Glutathione Stimulates Sulfated Estrogen Transport by Multidrug Resistance Protein 1*

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Multidrug resistance protein 1 (MRP1) is an ATP-binding cassette (ABC) transporter that transports a range of hydrophobic xenobiotics, as well as relatively hydrophilic organic anion conjugates. The protein is present at high levels in testicular Leydig and Sertoli cells. Studies with knockout mice suggest that MRP1 may protect germ cells from exposure to some cytotoxic xenobiotics, but potential endobiotic substrates in this organ have not been identified. Previously, we have shown certain D-ring, but not A-ring, estrogen glucuronides can act as competitive inhibitors of MRP1 mediated transport, suggesting that they are potential substrates for the protein. In the case of 17β-estradiol-17β-D-glucuronide, this has been confirmed by direct transport studies. The Leydig cell is the major site of estrogen conjugation in the testis. However, the principal products of conjugation are A-ring estrogen sulfates, which are then effluxed from the cell by an unknown transporter. To determine whether MRP1/mrp1 could fulfill this function, we used membrane vesicles from MRP1-transfected HeLa cells to assess this possibility. We found that estradiol and estrone 3-sulfate alone were poor competitors of MRP1-mediated transport of the cysteiny1 leukotriene, leukotriene C4. However, in the presence of reduced glutathione (GSH), their inhibitory potency was markedly increased. Direct transport studies using [35S]estrone 3-sulfate confirmed that the conjugated estrogen could be efficiently transported (Km = 0.73 μM, Vmax = 440 pmol mg-1 protein min-1), but only in the presence of either GSH or the nonreducing alkyl derivative, S-methyl GSH. In contrast to previous studies using vincristine as a substrate, we detected no reciprocal increase in MRP1-mediated GSH transport. These results provide the first example of GSH-stimulated, MRP1-mediated transport of a potential endogenous substrate and expand the range of MRP1 substrates whose transport is stimulated by GSH to include certain hydrophilic conjugated endobiotics, in addition to previously identified hydrophobic xenobiotics.

Human multidrug resistance protein (MRP)1 is a member of the ATP-binding cassette superfamily of transmembrane transporters, which was originally discovered by virtue of its association with drug resistance in tumor cells (1). It is now also known to be a primary active transporter of many conjugated organic anions (2). The first substrate shown to be actively transported by MRP1 using inside-out membrane vesicles was the glutathione-conjugated leukotriene, LTC4 (3, 4). Since then, the spectrum of molecules transported by MRP1 has been extended to include many other GSH conjugates, as well as several glucuronate and sulfate conjugates (5, 6). A number of unconjugated amphiphilic anions have also been demonstrated to be substrates for MRP1 (7). However, using an in vitro membrane vesicle system unmodified forms of the natural product drugs to which MRP1 confers resistance are not directly transported by this protein, although primary active transport of some of them has been observed in the presence of reduced glutathione (GSH) (8–11). Potential endogenous substrates of MRP1 that show a similar dependence on the presence of GSH have not been identified, although it has been observed that the intracellular levels of GSH are reduced in some drug selected and transfected cells overexpressing MRP1 in the absence of an exogenous substrate (12–14). In addition, GSH levels are elevated in some tissues in mrp1−/− mice (15). Whether this is attributable to the efflux of GSH in association with transport of endogenous compounds has not been established.

MRP1/mrp1 is highly expressed in the sex hormone-producing Leydig cells of the human and mouse testis (16, 17), as well as in Sertoli cells of the mouse (17). Both of these cell types have high levels of GSH S-transferase activity (18, 19) and are thought to contribute to detoxification in the testis by the formation of GS conjugates, which may be substrates for MRP1 or related transporters. Studies of mrp1−/− mice have already provided evidence that testicular mrp1 protects the local tissue against drug-induced damage (17). Thus, the testes of mrp1−/− mice treated with the anticancer drug, etoposide phosphate, showed aberrant spermatogenesis with no sign of meiotic divisions and an increased number of prematurely released round germ cells. In contrast, the same treatment of the wild-type mice produced only a partially distorted spermatogenesis and meiotic divisions still occurred.

