Photolabeling of Human and Murine Multidrug Resistance Protein 1 with the High Affinity Inhibitor \(^{125}\text{I}]\)LY475776 and Azidophenacyl-[\(35\text{S}\)]Glutathione*

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Multidrug resistance protein 1 (MRP1/ABCC1) is an ATP-dependent transporter of structurally diverse organic anion conjugates. The protein also actively transports a number of non-conjugated chemotherapeutic drugs and certain anionic conjugates by a presently poorly understood GSH-dependent mechanism. LY475776 is a newly developed \(^{125}\text{I}]-labeled azido tricyclic isoxazole that binds to MRP1 with high affinity and specificity in a GSH-dependent manner. The compound has also been shown to photolabel a site in the COOH-proximal region of MRP1’s third membrane spanning domain (MSD). It is presently not known where GSH interacts with the protein. Here, we demonstrate that the photolabile GSH derivative azidophenacyl-GSH can substitute functionally for GSH in supporting the photolabeling of MRP1 by LY475776 and the transport of another GSH-dependent substrate, estrone 3-sulfate. In contrast to LY475776, azidophenacyl-[\(35\text{S}\)]Glutathione* photolabels both halves of the protein. Photolabeling of the COOH-proximal site can be markedly stimulated by low concentrations of estrone 3-sulfate, suggestive of cooperativity between the binding of these two compounds. We show that photolabeling of the COOH-proximal site by LY475776 and the labeling of both NH$_2$- and COOH-proximal sites by azidophenacyl-GSH requires the cytoplasmic linker (CL3) region connecting the first and second MSDs of the protein, but not the first MSD itself. Although required for binding, CL3 is not photolabeled by azidophenacyl-GSH. Finally, we identify non-conserved amino acids in the third MSD that contribute to the high affinity with which LY475776 binds to MRP1.

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††† The abbreviations used are: MRP1, multidrug resistance protein 1; ABC, ATP-binding cassette; MSD, membrane spanning domain; CL3, cytoplasmic linker region; NBD, nucleotide binding domain; TM, transmembrane; LTC$_4$, leukotriene C$_4$; E$_{17}$β, 17β-estradiol 17-β-(D-glucuronide); HEK, human embryonic kidney; IACI, N-(hydrocinchonidin-8-yl)-4-azido-2-hydroxybenzamide; IAARh123, iodoaryl azido rhodamine 123; AG-A, agosterol-A.

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in predicted TM helix 17 and at least one residue in TM helix 14 are important in determining substrate specificity (28–30). Furthermore, it is possible to make compensatory mutations in these two helices, suggesting that they may be in spatial proximity to one another in the native protein (30).

As an alternative approach to identifying regions of the protein directly involved in substrate binding, MRPI has been photolabeled by its known high affinity physiological substrate LTC4 as well as by compounds that are less well characterized with respect to binding and transport (10, 12, 27, 31–34). Although these compounds compete with LTC4 for binding to MRPI, their affinities appear to be considerably lower, and they also interact with the distantly related P-glycoprotein, MRP1, whose affinities are generally lower, and both iodinated compounds have been shown to label the COOH-proximal halves of the protein (32, 33, 35) as does [3H]LTC4 (27). Both iodinated compounds have been shown to be restricted to a site in the COOH-proximal half of the protein (34). On the basis of these results it has been suggested that the site labeled by AG-A has a high affinity for drugs and that GSH binds to a site presumed to be in the NH2-terminal half of the protein, possibly involving the cytoplasmic linker (CL3) region between MSD1 and MSD2 (34). However, direct binding studies with photoactive derivatives of GSH have not been reported.

