The ATP-dependent glutathione S-conjugate export pump, named GS-X pump, has been shown to eliminate a potentially cytotoxic glutathione-platinum (GS-Pt) complex from tumor cells, thereby modulating glutathione (GSH)-associated resistance to cis-diaminedichloroplatinum(II) (cisplatin) (Ishikawa, T., and Ali-Osman, F. (1993) J. Biol. Chem. 268, 20116-20125). The present study provides evidence that the GS-X pump is functionally overexpressed in cisplatin-resistant human promyelocytic leukemia HL-60 (HL-60/R-CP) cells, in which the cellular GSH level was substantially enhanced. Indeed, the rate of ATP-dependent transport of the GS-Pt complex, measured with plasma membrane vesicles, was about 4-fold greater in HL-60/R-CP cells than in HL-60 cells. Three membrane proteins with apparent molecular masses of 200, 110, and 70 kDa were overexpressed in HL-60/R-CP cells, whereas F-glycoprotein (MDR1) was not immunologically detected in the membrane preparations from resistant and sensitive cells. Unlike in HL-60 cells, increased numbers of intracellular vesicles were observed in the cytoplasm of HL-60/R-CP cells. Fluorescence microscopy with syn-(CH₂CH₂)-1,5-diazabicyclo[3.3.0]-octa-3,6-dione-2,8-dione (monochlorobimane) revealed that the fluorescent glutathione S-conjugate accumulated in intracellular vesicles of the cisplatin-resistant cells in an energy-dependent manner. The GS-X pump is suggested to contribute to vesicel-mediated excretion of GSH-drug conjugates from cells. In addition, both HL-60 and HL-60/R-CP cells underwent cell differentiation in response to 12-O-tetradecanoylphorbol-13-acetate, retinoic acid, and dimethyl sulfoxide, resulting in proliferation arrest as well as a remarkable decrease in the c-myc mRNA levels. After cell differentiation, a significant decrease was observed in the activity of ATP-dependent transport of the GS-Pt complex in membrane vesicles prepared from both HL-60/R-CP and HL-60 cells. These results suggest that the expression of the GS-X pump in both cisplatin-resistant and -sensitive cells is related to cell proliferation.

The GS-X pump¹ is a novel ATP-dependent transporter that mediates the release of glutathione disulfide (GSSG), glutathione S-conjugates, cysteiny1 leukotrienes, and certain organic anions from normal and cancer cells (1). The function of the GS-X pump is critically linked to a variety of biological phenomena, such as oxidative stress, detoxification, inflammation, and modulation of cell proliferation (see Ref. 2 for recent review). The export of glutathione S-conjugates from cells is important not only in interorgan metabolism of glutathione (GSH) but also in reducing the intracellular accumulation of potentially cytotoxic GSH conjugates. Thus, the GS-X pump is called the "phase III" detoxification system for biotransformation of endo- and xenobiotics (2).

Accumulating evidence suggests that cellular GSH is a critical determinant in the tumor cell resistance to chemotherapeutic agents, such as nitrogen mustard, chloroethy1 nitrosoureas, and cisplatin (see Refs. 3-6 for review). Evidence that several GSH-drug conjugates are potentially cytotoxic suggests that the elimination of GSH-drug conjugates from tumor cells is an important factor for the cellular toxicity of anticancer drugs (7). We have recently shown that cisplatin reacts with intracellular GSH and that the resulting glutathione-platinum (GS-Pt) complex is actively exported from leukemia cells via the GS-X pump (7). Since the GS-Pt complex is a potential inhibitor for protein synthesis, the function of the GS-X pump is considered to modulate the resistance of human cancers to cisplatin (7, 8).

Cisplatin is an effective antitumor agent for treating various human cancers of the brain, head and neck, ovary, testis, and bladder (9). Its antitumor activity is attributed primarily to its ability to form DNA-platinum cross-link adducts (10, 11). Despite its clinical effectiveness, cellular drug resistance is a significant obstacle to a long term, sustained patient response to cisplatin-based therapy. Intracellular GSH would be a significant determinant in the resistance and cytotoxicity of cisplatin (12, 13); however, its exact molecular mechanisms are not fully understood. In the present study, we examined the role of the GS-X pump in cisplatin resistance, using a cisplatin-resistant variant of human leukemia HL-60 cells as a model system. Here we provide evidence that the GS-X pump is functionally overexpressed in cisplatin-resistant HL-60 cells and that the GS-X pump plays a significant part in vesicle-mediated excretion of GSH-drug conjugates from resistant cells.

Furthermore, we investigated the effect of cell differentiation on the activity of the GS-X pump to examine a potential link between the functional overexpression of the GS-X pump and cell proliferation. The HL-60 leukemia cell line (14) provides a useful model system for studying the role of proto-oncogenes in drug resistance as well as in cellular proliferation and differ-

¹ The abbreviations used are: GS-X pump, ATP-dependent glutathione S-conjugate export pump; cisplatin, cis-diaminedichloroplatinum(II); Me,S0, dimethyl sulfoxide; GS-Pt, bis(glutathionato)-platinum(II); LTC₄, leukotriene C₄; MRP, multidrug resistance-related protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoreses; TPA, 12-O-tetradecanoylphorbol-13-acetate.

