Structural Requirements for Functional Interaction of Glutathione Tripeptide Analogs with the Human Multidrug Resistance Protein 1 (MRP1)

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ABSTRACT

The human multidrug resistance protein 1 (MRP1) is a primary active transporter of reduced (GSH) and oxidized glutathione, as well as GSH-, glucuronate-, and sulfate-conjugated organic anions. In addition, the transport of certain MRP1 substrates is stimulated by the presence of GSH. To evaluate the structural features of GSH required for interaction with the protein, we investigated the ability of a series of GSH analogs to enhance GSH stimulatable transport of [3H]estrone 3-sulfate (E1SO4). We found that substitution of the γ-Glu residue with Gly, β-Asp, and α-Glu resulted in complete loss of transport stimulation. In contrast, substitution of Gly with Glu or β-Ala resulted in only a partial loss of stimulatory activity. E1SO4 transport activity surpassed GSH-stimulated levels in the presence of tripeptides in which Cys was substituted with the hydrophobic amino acids Leu, Phe, and homo-Phe. Moreover, polar substitutions of Cys did not enhance transport to the same extent as nonpolar substitutions of comparable size. γ-Glu-Leu-Gly was 1.6-fold more effective than GSH in stimulating E1SO4 uptake, and kinetic analysis indicated this was due to an increased Vmax. In addition, this tripeptide was shown to be a competitive inhibitor of apigenin-stimulated GSH transport (Ki value of 14 μM), confirming that it either interacts with the same site on MRP1 as GSH or that the binding of the two tripeptides is mutually exclusive. These data provide insight into the architecture of the GSH binding domain of MRP1.

GSH is a ubiquitous tripeptide (γ-Glu-Cys-Gly) that plays a critical role in many essential cellular processes (Hammond et al., 2001). Among the many functions of GSH is its central involvement in pathways that lead to the elimination of xenobiotics and certain endogenous metabolites. In this capacity, the key functional element of GSH is its nucleophilic cysteinyl thiol that can react with endogenous and exogenous compounds with electrophilic centers, resulting in the formation of a covalent bond. GSH conjugation can occur either spontaneously or through catalysis by a glutathione S-transferase (GST). Conjugation serves to increase the water solubility of the metabolite or xenobiotic, thus enhancing excretion, an important aspect of detoxification. The chemical structure of GSH is unusual with respect to the attachment of the Glu residue through its γ-COOH group to Cys (Fig.1).

This γ-COOH linkage substantially enhances the stability of GSH by increasing its resistance to cleavage by endogenous cellular peptidases (Lucente et al., 1998).

The human 190-kDa multidrug resistance protein MRP1 (gene symbol ABCC1) is a member of subfamily “C” of the ATP-binding cassette superfamily of transport proteins and was originally identified on the basis of its elevated expression in multidrug-resistant lung cancer cells (Cole et al., 1992; Leslie et al., 2001a). In transfected cell lines, MRP1 confers resistance to a broad range of natural product drugs as well as the folic acid antimetabolite methotrexate, and certain arsenic and antimony oxyanions (Cole et al., 1994; Grant et al., 1994; Chen et al., 1999; Hipfner et al., 1999; Hooijberg et al., 1999). In addition to its ability to confer drug resistance in tumor cells, MRP1 is a primary active transporter of GSH and glutathione disulfide, as well as GSH-, glucuronate-, and sulfate-conjugated organic anions of physiological and toxicological relevance (Hipfner et al., 1999; Paulusma et al., 1999; Leslie et al., 2001a). Potential physiological substrates include a mediator of inflammation, leukotriene C4, and the estrogen conjugate E1SO4 (Leier et al., 1994; Loe et al., 1996; Qian et al., 2001). Substrates of toxicological importance include the endo and exo GSH conjugates of...
the mycotoxin aflatoxin B\(_1\) and the GSH conjugate of the lipid peroxidation product 4-hydroxy trans-2-nonenal (Loe et al., 1997; Renes et al., 2000). Certain unmodified xenobiotics such as vincristine and aflatoxin B\(_1\) are also transported by MRP1 but their transport requires the presence of GSH (Versantvoort et al., 1995; Zaman et al., 1995; Loe et al., 1996, 1997, 1998; Rappa et al., 1997). GSH has also recently been reported to enhance or be required for MRP1-mediated transport of the conjugated steroid E\(_1\)SO\(_4\), the \(\beta\)-O-glucuronide of the carcino- genic 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and the glucuronide conjugate of the anticancer agent etoposide (Sakamoto et al., 1999; Leslie et al., 2001b; Qian et al., 2001). There is convincing evidence that GSH is cotransported with unmodified compounds, whereas this does not seem to be the case for at least two conjugated MRP1 substrates, E\(_1\)SO\(_4\) and NNAL-O-glucuronide.