In this study we have used various combinations of MRPI domains co-expressed in insect SF21 cells together with human and murine hybrid and mutant proteins to investigate the binding of a new and highly specific inhibitor of MRPI, LY475776. LY475776 is an iodinated azido tricyclic isoxazole that displays essentially complete dependence on GSH for binding to MRPI, which has a higher affinity for the protein than LTC4 (36, 37). Concurrent with these studies we have also examined the binding of azidothymidine-GSH, which shows that the binding of an acridine moiety in MRP1 and murine Mrp1 (43, chimeric Mrp1/1, Mrp1s (44), and mutant Mrp1 and mrp1 (28, 30). Mrp1 half molecules (amino acids 1–932 and 932–1531) were also cloned into the mammalian dual expression vector pBudCEV7 (Invitrogen). Membrane vesicles were isolated from transfected SF21 cells, membrane vesicles were prepared by nitrogen cavitation and purified by sucrose gradient centrifugation (12, 41). MRPI/mrplbuidied were constructed in the mammalian episomal expression vector pCEBV-7 and stably transfected in HEK293 cells (42). The transfected cell lines that have been described previously include: human MRPI and murine Mrp1 (43), chimeric Mrp1/Mrp1s (44), and mutant Mrp1 and mrp1 (28, 30). Mrp1 half molecules (amino acids 1–932 and 932–1531) were cloned into the mammalian dual expression vector pBudCEV7 (Invitrogen). Membrane vesicles were isolated from transiently transfected HEK293 cells. Membrane vesicles were prepared from HEK293 cells as described for SF21 cells.

Quantitation of MRPI Polypeptides and Membrane Vesicle Preparation—SDS-PAGE of membrane vesicle preparations was performed as described previously using 5–15% gradient gels (38, 45). Following transfer of the membrane proteins to Immobilon-P membranes (Millipore), MRPI polypeptides were detected using an enhanced chemiluminescence kit (PerkinElmer Life Sciences) and monoclonal antibodies QCRL-1 and MRPm6 for MRP1 and MRP1, which recognize a common epitope in MRP1 and mrp1 (46, 47).

 Vesicle Transport of LTC4 and Estrone 3-Sulfate—The ATP-dependent uptake of LTC4 and estrone 3-sulfate was measured using a rapid filtration assay as described previously (12, 16). Synthesis of Azidothymidine-[35S]GSH—Azidothymidine-[35S]GSH was prepared as described previously (48). In brief, 125 μCi of [35S]GSH was extracted with ethyl acetate to remove diethiothreitol before being added to a reaction mixture containing potassium phosphate buffer (50 mM, pH 7.4), 4-azidothymidine bromide (10 mM), GSH reductase (120 units), and ADP (1 mM). The reaction was allowed to proceed at 37 °C for 3 days. The products were separated by silica G thin layer chromatography using 1-propanol/water/acetic acid (12:5:1, v/v). The area on the thin layer chromatography plate containing the radioactivity with an Rf corresponding to unlabeled azidothymidine-GSH was scraped off and extracted with 400 μl of water six times. The extract was concentrated under a nitrogen stream.

Photolabeling of MRPI and Related Proteins with [125I]LY475776 or Azidothymidine-[35S]GSH—Membrane vesicles (75 μg of protein from SF21 cells or 50 μg of protein from HEK cells in 35 μl of 50 mM Tris-HCl, pH 7.4, 250 mM sucrose) were incubated with [125I]LY475776 (0.5 nM) at 37 °C or azidothymidine-[35S]GSH (0.5 μCi) at room temperature for 10 min and UV-irradiated for 5 min at 254 nm or 312 nm on ice, for LY475776 and or azidothymidine-[35S]GSH, respectively. Proteins were solubilized in Laemmli’s buffer and analyzed on 5–15% gradient gel for SDS-PAGE. The gel was then dried onto blotting paper and exposed to x-ray film at room temperature for detection of [125I]LY475776. For the azidothymidine-[35S]GSH-labeled proteins, gels were treated with Amplify (Amerham Biosciences), dried, and exposed to film at ~70°C. Exposure times were typically 1–3 days.

