0026-895X/08/7406-1630–1640$20.00
MOLECULAR PHARMACOLOGY
Vol. 74, No. 6
49080/3404400
Mol Pharmacol 74:1630–1640, 2008
Printed in U.S.A.

Mechanistic Differences between GSH Transport by Multidrug Resistance Protein 1 (MRP1/ABCC1) and GSH Modulation of MRP1-Mediated Transport[S]

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Received May 21, 2008; accepted September 2, 2008

ABSTRACT

Multidrug resistance protein 1 (MRP1/ABCC1) is an ATP-dependent polytopic membrane protein that transports many anticancer drugs and organic anions. Its transport mechanism is multifaceted, especially with respect to the participation of GSH. For example, vincristine is cotransported with GSH, estrone sulfate transport is stimulated by GSH, or MRP1 can transport GSH alone, and this can be stimulated by compounds such as verapamil or apigenin. Thus, the interactions between GSH and MRP1 are mechanistically complex. To examine the similarities and differences among the various GSH-associated mechanisms of MRP1 transport, we have measured first the effect of GSH and several GSH-associated substrates/modulators on the binding and hydrolysis of ATP by MRP1 using 8-azidoadenosine-5′-[^{32}P]-triphosphate ([^{32}P]azidoATP) analogs, and second the initial binding of GSH and GSH-associated substrates/modulators to MRP1. We observed that GSH or its nonreducing derivative *S*-methylGSH (*S*-mGSH), but none of the GSH-associated substrate/modulators, caused a significant increase in [γ-^{32}P]azidoATP labeling of MRP1. Moreover, GSH and *S*-mGSH decreased levels of orthovanadate-induced trapping of [α-^{32}P]azidoADP. [α-^{32}P]azidoADP.Vi trapping was also decreased by estone sulfate, whereas vincristine, verapamil, and apigenin had no apparent effects on nucleotide interactions with MRP1. Furthermore, estrone sulfate and *S*-mGSH enhanced the effect of each other 15- and 10-fold, respectively. Second, although GSH binding increased the apparent affinity of MRP1 for all GSH-associated substrates/modulators tested, only estrone sulfate had a reciprocal effect on the apparent affinity of MRP1 for GSH. Overall, these results indicate significant mechanistic differences between MRP1-mediated transport of GSH and the ability of GSH to modulate MRP1 transport.

The increased expression of multidrug resistance protein 1 (MRP1/ABCC1) in tumor cells causes resistance to chemotherapy (Cole et al., 1992; Deeley et al., 2006). MRP1, a member of the ATP-binding cassette (ABC) superfamily of transporters, which is also expressed in almost all normal tissues, uses the energy from ATP binding and hydrolysis to efflux a wide variety of drugs (e.g., anthracyclines, plant alkaloids, and antifolates) across the plasma membrane. In addition to transporting drugs, MRP1 actively effluxes many endogenous conjugated organic anions and metabolites of xenobiotics, and thus plays a physiological and a protective role in both normal and malignant tissues (Wijnholds et al., 2000; Leslie et al., 2005).

One striking feature of the transport mechanism of MRP1 function is its complex interaction(s) with the reducing tripeptide GSH. MRP1 mediates the cellular efflux of many GSH conjugates (Fig. 1A), including the proinflammatory cysteinyl leukotriene C_4 (LTC_4), which has been established to be a major physiological substrate of MRP1 (Loe et al., 1996b; Wijnholds et al., 1997). It also transports the GSH-conjugated metabolites of many xenobiotics and the pro-oxidant glutathione disulfide (GSSG) (Leier et al., 1996; Haimeur

This work was supported by grant MOP-10519 from the Canadian Institutes of Health Research (CIHR). A.R. was supported by a CIHR postdoctoral fellowship. S.P.C.C. is the Canada Research Chair in Cancer Biology and Bracken Chair in Genetics and Molecular Medicine.

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Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.108.049080.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: MRP1, multidrug resistance protein 1; ABC, ATP binding cassette; *S*-mGSH, *S*-methyl glutathione; GSSG, glutathione disulfide; LTC_4, leukotriene C_4; $E_2$17βG, estradiol glucuronide; MSD, membrane-spanning domain; NBD, nucleotide binding domain; DTT, dithiothreitol; azidoATP, 8-azidoadenosine triphosphate; Vi, orthovanadate; PAGE, polyacrylamide gel electrophoresis; NBS, nucleotide binding site; [γ-^{32}P]azidoATP, 8-azidoadenosine-5′-[γ-^{32}P]triphosphate; [α-^{32}P]azidoATP, 8-azidoadenosine-5′-[α-^{32}P]triphosphate.

et al., 2004; Leslie et al., 2005; Cole and Deeley, 2006). In addition, there are many organic anion substrates of MRP1, including methotrexate and estradiol glucuronide ($E_2 17\beta G$) and other glucuronides that are transported independently of GSH (Jedlitschky et al., 1996; Loe et al., 1996a; Bakos et al., 2000) (Fig. 1E). In contrast, efficient transport of several MRP1 substrates requires the presence of GSH. This mode of transport does not involve the formation of a conjugate, and the reducing ability of GSH is not required, because nonreducing tripeptide analogs such as ophthalmic acid and short-chain *S*-alkyl derivatives (e.g., *S*-methyl GSH, *S*-mGSH) can substitute for GSH (Loe et al., 1998, 2000; Leslie et al., 2001a, 2003a; Qian et al., 2001). Thus, the formation of GSH-conjugated substrates is not the only role that GSH plays in MRP1-mediated transport. The vinca alkaloid antineoplastic agent vincristine is only efficiently transported by MRP1 in the presence of GSH; on the other hand, vincristine stimulates the transport of GSH (Loe et al., 1996b, 1998), thus a cotransport mechanism has been proposed (Fig. 1B). A similar cotransport or cross-stimulated transport mechanism has also been proposed recently for GSH and the anthracene antineoplastic agent mitoxantrone (Morrow et al., 2006). Substrates such as estrone sulfate and NNAL-*O*-glucuronide (4-(nitrosamino)-1-(3-pyridyl)-1-butanol-*O*-glucuronide) are also transported at markedly increased levels when GSH is present (Leslie et al., 2001a; Qian et al., 2001). However, unlike vincristine and mitoxantrone, these substrates do not have a reciprocal stimulatory effect on GSH transport, and thus their transport by MRP1 is affected through a GSH-stimulated rather than cotransport mechanism (Fig. 1C).

There are also several classes of compounds that stimulate transmembrane transport of GSH by MRP1 (Fig. 1D). By itself, GSH is a relatively poor substrate of MRP1, but in the presence of phenylalkylamines such as verapamil or bioflavonoids like apigenin, its transport is significantly enhanced (Loe et al., 2000; Leslie et al., 2003b). However, unlike vincristine-stimulated GSH transport, there is no evidence that verapamil or apigenin are themselves transported. Thus, GSH transport by MRP1 seems to occur both by a cotransport or cross-stimulated mechanism and by a xenobiotic-stimulated mechanism.

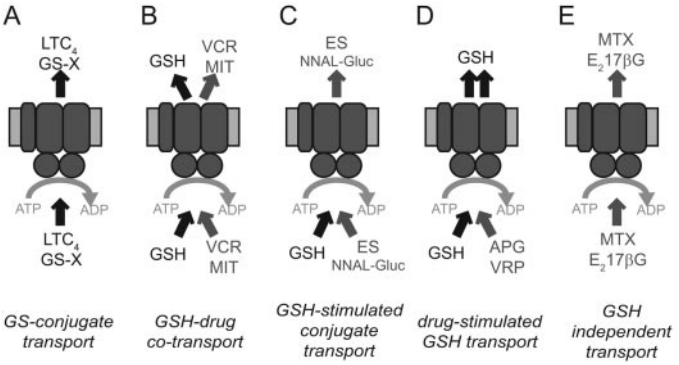

Fig. 1. Schematic representation of different modes of ATP-dependent transmembrane transport by MRP1 that involve GSH. A, transport of GSH conjugates; B, cotransport or cross-stimulated transport of GSH and drugs; C, GSH-stimulated transport of conjugated organic anions; D, modulator-stimulated transport of GSH; E, GSH-independent transport of organic anions. GS-X, GSH conjugate; VCR, vincristine; MIT, mitoxantrone; ES, estrone sulfate; NNAL-gluc, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol-*O*-glucuronide; APG, apigenin; VRP, verapamil; MTX, methotrexate.

