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IDENTIFICATION OF A GLUTATHIONE *S*-TRANSFERASE ASSOCIATED WITH MICROSOMES OF TUMOR CELLS RESISTANT TO NITROGEN MUSTARDS*

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Abstract—Walker 256 rat mammary carcinoma cells resistant to chlorambucil (WR) exhibited an approximate 4-fold increase in glutathione *S*-transferase (GST) activity using 1-chloro-2,4-dinitrobenzene as compared to the sensitive parent cell line (WS). WR cells maintained without biannual exposure to chlorambucil (WR_r) reverted to the sensitive phenotype and possessed GST levels equivalent to WS. Mitochondria, microsomes and cytosol were isolated from WS, WR and WR_r cell lines and analyzed for their GST composition. GST activity in each subcellular compartment of resistant cells was increased over the sensitive cells. Antibodies raised against total rat liver cytosolic GST crossreacted in resistant cells with two microsomal proteins (25.7 kD and 29 kD). The 29 kD protein was not detected in microsomal fractions from either WS or WR_r and this protein was found to be dissimilar from cytosolic GST subunits in its isoelectric point (pI 6.7) and migration on two-dimensional polyacrylamide gels. In addition, the 29 kD microsome-associated GST from WR cells was immunologically distinct from a 14 kD GST subunit previously identified in rat liver microsomes. These data implicate the induction of a specific microsomal GST subunit in WR cells following drug selection and suggest its potential involvement in the establishment of cellular resistance to chlorambucil.

The ability of glutathione *S*-transferases (GST‡) to catalyze the conjugation of a wide range of xenobiotics with glutathione suggests the critical function of GST in detoxification and cellular protection. Both an elevation of GST in several drug-resistant tumor cell lines [1–6] and the isolation of glutathione conjugated with the nitrogen mustard class of alkylating agents [7] have implicated the GSTs in the establishment of resistance to these agents. Previous studies from this laboratory have shown that Walker 256 rat mammary carcinoma cells which are resistant to chlorambucil (WR) possess increased levels of GST activity against 1-chloro-2,4-dinitrobenzene (CDNB) when compared to the sensitive parent cell line (WS) [1]. Characterization of the GST subunits in the cytosol of WR cells revealed an overproduction of a protein which reacted with antibodies against total rat liver GST and displayed an electrophoretic mobility similar to the cytosolic Yc subunit of rat liver [8].

The majority of cellular GST has been found to be cytosolic. These enzymes have also been detected in several subcellular organelles. In rat liver, two cytosolic isozymes of GST have been found associated with chromatin, specifically with U-SnRNPs [9], and 0.35% of total cytosolic GST activity is loosely bound to the outer mitochondrial membrane [10]. Morgenstern *et al.* [11] have identified a 14 kD protein that exhibits GST activity in the microsomes and outer mitochondrial membranes of rat liver [12]. Further characterization has established this transferase as distinct from cytosolic forms not only in molecular weight but also in immunoreactivity, amino acid sequence and inducibility by sulfhydryl reagents such as *N*-ethylmaleimide and iodoacetamide [13].

The existence of multiple isozymes of GST [14] and their localization in several subcellular compartments may reflect their functional diversity. The present study investigates the subcellular compartmentalization of GSTs in Walker 256 rat mammary carcinoma cells which are either sensitive or resistant to nitrogen mustards. In addition, the possible role of GST in the establishment of cellular resistance to chemotherapeutic agents has been addressed.

MATERIALS AND METHODS

Cell culture. Walker 256 rat mammary carcinoma cells were cultured in Dulbecco's minimal essential medium supplemented with 4 mM L-glutamine, 10% fetal calf serum and Kanamycin (91 μg/ml). Drug resistance was maintained in the WR cell population by exposing cells to chlorambucil (20 μg/ml) every 6 months. The revertant cell line (WR_r) was established in this laboratory by allowing a parallel culture of WR cells to grow for 1 year without periodic exposure to chlorambucil.

