This review addresses the recent molecular identification of several members of the glutathione S-conjugate (GS-X) pump family, a new class of ATP-binding cassette (ABC) transporters responsible for the elimination and/or sequestration of pharmacologically and agronomically important compounds in mammalian, yeast and plant cells. The molecular structure and function of GS-X pumps encoded by MRP, cMOAT, YCF1, and AtMRP genes, have been conserved throughout molecular evolution. The physiologic function of GS-X pumps is closely related with cellular detoxification, oxidative stress, inflammation, and cancer drug resistance. Coordinated expression of GS-X pump genes, e.g., MRP1 and YCF1, and γ-glutamylcysteine synthetase, a rate-limiting enzyme of cellular glutathione (GSH) biosynthesis, has been frequently observed.

**KEY WORDS:** ABC-transporter; GS-X pump; multidrug resistance associated protein (MRP); canalicular membrane multispecific organic anion transporter (cMOAT); yeast cadmium factor 1 (YCF1); γ-glutamylcysteine synthetase; glutathione.

**INTRODUCTION**

Plant and animal cells eliminate a broad range of lipophilic toxins from the cytosol after their conjugation with glutathione (GSH) (1–3). This transport process is mediated by a GS-X pump, a novel organic anion-transporting Mg$^{2+}$-ATPase. The term of “Gs-X pump” has been originally proposed based on its transport activity and high affinity toward glutathione S-conjugates (GS-conjugates), glutathione disulfide (GSSG), and cysteinyl leukotrienes (1). The kinetic properties and substrate specificity of the GS-X pump have been intensively studied using plasma membrane vesicles from different biological sources. Accumulating evidence suggests that the GS-X pump has a broad

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substrate specificity toward different types of organic anions and thereby plays a physiologically important role in inflammation, oxidative stress, xenobiotics metabolism, and tumor drug resistance. In plants, the GS-X pump is competent in the transport of GS-conjugates of the chloroacetanilide and triazine herbicides, metolachlor and simetryn, respectively, as well as GSSG and GS-conjugates of 1-chloro-2,4-dinitrobenzene (CDNB) and monochlorobimane (2, 3). It has recently been suggested that the GS-X pump is involved in the anthocyanin biosynthetic pathway (4).

During the past three years, there was a remarkable progress in our understanding of the molecular nature of the GS-X pumps. Overexpression of the multidrug resistance-associated protein (MRP1) gene (5, 6) in human cancer cells has been first reported to result in increased ATP-dependent GS-conjugate transport, thus demonstrating that the MRP1 gene encodes a human GS-X pump (7, 8). A liver specific GS-X pump, named cMOAT, has also been cloned from rat liver cDNA libraries and it exhibited an extensive homology with human MRP1 (9–11). Mutation of the cMOAT gene is implicated to be a cause of hereditary hyperbilirubinemia associated with defective function of the hepatic GS-X pump. The yeast cadmium factor (YCF1) gene from Saccharomyces cerevisiae has been identified on the basis of its ability to confer cadmium resistance (12). The YCF1 gene encodes an ATP-binding cassette (ABC) protein homologous to human MRP1, and the protein has recently been identified as vacuolar GS-X pump in yeast cells (13). Furthermore, two cDNAs, i.e., AtMRP1 and AtMRP2, encoding GS-X pump in a plant Arabidopsis thaliana have been cloned and they also show extensive sequence homologies to MRP1, cMOAT and YCF1 (Lu, Y.-P., Li, Z.-S., Rea, P. A., unpublished). Thus, evidence is accumulating to support the idea that the GS-X pumps comprise a multigene family in both the animal and plant kingdoms.

**GS-X PUMP AND GSH METABOLISM**

One of the major functions of the GS-X pump is excretion and/or sequestration of toxic compounds as a cellular protection system. The metabolism and detoxification of xenobiotics comprises three main stages: phases I, II and III (1). In phase I, xenobiotics and endogenous substances are oxidized, reduced or hydrolyzed to expose or introduce a functional group of the appropriate reactivity. Cytochrome P450s and mixed function oxidases are examples of phase I enzymes that confer the requisite electrophilicity on otherwise unreactive compounds for their subsequent metabolism by phase II enzymes. In phase II, the activated derivative is conjugated with GSH, glucuronic acid or sulfate by the action of GSH transferases (GSTs), UDP-glucuronyl transferases, and sulfotransferases. In the phase III, the resulting conjugates are transported out of the cytosol to the extracellular space or into intracellular compartments. The underlying principle is that the conjugation reaction confers negative charges on and increases the water solubility of the compound to promote its extrusion by the GS-X pump and other unidentified transporters.
The function of the GS-X pump is closely linked with cellular GSH metabolism. Glutathionation provides negative charges to compounds and thereby enables the substrate recognition by the GS-X pump. GSH bears two important features in its structure, namely, the γ-glutamate linkage and the SH-group, both of which are intimately linked to its intracellular stability and biological functions. The intracellular concentration of GSH in mammalian cells is 1–10 mM (14), which is even higher than the intracellular concentration of ATP. In many cells, GSH accounts for more than 90% of the total nonprotein sulfur. Such high intracellular concentration of GSH is made possible by the γ-Glu linkage structure, which protects the GSH molecule from protease cleavage. The SH-group of GSH is strongly nucleophilic and confers to the molecule the unique ability to react with a wide variety of agents including free radicals, reactive oxygen species, heavy metals, and cytotoxic electrophilic compounds, thereby playing a critical role of detoxification in living cells.