In addition to enhancing the transport of some MRP1 substrates, GSH increases the potency of several compounds to inhibit conjugated organic anion transport, despite the fact that GSH alone is a poor inhibitor (Leslie et al., 2001c). For example, vincristine and the Ca\(^{2+}\) channel antagonist verapamil are poor inhibitors of MRP1-mediated leukotriene C\(_4\) transport alone but in the presence of GSH, their inhibitory potency is increased more than 20-fold (Loe et al., 1996, 1998, 2000). Furthermore, it has been demonstrated that the binding of two MRP1-specific modulating agents, agosterol A and LY475776, is dependent on GSH (Ren et al., 2001; Mao et al., 2002). Interestingly, xenobiotics such as verapamil and several dietary flavonoids (including the flavone apigenin), stimulate GSH transport by MRP1 up to 4-fold without being transported themselves (Loe et al., 2000; Leslie et al., 2001c, 2003). Overall, the interaction between MRP1 and GSH is complex and presently is not well understood. However, it has been proposed that MRP1 contains at least one bipartite, if not multipartite, binding pocket to accommodate the hydrophobic and hydrophilic moieties of conjugated and unconjugated MRP1 substrates in combination with GSH (Loe et al., 1996; Heijn et al., 1997; Borst et al., 2000; Evers et al., 2000; Ito et al., 2001).

We have recently reported that ophthalmic acid, an endo- genously formed GSH analog that contains an \(\alpha\)-aminobutyrate (Abu) residue in place of cysteine, can support the transport of NNAL-O-glucuronide almost as well as GSH (Leslie et al., 2001b). Moreover, ophthalmic acid is as effective as GSH in supporting the photolabeling of the protein by the MRP1-specific modulator LY475776 (Mao et al., 2002). Thus, in contrast to GSH conjugation to electrophilic substrates by GSTs, the presence of a cysteinyl thiol group is not required for GSH-stimulated substrate transport by MRP1 or for the action of GSH-dependent MRP1 modulators. In the present study, we have extended our investigations to identify the structural features of GSH that enable it to modulate MRP1 transport activity. Thus, we have measured the ability of a series of GSH analogs and derivatives to substitute for GSH in enhancing the transport of \(^{3}\text{H}\)E\(_1\)SO\(_4\) as a model GSH-stimulatable substrate of MRP1.

**Materials and Methods**

**Materials.** \([6,7-^3\text{H}]\)Estrone sulfate (43 Ci mmol\(^{-1}\)) and [glycine 2-\(^2\text{H}\)]GSH (50 Ci mmol\(^{-1}\)) were purchased from PerkinElmer Life
Laval, QC, Canada). The tripeptides α-Glu-Cys-Gly, β-Asp-Cys-Gly, γ-Glu-Ala-Gly, γ-Glu-Val-Gly, γ-Glu-Leu-Gly, γ-Glu-Ser-Gly, γ-Glu-Thr-Gly, γ-Glu-Phe-Gly, γ-Glu-Met-Gly, γ-Glu-Tyr-Gly, γ-Glu-Trp-Gly, γ-Glu-benzyl-Cys-Gly (S-benzyl-GSH), and γ-Glu-homo-Phe-Gly were custom synthesized by Queen’s University Peptide Synthesis Laboratory (Kingston, ON, Canada). The purity of the peptides was confirmed to be ≥95% by high-performance liquid chromatography and their identities were confirmed by matrix-assisted laser desorption ionization mass spectroscopy. γ-Glu-α-Abu-Gly (ophthalamic acid), γ-Glu-Gly-Gly, γ-Glu-Cys-β-Ala ( homo-GSH), and Gly-Cys-Gly were from Bachem California (Torrance, CA). E1SO₄, GSH, and Gly-Cys-Gly were from Sigma Diagnostics (St. Louis, MO), and Gly-Cys-Gly was from Bachem California (Mississauga, ON, Canada). The disulfide of γ-Glu-Cys-Glu was precluded by a 10-fold molar excess of DTT at 21°C for 30 min before its addition to the transport assay described below. These tripeptides and their molecular volumes (obtained using the View-CHROMAT software) are listed in Table 1.