In addition to a protective role with respect to xenobiotic exposure, the relatively high levels of MRP1 in Leydig cells may also serve to protect the testis from the potential feminization of the ATP-binding cassette superfamily of transmembrane transporters, which was originally discovered by virtue of its association with drug resistance in tumor cells (1). It is now also known to be a primary active transporter of many conjugated organic anions (2). The first substrate shown to be actively transported by MRP1 using inside-out membrane vesicles was the glutathione-conjugated leukotriene, LTC4 (3, 4).

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§ The abbreviations used are: MRP, multidrug resistance protein; ABC, ATP-binding cassette; LTC4, leukotriene C4; E217b, 17β-estradiol-17β-D-glucuronide; E317b, 17β-estradiol-3β-D-glucuronide; E16a, 16α,17β-estriol 16β-D-glucuronide; GSH, reduced glutathione; GSSG, glutathione disulfide; DTT, dithiothreitol; mAb, monoclonal antibody; EST, estrogen sulfotransferase; DMEM, Dulbecco’s modified Eagle’s medium.
ing effects of endogenously produced estrogen conjugates. Estrogen is synthesized in the testis and is required for normal testicular function, as revealed by studies of mice in which the estrogen receptor α or P450 aromatase genes have been disrupted (20–22). MRPI has been shown to transport certain estrogen glucuronides but the major metabolite in Leydig cells is estrogen sulfate produced by estrogen sulfotransferase (EST) (5, 23). Using 3'-phosphoadenosine 5'-phosphosulfate as a sulfate donor, this sulfation takes place at the 3-hydroxyl group of the parent molecule, generating the more hydrophilic estrogen 3-sulfate. The relative hydrophlicity of estrogen 3-sulfate prevents its ready diffusion across the plasma membrane. Thus, it has been assumed that an export pump is involved in its efflux from the cell. Testicular expression of estrogen sulfotransferase is mainly localized in Leydig cells and is regulated by luteinizing hormone via modulation of cAMP levels (23, 24). Interestingly, the testes of 9–12-month-old est−/− mice displayed Leydig cell hypertrophy/hyperplasia, as well as seminiferous tubule disruption. The colocalization of MRPI and estrogen sulfotransferase in the testis prompted us to investigate whether estrogen sulfates were also substrates of MRPI. We found that estrogen sulfates alone were very poor substrates and competed poorly for transport and binding of the high affinity MRPI substrate LTC 4. However, in the presence of GSH, their inhibitory potency was significantly increased and GSH-enhanced direct transport of [3H]estrone sulfate could be easily detected. These results provide the first examples of potential endogenous MRPI substrates that depend on the presence of GSH for efficient transport and the first examples of conjugated endobiotics that display this type of dependence.

**EXPERIMENTAL PROCEDURES**

**Materials—**[6,7-3H]Estrone sulfate (53 Ci mmol−1), [2,4,6,7-3H]estradiol (87.6 Ci mmol−1), and [glycine-2-3H]GSH (42 Ci mmol−1) were purchased from PerkinElmer Life Sciences; [14,15-3H]LTC 4 (38 Ci mmol−1) were purchased from Amersham Pharmacia Biotech (Oakville, Ontario, Canada). Estrogen 3-sulfates, nucletides, GSH, verapamil, S-methyl GSH, glutathione disulfide (GSSG), 2-mercaptoethanol, and DTT were purchased from Sigma. Estrogen 3-sulfates were dissolved with H2O to prepare stock solutions (100 mg/ml) and diluted with transport buffer. The MRPI-specific murine monoclonal antibodies (mAbs) QCRI-1, QCRI-2, QCRI-3, and QCRI-4 have been described previously (25, 26).

**Cell Culture—**The HeLa T5 cells transfected with the pRCMV vector containing the MRPI coding sequence and the HeLa C1 cells transfected with empty pRCMV vector have been described previously (27). Both T5 and C1 were cultured in RPMI 1640 medium with 5% defined bovine cell serum and maintained in 400 μg/ml 1 Geneticin (G418). HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum.