(Received for publication, April 4, 1994, and in revised form, September 2, 1994)
entiation. Several mutations in specific proto-oncogenes have been identified in HL-60 cells (15). In particular, the c-myc gene is amplified 8- to 30-fold and highly expressed (16, 17), and this is associated with N-ras gene activation (18) and deletion of the p53 gene (19). HL-60 cells differentiate along the granulocytic pathway when they are treated with dimethyl sulfoxide (20, 21) or retinoid acid (22), and along the monocytic pathway when treated with phorbol esters (23) and vitamin D analogues (24). In the present study, the activity of the GS-X pump was found to be down-regulated by cell differentiation. Based on our results, we discuss a possible relationship between the expression of the GS-X pump and cell proliferation.

MATERIALS AND METHODS

Biochemicals, Enzymes, and Cells—GSH, GSSG, ATP, creatine phosphate, creatine kinase, phenylmethylsulfonyl fluoride, 1-γ-glutamyl-3-carboxyl-4-nitroanilide, and a random-primer labeling kit were purchased from Boehringer Mannheim (Mannheim, Germany). [3H]GSH and [3H]JTC were obtained from DuPont NEN. [3H]GTP was from Amersham Corp. Cisplatin was from Bristol-Myers Co. (Evansville, IN). RPMI 1640 medium, fetal calf serum, gentamycin, Me₂SO, all-trans-retinoic acid, TPA, acivicin, doxorubicin, vincristine, AMP, Sigma 104, succinic acid, p-nitrophenyl thymidine-5′-monophosphate acetate, phenolphthalein-glucuronic acid, and sodium dodecyl sulfate from Boehringer Mannheim (Mannheim, Germany). [3H]GSH was generated from [3H]GSH by a random priming method. Hybridization with the DNA probe (1 × 10⁶ counts/min/ml) was performed for 42 h at 37 °C in a mixture containing 5 × SSPE (750 mM sodium chloride, 5 mM EDTA, and 50 mM sodium phosphate, pH 7.4), 50% formamide, 5 × Denhardt's solution, 0.1% SDS, 10% dextran sulfate, and 200 μg/ml denatured salmon sperm DNA. After hybridization, the membrane was washed in 0.1% SDS, 2 × SSC (300 mM sodium chloride and 30 mM sodium citrate, pH 7.0) solution at room temperature for 20 min and subsequently in 0.1% SDS, 0.1 × SSC (15 mM sodium chloride and 1.5 mM sodium citrate, pH 7.0) solution at 55 °C for 30 min. The membranes were exposed to Kodak X-Omat AR films at −85 °C using intensifying screens.

Fluorescence Microscopy—Cells were incubated with 20 μM monochlorobimane (30) at 37 °C for 20 min. Subsequently, the cells were washed with ice-cold Hank's balanced salt solution and incubated in the monochlorobimane-free incubation medium at 37 °C. Next, the cells were placed on glass slide plates and observed without fixation under an epifluorescence Microscope, Vanox AH-2, equipped with a 20U1 excitation filter and a 17L420 barrier filter. The photographs were taken with Kodak Ektachrome ASA 400 film.

Flow Cytometry—The relative fluorescence intensity of cells was measured with an EPICS Elite fluorescence flow cytometer (Coulter Corp., Hialeah, FL) equipped with an air-cooled helium-cadmium laser for excitation and an 470-nm long-pass filter for emission. The emission intensity was converted from log fluorescence to linear fluorescence intensity. Every observation, at least 1 × 10⁴ cells were subjected to the flow cytometry.

Preparation of Plasma Membrane Vesicles from HL-60 Cells—In each preparation, HL-60 cells (5 × 10⁶ cells) were harvested by centrifugation and suspended in 50 ml of ice-cold PBS. After centrifugation at 40 × g for 5 min, the cell pellet was diluted 40-fold with a hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride). The cell lysate was then centrifuged at 100,000 × g for 30 min, and the resulting pellet was suspended in the hypotonic buffer and homogenized with a Potter-Elvehjem homogenizer. The crude membrane fraction was layered over 38% sucrose solution and centrifuged at 100,000 × g for 30 min. The turbid layer at the interface was collected, suspended in 250 μM sucrose containing 10 mM Tris-HCl, pH 7.4, and centrifuged at 100,000 × g for 20 min. The membrane fraction was collected and resuspended in a small volume (50-100 μl) of 250 mM sucrose containing 10 mM Tris-HCl, pH 7.4. Vesicles formed by passing the suspension through a 27-gauge needle were frozen in liquid N₂ and stored at −85 °C until used. Sialidase accessibility for the determination of inside-out vesicles was examined as described previously (21).

Measurement of Marker Enzyme Activities—γ-Glutamyl transferase activity was determined according to Tate and Meister (32) using 1-γ-glutamyl-3-carboxyl-4-nitroanilide and glycylglycine. The reaction was measured spectrophotometrically at 407 nm (ε = 9.9 mmols⁻¹ cm⁻¹) (33). 5′-Nucleotidase, alkaline phosphatase, and succinate dehydrogenase were assayed according to Golish et al. (34). Alkaline phosphodiesterase activity was determined according to Edelson and Erbs (35) using p-nitrophenyl thymidine-5′-monophosphate acetate. The reaction was measured by following the liberation of p-nitrophenol by 440 nm (ε = 12.0 mmols⁻¹ cm⁻¹) (36). β-Glucuronidase activity was determined according to Breitman et al. (37) using phenolphthalein-glucuronic acid, where phenolphthalein generated from the reaction was spectrophotometrically measured at 550 nm (ε = 26.6 mmols⁻¹ cm⁻¹) (38). All the assays were performed at 37 °C. Protein concentration was determined according to the protocol of the BCA protein assay provided by Pierce.
tion was carried out at 37 °C and the amount of \(^{3}H\)GS-Pt complex incorporated into the vesicles was measured by a rapid filtration technique as described previously (31).

