

GSH Inhibits Trypsinization of the C-terminal Half of Human MRP1*

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MRP1 is a 190-kDa membrane glycoprotein that confers multidrug resistance to tumor cells. The accumulated evidence has proved that GSH interacts with MRP1 and stimulates drug transport. However, the mechanism of GSH-dependent drug transport by MRP1 remains unclear. In this study, we used limited tryptic digestion of MRP1 in isolated membrane vesicles, in the presence and absence of GSH, to investigate the influence of GSH on MRP1 conformation. We found that GSH inhibited the generation of an ~35-kDa C-terminal tryptic fragment (including a C-terminal His tag) termed C2 from MRP1. This effect of GSH was not because of direct inhibition of trypsin activity, and agosterol A enhanced the inhibitory effect of GSH. The main cleavage site in MRP1 for the generation of the C2 fragment by trypsin resided between TMD2 and NBD2 of MRP1. Limited tryptic digestion of membrane vesicles expressing various truncated and co-expressed MRP1 fragments in the presence and absence of GSH revealed that GSH inhibited the production of the C2 fragment only in the presence of the L₀ region of MRP1. Thus the L₀ region is required for the inhibition of trypsinization of the C-terminal half of MRP1 by GSH. These findings, together with previous reports, suggest that GSH induces a conformational change at a site within the MRP1 that is indispensable for the interaction of MRP1 with its substrates.

MDR¹ is the major obstacle to successful cancer chemotherapy and is mediated by membrane proteins whose mechanism of action is not yet completely understood (1). MRP1 (multidrug

resistance protein 1) is a 190-kDa glycoprotein that belongs to a family of membrane proteins referred to as ATP-binding cassette (ABC) transporters that typically contain two transmembrane domains (TMD) and two nucleotide binding domains (NBD) (4). In most ABC transporters, hydrolysis of ATP by the NBDs is believed to provide energy for substrate transport (4). MRP1 is distinguished from the other ABC transporter, P-glycoprotein (P-gp), by an extra N-terminal TMD that is connected to the core region (Δ MRP) by a cytoplasmic linker region (L₀) (5). The human MRP1 is frequently overexpressed in cells whose MDR is not mediated by P-gp (2, 3). As an organic anion transporter, MRP1 actively transports a wide variety of diverse anionic compounds (6–8). It was also found that although GSH is a poor substrate for MRP1, it can stimulate the ATP-dependent transport of certain non-anionic organic drugs such as vincristine (VCR) (9), adriamycin (ADM) (10, 11), and aflatoxin B₁ (12) as well as certain endogenous hydrophilic anionic conjugates such as estrone 3-sulfate (13) and a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (14).

Loe *et al.* (9) have shown that GSH is required for vesicular transport of VCR by MRP1 and for inhibition by VCR of the transport of organic anions by MRP1. ATP-dependent uptake of [³H]GSH into membrane vesicles from MRP1-transfected cells was stimulated by VCR in a dose-dependent manner (9). This experiment, however, did not answer the question of whether GSH is an activator or a co-transported substrate of MRP1.

To clarify the role of GSH in MRP1 drug transport, we synthesized a photoaffinity analog of AG-A (azido-AG-A) that could reverse MRP1-mediated MDR (15). We reported recently (16) that GSH was required for the binding of azido-AG-A to the C-terminal half of MRP1. A similar conclusion was reached in another study by using a drug known as LY475776 (17). However, the mechanism of the stimulation of azido-AG-A binding to MRP1 by GSH remains unclear. Recently, it has been reported that a photoactive analog of GSH (azidophenacyl-[³⁵S]GSH) photolabels MRP1 at two sites that reside in the N- and C-terminal halves of the protein. No stimulation of photolabeling of MRP1 with azidophenacyl-[³⁵S]GSH could be detected following treatment with varying concentrations of LY475776 (18). These observations suggested that the first event in GSH-dependent drug transport is the interaction of GSH with MRP1. GSH may then induce a conformational change in MRP1 that is required for drug binding.

Ligand-induced conformational change is a common mechanism for the modulation of protein function (19). For example a cyclic pentapeptide ligand can induce a conformational change

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¹ The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; MRP1, the human multidrug resistance protein; ABC transporter, ATP-binding cassette transporter; TMD, transmembrane domain; NBD, nucleotide-binding domain; GSH, glutathione; VCR, vincristine; ADM, adriamycin; LTC₄, leukotriene C₄; AG-A, Agosterol A; BAEE, N-(α -benzoyl)-L-arginine ethyl ester; mAb, monoclonal antibody; DTT, dithiothreitol.