**Membrane Vesicle Preparation and Transport Studies—**Plasma membrane vesicles were prepared as described (8). Briefly, cell pellets were covered with buffer containing 50 mM Tris-HCl, 250 mM sucrose, 0.25 mM CaCl2, and protease inhibitors and frozen at −70 °C overnight. The cells were then thawed and disrupted by N2 cavitation (10-min equilibrium at 200 psi). EDTA was added to 1 mM before centrifugation at 500 × g for 15 min to remove cell debris. To increase the yield of membrane vesicles, the resulting pellet was washed once with 5 ml of transport buffer (50 mM Tris-HCl and 250 mM sucrose, pH 7.5) and centrifuged again. The supernatants were pooled, layered over 35% (w/v) sucrose in 10 mM Tris-HCl (pH 7.5), and centrifuged at 100,000 × g for 1 h. The interface was collected in washing buffer (10 mM Tris-HCl and 25 mM sucrose) and followed by centrifugation at 100,000 × g for 30 min. The membrane pellet resuspended in transport buffer and passed 10 times through a 27.5-gauge needle for vesicle formation. The membrane vesicles were then aliquoted and stored at −70 °C until use.

ATP-dependent uptake of [3H]LTC 4 into the inside-out membrane vesicles was measured by a rapid filtration technique (8). In standard transport assays, 2.5 μg of membrane vesicles were used in a 25-μl reaction volume and incubated at 23 °C in the presence of 50 nM

\[ [3H]LTC_4, 10 \text{mM MgCl}_2, \text{and } 4 \text{mM ATP or AMP in transport buffer.} \]

Where indicated, GSH was added to 1 mM unless otherwise stated. Uptake was stopped in rapid dilution in ice-cold buffer and followed by filtration through glass fiber (type A/E) filters (Gelman Sciences, Dorval, Quebec, Canada) that had been presoaked overnight at 50 °C. In transport buffer, LTC 4 at initial concentration of 400 nM, carried over 200 μl of avidin-gold particles that adhered to the amount of [3H]LTC 4 that remained bound to the filter in the absence of the membrane vesicles, which was usually less than 5–10% of the total radioactivity. For kinetic analysis of LTC 4 transport in the presence of estrogen sulfates and/or GSH, LTC 4 was included at concentrations ranging from 16 nM to 1 μM and ATP-dependent [3H]LTC 4 uptake was determined as above.

**MRPI-mediated Glutathione-enhanced Estrogen Sulfate Transport**

ATP-dependent uptake of [3H]estrone 3-sulfate was measured by rapid filtration as above, except that the incubation temperature was 37 °C and substrate concentration was 300 nM unless otherwise indicated. Uptake was stopped after 60 s or at the time indicated by rapid dilution in ice-cold buffer, and the reaction mixture was filtered through glass fiber filters. Where indicated, MRPI-specific mAbs were added to 10 μg/ml and preincubated with membrane vesicles on ice for 1 h. All data were corrected by subtracting nonspecific binding of [3H]estrone 3-sulfate to the filter, which was usually less than 5% of the total radioactivity. For kinetic analysis of GSH-enhanced estrone 3-sulfate transport in the absence or presence of LTC 4, estrone 3-sulfate was included at concentrations ranging from 125 nM to 16 μM and ATP-dependent estrone 3-sulfate uptake was determined as above.

Transport of [3H]GSH into the membrane vesicles was also measured by rapid filtration as above. In a 50-μl reaction volume, 22 μg of membrane vesicle protein were incubated at 37 °C for 20 min in the presence of 100 μM [3H]GSH (80 or 288 nCi/reaction), 10 mM DTT, 10 mM MgCl2, and 4 mM ATP or AMP in transport buffer. Estrone 3-sulfate or estradiol 3-sulfate was added to several concentrations ranging from 0.2 to 20 μM. Verapamil was used as a positive control for stimulation of [3H]GSH transport (28) and was added to 100 μM. All data were corrected by subtracting the amount of [3H]GSH that remained bound to the filter in the presence of 4 mM AMP, which was usually less than 5% of the total radioactivity.

**Photofluorophotigraphy of MRPI with [3H]LTC 4 and Inhibition of Transport by Estrogen Sulfates—**Membrane vesicles (75 μg of membrane protein in 35 μl) were incubated with [3H]LTC 4 (0.25 μCi, 200 nM) in the absence or presence of various concentrations of estrone 3-sulfate or estradiol 3-sulfate at room temperature for 10 min and frozen in liquid nitrogen. Samples were alternately irradiated for 30 s at 312 nm in a Stratallinker, followed by snap-freezing in liquid nitrogen, for a total of 10 min. Radiolabeled vesicles were solubilized in Laemmli’s buffer and analyzed by 7.5% gel SDS-PAGE. Silver staining of the gel was fixed in isopropanol:water:acetic acid (25:65:10) for 30 min and then soaked in Amplify for 15–20 min. After drying under vacuum at 80 °C, the gel was placed in close contact with x-ray film at −70 °C for 2 weeks (8).

