ATP-dependent glutathione disulphide transport mediated by the *MRP* gene-encoded conjugate export pump

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We have previously shown that the multidrug resistance protein (MRP) mediates the ATP-dependent membrane transport of the endogenous glutathione conjugate leukotriene C_4 (LTC_4) and of structurally related anionic conjugates of lipophilic compounds [Jedlitschky, Leier, Buchholz, Center and Keppler (1994) Cancer Res. **54**, 4833–4836; Leier, Jedlitschky, Buchholz, Cole, Deeley and Keppler (1994) J. Biol. Chem. **269**, 27807–27810]. We demonstrate in the present study that MRP also mediates the ATP-dependent transport of GSSG, as shown in membrane vesicles from human leukaemia cells overexpressing MRP (HL60/ADR cells) or HeLa cells transfected with an *MRP* expression vector (HeLa T5 cells) in comparison with the respective parental or control cells. The K_m value for ATP-dependent transport of GSSG was 93 ± 26 μM (mean value $\pm$ S.D., $n = 5$) in membrane vesicles from HeLa T5 cells. GSH, at a concentration of 100 μM, was not a substrate for any significant ATP-dependent MRP-mediated transport. The transport of GSSG was competitively inhibited by LTC_4, by the leukotriene D_4 receptor antagonist 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio)propanoic acid (MK 571) and by *S*-decylglutathione, with K_i values of 0.3, 0.6 and 0.7 μM respectively. These studies identify MRP as the membrane glycoprotein which mediates the ATP-dependent export of GSSG from these cells.

INTRODUCTION

Glutathione plays an important role in the cellular defence against oxidative stress. GSH serves as a reductant in the metabolism of hydrogen peroxide and organic hydroperoxides catalysed by glutathione peroxidases. Increased oxidation of GSH to GSSG may be followed by its excretion from the cells [1]. In addition, GSH may be involved in the inactivation of electrophilic compounds by the formation of glutathione S-conjugates catalysed by glutathione S-transferases. Primary-active membrane transport of GSSG and of glutathione S-conjugates was originally established in inside-out oriented vesicles from human erythrocytes [2,3] and subsequently studied by kinetic measurements in the plasma-membrane fractions from various tissues [4–13]. Controversy persists with respect to the relationship between ATP-dependent transport of GSSG and of glutathione S-conjugates such as *S*-(2,4-dinitrophenyl)glutathione (DNP-SG). In a number of studies in several tissues, reciprocal inhibition of ATP-dependent transport of DNP-SG and GSSG has been reported [3–5,7–9,11], consistent with a common transport system. However, separate transport systems have been proposed as well [14].

Based on our previous work in mastocytoma cells [15], we demonstrated recently in human leukaemia cells overexpressing the multidrug resistance protein (MRP) and in HeLa cells transfected with an MRP expression vector, that MRP is an ATP-dependent export pump for the endogenous glutathione S-conjugate leukotriene C_4 (LTC_4) and for structurally related conjugates of lipophilic compounds with glutathione and several other anionic residues [16,17]. A similar conclusion was reached more recently by another group [18]. The *MRP* gene encodes an integral membrane glycoprotein belonging to the ATP-binding cassette (ABC) superfamily of membrane transporters [19,20]. Overexpression of MRP has been shown to confer a multidrug resistance phenotype on transfected cells [21] and has been found in a number of human tumour cell lines that do not overexpress the MDR1 P-glycoprotein [19,20,22-26]. Recently, examples of multidrug resistant cell lines overexpressing both MDR1 P-glycoprotein and MRP have also been described [27].

In the present study we used membrane vesicles from MRP-overexpressing cells, and the respective control cells, as a direct approach to investigate whether the *MRP* gene-encoded conjugate export pump mediates the ATP-dependent transport of GSSG in addition to several amphiphilic anionic conjugates established earlier as substrates for MRP-mediated ATP-dependent membrane transport [16,17].