We next investigated whether the inhibitory effect of GSH on trypsin digestion of MRP1 might be due to a decreased enzymatic activity of trypsin in the presence of GSH. We therefore assayed the effect of GSH on trypsin activity by using BAEE as a substrate. As controls, we also tested the effect of other reducing agents such as cysteine and DTT, the nonreducing GSH analogs methyl-GSH and ethyl-GSH, and glycine and glutamic acids (the amino acids that constitute GSH) on trypsin activity. The mean of three independent experiments was analyzed. As shown in Table I, agents with reducing activities do inhibit trypsin activity. GSH, cysteine, and DTT at a concentration of 10 mM decreased trypsin activity by 15.5, 16.8,

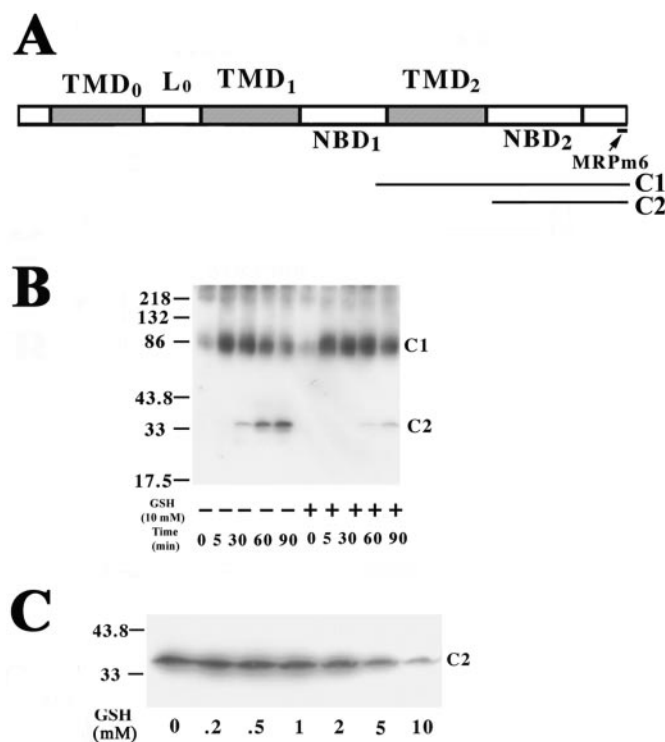


FIG. 1. GSH inhibits the generation of a C-terminal tryptic fragment of MRP1. A, schematic diagram depicting the localization of the TMD, NBD, and L subdomains of MRP1; the epitope recognized by the m6 mAb, and the tryptic fragments of 67 (C1) and 35 kDa (C2). B, KB/MRP membrane vesicles (50 μ g of protein) were digested with trypsin at 20 ng/ml for 0–90 min in the presence (+) or absence (–) of 10 mM GSH. Trypsinization was terminated by the addition of soybean trypsin inhibitor at a final concentration of 100 μ g/ml. Tryptic fragments were separated on 8.5% SDS-PAGE and immunoblotted with the C-terminal antibody m6. C, KB/MRP membrane vesicles (50 μ g of protein) were digested with trypsin at 20 ng/ml for 90 min in the absence or presence of GSH concentrations ranging from 0.2 to 10 mM and immunoblotted with m6. C1 indicates the 67-kDa C-terminal region of MRP1. C2 indicates the C-terminal tryptic fragment whose generation is suppressed by GSH. Molecular weight markers are shown at left.

TABLE I
Modulation of trypsin activity by GSH and other agents

Trypsin activity was assayed using BAEE as substrate, as described under "Experimental Procedures," in the absence (control) and in the presence of various agents. Residual trypsin activity (mean of three experiments \pm S.E.) was calculated as described under "Experimental Procedures."

Agents	Residual activity
	% control
Control	100
50 mM GSH	53.5 \pm 6.8
10 mM GSH	84.5 \pm 3.5
2 mM GSH	95.4 \pm 1.3
10 mM <i>S</i> -methyl GSH	96.3 \pm 3.9
10 mM <i>S</i> -ethyl GSH	100.7 \pm 4.9
10 mM glycine	98.1 \pm 0.9
10 mM glutamic acid	100.7 \pm 0.5
10 mM cysteine	83.2 \pm 0.3
10 mM DTT	34.6 \pm 5.6
1 mM AG-A	105.3 \pm 2.9
1 mM VCR	95.7 \pm 6.0

and 65.4%, respectively. In contrast, methyl-GSH at a concentration of 10 mM decreased trypsin activity by only 3.7%, and ethyl-GSH at a concentration of 10 mM had no effect on trypsin activity. We therefore determined whether 10 mM methyl-GSH or 10 mM ethyl-GSH could also suppress the generation of the C2 tryptic fragment of MRP1. As shown in Fig. 2A, both meth-