The above observations make it clear that the interactions between MRP1 and GSH are complex, and it is still not understood how one tripeptide can interact with MRP1 in so many apparently different ways depending on what other substrates or modulators are present. Active transport of a molecule across the membrane by MRP1 and other ABC transporters is a complex process involving extensive interdomain communication between the membrane-spanning domains (MSDs) where the substrates are presumed to bind and the cytosolic nucleotide binding domains (NBDs) where ATP binds and is hydrolyzed. For MRP1 (and MRP2), this process is further complicated by the interactions of these transporters with GSH. Our overall goal is to elucidate molecular details of the similarities and differences among the various GSH-associated mechanisms of MRP1 transport. We have studied previously the effect of ATP binding and hydrolysis on GSH-stimulated estrone sulfate binding and transport, that is, on communication from the NBDs to the MSDs (Rothnie et al., 2006). In the present study, we have examined signaling in the reverse direction, determining what effects GSH and the various GSH-associated substrates and modulators of MRP1 have on the binding and/or hydrolysis of ATP by this ABCC transporter.

Materials and Methods

Materials. 8-Azidoadenosine-5′-[γ-^{32}P]triphosphate ([γ-^{32}P]-azidoATP) and 8-azidoadenosine-5′-[α-^{32}P]triphosphate ([α-^{32}P]-azidoATP) were purchased from ALT BioScience (Lexington, KY). [14,15,19,20-^{3}H(N)]LTC_4 was from PerkinElmer Life Sciences (Danvers, MA), and LTC_4 was from Calbiochem (La Jolla, CA). Diphenylcarbamylchloride-treated trypsin was from MP Biomedicals (Irvine, CA). Monoclonal antibodies MRPm6 and MRPr1 were kind gifts from Drs. R. J. Scheper and G. L. Scheffer (Free University, Amsterdam, the Netherlands). Monoclonal antibody QCRL-1 was derived in our laboratory (Hipfner et al., 1996). Amplify fluorography solution was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). GSH, *S*-mGSH, GSSG, ATP, sodium orthovanadate, $E_2 17\beta G$, estrone sulfate, vincristine sulfate, verapamil hydrochloride, apigenin, DTT, and *N*-α-benzoyl-L-arginine ethyl ester were from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Membrane Preparation. The doxorubicin-selected, MRP1-overexpressing multidrug-resistant small-cell lung cancer cell line H69AR was cultured, and plasma membranes were prepared as described previously (Cole et al., 1992; Rothnie et al., 2006).

[γ-^{32}P]AzidoATP Binding to MRP1 in the Presence of Substrates/Modulator. [γ-^{32}P]AzidoATP labeling of MRP1 was carried out essentially as described previously (Gao et al., 2000; Conseil et al., 2006). In brief, cell membranes (10 μg of protein) in buffer 1 (50 mM HEPES, pH 7.4, and 250 mM sucrose) were incubated for 10 min on ice with various concentrations (0–10 mM) of *S*-mGSH or GSH in the presence or absence of 30 μM estrone sulfate/100 μM vincristine/100 μM verapamil/30 μM apigenin or with various concentrations (0–100 μM) of estrone sulfate/vincristine/verapamil/apigenin, plus or minus 5 mM *S*-mGSH. [γ-^{32}P]AzidoATP (5 μM) and $MgCl_2$ (5 mM) were added and samples incubated on ice for a further 5 min. Samples were then exposed to UV light on ice (302 nm, 8 min). The reactions were stopped by the addition of ice-cold buffer 2 (50 mM Tris, pH 7.4, 0.1 mM EGTA, and 5 mM $MgCl_2$), and the membranes were centrifuged at 25,000*g* for 15 min at 4°C. The pellets were washed once more, resuspended in 20 μl of buffer 3 (50 mM, Tris pH 7.4, and 250 mM sucrose) supplemented with Laemmli sample buffer, subjected to SDS-PAGE, dried, and then exposed to film.

Orthovanadate-Induced Trapping of [α-^{32}P]AzidoADP by MRP1 in the Presence of Substrates/Modulators. Trapping of

[α-^{32}P]azidoADP by MRP1 was performed essentially as described previously (Leslie et al., 2001b; Conseil et al., 2006). In brief, membranes (10 μg of protein) in buffer 1 were incubated for 10 min on ice in the presence of various substrates/modulators: LTC_4 (0–3 μM), $E_2$17βG (0–100 μM), GSSG (0–1 mM), estrone sulfate (0–100 μM), vincristine (0–100 μM), verapamil (0–100 μM), apigenin (0–100 μM), or *S*-mGSH (0–3 mM), or combinations of these substrates/modulators, as described in the figure legends. [α-^{32}P]AzidoATP (5 μM), $MgCl_2$ (5 mM), and freshly prepared sodium orthovanadate (1 mM) were added, and samples were incubated at 37°C for 15 min. The reactions were stopped by the addition of ice-cold buffer 2, and untrapped nucleotide was removed by centrifugation (25,000*g*, 15 min, 4°C). The membrane pellets were washed again, resuspended in 20 μl of buffer 3, and exposed to UV light on ice (302 nm, 8 min) before being subjected to SDS-PAGE, dried and exposed to film as above.

Limited Trypsin Digestion of MRP1 in the Presence of Substrates/Modulators. Limited trypsin digestions were carried out as described previously (Rothnie et al., 2006). In brief, cell membranes (0.25 mg protein/ml) in 50 mM HEPES, pH 7.4, were incubated alone, or in the presence of either 10 mM DTT, 100 μM estrone sulfate/vincristine/verapamil/apigenin, 10 mM GSH (plus 10 mM DTT), or 10 mM *S*-mGSH, or combinations of these reagents, for 30 min on ice. Diphenylcarbamylchloride-treated trypsin was then added at trypsin/protein ratios of 1:5000 to 2.5:1 (w/w) for 15 min at 37°C. Samples (2 μg of protein) were resolved on a 7% acrylamide gel and immunoblotted using monoclonal antibodies MRPm6 (1:1000), MRPr1 (1:5000), and QCRL-1 (1:5000) to detect the major tryptic fragments of MRP1 (Hipfner et al., 1996, 1998). To exclude the possibility that the substrates/modulators, GSH, and/or DTT had any effect by themselves on the activity of the trypsin used in the limited digests, their effect on the trypsin digestion of the model substrate *N*-α-benzoyl-L-arginine ethyl ester was monitored as described previously (Rothnie et al., 2006).

ATP-Dependent [^{3}H]LTC_4 Transport by MRP1 in the Presence of GSH and Other Substrates/Modulators. [^{3}H]LTC_4 transport assays were carried out essentially as described previously (Loe et al., 1996b; Conseil et al., 2006). In brief, 2 μg of membrane protein in buffer 1 was incubated at 23°C for 1 min with 50 nM [^{3}H]LTC_4 (20 nCi per point), 10 mM $MgCl_2$, 10 mM DTT, 4 mM ATP (plus an ATP-regenerating system consisting of creatine kinase and creatine phosphate) or 4 mM AMP, and 0 to 10 mM GSH ± 10 μM estrone sulfate/100 μM vincristine/100 μM verapamil/30 μM apigenin. The reaction was stopped by dilution in ice-cold buffer 3, rapidly filtered through a PerkinElmer unifilter GF/B plate using a Packard Filtermate Harvester (PerkinElmer), and washed twice. Tritium bound to the filter plates was quantified using a PerkinElmer Top Count NXT Microplate Scintillation counter. Uptake in the presence of AMP was subtracted from uptake in the presence of ATP to determine ATP-dependent transport.

[^{3}H]LTC_4 Photolabeling of MRP1 in the Presence of GSH and GSH-Associated Substrates/Modulators. [^{3}H]LTC_4 photolabeling of MRP1 was carried out essentially as described previously (Conseil et al., 2006). In brief, cell membranes (20 μg of protein) were incubated for 30 min at room temperature in buffer 1 with [^{3}H]LTC_4 (60 nCi, 200 nM), 10 mM $MgCl_2$, 10 mM DTT, and either various concentrations of GSH (0–10 mM) in the presence or absence of 10 μM estrone sulfate/100 μM vincristine/100 μM verapamil/30 μM apigenin or various concentrations (0–300 μM) of estrone sulfate/vincristine/verapamil/apigenin plus or minus 1 mM GSH. Samples were then snap-frozen in liquid N_2, and alternately irradiated (302 nm, 8 cm, 1 min) and snap-frozen, 10 times. Laemmli sample buffer was added and samples subjected to SDS-PAGE. Gels were fixed (25% isopropanol/10% acetic acid, 20 min), incubated in Amplify solution (30 min), dried, and exposed to film at −70°C for 2 to 8 weeks.