The biosynthesis of GSH takes place in the cytosol. The reaction consists of two steps catalyzed by γ-glutamylcysteine synthetase and by GSH synthetase, where each step requires one molecule by ATP (Fig. 1). γ-Glutamylcysteine synthetase (γ-GCS) catalyzes the first step (reaction 1) in which an amide linkage is formed between the amino group of cysteine and the γ-carboxyl group of glutamate.

![Fig. 1. Schematic illustration for the biosynthesis of GSH, conjugation of GSH with electrophilic compounds or heavy metals (X), and ATP-dependent transport of GSH conjugates (GS-X) via the GS-X pump. In the animal cell, GS-X is exported across the plasma membrane or compartmentalized into intracellular vesicles and subsequently released from the cell by exocytosis. In the yeast and plant cell, GS-X is transported by the GS-X pump into the vacuole.](image-url)
glutamate. In the second step, GSH synthetase catalyzes the reaction between glycine and the cysteine carboxyl group of γ-glutamylcysteine:

\[
\text{L-Glu} + \text{L-Cys} + \text{ATP} \rightarrow \gamma\text{-glutamylcysteine} + \text{ADP} + \text{Pi} \quad \text{(Reaction 1)}
\]

\[
\gamma\text{-Glutamylcysteine} + \text{L-Gly} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{Pi} \quad \text{(Reaction 2)}
\]

The reaction catalyzed by γ-GCS is the rate-limiting step of GSH biosynthesis and is controlled by negative feedback from its end product, GSH, via nonallosteric competitive inhibition (15).

The pathway of GSH-mediated drug inactivation is a biologically “expensive” mechanism. The synthesis of GSH and the export of GS-conjugates from the cytosol requires at least three molecules of ATP in order to metabolize 1 mol of the drug molecule. In the case of the detoxification of cadmium, at least five molecules of ATP are required, namely, four molecules of ATP for the synthesis of two molecules of GSH and at least one molecule of ATP for the export of bis-glutathionato cadmium(II), a glutathione-cadmium chelate complex. This compares unfavorably with the glycolytic pathway, in which only two molecules of ATP are gained from one molecule of glucose. Nevertheless, the fact that the GS-X pump is ubiquitously distributed in the plant and animal kingdoms strongly suggests that the GSH-associated metabolism and transport pathway is fundamentally important for the survival of living cells.

It is estimated that GSH biosynthesis originated about 3.5 billion years ago. GSH is found in the vast majority of eukaryotes, whereas in eubacteria GSH biosynthesis is limited to only two groups, i.e., cyanobacteria and purple bacteria (16). The former appeared on the earth about 35 billion years ago and was capable of oxygenic photosynthesis. The cyanobacteria is the group considered to have given rise to plant chloroplasts, whereas the purple bacteria is considered to have introduced the ancestor responsible for eukaryotic mitochondria. GSH production appears to be closely associated with those prokaryotes responsible for the oxygen-producing and oxygen-utilizing pathways of eukaryotes, suggesting that the ability of GSH biosynthesis may have been acquired by eukaryotes in those endosymbiotic process that give rise to chloroplast and mitochondria (16). In fact, GSH plays a pivotal role in protection of living cells under oxidative stress (17). Intracellular glutathione disulfide (GSSG) is maintained at low levels (less than 3% of total GSH pool) by the action of GSSG reductase, whereas enhanced GSH peroxidase reaction (e.g., via redox-cycling of quinone compounds) leads to an increase in the cellular GSSG level and GSSG efflux from cells. GSSG efflux has been observed in many different organs and cell types, including erythrocytes, liver, lung, and heart. It is important to note that studies on the GS-X pump started with the discovery of GSSG efflux from human erythrocytes.

In 1969, the frontier work was made by Srivastava and Beutler who reported that GSSG from human erythrocytes is an unidirectional and energy-dependent process (18). GSSG transport occurred even against a concentration gradient of GSSG and the transport was halted almost entirely by exhausting endogenous ATP by preliminary incubation of erythrocytes in a glucose-free medium for
The GS-X Pump

eight hours or by the presence of fluoride in the incubation medium. Their report provided the first evidence that GSSG efflux is mediated not by simple diffusion but by active membrane transport. Although they suggested energy-dependence of GSSG transport, it was not elucidated whether ATP is directly required. Eleven years later, 1980, the transport of GSSG across the plasma membrane was proven to be an ATP-dependent “primary” active process in inside-out membrane vesicles from human erythrocytes (19). Subsequently, ATP-dependent GSH conjugate transport was demonstrated by Board (20), Kondo et al. (21) and Labelle et al. (22).