Electrophoresis and Immunoblotting—SDS-PAGE was carried out according to the method of Laemmli (39) using 10%-or 12%-acylamide gels. After electrophoresis, proteins were either stained by Coomassie Blue R-250 or electrophoretically transferred to Immobilon™-P membranes (Millipore, Bedford MA) in a solution containing 25 mM Tris, 192 mM glycine, pH 8.3, at 4 °C with a constant current of 150 mA for 8 h, according to the method of Towbin et al. (40). The active site of the protein-transferred membrane was blocked by 3% (v/v) gelatin in 0.9% NaCl containing 10 mM Tris-HCl, pH 7.7, and 0.05% (v/v) Tween 20 at 37 °C for 2 h. The membrane was then incubated with a rabbit polyclonal antibody raised against P-glycoprotein (Oncogene Science, Manhasset, NY) in 0.9% NaCl, 10 mM Tris-HCl, pH 7.4, 1% gelatin, 0.05% Tween 20 at 37 °C for 2 h. After washing the membrane with the same buffer, the membrane was incubated with goat anti-(rabbit-IgG)-horseradish peroxidase conjugate (Oncogene Science) in the same buffer, the membrane was incubated with goat anti-(rabbit-IgG)-horseradish peroxidase conjugate (Oncogene Science) in the same buffer at 37 °C for 2 h.

Fig. 1. Dose-response relationships for HL-60 (■) and HL-60/R-CP (⊙) cells to cisplatin and doxorubicin. The cells (0.15 x 10^6 cells/ml) were incubated in 100 μl of the culture medium containing cisplatin (0, 0.3, 1, 3, 10, 30, and 100 μM) or doxorubicin (0, 0.03, 0.1, 0.3, 1, 3, and 10 μM) in 96-well plates in a humidified tissue-culture chamber (37 °C, 5% CO₂). After 72 h, the cell density of surviving cells (trypan blue-negative) was counted with a hemocytometer. C₅₀ is the cell density after treatment with cisplatin or doxorubicin at concentration C, the cell density of the untreated control. 1.13 x 10^6 cells/ml for HL-60 cells and 1.18 x 10^6 cells/ml for HL-60/R-CP cells after the 72-h incubation. Data are expressed as mean ± S.E. (n = 3).

The activity of glutathione S-transferase toward 1-chloro-2,4-dinitrophenol was 143 ± 10 and 139 ± 12 nmol/min x mg protein⁻¹ (n = 3) in HL-60 and HL-60/R-CP cells, respectively. Myeloperoxidase activity determined with guaiacol was 13.8 ± 0.9 and 12.2 ± 1.1 units x mg protein⁻¹ (n = 3) in HL-60 and HL-60/R-CP cells, respectively (1 unit = [ΔAbsorbance at 470 nm] x min⁻¹). Thus, between the cisplatin-sensitive and -resistant cells, no significant difference was observed in those enzyme activities.

ATP-dependent Transport of GS-Pt Complex in Cisplatin-resistant and -sensitive Cells—In our previous study, the transport of the GS-Pt complex across the plasma membrane of murine leukemia L1210 cells was found to be an ATP-dependent process mediated by the GS-X pump (7). In order to determine the ATP-dependent transport of the GS-Pt complex in cisplatin-sensitive and -resistant cells, plasma membrane vesicles were prepared from HL-60 and HL-60/R-CP cells by sucrose-density gradient centrifugation. The enrichment of the plasma membrane in the preparations was examined by measuring the specific activities of marker enzymes (Table I). The specific activities of plasma membrane enzymes, i.e. γ-glutamyl transferase, alkaline phosphatase, alkaline phosphodiesterase, and 5'-nucleotidase, in the membrane preparations were 20-27-fold higher than those in the cell homogenate, whereas the specific activity of succinate dehydrogenase, a mitochondrial enzyme, was about 10% of the cell homogenate. As indicated by the activity of β-glucuronidase, the lysosome membrane was slightly contaminated in the plasma membrane preparations. Taken together, these results suggest a high enrichment of the plasma membrane in the membrane preparations from both HL-60 and HL-60/R-CP cells. Based on the sialidase accessibility, 45-55% of the total population of the membrane vesicles was estimated to be inside-out.

Fig. 2 demonstrates the time courses of transport of the \(^{3}H\)-labeled GS-Pt complex into membrane vesicles prepared from HL-60/R-CP and HL-60 cells. The rate of the ATP-dependent transport of the GS-Pt complex was about 4-fold greater in the membrane vesicles from HL-60/R-CP cells than in those from HL-60 cells, whereas the apparent K₅₀ value for the GS-Pt complex was unchanged (130 μM for the membrane vesicle preparations from resistant and sensitive cells). In addition, ATP-dependent transport of LTP₄, an endogenous substrate of the GS-X pump (1), was also 5-fold higher in the membrane vesicles from HL-60/R-CP cells than in those from HL-60 cells (data not shown). These results suggest that the GS-X pump was functionally overexpressed in the cisplatin-resistant cells.
HL60 and HL-GO/R-CP cells were determined as described under "Materials and Methods." The specific activities are expressed as mean ± S.E., n = 3. In parenthesis, the relative enrichment is shown as the ratio of the specific activity of the corresponding enzyme in the plasma membrane preparations over the specific activity in the homogenates.