Colony forming assays. The toxicity of chlorambucil to the Walker cell lines was assessed using a colony forming assay in soft agar as described

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‡ Abbreviations: GST, glutathione *S*-transferase; WR, Walker resistant cells; CDNB, 1-chloro-2,4-dinitrobenzene; WS, Walker sensitive cells; WR_r, Walker resistant revertants; SDS, sodium dodecyl sulfate; pI, isoelectric point; and Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

previously [15]. Briefly, cells were treated with chlorambucil (10–300 μM) for 2 hr, suspended in Dulbecco's minimal essential medium and plated into a 3% agar-complete medium mixture. After 1 week, cell colonies were stained with 1% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride hydrate, and colonies of greater than 32 cells were counted using a Biotran III automatic totalizer (New Brunswick Scientific, Edison, NJ).

Subcellular fractionation. All cells were harvested in mid-log phase at a density of 4.6–9.5 $\times$ 10^5/ml (total yield = 2.4–5.7 $\times$ 10^9 cells). Cells were centrifuged for 10 min at 2000 rpm (4°), and the cell pellet was rinsed with Hanks' balanced salt solution. The washed cell pellet was resuspended in approximately 20 ml of isolation medium (200 mM D-mannitol, 70 mM sucrose, 2 mM potassium Hepes, pH 7.4) and homogenized with a teflon homogenizer using six complete strokes at a rheostat setting of 60. All procedures were conducted at 4°, and a 1-ml aliquot from each step was stored at −70° for enzymatic analyses. Mitochondria were isolated according to Greenawalt [16]. The cell lysate was spun at 660 *g* for 15 min to remove cell debris, nuclei and plasma membranes. The pellet was washed with isolation medium and the combined supernatant fractions were centrifuged at 10,000 *g* for 15 min at 4° to pellet mitochondria. The mitochondrial pellet was rinsed and finally resuspended in isolation medium. The combined supernatant fractions at 10,000 *g* were centrifuged through an equal volume of 0.25 M sucrose at 105,000 *g* for 65 min to separate microsomes from cell cytosol. The microsomal pellet was resuspended in 0.25 M sucrose. The protein concentration of each fraction was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine gamma-globulin as standard.

Enzyme assays. All samples were sonicated using a Sonic Dismembrator (Artek, Farmingdale, NY) for 30 sec at setting 60. Total GST activity was quantified with the substrate 1-chloro-2,4-dinitrobenzene [17]. Cytosolic contamination of cell fractions was monitored using a spectrophotometric assay for lactate dehydrogenase (Diagnostics Kit, Sigma Chemical Co., St Louis, MO).

Gel electrophoresis. Cell fractions were analyzed on 12% polyacrylamide slab gels containing sodium dodecyl sulfate (SDS) according to Laemmli [18]. Samples were solubilized in SDS sample buffer (0.0625 M Tris, 5% β-mercaptoethanol, 10% glycerol and 2% SDS), boiled for 3–5 min, and applied to the gels at either equivalent protein content or equivalent GST activity. Following electrophoresis, gels were stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol/10% acetic acid and destained in 25% methanol/10% acetic acid.

For two-dimensional gels, proteins were separated in the first dimension on 12-cm tube gels containing 9 M urea, 4% acrylamide and 5% ampholines, pH 3.5 to 10 (Serva, Westbury, NY) [19]. Preparations were electrophoresed under nonequilibrium conditions with samples loaded at the acidic end and run for 4 hr at 400 V [20]. Upon completion, gels were removed from the tubes and stored at −70° until use [19]. Following equilibration, tube gels were joined to the slab gel (12% SDS polyacrylamide) with 1% agarose and electrophoresed as described for one-dimensional gels. All samples were run in duplicate with one gel stained with silver [21] and the other transferred to nitrocellulose.