In 1984, we have reported that of GSSG and GSH conjugates are released from the isolated perfused heart (23). The heart is the organ continuously exposed to highly oxygenated blood from the lung. Cardiomyopathy resulting from oxidative damage inflicted by hyperoxia or administration of certain anticancer drugs, e.g., doxorubicin, has been described. GSSG efflux was suggested to be an important defense mechanism against oxidative stress (24). The relationship of GSSG efflux rate vs. cytosolic free ATP/ADP ratio shows that GSSG efflux rate is half-maximal at (ATP/ADP)$_{rec}=10^{25}$, suggesting an ATP-dependent transport process. GSSG efflux from the heart was not affected by epinephrine, nor-epinephrine or dibutyryl cyclic AMP, suggesting the GSSG transport is independent of $\alpha$- or $\beta$-adrenergic hormonal regulations (25). Using plasma membrane vesicles prepared from rat hearts, we have demonstrated ATP-dependent primary active transport of GSSG and GS-conjugates (26). Cardiac GS-X pump was shown to have high affinities toward GS-conjugates with a long aliphatic carbon chain (25). We first provided evidence that leukotriene C$_4$ (LTC$_4$), a pro-inflammatory mediator, is an endogenous substrate for the GS-X pump (26, 27).

In the liver, GSSG and GS-conjugates are predominantly excreted into bile. Akerboom et al. reported that hepatobiliary transport of GSSG was inhibited by GS-conjugates, suggesting the existence of a common transport system for hepatobiliary transport of both GSSG and GS-conjugates (28). Although the membrane potential had been speculated to be a potential driving force for the transport of GSSG and GSH conjugates (29), Kobayashi and his coworkers provided evidence for ATP-dependent primary active transport of S-(2,4-dinitrophenyl)-glutathione (GS-DNP) using rat hepatocyte canalicular membrane vesicles (30). Moreover, Koabayashi et al. reported that transport of $p$-nitrophenyl-glucuronide across rat liver canalicular membrane was an ATP-dependent process (31). Inhibition of the transport of the glucuronide conjugate by GS-DNP suggested that the hepatobiliary elimination of glucuronide conjugates is mediated by the hepatic GS-X pump (31).

The discovery of two strains of jaundiced mutant Wistar and Sprague-Dawley rats, i.e., TR$^-$ and EHBR, greatly enhanced the study of the hepatic GS-X pump. The mutant rats manifest predominantly conjugated hyperbilirubinemia and are defective in the biliary secretion of GSH, GSSG, GS-conjugates (32–34), GSH-metal complexes (35), bilirubin-glucuronide conjugates (36) and cysteinyl leukotrienes (37, 38), as well as organic anions, e.g., bromosulphthalein, indo-
cyanine green and dibromosulfophthalein (39, 40). These findings stimulated the idea that the hepatobiliary GS-X pump has a broad substrate specificity toward different organic anions. Thus, the hepatic GS-X pump is also called “canalicular multispecific organic anion transporter (cMOAT)”. The functional defect of the hepatic GS-X pump or cMOAT is inherited with characteristics of an autosomal recessive abnormality, and those mutant rats are regarded as animal models of the Dubin–Johnson syndrome in humans. Interestingly, erythrocyte membranes from patients of the Dubin–Johnson syndrome and from the TR⁺ mutant rats exhibited ATP-dependent transport of GS-DNP and GSSG as normal (41). Thus, it has been suggested that erythrocyte and hepatic GS-X pumps are encoded by distinct genes. This hypothesis has recently been verified by molecular identification of hepatic and extrahepatic GS-X pump genes, i.e. cMOAT and MRP1 (see below).

The existence of the GS-X pumps in yeast and plants has also been reported by St-Pierre et al. (42), Martinoaia et al. (2) and Li et al. (3). In yeast and plant cells, instead of excretion, conjugates of xenobiotics are stored in the vacuole. The GS-conjugates of 1-chloro-2,4-dinitrobenzene (CDNB), N-ethylmaleimide and metalachlor were barely taken up by isolated vacuoles in the absence of Mg²⁺ and ATP. In the presence of Mg²⁺ and ATP, however, uptake was markedly enhanced. ATP-dependent accumulation of GS-conjugates was inhibited by vanadate, but not by bafilomycin, a specific inhibitor of the vacuolar H⁺-ATPases (3). Thus, ATP-dependent accumulation of GS-conjugates in the vacuole is independent of H⁺ gradient across the membrane. Furthermore, GSSG was also, actively transported into isolated vacuoles, while uptake of GSH was only marginal (43). The uptake of GSSG was completely inhibited by the GS-conjugate of the herbicide metalachlor, indicating that transport of GSSG is mediated by a vacuolar GS-X pump. In many functional respects, vacuolar GS-X pumps of yeast and plants show a striking resemblance to the GS-X pump found in rats and humans.