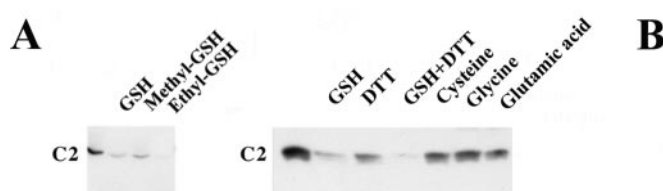


FIG. 2. Modulation of tryptic digestion of MRP1 by GSH and other agents. KB/MRP membrane vesicles (50 μ g of protein) were digested with trypsin at 20 ng/ml for 60 min in the absence (control) or presence of 10 mM GSH, 10 mM methyl-GSH, or 10 mM ethyl-GSH (A) or 10 mM DTT, 10 mM GSH + 10 mM DTT, 10 mM glycine, 10 mM cysteine, or 10 mM glutamic acid (B) and immunoblotted with m6. The C2 tryptic digestion fragment is indicated.

yl- and ethyl-GSH inhibited the generation of the C2 tryptic fragment to a similar extent as GSH. In contrast, 10 mM DTT had a lesser effect on the generation of the C2 fragment, even though it inhibited trypsin activity to a greater extent than the GSH derivatives. Similarly the addition of cysteine, glycine, or glutamic acid, the amino acids that constitute GSH, had a much weaker effect on the generation of the C2 fragment by trypsin, even though cysteine could inhibit trypsin activity to a similar extent as GSH (Fig. 2B).

These data are consistent with our previous observation that GSH stimulates azido-AG-A photolabeling of MRP1 independently of its reducing activity (16). They also indicate that GSH inhibition of the generation of the C2 tryptic fragment is not due to the direct inhibition of trypsin activity. The most likely explanation is that the binding of GSH to MRP1 may inhibit access of trypsin to MRP1 and the consequent cleavage reaction leading to generation of the C2 band.

We have found previously (30) that GSH was required for the binding of a photoactive analog of AG-A to the C-terminal half of MRP1, and that amino acids 1223–1295 of MRP1 were required for GSH-dependent binding of AG-A to MRP1. Based on the molecular size of the C2 fragment, the cleavage site that leads to the generation of C2 must be close to this region. Therefore, we investigated whether the VCR that is transported by MRP1 in the presence of GSH or AG-A might affect the generation of the C2 fragment. Limited trypsinization of MRP1 was conducted in the presence of the drugs alone or in combination with GSH, and the generation of the C2 fragment was monitored. As indicated in Fig. 3A, the addition of AG-A or VCR alone had no effect on the generation of the C2 fragment by trypsin. However, when AG-A was added in combination with GSH, AG-A considerably attenuated the generation of the C2 fragment (Fig. 3A). The effect of various concentrations of AG-A, ranging from 10 μ M to 1 mM, on the generation of the C2 band in the presence of 2 mM GSH was investigated (Fig. 3B). Increasing AG-A concentrations enhanced the suppressive effect of 2 mM GSH on the tryptic generation of the C2 fragment. Because AG-A does not inhibit trypsin activity as shown in Table I, AG-A must enhance the suppressive effect of GSH by a different mechanism.

The GSH-sensitive Tryptic Site Is Located in the Cytoplasmic Region of MRP1 between TMD₂ and NBD₂—We next investigated the location in MRP1 of the cleavage site that generates the C2 fragment. Because C2 has a molecular mass of about 35 kDa in SDS-PAGE, the cleavage site that generates the C2 fragment should be located about 350 amino acids upstream of the C-terminal end (1531) of MRP1. This would localize the C2 tryptic site within TMD₂ of MRP1. If this is the case, then the C2 fragment should be associated with the membrane fraction. We therefore investigated the potential membrane association of the cleaved C2 fragment. KB/MRP membrane vesicles were digested with trypsin at 20 ng/ml for 30 min and then centri-