MATERIALS AND METHODS

Materials

[14,15,19,20-^{3}H]LTC_4 (4.7 TBq/mmol) and [glycine-2-^{3}H]glutathione ([^{3}H]GSH; 1.5 TBq/mmol) were obtained from Du Pont–New England Nuclear (Boston, MA, U.S.A.). Unlabelled LTC_4 was from Amersham Buchler (Braunschweig, Germany). Unlabelled GSSG and *S*-decylglutathione were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The LTD_4 receptor antagonist 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio)propanoic acid (MK 571) [28] was provided by Dr. A. W. Ford-Hutchinson (Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). G418 (geneticin) was from Boehringer Mannheim Biochemica (Mannheim, Germany). Nitrocellulose

Abbreviations used: AMP-PCP, adenosine 5′-[β,γ-methylene]-triphosphate; BCM, bile canalicular membrane preparation; DTT, dithiothreitol; ABC, ATP-binding cassette; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; MRP, multidrug resistance protein (multidrug resistance-associated protein); HeLa C1 cells, HeLa cells transfected with the pRc/CMV vector [21]; HeLa T5 cells, HeLa cells transfected with a vector containing the MRP coding sequence [21]; HL60 cells, drug-sensitive parental human leukaemia cells; HL60/ADR cells, human leukaemia cells selected for resistance to adriamycin (doxorubicin) [23]; LTC_4, leukotriene C_4; MK 571, 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]thio)-propanoic acid.

filters (pore size 0.2 μm) were from Schleicher & Schüll (Dassel, Germany).

Preparation of [^{3}H]GSSG

After removal of dithiothreitol (DTT) from the [^{3}H]GSH solution by ethyl acetate extraction, [^{3}H]GSSG was synthesized non-enzymically in a 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM H_2O_2 [29]. The purity of [^{3}H]GSSG was tested by HPLC separation using an NH_2-Spherisorb column (cti, Idstein, Germany) and a water/methanol/acetic acid gradient for elution [30].

Cells

The non-P-glycoprotein-expressing doxorubicin-resistant HL60/ADR cells and the sensitive parental HL60 cells [23,31,32], as well as the transfected HeLa cells, were grown in RPMI medium with 10 % fetal calf serum in a humidified incubator (5 % CO_2, 37 °C). The doxorubicin-resistant HL60/ADR cells were cultured in the presence of 200 nM daunorubicin until 3 days before membrane preparation. HeLa cells transfected with the pRc/CMV vector (HeLa C1 cells) or with the vector containing the MRP coding sequence (HeLa T5 cells) were selected by their ability to grow in the presence of G418, which selected for cells that expressed the neomycin-resistance gene encoded by the pRc/CMV vector [21].

Preparation of plasma-membrane vesicles

The HL60 or HeLa cells were harvested from the cell culture by centrifugation and plasma-membrane vesicles were prepared as described [15–17,33]. In brief, the cells were lysed by incubation in hypotonic buffer [0.5 mM sodium phosphate (pH 7.0)/0.1 mM EDTA supplemented with proteinase inhibitors] for 1.5–2 h followed by homogenization with a Potter–Elvehjem homogenizer. After centrifugation of the homogenate at 12000 ***g*** (10 min, 4 °C) the post-nuclear supernatant was centrifuged at 100000 ***g*** (45 min, 4 °C). The resulting pellet was suspended in incubation buffer (250 mM sucrose/10 mM Tris/HCl, pH 7.4) homogenized with a tight-fitting Dounce homogenizer and layered over 38 % sucrose in 5 mM Hepes/KOH, pH 7.4. After centrifugation at 280000 ***g*** (2 h, 4 °C) the interfaces were collected, washed by centrifugation in the incubation buffer (100000 ***g***; 50 min, 4 °C) and passed 20 times through a 27-gauge needle for vesicle formation.

The membrane fraction enriched in bile canalicular membranes (BCM) from rat liver was prepared as described [34,35].

Vesicles transport studies

ATP-dependent transport of [^{3}H]GSSG into membrane vesicles was measured by rapid filtration [15]. Membrane vesicles (30 μg of protein from bile canalicular or HL60 cell membrane preparations and 20 μg of protein from HeLa cell membrane preparations) were incubated in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase and [^{3}H]GSSG, in an incubation buffer containing 250 mM sucrose and 10 mM Tris/HCl, pH 7.4. The GSSG concentration was 100 μM for the transport assays comparing the different cell types, and in the range 20–500 μM for determination of K_i and K_m values. Inhibitor concentrations are indicated in the legends of the respective Figures. The final incubation volume was 110 μl. Aliquots of 20 μl were taken at the times indicated and diluted in 1 ml of ice-cold incubation buffer. The diluted samples were filtered immediately through nitrocellulose filters (0.2 μm pore size), which were pre-soaked in incubation buffer, by the use of a rapid filtration device, and rinsed twice with 5 ml of incubation buffer. Filters were dissolved in liquid scintillation fluid and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of the non-hydrolysable ATP-analogue adenosine 5′-[β,γ-methylene]-triphosphate (AMP-PCP). Rates of net ATP-dependent transport were calculated by subtracting values obtained in the presence of AMP-PCP as a blank from those in the presence of ATP.