Data Analysis. Autoradiographs from both [^{32}P]azidoATP and [^{3}H]LTC_4 labeling experiments were analyzed by densitometry using the program Image J (National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). All nonlinear regression analysis was carried out using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). Data sets are composed of a minimum of three independent experiments. Statistical comparisons were carried out using either the Student's *t* test, comparing two sets of conditions, or one-way analysis of variance with a Tukey post hoc test, comparing more than two sets of conditions. Differences were considered statistically significant when $p < 0.05$.

Results

S-mGSH but Not Substrate (Estrone Sulfate, Vincristine) or Modulator (Apigenin, Verapamil) Increases Binding of [γ-^{32}P]AzidoATP to MRP1. To begin investigating the interdomain communication that occurs during MRP1-mediated transport, we first wanted to determine whether GSH and the various GSH-associated substrates/modulators affect the binding of ATP to MRP1. The ATP analog azidoATP, which has been shown previously to support the transport activity of MRP1 comparably with ATP itself (Gao et al., 2000; Nagata et al., 2000), was used for this purpose because it can be covalently attached to MRP1 by exposure to UV light. Photolabeling reactions were carried out at 4°C to limit any hydrolysis, and use of a γ-^{32}P-labeled analog ensured that only nonhydrolyzed azidoATP bound to MRP1 was detected. However, because the azido group reacts chemically with DTT (which is typically included to ensure that GSH remains in the reduced form), the nonreducing *S*-mGSH analog was used in place of GSH so that DTT was not needed.

As shown in Fig. 2A, the amount of [γ-^{32}P]azidoATP labeling of MRP1 increased significantly with increasing concentrations of *S*-mGSH. Thus, in the presence of 3 mM *S*-mGSH, labeling of MRP1 by [γ-^{32}P]azidoATP was 1.5 ± 0.1-fold higher than in the absence of *S*-mGSH ($p < 0.01$), and at 10 mM *S*-mGSH, labeling was 3.4 ± 0.2-fold higher ($p < 0.001$). In contrast, the addition of substrates estrone sulfate or vincristine, or modulators verapamil or apigenin, even at concentrations far in excess of those of pharmacological or physiological relevance, had no effect on the level of [γ-^{32}P]-azidoATP labeling of MRP1, whether added alone or in the presence of *S*-mGSH (Fig. 2, B–I).

GSH Binding to MRP1 Causes a Change in MRP1 Tryptic Fragmentation, whereas Substrates and Modulators Do Not. We and others have shown previously that the binding of GSH or *S*-mGSH to MRP1 causes a conformational change in the transporter, as measured primarily by changes in the fragment pattern after limited trypsinolysis (Manciu et al., 2003; Ren et al., 2005; Rothnie et al., 2006). In contrast, we have now determined that estrone sulfate, vincristine, verapamil, and apigenin (all at 100 μM) have no significant effect on the trypsin digestion pattern of MRP1 (Fig. 3A; data not shown). Furthermore, when added together with GSH or *S*-mGSH, these substrates and modulators had no additional effect beyond that of GSH or *S*-mGSH alone (Fig. 3, B and C; data not shown).

S-mGSH and Estrone Sulfate Cause a Decrease in [α-^{32}P]AzidoADP.Vi Trapping by MRP1. We next wished to determine whether GSH and various GSH-associated substrates/modulators affected the next step in the catalytic cycle of MRP1 (i.e., the hydrolysis of ATP to ADP.Pi). This intermediate state can be mimicked by adding sodium or-

thovanadate (Vi) with the [α-^{32}P]azidoATP in the labeling reaction mixture. When carried out under conditions permitting hydrolysis, Vi replaces the rapidly dissociated P_i to form a more stable azidoADP.Vi complex, which can then be "trapped" by UV cross-linking (Urbatsch et al., 1995).

As shown in Fig. 4A, there was no detectable labeling of MRP1 by [α-^{32}P]azidoATP under hydrolysis conditions in the absence of Vi (first lane); however, in the presence of 1 mM Vi (remaining lanes), significant labeling of MRP1 was observed as expected. The addition of *S*-mGSH alone decreased the level of [α-^{32}P]azidoADP.Vi trapping by MRP1 in a concentration-dependent manner (Fig. 4, A and B), as we observed previously for GSH (Leslie et al., 2001b). Likewise, when added alone, estrone sulfate also caused a decrease in [α-^{32}P]azidoADP.Vi trapping by MRP1 (Fig. 4, C and D). However, when *S*-mGSH and estrone sulfate were added in combination, they enhanced the effect of each other. Thus, *S*-mGSH decreased the level of [α-^{32}P]azidoADP.Vi trapping with an IC_{50} value of 3.4 $\pm$ 0.5 mM, and in the presence of 10 μM estrone sulfate, the apparent potency of *S*-mGSH increased approximately 15-fold (IC_{50} = 0.22 $\pm$ 0.05 mM, $p <$ 0.05). Likewise, estrone sulfate decreased the level of [α-^{32}P]azidoADP.Vi trapping by MRP1 with an IC_{50} value of 50 $\pm$ 5 μM, and in the presence of 3 mM *S*-mGSH, the IC_{50}