Membrane Vesicle Preparation and Immunoblotting. Membrane vesicles were prepared as described previously (Loe et al., 2006). Briefly, cells were homogenized in buffer containing 250 mM sucrose/10 mM Tris, pH 7.4, and EDTA added to 1 mM. The suspension was centrifuged at 8000 g for 1 h, the interface was removed and then resuspended by vigorous syringing with a 27-gauge needle. Protein concentration was determined using a Bradford assay (Bio-Rad, Mississauga, ON, Canada), and aliquots of membrane vesicles were stored at −70°C. Relative levels of MRP1 protein in membrane vesicles were determined by immunoblot analysis as described previously, with the human MRP1-specific monoclonal antibody QCR1-1 (Hipfner et al., 1996).

<table>
<thead>
<tr>
<th>Substituted Amino Acid</th>
<th>Tripeptide</th>
<th>Molecular Volume</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>γ-Glu-Gly-Cys-Gly (GSH)</td>
<td>212</td>
</tr>
<tr>
<td>α-Glu</td>
<td>Gly-Cys-Gly</td>
<td>212</td>
</tr>
<tr>
<td>β-Asp-Cys-Gly</td>
<td>199</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>γ-Glu-Cys-β-Ala</td>
<td>225</td>
</tr>
<tr>
<td>γ-Glu-Cys-α-Ala</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>γ-Glu-Abu-Gly</td>
<td>206</td>
</tr>
<tr>
<td>γ-Glu-Gly</td>
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</tr>
<tr>
<td>γ-Glu-Ala</td>
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<tr>
<td>γ-Glu-Val</td>
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<td>γ-Glu-Leu</td>
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<tr>
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</tr>
<tr>
<td>γ-Glu-Thr</td>
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<tr>
<td>γ-Glu-Met</td>
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<td></td>
</tr>
<tr>
<td>S-methyl GSH</td>
<td>228</td>
<td></td>
</tr>
<tr>
<td>S-ethyl GSH</td>
<td>238</td>
<td></td>
</tr>
<tr>
<td>γ-Glu-benzyl-Cys-Gly (S-benzyl-GSH)</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>γ-Glu-homo-Phe-Gly</td>
<td>261</td>
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<tr>
<td>γ-Glu-benzyl-Cys-Gly (S-benzyl-GSH)</td>
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<td></td>
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<tr>
<td>S-(p-Azidophenacyl)-GSH</td>
<td>319</td>
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</table>

Results

Effect of γ-Glu Substitutions on GSH-Stimulated [3H]E1SO₄ Uptake by MRP1. To determine whether the γ-Glu residue in GSH (γ-Glu-Cys-Gly) was important for the ability of the tripeptide to enhance E1SO₄ uptake by MRP1, uptake was measured using membrane vesicles prepared from MRP1-transfected HeLa cells in the presence of three GSH analogs containing a modified γ-Glu residue. Consistent with previous reports, GSH (1 mM) stimulated E1SO₄ uptake approximately 6- to 7-fold compared with basal uptake in the absence of this tripeptide (Figs. 2, 4, and 5) (Qian et al., 2001; Conrad et al., 2002). Shortening the γ-Glu side chain or removing it altogether by substituting γ-Glu with β-Asp and Gly, respectively, resulted in tripeptides with side chain molecular volumes 94 and 76% that of GSH (Table 1). However, neither tripeptide stimulated E1SO₄ uptake to levels above...
Effect of GSH Derivatives on \[^{3}H\]E1SO\(_4\) Uptake by MRP1. To investigate the importance of the Gly residue for GSH interaction with MRP1, \[^{3}H\]E1SO\(_4\) uptake was measured in the presence of \(\gamma\)-Glu-Cys-\(\beta\)-Ala and the oxidized and reduced forms of \(\gamma\)-Glu-Cys-\(\alpha\)-Glu. Levels of E\(_1\)SO\(_4\) uptake were approximately 80% of those observed in the presence of GSH when Gly was substituted with \(\beta\)-Ala (\(\gamma\)-Glu-Cys-\(\beta\)-Ala) (molecular volume 225 Å\(^3\)), an amino acid derivative that has one more methylene group than Gly, which increases the molecular volume of this side chain from 3 to 15 Å\(^3\) (Fig. 2B). The bulky \(\gamma\)-Glu-Cys-\(\alpha\)-Glu was tested in its oxidized/disulfide form and also after it had been reduced with DTT. The reduced form stimulated uptake to levels that were approximately 30% of those in the presence of GSH, whereas oxidized \(\gamma\)-Glu-Cys-\(\alpha\)-Glu had no stimulating effect at all (Fig. 2B). This observation suggests that at least in the presence of E\(_1\)SO\(_4\), there is a limit to the bulk of the side chain at the Gly position of GSH that can be accommodated by the GSH binding site of MRP1.

