and endoglycosidase H (Endo H) (New England Biolabs, Pickering, ON, Canada), respectively, from the diluted membrane vesicles was performed as previously described (Hipfner et al., 1997). Subsequent immunoblotting was performed as described above.

Immunoreactivity of HeLa-MRP1 and HEK-MRP1 with mAb QCRL-1. SDS-PAGE and immunoblotting were done as described, with the following exceptions. Where indicated, samples were prepared in Laemmli buffer containing PIC, and immunoblotting was done in 4% bovine serum albumin with 100 μM sodium orthovanadate. Blots were then stripped and treated with calf intestinal alkaline phosphatase (CIP, 20 U ml^{-1}) for 30 minutes at 37°C and then reprobed for MRP1 with mAb QCRL-1. For comparison of HeLa-MRP1 with HEK-MRP1 levels, equal loading was confirmed with amido black staining of the blots instead of the Na^+/K^+ -ATPase, which had different levels between the cell lines.

Results

Kinetic Analysis of As(GS)₃ Transport by WT-MRP1 Expressed in HeLa and HEK293 Cells. During a study of MRP1 mutants transiently expressed in HEK293 cells, we observed a 12-fold difference in K_m value for WT MRP1-mediated As(GS)₃ vesicular transport compared with previously published data (Leslie et al., 2004; Shukalek and Leslie, 2011). To investigate this observation further, kinetic parameters were determined for As(GS)₃ transport by MRP1-enriched vesicles prepared from HeLa-WT-MRP1 and HEK-WT-MRP1 cells (Fig. 1, A and B; Table 1). For HeLa-WT-MRP1 vesicles, the K_m for As(GS)₃ was $0.32 \pm 0.03 \mu\text{M}$ (Fig. 1A; Table 1), consistent with the previously published value, $0.32 \pm 0.08 \mu\text{M}$, determined using H69AR human lung cancer cell membrane vesicles (Leslie et al., 2004). In contrast, the K_m value for As(GS)₃ transport determined using HEK-WT-MRP1 vesicles was $3.8 \pm 1.2 \mu\text{M}$ (Fig. 1B; Table 2). The capacity of As(GS)₃ transport by WT-MRP1 was also significantly different between the HeLa and HEK293 membrane vesicles, with a 7-fold higher V_{max} for HEK-WT-MRP1 versus HeLa-WT-MRP1 ($42 \pm 17 \text{ pmol}/\text{mg}$ per minute versus $307 \pm 118 \text{ pmol}/\text{mg}$ per minute, respectively) after normalization for MRP1 protein levels (Fig. 1, A and B; Table 1; and Supplemental Table 2).

Kinetic Analysis of E₂17 β G Transport by WT-MRP1 Expressed in HeLa and HEK293 Cells. The large difference between K_m values for As(GS)₃ transport between HEK- and HeLa-WT-MRP1 cell lines was not expected because this cell line difference had not been observed for other MRP1 substrates (Loe et al., 1996a,b; Stride et al., 1997). For example, the K_m value of $105 \pm 31 \text{ nM}$ for LTC₄ determined using membrane vesicles prepared from WT-MRP1 transfected HeLa (T14) cells was very similar to the K_m of 98 nM determined using WT-MRP1 transfected HEK293 cells (Loe et al., 1996b). Furthermore, the K_m values for E₂17 β G determined using membrane vesicles from the same cell lines were almost identical (2.5 μM in HeLa and 2.9 μM in HEK293) (Loe et al., 1996a; Stride et al., 1997). Consistent with the published data, the K_m value of E₂17 β G for HeLa-WT-MRP1 in the present study was $4.0 \pm 2.6 \mu\text{M}$ and for

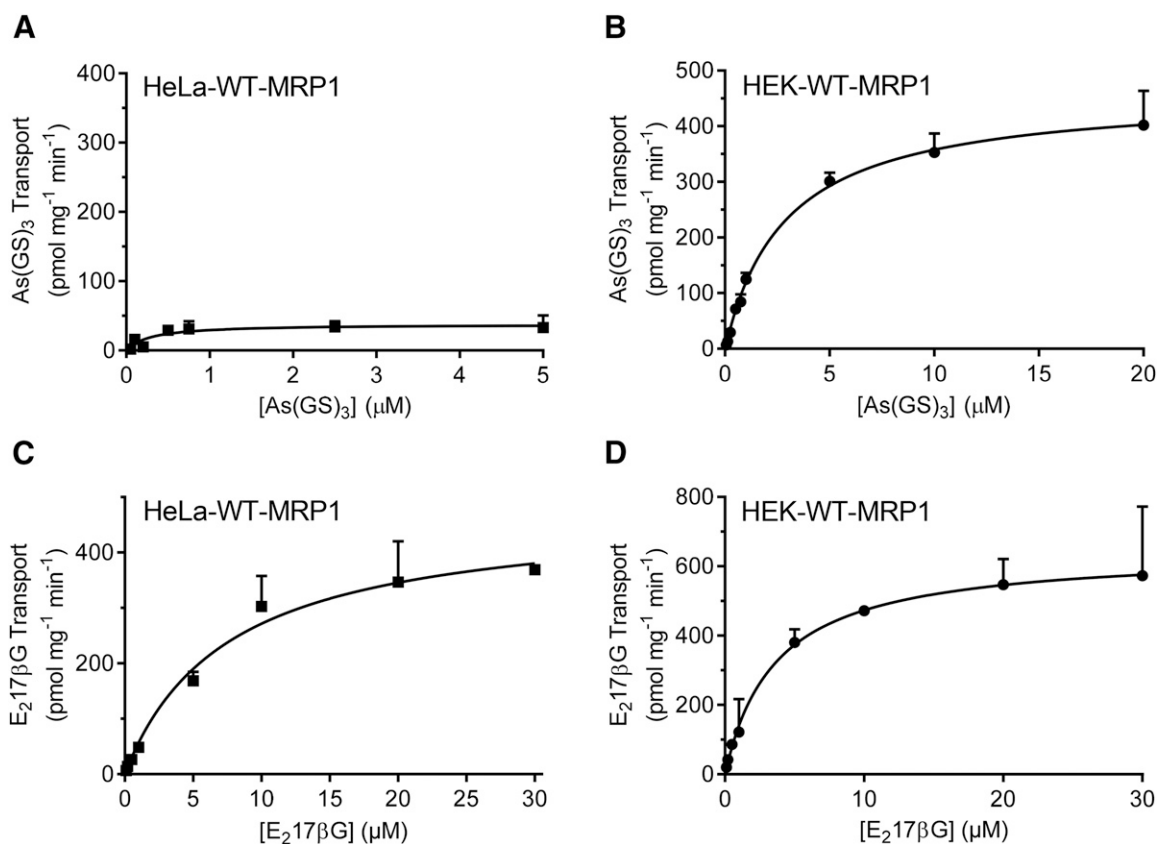


Fig. 1. Kinetic analysis of $\text{As}(\text{GS})_3$ and $\text{E}_217\beta\text{G}$ transport by WT-MRP1-enriched membrane vesicles prepared from HeLa and HEK293 cells. ATP-dependent uptake of (A, B) $\text{As}(\text{GS})_3$ (0.05–20 μM , 30–120 nCi) or (C, D) $\text{E}_217\beta\text{G}$ (0.1–30 μM , 30–80 nCi) was measured for 1 and 1.5 minutes, respectively, at 37°C using membrane vesicles prepared from (A, C) HeLa-WT-MRP1 or (B, D) HEK-WT-MRP1 cells. Kinetic values were determined using Michaelis-Menten nonlinear regression analysis (Graphpad Prism). Data points represent means (+ S.D.) of triplicate determinations in a single experiment. Similar results were obtained in at least two additional independent experiments and mean kinetic parameters for $\text{As}(\text{GS})_3$ transport are summarized in Table 1. All data were corrected for MRP1 levels relative to HEK-WT-MRP1.