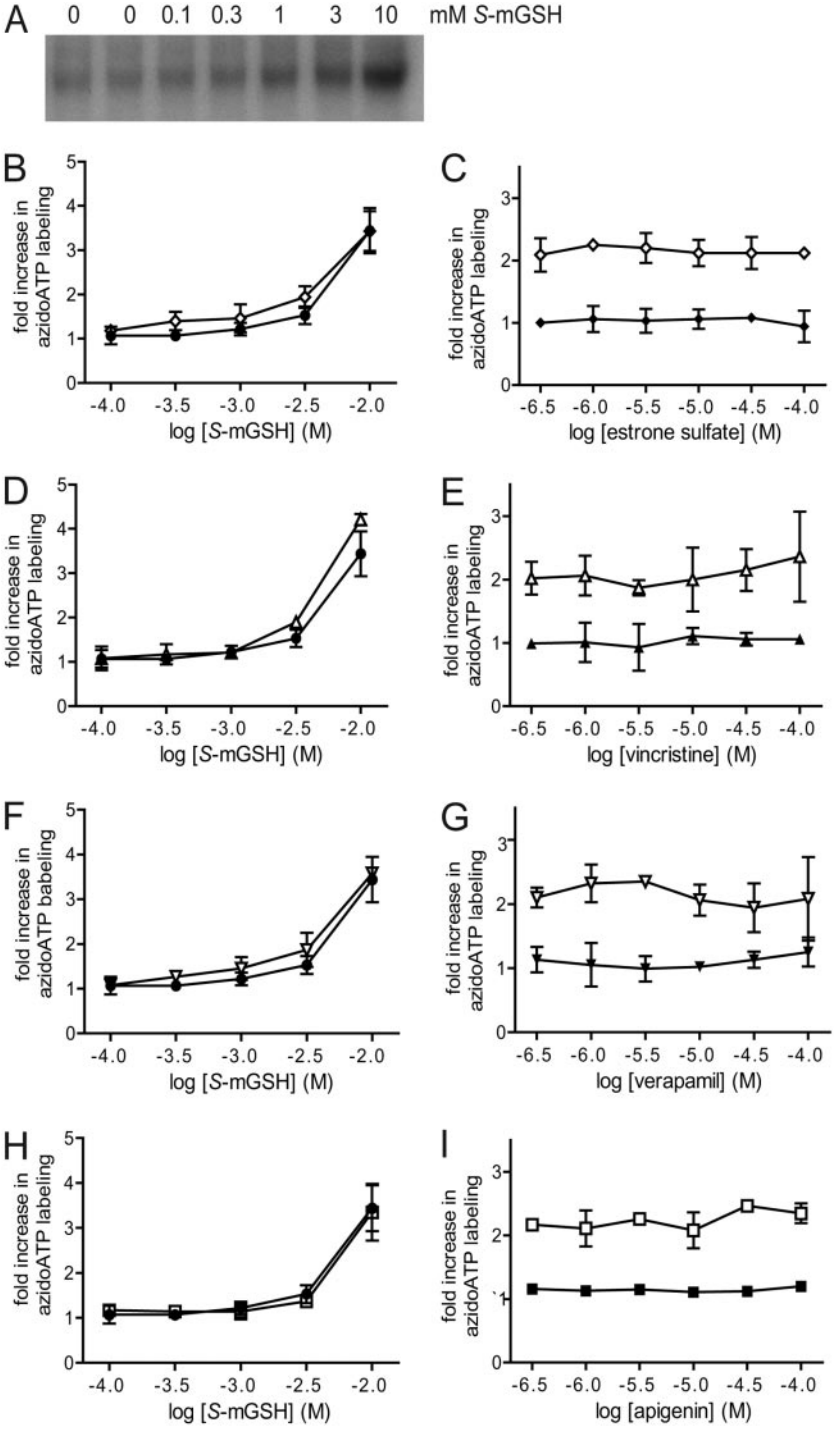

Fig. 2. [γ-^{32}P]azidoATP labeling of MRP1 in the presence of *S*-mGSH and/or GSH-associated substrates and modulators. A, autoradiograph showing labeling of MRP1 (10 μg of membrane protein) after incubation of H69AR membranes with [γ-^{32}P]azidoATP under nonhydrolytic conditions (4°C) in the presence of various concentrations (0–10 mM) of *S*-mGSH. B, D, F, and H, quantification of autoradiographs as shown in A for H69AR membranes incubated with [γ-^{32}P]azidoATP and *S*-mGSH alone (closed symbols) or in the presence (open symbols) of 30 μM estrone sulfate (B), 100 μM vincristine (D), 100 μM verapamil (F), or 30 μM apigenin (H). C, E, G, and I, quantification of autoradiographs of [γ-^{32}P]azidoATP labeling of MRP1 in the presence of various concentrations (0.3–100 μM) of estrone sulfate (D), vincristine (E), verapamil (G), or apigenin (I), plus (open symbols) or minus (closed symbols) 5 mM *S*-mGSH. Data points are mean ($\pm$ S.D.) from at least three independent experiments.

value was approximately 10-fold lower (5.3 ± 1.7 μM, $p < 0.01$).

It is often assumed that binding of transported substrates will stimulate the ATPase activity of an ABC transporter and that azidoADP.Vi trapping can be used as a measure of this ATP hydrolysis. Indeed, several studies have shown that binding of at least some substrates of MRP1 can increase its rate of ATP hydrolysis (Chang et al., 1997; Mao et al., 1999; Hooijberg et al., 2000; Manciu et al., 2003). However, *S*-mGSH and estrone sulfate, which are both transport substrates of MRP1, decreased the level of [α-^{32}P]ADP.Vi trapping (Fig. 4). For this reason, we examined the effect of some other substrates on [α-^{32}P]azidoADP.Vi trapping by MRP1. Figure 5 shows the effect of increasing concentrations of LTC_4, GSSG, and $E_2$17βG on the amount of trapped [α-^{32}P]-azidoADP. LTC_4 and GSSG both increased azidoADP.Vi trapping in a concentration-dependent manner, with LTC_4 (which is known to be the higher-affinity substrate of MRP1) being both more potent and causing the larger increase. In contrast, $E_2$17βG caused a significant concentration-dependent decrease in [α-^{32}P]azidoADP.Vi trapped product, as we reported previously (Létourneau et al., 2008). These observations indicate that the level of [α-^{32}P]azidoADP.Vi trapping by MRP1 in the presence of several of its substrates does not necessarily correlate with its ability to transport the substrate.

Effect of *S*-mGSH on [γ-^{32}P]AzidoATP Labeling of, and [α-^{32}P]AzidoADP.Vi Trapping at, NBD1 and NBD2. The results shown in Figs. 2 and 4 depict the level of radiolabel bound to full-length MRP1, and thus the signals reflect total binding to both NBDs. Based on a growing body of biochemical evidence and recently solved crystal structures

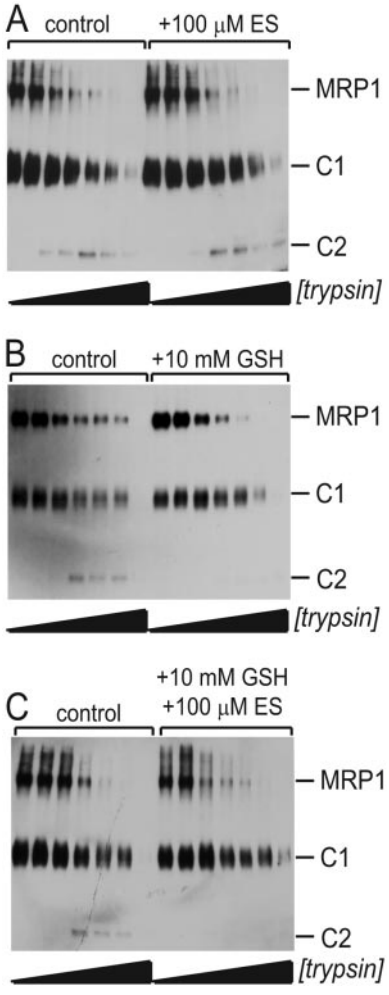

Fig. 3. Tryptic digestion profiles of MRP1 in the presence and absence of estrone sulfate and GSH. MRP1-containing membrane protein (0.25 mg/ml) was preincubated alone or with estrone sulfate (100 μM) (A), alone or with 10 mM GSH (plus 10 mM DTT) (B), or alone or with estrone sulfate (100 μM) and 10 mM GSH (plus 10 mM DTT) (C) for 30 min on ice before the addition of trypsin at trypsin/protein ratios of 2.5:1 to 1:5000 (w/w) for 15 min at 37°C. Samples (2 μg of protein) were resolved on a 7% acrylamide gel and immunoblotted with monoclonal antibody MRPm6 (1:1000), which detects an epitope in the COOH terminus of MRP1 as described under *Materials and Methods*. The position of the intact 190-kDa MRP1, the larger (approximately 70 kDa) COOH-proximal half (C1) of MRP1, and its smaller COOH-terminal (C2) tryptic fragment are indicated. ES, estrone sulfate.

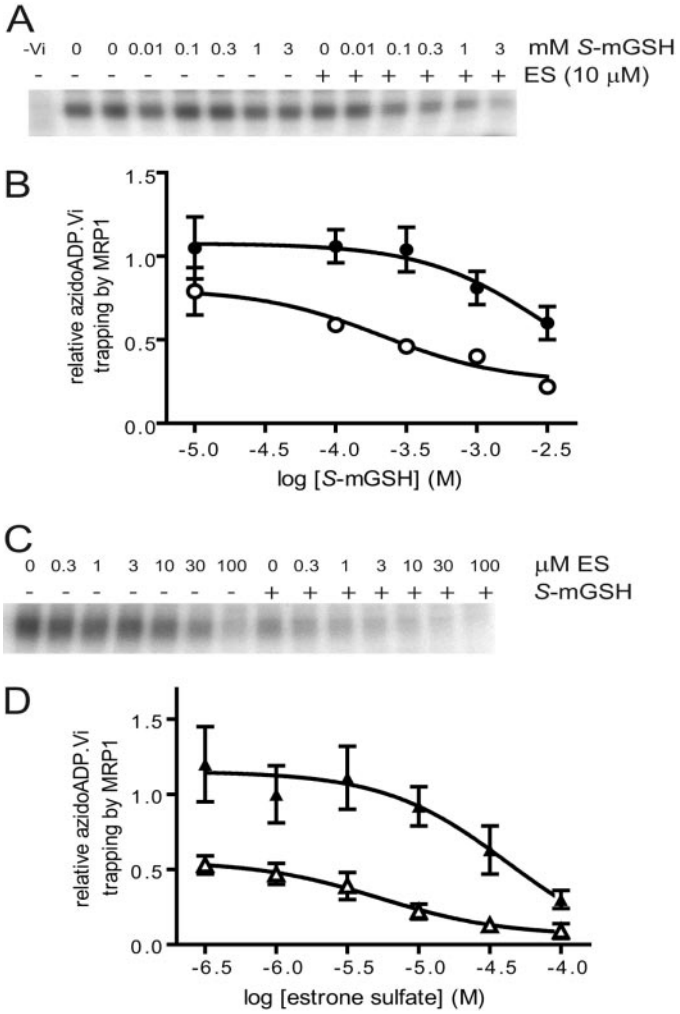

Fig. 4. Effect of *S*-mGSH and/or estrone sulfate on [α-^{32}P]azidoADP.Vi trapping by MRP1. representative autoradiograph (A) and quantification of autoradiographs (B) showing orthovanadate-induced trapping of [α-^{32}P]azidoADP by MRP1 (10 μg of membrane protein) under conditions permitting ATP hydrolysis (1 mM Vi, 37°C, 15 min) in the presence of various concentrations of *S*-mGSH, plus (○) or minus (●) 10 μM estrone sulfate. Note that in A, orthovanadate (Vi) is absent in the first lane but present in the remaining lanes. Representative autoradiograph (C) and quantification of autoradiographs (D) showing [α-^{32}P]azidoADP.Vi trapping by MRP1 in the presence of various concentrations of estrone sulfate plus (△) or minus (▲) 3 mM *S*-mGSH. Data points are mean (± S.D.) from at least three independent experiments. ES, estrone sulfate.