Effect of Nonaromatic and Nonsulfur-Containing Cys Substitutions on GSH-Stimulated \[^{3}H\]E1SO\(_4\) Uptake by MRP1. We have previously reported that ophthalmic acid, an analog of GSH-containing Abu (side chain molecular volume 28 Å\(^3\)), instead of Cys (side chain molecular volume 36 Å\(^3\)), can support the transport of NNAL-G-glucuronide (Leslie et al., 2001b) and photolabeling by LY475776 (Mao et al., 2002), demonstrating that the thiol group of the Cys residue is not required for the effective interaction of GSH with MRP1. We now show that ophthalmic acid also stimulates E\(_1\)SO\(_4\) transport to levels that are approximately 90% of those observed in the presence of GSH (Fig. 4A). Other GSH analogs containing nonaromatic nonsulfur-containing substituents in place of Cys (Fig. 3A) also stimulated \[^{3}H\]E1SO\(_4\) uptake but with differing abilities. Reducing the Abu by a single methylene group through substitution with Ala, which has a side chain of molecular volume less than 50% of Cys, still resulted in a peptide (\(\gamma\)-Glu-Ala-Gly) capable of supporting E\(_1\)SO\(_4\) with an uptake activity approximately 65% that of GSH. Complete elimination of the side chain through substitution of Cys with Gly (\(\gamma\)-Glu-Gly-Gly) reduced E\(_1\)SO\(_4\) transport stimulatory activity by approximately half compared with GSH (Fig. 4A).

E\(_1\)SO\(_4\) uptake levels in the presence of tripeptides that contained Cys substitutions with amino acids of increasing hydrophobicity were comparable with or greater than GSH-stimulated uptake levels. Thus, transport in the presence of peptides in which Cys was substituted with Val (side chain molecular volume 42 Å\(^3\)) and the larger Leu (side chain molecular volume 54 Å\(^3\)) was approximately 100 and 160%, respectively, of that observed in the presence of GSH. In contrast, \[^{3}H\]E\(_1\)SO\(_4\) transport uptake was reduced to approximately 40 and 60%, of GSH levels in the presence of tripeptides in which the Cys residue of GSH was replaced with Ser (side chain molecular volume 23 Å\(^3\)) (\(\gamma\)-Glu-Ser-Gly) and Thr (side chain molecular volume 37 Å\(^3\)) (\(\gamma\)-Glu-Thr-Gly), respectively, both of which are more hydrophilic residues with side chains of comparable molecular volumes to Cys (Fig. 4A).

These results show that both molecular volume and hydrophobicity of the amino acid side chain in the Cys position of GSH are important for interaction with the GSH binding pocket of MRP1 because there was a positive correlation...
between these properties and the relative ability to stimulate E_{1}SO_{4} uptake activity.

Effect of Nonaromatic Sulfur-Containing Cys Substitutions on GSH-Stimulated \[^{3}H\]E_{1}SO_{4} Transport by MRP1. We have previously reported that short-chain S-alkyl derivatives of GSH support NNAL-O-glucuronide, vincristine, and E_{1}SO_{4} transport by MRP1 as well as supporting the photolabeling of MRP1 by the tricyclic isoxazole modulator LY475776 (Loe et al., 1998; Leslie et al., 2001b; Qian et al., 2001; Mao et al., 2002). It was therefore of interest to examine the activity of several other tripeptides with nonaromatic S-linked substitutions that increase the steric bulk of this region of the tripeptide (Fig. 3B). Under the conditions of this study, S-methyl GSH (1 mM) (side chain molecular volume 48 Å\(^3\)) stimulated E_{1}SO_{4} uptake to a level that was 1.7-fold greater than GSH (Fig. 4B) and similar to the stimulation observed with \[^{1}H\]9253-Glu-Leu-Gly, which is of comparable molecular volume (side chain 54 Å\(^3\)) (Fig. 4A). The bulkier S-ethyl GSH (side chain 61 Å\(^3\)) was an even more potent stimulator, increasing E_{1}SO_{4} uptake approximately 2.5-fold more than GSH (Fig. 4B). In contrast, E_{1}SO_{4} uptake in the presence of \[^{1}H\]9253-Glu-Met-Gly, which has the same molecular formula and volume but different molecular shape from S-ethyl GSH, was slightly less (83 ± 6%) than that in the presence of GSH (Fig. 4B). The difference in the ability of S-ethyl GSH and \[^{1}H\]9253-Glu-Met-Gly to stimulate E_{1}SO_{4} uptake suggests that in addition to bulk and hydrophobicity, the molecular dimensions of the amino acid side chain at this position is important for maximal stimulation of MRP1 transport activity.