To determine the isoelectric point of various GSTs, one-dimensional flatbed isoelectric focusing was performed using an Isobox (Hoefer Scientific, San Francisco, CA). Polyacrylamide gels [5.5% acrylamide, 5.5% ampholines, pH 3.5 to 10 (Serva), 10% glycerol and 0.2% Temed] were aged at 4° overnight and prefocused for approximately 1 hr at 60 W using wicks saturated with 0.02 M acetic acid and 0.02 M NaOH. Samples were focused for 2 hr at 1000 V and subsequently transferred to nitrocellulose.

Antibody production. Polyclonal antibodies were generated against total cytosolic GST from rat liver (Sigma Chemical Co.) as described previously [8]. Polyclonal antisera directed against rat liver microsomal transferase (14 kD) [22] was provided by Dr Ralf Morgenstern, Stockholm Universitet, Stockholm, Sweden.

Western blot analysis. Gels were electrophoretically transferred to nitrocellulose overnight at 30 V in 25 mM Tris/192 mM glycine (pH 8.3) containing 20% methanol [23]. Nitrocellulose membranes were placed in buffer A (50 mM Tris, 0.4 M NaCl, pH 7.5) containing 0.05% Tween 20 and rinsed. All rinses were for 45 min with three changes of buffer and all incubations were for 1 hr. Membranes were incubated with buffer A containing 3% bovine serum albumin followed by antibody against rat liver GST in the same buffer (diluted 1:500). Membranes were rinsed and incubated with peroxidase conjugated goat anti-rabbit IgG (Bio-Rad) in buffer A containing 3% bovine serum albumin. After rinsing, blots were developed using 1-chloro-4-napthol (Bio-Rad) and H_2O_2 in buffer A containing 11% methanol. The reaction was stopped by rinsing with water.

RESULTS

Prior to transferase analysis, Walker 256 cell lines were assessed for resistance to chlorambucil by determining the effect of drug exposure on the number of colonies formed in agar. The inhibition of colony formation observed at any drug concentration was significantly less for WR cells than for the sensitive parent cell line WS (Fig. 1). When a subpopulation of WR cells was grown for 1 year without routine biannual exposure to chlorambucil, cell survival paralleled that of WS cells. These cells represented WR revertants, designated WR_r.

Data from this laboratory [8] have suggested that higher levels of GST activity in WR cells are due to the elevation of a 29 kD cytosolic subunit. To determine if the increased GST activity in WR cells was due exclusively to this cytosolic subunit, subcellular components of each cell line (WR, WS and WR_r) were isolated and assayed for GST activity. Cells were separated into three fractions by differential centrifugation and each was examined for cytosolic contamination. The total GST content of each cell fraction was quantified using CDNB as substrate. As shown in Table 1, GST activity was approximately 4-fold higher for WR cell homo-

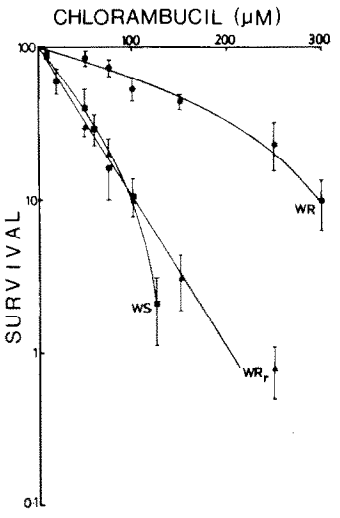

Fig. 1. Cytotoxicity curves demonstrating the effect of chlorambucil on the colony forming ability of Walker cells. Each cell line (WS, WR and WR$_r$) was exposed to 0–300 μM chlorambucil for 2 hr. The number of surviving colonies was determined and expressed as a percentage of the untreated control.

genates as compared to those of the WS and WR$_r$ lines. This elevation of GST over both the sensitive and revertant cell lines was not restricted to any one particular subcellular fraction but rather the result of a generalized increase in each compartment. In addition, lysates from WS and WR$_r$ cells did not differ in total GST activity when compared. For all cell lines, approximately 50% of the GST activity recovered from each fractionation procedure was cytosolic.