**Table 1**

| Marker enzyme         | Specific activity in plasma membrane preparation |
|-----------------------|--------------------------------------------------|
|                       | HL-60 | HL-60/R-CP |
| Plasma membrane       |       |           |
| γ-Glutamyltransferase | 148 ± 21 (21.4) | 488 ± 47 (27.1) |
| Alkaline phosphodiesterase | 8.3 ± 1.0 (21.2) | 9.8 ± 1.1 (25.1) |
| Alkaline phosphatase  | 8.4 ± 0.6 (23.1) | 7.3 ± 0.6 (22.5) |
| 5'-Nucleotidase       | 2.1 ± 0.2 (24.8) | 1.7 ± 0.2 (20.4) |
| Lysosome membrane     |       |           |
| β-Glucuronidase       | 2.2 ± 0.1 (3.4) | 1.8 ± 0.1 (3.4) |
| Mitochondria inner membrane |       |           |
| Succinate dehydrogenase | 0.5 ± 0.1 (0.12) | 0.6 ± 0.1 (0.11) |

**Fig. 2** ATP-dependent uptake of GS-Pt complex by membrane vesicles from HL-60 and HL-60/R-CP cells. Membrane vesicles from HL-60 and HL-60/R-CP cells were prepared as described under “Materials and Methods.” [3H]GS-Pt complex was synthesized by incubating cisplatin with [3H]GSH and isolated by QAE-Sephadex anion-exchange chromatography. The standard incubation medium for the transport experiment contained the membrane vesicles (50 μg of protein), 200 μM [3H]GS-Pt complex, 0.25 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM creatine phosphate, and 100 μg/ml creatine kinase and either 1 mM (C) or 0 μM (A) ATP, in a final volume of 110 μl. The reaction was started by adding the [3H]GS-Pt complex to the incubation medium. The reaction was carried out at 37°C, and the [3H]GS-Pt complex incorporated into the vesicles was measured by a rapid filtration technique. Data are expressed as mean ± S.E. (n = 3).

**Fig. 3** Alteration of plasma membrane proteins in HL-60/R-CP cells. A, SDS-PAGE of plasma membrane preparations from HL-60 and HL-60/R-CP cells. Plasma membranes were prepared by sucrose-density gradient centrifugation as described under “Materials and Methods.” The membrane proteins (a total of 18 μg) were separated by SDS-PAGE (12% acrylamide gel) and stained with Coomassie Blue R-250. B, immunoblot analysis of P-glycoprotein (P-gp) in plasma membranes of HL-60/R-CP and C11D/VCV cells. Membrane proteins (a total of 18 μg) were separated by SDS-PAGE (10% acrylamide gel). Thereafter, proteins were either stained with Coomassie Blue or electrophoretically transferred to an Immobilon membrane. P-glycoprotein on the membrane was detected by a specific antibody as described under “Materials and Methods.”

**Fig. 4** Microscopic photographs of HL-60 and HL-60/R-CP cells after May-Giemsa’s staining. Magnification, x800.

Alterations in Membrane Proteins in Cisplatin-resistant Cells—SDS-PAGE of the plasma membrane preparations from HL-60 and HL-60/R-CP cells demonstrated that at least three membrane proteins with apparent molecular masses of 200, 110, and 70 kDa, respectively, were overexpressed in the cisplatin-resistant cells (Fig. 3A). Immunoblot analysis showed that P-glycoprotein was not expressed in the cisplatin-resistant cells, whereas it was clearly expressed in the plasma membrane from vincristine-resistant C11D cells (C11D/VCV), as the positive control (Fig. 3B).

**Fig. 3A**

**Fig. 3B**

**Fig. 4**

**Biological Function of Intracellular Vesicles in Cisplatin-resistant Cells**—We have tested the above stated hypothesis by fluorescence microscopy. Monochlorobimane (30), a nonfluorescent compound, is specifically conjugated with GSH in the cell by the action of glutathione S-transferases, and the resulting glutathione S-conjugate exhibits intense fluorescence (excitation at 370–385 nm; emission at 477–484 nm) (42, 43). Since the GS-X pump has a broad substrate specificity toward a variety of glutathione S-conjugates (1, 2), the compound made level was significantly increased in the HL-60/R-CP cells of this study. This result (Fig. 4) therefore implied a potential link between the formation in the present intracellular vesicles and the GSH-associated biotransformation of cisplatin in HL-60/R-CP cells. Based on our current knowledge, it could be hypothesized that the GS-X pump formed in cells accumulates in the vesicles and is subsequently excreted via exocytosis and that the GS-X pump is supposed to be involved in the transport of the GS-Pt complex from the cytosol space into the vesicles.
A jugate was inhibited by monochlorobimane at intracellular vesicles. Olympus Fluorescence Microscope, Vanox AH-2. B, effect of ATP depletion (left) and monensin (right) on vesicular accumulation of the glutathione-bimane conjugate in HL-60/R-CP cells. The cells were incubated with 20 μM monochlorobimane at 37 °C for different periods as indicated (0, 45, and 90 min). Fluorescence photographs were taken in the same manner as described in Fig. 5.