[^{3}H]GSH was tested as a substrate for transport at a concentration of 100 μM. Its purity and lack of contamination by [^{3}H]GSSG were controlled by HPLC. Transport assays for [^{3}H]GSH were performed in the presence of 100 μM DTT. At this concentration, DTT had no effect on the ATP-dependent transport of [^{3}H]LTC_4 and of [^{3}H]GSSG. In the experiments with [^{3}H]GSSG only the ATP-independent binding to the membranes and filters was reduced by DTT.

RESULTS

[^{3}H]GSSG transport by canalicular membrane vesicles from liver

Our assay conditions for ATP-dependent transport of [^{3}H]GSSG were optimized in BCM vesicles from rat liver, where GSSG transport had been established earlier [7,10]. [^{3}H]GSSG uptake into the membrane vesicles was due to an ATP-dependent and an ATP-independent process, as shown by the [^{3}H]GSSG accumulation in the presence of ATP as well as in the presence of the non-hydrolysable ATP analogue AMP-PCP (Figure 1). After 15 min of incubation with 100 μM [^{3}H]GSSG the vesicle-associated radioactivity was 6.4 ± 0.7 nmol·mg of protein^{-1} in the presence of ATP and 2.0 ± 0.7 nmol·mg of protein^{-1} in the presence of AMP-PCP. The initial rate of net ATP-dependent

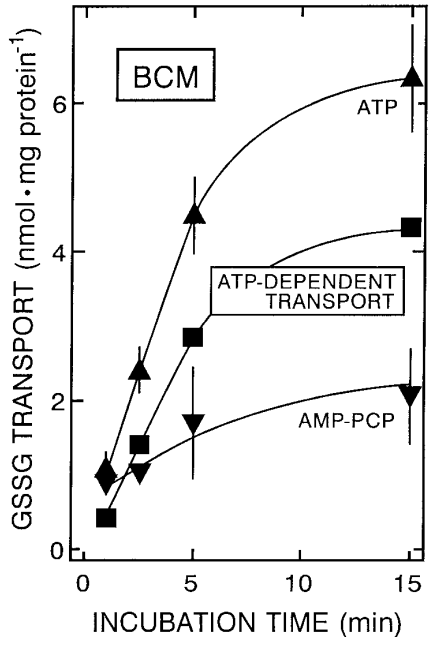

Figure 1 [^{3}H]GSSG transport into bile canalicular membrane vesicles (BCM) from rat liver

Membrane vesicles (30 μg of protein) were incubated with [^{3}H]GSSG (100 μM) and the vesicle-associated radioactivity was determined by a rapid filtration technique as described in the Materials and methods section. The rate of net ATP-dependent transport (■) was calculated by subtracting transport in the presence of 4 mM AMP-PCP (▼), as a blank, from transport in the presence of 4 mM ATP (▲). Data represent mean values ± S.D. from four experiments.

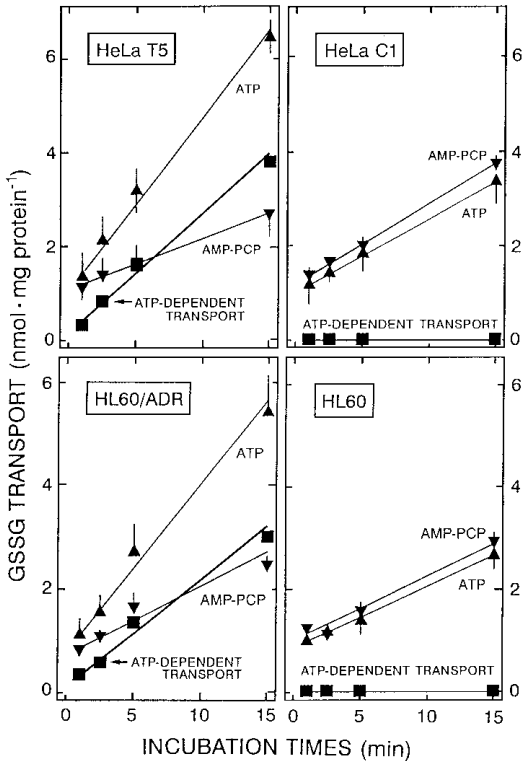

Figure 2 [^{3}H]GSSG transport into membrane vesicles from MRP-overexpressing and control cells