Effect of Cys Substitutions with Aromatic Amino Acids on GSH-Stimulated E_{1}SO_{4} Transport by MRP1. The ability of several Cys-substituted tripeptides with extended hydrophobic side chains to stimulate E_{1}SO_{4} uptake to levels similar to or greater than GSH prompted us to test additional GSH analogs with bulkier, aromatic side chains at this position, including Phe, homo-Phe, benzyl-Cys, Tyr, and Trp (Fig. 5). Substitution of Cys with Phe (\[^{1}H\]9253-Glu-Phe-Gly), which increases the molecular volume of the side chain from 36 to 74 Å\(^3\) resulted in a 1.3-fold increase in estrone 3-sulfate uptake compared with GSH (Fig. 5). Substitution of Cys with Phe (\[^{1}H\]9253-Glu-Phe-Gly), which increases the molecular volume of the side chain from 36 to 74 Å\(^3\) resulted in a 1.3-fold increase in estrone 3-sulfate uptake compared with GSH (Fig. 5). Similarly, when the length of the alkyl side chain before the link between the peptide backbone and the aromatic substituent was increased by one methylene group, the resulting tripeptide (\[^{1}H\]9253-Glu-homo-Phe-Gly) stimulated uptake 1.4-fold more than GSH. On the other hand, when Cys was replaced with benzyl-Cys, an amino acid with an even longer alkyl linker chain and larger molecular volume (94 Å\(^3\)), the resulting tripeptide (\[^{1}H\]9253-Glu-benzyl-Cys-
Gly or S-benzyl GSH) stimulated uptake to a level comparable with that in the presence of GSH (Fig. 5).

Despite the fact that γ-Glu-Phe-Gly stimulated E$_1$SO$_4$ uptake more than GSH, the addition of a hydroxyl group to the aromatic ring by substituting Cys with Tyr (γ-Glu-Tyr-Gly), which increases the side chain molecular volume to 82 Å$^3$, substantially reduced the stimulating activity of the tripeptide to only 25% that of GSH. Similarly, γ-Glu-Trp-Gly (Trp side chain molecular volume 100 Å$^3$), which is comparable in size with γ-Glu-benzyl-Cys-Gly, stimulated uptake only 2-fold above the low basal uptake level in the absence of GSH, with an activity approximately 30% of that of GSH (Fig. 5). Thus, as observed for the Cys→Ser-substituted and Cys→Thr-substituted tripeptides, the introduction of an H-bonding polar amino acid diminished the interaction of the tripeptide with the GSH binding pocket of MRP1 even if the side chain is aromatic.

S-(p-Azidophenacyl)-GSH has been widely used for photoaffinity-labeling studies of GSH-dependent enzymes, as well as more recently of MRP1, and has a significantly larger total and side chain molecular volume (319 and 144 Å$^3$, respectively) than GSH (Seddon and Douglas, 1980; Ciaccio et al., 1996; Whalen et al., 1996; Qian et al., 2002). This compound was only testable at a maximal concentration of 100 μM because of its low solubility in the aqueous transport assay mixture. At this concentration, this tripeptide stimulated E$_1$SO$_4$ uptake 2-fold above the low level basal activity, which was comparable with the stimulation in the presence 100 μM GSH but 5- to 6-fold less than in the presence of 1 mM GSH (Fig. 5C).

**Effect of γ-Glu-Leu-Gly on Kinetic Parameters of [³H]E$_1$SO$_4$ Uptake by MRP1.** The most potent nonsulfur-containing analog of GSH, γ-Glu-Leu-Gly, was further characterized by determining its effect on the kinetic parameters...
of E1SO4 transport. Uptake was measured at seven different concentrations (0.7–12 μM) of [3H]E1SO4 in the presence of γ-Glu-Leu-Gly (1 mM) or GSH (1 mM), and \( K_m \) and \( V_{max} \) values were obtained from Eadie-Hofstee plots of the data (Fig. 6). In the presence of GSH, the \( K_m \) and \( V_{max} \) values for E1SO4 were 436 nM and 127 pmol mg\(^{-1}\) min\(^{-1}\), respectively. In the presence of γ-Glu-Leu-Gly, the \( K_m \) value was comparable at 479 nM but the \( V_{max} \) value was increased 1.5-fold to 192 pmol mg\(^{-1}\) min\(^{-1}\).