HEK-WT-MRP1, $2.5 \pm 0.8 \mu\text{M}$, values that are not significantly different from each other (Fig. 1, C and D). These data suggest that a difference in MRP1 exists between the two

TABLE 1

Summary of kinetic values of $\text{As}(\text{GS})_3$ transport by WT-MRP1, SF-MRP1, and Asn19/23Gln-MRP1-enriched membrane vesicles from HeLa and HEK293 cells prepared in the presence or absence of a phosphatase inhibitor cocktail (PIC)

	$K_m \pm \text{S.D.} (\mu\text{M})$	$^aV_{max} \pm \text{S.D.} (\text{pmol mg}^{-1} \text{min}^{-1})$	<i>N</i>
HeLa			
WT-MRP1	$0.32 \pm 0.03^*$	$42 \pm 17^{**}$	4
WT-MRP1 + PIC	6.0 ± 1.3	505 ± 292	3
SF-MRP1	$0.3 \pm 0.1^*$	$44 \pm 15^{**}$	5
SF-MRP1 + PIC	3.2 ± 1.4	609 ± 251	5
HEK293			
WT-MRP1	3.8 ± 1.2	307 ± 118	8
WT-MRP1 + PIC	3.6 ± 0.8	270 ± 156	4
SF-MRP1	$0.6 \pm 0.4^*$	$38 \pm 21^{**}$	4
SF-MRP1 + PIC	3.3 ± 1.8	195 ± 81	5
N19/23Q-MRP1	$0.5 \pm 0.2^*$	$37 \pm 9^{**}$	3
N19/23Q-MRP1 + PIC	4.2 ± 1.6	300 ± 174	6

HEK, human embryonic kidney 293 (cell line); MRP1, multidrug resistance protein 1; SF, sugar-free; WT, wild-type.

^a V_{max} values were corrected for levels of WT-MRP1, SF-MRP1, and Asn19/23Gln relative to HEK-WT-MRP1 using densitometry (ImageJ Software) analysis of at least three different immunoblots (see Supplemental Table 2).

* K_m value significantly different ($P < 0.01$) from HEK-WT-MRP1 (one way analysis of variance, Dunnett's multiple comparison test).

** V_{max} value significantly different ($P < 0.01$) than for HEK-WT-MRP1 (one way analysis of variance, Dunnett's multiple comparison test).

cell lines that influences the affinity of $\text{As}(\text{GS})_3$ for MRP1, but not $\text{E}_217\beta\text{G}$ or LTC_4 .

Molecular Weight Difference between MRP1 Expressed in HeLa and HEK293 Cells. To investigate the differences between WT-MRP1 expressed in HeLa versus HEK293 cells, the respective relative electrophoretic mobility was determined using SDS-PAGE followed by immunoblot analysis. Consistent with previous results, we observed that WT-MRP1 expressed in HeLa cells migrated at ~190 kDa, whereas HEK-WT-MRP1 migrated at ~180 kDa (lanes 5 and 1, respectively, Fig. 2) (Stride et al., 1997). To determine whether this shift in molecular weight was due to glycosylation, MRP1-enriched vesicles prepared from HEK293 and HeLa cells were treated with different glycosidase enzymes (Fig. 2). Digestion with Endo H caused no shifts in MRP1 mobility, indicating that MRP1 within the plasma membrane-enriched vesicles was modified by complex glycosylation. On the other hand, complete removal of *N*-linked glycosylation using PNGase F resulted in the shift of HEK-WT-MRP1 and HeLa-WT-MRP1 to comparable mobilities of ~170 kDa. PNGase F-treated WT-MRP1 had the same mobility as the SF-MRP1 (the Asn19/23/1006Gln-MRP1 mutant that lacks glycosylation) expressed in HeLa and HEK293 cells. Thus, HeLa- and HEK-WT-MRP1 were fully glycosylated; however, differential glycosylation was responsible for the lower molecular weight of HEK-WT-MRP1 compared with HeLa-WT-MRP1.

TABLE 2

Summary of As(GS)₃ transport kinetic parameters for HEK-MRP1 mutated at Tyr920 and Ser921

	K_m (\pm S.D.)	V_{max} (corrected) ^a (\pm S.D.)	<i>N</i>
	μM	$\text{pmol mg}^{-1} \text{min}^{-1}$	
WT	3.8 ± 1.2	307 ± 118	8
Y920F/S921A	$0.4 \pm 0.1^*$	$28 \pm 16^{**}$	3
Y920F	3.9 ± 1.0	125 ± 54	3
S921A	3.7 ± 1.3	122 ± 42	3
Y920E/S921E	3.6 ± 0.8	135 ± 33	4
N19/23Q/Y920E/S921E	6.4 ± 1.9	162 ± 58	5

HEK, human embryonic kidney 293 (cell line); MRP1, multidrug resistance protein 1; SF, sugar-free; WT, wild-type.

^a V_{max} values were corrected for levels of mutant MRP1 relative to WT-MRP1 using densitometry (ImageJ Software) of at least three different immunoblots (see Supplemental Table 2).

* K_m value significantly different ($P < 0.01$) from HEK-WT-MRP1 (one-way analysis of variance, Dunnett's multiple comparison test).

** V_{max} value significantly different ($P < 0.01$) from HEK-WT-MRP1 (one-way analysis of variance, Dunnett's multiple comparison test).

To determine whether phosphorylation could also contribute to differences in the molecular weights of HEK-WT-MRP1 and HeLa-WT-MRP1, MRP1-enriched membrane vesicles were dephosphorylated by treatment with CIP. No shift in the molecular weight of either WT-MRP1 was detected (data not shown); however, the molecular weight of MRP1 (190 kDa) is quite large relative to the molecular weight of a phosphate group (95 Da), making it difficult to draw conclusions from this observation.

Kinetic Parameters of As(GS)₃ Transport by SF-MRP1 Expressed in HeLa and HEK293 Cells. After establishing that the difference in molecular weight between WT-MRP1 expressed in HeLa versus HEK293 cells was associated with differential *N*-glycosylation, the functional relevance of this finding was investigated. Consequently, K_m and V_{max} values were determined for As(GS)₃ transport using HeLa- and HEK-SF-MRP1-enriched membrane vesicles. Unexpectedly, HeLa-SF-MRP1 and HEK-SF-MRP1 displayed similar high-affinity (K_m $0.3 \pm 0.1 \mu\text{M}$ and $0.6 \pm 0.4 \mu\text{M}$, respectively) and low-capacity (V_{max} $44 \pm 15 \text{ pmol/mg per minute}$ and $38 \pm 21 \text{ pmol/mg per minute}$, respectively) kinetic parameters, similar to those of HeLa-WT-MRP1 (Fig. 3, A and B; Table 1). This finding indicated that *N*-glycosylation influenced transport of As(GS)₃ by MRP1.

Kinetic Analysis of E₂17 β G Transport by SF-MRP1 Expressed in HeLa and HEK293 Cells. Previous work on SF-MRP1 expressed in HeLa cells showed an increase in capacity of LTC₄ transport relative to HeLa-WT-MRP1 but no change in affinity (K_m) (Weigl, 2005). To determine whether the shift in affinity observed for SF-MRP1 expressed in HEK293 cells occurs for other substrates, the kinetic parameters of E₂17 β G transport by SF-MRP1 expressed in HeLa and HEK293 cells were determined. Consistent with the values for HeLa-WT-MRP1 and HEK-WT-MRP1 and the published data, the K_m value of E₂17 β G transport for HeLa-SF-MRP1 was $4.3 \mu\text{M}$ and HEK-SF-MRP1 was $5.9 \mu\text{M}$ (Fig. 3, C and D). These data suggest that although differential glycosylation of WT-MRP1 expressed in HEK293 versus HeLa cells is responsible for the shift in As(GS)₃ transport kinetic parameters, it does not influence the affinity of MRP1 for E₂17 β G.