RESULTS

Photolabeling of Truncated and Co-expressed MRPI Fragments with [125I]LY475776—The ability of [125I]LY475776 to label MRPI was confirmed using plasma membrane vesicles from SF21 insect cells and stable transfectants of human embryonic kidney (HEK) cells expressing the full-length protein. As observed previously using membranes from multidrug resistant H69AR cells, photolabeling was specific for a protein of the anticipated size of MRPI and was detectable only in the presence of GSH (Fig. 1, A and C, respectively) (36). No labeling
HEK cells co-expressing MRP1 and comparable analysis of membranes from Sf21 cells expressing a site in the NH2-proximal half of MRP1 and weaker labeling of a site in the COOH-proximal half (27). We have also shown that MRP1 and vincristine markedly and reciprocally increase the apparent affinity between binding of the two substrates. In contrast, other substrates such as estrone sulfate and 4-methylnitrosaminol-1-(3-pyridyl)-1-butanol-3-glucuronide (NNAL) glucuronide, which are also transported by MRP1 in a GSH-dependent manner, appear not to have a reciprocal effect on GSH transport (14, 16). To directly examine GSH binding, we synthesized the photoactivatable analog azidophenacyl-GSH that has been shown previously to bind to glutathione S-transferase and to substitute for GSH in supporting the transport of some MRP1 substrates (Fig. 1B, right panel) (17). Thus, the weak photolabeling of the NH2-half of the protein appears to be the result of a specific GSH-dependent interaction.

Previous labeling and transport studies have shown that MSD1 extending from amino acid 1 to 204 is not required for the binding and transport of [3H]LTC4 (26, 27). Deletion of part of CL3 between amino acids 204 and 281 essentially abolishes photolabeling of the region containing MSD2 and NBD1, but its removal has less of an effect on labeling of the COOH-proximal site (27, 29). Consequently, we examined the extent to which binding and photolabeling with [125I]LY475776 was dependent on the presence of both MSD1 and CL3. MRP1 lacking amino acids 1–204 could be efficiently photolabeled (Fig. 1C, left panel). However, truncation to amino acid 280 essentially eliminated labeling, and the low level of labeling detected was unaffected by GSH (Fig. 1C). As with LTC4, co-expression of MRP1932–1531 with a fragment comprised of the missing NH2-proximal amino acids restored strong GSH-dependent labeling of the larger fragment despite the fact that LY475776 primarily labels the COOH-proximal region of the protein (Fig. 1C, right panel).

Azidophenacyl-GSH Can Substitute Functionally for GSH in Enhancing the Transport of Estrone Sulfate by MRP1 and Photolabeling of the Protein by LY475776—Previous studies have shown that some MRP1 substrates that display a dependence on GSH for transport, such as vincristine, reciprocally stimulate the transport of GSH (17). These studies have also shown that GSH and vincristine markedly and reciprocally increase the apparent affinity between binding of the two substrates. In contrast, other substrates such as estrone sulfate and 4-methylnitrosaminol-1-(3-pyrindyl)-1-butanol-3-glucuronide (NNAL) glucuronide, which are also transported by MRP1 in a GSH-dependent manner, appear not to have a reciprocal effect on GSH transport (14, 16). To directly examine GSH binding, we synthesized the photoactivatable analog azidophenacyl-GSH that has been shown previously to bind to glutathione S-transferase and to substitute for GSH in supporting the transport of estrone sulfate and to enhance MRP1 binding of LY475776. Azidophenacyl-GSH at 100 μM stimulated estrone sulfate transport ~2.5-fold, a level of stimulation similar to that obtained previously with the same concentration of GSH (Fig. 2A) (16). The derivative also stimulated photolabeling of full-length MRP1 expressed in Sf21 cells, despite the fact that its solubility limited the concentration used to 100 μM (Fig. 2A). Thus, azidophenacyl-GSH is able to substitute for its parent molecule, both with respect to enhancing estrone sulfate transport and LY475776 binding. We then examined the ability of azidophenacyl-[35S]GSH to photoactivate MRP1. As shown in Fig. 2B, intact MRP1 in Sf21 membranes was clearly labeled with azidophenacyl-[35S]GSH. Unlabeled GSH (5 mM) significantly inhibited but did not abolish the labeling. However, LTC4 (8 μM) completely blocked azidophenacyl-[35S]GSH labeling of the protein, indicating that the binding was specific and that both molecules occupy the same or mutually exclusive binding sites.