Modulation of Apigenin-Stimulated [3H]GSH Uptake by γ-Glu-Leu-Gly. To further characterize the interaction of γ-Glu-Leu-Gly with MRP1, the ability of this tripeptide to inhibit apigenin-stimulated [3H]GSH uptake was examined. As shown in Fig. 7A, basal [3H]GSH uptake by HepG2 MRPI-enriched membrane vesicles was 42 ± 7 pmol mg\(^{-1}\) min\(^{-1}\), and this uptake was stimulated 5-fold to 235 ± 12 pmol mg\(^{-1}\) min\(^{-1}\) in the presence of apigenin (30 μM) (Fig. 7A). Apigenin-stimulated [3H]GSH uptake was reduced by γ-Glu-Leu-Gly in a concentration-dependent manner with approximately 60% inhibition observed at 100 μM (Fig. 7A), a concentration equal to the initial concentration of [3H]GSH in the uptake assay. The IC\(_{50}\) value for γ-Glu-Leu-Gly was estimated to be 60 μM (Fig. 7A).

The mode of γ-Glu-Leu-Gly inhibition of [3H]GSH uptake was characterized by determination of kinetic parameters. The \( K_m \) and \( V_{max} \) values obtained from Eadie-Hofstee plots for apigenin-stimulated GSH uptake were 69 μM and 710 pmol mg\(^{-1}\) min\(^{-1}\), respectively. In the presence of γ-Glu-Leu-Gly (60 μM), the apparent \( K_m \) value for GSH uptake was increased more than 5-fold to 362 μM, whereas the \( V_{max} \) (768 pmol mg\(^{-1}\) min\(^{-1}\)) was similar to that in the absence of γ-Glu-Leu-Gly. These results indicate γ-Glu-Leu-Gly is a potent competitive inhibitor of apigenin stimulated [3H]GSH uptake with a \( K_i \) value of 14 μM (Fig. 7B).

Discussion

In the present study, we found that the molecular volume of the γ-Glu side chain in GSH and its γ-COOH linkage to Cys were critical for effective interaction with the GSH binding pocket of MRPI because tripeptides in which the side chain was eliminated or shortened or in which the linkage...
COOH and NH₂ groups are important determinants of GSH activity. Our observation that the removal of the NH₂ group reduces the catalytic activity. Several studies of GSTs using dipeptides indicate that the carboxylate moiety (GABA-) substantially different (Fig. 1). Thus, although an amino acid is required at the Gly residue of GSH could be altered, no longer stimulated EₛSO₄ uptake. The γ-Glu residue has been shown to be the main binding determinant of GSH for several rat liver GST isoenzymes (Adang et al., 1988). Thus, similar to our findings with MRPI-mediated transport of EₛSO₄, β-Asp-Cys-Gly was not a substrate for any of the four GST isoenzymes tested. However, in contrast to our observations with MRPI, some level of GST activity was maintained in the presence of α-Glu-Cys-Gly (Adang et al., 1990). Regardless of the site of addition (γ or α-COOH) of the Glu residue, a free COOH group is present at the same distance from the peptide bond in α-Glu-Cys-Gly and γ-Glu-Cys-Gly (GSH), whereas the position of the free NH₂ group is substantially different (Fig. 1). Several studies of GSTs using the decarboxylated and deaminated analogs of GSH GABA-Cys-Gly and glutaric acid-Cys-Gly, respectively; in the presence of γ-Glu-Leu-Gly, the Kᵥ and Vₘₐₓ values were 479 μM and 192 pmol mg⁻¹ min⁻¹, respectively. Data points are the means (± S.D.) of triplicate determinations in a typical experiment. Similar results were obtained in three additional independent experiments.

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We also found that the Gly residue of GSH could be altered significantly while still retaining some level of stimulatory activity. Previous studies demonstrated that the dipeptides γ-Glu-Cys and Cys-Gly do not support vincristine transport by MRPI, suggesting that a tripeptide is required (Loo et al., 1998). Thus, although an amino acid is required at the Gly position, the molecular volume of the side chain is not critical for interaction with the GSH binding site of MRPI. For example, the γ-Glu-Cys-β-Ala contains an additional methylene group within the COOH terminus of this tripeptide, yet this peptide was just as effective as GSH in stimulating EₛSO₄ transport. Even γ-Glu-Cys-α-Glu-stimulated transport although only 30% as effectively as GSH. The disulfide form of γ-Glu-Cys-α-Glu was not expected to support transport of EₛSO₄ because the disulfide form of GSH (glutathione disulfide) did not (Qian et al., 2001).