of bacterial ABC proteins, the two NBDs of MRP1 and other mammalian transporters are presumed to form a "sandwich" dimer with two composite nucleotide binding sites (NBS), each of which contains the Walker A and Walker B motifs of the one NBD and the "C" signature motif of the other (Dawson and Locher, 2007). Depending on the transporter, the two NBSs are more ("consensus") or less ("degenerate" or "nonconsensus") similar, as reflected by their sequences and the functional interchangeability or noninterchangeability of the two NBDs. For MRP1, initial binding of ATP occurs predominantly at its nonconsensus NBS1 (composed of NBD1 Walker A and B, and the atypical NBD2 "C" signature motifs), whereas ATP hydrolysis occurs predominantly at the more typical consensus NBS2. To examine the effect of *S*-mGSH on the relative levels of [γ-^{32}P]azidoATP binding and [α-^{32}P]azidoADP.Vi trapping at the two NBDs, the intact protein was photolabeled in the presence of this tripeptide, and then controlled proteolytic degradation was allowed to occur. Previous studies have shown that because of a hypersensitive cleavage site in its linker region (Hipfner et al., 1996), MRP1 undergoes limited degradation under mild conditions, which is characterized by the appearance of two fragments corresponding to the 120-kDa NH_2-proximal and the 75-kDa COOH-proximal halves of the transporter.

As expected, in the absence of *S*-mGSH and substrate, more [γ-^{32}P]azidoATP binding was detected at NBD1/NBS1, with very little detected at NBD2/NBS2 (Fig. 6) (Gao et al., 2000; Hou et al., 2000). However, in the presence of *S*-mGSH, the expected increase in [γ-^{32}P]azidoATP labeling seems to occur solely at NBD1/NBS1. In contrast, [α-^{32}P]azidoADP.Vi trapping in the absence of *S*-mGSH or substrate occurs predominantly at the consensus NBS2 as expected (Gao et al., 2000; Hou et al., 2000; Nagata et al., 2000). However, in the presence of *S*-mGSH and/or estrone sulfate, the reduction in [α-^{32}P]azidoADP.Vi trapping occurs at both NBDs/NBSs.

The Substrate Vincristine and Modulators Verapamil and Apigenin Have No Effect on [α-^{32}P]ADP.Vi Trapping by MRP1. In contrast to estrone sulfate, Fig. 7 shows that the other GSH-associated substrate vincristine and the modulators verapamil and apigenin had no effect on the relative levels of [α-^{32}P]azidoADP.Vi trapping by MRP1, either alone or in combination with *S*-mGSH. To exclude the possibility that the absence of any effect was caused by using *S*-mGSH rather than GSH itself, experiments were repeated using GSH solutions that were freshly prepared and used immediately and thus not requiring DTT, which can interact chemically with the azido group. However, as shown in Fig. 8, GSH had the same effect on [α-^{32}P]azidoADP.Vi trapping by MRP1 as *S*-mGSH. Furthermore, when GSH was used rather than *S*-mGSH, vincristine, verapamil, and apigenin still had no effect on the level of [α-^{32}P]azidoADP.Vi trapping (data not shown).

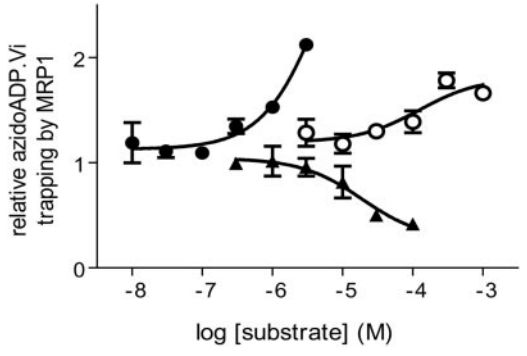

Fig. 5. The effect of transported substrates LTC_4, $E_2 17\beta G$ and GSSG on orthovanadate-induced trapping of azidoADP by MRP1. Quantification of autoradiographs showing [α^{32}-P]azidoADP.Vi trapping by MRP1 after incubation of H69AR membranes (10 μg of protein) under conditions permitting ATP hydrolysis (1 mM Vi, 37°C, 15 min) in the presence of 0.01 to 3 μM LTC_4 (●), 0.3 to 100 μM $E_2 17\beta G$ (▲), or 0.003 to 1 mM GSSG (○). Data points are mean (± S.D.) from three independent experiments.

Vesicular Uptake Assays Suggest that GSH-Associated Substrates/Modulators Increase MRP1 Affinity for GSH. We have reported previously that the GSH-associated substrates/modulators estrone sulfate, vincristine, verapamil, and apigenin all cause an increase in the apparent affinity of MRP1 for GSH, as measured by ATP-dependent vesicular uptake assays (Loe et al., 1998, 2000; Leslie et al., 2001b). These vesicular uptake assays were repeated because the cell line used in the present study is different from that used in the previous studies. However, comparable results were obtained (data not shown). Therefore, it was anticipated that these substrates would enhance the ability of GSH to decrease Vi-induced [α-^{32}P]azidoADP trapping, even if they had no direct effect themselves. However, the results described above (Figs. 4 and 7) show that only estrone sulfate enhanced the inhibitory effect of GSH on the Vi-induced trapping of [α-^{32}P]azidoADP by MRP1.

GSH Enhances the Ability of the GSH-Associated Substrates/Modulators to Displace LTC_4 Binding. Vesicular uptake assays provide an overall measure of the complete transport cycle of MRP1. In an attempt to explain why vincristine, verapamil, and apigenin seem to increase the affinity for GSH (or *S*-mGSH) yet do not enhance the effect of GSH (or *S*-mGSH) on ADP.Vi trapping, we chose to examine the initial binding of substrates/modulators and GSH to MRP1. In the absence of direct radioligand binding assays, which for all MRP1 substrates/modulators except estrone sulfate have proved technically too problematic to be reliable, we examined the ability of GSH and the various GSH-associated substrates/modulators to displace labeling of MRP1 by

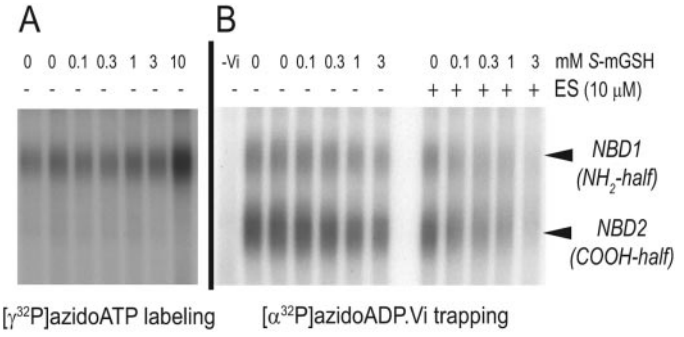

Fig. 6. Effect of *S*-mGSH and estrone sulfate on [γ-^{32}P]azidoATP labeling and orthovanadate-induced trapping of [α-^{32}P]azidoADP by NBD1/NBS1 and NBD2/NBS2 of MRP1. A, autoradiograph showing the relative levels of [γ-^{32}P]azidoATP bound to the NH_2- (NBD1) or COOH- (NBD2) proximal halves of MRP1, in the presence of various concentrations of *S*-mGSH. B, autoradiograph showing the relative levels of [α-^{32}P]azidoADP trapped by the NH_2- (NBD1) or COOH- (NBD2) proximal halves of MRP1 after incubation of H69AR membranes with [α-^{32}P]azidoATP under conditions permitting hydrolysis (1 mM Vi, 37°C, 15 min) in the presence of various concentrations of *S*-mGSH, plus or minus 10 μM estrone sulfate (ES). MRP1 was cleaved into its NH_2- and COOH-proximal halves after UV cross-linking by allowing limited proteolysis by prolonged incubation of the ^{32}P-labeled membranes at 37°C.

its intrinsically photoactive substrate [^{3}H]LTC$_4$. Figure 9A shows, as we demonstrated previously (Qian et al., 2001), that labeling of MRP1 with [^{3}H]LTC$_4$ can be displaced by high concentrations (>10 μM) of estrone sulfate. The addition of GSH (1 mM) caused an approximately 25-fold increase in the inhibitory potency of estrone sulfate. Similar results were obtained for vincristine and apigenin (Fig. 9, B and D). On its own, verapamil had no effect on [^{3}H]LTC$_4$ labeling; however, in the presence of GSH, it was able to effectively displace LTC$_4$ (Fig. 9C). Thus, GSH enhanced the ability of all four GSH-associated substrates/modulators to displace LTC$_4$, which agrees well with our previous ATP-dependent vesicular transport data (Loe et al., 1998, 2000; Leslie et al., 2001b; Qian et al., 2001).