Given the extreme GSH dependence of MRP1 labeling by LY475776, we determined whether the compound reciprocally stimulated labeling by azidophenacyl-[35S]GSH. No stimulation of photolabeling of MRP1 by azidophenacyl-[35S]GSH could be detected over a wide range of concentrations of LY475776. Instead, concentration-dependent inhibition of photolabeling was observed between 100 nM and 1 μM, which was...
with $^{125}$ILY475776, but azidophenacyl-GSH was used in place of GSH. Vesicles prepared from MRP1-transfected HEK cells were photolabeled in the absence of 0.1 mM azidophenacyl-GSH ($^{125}$I$^{1}$estrone sulfate (data not shown). In contrast, photolabeling of MRP1 by azidophenacyl-$^{35}$S[GSH was modestly increased in the presence of GSH. No stimulation of binding to either site was detected when a comparable experiment was carried out with estrone sulfate (data not shown).

We also determined whether the stimulation of photolabeling of the intact protein by estrone sulfate affected both sites equivalently. The conjugated estrogen markedly stimulated labeling of the COOH-proximal half of the protein in a concentration-dependent fashion with relatively little change in the extent of labeling of the NH$_2$-proximal site (Fig. 4C). The increase in photolabeling of the COOH-proximal site was readily observed at a concentration of 1 $\mu$M estrone sulfate that approximates the $K_{m}$ for transport of this compound in the presence of GSH. No stimulation of binding to either site was detected when a comparable experiment was carried out with LY475776 in place of estrone sulfate (data not shown).

$^{35}$S$^{1}$LY475776 Photolabels MRP1 More Efficiently Than Its Murine Ortholog, mrp1—Although MRP1 and its murine ortholog are highly conserved, they differ with respect to their ability to confer resistance to several anthracyclines and to transport some estrogen conjugates, such as E$_2$17$\beta$G and estrone sulfate (43, 51). We have shown that sequence variations in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44). Because this region of the protein contains the site that is strongly labeled in the COOH-proximal third of the two proteins play a major role in these functional differences (28, 30, 44).

To further define the structural basis of the difference in
In the presence of various concentrations of LY475776 and in the absence of a hybrid containing the NH2-proximal two-thirds of the characterized human and murine hybrid proteins (44). Photolabeling of MRP1 was much weaker than that of the human protein, consistent with the inhibition studies, these experiments revealed that labeling of MRP1 was indistinguishable from wild type mrp1. However, photolabeling of the complementary hybrid containing the amino acids 959–1531 of MRP1 was clearly enhanced relative to the wild type murine protein (Fig. 6B). To further define the region responsible for increased photolabeling, we used two additional hybrids that contained either amino acids 959–1187 or 1188–1531 of MRP1. Although neither was photolabeled to the same extent as the hybrid containing the entire region of MRP1 from 959–1531, labeling of the hybrid containing MRP1 959–1187 was enhanced relative to wild type mrp1 (Fig. 5B). No enhancement was observed with the hybrid containing MRP1 1188–1531. Qualitatively, these results are similar to those we obtained when examining anthracycline resistance, which indicated that residues specific to the human protein in the region between amino acids 959 and 1187 are important for resistance to this class of drugs (44).

Identification of Individual Amino Acids Involved in Binding of LY475776

Within the region between amino acids 959 and 1187 of MRP1, we have identified one non-conserved amino acid that is essential for conferring anthracycline resistance (28). Conversion of Glu-1089 in MRP1 to Gln, the corresponding amino acid in mrp1, abolished the ability to confer anthracycline resistance, whereas the reciprocal mutation in mrp1 created a protein that could confer resistance to this class of drugs (28). Consequently, we examined the ability of these two mutant proteins to bind both azido-[125I]LY475776 and azidophenacyl-[35S]GSH. The extent of [125I]LY475776 labeling of Mrp1A1239T with [125I]LY475776 and azidophenacyl-[35S]GSH was slightly enhanced when compared with wild type mrp1 (Fig. 6A, left panel). These mutations neither affected the ability of MRP1 or mrp1 to transport LTC4 nor affected labeling by azidophenacyl-[35S]GSH (Fig. 6A, right panel) (28).