Cys is commonly thought of as being the most physiologically active amino acid of GSH, and indeed, as mentioned previously, replacement of this residue results in a tripeptide that cannot be used by GSTs in conjugation reactions (Adang et al., 1990). In contrast, our studies show that it can be modified dramatically and still retain the ability to enhance EₛSO₄ transport by MRPI. Significant levels of transport stimulation were observed even with a peptide having no side chain at this position (γ-Glu-Gly-Gly), demonstrating that not only is the thiol group not essential for its interaction with MRPI but also no space-filling side chain is required.

Tripeptides in which the Cys residue was replaced with residues of increasing hydrophobicity, including those with longer side chains (Val, Leu, methyl-Cys, and ethyl-Cys) and bulkier aromatic side chains (Phe, homo-Phe, and benzyl-Cys), maintained or exceeded the ability of GSH to stimulate EₛSO₄ transport. In contrast, tripeptides with polar amino acid substitutions of the Cys residue were less able to support transport, even when the substituted side chain occupied a molecular volume comparable with Cys. Thus, the conservatively substituted γ-Glu-Ser-Gly stimulated transport only half as effectively as GSH. Furthermore, whereas γ-Glu-Val-Gly stimulated transport just as well as GSH, the more polar γ-Glu-Thr-Gly was much less effective. Similarly, γ-Glu-Phe-Gly exceeded GSH in its ability to stimulate EₛSO₄ uptake, whereas γ-Glu-Tyr-Gly did not stimulate uptake at all. Finally, γ-Glu-Trp-Gly did not stimulate uptake to any extent despite the fact that a tripeptide with an equally bulky but nonpolar side chain, γ-S-benzyl-GSH, did so at a level similar to GSH. This is likely due to the hydrogen-bonding capabilities of the Trp indole ring (Gallivan and Dougherty, 1999) because peptides substituted with other polar amino acids such as Ser, Thr, or Tyr were also ineffective at stimulating transport. In addition, although the molecular volume of Trp is similar to benzyl-Cys, it has a very different molecular shape and therefore reduced steric complementarity could also be a factor in the poor interaction of γ-Glu-Trp-Gly with the GSH binding pocket of MRPI.

Of particular interest was our observation that γ-Glu-Met-Gly stimulated EₛSO₄ transport almost as well as GSH, whereas S-ethyl GSH, which has the same molecular formula and volume and similar overall polarity as γ-Glu-Met-Gly, caused a significant hyperstimulation (2.5-fold). This indicates that although the molecular volume of the side chain at the Cys position is important for GSH analog interaction with MRPI, some specificity exists with respect to the location of the sulfur atom. The importance of the location of the sulfur atom could be simply due to a change in shape of the functional group, reducing the steric complementarity, or it could be related to the optimal placement of the sulfur atom for critical bonding interactions with amino acids in the GSH binding pocket of MRPI. Overall, the lack of stimulation of
E$_2$SO$_4$ uptake by Cys-substituted tripeptides with polar amino acid side chains and the hyperstimulation of uptake by Cys-substituted tripeptides with large hydrophobic side chains provides strong evidence that the Cys residue of GSH is positioned to interact with hydrophobic residues in the GSH binding region of MRP1. This is in contrast to the conjugating GSTs where the thiol group of the Cys residue typically forms hydrogen bonds with the hydroxyl group of polar residues such as Tyr or Ser in the active site of the enzyme (Dirr et al., 1994).

Kinetic analyses of [H]$^3$E$_2$SO$_4$ uptake showed that the apparent affinity ($K_m$) of MRP1 for this substrate was similar in the presence of GSH or γ-Glu-Leu-Gly; however, the transport efficiency was 1.5-fold higher in the presence of the Leu-substituted analog. Further kinetic analyses showed that γ-Glu-Leu-Gly was a competitive inhibitor of apigenin-stimulated [H]$^3$GSH transport ($K_i$ value of 14 μM), indicating that γ-Glu-Leu-Gly and GSH likely bind to the same or at least overlapping sites on MRP1. The $K_m$ value for γ-Glu-Leu-Gly is lower than the $V_{max}$ value for apigenin-stimulated GSH uptake, which is approximately 70 μM (Leslie et al., 2003). This implies that γ-Glu-Leu-Gly binds MRP1 with high affinity and could potentially be a substrate for this transporter. It is also possible that γ-Glu-Leu-Gly binds to MRP1 with such high affinity that it may be poorly transported across the membrane. If an ordered binding mechanism occurs (i.e., the tripeptide binds first causing a conformational change in MRP1 that is required for subsequent interaction with E$_2$SO$_4$), it could be that tripeptides such as γ-Glu-Leu-Gly and S-ethyl-GSH have a stronger affinity for MRP1 than