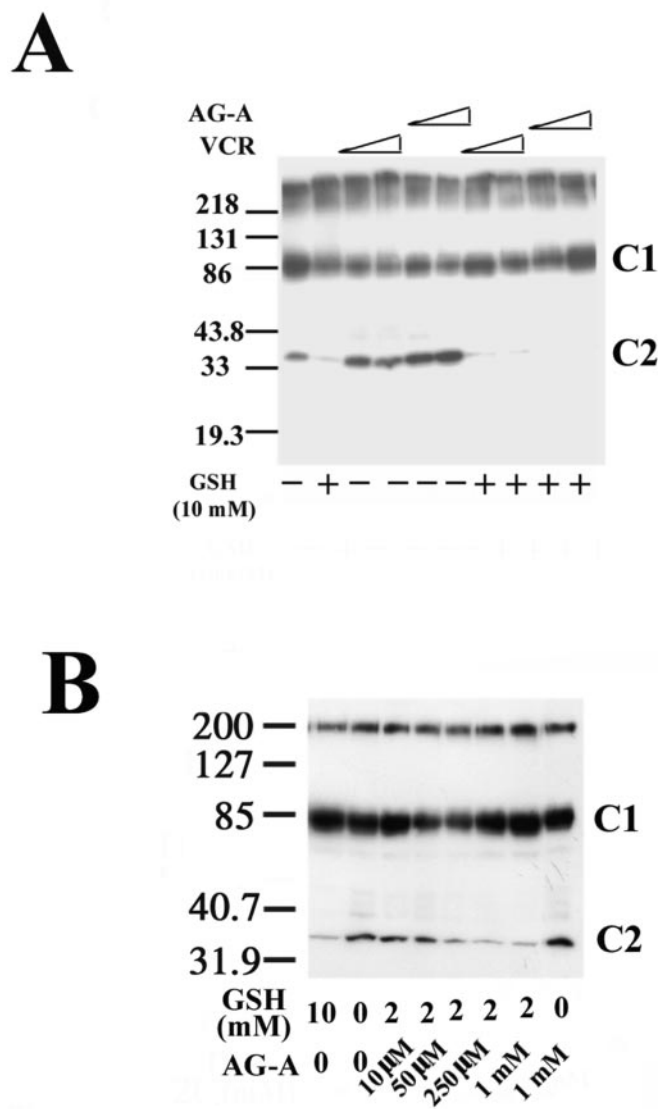


FIG. 3. AG-A enhances the GSH inhibition of the generation of the C2 tryptic fragment. Limited trypsinization of KB/MRP membrane vesicles was carried out as described in the legend to Fig. 1 in the absence (–) or presence (+) of 10 mM GSH supplemented with VCR or AG-A as indicated (A). The effect of 10 mM GSH or of 2 mM GSH together with increasing concentrations of AG-A ranging from 10 μ M to 1 mM on the generation of the C2 fragment are shown in B. C2 fragments were detected by immunoblotting with the m6 mAb. Molecular weight markers are shown at left.

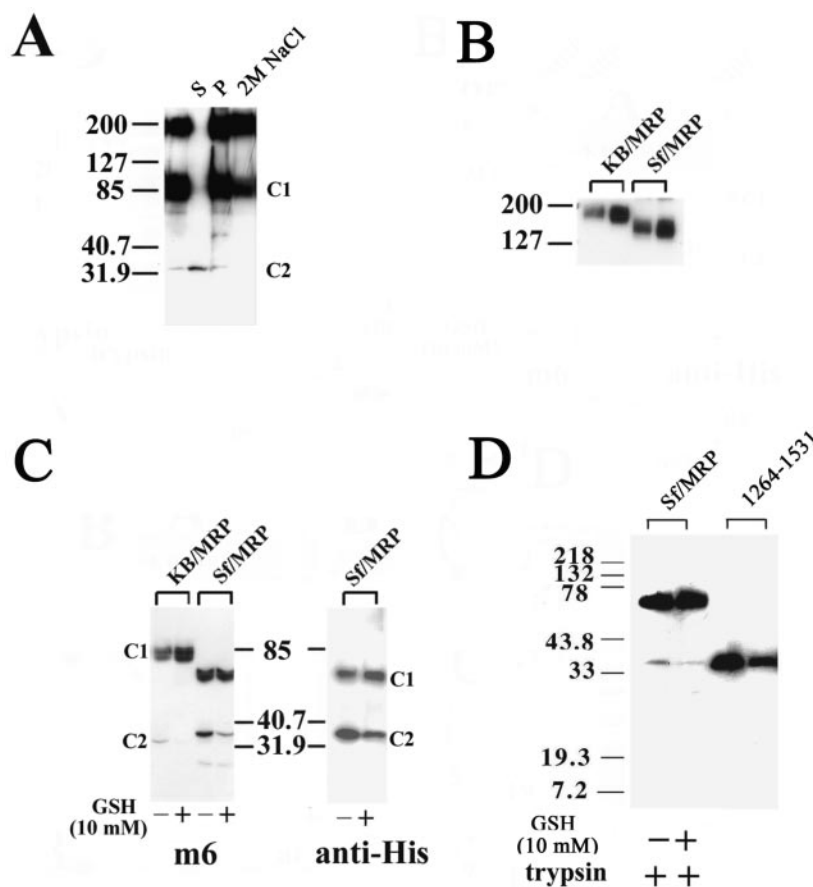
fuged to separate the membrane and soluble fractions. As indicated in Fig. 4A, most of the C2 tryptic fragments were detected in the soluble fraction, and the residual membrane-bound C2 fragments were released from the membrane by washing with the high concentration of NaCl, indicating that the tryptic site suppressed by GSH must be located in the cytoplasmic region of MRP1 between TMD₂ and NBD₂.