To further investigate the labeling characteristics of LY475776, we carried out photolabeling experiments with mrp1 and MRP1 mutant proteins in which we had exchanged corresponding non-conserved amino acids in TM14, Thr-1242 in MRP1 to Ala as in mrp1 (Fig. 6B, left panel). These residues have little effect on LTC4 transport (30, 52). Conversion of Thr-1242 in MRP1 to Ala as in mrp1, significantly decreased the extent of [125I]LY475776 labeling of the protein, and the reciprocal mutation slightly increased labeling of the murine protein (Fig 6B, left panel). In contrast, when the same preparations of HEK membranes were photolabeled with azidophenacyl-[35S]GSH, the extent of labeling of T1242A Mrp1 was higher than that of the wild type protein. However, the relative levels of labeling paralleled the expression of the wild type and mutant proteins, as detected by Western blotting.

**FIG. 5.** Effect of LY475776 on LTC4 transport by MRP1 and murine mrp1 and photolabeling of membrane vesicles containing wild type MRP1 or mrp1 and various hybrid proteins. A, membrane vesicles were prepared from stably transfected HEK cells expressing MRP1 or mrp1, and the ATP-dependent uptake of [3H]LTC4 was determined after 1 min at 22 °C. The assay was repeated in the presence of various concentrations of LY475776 and in the absence (■, MRP1; □, Mrp1) or presence (○, MRP1; △, mrp1) of 1 mM GSH. Shown is the [3H]LTC4 uptake in the presence of LY475776 expressed as a percent of uptake with no LY475776 addition. Data points are means (± S.D.) of triplicate determinations in a single experiment. B, upper panel, membrane vesicles were prepared from stably transfected HEK cells expressing MRP1, mrp1, and various hybrid proteins. An aliquot (4 μg) was subjected to SDS-PAGE electrophoresis using a 5–15% gradient gel and immunoblotted using the rat monoclonal antibody (MAb), MRPr-1, which reacts equivalently with MRP1 and Mrp1. lower panel, samples of the same preparations of membranes used for immunoblotting were photolabeled in the absence and presence of 1 mM GSH.

**FIG. 6.** Photolabeling of MRP1/mrp1 point mutations by [125I]LY475776 and azidophenacyl-[35S]GSH. Membrane vesicles were prepared from stably transfected HEK cells overexpressing MRP1 or mrp1 or proteins mutated at single amino acid residues in TM14 (MRP1S1233A, Mrp1S1233A and TM17 (MRP1T1242A, Mrp1A1239T, MRP1Y1243F, Mrp1A1239T, MRP1S1235A, Mrp1A1239T, MRP1S1235A, Mrp1A1239T, MRP1E1089Q, Mrp1Q1086E) and TM17 (MRP1T1241A, Mrp1A1239T, Mrp1Y1243F) and photolabeled with [125I]LY475776 or azidophenacyl-[35S]GSH. A, photolabeling of TM14 mutants Mrp1A1239T with [125I]LY475776 and azidophenacyl-[35S]GSH. B, photolabeling of TM17 mutants MRP1T1242A and mrp1A1239T with [125I]LY475776 and azidophenacyl-[35S]GSH.
Photolabeling of MRP1 by LY475776 and Azidophenacyl-GSH

DISCUSSION

Photolabeling studies of MRP1 using azido derivatives of IACI and IAARh123 followed by partial proteolysis have identified two preferential sites of cross-linking involving peptides containing TM helices 10 and 11 or TM 16 and 17 (31–33). Our previous photolabeling studies of MRP1 have shown that LTC4, like IACI and IAARh123, labels sites in MSD2 and MSD3 (27).