Estrone Sulfate Enhances the Ability of GSH to Displace LTC$_4$ Binding, but Vincristine, Verapamil, and Apigenin Do Not. The above results suggest that GSH binding increases the affinity of MRP1 for its GSH-associated substrates/modulators. To determine whether the reverse is true (i.e., that estrone sulfate, vincristine, verapamil, or apigenin binding increases the affinity of MRP1 for GSH), a second set of [^{3}H]LTC$_4$ displacement experiments was carried out. As shown in Fig. 10A, [^{3}H]LTC$_4$ labeling of MRP1 was inhibited by GSH with an IC$_{50}$ of 1.5 $\pm$ 0.2 mM. The addition of 10 μM estrone sulfate enhanced the displacement and reduced the IC$_{50}$ 8-fold to 0.18 $\pm$ 0.05 mM ($p < 0.01$). In contrast, vincristine (100 μM), verapamil (100 μM), and apigenin (30 μM) had no effect on the ability of GSH to displace [^{3}H]LTC$_4$ (IC$_{50}$ values of 1.1 $\pm$ 0.2, 1.3 $\pm$ 0.1, and 1.1 $\pm$ 0.1 mM, respectively). Thus, estrone sulfate is the only one of the four GSH-associated substrates/modulators that seems to increase the affinity of MRP1 for GSH, at least at the initial step of substrate binding.

Discussion

In the present study, we have examined the effect of GSH and various GSH-associated substrates/modulators on the binding and hydrolysis of ATP by MRP1. The coupling between transport of a substrate by MRP1 and hydrolysis of ATP is not well understood, and even more poorly understood is the role GSH may play in this coupling. For this reason, we sought to determine whether the role of GSH was the same for each of the GSH-associated classes of transport that are

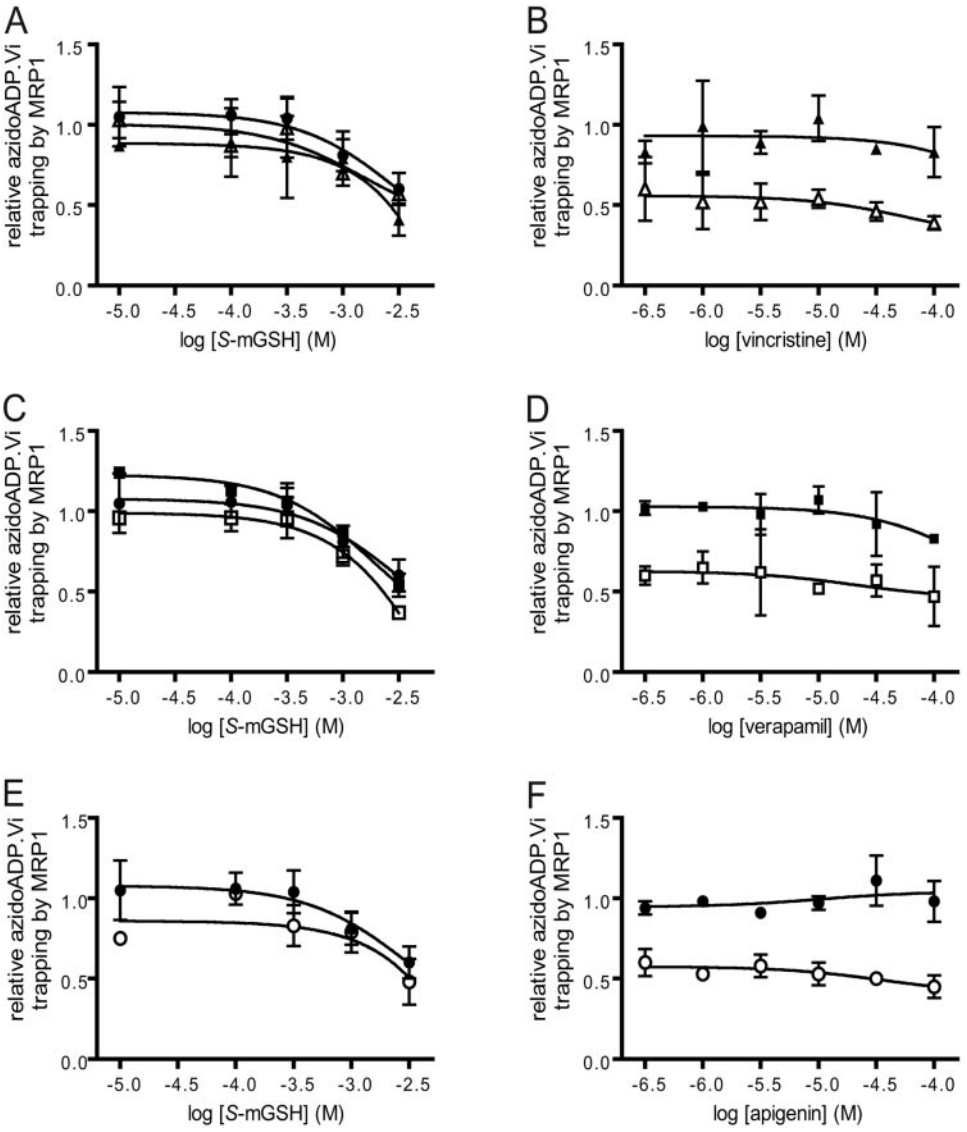

Fig. 7. Verapamil, vincristine, or apigenin have no effect on orthovanadate-induced trapping of [α-^{32}P]azidoADP by MRP1. A, C, and E, quantification of levels of [α-^{32}P]azidoADP.Vi trapping by MRP1 (10 μg of membrane protein) under conditions permitting ATP hydrolysis (1 mM Vi, 37°C, 15 min), in the presence of various concentrations of *S*-mGSH, in the absence (●) or presence of 10 μM (▲) or 100 μM (△) vincristine (A), 10 μM (■) or 100 μM (□) verapamil (B), or 30 μM apigenin (○) (E). B, D, and F, [α-^{32}P]-AzidoADP.Vi trapping by MRP1 in the presence of various concentrations of vincristine (B), verapamil (D), or apigenin (F) with (open symbols) or without (closed symbols) 3 mM *S*-mGSH. Data points are mean ($\pm$ S.D.) from at least three independent experiments.

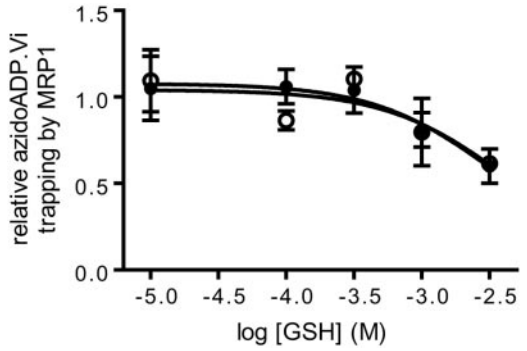

Fig. 8. Comparison of the effects of *S*-mGSH and GSH on orthovanadate-induced trapping of [α-^{32}P]azidoADP by MRP1. [α-^{32}P]AzidoADP.Vi trapping by MRP1 (10 μg of membrane protein) under conditions permitting ATP hydrolysis (1 mM Vi, 37°C, 15 min) was carried out in the presence of various concentrations of *S*-mGSH (closed symbols) and GSH (open symbols) as described under *Materials and Methods*.

depicted in Fig. 1. We also examined the initial binding of GSH and selected GSH-associated substrates/modulators to MRP1, determined the effect they have on each other, and used trypsin digestion profiles to examine conformational changes in MRP1 that might be induced by these compounds.