To localize more exactly the cleavage site that generates the C2 fragment, we made use of a C-terminally His-tagged MRP1 expressed in Sf21 insect cells. This expression system allows dissection of the functional domains of MRP1. We have shown previously that a 10xHis tag inserted into the C terminus of MRP1 has no effect on the function of the transporter (26). We first determined the expression of the His-tagged MRP1 in insect cells. Because MRP1 in Sf21 insect cells is less glycosylated than in KB/MRP cells, it migrates as an ~170-kDa band (26) (Fig. 4B). We then determined whether trypsin digestion of insect-expressed MRP1 could generate a C2 fragment similar

to that generated in KB/MRP cells and, if so, whether this digestion was GSH-sensitive. KB/MRP and Sf/MRP membrane vesicles (50 μ g of protein) were therefore digested with trypsin at 20 ng/ml for 90 min in the presence or absence of 10 mM GSH. Tryptic fragments were immunoblotted with either the C-terminal antibody m6 or an anti-His tag mAb as appropriate. As shown in Fig. 4C, the C2 fragment was detected with both of these antibodies, although the size of the C2 band derived from Sf/MRP membrane vesicles was slightly larger than that from KB/MRP probably due to the presence of the 10xHis tag in the C terminus of MRP1. The generation of the C2 fragment in Sf/MRP membrane vesicles was also inhibited in the presence of 10 mM GSH (Fig. 4C). Thus the His-tagged MRP1 expressed in insect cells can be used as a model of GSH suppression of MRP tryptic digestion. Because amino acid sequencing of the C2 fragment was difficult (data not shown) and the C₂ fragment was not associated with membrane, the cleavage site probably resides within the cytoplasmic region close to the C terminus of TM17. We expressed a C-terminal fragment, MRP1-(1264–1531), containing a His tag in Sf21 insect cells. MRP1-(1264–1531) had an electrophoretic migration similar to that of the C2 tryptic fragment generated from Sf/MRP membrane vesicles (Fig. 4D). These data suggested that the main cleavage site in MRP1 for the generation of the C2 fragment resides between TMD₂ and NBD₂.

L₀ Region of MRP1 Is Required for Attenuation of the C2 Generation by GSH—It has been reported that the cytoplasmic region L₀ between TMD₀ and TMD₁ of MRP1 is important for MRP1 function (16, 31, 32). We have shown previously that L₀ is required for GSH-dependent binding of a photoanalogue of AG-A to the C-terminal half of the protein, and we have suggested that L₀ is the interaction site of GSH on MRP1 (16). In this study, we found that GSH inhibited the cleavage of MRP1 in a region between TMD₂ and NBD₂. To investigate whether the L₀ region is required for the GSH inhibition of generation of the C2 fragment by trypsin, a series of truncated MRP1 constructs and co-expressed MRP1 fragments transfected into Sf21 insect cells was examined. The construction and expression of these mutant proteins in insect cells was described in detail in our previous study (16). A schematic diagram of the constructs is shown in Fig. 5A, and the expression level of the various constructs is shown in Fig. 5B. Under the conditions used for the infection, the expression level of the various constructs was comparable either when they were expressed alone or co-expressed from a dual vector. Membrane vesicles were digested with trypsin in the presence and absence of GSH and immunoblotted with the MRP1 m6 antibody for detection of the C2 tryptic fragment. In membrane vesicles expressing C_{932–1531} or Δ MRP alone, C2 bands were generated by trypsin digestion, but their generation was not affected by GSH. In contrast, when C_{932–1531} and Δ MRP constructs were co-expressed with N_{1–932} and TMD₀L₀ fragments, respectively, the generation of the C2 fragment by trypsin was attenuated in the presence of GSH (Fig. 5C). These data indicate that TMD₀L₀ is required for GSH-mediated suppression of the generation of the C2 fragment by trypsin. To investigate the role of TMD₀ in GSH-mediated suppression, we created a construct, L₀ Δ MRP (amino acids 203–1531), that lacked the first transmembrane domain of MRP1. Membrane vesicles expressing L₀ Δ MRP were digested with trypsin in the presence and absence of GSH and immunoblotted with the anti-MRP antibody m6. GSH could only weakly inhibit the generation of the C2 fragment by trypsin from this construct, and the inhibition was less than that observed following incubation of GSH with wild type MRP1. These results suggested that both the TMD₀ and L₀ regions of MRP1 are required for GSH-induced suppression of the gener-