Membrane vesicles from HeLa cells transfected with the MRP expression vector (*HeLa T5*; upper left panel) or with the parental vector (*HeLa C1*; upper right panel), and from drug-selected *MRP*-overexpressing HL60/ADR (lower left panel) or parental HL60 (lower right panel) cells were incubated with [^{3}H]GSSG (100 μM). The vesicle-associated radioactivity was determined by a rapid filtration technique and the rate of net ATP-dependent transport (■) was calculated by subtracting transport in the presence of 4 mM AMP-PCP (▼), as a blank, from transport in the presence of 4 mM ATP (▲). Data represent mean values ± S.D. from four experiments.

transport, calculated by subtracting the transport rate in the presence of AMP-PCP from that in the presence of ATP, was 0.6 nmol·mg protein^{-1}·min^{-1} (Figure 1). These studies indicated the importance of the use of a non-hydrolysable ATP analogue replacing ATP in the blank assay for all [^{3}H]GSSG transport measurements. The K_m value for ATP-dependent [^{3}H]GSSG transport in rat liver BCM amounted to 111 μM. The LTD_4 receptor antagonist MK 571 was a competitive inhibitor of GSSG transport in BCM vesicles with a K_i value of 1.0 μM.

Comparison of [^{3}H]GSSG transport in MRP-overexpressing and control cells

Membrane vesicles from the MRP-overexpressing cells were used to investigate the involvement of the *MRP*-encoded conjugate export pump in the transport of GSSG. ATP-dependent [^{3}H]GSSG accumulation was determined in membrane vesicles from the MRP-overexpressing drug-selected HL60/ADR cells, from *MRP*-transfected HeLa T5 cells, as well as from the respective control cells (Figure 2). Membrane vesicles from the control cell lines showed no significant ATP-dependent [^{3}H]GSSG transport and, at a concentration of 100 μM [^{3}H]GSSG, the vesicle-associated radioactivity was in the same range in the presence of ATP and in the presence of AMP-PCP (Figure 2). After 15 min of incubation, 2.9 ± 0.2 nmol [^{3}H]GSSG·mg of protein^{-1} (AMP-PCP) and 2.7 ± 0.3 nmol [^{3}H]GSSG·mg of protein^{-1} (ATP) for membrane vesicles from HL60, and 3.7 ± 0.2 nmol·mg of protein^{-1} (AMP-PCP) and 3.4 ± 0.5 nmol·mg of protein^{-1} (ATP) for membrane vesicles from HeLa C1 cells, were measured. In contrast, membrane vesicles from both MRP-overexpressing cell lines showed an ATP-stimulated [^{3}H]GSSG transport into the vesicles (Figure 2). The [^{3}H]GSSG accumulation after 15 min of incubation was 5.5 ± 0.7 nmol·mg of protein^{-1} for HL60/ADR and 6.5 ± 0.3 nmol·mg of protein^{-1} for HeLa T5 cells. The ATP-independent [^{3}H]GSSG accumulation rates in the presence of AMP-PCP in membrane vesicles from these cell lines were in the same range as those in membranes from the respective parental or control cells (2.4 ± 0.2 nmol·mg of protein^{-1} for HL60/ADR and 2.7 ± 0.5 nmol·mg of protein^{-1} for HeLa T5). Net ATP-dependent transport rates of 0.2 nmol·min^{-1}·mg of protein^{-1} for HL60/ADR membranes and 0.23 nmol·min^{-1}·mg protein^{-1} for HeLa T5 membranes were calculated by subtracting the respective vesicle-associated radioactivity in the presence of AMP-PCP from values in the presence of ATP. [^{3}H]GSH, at a concentration of 100 μM, was not a substrate for ATP-dependent transport by HeLa T5 membrane vesicles under our experimental conditions.