In our initial series of experiments, we observed that both GSH and *S*-mGSH caused a significant increase in the labeling of MRP1 by [γ-^{32}P]azidoATP. We and others have also shown previously that GSH or *S*-mGSH binding to MRP1 causes a conformational change in MRP1, apparently at the COOH end of the transporter (Manciu et al., 2003; Ren et al., 2005; Rothnie et al., 2006). Thus, it is conceivable that this conformational change in the protein is responsible for communicating GSH binding to the NBSs, which leads to increased ATP binding at NBD1/NBS1. Recent homology models of the three-dimensional structure of MRP1 (and other mammalian ABC proteins) indicate that the transmembrane helices exhibit significant twisting such that NBD1 interacts directly with the cytosolic loop between transmembrane he-

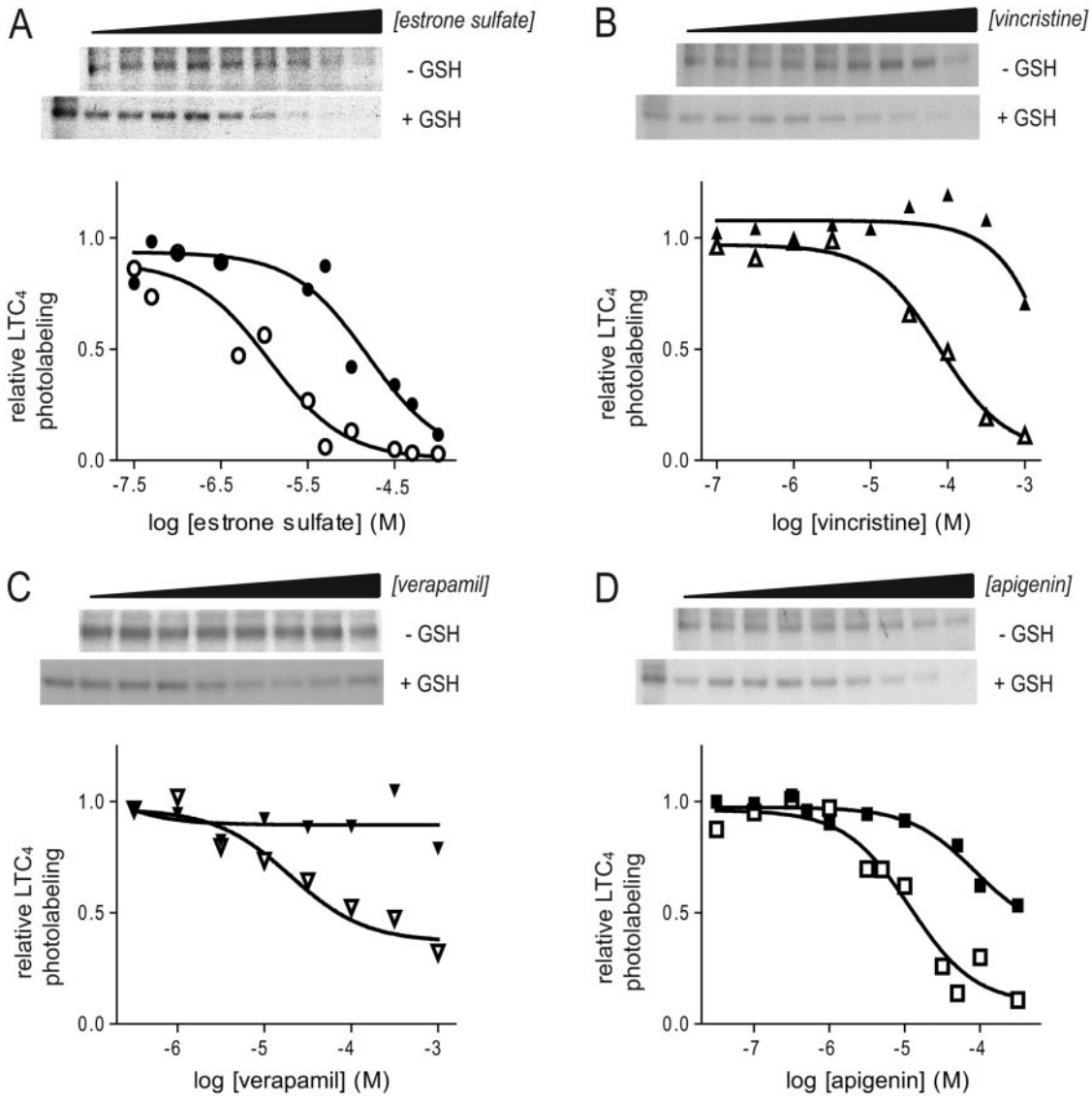

Fig. 9. [^{3}H]LTC$_4$ photolabeling of MRP1 and displacement by GSH-associated substrates and modulators. Shown are representative autoradiographs and quantification of levels of [^{3}H]LTC$_4$ photolabeling of MRP1 (20 μg of membrane protein, 200 nM [^{3}H]LTC$_4$ for 30 min at room temperature) in the presence of 0.03 to 100 μM estrone sulfate (A), 0.1 to 1000 μM vincristine (B), 0.3 to 1000 μM verapamil (C), or 0.03 to 300 μM apigenin (D) in the absence (closed symbols) or presence (open symbols) of 1 mM GSH.

lices 15 and 16 of MSD2 (Dawson and Locher, 2007; DeGorter et al., 2008), which seems likely to be the means by which signaling between the COOH-proximal end of MRP1 and NBD1/NBS1 occurs.

When examining the initial binding of substrates to MRP1, we observed that GSH and estrone sulfate alone were both able to displace LTC_4, as reported previously (Qian et al., 2001), and binding of either one led to an increased affinity for the other. Based on results from radioligand binding assays, we have suggested that binding of GSH opens up a high-affinity binding site (K_d = 0.6 μM) for estrone sulfate (Rothnie et al., 2006). Only high-affinity binding can be measured by ligand binding assays because of technical limitations, and this requires the GSH to bind to MRP1 first, but, as noted earlier, estrone sulfate can bind and be transported by MRP1 in the absence of GSH, albeit at rather low affinity and efficiency (Qian et al., 2001; Leslie et al., 2003a). Thus, low-affinity binding of estrone sulfate seems sufficient to cause an increase in MRP1's affinity for GSH, presumably by causing a conformational change that is not detectable using our limited trypsin digestion protocols. This may be because it occurs within a nonaccessible, membrane-embedded region of the transporter or because it affects regions of MRP1 that are not recognized by the antibodies used. Thus, it would seem that the GSH and estrone sulfate binding sites of MRP1 are allosterically linked, and the communication between them is bidirectional.

Vincristine and apigenin were able to displace LTC_4, but only when added at very high concentrations, suggesting that MRP1 has a relatively low affinity for these compounds. The affinity of MRP1 for verapamil seems even lower because no displacement of LTC_4 was detected in the presence of this modulator. Nevertheless, the addition of GSH markedly increased the affinity of MRP1 for all three of these compounds, whereas none of the three had a reciprocal effect on the affinity of MRP1 for GSH. Thus, there is no evidence of any kind of conformational change occurring upon binding of vincristine, apigenin, or verapamil, suggesting that signaling from the GSH binding site to the binding site(s) of these substrates/modulators (in contrast to estrone sulfate) may be unidirectional.