Kinetic Analysis of As(GS)₃ Transport by Glycosylation Mutants of MRP1 Expressed in HEK293 Cells. As a result of the changes in As(GS)₃ kinetic parameters observed

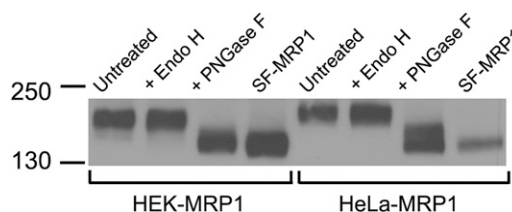


Fig. 2. Glycosylation status of WT-MRP1 stably expressed in HEK293 and HeLa cells. Plasma membrane preparations from WT-MRP1 expressed in HEK293 and HeLa cells were incubated with or without the deglycosylation enzymes Endo H and PNGaseF, separated by 8% SDS-PAGE and immunoblotted with the anti-MRP1 specific rat mAb MRPr1 (epitope mapped to amino acids 238–247) (Hipfner et al., 1998). The sugar-free (SF)-MRP1 mutant was included as a control for comparison purposes.

for SF-MRP1, the influence of glycosylation was further investigated. Single Asn19Gln, Asn23Gln, Asn1006Gln, and double Asn19/23Gln mutants of MRP1 were generated and expressed in HEK293 cells, and membrane vesicles were prepared and immunoblotted. A modest reduction in molecular weight for each single mutant and a more pronounced shift for the double Asn19/23Gln mutant were observed, indicating that all the *N*-glycosylation sites were used in HEK293 cells (Fig. 4A). Characterization of As(GS)₃ transport revealed that the kinetic parameters for the single glycosylation mutants were similar to HEK-WT-MRP1, with high K_m (range $3\text{--}5 \mu\text{M}$) and V_{max} (range, $385\text{--}460 \text{ pmol/mg protein per minute}$) (data not shown); however, the K_m and V_{max} values ($0.5 \pm 0.2 \mu\text{M}$ and $37 \pm 9 \text{ pmol/mg per minute}$, respectively) for the double Asn19/23Gln mutant were substantially decreased (Fig. 4B; Table 1) and not significantly different from HeLa-WT-MRP1. This finding suggested that differences in glycosylation at Asn19 and Asn23 contributed to the differences in As(GS)₃ transport by HEK-WT-MRP1 and HeLa-WT-MRP1.

Kinetic Analysis of E₂17 β G Transport by MRP1-Asn19/23Gln Expressed in HEK293 Cells. To ensure that, as for the fully glycosylated mutant, the Asn-to-Gln mutation of the first two glycosylation sites did not affect the transport affinity for every MRP1 substrate, E₂17 β G kinetic parameters were determined for MRP1-Asn19/23Gln expressed in HEK293 cells (Fig. 4C). The K_m value was $2.4 \mu\text{M}$, similar to that previously published for HEK-WT-MRP1 and HeLa-WT-MRP1 (Loe et al., 1996a; Stride et al., 1997) and determined for HEK-WT-MRP1, HEK-SF-MRP1, HeLa-WT-MRP1, and HeLa-SF-MRP1 by us (Fig. 1, C and D, and Fig. 3, C and D). Thus, the effect of glycosylation on transport by MRP1 is substrate-selective.

Kinetic Analysis of As(GS)₃ Transport by WT-MRP1 in the Presence of Phosphatase Inhibitors. In parallel with the kinetic characterization of As(GS)₃ transport by SF-MRP1, the influence of phosphorylation was also investigated. Thus, kinetic parameters of As(GS)₃ transport by WT-MRP1-enriched membrane vesicles prepared in the presence of a PIC were determined. MRP1-enriched vesicles prepared from HeLa and HEK293 cells, in the presence of a PIC, displayed a high K_m ($6.0 \pm 1.3 \mu\text{M}$ and $3.6 \pm 0.8 \mu\text{M}$, respectively) and V_{max} ($505 \pm 292 \text{ pmol/mg per minute}$ and $270 \pm 156 \text{ pmol/mg per minute}$, respectively), parameters that are comparable to HEK-WT-MRP1 in the absence of PIC (Fig. 5, A and B; Table 1). These results suggest that when MRP1 phosphorylation is maintained, MRP1 transports As(GS)₃ with lower affinity and higher capacity.

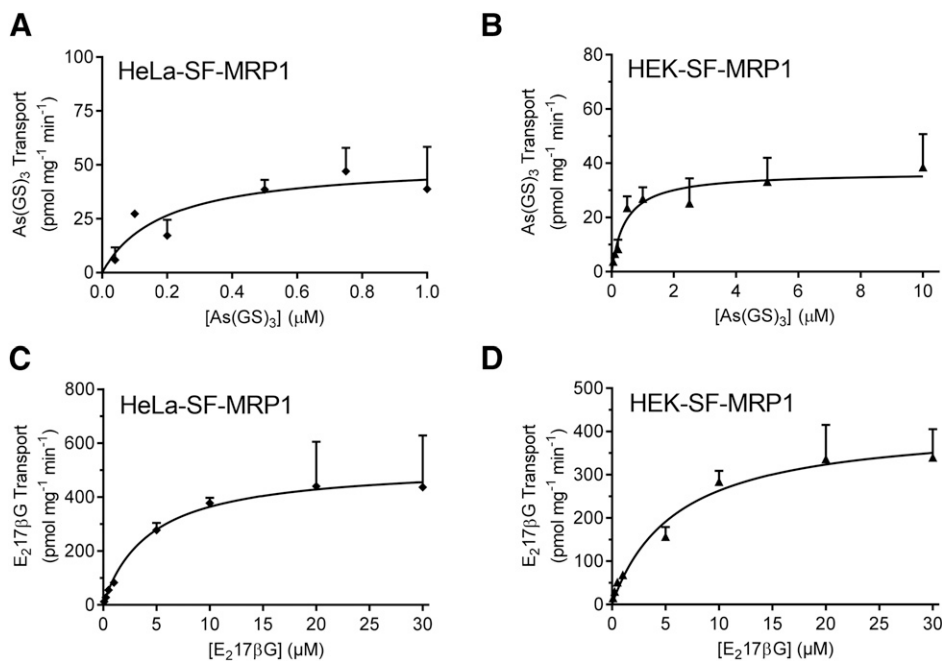


Fig. 3. Kinetics of $\text{As}(\text{GS})_3$ and $\text{E}_217\beta\text{G}$ transport by SF-MRP1-enriched membrane vesicles prepared from HeLa and HEK293 cells. ATP-dependent uptake of (A, B) $\text{As}(\text{GS})_3$ (0.05–10 μM , 30–120 nCi) or (C, D) $\text{E}_217\beta\text{G}$ (0.1–30 μM , 30–80 nCi) was measured for 1 minute and 1.5 minutes, respectively, at 37°C using membrane vesicles prepared from (A, C) HeLa-SF-MRP1 or (B, D) HEK-SF-MRP1 cells. Kinetic values were determined using Michaelis-Menten nonlinear regression analysis (Graphpad Prism). Data points represent means (+S.D.) of triplicate determinations in a single experiment and were corrected for MRP1 levels relative to HEK-WT-MRP1. For (A and B), similar results were obtained in at least three additional experiments, and mean kinetic parameters for $\text{As}(\text{GS})_3$ transport are summarized in Table 1.

Kinetic Analysis of $\text{As}(\text{GS})_3$ Transport by Deglycosylated Phosphorylated MRP1. To determine whether the influence of glycosylation and phosphorylation on $\text{As}(\text{GS})_3$ transport kinetics by MRP1 were interdependent, K_m and V_{max} values were determined using SF- and Asn19/23Gln-MRP1-enriched membrane vesicles prepared in the presence of a PIC. Under these conditions, HeLa-SF-MRP1, HEK-SF-MRP1, and HEK-Asn19/23Gln-MRP1-enriched membrane vesicles had high K_m ($3.2 \pm 1.4 \mu\text{M}$, $3.3 \pm 1.8 \mu\text{M}$, and $4.2 \pm 1.6 \mu\text{M}$, respectively) and high V_{max} ($609 \pm 251 \text{ pmol/mg per minute}$, $195 \pm 81 \text{ pmol/mg per minute}$, and $300 \pm 174 \text{ pmol/mg per minute}$, respectively) values (Fig. 5, C–E; Table 1). These

values were not significantly different from those for HEK-WT-MRP1 in the presence or absence of PIC. Therefore, maintaining phosphorylation of deglycosylated MRP1 results in lower-affinity, higher-capacity transport of $\text{As}(\text{GS})_3$, regardless of the cell line.