**MOLECULAR IDENTIFICATION OF GS-X PUMPS**

Identification of the GS-X pump in human, rat, yeast, and plants at the molecular level has served to confirm its wide distribution and demonstrate that GS-conjugate transporters constitute a multigene family within the ABC transporter superfamily. In 1994, Müller et al. and Leier et al. provided evidence that overexpression of MRP1 gene, first isolated from human small cell lung cancer cell lines (5, 6), conferred increased Mg-ATP-dependent GSH conjugate transport (7, 8), thus demonstrating that MRP1 gene encodes a human GS-X pump. Since then, other closely related genes have been characterized. The liver-specific GS-X pump, i.e., cMOAT, has been cloned from cDNA libraries of TR⁻ and EBHR rat liver (9–11). The yeast cadmium factor (YCF1) gene, which was originally isolated on the basis of its ability to confer cadmium resistance (12), was found to be a vacuolar GS-X pump in *Saccharomyces cerevisiae* (13). Most recently, two genes (AtMRP1, AtMRP2) encoding plant GS-X pumps have been isolated from
a vascular plant, *Arabidopsis thaliana*, and they exhibit extensive homology with MRP1, YCF1 and cMOAT (Lu, Y.-P., Li, Z.-S., Rea, P. A., unpublished data).

Sequence comparisons of MRP1, cMOAT, YCF1, AtMRP1 and AtMRP2 with other members of the ABC transporter superfamily reveal two major subgroups (Fig. 2). One consists of MRP1 (5, 6, 44), cMOAT (9–11, 45), YCF1 (12), AtMRP1, AtMRP2 (Lu, Y.-P. Li, Z.-S., Rea, P. A., unpublished data), SUR (46), EBCR (47), Yrs/Yorl (48), the *Leishmania* P-glycoprotein-related molecule (Lei/PgpA) (49–51) and the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (52). The other consists of the P-glycoprotein (MDR) (53, 54), major histocompatibility complex transporters (TAP1 and TAP2) (55) and STE6 (56). Of all the ABC transporters defined to date, MRP1, cMOAT,
YCF1, AtMRP1 and AtMRP2 are known to have GS-X pump activity. Unlike the similarities between the GS-X pump subgroup, Lei/PgpA and CFTR, which center on the nucleotide-binding folds (NBFs), those within the GS-X pump family are found throughout the sequence. GS-X pump family members are 40–45% identical (60–65% similar) at the amino acid level, possess NBFs with an equivalent spacing of conserved residues and are collinear with respect to the location, extent and alternation of putative transmembrane spans and extramembrane domains. Two features of members of the GS-X pump family that distinguish them from other ABC transporters are their possession of a central truncated CFTR-like “regulatory” domain, rich in charged amino acid residues, and an approximately 200 amino acid residue N-terminal extension.

The conclusion that MRP1 gene encodes a human GS-X pump transporting multivalent organic anions is deduced from the following observations: (a) ATP-dependent transport of LTC₄ and GS-conjugates observed in plasma membrane vesicles from MRP1-overexpressing, doxorubicin-resistance HL60/ADR cells was greater than that observed in drug-sensitive cells (57). (b) A 190-kDa membrane protein was detected by [³H]LTC₄ photoaffinity labeling in the plasma membrane from HL60/ADR cells (57). (c) The [³H]LTC₄-labeled 190 kDa membrane protein was immunoprecipitated by MRP-specific monoclonal antibody (57): (d) Plasma membrane vesicles prepared from MRP-transfected cells exhibited ATP-dependent transport of LTC₄, GS-DNP, GSSG, 17-β-estradiol-17-(β-D-glucuronate, and sulfate conjugates (7, 8, 58–61). (e) MRP-Mediated transport of [³H]LTC₄ was strongly inhibited by GSH conjugates carrying a long aliphatic carbon chain, but not inhibited by doxorubicin (60), being consistent with our previous observations on GS-X pump function (1, 24, 26, 27). (f) MRP is expressed in a variety of normal tissues and cell types, including heart, skeletal muscle, lung, adrenal gland, erythrocytes, and macrophages (62), however its expression level in the liver is very low (6, 62).

cMOAT, the liver-specific GS-X pump, was cloned from cDNA libraries of rat liver (9–11), and from a cisplatin-resistant human epidermoid cancer cell line, KB/KCP4 (44). While MRP1 gene is localized on 16p13.12–13 (5), human cMOAT gene was found on 10q24 (44), thus demonstrating distinct localization of hepatic and extrahepatic GS-X pump genes. Rat cMOAT consists of 1541 amino acids, exhibiting highest overall identity to human MRP1 (47.6%), YCF1 (41.8%), and CFTR (30.2%) (9–11). cMOAT is almost exclusively expressed in the liver and to a lesser extent in the duodenum, jejunum and kidney, whereas none is detectable in the brain, heart, lung, testis, and skeletal muscle (9, 11). Double immunofluorescence and confocal laser scanning microscopy revealed exclusive localization of cMOAT at the canalicular membrane domain of hepatocytes in the normal rat, but its loss in TR⁻ and EHB rat (10). No cMOAT mRNA was detectable in these mutant rats (9–11). In TR⁻ rat, a single-nucleotide deletion at amino acid 393 resulted in the introduction of the stop codon at amino acid 401 (9). In EHB rat, one base pair replacement from G to A at nucleotide 2564 resulted in the introduction of the premature stop codon at the corresponding amino acid 855 in all tissues examined (11). Since EHB and TR⁻ are allelic mutants and both strains exhibit the autosomal
recessive inheritance in hepatobiliary excretion of organic anions, it is concluded that the impaired expression of this hepatic GS-X pump, cMOAT, is related to the pathogenesis of hyperbilirubinemia in the mutant rats. Genetic alterations associated with Dubin–Johnson syndrome in humans remain to be elucidated.