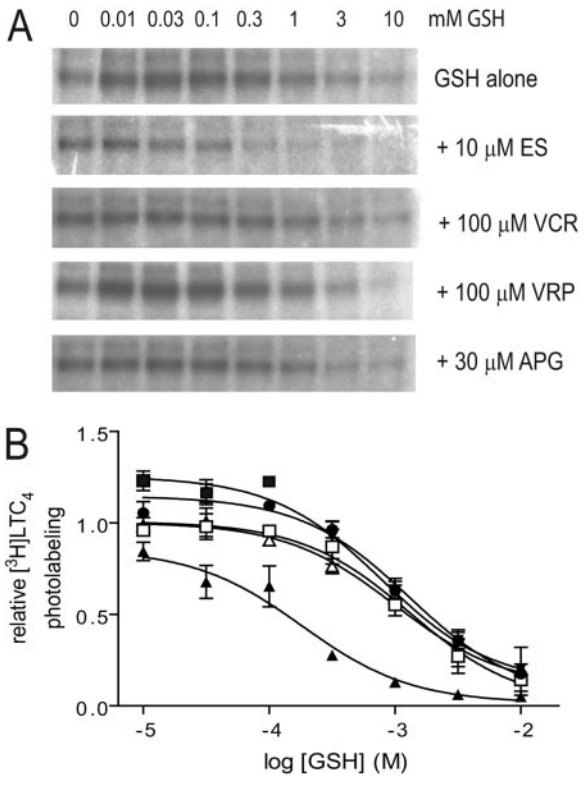

Fig. 10. GSH and substrate/modulator displacement of [^{3}H]LTC_4 photolabeling of MRP1. Representative autoradiographs (A) and quantification of levels (B) of [^{3}H]LTC_4 photolabeling of MRP1 and displacement by 0.01 to 10 mM GSH alone (●) or together with 10 μM estrone sulfate (▲), 100 μM vincristine (△), 100 μM verapamil (■), or 30 μM apigenin (□). Data points are mean (± S.E.) from at least three independent experiments. ES, estrone sulfate; VCR, vincristine; VRP, verapamil; APG, apigenin.

The results of the LTC_4 displacement experiments highlight an important difference between measuring ATP-dependent substrate transport and initial substrate binding by MRP1, and probably other ABC transporters as well. Thus, when measuring the inhibition of MRP1-mediated ATP-dependent LTC_4 transport by GSH in the presence or absence of vincristine/verapamil/apigenin, the results obtained were strikingly different from those obtained from the simpler LTC_4 displacement assays that were carried out in the absence of any nucleotide. Transmembrane transport of a molecule is a complex process that first involves substrate binding at high affinity on the cytosolic side of the membrane, followed by a reorientation to the extracellular face of the membrane where substrate affinity is decreased so that it may be released. The substrate binding site(s) must then return to its initial high-affinity state (Tanford, 1983). For MRP1 and other drug-transporting ABC proteins, this transport process is coupled to the process of binding and hydrolysis of ATP. Vesicular uptake or cellular transport assays provide an overall measure of both processes but do not allow for a detailed analysis of any of the individual steps, including initial substrate binding.

As reported previously, we confirmed that binding of GSH or *S*-mGSH reduces vanadate-induced azidoADP trapping by MRP1 (Leslie et al., 2001b). It is generally presumed that binding of a substrate to an ABC transporter stimulates ATP hydrolysis, which in turn stimulates the transport of the substrate across the membrane. This stimulation of ATP hydrolysis has also generally been assumed to be detected by an increase in trapping of azidoADP by the transporter in the presence of orthovanadate. The decrease in azidoADP.Vi trapping by MRP1 that we observed in the presence of $E_2$17βG, GSH, and/or estrone sulfate seems contradictory to these assumptions, suggesting that they may represent an oversimplified interpretation of what the vanadate-induced trapping of azidoADP reflects biochemically. We and others have shown previously that the MRP1 substrates LTC_4 and GSSG stimulate the ATPase activity of the purified transporter, although rather weakly (Chang et al., 1997; Mao et al., 1999; Leslie et al., 2001b). Earlier studies have also shown that LTC_4 and GSSG increase vanadate-induced azidoADP trapping by MRP1 (Taguchi et al., 1997; Bakos et al., 1998; Gao et al., 2000; Leslie et al., 2001b). On the other hand, we have observed that $E_2$17βG significantly decreases azidoADP.Vi trapping (Létourneau et al., 2008). Likewise, we have found that *S*-mGSH (and GSH) also decrease azidoADP.Vi trapping, whereas reports of the ability of GSH to stimulate ATP hydrolysis as measured by ATPase assays are variable (Chang et al., 1997; Mao et al., 1999; Hooijberg et al.,

2000; Leslie et al., 2001b; Manciu et al., 2003). We obtained evidence recently that rather than reflecting reduced ATP hydrolysis, the decreased azidoADP.Vi trapping in the presence of $E_2$17βG involves an enhanced rate of posthydrolysis release of ADP (Létourneau et al., 2008). This would reduce the time the NBS is occupied by ADP, which could explain a lower level of Vi-induced trapping of the dinucleotide at NBS2. On the other hand, $E_2$17βG (or GSH/*S*-mGSH) binding may cause a conformational change in the NBDs/NBSs that does not favor the formation of a stable ADP.Vi complex. Whatever the explanation, it seems clear that the interpretation of azidoADP.Vi trapping experiments in the presence of substrates or modulators, at least in the case of MRP1, is not as straightforward as believed previously. Thus, caution should be exercised when interpreting results from these types of experiments.

That binding of GSH to MRP1 results in both increased labeling of NBS1 by azidoATP and decreased azidoADP.Vi trapping at NBS2 initially seems somewhat counterproductive. Just how and why a conformation change induced by GSH binding has a seemingly positive effect at one NBS and an apparently negative effect at the other NBS is unclear. One possibility is that the sequence differences between the two NBSs are sufficient to result in an induced conformational change having opposite effects on the two sites. However, as discussed above, we believe that the azidoADP.Vi trapping experiments do not simply represent the level of ATP hydrolysis, and thus, the effect of GSH on NBS2 does not necessarily reflect an inhibition of ATPase activity.

The substrate estrone sulfate, like GSH/*S*-mGSH and $E_2$17βG, caused a decrease in azidoADP.Vi trapping by MRP1, and when added together, estrone sulfate and *S*-mGSH enhanced the effect of each other. Thus, it seems that estrone sulfate binding not only results in signaling to the GSH binding site of MRP1 (and vice versa) but also signaling to the NBSs. In contrast, the substrate vincristine and modulators verapamil and apigenin had no effect on azidoADP.Vi trapping, and when added with GSH/*S*-mGSH, no effect beyond that of the tripeptide alone was detected. These results correlate well with previous studies, in which the effect of these compounds on ATPase activity was measured directly, and no significant effect of several drugs, including vincristine and apigenin, either alone or in the presence of GSH, was observed (Mao et al., 1999; Hooijberg et al., 2000; Manciu et al., 2003). Thus, there is no convincing evidence that binding of these three compounds alone to MRP1 results in direct signaling to the NBSs.

One aspect of our present observations that is difficult to rationalize is how vincristine, verapamil, and apigenin enhance GSH transport by MRP1 if it is not by increasing initial binding of GSH or by stimulating the binding or hydrolysis of ATP. This is particularly difficult to understand for apigenin and verapamil, which are not themselves even transported by MRP1. It is possible that these modulators cause GSH to dissociate from MRP1 more rapidly after reconfiguration of the protein to expose the GSH binding site to the other side of the membrane. On the other hand, the presence of verapamil/apigenin bound to MRP1 may facilitate the resetting of the protein after release of the GSH, so that another round of transport can occur. Recent studies relevant to this suggestion indicate that although ATP hydrolysis at NBS1 is very low, release of nucleotide from this NBS may be the rate-limiting step, at least in the LTC_4 transport process (Yang et al., 2005). Further analyses are required to distinguish between these and other possibilities.

In conclusion, our data here, together with those published previously (Rothnie et al., 2006), indicate that GSH binding to MRP1 causes a conformational change that seems to result in both an increase in ATP binding and an increase in the apparent affinity for the GSH-associated substrates vincristine and estrone sulfate and modulators verapamil and apigenin. However, these four GSH-associated substrates/modulators can then be classified into one of two groups (Supplemental Fig. 1). The first group is composed of vincristine, verapamil, and apigenin. Although GSH increases the apparent affinity of MRP1 for these compounds, they have no reciprocal effect on GSH binding. In addition, none of these compounds has any direct effect on the interaction of the transporter with nucleotide. In the second group, which is presently composed of estrone sulfate, GSH increases the apparent affinity of MRP1 for this substrate and vice versa, and both GSH and the substrate have an effect on Vi-induced trapping of azidoADP. For the first group (vincristine, verapamil, and apigenin), GSH is transported across the membrane, but for the second group (estrone sulfate), GSH is not transported but simply acts as a modulator of efflux activity (Fig. 1). Thus, our results provide strong evidence that MRP1-mediated transport of GSH and GSH-modulated transport are mechanistically distinct.

Acknowledgments

We thank Kathy Sparks for assistance with cell culture. We also thank Drs. R. J. Scheper and G. L. Scheffer (Free University, Amsterdam, the Netherlands) for providing antibodies MRPm6 and MRPr1. We also thank Dr. Kazumitsu Ueda (Kyoto University) for useful discussions.

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