Effect of Phosphorylation on Limited Trypsinolysis of Deglycosylated MRP1. After demonstrating that the inclusion of a PIC during membrane vesicle preparation results in lower-affinity, higher-capacity transport of $\text{As}(\text{GS})_3$ by WT-MRP1 and SF-MRP1, regardless of the cell type, experiments were designed to narrow the region of phosphorylation maintained in the presence of PIC. This was necessary

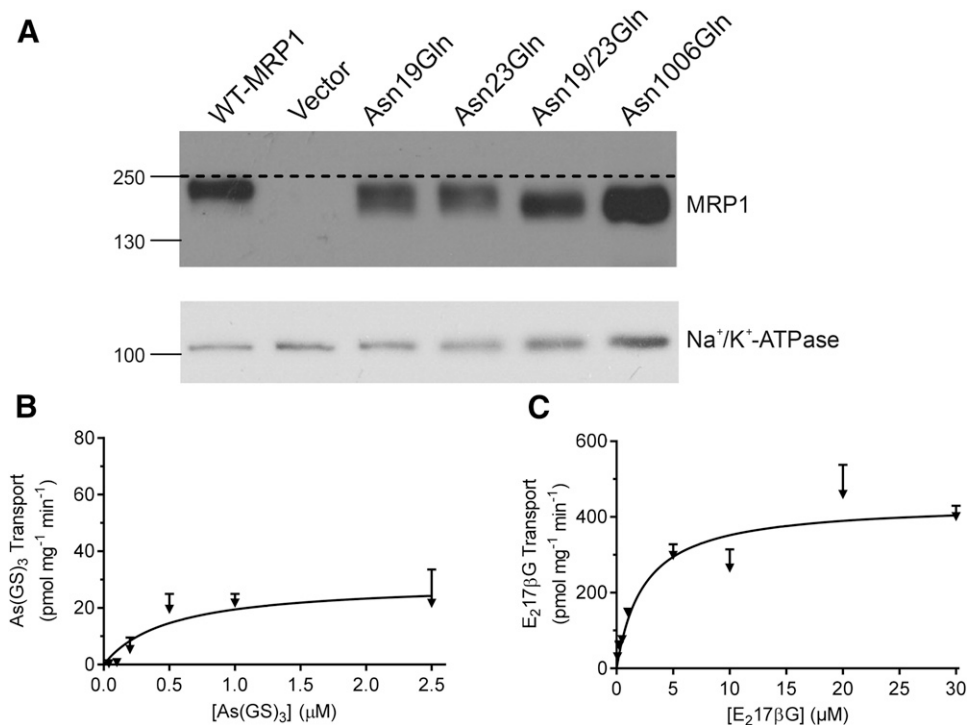


Fig. 4. Expression of MRP1 glycosylation mutants in HEK293 cells and kinetic analysis of $\text{As}(\text{GS})_3$ and $\text{E}_217\beta\text{G}$ transport by MRP1-N19/23Q. (A) Plasma membrane-enriched fractions containing various N-linked glycosylation site mutants of MRP1 were resolved by 7% SDS-PAGE and immunoblotted with the anti-MRP1-specific mAb MRP1. Blots were then probed with the anti- Na^+/K^+ -ATPase-specific pAb H-300. A hatched line is present to emphasize the slight shift in molecular weight between the WT-MRP1 and the glycosylation mutants. (B, C) MRP1-N19/23Q-enriched plasma membrane vesicles prepared from HEK293 cells were used to measure transport of (B) $\text{As}(\text{GS})_3$ (0.05–2.5 μM , 30–80 nCi) and (C) $\text{E}_217\beta\text{G}$ (0.1–30 μM , 30–80 nCi) at 1 minute and 1.5 minutes, respectively, at 37°C. Kinetic values were determined using Michaelis-Menten nonlinear regression analysis (Graphpad Prism). Data points represent means (+S.D.) in a single experiment, and similar results were obtained in two additional experiments with mean kinetic parameters for $\text{As}(\text{GS})_3$ transport summarized in Table 1. All data were corrected for MRP1 levels relative to HEK-WT-MRP1.

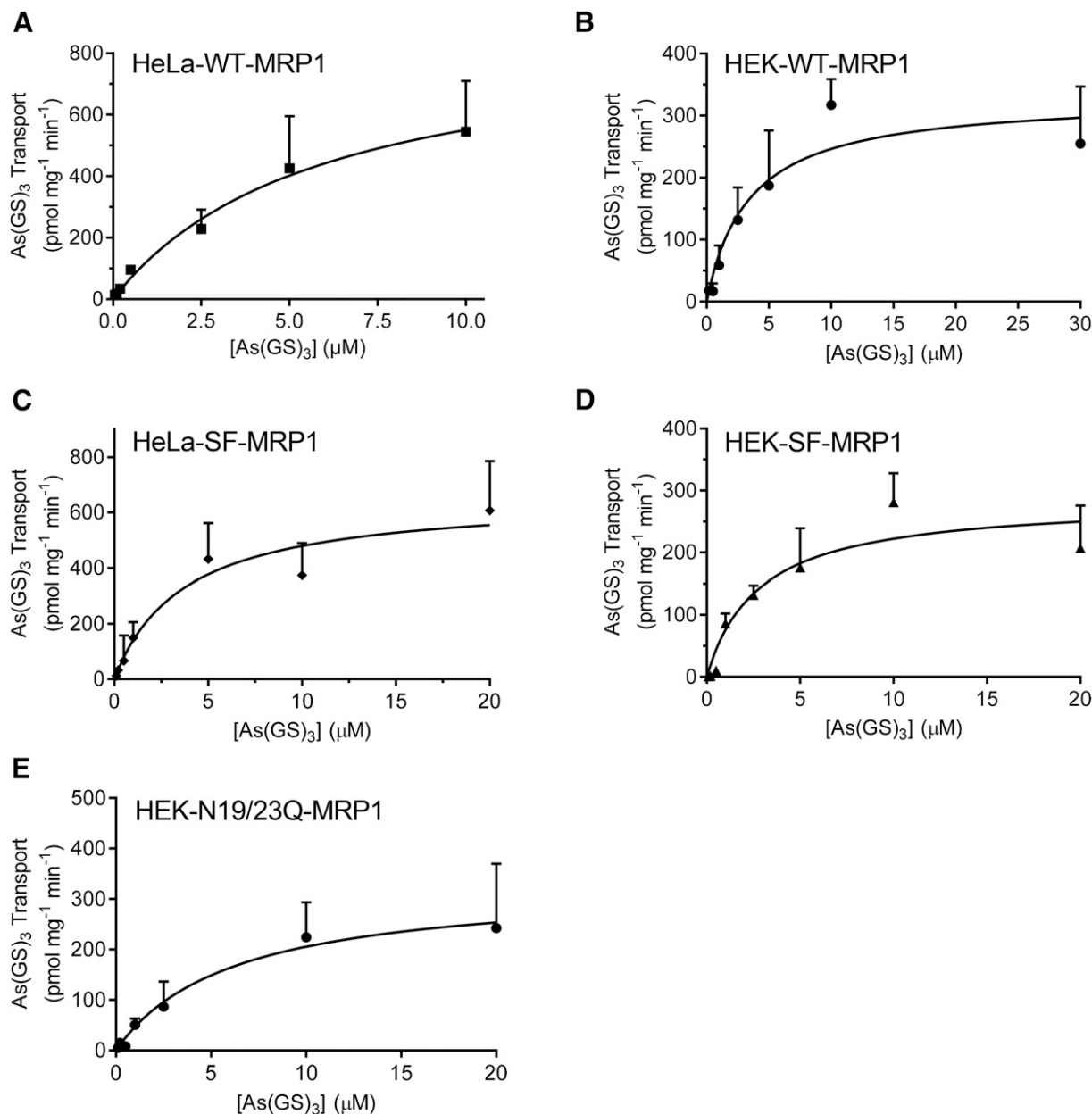


Fig. 5. Kinetics of $\text{As}(\text{GS})_3$ transport by WT-MRP1, SF-MRP1, and N19/23Q-MRP1-enriched membrane vesicles prepared in the presence of a PIC from HeLa and/or HEK293 cells. $\text{As}(\text{GS})_3$ (0.05–30 μM , 30–120 nCi) transport by MRP1-enriched membrane vesicles prepared in the presence of a PIC from (A) HeLa-WT-MRP1, (B) HEK-WT-MRP1, (C) HeLa-SF-MRP1, (D) HEK-SF-MRP1, and (E) HEK-N19/23Q-MRP1 was measured for 1 minute at 37°C. Kinetic values were determined using Michaelis-Menten nonlinear regression analysis (Graphpad Prism). Data points represent means (+S.D.) in a single experiment, and similar results were obtained in at least two additional experiments. Mean kinetic parameters are summarized in Table 1. All data were corrected for MRP1 levels relative to HEK-WT-MRP1.

because there are more than 50 predicted phospho-Ser, -Thr, and Tyr sites in the cytosolic domains of MRP1 (NetPhos 2.0) (Blom et al., 1999). To do this, we took advantage of the well characterized pattern of MRP1 fragments obtained after controlled exposure to trypsin and their detection with several regionally directed, MRP1-specific antibodies (Supplemental Fig. 1A) (Hipfner et al., 1997; Mao et al., 2002). Thus, limited trypsinolysis was used to produce small-molecular-weight fragments of MRP1 to visualize mobility shifts, in the presence or absence of PIC, owing to phosphorylation (Supplemental Fig. 1). Tryptic fragments of MRP1 were detected with MRP1-specific antibodies against the NH_2 -proximal (N1 and N2 with mAb MRPr1; N1 and N3 with pAb MRP-1) and COOH-

proximal (C1 with mAb QCRL-1; C1 and C2 with mAb MRPr6) regions of the transporter (Supplemental Fig. 1A).