In contrast to the results obtained with compounds that bind and photolabel MRP1 in a GSH-independent manner, the azido derivative of AG-A in the presence of GSH labels only the COOH-proximal half of the protein (34). AG-A competitively inhibits LTC4 uptake by MRP1-enriched membrane vesicles with a Ki of 30 μM (34). Whether or not AG-A is actually transported by the protein has not been established. Based on the profile of photolabeling observed with the azido derivative of AG-A, it has been proposed that the binding of GSH to a site in the NH2-half of MRP1, possibly CL3, enhances the binding of a second substrate, such as AG-A, to a site in MSD3 (34).

LY475776 is structurally unrelated to AG-A but also binds to MRP1 in a GSH-dependent manner (36). However, the EC50 for inhibition of LTC4 transport by MRP1 is ~20 nM, compared with a Ki of 30 μM for AG-A (53). LY475776 is also specific for MRP1 and does not photolabel the MRP1 homologues MRP2 and MRP3, or P-glycoprotein (P-gp)3,4. Recent proteolysis studies in which [125I]LY475776 has been used to photolabel MRP1 expressed in the multidrug resistant human small cell lung cancer cell line, H69AR, have revealed predominant labeling of a tryptic peptide containing TM helices 16 and 17 in addition to two minor fragments thought to contain TM helices 12 and 13 and 14 and 15 (36). Consistent with this observation, photolabeling studies with co-expressed baculovirus-encoded MRP1932 and MRP3932,1531 clearly indicate that the compound strongly and preferentially photolabels a site in the COOH-proximal fragment in a GSH-dependent manner. These studies also establish that co-expression with the NH2-proximal portion of the protein is a prerequisite for any photolabeling to occur. Thus, the pattern of labeling is similar to that reported for azido AG-A (34). However, using the co-expressed fragments of MRP1, we also detected GSH and S-methyl-GSH-dependent photolabeling of MRP1932. Although very weak relative to the labeling of the COOH-proximal site, dependence on the presence of GSH or a non-reducing analog such as S-methyl-GSH suggests that the binding is specific. If so, it implies that the NH2-proximal site is not simply a site to which GSH alone binds and facilitates the binding of a GSH-dependent substrate to a site in MSD3, as proposed (34).

We have attempted to gain more information about the binding characteristics of GSH by using the photoactivatable derivative, azidophenacyl-[35S]GSH. Azidophenacyl-GSH has been reported previously to photolabel intact MRP1 (50). However, its ability to substitute for GSH in stimulating transport of other MRP1 substrates had not been demonstrated. Previous studies have shown that a number of GSH derivatives including the alkyl derivatives, S-methyl and S-ethyl-GSH, and tripeptides lacking the cysteine sulfur atom can substitute for GSH in enhancing the transport of some substrates (14, 17).

Derivatives with longer alkyl chains fail to stimulate transport of GSH-dependent substrates and may inhibit transport of some GSH-independent substrates. Consequently, prior to using azidophenacyl-GSH for photolabeling studies, we first ascertained whether it could substitute functionally for GSH. Our studies show that the compound stimulates the transport of estrone sulfate and that it can also substitute for GSH in enhancing photolabeling of MRP1 by LY475776. Thus, azidophenacyl-GSH retains the ability to stimulate binding or transport of at least these two compounds. The photolabeling of intact MRP1 by azidophenacyl-[35S]GSH was also efficiently blocked by the glutathione-conjugated substrate, LTC4, indicating that the labeling observed was specific.

Our data demonstrate that the photolabeling profile of azidophenacyl-GSH is similar to that of LTC4 and that the compound labels sites in both halves of the protein (27). Although labeling of the two locations by both compounds requires the presence of CL3, neither photolabel this region. Consequently, it remains to be determined whether CL3 interacts directly with either GSH or the GSH moiety of LTC4, as proposed (36). CL3 may have a more general function in maintaining the architecture of the protein, because its removal abolishes trafficking of MRP1 in mammalian cells and eliminates binding and/or transport of GS-conjugates, GSH-dependent substrates and other conjugated substances such as E217βG (27, 34, 39, 54).