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**RESULTS**

**Inhibition of MRPI-mediated LTC 4 Transport by Estrogen Sulfates—**To determine whether estrogen sulfates were potential substrates for MRPI, we initially tested the ability of estrone and estradiol 3-sulfate to compete for transport of the high affinity MRPI substrate, LTC 4. Neither estrogen sulfate...
 alone was a potent inhibitor of \[^{3}H\]LTC\(_{4}\) uptake by vesicles prepared from MRPI-transfected HeLa T5 (HeLa-MRP) cells (Fig. 1, A and B). At lower concentrations of the conjugated estrogens (20–200 nM), a reproducible 25–50% increase in the rate of LTC\(_{4}\) transport was observed with significant inhibition of transport being observed when concentrations of the estrogen sulfates reached more than 20 \(\mu M\). Previously, GSH has been shown to increase the inhibitory potency of some hydrophobic xenobiotics, which are themselves poor competitive inhibitors of LTC\(_{4}\) transport (8). However, no effect on the transport of conjugated endobiotics has been reported. We found that addition of 1 mM GSH markedly increased the inhibitory potency of the sulfated estrogens, resulting in a decrease in IC\(_{50}\) values from 31 and 50 \(\mu M\) to 0.2 and 0.3 \(\mu M\), for estrone sulfate and estradiol sulfate, respectively (Fig. 1, A and B).

To determine whether inhibition of LTC\(_{4}\) transport by the estrogen sulfates was competitive, Eadie-Hofstee plots of LTC\(_{4}\) transport in the absence or presence of 2 \(\mu M\) estrone sulfate were constructed. At this concentration, estrone sulfate alone inhibited \[^{3}H\]LTC\(_{4}\) transport by \(\sim 10\%\) while estradiol sulfate stimulated transport by \(\sim 25\%\) (Fig. 1, A and B). However, in the presence of 1 mM GSH, both estrone and estradiol sulfate behaved as competitive inhibitors of \[^{3}H\]LTC\(_{4}\) transport, with apparent \(K_i\) values of 0.45 and 0.38 \(\mu M\), respectively (Fig. 1, C and D).

**Attenuation of \[^{3}H\]LTC\(_{4}\) Photolabeling of MRP1 by Estradiol Sulfates**—To determine whether inhibition of LTC\(_{4}\) transport was the result of direct competition for binding, we examined the ability of the conjugated estrogens to inhibit \[^{3}H\]LTC\(_{4}\) photolabeling of MRP1 in T5 membrane vesicles in the absence or presence of GSH. GSH alone weakly inhibited photolabeling with \[^{3}H\]LTC\(_{4}\) (Fig. 2A), decreasing labeling by 20–25% at a concentration of 1 mM. Low concentrations of estrone or estradiol sulfate alone (0.2 \(\mu M\)) resulted in small but reproducible increases in \[^{3}H\]LTC\(_{4}\) photolabeling of MRP1 (Fig. 2B), while higher concentrations either decreased (20 \(\mu M\)) or abolished (200 \(\mu M\)) labeling. The enhanced photolabeling of MRP1 observed with low concentrations (0.2–2 \(\mu M\)) of sulfated estrogens alone was not apparent in the presence of 1 mM GSH (Fig. 2B), and \[^{3}H\]LTC\(_{4}\) labeling was completely abolished in the presence of 1 mM GSH and 20 \(\mu M\) estrone sulfate or estradiol sulfate (Fig. 2B). Furthermore, the effect of GSH could be fully mimicked by a nonreducing GSH derivative, S-methyl GSH (Fig. 2C), indicating that GSH-enhanced inhibition of \[^{3}H\]LTC\(_{4}\) labeling of MRP1 was not caused by a change in the redox state of the protein.