was more prominent in HL-60/R-CP cells than in HL-60 cells. Fig. 6 shows the fluorescence microscopy results of the sensitive and resistant cells at 0, 45, and 90 min. Just after the incubation with monochlorobimane, the fluorescence was detected almost homogeneously within the cells (time, 0 min; Fig. 6). After 45 min, the accumulation of fluorescence in the vesicles was observed in about 50% of the total resistant cells, and after 90 min, it was detected in almost 100% of the cells. It is very important to note that the fluorescence intensity of HL-60/R-CP cells decreased dramatically during this period (0–90 min), although the initial fluorescence intensity in the resistant cells was even higher than that in HL-60 cells because of the higher intracellular GSH content (Fig. 6). This is more clearly shown by flow cytometry as a shift of the fluorescence intensity peak (Fig. 7). In the sensitive parent cells, on the other hand, the fluorescence intensity and its intracellular distribution was only slightly changed during the same period (0–90 min) (Figs. 6 and 7). Thus, these data strongly suggest that the cisplatin-resistant cells excrete the glutathione S-conjugate more effectively than the sensitive parent cells. In addition to the above-mentioned observations, just after incubation with monochlorobimane, HL-60/R-CP cells exhibited a greater heterogeneity in the fluorescence intensity than HL-60 cells (0 min in Fig. 7). This suggests differences in single-cell GSH content and/or in the rate of glutathione S-conjugate efflux among the cells. Likewise, heterogeneity in single-cell GSH content was previously reported in cells obtained by disaggregation of a biopsy of human renal cell carcinoma (42).

**Effect of Retinoic Acid, MeSO, and TPA on Cisplatin-resistant Cells**—The doubling times of HL-60 and HL-60/R-CP cells were 29.8 ± 0.2 and 30.1 ± 0.2 h, respectively; there was no significant difference in their proliferation rates. By far, it had been well documented that HL-60 cells differentiate along the granulocytic pathway when they are treated with retinoic acid (22) and Me2SO (20, 21) or along the monocytic pathway when
treated with TPA (23). However, it was not known whether the cisplatin-resistant cells established in this study maintain such properties or have lost, through the selection process, the biological response to those differentiation-inducing agents. Thus, the potency of HL-60/R-CP cells for cell differentiation was examined as below.

When HL-60/R-CP cells were incubated with retinoic acid, Me$_2$SO, or TPA, the cells arrested their proliferation and, concomitantly, the mRNA level of c-myc decreased substantially. Northern blot analysis clearly demonstrated that the mRNA level of c-myc started to decrease 1 h after the onset of incubation with 1 μM all-trans-retinoic acid (Fig. 6A). After 6 h, c-myc mRNA had diminished almost completely. The mRNA level of β-actin remained constant throughout the incubation period (up to 12 h) (data not shown). Similar results were also obtained by incubation with 1.3% (v/v) Me$_2$SO. When the cells were incubated with 32 μM TPA, the c-myc expression level dropped remarkably between 2 and 6 h of the incubation period (Fig. 6B). The response of HL-60/R-CP cells to these differentiation-inducing agents was virtually identical to that observed for HL-60 cells. Thus, these results confirm that the cisplatin-resistant cells maintain the biological properties of the sensitive parent cells with respect to cell proliferation and differentiation.

Effect of Cell Differentiation on GS-X Pump Activity—Based on the results we obtained regarding cell differentiation, we examined the effect of differentiation on the activity of the GS-X pump in both HL-60 and HL-60/R-CP cells. Cell differentiation was induced by retinoic acid, Me$_2$SO, or TPA, as described above. After 72 h, the activity of ATP-dependent transport of the GS-Pt complex in the plasma membrane vesicles was determined. Table II summarizes the effect of the differentiation-inducing agents on the transport activity. After cell differentiation, the activity of ATP-dependent transport of GS-Pt complex in plasma membrane vesicles significantly decreased in both HL-60 and HL-60/R-CP cells. In particular, in HL-60 cells, a remarkable decrease was observed with retinoic acid; the transport activity in the differentiated cells was 30% of that of the undifferentiated cells. This decrease was not due to different populations of inside-out vesicles in the preparations (inside-out vesicles, 52% for differentiated cells; 46% for undifferentiated cells).

DISCUSSION

Drug resistance is a biological response of tumor cells to chemotherapeutic agents and represents a constitut of different resistance determinants. Identifying and modulating these determinants is important for understanding the biological nature of drug resistance and solving the associated problems in human cancer chemotherapy. Recent studies of the multidrug resistance phenotype of tumor cells have led to the discovery of P-glycoprotein, a 170-kDa plasma membrane glycoprotein that mediates the efflux of anticancer drugs such as doxorubicin, vincristine, and taxol (44, 45). The overexpression of this export pump in tumor cells has been found to be closely associated with several multidrug resistance phenotypes. More recently, another type of drug transporter, the multidrug resistance-associated protein (MRP), has been identified in doxorubicin-resistant small cell lung cancer in humans (46). MRP mediates the transport of doxorubicin into specific extranuclear compartments (46).