YCF1 gene encodes a yeast vacuolar GS-X pump consisting of 1,515 amino acids with extensive homology to MRP1 (42.6% identity, 63.3% similarity) and CFTR (31% identity, 56.7% similarity). The protein encoded by YCF1 gene is required for cadmium resistance in S. cerevisiae. Another type of cadmium resistance gene designated HMT1 has been identified in Schizosaccharomyces pombe, and this protein contains a single transmembrane domain and nucleotide binding fold (63, 64). Sequence comparison reveals that HMT1 belong to a subclass of ABC transporters which is distinct from YCF1, MRP1, cMOAT and CFTR. S. cerevisiae, cells harboring a deletion of the YCF1 gene are hypersensitive to cadmium compared to wild type cells. Mutagenesis experiments have demonstrated that conserved amino acid residues, functionally critical in MRP1 and CFTR, play a vital role in YCF1-mediated cadmium resistance (12). Mutagenesis of phenylalanine 713 in the NBF1 of YCF1 completely abolished in cadmium detoxification. Furthermore, substitution of a serine to alanine residue in a potential protein kinase a phosphorylation site in a central region of YCF1, which displays sequence similarity to the central regulatory domain of MRP1 and CFTR, also rendered YCF1 nonfunctional, suggesting that phosphorylation of the serine residue is critical for GS-X pump activity of YCF1.

**TRANSPORT OF GSH-METAL COMPLEXES BY GS-X PUMP**

The GS-X pump plays an important role in excretion or sequestration of heavy metals and electrophilic compounds after conjugation with cellular GSH. Li et al. (13, 65) have recently provided evidence that YCF1 sequesters a GSH-cadmium complex, i.e. bis-glutathionato cadmium(II), as well as GSH-conjugates from the cytosol into the vacuole in the yeast. Disruption of the YCF1 gene resulted in yeast strains (DTY167 and DTY168) hypersensitive to cadmium (12). While wild type cells mediate vacuolar accumulation of the fluorescent GS-conjugate of monochlorobimane, the mutant DTY167 cells lack the activity of vacuolar accumulation (13). Introduction of plasmid borne, epitope-tagged gene encoding functional YCF1 and DTY167 cells restored cellular resistance to cadmium concomitant with the activity for ATP-dependent transport of DNP-SG and bis-glutathionato cadmium(II) (GS2-Cd) in vacuolar membrane vesicles (13, 65). ATP-dependent transport of GS2-Cd in vacuolar membrane vesicles was inhibited by GS-DNP, suggesting a common pathway for the transport of those compounds (65). YCF1 mRNA level as well as GS-X pump activity in DTY165 cells (wild type) was dramatically enhanced by pretreatment with 200 μM CdSO4 or 150 μM CDNB for 24 h (65).

We previously demonstrated that human GS-X pump encoded by the MRP1 gene eliminates a GSH-platinum (GS2-Pt) complex, bis-glutathionato
platinum(II), from cancer cells (66). The Km value for the GS2-Pt complex was estimated to be 100 μM, being similar to that for GSSG (67). The MRP1 gene is overexpressed in cisplatin-resistant human leukemia HL-60/R-CP cells, whereas no gene amplification was detected (67,68). Instead, MRP1 mRNA level in HL-60/R-CP cells was significantly induced within 48 h by incubation of cells with cisplatin, cadmium, zinc or arsenite (68). Tommasini et al. has recently reported that transfection of the human MRP cDNA in yeast mutant DTY168 cells restored cadmium resistance to the wild-type level (69), suggesting that MRP1 functionally complements YCF1. MRP1-transfected cancer cells are reportedly resistant to arsenite and antimony (70). MRP1-mediated efflux of arsenite accompanies GSH efflux, supporting the hypothesis that MRP1 transports the GSH-arsenite complex (71). Through the reaction with cellular GSH, heavy metals are converted to organic anions. The multivalent negative charge is crucial for the recognition of substrates by GS-X pumps, where one molecule of heavy metal must be ligated by at least two molecules of GSH. Mono(glutathionato)-cadmium is not substrate for YCF1 (65) or MRP1 (Ishikawa, T. unpublished observation). Furthermore, it is important to note that the overexpression of the MRP1 gene per se does not necessarily result in cellular resistance to heavy metals. In the case of cisplatin, formation of the GS2-Pt complex in the cells is slow and this is the rate limiting step of the over-all reaction including the export process (66), whereas the reaction of cadmium with GSH is much faster (65). High cellular GSH levels are important to propel the interaction of heavy metals with GSH and to shift the thermodynamic equilibrium toward the formation of GSH-heavy metal complexes. γ-Glutamylcysteine synthetase (γ-GCS) plays an important role in the regulation of cellular GSH levels, thereby modulating the GS-X pump function.