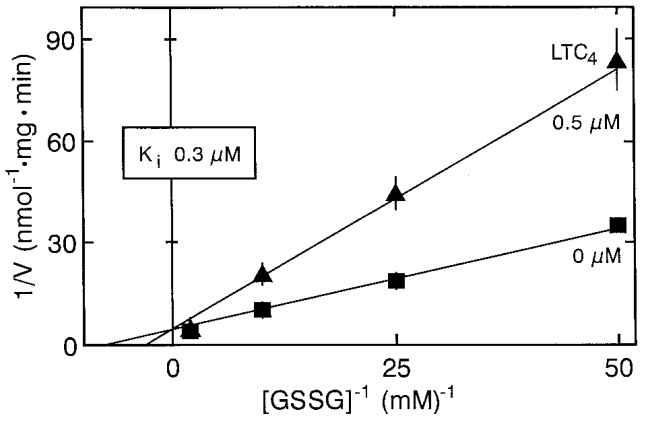

Figure 3 ATP-dependent transport of [^{3}H]GSSG into membrane vesicles from *MRP*-transfected HeLa T5 cells and its competitive inhibition by LTC_4

Double-reciprocal Lineweaver–Burk plot with GSSG concentrations ranging from 20 to 500 μM in the presence of 0.5 μM LTC_4. Mean values ± S.D. from four determinations.

Kinetic characterization of the MRP-mediated ATP-dependent [^{3}H]GSSG transport in membrane vesicles from MRP-overexpressing HeLa T5 and HL60/ADR cells

ATP-dependent MRP-mediated [^{3}H]GSSG transport in membrane vesicles from *MRP*-transfected HeLa T5 cells indicated a K_m of 93 ± 26 μM and a $V_{max.}$ of 0.5 ± 0.2 nmol·min^{-1}·mg of protein^{-1} (mean values ± S.D., $n = 5$). The K_m value for this transport in membrane vesicles from the MRP-overexpressing HL60/ADR cells was 64 ± 7 μM (mean value ± S.D., $n = 3$).

Inhibition of MRP-mediated ATP-dependent [^{3}H]GSSG transport by LTC_4 and structural analogues

Established substrates and inhibitors [16,17] served in the further characterization of MRP-mediated [^{3}H]GSSG transport. LTC_4, an endogenous glutathione conjugate and high-affinity MRP substrate, inhibited [^{3}H]GSSG transport in membrane vesicles from *MRP*-transfected HeLa T5 cells competitively with a K_i of

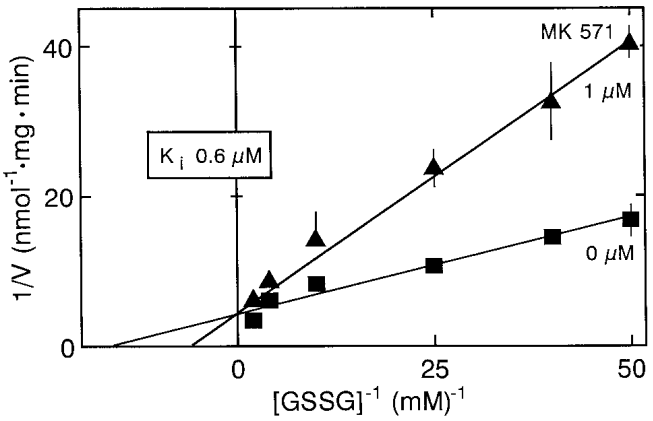

Figure 4 ATP-dependent transport of [^{3}H]GSSG into membrane vesicles from MRP-overexpressing HL60/ADR cells and its competitive inhibition by the LTD_4 receptor antagonist MK 571

Double-reciprocal Lineweaver–Burk plot with GSSG concentrations ranging from 20 to 500 μM in the presence of 1 μM MK 571. Mean values $\pm$ S.D. from four determinations.

0.3 μM (Figure 3). On the other hand, MRP-mediated transport of [^{3}H]LTC_4 at a concentration of 50 nM was inhibited by GSSG with an IC_{50} value of about 180 μM. Furthermore, ATP-dependent [^{3}H]GSSG transport in membrane vesicles from MRP-overexpressing HL60/ADR cells was studied in the presence of the LTD_4 receptor antagonist MK 571, a potent inhibitor of MRP-mediated LTC_4 transport [15–17,33] and in the presence of the synthetic amphiphilic glutathione S-conjugate, *S*-decylglutathione. These compounds inhibited [^{3}H]GSSG transport competitively with K_i values of 0.6 μM for MK 571 (Figure 4) and 0.7 μM for *S*-decylglutathione (results not shown).