Intracellular GSH is a critical determinant in the drug resistance of human tumors, especially to alkylating anticancer agents such as nitrogen mustard, bifunctional nitrosoureas, and cisplatin (3-6). Increased cellular GSH confers tumor cell resistance to those agents, whereas depletion of cellular GSH results in a reversal of the cellular sensitivity. While much attention has hitherto been paid to the function of cellular GSH in the resistance mechanism, evidence is gradually accumulating to demonstrate the important role of the GS-X pump in modulation of GSH-associated drug resistance (47). Continuous elimination of GSH conjugates from cells is an important mechanism of reducing intracellular accumulation of glutathione S-conjugates. The GS-Pt complex generated from the conjugation reaction of cisplatin with intracellular GSH is potentially cytotoxic. The GS-X pump is proposed to play a major role in eliminating of the GS-Pt complex from cancer cells (7). The present study provides evidence that the GS-X pump is functionally overexpressed in the cisplatin-resistant variant of HL-60 cells, i.e. HL-60/R-CP. It is noteworthy that the enhanced activity of the GS-X pump in our study did not correlate with the cellular sensitivity to doxorubicin (Fig. 1) or vincristine (data not shown). Furthermore, P-glycoprotein (MDR1) was not immunologically detected in the plasma membrane preparation of HL-60/R-CP cells (Fig. 3B). These results support our conclusion (48) that the GS-X pump is distinct from P-glycoprotein (MDR1).

As indicated in Figs. 5 and 6, the GS-X pump may play a key role in the accumulation of GSH-drug conjugates in the intracellular vesicles. Intracellular compartmentalization of drug metabolites is considered to be one of the significant mechanisms in drug resistance. In many cases, drug-resistant tumor cells are more vesicular than are sensitive cells, suggesting that intracellular vesicles may sequester drugs or drug metabolites in a nontoxic site and/or efflux them by means of exocytosis (49). Using monochlorobimane, we demonstrated that accumulation of the fluorescent glutathione S-conjugate in intracellular vesicles was more prominent in HL-60/R-CP cells than in HL-60 cells (Fig. 6). Moreover, the HL-60/R-CP cells excreted the glutathione S-conjugate more effectively than the sensitive cells (Fig. 6). The vesicular accumulation was ATP dependent (Fig. 5B), and the vesicular trafficking was inhibited by monensin (Fig. 5B). Based on these results, we hypothesize that, in resistant cells, glutathione S-conjugates of drugs are accumulated in intracellular vesicles by the GS-X pump and subsequently excreted by exocytosis (see Fig. 9 for schematic illustration). The accumulation of the glutathione S-conjugate of monochlorobimane in intracellular vesicles was also observed in cultured rat hepatocytes (43). The hepatic process
is mediated by a canalicular multispecific organic anion transporter identical to the GS-X pump. Importantly, the vesicular accumulation does not occur in hepatocytes from mutant rats in which the hepatic export pump is functionally defective (43). In plant cells, on the other hand, it has recently been reported that glutathione S-conjugates of phytotoxic foreign compounds are transported into intracellular vacuoles. The transport into the vacuoles is mediated by a specific ATPase that is very similar to the GS-X pump in animal cells (50). Thus, it is conceivable that the GS-X pump and/or its similar transporters are involved in such subcellular compartmentalization of glutathione S-conjugates in animal as well as plant cells.

At present, the molecular structure of the GS-X pump is not known. In HL-60/R-CP cells, three membrane proteins with apparent molecular masses of 200, 110, and 70 kDa, respectively, were found to be overexpressed. Kawai et al. (51) previously reported that a 200-kDa membrane glycoprotein is overexpressed in cisplatin-resistant sublines of murine lymphoma cells. The expression of the 200-kDa protein was correlated with reduced accumulation of platinum in lymphoma cells (51), but the function of the protein remains unknown. It has slowly become apparent that the GS-X pump plays a role in the elimination of a variety of glutathione-drug conjugates (1, 2) as well as heavy metal-glutathione complexes (7, 52, 53) from cells. Heavy metal tolerance in fission yeast has recently been found to be closely related to the expression of an ATP-binding cassette-type vacuolar membrane transporter, named HMT1 (54). Fission yeast strains harboring an HMT1-expressing multicopy plasmid also exhibited metal tolerance to cadmium, im-

**FIG. 8.** Effect of retinoic acid (A) and TPA (B) on the mRNA level of c-my  in HL-60 and HL-60/R-CP cells. Cells were incubated with 1 μM all-trans-retinoic acid (A) or 32 nM TPA (B). At different time points indicated in the figure, aliquots of the cell suspension containing 1 × 10⁷ cells were withdrawn, and total cellular RNA was extracted. The RNA (10 μg/lane) was then fractionated by electrophoresis in 1.0% (w/v) agarose gels and visualized by ethidium bromide (bottom). Northern hybridization (top) with the ³²P-labeled c-my probe was carried out as described under “Materials and Methods.”

**TABLE II**

| Cells          | Addition | ATP-dependent GS-Pt transport  
| ---------------|----------|-------------------------------|
|                |          | pmol/min/mg protein          |
| HL-60          | None     | 20.5 ± 1.7                   |
|                | RA       | 6.0 ± 1.2                    |
|                | Me₂SO    | 9.0 ± 1.4                    |
|                | TPA      | 10.1 ± 1.0                   |
| HL-60/R-CP     | None     | 80.1 ± 7.3                   |
|                | RA       | 57.3 ± 4.1                   |
|                | Me₂SO    | 61.7 ± 7.1                   |
|                | TPA      | 51.8 ± 2.4                   |