To determine if the resistance phenotype was accompanied by an alteration in the GST composition of the subcellular fractions, protein preparations from each compartment listed in Table 1 were separated on 12% SDS polyacrylamide gels, transferred to nitrocellulose, and incubated with GST antibodies. An apparent difference in GST composition between sensitive and resistant cell lines was observed in the microsomal compartment. Western blot analyses using antibodies against total rat liver cytosolic GST identified two crossreactive microsomal proteins (M_r 25.7 kD and 29 kD) in WR cells and only the 25.7 kD protein in WS and WR$_r$ (Fig. 2). These results were obtained when samples were compared at equivalent levels of GST activity (2 nmol/min) or at equal protein amounts (75 μg) (Fig. 2). This 29 kD microsome-associated form of GST was dissimiliar to that reported by Morgenstern *et al.* [22] in estimated molecular weight (14 kD) and immunoreactivity. Polyclonal antibodies directed against the 14 kD microsomal GST recognized a protein of equivalent molecular weight in Walker cell microsomes (WS and WR), but they did not crossreact with the 29 kD GST identified in the microsomes of WR cells (data not shown). In addition, treatment of microsomal preparations with *N*-ethylmaleimide did not cause an induction of GST activity (Fig. 3) as was reported for the 14 kD protein [24]. The decrease in cytosolic GST activity after *N*-ethylmaleimide exposure, as shown in C and D, has been reported by others [22].

Further separation of microsomal proteins was accomplished by two-dimensional gel electrophoresis. Western blot analyses using antibodies against rat liver GST confirmed the presence of the additional GST subunit in resistant lines previously observed on one-dimensional gels (Fig. 4). Two proteins, one acidic and one neutral, in the molecular weight range of the GST subunits (23–30 kD), were detected by immunoblotting. However, only one acidic subunit was observed in WS and WR$_r$ microsomal preparations. Use of a cytosolic enzyme marker demonstrated that the WR microsomal samples were relatively free of cytosolic contamination. Total lactate dehydrogenase activity was elevated 38-fold in WR cytosol relative to microsomes (Table 2). Using flat-bed isoelectric focusing, the pI of this neutral microsomal protein was estimated to be 6.67 as compared to 6.50 for a WR cytosolic subunit of closest pI (Table 3).

DISCUSSION

The present study represents the first characterization of the subcellular distribution of GST in tumor cells. Walker 256 rat mammary carcinoma cells resistant to chlorambucil possessed a 29 kD GST subunit not detected in either the sensitive parent cell line or the revertant cell line. A second GST subunit was associated with the microsomes of both sensitive and resistant cells.

Table 1. Compartmentalization of glutathione *S*-transferase activity

Sample	GST specific activity (nmol/min/mg protein)		
	WR	WS	WR$_r$
Homogenate	40.65 ± 6.5 (100)	11.29 ± 2.0 (100)	11.76 ± 3.5 (100)
Mitochondria (P-10)	25.59 ± 11.3 (1.6)	6.29 ± 2.4 (2.5)	6.94 ± 2.3 (4.8)
Microsomes (P-105)	22.86 ± 9.3 (2.2)	4.71 ± 1.6 (1.7)	4.64 ± 0.8 (0.8)
Cytosol (S-105)	52.5 (49.9)	14.91 ± 5.4 (57.9)	16.90 ± 8.2 (45.0)

Values represent average specific activity against the substrate 1-chloro-2,4-dinitrobenzene (CDNB) ± the standard deviation for three separate experiments. The percent of total activity recovered in each compartment is given in parentheses.