![Fig. 7. Modulation of apigenin-stimulated [H]$^3$GSH uptake by γ-Glu-Leu-Gly.](image)

- **A** uptake was measured at an initial [H]$^3$GSH concentration of 100 μM (120 nCi) in the absence (open column) and presence (shaded column) of apigenin (30 μM) with γ-Glu-Leu-Gly indicated. Columns represent the means of triplicate determinations (± S.D.) in a typical experiment.
- **B** uptake of [H]$^3$GSH over a range of substrate concentrations (10–1500 μM) was measured in the presence of apigenin (30 μM) with 0 μM (●) and 60 μM (▲) of γ-Glu-Leu-Gly. Symbols represent the means of triplicate determinations (± S.D.) in a typical experiment. Eadie-Hofstee plots were generated and kinetic parameters determined (inset). In the absence of γ-Glu-Leu-Gly, the $K_m$ and $V_{max}$ values for apigenin-stimulated GSH transport were 69 μM and 710 pmol mg$^{-1}$ min$^{-1}$, respectively; in the presence of γ-Glu-Leu-Gly, the apparent $K_m$ and $V_{max}$ values were 362 μM and 768 pmol mg$^{-1}$ min$^{-1}$, respectively. The $K_i$ (γ-Glu-Leu-Gly) was determined to be 14 μM.
GSH and cause a protein conformational change more favorable for $\text{E}_2\text{SO}_4$ transport. We have previously proposed that GSH may stimulate transport by masking a site in MRP1 that diminishes the affinity of the protein for certain substrates (Leslie et al., 2001b). Tripeptides such as $\gamma$-Glu-Leu-Gly could play a similar “masking role”. However, this would make it difficult to explain how $\gamma$-Glu-Leu-Gly competitively inhibits apigenin-stimulated GSH transport, unless the masking site overlaps with the site of GSH interaction before transport.

We have previously reported that certain short-chain $\text{S}$-alkyl derivatives of GSH support MRP1-mediated transport of vincristine and $\text{NNAL-O-glucuronide}$ (Loe et al., 1998; Leslie et al., 2001b) as well as photoactivating by the MRP1-specific chemosensitizing agent LY475776 photoactivating of MRP1 approximately as well as GSH and in the case of vincristine, $\text{S}$-methyl GSH supports uptake to levels that are only 30% of those observed with GSH (Loe et al., 1998; Leslie et al., 2001b; Qian et al., 2001). In the current study, we have found that $\text{S}$-ethyl GSH is even more effective than $\text{S}$-methyl GSH at stimulating $\text{E}_2\text{SO}_4$ uptake. In contrast, this analog was less effective than $\text{S}$-methyl GSH in supporting vincristine uptake (Loe et al., 1998). Thus, $\text{E}_2\text{SO}_4$ transport is stimulated much more effectively by the $\text{S}$-alkyl derivatives than vincristine transport. The reason for these differences is uncertain but presumably relates to some physical properties of these substrates. Vincristine occupies a molecular volume of 615 Å$^3$, which is significantly greater than that of $\text{E}_2\text{SO}_4$ (239 Å$^3$), $\text{NNAL-O-glucuronide}$ (271 Å$^3$), and LY475776 (366 Å$^3$). Thus, it is possible that the presence of vincristine within the substrate binding pocket reduces the interaction of the bulkier $\text{S}$-alkyl derivatives simply through steric hindrance. It follows, therefore, that even though $\gamma$-Glu-Leu-Gly is a potent stimulator of $\text{E}_2\text{SO}_4$ transport and a clear competitive inhibitor of apigenin-stimulated GSH transport, it is not necessarily expected that this tripeptide would stimulate transport or binding of other GSH-dependent MRP1 substrates to the same extent. Thus, if one envisions GSH and substrate fitting into a binding pocket with multiple binding coordinates, it may be that the significantly greater bulk of vincristine (compared with $\text{E}_2\text{SO}_4$) could prevent a GSH analog with a larger molecular volume from being accommodated in the same site. Studies are in progress to test this hypothesis. We conclude that in addition to molecular volume, other factors contribute to the functional interaction of GSH and its analogs with MRP1, including molecular geometry and the positioning of hydrogen bonding donor and acceptor atoms.

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