**MRP1-mediated Glutathione-enhanced Estrogen Sulfate Transport**

**Fig. 2.** Photoaffinity labeling of MRPI-enriched membrane vesicles by \[^{3}H\]LTC\(_{4}\) and inhibition of labeling by estrone 3-sulfate and estradiol 3-sulfate in the absence and presence of GSH. MRPI-enriched T5 membrane vesicles (75 \(\mu g\)) were incubated with \[^{3}H\]LTC\(_{4}\) (200 nM, 0.25 \(\muCi\)) alone or in the presence of various concentrations of competitors at 22 °C for 10 min. Samples were irradiated at 312 nm prior to being subjected to SDS-polyacrylamide gel electrophoresis and fluorography (see "Experimental Procedures"). Panel A, effect of GSH (0.3–3 mM) and S-methyl GSH (0.3–3 mM) alone on \[^{3}H\]LTC\(_{4}\) labeling of MRPI. Panel B, inhibition of \[^{3}H\]LTC\(_{4}\) labeling by estrone sulfate or estradiol sulfate (0.2–200 \(\mu M\)) alone and by the sulfates (0.2–20 \(\mu M\)) in the presence of 1 mM GSH. Panel C, effect of a combination of 1 mM S-methyl GSH with several concentrations of estrone sulfate (0.2–20 \(\mu M\)) on \[^{3}H\]LTC\(_{4}\) labeling of MRPI. S-MeGSH, S-methyl GSH.
estrone 3-sulfate was very low (5–6 pmol mg⁻¹ protein min⁻¹). However, an approximate 10-fold increase in the rate of transport was observed when 1 mM GSH was present (Fig. 3A). GSH-stimulated transport was linear for up to 60 s at a rate of ~60 pmol mg⁻¹ protein min⁻¹ with an initial concentration of 300 nM estrone 3-sulfate. In the presence of 4 mM AMP and 1 mM GSH, estrone 3-sulfate uptake by the vesicles was 2–4 pmol mg⁻¹ protein min⁻¹. ATP-dependent and GSH-stimulated estrone 3-sulfate transport was not detected with membrane vesicles prepared from vector-transfected HeLa C1 control cells (Fig. 3B).

To confirm that vesicle-associated estrone sulfate was indicative of transport into the vesicle lumen, rather than surface or intramembrane binding, we determined the effect of increasing extravesicular osmolarity. As expected for true transport, the amount of vesicle-associated [³H]estrone sulfate decreased with increasing concentrations of sucrose extrapolating to zero at infinite osmolarity (Fig. 4A).

To confirm that transport was MRP1-mediated, we used previously characterized conformation-dependent MRP1-specific mAbs, which have been shown to inhibit MRP1-mediated transport of LTC₄ and other MRP1 substrates (8–10, 26, 28, 32). As observed for other MRP1 substrates, three mAbs that recognize distinct conformation-dependent epitopes in the first (mAbs QCRL-2 and QCRL-3) and second (mAb QCRL-4) nucleotide-binding domains of MRP1 (26) completely inhibited ATP-dependent and GSH-stimulated estrone sulfate transport by T5 vesicles (Fig. 4B). In contrast, mAbs QCRL-1, which recognizes a linear epitope in the linker region of MRP1(33), had no inhibitory effect.

To determine the kinetic parameters of estrone sulfate transport and the effect of GSH, rates of ATP-dependent uptake in the presence and absence of 1 mM GSH were determined at several concentrations of estrone 3-sulfate (125 nM to 16 μM) to obtain Kₘ and Vₘₐₓ values (Fig. 3C). A nonlinear regression analysis of the data yielded an apparent Kₘ of 0.73 ± 0.17 μM for estrone 3-sulfate and a Vₘₐₓ of 440 ± 27 pmol mg⁻¹ protein min⁻¹ in the presence of GSH, and an apparent Kₘ of 4.2 ± 1.1 μM for estrone 3-sulfate and a Vₘₐₓ of 107 ± 10 pmol mg⁻¹ protein min⁻¹ in its absence. The inset shows Eadie-Hofstee transformation of the data (Fig. 3C).

When the nonreducing S-methyl GSH was used in place of GSH, 1 mM S-methyl GSH stimulated estrone 3-sulfate uptake by T5 membrane vesicles, somewhat more effectively than the same concentration of GSH (Fig. 5A). In contrast, the sulfhydryl reducing agents DTT (10 mM) and 2-mercaptoethanol (5 mM) and oxidized glutathione GSSG (0.05 mM) had no effect on estrone 3-sulfate transport. Since S-methyl GSH appeared to be a more potent stimulator of estrone sulfate transport than GSH, rates of transport were determined as a function of GSH or S-methyl GSH concentration (Fig. 5B). Both compounds exhibited a similar concentration dependence with 50% of maximal stimulation being observed at a concentration of ~0.5 mM.
However, the maximal rate of estrone sulfate transport obtained with S-methyl GSH was 2.5-fold greater than with GSH itself.