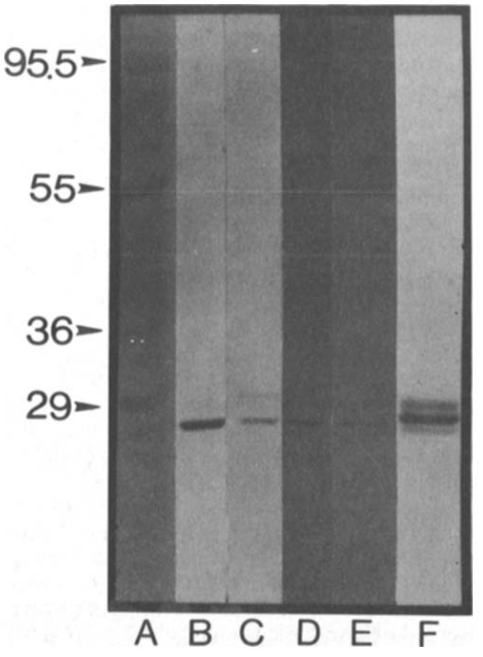

Fig. 2. Western blot analyses of microsomal preparations from Walker sensitive (lanes B and D) and resistant (lanes C and E) cells. Samples were compared at both equivalent levels of GST activity (2 nmol/min) against CDNB (lanes B and C) and equal amounts of protein (75 μg) (lanes D and E). Lane A contains molecular weight markers prestained with Coomassie (Diversified Biotech, Newton Centre, MA). Lane F represents rat liver cytosolic GST used as a marker.

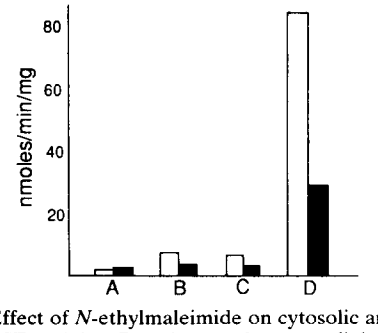

Fig. 3. Effect of *N*-ethylmaleimide on cytosolic and microsomal GST activity. Protein samples were dialyzed overnight against saline (4°) to remove glutathione, and 150 μg protein was incubated with (shaded areas) or without (unshaded areas) 1 mM *N*-ethylmaleimide for 10 min at room temperature. GST activity was assessed according to Habig *et al.* [17] using CDNB as substrate except that the glutathione concentration in the reaction mixture was increased to 3 mM. GST activity is expressed as nanomoles per minute per milligram protein. Key: (A) WS microsomes, (B) WR microsomes, (C) WS cytosol, and (D) WR cytosol.

The GST subunit identified in all microsomal preparations (WS, WR_r and WR) appeared to be equivalent to a cytosolic form in both its isoelectric point (pI 5.1) and molecular weight (23 kD). Several investigators have reported a similar association of cytosolic GST with rat liver microsomes. Morgenstern and DePierre [13] estimated that 63% of total microsomal GST activity is attributable to cytosolic isozymes. Friedberg *et al.* [25] identified three forms of GST with isoelectric points similar to cytoplasmic GSTs in microsomal membranes. Lastly, Lee and McKinney [26] have reported that the microsomal and cytosolic GSTs from mouse liver are comparable with respect to several properties including molecular weight, immunological reactivity, isoelectric point and enzymatic activity.

The potential for cross-contamination of proteins during cellular fractionation exists in all subcellular compartmentalization studies. Use of a cytosolic marker enzyme, lactate dehydrogenase, suggested that the commonality in GST seen in cytosolic and microsomal compartments was not due to cytosolic contamination of the microsomal preparations. Second, crossreactivity of an antibody against the 14 kD microsomal GST (not found in cytosol) with only microsomal fractions served as an additional indicator of the purity of the isolated microsomal proteins.

The 29 kD microsomal GST found only in WR cells had an isoelectric point distinct from the cytosolic GST subunit overproduced in these same cells following chlorambucil selection [8]. The overproduced microsomal GST had an isoelectric point of 6.67 while the elevated cytosolic subunit had an isoelectric point of 6.50. The degree of variability in pI estimation of cytosolic GSTs common to both WR and WS was always equal to or less than 0.03 units

Table 2. Marker enzyme analysis

Sample	Lactate dehydrogenase activity (units/mg protein)	(total units)
Homogenate	1.12	1742.70
Mitochondria (P-10)	0.84	14.02
Microsomes (P-105)	0.68	39.28
Cytosol (S-105)	3.95	1477.85

One unit of enzyme activity equals the amount of lactate dehydrogenase that will catalyze the production of one micromole of NADPH per minute. Values represent an average of duplicate assays performed on two separate preparations of WR cells.