FIG. 4. The GSH-sensitive tryptic site is located in the cytoplasmic region between TMD₂ and NBD₂. A, KB/MRP membrane vesicles were digested with trypsin as described in the legend to Fig. 1. The trypsin reaction was stopped by the addition of soybean trypsin inhibitor at a final concentration of 100 μ g/ml. Membrane vesicles were then centrifuged for 30 min at $20,000 \times g$ at 4 °C. Soluble (S) and pellet (P) fractions, or pellet washed with 1 ml of cold stop solution containing 2 M NaCl, were separated on 8.5% SDS-PAGE and immunoblotted with the m6 antibody. B, MRP1 tagged with 10xHis was expressed in Sf21 insect cells. Membrane vesicles from KB/MRP and Sf/MRP cells (5 and 10 μ g protein, left and right lanes, respectively, of each sample) were separated on 7.5% SDS-PAGE and immunoblotted with the MRP1 mAb. C, Sf/MRP and KB/MRP membrane vesicles (50 μ g of protein) were digested with trypsin at 20 ng/ml for 60 min in the absence and presence of 10 mM GSH. Soybean trypsin inhibitor at a final concentration of 100 μ g/ml was employed to terminate the trypsin activity. Tryptic fragments were separated on 8.5% SDS-PAGE and immunoblotted with the m6 (left) or an anti-His monoclonal antibody (right). D, Sf/MRP membrane vesicles were digested with trypsin in the presence or absence of GSH as described above. The C2 tryptic fragment from Sf/MRP membrane vesicles, as well as the MRP1-(1264–1531) fragment purified from Sf21 insect cells by metal chromatography (1 μ g, right lane, and 2 μ g, left lane for each fragment), were separated on 11% SDS-PAGE and immunoblotted with the m6 antibody.



ation of the C2 fragment by trypsin. The accumulated evidence indicates that the tryptic cleavage site that generates the C2 fragment must be located in the cytoplasmic region of MRP1 between TMD₂ and NBD₂.

DISCUSSION

GSH stimulates ATP-dependent transport of various agents by MRP1 (9–14). The mechanism by which GSH enhances the transport of these agents is still unknown. It has been suggested that MRP1 co-transporters these compounds with GSH or that GSH in some way activates MRP1 thereby facilitating substrate binding and/or transport. Elucidation of the mechanism by which GSH facilitates drug transport would be useful for the design of drug strategies for anti-tumor therapy. We have synthesized previously a photoanalog of AG-A, and by using this analog, we found that GSH stimulates AG-A binding to the C-terminal half of MRP1 (16). LY475776, a photoanalog structurally unrelated to AG-A, also binds to the C-terminal half of MRP1 in a GSH-dependent manner (17, 18). However, the mechanism by which GSH enhances drug binding by MRP1 remains unclear. Azidophenacyl-[³⁵S]GSH stimulated LY475776 binding to the C-terminal half of MRP1 and photolabeled MRP1 at two sites that reside in the N- and C-terminal half of the protein. However, LY475776 could not stimulate azidophenacyl-[³⁵S]GSH photolabeling of MRP1. These results suggested that GSH binding to MRP1 precedes the drug binding (17, 18).

Induction of a conformational change in MRP1 by GSH has been suggested as a mechanism by which GSH might modulate MRP1 activity. Limited trypsin digestion is an approach that has been successfully used to investigate nucleotide-induced conformational changes of various transport ATPases (23, 33–35). Manciu *et al.* (36) demonstrated that GSH had a marked

protective effect against the digestion of MRP1 by trypsin. Manciu *et al.* (36) also observed a drastic modification of the accessibility of MRP1 toward the external environment and/or the stability of the conformational state of MRP1 in the presence of GSH by monitoring the amide hydrogen exchange. They also suggested that this conformational change affected the structural organization of the cytosolic domain of MRP1 (36). However, it is still not known which region of the MRP1 is directly altered by GSH.

In order to address this question, we used limited trypsinization of MRP1 in inside-out membrane vesicles as an assay to monitor potential GSH-induced conformational changes of MRP1 and to localize the region of MRP1 directly modulated by GSH. The addition of GSH suppressed the generation of an ~35-kDa C-terminal fragment (C2) by trypsin, which could be detected by an MRP1-specific C-terminal monoclonal antibody m6. Although trypsin activity can be inhibited in the presence of agents with reducing activity, GSH-induced inhibition of the generation of the C2 fragment by trypsin did not appear to be due to a GSH-induced decrease in trypsin activity for the following reasons. First, GSH derivatives without reducing activity such as *S*-methyl-GSH and *S*-ethyl-GSH had no effect on trypsin activity but did inhibit the generation of the C2 fragment by trypsin. Second, DTT, a reducing reagent that inhibited trypsin activity to a level four times lower than did a similar concentration of GSH, had little effect on the generation of the C2 fragment by trypsin. Third, generation of the C2 fragment by trypsin was not altered by the addition of GSH to membrane vesicles expressing the Δ MRP and C_{932–1531} constructs.