In cisplatin-resistant human epidermoid carcinoma KCP-4 cells, Taniguchi et al. (45) and Chuman et al. (72) have recently shown that cMOAT may function as a transporter for GS2-Pt complex to reduce cellular accumulate of cisplatin. MRP1 gene was not expressed in their cisplatin-resistant cell line (45,72). To our knowledge, the paper by Taniguchi et al. (45) is the first report demonstrating the expression of cMOAT gene in human cancer cells. In conjunction with our previous studies (66–68), these reports suggest that GS-X pumps encoded by MRP1 and cMOAT genes have an extensive overlapping substrate specificity toward organic anions, including GSH-metal complexes.

Hepatobiliary transport of GSH is critically involved in the biliary excretion of the physiologically important copper and zinc, as well as the toxic metals, such as arsenic, mercury, cadmium, lead, antimony and bismuth (73–80). It has been shown that excretion of these metals in bile is increased when biliary excretion of endogenous GSH is enhanced (73, 75). In contrast, biliary excretion is diminished when hepatobiliary transport of GSH is decreased by agents that depletes hepatic GSH (e.g., diethylmalate) or inhibit of GSH transport from liver to bile (e.g., sulfobromophthalein and indocyanine green) (77–79). In transport mutant (GY or TR−) rats, hepatobiliary transport of GSH and intravenously injected metals, e.g., copper, zinc, and selenium, was severely impaired (35,80). The underlying mechanism for the GSH-dependent biliary excretion of those metals may involve
(a) complexation of metals with hepatic GSH, and (b) subsequent hepatobiliary transport of GSH-metal complexes mediated by cMOAT.

**SEQUESTRATION BY GS-X PUMP**

Plant GS-X pump plays a role in vacuolar sequestration of pigments, natural herbicides, allelochemicals and pathogen-related compounds (2, 3) (Fig. 1). Recent functional analyses of the maize gene, Bronze-2, which participates in anthocyanin pigment biosynthesis, have provided compelling evidence that endogenous compounds are metabolized and sequestered into the vacuole in a manner similar to xenobiotics (4). Anthocyanins share a common biosynthetic origin and core structure based on cyanidin-3-glucoside (81). It is through the species-specific modification of cyanidin-3-glucoside by hydroxylation, methylation, glucosylation and acetylation that the wide spectrum of red, blue, and purple colors in the vacuoles of flowers, fruits, and leaves is produced. Walbot and colleagues (4) have recently shown that the Bronze-2 (GZ-2) gene in maize encodes a GST gene responsible for conjugating anthocyanin with GSH. bz-2 mutants, on the other hand, are unable to pump pigments from the cytosol into the vacuole lumen, being due to defective in the glutathionation of anthocyanins (4). Thus, GS-conjugation of anthocyanin appears to be a prerequisite for the vacuolar accumulation of anthocyanin pigments and the vacuolar GS-X pump may transport the GS-conjugation of anthocyanin into the lumen.

Yeast and animal cells also sequestrate GS-conjugates from the cytosol into subcellular compartments. Using monochlorobimane, its fluorescent GS-conjugate was demonstrated to be sequestrated into endosomal intracellular vesicles in the primary culture of rat hepatocytes (82) and cisplatin-resistant HL-60/R-CP cells (67). In yeast cells, the fluorescent conjugate was accumulated in the central vacuole. The involvement of cMOAT, MRP1, and YCF1 in sequestration of GS-conjugates is supported by the following observations: (a) Monochlorobimane, a nonfluorescent compound, is specifically conjugated with GSH in the cell by the action of GSTs, and the resulting fluorescent GS-conjugates is a substrate for the GS-X pump. (b) When cultured normal and TR- rat hepatocytes were loaded with the GS-conjugate of bimane, both cell types displayed a strong cytosolic fluorescence. Normal rat cells completely lost the cytosolic fluorescence during incubation in monochlorobimane-free medium because of excretion of the GS-conjugate. However, fluorescent vesicles were observed in the perinuclear region and around a canaliculus. In contrast, TR-cells lost their cytosolic fluorescence more slowly and completely lacked vesicular fluorescence (82). (c) Likewise, the accumulation of fluorescence in intracellular vesicles was more prominent in HL-69/R-CP cells that in HL-60 cells. The cisplatin-resistant HL-60/R-CP cells expressed MRP1 gene at high levels. The vesicular accumulation of the fluorescent GS-conjugate in HL-60/R-CP cells was inhibited by ATP depletion (67). (d) In cultured hepatocytes and cisplatin-resistant HL-60/R-CP cells, vesicular fluorescence was significantly increased by preincubation with monensin or methylamine that interfere with
vesicular trafficking out of the trans-Golgi complex (82, 67), suggesting that GS-conjugates accumulated in intracellular vesicles are excreted by exocytosis. (e) DTY165 (wild type) cells exhibited the vacuolar accumulation of the fluorescent GS-conjugate, whereas such vacuolar accumulation was abolished in DTY167 (yclΔ strain) cells (13). The capacity for ATP-dependent, uncoupler-insensitive DNP-SG uptake was strictly associated with vacuolar membrane vesicles purified from DTY165 cells (13). These results support the idea that intracellular sequestration of GS-conjugate occurs in plant, yeast, and animal cells, and that the GS-X pump mediates "storage export" of GS-conjugates, and probably other multivalent organic anions (2).