Despite the extreme GSH dependence of photolabeling of MRP1 by [125I]LY475776 and its preferential binding to the COOH-proximal site, no reciprocal enhancement of photolabeling by azidophenacyl-[35S]GSH was observed. In addition, we detected no stimulation of GSH transport by LY475776. These and other observations are consistent with the possibility that the interaction of GSH or a derivative with MRP1 is a prerequisite for binding of Ly465776. They also suggest that the binding of LY475776, unlike estrone 3-sulfate, does not cooperatively enhance the binding of GSH. Thus, LY475776 may bind with high affinity to a conformation of the protein induced by interaction of GSH with one or both sites photolabeled by azidophenacyl-GSH. The exceptionally high apparent affinity of LY475776 for MRP1 in the presence of GSH, and its lack of reciprocal stimulation of the binding of azidophenacyl-GSH, may indicate that it binds without inducing any further change in the conformation of the protein.

In contrast to LY475776, estrone sulfate can be transported by MRP1 in the absence of GSH, although relatively poorly, and thus alone can clearly bind to the protein (16). The presence of GSH decreases the Km and increases the Vmax for estrone sulfate transport 3–4-fold (16). Studies presented here using dual-expressed half-molecules indicate that photolabeling by azidophenacyl-[35S]GSH, particularly of the COOH-proximal site, is enhanced in the presence of estrone sulfate. We have shown previously that S-methyl-GSH preferentially inhibits LTC4 labeling of the NH2-proximal site in MRP1, whereas low concentrations weakly stimulate labeling of the COOH-proximal site (27). In addition, low concentrations of estrone sulfate also stimulate LTC4 transport (16). These observations suggest that initial binding of GSH or a substrate such as estrone sulfate to the NH2-proximal site facilitates binding to the COOH-proximal site. The fact that trapping of the protein in a transition state selectively decreases binding of LTC4 to the NH2-proximal site is also consistent with this suggestion (27).

We have established that among MRP homologs LY475776 is highly specific for MRP1.2,3 Studies described here reveal that the compound also binds with 5–6-fold higher affinity to MRP1 when compared with its highly conserved murine ortholog, and
that labeling was enhanced in hybrid proteins containing all or part of MSD3 from the human protein. These findings are consistent with very recent partial trypsinolysis studies indicating that LY475776 preferentially photolabels a proteolytic fragment encompassing TM helices 16 and 17 (36). Weak labeling of two additional peptides was also detected in these studies. Based on their size, the fragments may contain TM helices 12 and 13 and 14 and 15. Studies with MRPI/mrp1 hybrid proteins suggest that the latter region, although not strongly photolabeled, is important for high affinity binding of LY475776. Previously, we have shown that a non-conserved amino acid in this region, Glu-1089, in predicted TM14 of MRPI is essential for the ability of the human protein to confer anthracycline resistance (28). This residue, which is replaced by glutamine in mrp1, is also required for high affinity binding of LY475776. Comparative studies of MRPI and murine mrp1 have also identified a second non-conserved residue in TM17, Thr-1242 in MRPI, that is required for efficient transport of E217βG (30). Mutation of Thr-1242 to Ala, as found at the corresponding position in murine mrp1, decreases the ability to transport E217βG and to confer drug resistance, but does not affect LTC4 transport. This mutation in the human protein also decreases photolabeling by LY475776, but as observed with the mutation in TM14, has no effect on photolabeling by azido-decylglycine (27). Thus, binding of LY475776 shares a requirement for some residues that are involved in the binding and or transport of chemotherapeutic drugs and some conjugated steroids, but which appear not to be involved in the binding of the two GSH conjugates, LTC4 and azido-decylglycine. Whether there is a specific subset of residues involved in the binding of GSH-conjugates that is distinct from those that contribute to the binding of other conjugated and non-conjugated substrates is not yet known.

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