To localize the site in MRP1 directly affected by GSH binding, we examined the generation of the C2 fragment by trypsin

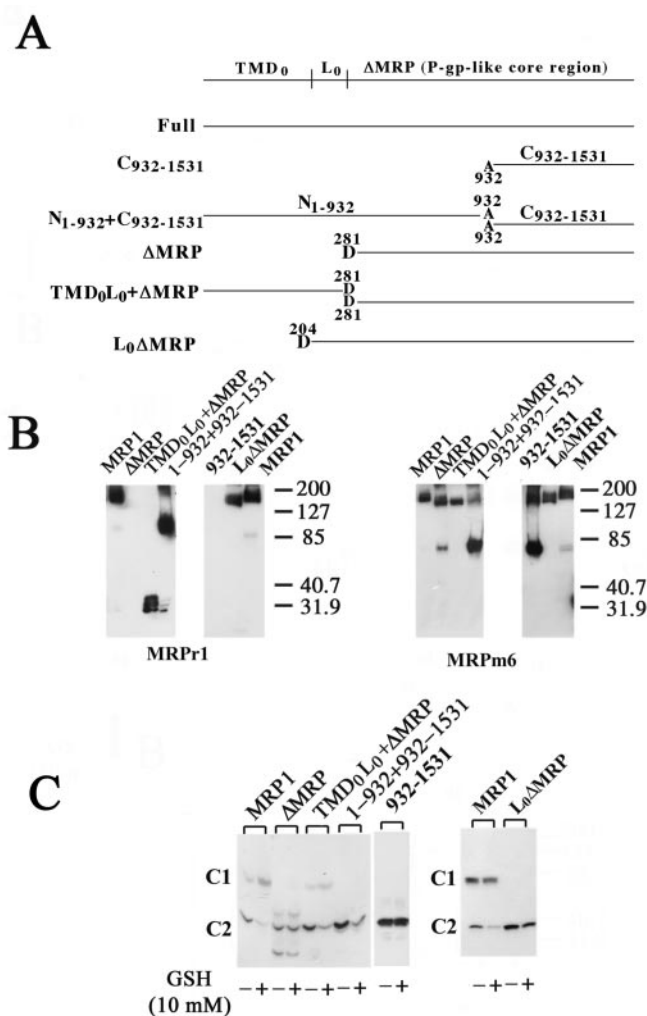


FIG. 5. The L₀ region of MRP1 is required for the GSH-induced attenuation of the generation of the C2 tryptic fragment. *A*, schematic diagram indicating the N-terminal domain (TMD₀) of MRP1 that is connected to the P-gp-like core by a cytoplasmic linker (L₀). Sf21-expressed MRP1 constructs are indicated. N₁₋₉₃₂ + C₉₃₂₋₁₅₃₁ and TMD₀L₀ + ΔMRP represent two fragments of MRP1 co-expressed by the dual expression vector. *B*, membrane vesicles were prepared from Sf21 insect cells infected with recombinant baculovirus encoding either full-length MRP1 or the indicated truncated or co-expressed MRP1 fragments. Membrane proteins (10 μg) from each sample were subjected to 8.5% SDS-PAGE and immunoblotted with the anti-N-terminal antibody r1 to detect MRP1, N₁₋₉₃₂, and TMD₀L₀ (left) or with the anti-C-terminal antibody m6 to detect MRP1, C₉₃₂₋₁₅₃₁, and ΔMRP (right). Molecular weight markers are shown at right. *C*, membrane vesicles (50 μg of protein) expressing various truncated and co-expressed MRP1 constructs as indicated were digested with trypsin in the absence or presence of 10 mM GSH, as described in the legend to Fig. 1, and immunoblotted with the m6 antibody.

using MRP1 sub-domains expressed singly or together in an insect expression system. We have reported previously that neither the core P-gp-like region of MRP1 (ΔMRP) nor the C-terminal half of MRP1 (MRP1-(932–1531)), when expressed alone, could transport LTC₄ or bind azido-AG-A in a GSH-dependent manner (16). However, in membrane vesicles dually expressing ΔMRP and TMD₀L₀ or N₁₋₉₃₂ and C₉₃₂₋₁₅₃₁, GSH-dependent binding of azido-AG-A to these reconstituted transporters was observed. In this study, we found that limited trypsinization of membrane vesicles expressing ΔMRP or C₉₃₂₋₁₅₃₁ alone generated the C2 fragment. However, even high concentrations of GSH were unable to inhibit the generation of the C2 fragment by trypsin from these constructs. On the other hand, in membrane vesicles co-expressing ΔMRP and TMD₀L₀

or N₁₋₉₃₂ and C₉₃₂₋₁₅₃₁, C2 generation by trypsin was reduced by GSH. In membrane vesicles expressing L₀ΔMRP, GSH inhibition of the generation of the C2 fragment by trypsin was much less than in membrane vesicles expressing wild type MRP1. Taken together with the previous finding that GSH might interact with the L₀ region of MRP1, these data suggested that the L₀ region of MRP1 is required for the GSH inhibition of generation of the C2 fragment by trypsin and that the TMD₀ region might enhance the function of L₀ in mediating this suppression (16).