Inhibition of Estrone 3-Sulfate Transport by LTC4—Since we found that estrogen sulfates competitively inhibited MRPI-mediated [3H]estrone 3-sulfate transport in the presence of GSH, we determined whether the reverse was also true. Thus, the ability of LTC4 to inhibit [3H]estrone 3-sulfate transport by MRPI was examined in T5 vesicles. GSH-enhanced estrone 3-sulfate uptake was inhibited by LTC4 in a dose-dependent manner (Fig. 6A). An Eadie-Hofstee plot of estrone 3-sulfate uptake in the presence of 0.2 μM LTC4 indicated that the inhibition was competitive, with an apparent Kᵢ (LTC4) of 0.2 μM (Fig. 6B).

Effect of Sulfated Estrogens on MRP1-mediated (3H)GSH Transport—Previous studies of the GSH stimulus of the unconjugated xenobiotic vincristine revealed a reciprocal stimulation of GSH transport by the drug, suggesting a cotransport mechanism (10). In addition, verapamil was shown to markedly stimulate GSH transport by MRPI, but in this case no transport of verapamil could be detected (28). Consequently, we examined the ability of the estrogen sulfates to stimulate GSH transport using verapamil as a positive control. As expected, verapamil at 100 μM significantly enhanced GSH transport by about 3-fold. In the first set of experiments, 80 nCi of [3H]GSH was used in each reaction and no stimulation of GSH transport was observed with either estrogen sulfate at 0.2 and 2 μM. A modest stimulation (25%) was observed at 20 μM but only with estrone sulfate (Fig. 7A). In a second set of experiments, the amount of [3H]GSH was increased to 288 nCi/
FIG. 7. Effect of estrogen sulfates on [3H]GSH uptake by T5 membrane vesicles. Panel A, membrane vesicles (22 μg of protein) were incubated with 100 μM [3H]GSH (80 nCi/reaction) for 20 min at 37 °C in the presence of 5 mM DTT, 4 mM ATP, and the indicated concentrations (0.2–20 μM) of estrone 3-sulfate (E1S) or estradiol 3-sulfate (E2S). Verapamil (VLP, 100 μM) was used as a positive control stimulating MRP1-mediated GSH transport. Control (CON) represents ATP-dependent GSH transport in the absence of a second substrate. All data were corrected by subtracting [3H]GSH uptake in the presence of 4 mM AMP, and bars represent the means (± S.E.) of triplicate determinations in a single experiment. Panel B, experimental conditions were the same as above except that the amount of [3H] GSH was increased to 288 nCi/reaction. Control (CON) represents ATP-dependent GSH transport in the absence of a second substrate. Estrone 3-sulfate (E1S) and estradiol 3-sulfate (E2S) were added to 100 μM. All data were corrected by subtracting [3H] GSH uptake in the presence of 4 mM AMP, and bars represent the means (± S.E.) of triplicate determinations in a single experiment.

FIG. 8. [3H]Estradiol sulfate accumulation by intact HEK cells transiently expressing est or est and MRP1. Untransfected control HEK cells and those transfected with the expression vector pCDNA3 containing est cDNA alone or in combination with pCEBV7-MRP1 for 66 h were harvested and incubated with 500 nM [3H]estradiol in serum-free DMEM at 37 °C for 20 min. [3H]Estradiol sulfate accumulation was then determined as described under “Experimental Procedures.” Bars represent means (± S.E.) of triplicate determinations in a single experiment. *, p < 0.01 when compared with untransfected control cells; **, p < 0.01 when compared with the cells transfected with pCDNA3-est alone. The other half of aliquots of suspended cells were sonicated to prepare cytosolic proteins for est activity assay. An ~10-fold increase in the activity was found in the est-transfected cells, and MRP1 expression did not affect the activity.