Because of the striking difference between $\text{As}(\text{GS})_3$ transport kinetics in vesicles prepared from HEK-SF-MRP1 in the presence and absence of PIC, the molecular weights of tryptic fragments of HEK-SF-MRP1 prepared under the two conditions were compared. As shown in Supplemental Fig. 1, B and C, a small shift in N1 mobility was detected with both MRPr1 and MRP-1 antibodies, suggesting that the N1 fragment contains a site that is phosphorylated when vesicles are prepared in the presence of PIC. Somewhat unexpectedly, there was no detectable corresponding shift in the N2 (Supplemental Fig. 1B) or N3 (Supplemental Fig. 1C)

fragments of HEK-SF-MRP1. These observations suggested that the phosphorylation site resided in the area of N1 cleaved between N2 and N3 or the cleaved COOH-terminal portion of N3 lost due to further trypsinolysis of the N2 and N3 fragments. Similar results to HEK-SF-MRP1 were obtained for HEK-N19/23Q-MRP1 in the presence and absence of PIC (data not shown). No shifts were observed for the C1 or C2 of the COOH-terminal fragments as detected with mAbs MRPm6 and QCRL-1 (data not shown). Taken together, the limited tryptic digest data suggested that the phosphorylated residues that are maintained by PIC treatment during membrane vesicle preparation reside within the N1 fragment and, more specifically, in the regions cleaved by further trypsin digestion: CL3 connecting MSD0 to MSD1 and/or the linker region on the COOH-terminus of the NH₂-proximal half of MRP1 (Supplemental Fig. 1A; Fig. 6A).

Analysis of As(GS)₃ Transport by MRP1 Mutated at Potential Phosphorylation Sites in CL3. To explore the possibility that phosphorylation of one or more amino acids in CL3 (spanning approximately amino acids 193–320) connecting MSD0 to MSD1 could be responsible for modulating the affinity and capacity of MRP1 for As(GS)₃, a site-directed mutagenesis-based approach was used. The mAb MRPr1 reliably detects the N1 and N2 fragments (but not N3) after limited trypsinolysis of MRP1, indicating that its epitope remains intact during trypsin digestion. Therefore, if loss of a phosphorylation site between N2 and N3 during trypsinolysis occurs (as suggested by the results shown in Supplemental Fig. 1, B and C), it must occur COOH-proximal to Glu247 since the MRPr1 epitope has been mapped to amino acids 238–247 (Hipfner et al., 1998). There are seven Ser, three Thr, and one Tyr residues between amino acids 248 and 320 of human MRP1. Ser304, Ser312, and Thr320 were excluded because they are located far from the junction of the N2 and N3 fragments. To determine whether the remaining Thr249, Ser250, Thr268, Tyr277, Ser278, Ser279, Ser288, and/or Ser289 were important for determining the affinity and capacity of MRP1 for As(GS)₃, they were individually mutated to Ala. All Ala-substituted mutants were expressed at comparable levels to WT-MRP1 in HEK293 cells (Fig. 6). In addition, As(GS)₃ transport for all eight mutants exhibited high K_m (range, 3.1–13 μ M) and V_{max} (range, 145–610 pmol/mg per minute) values similar to those of HEK-WT-MRP1 (data not shown). To address the possibility that multiple residues in this region must be phosphorylated in order for a shift in kinetics to be observed, all eight potential phosphorylation sites were mutated in a single expression construct (i.e., MRP1-Thr249Ala/Ser250Ala/Thr268Ala/Tyr277Ala/Ser278Ala/Ser279Ala/Ser288Ala/Ser289Ala or MRP1_8X_CL3). Characterization of As(GS)₃ kinetics for HEK-MRP1_8X_CL3 revealed moderately lower K_m of $2.0 \pm 0.7 \mu$ M and V_{max} of 177 ± 90 pmol/mg per minute values versus HEK-WT-MRP1; however, they were not significantly different. Thus, phosphorylation of the Ser/Thr/Tyr residues within CL3 did not account for the differences in As(GS)₃ transport kinetics between the HEK293 and HeLa cell lines.

Analysis of As(GS)₃ Transport by MRP1 Mutated at Potential Phosphorylation Sites in the Linker Region between NBD1 and MSD2. To determine whether the PIC-dependent shift in the molecular weight of the N1 fragment of MRP1 (Supplemental Fig. 1, B and C) was due to phosphorylation at the COOH terminus of NBD1, a site-directed mutagenesis study of this region was initiated. The linker region

between NBD1 and MSD2 is hypersensitive to proteases and is also the site of the epitope detected by the MRP1-specific mAb QCRL-1 (amino acids 918–924) and thus represents the boundary between the N1 and C1 tryptic halves of MRP1 (Hipfner et al., 1996). Consequently, as a starting point, 11 potential phosphorylation sites between Ser905 and Thr931 were mutated either individually (Ser905Ala, Ser915Ala, Ser916Ala, Ser917Ala, Ser925Ala, Ser930Ala, Thr931Ala), or (to increase screening efficiency) as double mutants (Ser918/919-Ala and Tyr920Phe/Ser921Ala). All single and double mutants were expressed at levels comparable to WT-MRP1 in HEK293 cells (Fig. 6). The Ser916Ala and Thr931Ala mutants exhibited 1.3- and 1.5-fold higher As(GS)₃ (2.5 μ M; 80 nCi) transport activity than WT-MRP1 (data not shown) and were not characterized kinetically. All other linker region mutants exhibited high K_m (range, 2–5.7 μ M) and V_{max} (range, 81–323 pmol/mg protein per minute) values similar to those of HEK-WT-MRP1 (data not shown), with one exception (Fig. 7A; Table 2). Thus, the Tyr920Phe/Ser921Ala-MRP1 double mutant exhibited As(GS)₃ transport kinetic parameters (K_m of $0.4 \pm 0.1 \mu$ M and V_{max} of 28 ± 16 pmol/mg per minute) very similar to those of HeLa-WT-MRP1 and HeLa/HEK-SF-MRP1, suggesting that phosphorylation of one or both of Tyr920 and Ser921 was critical for regulating the affinity and transport capacity of MRP1 for As(GS)₃.

To determine whether phosphorylation of Tyr920, Ser921, or both were required for the shift in As(GS)₃ kinetic parameters, single mutants were generated and characterized (Table 2). The kinetic parameters for both single mutants Tyr920Phe-MRP1 (K_m of $3.9 \pm 1.0 \mu$ M and V_{max} of 125 ± 54 pmol/mg per minute) and Ser921Ala-MRP1 (K_m of $3.7 \pm 1.3 \mu$ M and V_{max} of 122 ± 42 pmol/mg per minute) were higher than those of the double mutant and were not significantly different from HEK-WT-MRP1, suggesting that both Tyr920 and Ser921 must be mutated to observe the reduction of As(GS)₃ K_m and V_{max} values.

To mimic phosphorylation of Tyr920 and Ser921, both amino acids were also replaced with a negatively charged Glu residue. Consistent with phosphorylation of these amino acids being responsible for modulation of As(GS)₃ transport by MRP1, the HEK-Tyr920Glu/Ser921Glu-MRP1 exhibited As(GS)₃ transport kinetic parameters (K_m of $3.6 \pm 0.8 \mu$ M and V_{max} of 135 ± 33 pmol/mg per minute) similar to those of HEK-WT-MRP1 (Fig. 7B; Table 2).