**FIG. 9.** Schematic illustration for vesicle-mediated excretion of GSH-drug conjugates from cancer cells. DNA-reacting, electrophilic agents (X) are conjugated with cellular GSH to form GSH-drug conjugates (GS-X). The GSH-drug conjugates are subsequently transported into intracellular vesicles via the GS-X pump. The vesicles are fused with the plasma membrane, and GSH-drug conjugates are released from the cell by exocytosis. Through the fusion of the vesicular membrane with the plasma membrane, the GS-X pump is translocated to the plasma membrane.
pH-sensitive compartmentalization of heavy metals (or heavy metal complexes) in vacuoles (54). In the fission yeast, cadmium is chelated with phyrochylatins (55) or cadmium-binding peptides (56), which are enzymatically synthesized from GSH (57). In mammalian cells, the reaction of cadmium with GSH is a first-line defense mechanism (58, 59). Interestingly, HL-60/R-CP cells were 5-fold more resistant to cadmium than HL-60 cells (IC<sub>50</sub> = 21 μM HL-60 cells versus 110 μM HL-60/R-CP cells).<sup>2</sup> It is, therefore, tempting to speculate about a functional and structural relationship between HMT1 in the fission yeast and the GS-X pump in mammalian cells with respect to their role in the ATP-dependent transport of heavy metal-thiol complexes.

Besides identifying the GS-X pump molecule, it would also be important to understand how the expression of the GS-X pump gene or genes is regulated in drug-resistant and -sensitive tumor cells. A significant increase was observed in both the cellular GSH level and the activity of the GS-X pump in HL-60/R-CP cells (the present study). The mRNA level of γ-glutamylcysteine synthetase (GS) was up-50-fold higher in HL-60/R-CP cells (the present study). The mRNA level of the promoter of P-glycoprotein (MDR1) in NIH3T3 cells (68). In heat shock reportedly leads to an increased expression of y-glutamyltransferase activity (12). The latter enzyme catalyzes the first step of the catalysis of GSH conjugates exported from cells via the GS-X pump. Thus, it is suggested that the expression of GSH-metabolizing enzymes involved in biosynthesis, transport, and catalysis of GSH, is possibly regulated by certain master switches. For instance, heat shock reportedly leads to an increased expression of γ-glutamylcysteine synthetase and reportedly enhanced the efflux of glutathione S-conjugate transport, suggesting MRP-mediated efflux of glutathione S-conjugates from cells. Thus, MRP is considered to be a first-line defense mechanism involving a relationship between HMT1-mediated transport and compartmentalization of heavy metals (or heavy metal complexes).

### References

1. Ishikawa, T. (1993) in Structure and Function of Glutathione S-Transferases (Tew, K. D., Pickford, C. B., Mamline, T. J., Mannervik, B., and Royce, J., eds) pp. 211–241 CRC Press, Boca Raton.
2. Ishikawa, T. (1992) Trends Biochem. Sci. 17, 465–468.
3. Meister, M. (1991) Pharmacol. Ther. 51, 135–194.
4. Hamilton, T. C., Winker, M. A., Louie, K. M., Ramirez, K. M., and Erbs, C. (1979) Arch. Biochem. Biophys. 198, 77–86.
5. Edelson, P. (1974) J. Am. Chem. Soc. 96, 7593–7602.
6. Anick, B. A., and Nathan, C. (1984) J. Exp. Med. 159, 124–136.
7. Tietze, F. (1969) Anal. Chem. 41, 880–891.
8. Kuo, M. T. (1992) Molecular Pathology) for kindly providing a vincristine-resistant subclone of HL-60 cells. Generous support by Dr. Tatsushi Assano (Department of Cell Biology) as well as Drs. Aria Asano and Francia Ali-Osman (Department of Experimental Pediatrics) in the fluorescence microscopy study is gratefully acknowledged. We thank Karen M. Ramirez for her support in the flow cytometry analysis as well as Lois Craft for her assistance in the preparation of the manuscript.

**Acknowledgments**—We thank Dr. M. Tien Kuo (Department of Molecular Pathology) for kindly providing a vincristine-resistant subclone of HL-60/R-CP cells).2

### Related to Cell Proliferation

These findings become even more interesting when we consider facts that retinoic acid is widely used in the treatment of human cancers (69) and that TPA sensitizes cisplatin-resistant human ovarian carcinoma cells (70). In addition, the expression of the human GST π gene is down-regulated by retinoic acid (71).

We have first demonstrated that LTC₄ and its metabolites are endogenous substrates for the GS-X pump (72–74). LTC₄ production in leukemia cells was reported to be enhanced after terminal cell differentiation (75–77). The present finding, however, suggests that the regulation of the GS-X pump expression in HL-60 cells is more closely associated with cellular detoxification rather than with leukotriene biosynthesis.

**Note Added in Proof**—After submission of this manuscript, a significant progress was made in the molecular identification of the GS-X pump. A recent study by Muller et al. provides important evidence that overexpression of the multidrug resistance-associated protein (MRP) gene in human cancer cells results in increased ATP-dependent glutathione S-conjugate transport (78), suggesting MRP-mediated efflux of glutathione S-conjugates from cells. Thus, MRP is considered to be a member of the GS-X pump family in animal and plant kingdoms. The expression of MRP in HL-60/R-CP cells is currently being examined in our laboratory.