The K_m value (0.73 μM) for MRP1-mediated and GSH-enhanced estrone sulfate transport is comparable with that for organic anion transporter 3 (OAT3)- or organic anion transporter 4 (OAT4)-mediated transport of estrone sulfate (34, 35). However, these latter transporters have a more restricted tissue distribution than MRP1/mrp1 and are involved in the uptake rather than efflux of organic anions. The relatively high coexpression of MRP1/mrp1 with EST in Leydig cells suggests that MRP1 is likely to be involved in estrogen sulfate efflux from the testis. Since estrone sulfate can be converted back into estrone by estrogen sulfatase, efficient removal of estrone sulfate by an export pump is expected to be important for maintenance of low estrogen levels in organs such as the testis. Studies of murp1−/− mice treated with etoposide phosphate strongly suggest that murp1 may also protect the testis from exposure to cytotoxic xenobiotics (17). In addition, the recent report that EST is able to sulfate environmental estrogens, such as bis-, 4-octyl-, and p-nonylphenols, raises the possibility that MRP1 or related proteins could play a role in the cellular elimination of the conjugates of these estrogenic compounds (36).

The requirement of GSH for efficient transport of the sulfated estrogen provides the first example of GSH-enhanced transport of a conjugated endogenous substrate by MRP1. Previous studies have shown that GSH is required for the transport of some xenobiotics including unmodified chemotherapeutic agents such as vincristine (8–10), daunorubicin (9, 11) and aflatoxin B_1 (32), and enhances transport of etoposide conjugated with glucuronate (37). GSH has been reported recently to stimulate transport of [3H]luteolin 7-O-diglucuronyl-4′-O-glucuronide in plant leaves, but the transporter involved has not been characterized structurally. However, it appears functionally to be a membrane-potential sensitive member of the ABC superfamily (38).

We have previously proposed that MRP1 contains a bipartite site to which the hydrophobic and anionic moieties of its conjugated substrates bind (8). Studies of the GSH-stimulated transport of vincristine, and the influence of GSH on the ability of vincristine to compete for LTC_4 transport by MRP1, indicate...
that GSH not only increases the $V_{\text{max}}$ for the drug but also the affinity with which it interacts with the protein, as reflected by an approximate 20-fold increase in its inhibitory potency. These studies also indicated that vincristine reciprocally increases the affinity of MRP1 for GSH (10). The data suggest that initial low affinity binding of either GSH or drug by MRP1 induces a conformational change in the protein such that high affinity binding of the cotransported substrate can occur. In this respect, the model differs from the two-site model proposed by Borst et al. (39) in which the protein is envisaged to have two binding sites: one that has a relatively high affinity for drug and low affinity for GSH and another with high affinity for GSH and low affinity for drug (39). Although this model may explain the ability of drug and GSH to reciprocally stimulate cotransport, it is difficult to explain why GSH markedly increases the ability of drug to competitively inhibit transport of high affinity, conjugated substrates such as LTC4. Consistent with the existence of a low affinity site for GSH alone, our photoaffinity experiments with [3H]LTC4 indicate that GSH and S-methyl GSH decrease [3H]LTC4 labeling of MRP1 in a concentration-dependent manner in the millimolar range, suggesting that the binding sites for LTC4 encompass a low affinity site to which GSH (or S-methyl GSH) can bind.

Recent studies of drug binding and transport by the bacterial, homodimeric ABC multidrug resistance protein, LmrA, suggest positive cooperativity between allosterically linked low and high affinity substrate binding sites (40). A “two-cylinder engine” model, which embodies the alternating catalytic sites model proposed by Senior et al. (41), has been proposed in which the transport of drug from an high affinity intracellular site to a low affinity extracellular site on the same subunit of the homodimer is driven by the alternating hydrolysis of ATP at one or the other nucleotide binding domain. Thus, the two subunits cycle with respect to exposure of high or low affinity sites (40).

The evidence for positive cooperativity is based in part on the observation that two substrates that compete for transport at high concentrations reciprocally stimulate transport of each other at lower concentrations. Unlike the bacterial homodimeric transporters and transporters such as P-glycoprotein, the nucleotide binding domains of the MRP-related proteins are relatively divergent. Evidence to date suggests that they are also not functionally equivalent and may not alternate catalytically (42). Similarly, there is no evidence of primary structure conservation between the NH2 and COOH-proximal membrane-spanning domains of MRP1. However, we have consistently observed that low concentrations of the estrogen sulfates in the absence of GSH stimulate LTC4 transport and binding, as evidenced by photocross-linking studies, while they compete at higher concentrations. Thus, the data are also consistent with the existence of interacting binding sites being present on MRP1. However, since both the estrogen sulfates and LTC4 are relatively hydrophilic and the duration of transport assays is extremely short, the stimulation observed suggests that the sites are accessible from the cytoplasmic face of the membrane. In addition, the stimulation of binding occurs in the complete absence of nucleotide, indicating that if allosteric changes in structure occur following initial interaction with substrate, they do not require the binding and/or hydrolysis of ATP.