Transport of E₂17 β G and Methotrexate by HEK-Tyr920Phe/Ser921Ala-MRP1. To ensure that the double Tyr920Phe/Ser921Ala mutation did not just generally disrupt MRP1 function, kinetic parameters for E₂17 β G transport by this mutant were determined. Similar to values determined for HeLa- and HEK-WT-MRP1 in this and previously published studies, E₂17 β G was transported by HEK-Tyr920Phe/Ser921Ala-MRP1 with a K_m of 2.3 μ M and V_{max} of 424 pmol/mg per minute (compare Fig. 7C with Fig. 1, C and D) (Loe et al., 1996a; Stride et al., 1997). Similarly, methotrexate (100 μ M, 250 nCi) transport by HEK-Tyr920Phe/Ser921Ala-MRP1 and WT-MRP1 was the same (Fig. 7D). Methotrexate transport by the phosphorylation mimicking Tyr920Glu/Ser921Glu-MRP1 mutant was also no different from those of WT-MRP1 (Fig. 7D).

As(GS)₃ Transport by HEK-Asn19/23Gln/Tyr920Glu/Ser921Glu-MRP1. To determine whether the phosphorylation-mimicking Tyr920Glu/Ser921Glu substitutions could convert

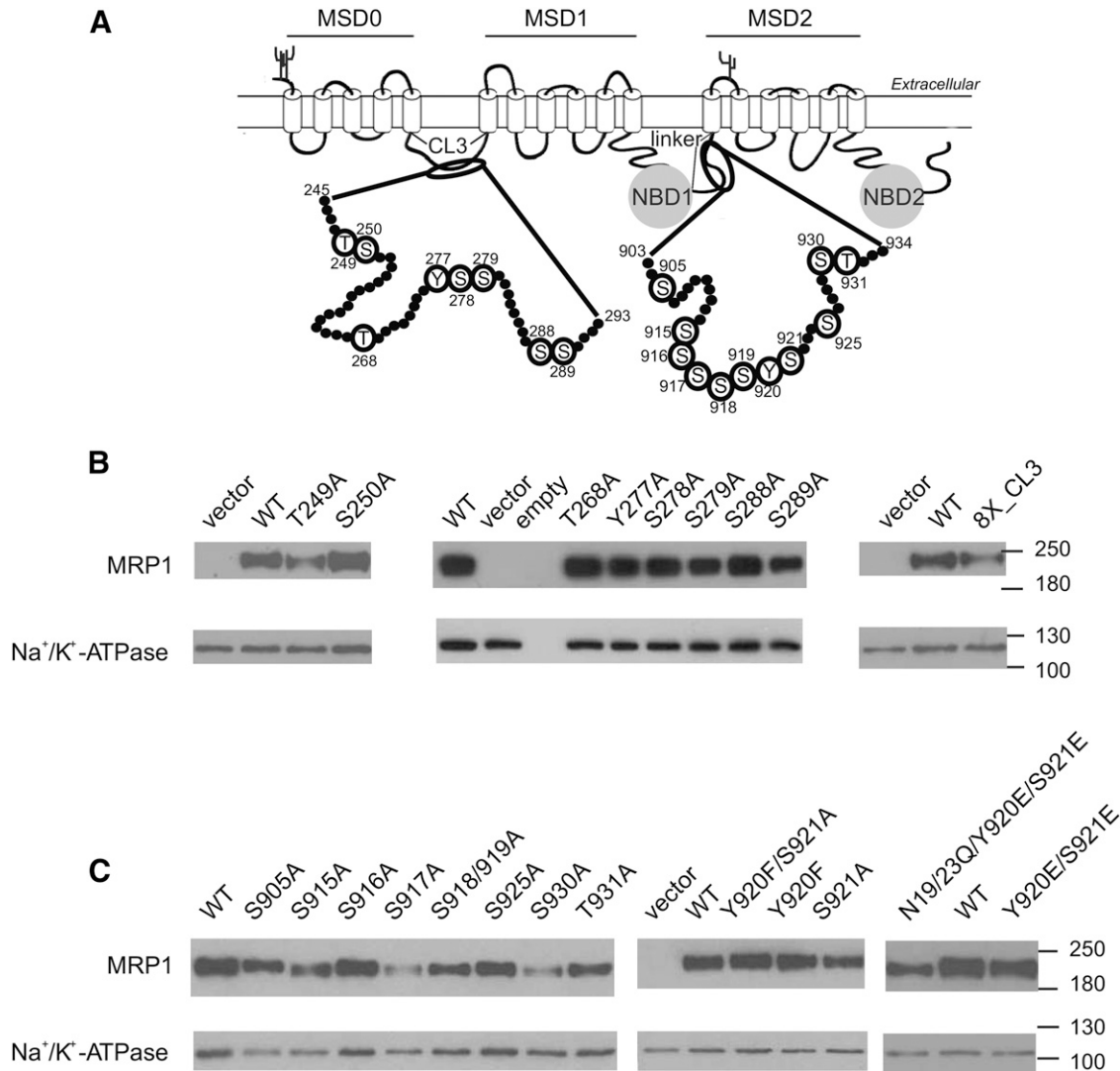


Fig. 6. Predicted location of selected potential Ser/Thr/Tyr phosphorylation sites in human MRP1 and immunoblots of MRP1 mutated at these residues. (A) Shown is a predicted secondary structure of MRP1. The locations of the 19 potential phosphorylation sites mutated in this study are indicated. (B, C) Representative immunoblots of membrane vesicles prepared from HEK293 cells expressing empty vector, WT-MRP1, and potential single and double phosphorylation-site MRP1 mutants: (B) CL3 mutants; or (C) mutants in the linker region between NBD1 and MSD2. MRP1 proteins were detected with mAb MRPm6, and then blots were probed with the anti- Na^+/K^+ -ATPase antibody H-300 (as a loading control).

the Asn19/23Gln-MRP1 transport kinetics from a high-affinity, low-capacity to lower-affinity, higher-capacity transporter of $\text{As}(\text{GS})_3$, the quadruple Asn19/23Gln/Tyr920Glu/Ser921Glu-MRP1 mutant was generated and expressed in HEK293 cells. Membrane vesicles enriched for this quadruple MRP1 mutant had kinetic parameters (K_m $6.4 \pm 1.9 \mu\text{M}$ and V_{max} of $162 \pm 58 \text{ pmol/mg per minute}$) similar to HEK-WT-MRP1 (Fig. 7E; Table 2), suggesting that the phosphorylation-mimicking substitutions restored the ability of the double *N*-glycosylation Asn19/23Gln mutant to transport $\text{As}(\text{GS})_3$ with lower affinity and higher capacity. These results strongly suggest that it is phosphorylation of Tyr920 and Ser921, rather than *N*-glycosylation, that (directly) regulates $\text{As}(\text{GS})_3$ transport capacity and affinity.

Effect of Phosphorylation on Immunoreactivity of MRP1 with mAb QCRL-1. Kinetic parameters for HeLa-WT-MRP1 were not significantly different from those of the dephosphorylated HEK-Tyr920Phe/Ser921Ala-MRP1 mutant,

supporting the idea that HEK-WT-MRP1 and HeLa-WT-MRP1 are differentially phosphorylated at these amino acids (Tables 1 and 2). For this reason, the region of differential phosphorylation of HeLa-WT-MRP1 and HEK-WT-MRP1 was explored using the MRP1-specific mAb QCRL-1, whose epitope spans MRP1 amino acids 918–924 (and contains Tyr920 and Ser921) (Hipfner et al., 1996). Thus, immunoblots were done on samples prepared in the presence and absence of PIC to determine whether the reactivity of QCRL-1 with HeLa-MRP1 and HEK-MRP1 vesicles was affected by phosphorylation (Supplemental Fig. 2, top panel). These immunoblots were then stripped, treated with CIP, and blotted again with QCRL-1 so that reactivity under the two conditions could be compared (Supplemental Fig. 2, middle panel). Before CIP treatment, HEK-MRP1 levels were 60% lower than HeLa-MRP1 levels ($P < 0.02$) (Supplemental Fig. 2, top panel). After CIP treatment, HEK-MRP1 levels were 5- \pm 2-fold higher than HeLa-MRP1 levels ($P < 0.02$) (Supplemental Fig. 2, middle panel). These results indicate