**REGULATION OF GS-X PUMP GENE EXPRESSION**

At present, information on molecular mechanisms involved in the regulation of GS-X pump gene expression is substantially limited. GS-X pump activity in plant cells was shown to be significantly enhanced by CDNB, a substrate for GSTs (83). This increase of GS-X pump activity could be ascribed to enhanced *de novo* synthesis of GS-X pump protein and/or increased recruitment of GS-X pump molecules in the vacuolar membrane.

YCF1 in the yeast was shown to be induced by pretreatment with cadmium or CDNB (65). RNase protection assays of YCF1 expression in DTY165 cells revealed that YCF1 mRNA levels were increased by 1.9- and 2.5-fold by 200 μM CdSO4 and 150 μM CDNB, respectively. Concomitantly, the GS-X pump activity assessed by ATP-dependent GS2-CD and GS-DNP was increased to similar extents. Wu and Moye-Rowley have revealed that the YCF1 gene and the GSH1 gene encoding γ-GCS are coordinately regulated by γAP-1 which encodes yeast transcription factor AP-1 (84). Transcriptional activation of these genes mediated by γAP-1 is essential for cadmium resistance in yeast cells (85). Both organic electrophiles and heavy metals induce YCF1 expression and γAP-1 protein, a member of the basic-leucine zipper (BZIP) family of transcription factors, transcriptionally activates YCF1 and GSH1 genes (84, 86).

In cisplatin-resistant HL-60/R-CP cells, our group has demonstrated that MRP1 and γ-GCS genes are coordinately induced by cisplatin and heavy metals, such as arsenite, cadmium, and zinc (68). In addition, human glioma A172 cells pretreated with ACNU for 24 hours enhanced mRNA levels of both MRP1 and γ-GCS by 3- to 5-fold (87). These results strongly suggest that expression of MRP1 gene is closely related with cellular GSH biosynthesis and that certain common factor(s) may regulate the expression of both MRP and γ-GCS genes. Since *cis*-regulatory elements including AP-1 binding sites have been identified in the promoter regions of human γ-GCS and MRP genes (88–90) (Fig. 3), it is extremely interesting to examine whether AP-1 is responsible to transcriptionally regulate the expression of MRP1 and γ-GCS genes in mammalian cells.

The fact that MRP1 and γ-GCS can be coordinately induced by cisplatin, 1-(4-amino-2-methyl-5-pyridinyl)methyl-3-(2-chloroethyl)3-nitrosourea (ACNU), and heavy metals provide important information to our understanding of how
drug resistance genes are acutely induced upon drug treatments. Unlike cell culture studies where drug-resistant variants are usually obtained through continuous drug exposure, such transient induction of drug-resistance gene expression is more directly related to cancer chemotherapeutic protocols. Interestingly, human colorectal cancers frequently overexpress MRP1 mRNA (91) (Fig. 4). Because the patients involved in the study had not been treated with chemotherapeutic agents, it is suggested that up-regulation of different drug genes are associated with different human cancers and, more importantly, development of drug resistance in these cancers is an intrinsic mechanism. Because intrinsic drug resistance is the major factor controlling efficacy of chemotherapy in cancer treatment, a better understanding of how different drug-resistance genes are regulated in different tumor systems is of great importance for development of effective strategies to circumvent drug resistance in cancer chemotherapy.

MECHANISMS OF ANTITUMOR DRUG RESISTANCE MEDIATED BY MRP1/GS-X PUMP

Human MRP1 cDNA was originally isolated from a doxorubicin-selected, multidrug resistant lung cancer cell line (5). Overexpression of MRP1 has been subsequently observed in many doxorubicin–resistant cell lines. Furthermore, transfection of expression vectors harboring human MRP1 cDNA reportedly confers resistance to doxorubicin and many other antitumor drugs, e.g., vincristine, vinblastine, and VP-16, in otherwise drug-sensitive cell lines (70). These
results suggest the important role of MRP1 expression and drug resistance in cultured mammalian cells. To date, however, there is little knowledge about the mechanism by which GSH participates in MRP-mediated drug resistance. These antitumor agents are poor substrates in membrane vesicles transport assay prepared from MRP1-overexpressing cells and also poor inhibitors for LTC4 transport mediated by MRP1. On the other hand, in the presence of GSH, inhibitions of ATP-dependent transport of LTC4 by certain antitumor drug (e.g., vincristine, VP-16 and Taxol) can be enhanced (60). These findings are consistant, in part, with the notion that the presence of GSH can facilitate MRP-mediated drug transport.