DISCUSSION

Kinetic studies by different groups using plasma-membrane vesicles from entirely different cell types, including hepatocyte canalicular membranes, suggested the existence of a common transport system for GSSG and glutathione S-conjugates [3–5,7–9,11]. The identification of MRP as a glutathione S-conjugate export pump [16–18] and the availability of transfected cell lines overexpressing MRP [21] has now allowed the establishment of the identity of this ATP-dependent transport system for GSSG (Figure 2). We have demonstrated the direct correlation between MRP expression and ATP-dependent GSSG transport, using membrane vesicles from MRP-overexpressing *MRP*-transfected as well as drug-selected cells, in comparison with membrane vesicles from the respective control cells (Figure 2). ATP-dependent transport of DNP-SG across the hepatocyte canalicular membrane is also competitively inhibited by GSSG [4,7] and, as analysed during the present study, by the LTD_4 receptor antagonist MK 571 ($K_i = 1$ μM). Furthermore, both GSSG and DNP-SG are not secreted into the bile of transport-deficient GY/TR$^-$ mutant rats [36], which lack the ATP-dependent conjugate transport in the canalicular membrane [6]. It will be of interest, therefore, to demonstrate whether an MRP homologue is expressed in the canalicular membrane of rat liver. For the analysis of accurate ATP-dependent GSSG transport the use of a non-hydrolysable ATP analogue, such as AMP-PCP, as a blank was important, both for hepatocyte canalicular membrane vesicles (Figure 1) and for membrane vesicles from MRP-overexpressing tumour cell lines, in order to correct for ATP-independent transport and/or non-specific binding of [^{3}H]GSSG to the membranes.

Potent competitive inhibition of ATP-dependent GSSG transport by established substrates and inhibitors of MRP-mediated transport further supports our conclusion that GSSG is transported by MRP (Figures 3 and 4). MK 571 acts as a potent inhibitor of MRP-mediated transport [16,17] and LTC_4 is a high-affinity substrate with a K_m of 97 nM [17]. Both compounds were competitive inhibitors of ATP-dependent GSSG transport with K_i values of 0.6 and 0.3 μM respectively (Figures 3 and 4).

For both GSSG and DNP-SG, distinct high- and low-affinity transport systems have been kinetically characterized [2,3,8,11–13,37]. However, in view of the GSSG concentrations in intact cells [38], the transport system with the low K_m would be the predominantly active one under most conditions. The K_m values determined for MRP-mediated ATP-dependent GSSG transport in membrane vesicles from the MRP-overexpressing HL60/ADR and HeLa T5 cells were 64 ± 7 and 93 ± 26 μM respectively. This difference between the K_m values for the two cell lines may be due to differences in the membrane composition of both vesicle preparations rather than to differences in MRP itself. The K_m values are in the same range as the K_m for the high-affinity ATP-dependent GSSG transport system in membrane vesicles from human erythrocytes [2].

Candidate proteins involved in the transport of glutathione S-conjugates have been isolated on the basis of an ATPase activity stimulated by GSSG or DNP-SG [39,40]. These proteins with molecular masses of 38–82 kDa are smaller than typical transmembrane transporters of the ABC family [41]. Furthermore, a direct link between the ATP-dependent transport and the isolated ATPase was not established.

MRP, which represents an ATP-binding transmembrane glycophosphoprotein of about 190 kDa, was identified as a cause of non-P-glycoprotein-mediated resistance to anti-cancer drugs, including anthracyclines [19–21,23,25,26]. The mechanism of anthracycline-induced cytotoxicity appears to be linked in part to its enzymic reductive activation to a semiquinone free radical, with subsequent generation of toxic radicals [42]. Increased oxidation of GSH by glutathione peroxidase may lead to enhanced formation of GSSG, which is now shown to be a substrate for the *MRP* gene-encoded conjugate export pump transporting GSSG into the extracellular space (Figure 2). Further studies should clarify whether additional proteins exist for the membrane transport of GSSG or whether the *MRP* gene encodes the only type of GSSG transporter in mammalian cells.

This work was supported in part by the Deutsche Forschungsgemeinschaft through SFB 352, Heidelberg, Germany.

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Received 29 August 1995/18 October 1995; accepted 30 October 1995