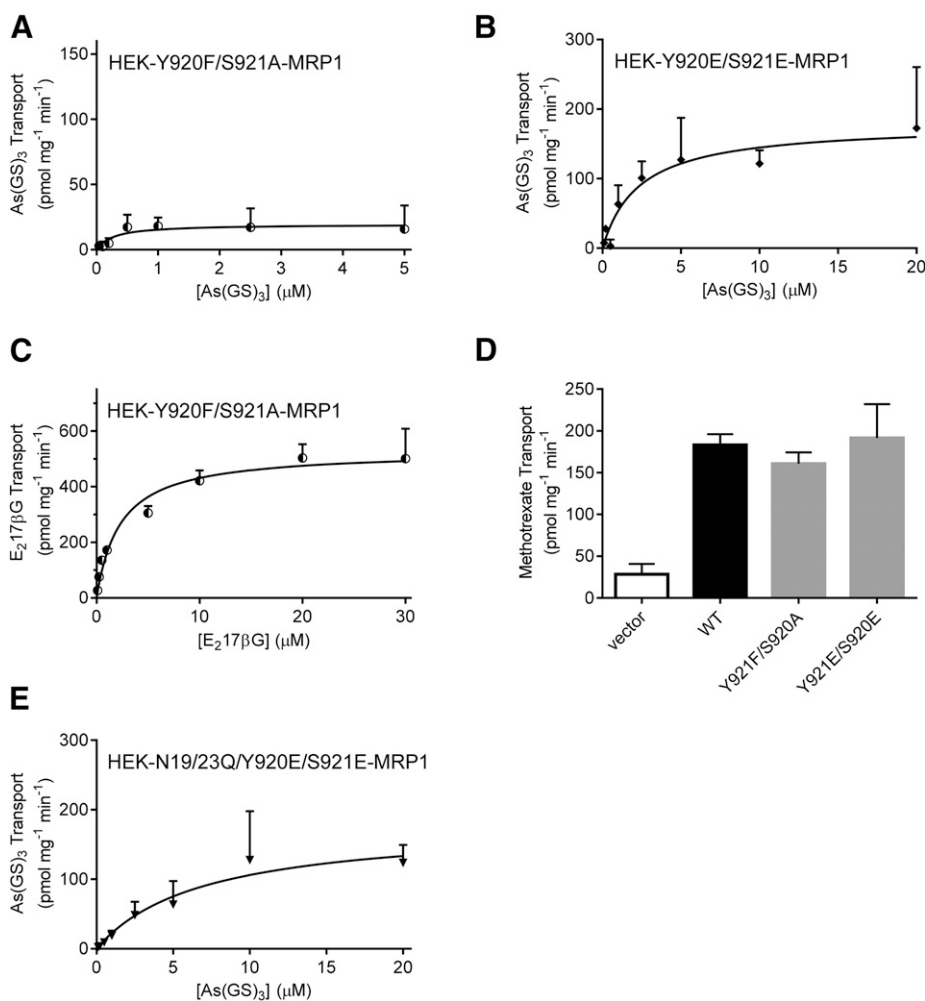


Fig. 7. As(GS)₃, E₂17βG, and/or methotrexate transport by Tyr920Phe/Ser921Ala-MRP1, Tyr920Glu/Ser921Glu-MRP1, and Asn19/23Gln/Tyr920Glu/Ser921Glu-MRP1 enriched membrane vesicles prepared from HEK293 cells. ATP-dependent uptake of (A, B, and E) As(GS)₃ (0.05–20 μM), (C) E₂17βG (0.1–30 μM, 30–80 nCi), or (D) methotrexate (100 μM, 250 nCi) was measured for 1, 1.5, and 20 minutes, respectively, at 37°C using membrane vesicles prepared from HEK293 cells expressing the indicated *N*-glycosylation and phosphomimetic mutant MRP1 proteins. Data points and bars represent means (+ S.D.) of triplicate determinations in a single experiment, and similar results were obtained in at least one additional experiment. All data were corrected for MRP1 levels relative to HEK-WT-MRP1. Kinetic values were determined using Michaelis-Menten nonlinear regression analysis (Graphpad Prism). Mean kinetic parameters for As(GS)₃ transport are summarized in Table 2.

that QCRL-1 reacts more strongly with dephosphorylated MRP1. They also strongly suggest that the region of MRP1 corresponding to the QCRL-1 epitope (918–924) in HEK293 and HeLa membrane vesicles is differentially phosphorylated. The difference in As(GS)₃ transport kinetics between HeLa-MRP1 prepared in the presence and absence of PIC was considerable, whereas no significant difference was observed under these conditions for HEK-MRP1 (Table 1). Importantly, the relative QCRL-1 reactivity was consistent with the relative differences in As(GS)₃ transport. Thus, before CIP treatment, HeLa-MRP1 (–PIC) levels were 1.7- ± 0.5-fold higher than HeLa-MRP1 (+PIC) levels ($P < 0.02$), indicating that HeLa-MRP1 (+PIC) QCRL-1 epitope was phosphorylated to a greater extent than HeLa-MRP1 (–PIC). After CIP treatment and reprobings blots with QCRL-1, the difference in MRP1 signal intensity between HeLa-MRP1 (+PIC) and HeLa-MRP1 (–PIC) was lost. As observed for As(GS)₃ transport (Table 1, Figs. 1B and 5B), there was no difference in QCRL-1 immunoreactivity between HEK-MRP1 with or without PIC (Supplemental Fig. 2, top panel). Overall, these observations suggest that the QCRL-1 epitope (containing Tyr920/Ser921) is phosphorylated in HEK-MRP1 (±PIC) and HeLa-MRP1 (+PIC) to a greater extent than HeLa-MRP1 (–PIC), and under these conditions, higher As(GS)₃ K_m and V_{max} values are also observed.

Discussion

The results presented in this study suggest that the substantial differences in the kinetics of As(GS)₃ transport by WT-MRP1-enriched membrane vesicles isolated from HEK293 and HeLa cells are caused by cell line-specific differences in MRP1 post-translational modification. Specifically, MRP1 affinity and capacity for As(GS)₃ are influenced by glycosylation of Asn19 and Asn23 and phosphorylation of Tyr920 and Ser921. Our data also indicate that cross-talk occurs between MRP1 *N*-glycosylation and phosphorylation, with phosphorylation dictating the affinity and capacity for As(GS)₃ and *N*-glycosylation influencing the phosphorylation status. Finally, since transport of other organic anion substrates (i.e., E₂17βG and methotrexate) of MRP1 are unaffected by Tyr920/Ser921 phosphorylation, this post-translational regulation appears to be a substrate-selective phenomenon. We propose that phosphorylation of MRP1 at Tyr920 and Ser921 allows a switch from high-affinity, low-capacity to low-affinity, high-capacity, resulting in the transport of arsenic by MRP1 over a wide concentration range.

The cell line differences in MRP1 transport kinetics for As(GS)₃ were unexpected because almost identical kinetic parameters had previously been reported using MRP1-enriched membrane vesicles isolated from HEK293 and HeLa cell lines for the prototypical MRP1 substrates E₂17βG and

LTC₄ (Loe et al., 1996a,b; Stride et al., 1997). Kinetic parameters of E₂17βG were also confirmed in this study to be very similar between the two cell lines (Fig. 1, C and D). Consistent with our observation that Tyr920 and Ser921 modulates MRP1 transport of As(GS)₃ selectively, amino acids 859–931 in the MRP1 linker region joining NBD1 to MSD2 have been shown previously to be nonessential for LTC₄ binding and transport (Gao et al., 1998). Furthermore, no difference was found for methotrexate transport activity between HEK-WT-MRP1 and the HEK-Tyr920Phe/Ser921Ala or HEK-Tyr920Glu/Ser921Glu mutants (Fig. 7D). Thus, our data support the conclusion that the As(GS)₃ binding site on MRP1 contains elements (i.e., contact amino acids) that distinguish it from the binding site(s) of not only E₂17βG and methotrexate, but also LTC₄ and likely other organic anions. This is consistent with previous reports that individual substrates of MRP1 establish their own distinct and often mutually exclusive interactions with amino acids (Cole and Deeley, 2006; Deeley and Cole, 2006; Maeno et al., 2009). Interestingly, casein kinase 2α-dependent phosphorylation of MRP1 at Thr249 in CL3 has been reported to stimulate the transport of LTC₄ and E₂17βG, as well as reduce doxorubicin accumulation and cytotoxicity (Stolarczyk et al., 2012). In contrast, mutation of Thr249 to Ala had no significant effect on As(GS)₃ transport (K_m 5.1 ± 3.5 μM, V_{max} 404 ± 267 pmol/mg per minute in HEK), providing further support for a unique As(GS)₃ binding site and a novel mechanism of MRP1 regulation. Kinases and phosphatases responsible for the phosphorylation and dephosphorylation, respectively, of Tyr920 and Ser921 are potential therapeutic targets for increasing MRP1 function after environmental arsenic exposure or reducing MRP1 activity during As₂O₃ therapy.