In some respects, the results we have obtained in the present study with estrone sulfate are similar to previous observations on the effect of GSH on rate of transport and apparent binding affinity of unmodified hydrophobic substrates such as vincristine, despite the fact that the compound is conjugated. However, in the case of vincristine, aflatoxin B1, and daunorubicin, it has not been possible to determine kinetic parameters of transport in the absence of GSH. Our data demonstrate that GSH decreases the $K_m$ for estrone sulfate from 4.2 to 0.73 $\mu$M and increases $V_{\text{max}}$ from 107 to 440 pmol min$^{-1}$ mg protein$^{-1}$. In addition, GSH also markedly enhanced the ability of estrone sulfate to inhibit photolabeling of MRP1 with LTC4. In these experiments, the GSH-enhanced inhibitory potency of estrone sulfate was obtained in the absence of ATP, indicating that any induced change in affinity for the conjugated estrogen occurs without a requirement for either nucleotide binding or hydrolysis.

Earlier studies with conjugated estrogens indicated that E217G could compete for LTC4 binding and transport by MRP1 while estrogens conjugated with glucuronide at the 3 position of the A-ring are very poor inhibitors (5). These studies also revealed that a change in site of glucuronidation from the 17β to 16α position on the D-ring markedly decreased the affinity for the protein, as judged by the difference between the $K_v$ values for E217G (1.4 $\mu$M) and E16αG (45 $\mu$M) as inhibitors of E217G transport. Taken together, the data indicate that strict structural requirements with respect to the site of glucuronidation of the steroid nucleus must be met to interact with MRP1 as a substrate or competitive inhibitor. Sulfation at the 3-position of the A-ring of E217G had little or no effect on its ability to compete for transport, implying that the presence of the sulfate group neither precluded nor enhanced interaction with the protein. In contrast, the conjugated bile salt glycolithocholate 3-sulfate was an effective inhibitor of E217G transport when compared with bile salts that were not conjugated at this position of the A-ring. This is consistent with the possibility that sulfation of the A-ring, in the absence of additional anionic conjugation, might enhance interaction with MRP1. A low level of transport of estrone sulfate could be detected in the absence of GSH, suggesting that sulfation resulted in the formation of a relatively low affinity, low capacity substrate in which the sulfate presumably does not prevent GSH from interacting with the protein, either because it binds to a different site or because it interacts only weakly with the GSH binding site. This latter possibility would be consistent with the inhibition of basal MRP1-mediated GSH transport by HeLa T5 vesicles observed at high concentrations of estradiol sulfate. However, the GSH has no effect on the inhibitory potency of other A-ring conjugates, such as E3βG or on the transport of E217G, or estradiol itself (data not shown). Thus with respect to stimulation of transport by MRP1, it remains difficult to predict which conjugated or nonconjugated compounds might be affected by the presence of GSH.

It has been observed that GSH levels are increased in some tissues of mrg knockout mice (15) and decreased in drug-selected or transfected cells that overexpress MRP1 (12, 13), suggesting that MRP1 might efflux GSH either alone or via cotransport with currently unidentified, endogenous substrates. The stimulation of estrogen sulfate transport by GSH suggested that these compounds might be candidates for such substrates. Thus far, vesicle transport studies have provided strong evidence for cotransport of GSH with some xenobiotics (15, 10). However, in other cases, it has not been possible to detect a xenobiotic-dependent stimulation of GSH transport (10, 11). In addition, we have shown that some compounds, such as verapamil, can markedly stimulate MRP1-mediated GSH transport with no detectable net transport of the compound itself (28).

Despite the readily demonstrable GSH stimulation of estrone sulfate transport, we have not been able to detect reciprocal stimulation of GSH transport by the conjugated estrogen. No significant increase in GSH uptake by membrane vesicles
MRP1-mediated Glutathione-enhanced Estrogen Sulfate Transport

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