GSH-induced suppression of tryptic cleavage is specific for the generation of the C2 tryptic fragment. In this study we used several approaches to determine exactly the localization of the cleavage site in MRP1 leading to the generation of the C2 tryptic fragment. Immunoblotting with specific antibodies to either the C-terminal end of MRP1 or the His tag of a C-terminal 10xHis-tagged MRP1 indicated that the C2 peptide represents the C-terminal tail of MRP1. Furthermore, the C2 fragment was not associated with the membrane fraction following limited trypsin treatment. These data suggested that the C2 fragment was derived from the C-terminal cytoplasmic region of MRP1. Based on the molecular size of the C2 fragment, this region would include the NBD₂ domain and would suggest that the cleavage site probably lies within the cytoplasmic region close to the C terminus of TM17 of MRP1.

Amino acid sequencing of the purified C2 fragment was difficult, probably because the C2 band in SDS-PAGE contained several different tryptic fragments. In fact, there are several basic amino acids, Arg¹²⁴⁹, Arg¹²⁶³, Lys¹²⁶⁵, Lys¹²⁷², and Arg¹²⁹², in the cytoplasmic region adjacent to the C terminus of the predicted TM17 that could serve as tryptic digestion sites. We had previously mutated Arg¹²⁴⁹ to Ala (32) and therefore tested this mutant for the generation of the C2 fragment following trypsin digestion. This mutation did not alter the generation of the C2 fragment (data not shown), suggesting that Arg¹²⁴⁹ is not the cleavage site in the C-terminal half of MRP1. The closest arginine to Arg¹²⁴⁹ in the cytoplasmic region between TM17 and NBD₂ is Arg¹²⁶³. We therefore expressed a C-terminal fragment, MRP1-(1264–1531) containing a 10xHis tag in Sf21 insect cells, and we determined its migration rate in SDS-PAGE compared with the C2 tryptic fragment. The migration rates were similar suggesting that Arg¹²⁶³ may be the main cleavage site in MRP1 responsible for the generation of the C2 fragment. The predicted molecular weight of the MRP1-(1264–1531) fragment is 30,022.25, whereas predicted molecular weight of the MRP1-(1264–1531) fragment containing the 10xHis tag is 31,393.66. This is in contrast to the molecular weights observed for the C2 fragments generated from KB/MRP, Sf/MRP, and for the MRP1-(1264–1531) fragment when analyzed by SDS-PAGE in which they migrate as a 35-, 36-, and 36-kDa band, respectively. The slower migration of these peptides on SDS-PAGE compared with the predicted molecular weight may perhaps be attributed to post-translational modifications (25).

We have found previously that a GSH-dependent drug-binding site may be located within the region encompassed by amino acids 1223–1295 in MRP1 (30). We therefore investigated whether the drug AG-A might affect the generation of the C2 fragment. We found that AG-A could inhibit the generation of the C2 tryptic fragment in the presence of GSH. Thus GSH-dependent binding of AG-A itself and/or a GSH-induced conformational change of the drug-binding site may have prevented the access of trypsin to the cleavage site. In a previous study by Manciu *et al.* (36), MRP1 was less efficiently digested by trypsin in the presence of both GSH and ADM or GSH and 3'-(3-methoxymorpholino)doxorubicin, but the digestion profile

was very similar to that obtained with GSH alone. They therefore concluded that only GSH affects the conformation of MRP1 (36). The discrepancy between their study and our data may perhaps be attributed to the difference in the drugs used and/or the systems used to detect the tryptic fragments. The previous study used silver staining to detect tryptic fragments. However in our study tryptic fragments were detected by the more sensitive method of immunoblot analysis with a specific monoclonal antibody against the C terminus of MRP1.

We cannot completely rule out the possibility that GSH might interact with the drug-binding site of MRP1 only in the presence of TMD₀L₀ or L₀, thereby inhibiting access of trypsin to the trypsin-sensitive site without inducing any conformational change in MRP1. However, our findings, together with the reports described previously, strongly suggest that GSH binding to MRP1 induces a conformational change that affects the structural organization of the drug-binding site and facilitates the binding of AG-A.

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