Our finding that MRP1-Tyr920 is phosphorylated is in contrast to an earlier study in which phosphoamino acid analysis of MRP1 immunoprecipitated from HL60/ADR cells indicated that only Ser residues (but not Tyr or Thr residues) on the transporter are phosphorylated (Ma et al., 1995). The reasons for the different observations of the two studies are uncertain; however, Ma et al. (1995) included the protein tyrosine phosphatase inhibitor sodium vanadate in their lysis buffer, and since EDTA (a chelator of vanadate) was also present, protein tyrosine phosphatase inhibition was likely incomplete (Ma et al., 1995; Huyer et al., 1997). Moreover, there are currently 70 records of MRP1-Tyr920 phosphorylation in the PhosphoSitePlus database (www.PhosphositePlus.com), providing strong support for its utilization (Hornbeck et al., 2015).

Initially, it was surprising to us that glycosylation influenced the affinity of MRP1 for As(GS)₃. Although *N*-linked glycosylation is well known to influence the localization, stability, and expression of a variety of membrane proteins, there are few examples of it affecting substrate binding by transporters (Filippo et al., 2011; Moremen et al., 2012; Scott and Panin, 2014). Our observation that SF-MRP1 and Asn19/23Gln-MRP1 membrane vesicles prepared in the presence of a PIC had low-affinity, high-capacity As(GS)₃ transport, regardless of the cell line from which they were prepared, suggested that glycosylation might be influencing the phosphorylation status of MRP1 rather than directly altering As(GS)₃ affinity and capacity. Membrane vesicles prepared from Asn19/23Gln/Tyr920Glu/Ser921Glu-MRP1 expressed in HEK293 cells had low-affinity, high-capacity As(GS)₃ transport (Fig. 7E; Table 2), consistent with phosphorylation

dictating MRP1's As(GS)₃ affinity and capacity rather than glycosylation.

How glycosylation of Asn19 and Asn23 at the extracellular NH₂-terminus of MRP1 influences Tyr920 and Ser921 phosphorylation in the cytosolic linker region between NBD1 and MSD2 remains to be determined. One possibility is that the size and content of the glycans at the NH₂-terminus could influence the conformation of MRP1 and alter the accessibility of its linker region to kinases and/or phosphatases. Indeed, some evidence has been reported, suggesting that the intracellular domains can influence the extracellular NH₂-terminus of MRP1. For example, evidence from antibody binding to the NH₂-terminus suggests that ATP-binding to the cytosolic NBDs of MRP1 results in a conformational change propagated to the NH₂-terminal domain (Binyamin et al., 2005). Interestingly, reduced *N*-linked glycosylation of MRP1 has been reported in an oxaliplatin-selected ovarian carcinoma cell line relative to its parental cell line (Beretta et al., 2010). The sugar group content of glycosylation has been reported to differ for other *N*-glycosylated proteins between different cell and tissue types and during disease states (Fujimori-Arai et al., 1997; Peng et al., 2003; Fujikura et al., 2011; Bull et al., 2014; Ferris et al., 2014). Thus, it is possible that the composition of MRP1 glycosylation could vary between different healthy tissue types and different disease states, which in turn could alter MRP1 phosphorylation at Tyr920 and Ser921. This has the potential to ultimately impact the detoxification of arsenic as well as any other (yet to be identified) substrates that involve Tyr920 and Ser921 in MRP1 for binding and transport.

The relative resistance of HEK293 and HeLa cells stably expressing MRP1 to As^{III} is similar (~2- and 3-fold, respectively) (Cole et al., 1994; Stride et al., 1997; Carew et al., 2011), suggesting that phosphorylation of Tyr920 and Ser921 may occur to the same extent in the two cell lines under certain conditions. This is consistent with the presence of PIC during the vesicle preparation resulting in low-affinity, high-capacity As(GS)₃ transport function, regardless of the cell line. Thus, although the kinases responsible for Tyr920 and Ser921 phosphorylation are important to identify, we think it is more likely that the differences between HEK-WT-MRP1 and HeLa-WT-MRP1 membrane vesicles are caused by differences in susceptibility to a phosphatase, potentially as a result of a conformational change in the linker region induced by differential glycosylation.

It is also possible that the HEK293 and HeLa cells express different phosphatases, and HEK-WT-MRP1 retains Tyr920 and Ser921 phosphorylation because the phosphatase(s) responsible for cleaving these sites is/are not expressed. Interestingly, multiple studies have shown that arsenic can influence both kinase and phosphatase signaling pathways. For example, arsenic can activate mitogen-activated protein kinase, c-Jun N-terminal kinase, and PI3K/Akt/mTOR pathways (Verma et al., 2002; Gafis et al., 2006; Plataniias, 2009; Goussetis and Plataniias, 2010; Beauchamp et al., 2015). Phosphoproteomic studies comparing untreated and arsenic-treated cell lines have also shown a general increase in protein phosphorylation through these pathways (Alp et al., 2010; Wen et al., 2010). Arsenic is also known to inhibit certain phosphatases, including protein tyrosine phosphatase 1B and the transmembrane tyrosine phosphatase CD45 (Rehman et al., 2012). Thus, we suggest that exposure of cells to arsenic

results in a shift to a pro-phosphorylation state, which in turn promotes MRP1 to switch from a high-affinity, low-capacity to a low-affinity, high-capacity As(GS)₃ transporter.

The success of As₂O₃ in the treatment of acute promyelocytic leukemia has generated substantial interest in its use as a therapeutic for other leukemias and solid tumors (Platanias, 2009; Subbarayan and Ardalan, 2014). In vitro results are promising, but clinical success has been limited by the higher doses of As₂O₃ required for induction of non-acute promyelocytic leukemia tumor cell death (Platanias, 2009). Considerable effort has been made to target kinase pathways that are activated by As₂O₃ to increase the efficacy of this agent (Verma et al., 2002; Gafis et al., 2006; Dolniak et al., 2008; McNeer et al., 2010; Galvin et al., 2013; Beauchamp et al., 2015; Lozano-Santos et al., 2015). It may be that the kinase pathways responsible for phosphorylation of Tyr920 and Ser921 of MRP1 overlap with the kinase pathways activated by As₂O₃. If this is true, then the administration of kinase inhibitors would not only increase the antileukemic properties of As₂O₃ but would also prevent Tyr920 and Ser921 phosphorylation, resulting in saturation of MRP1 transport and increased As₂O₃ accumulation within tumor cells.

In conclusion, the results of the present study show that both N-glycosylation and Ser/Tyr phosphorylation are critical for the regulation of MRP1-mediated transport of As(GS)₃ and could considerably alter the handling of arsenic by cells and tissues. Thus, phosphorylation of MRP1 presents a novel and potential high-impact cellular defense mechanism, based on its ability to switch cellular arsenic efflux from a high-affinity, low-capacity process (important during low levels of arsenic exposure), to a lower-affinity but higher-capacity process under conditions of higher arsenic exposure. Our findings have important implications for the future design of therapeutic agents that can modulate the phosphorylation status of MRP1 to increase its transport capacity during arsenic poisoning and reduce its transport capacity during administration of arsenic-based chemotherapeutics.

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Authorship Contributions

Participated in research design: Shukalek, Leslie.

Conducted experiments: Shukalek, Swanlund, Rousseau, Marensi.

Contributed new reagents or analytic tools: Shukalek, Swanlund, Rousseau, Weigl, Cole, Leslie.

Performed data analysis: Shukalek, Swanlund, Leslie.

Wrote or contributed to the writing of the manuscript: Shukalek, Cole, Leslie.

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