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2. T. Ishikawa, unpublished results.
GS-X Pump in Cisplatin-resistant Leukemia Cells

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37. Brittinger, G., Hirschhorn, R., Douglas, S. D., and Weissmann, G. (1986) J. Cell Biol. 37, 394–411
38. Talalay, P., Fishman, W. H., and Huggins, C. (1946) J. Biol. Chem. 166, 757–772
39. Laemmli, U. K. (1970) Nature 237, 685–688
40. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
41. Teeter, L. A., Sanford, J. A., Sen, S., Stallinga, R. L., Stelilano, M. J., and Kuo, M. T. (1986) Mol. Cell. Biol. 6, 4264–4273
42. Shreve, D. C., Bumpa, E. A., and Rice, G. C. (1988) J. Biol. Chem. 263, 14107–14114
43. Oude Eferink, R., Bakker, C. K. K., Boelens, H., Middelkoop, E., Ottenhoff, R., Heijs, M., and Jansen, P. L. M. (1993) Hepatology 17, 543–444
44. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171
45. Gettesman, M. M., and Pestan, I. (1988) J. Biol. Chem. 263, 12166–12166
46. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Dunrue, A. M. V., and Deyee, R. G. (1992) Science 258, 1640–1654
47. Ishikawa, T., and Ali-Osman, F. (1994) in Drug Transport in Antimicrobial and Anticancer Chemotherapy (Georgopapadakou, N. H., ed) Marcel Dekker, New York, in press
48. Ishikawa, T. (1990) Trends Biochem. Sci. 15, 219–220
49. Dietel, M. (1993) Cancer Res. 53, 2683–2686
50. Martinou, E., Grill, E., Tommasini, R., Keus, K., and Amrheim, N. (1993) Nature 364, 247–249
51. Kawai, K., Nakatani, N., Georges, E., and Ling, V. (1990) J. Biol. Chem. 265, 13137–13142
52. Ballatori, N., and Clarke, T. W. (1983) Am. J. Physiol. 244, G435–G441
53. Gervazia, A. Verge, F., and Gregus, Z. (1991) Biochem. Pharmacol. 41, 937–944
54. Ortz, D. F., Krepel, L., Speiser, D. M., Scheel, G., McDonald, G., and Ow, D. W. (1992) EMBO J. 11, 3481–3489
55. Grill, E., Winnacker, E. L., and Zenk, M. H. (1985) Science 230, 674–676
56. Murasugi, A., Waldb, C., and Hayashi, I. (1981) Biochem. Biophys. Res. Commun. 103, 1021–1026
57. Grill, E., Löffler, S., Winnacker, E. L., and Zuck, M. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6858–6862
58. Perrin, D. B., and Want, A. E. (1971) Biochim. Biophys. Acta 230, 96–104
59. Singh, H. K., Anderson, M. E., and Meister, A. (1987) FEBS L. 1, 220–223
60. Kondo, T., Yoshida, K., Urasa, Y., Goto, S., Goss, S., and Taniguchi, N. (1993) J. Biol. Chem. 268, 20386–20372
61. Morimoto, R. I., Sarge, K. D., and Abravaya, K. (1992) J. Biol. Chem. 267, 21987–21990
62. Oesterreich, S., Schunk, R., Benndorf, R., and Bielka, H. (1991) Biochem. Biophys. Res. Commun. 180, 243–249
63. Isonishi, S., Hom, D. K., Thebaud, B., Mann, S. C., Andrews, P. A., Basu, A., Lazo, J. S., Eastman, A., and Howell, S. B. (1991) Cancer Res. 51, 5903–5909
64. Kashiwagi-Sabet, M., Wang, W., and Scanlon, K. J. (1990) J. Biol. Chem. 265, 11285–11288
65. Kashiwagi-Sabet, M., Lu, Y., Leong, L., Haedicke, K., and Scanlon, K. J. (1990) Eur. J. Cell Biol. 50, 393–399
66. Morrow, C. S., Cowan, K. H., and Goldsmith, M. E. (1989) Gene (Amst.) 75, 3–11
67. Teeter, L. D., Eckerberg, T., Tsei, Y., and Kuo, M. T. (1991) Cell Growth Differ. 2, 429–437
68. Chin, K. V., Ueda, K., Pastan, I., and Gottesman, M. M. (1992) Science 255, 469–472
69. Lotan, R. (1991) Semin. Cancer Biol. 2, 197–208
70. Isonishi, S., Andrews, P. A., and Howell, S. B. (1990) J. Biol. Chem. 265, 3623–3627
71. Xia, C., Taylor, B. J., Spencer, S. R., and Ketterer, B. (1993) Biochem. J. 292, 845–850
72. Ishikawa, T. (1999) FEBS Lett. 466, 177–180
73. Ishikawa, T., Kobayashi, K., Segawa, Y., and Hayashi, K. (1989) FEBS Lett. 265, 95–98
74. Ishikawa, T., Müller, M., Kontsevann, C., Schaub, T., and Keppler, D. (1990) J. Biol. Chem. 265, 19273–19279
75. Soderström, M., Bolling, A., and Hammarström, S. (1992) Biochem. Biophy. Res. Commun. 180, 1043–1049
76. Lam, B. X., Xu, X., Aljoska, M. S., and Austen, F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11598–11602
77. Nicholson, C. W., Ali, A., Klemka, M. W., Munday, N. A., Zhang, R. J., and Ford-Hutchinson, A. W. (1992) J. Biol. Chem. 267, 17649–17657
78. Müller, M., Meijer, C., Zaman, G. J. R., Borst, P., Schepers, R. J., Mulder, N. H., de Vries, E. G. E., and Jansen, P. L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, in press