It was reported that overexpression of MRP1 by transfection of its cDNA resulted in increased resistance to doxorubicin in the transfected cells. However, plasma membrane vesicles prepared from the transfected cells failed to display transport activity of doxorubicin (59, 60; cf. 92). In our study using membrane vesicles, doxorubicin per se was not the direct substrate for the GS-X pump encoded by the MRP1 gene. ATP-dependent transport of GS-conjugates and LTC4 in plasma membrane vesicles prepared from MRP1 cDNA-transfected cells are not inhibited by doxorubicin (93). Moreover, the 190-kDa protein in MRP1-overexpressing H69AR cells was not labeled with a photoaffinity analog of doxorubicin (93). On the other hand, cellular GSH has been shown to be a critical factor for the export of daunorubicin by MRP1 (71). Non-P-glycoprotein-
mediated resistance to doxorubicin is related to cellular GSH levels and GSH-metabolizing enzyme systems (94). Thus, it is conceivable that GSH may form conjugates with the antitumor drug prior to transport.

We previously proposed a hypothetical scheme to explain that glutathionated doxorubicin may be recognized by MRP1/GS-X pump as shown in Fig. 5. In this scheme, two-electron reduction of anthracyclines yields active intermediates. Reductive activation of anthracycline compounds is known to lead to covalent binding to proteins and nucleic acids (96). Mitochondrial sulfhydryl groups are modified by doxorubicin aglycones (97). In mammalian cells, two-electron reduction of quinone compounds is catalyzed by DT-diaphorase [NAD(P)H:quinone oxidoreductase], and this enzyme activity is greatly

![Figure 5](image-url)

**Fig. 5.** A putative metabolic pathway of the reductive bioactivation and GSH conjugation of doxorubicin as well as the subsequent transport of the GS-conjugate of doxorubicin aglycon.
increased in the preneoplastic state (98). The reduced doxorubicin readily
undergoes deglycosylation. The C-7 of the aglycone intermediate is electrophilic
and can react with nucleophiles, such as GSH, cysteine, N-acetyl cysteine, and
protein thiol groups. The reaction of the aglycone with GSH may be catalyzed by
GSTs. The GS-conjugates thus formed are recognized by the MRP1 as its
substrates and transported either immediately or after oxidation by molecular
oxygen and/or by autocatalysis with the original drug. This scheme indicates that
both the MRP1 and the metabolic enzyme system including DT-diaphorase and
GSTs, as well as intracellular GSH, may be critically involved in the mechanism
underlying doxorubicin resistance.

To test our hypothesis, we have prepared doxorubicin- and daunorubicin-
conjugates by linking the cysteine residue of GSH to aglycon of these anthracycl-
ines at C-7 or C-14 position. The conjugates were purified by HPLC and the
structures of the conjugates were confirmed by NMR and MS spectra. Being
consistent with our previous findings, doxorubicin and daunorubicin did not
inhibit ATP-dependent LTC4 transport in plasma membrane vesicles prepared
from MRP1-overexpressing SR3A cells. In contrast, GSH-conjugates of
doxorubicin- and daunorubicin-aglycons competitively inhibited LTC4 transport
(95), suggesting that glutathionation of anthracyclines, or addition of negative
charges on them, may facilitate the transport of these antitumor drugs in
MRP1-overexpressing cancer cells.

While glutathionation of doxorubicin and daunomycin may be a straightforward
mechanism of MRP1-mediated resistance to these antitumor drugs. It
remains possible that GSH may be transiently associated with these antitumor
drugs and/or MRP thereby creating favorable conformational conditions for drug
transport, in viewing the fact that GSH-doxorubicin complex has not been
detected in the culture medium of MRP1-overexpressing cells. In any event, the
molecular bases involved in MRP1-associated overexpressing cells. In any event,
the molecular bases involved in MRP1-associated drug resistance remain to be
critically investigated.

CONCLUDING REMARKS

GS-X pumps in plants and animals exhibits a striking resemblance in the
function and molecular structure, thereby comprising a novel ABC transporter
family. It is important to note that the GS-X pumps are expressed in normal
tissues and cell types and their function is closely associated with the cellular
protection mechanism. The glutathione moiety is an important property for
maximal affinity to the active site of the GS-X pumps, whereas the moiety itself is
not considered to be a structural determinant. Thus, GS-X pumps have a broad
substrate specificity toward different types of substrates that contain a large
hydrophobic moiety and two negative charges. Like cMOAT, human MRP1
transported not only GS-conjugates, glucuronide and sulfate conjugates, such as
17β-glucuronosyl estradiol, glucuronosyl etoposide, 3α-sulfatolithocholy taurine.
The GS-X Pump

These findings are in support of the idea that the GS-X pump is a member of the "phase III" detoxification system.

Recent molecular identification of the GS-X pump in human, rat, yeast and plants has shed light on the genetic diversity of the GS-X pump family. Regulation of GS-X pump gene expression is coming into the focus. Site-directed mutation techniques would provide powerful tool to study the gene regulation machinery, as well as the structure–activity relationship of the GS-X pump molecule. The next important goal would be to understand the molecular mechanism involved in the energy transfer within the pump molecule from ATP hydrolysis to the transport of organic anions. Furthermore, knock-out or transgenic animals and plants may uncover unknown physiological functions of the GS-X pump. Our recent study suggests that the GS-X pump may modulate cell cycle arrest of human cancer cells induced by anticancer prostaglandins (99,100). The GS-X pump is considered to be a novel and important target of drug discovery. In this context, we have just entered the new era of GS-X pump research that propels both basic and applied sciences.

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