Table 3. Isoelectric points of microsomal and cytosolic GSTs

	pI	
	WS	WR
Microsomes	5.10	5.13
		6.67
Cytosol	5.10	5.07
	5.16	5.13
	6.47	6.50

Isoelectric points were determined using flat bed isoelectric focusing and standards ranging in pI from 3.55 to 9.30 (Sigma Chemical Co.)

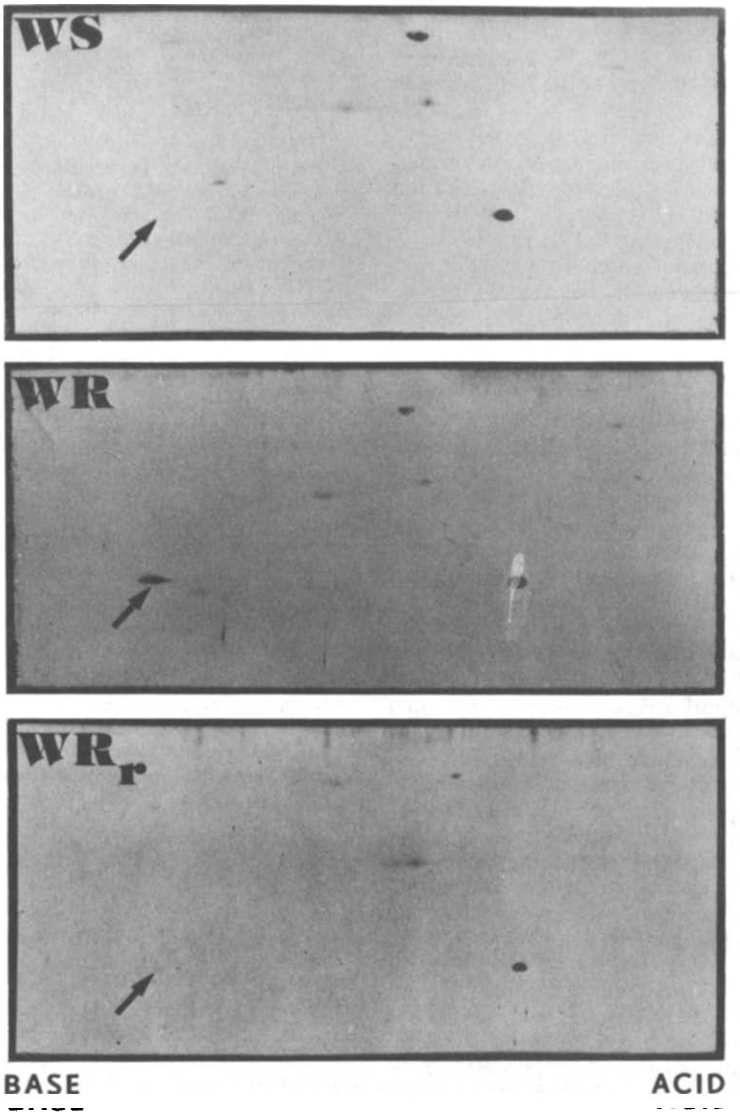

Fig. 4. Western blots of microsomal fractions separated by two-dimensional gel electrophoresis. Protein samples (125 μg) were separated in the first dimension using non-equilibrium isoelectric focusing (pH gradient of 4.2 to 9.5) and in the second dimension on a 12% SDS polyacrylamide gel. Proteins were electrophoretically transferred from gels to nitrocellulose and analyzed as described in the legend of Fig. 2. Arrows mark the position of the 29 kD microsomal GST in WR cells and the expected position (absent) in WS and WR$_r$ cells.

(Table 3), suggesting that a difference in isoelectric points of 0.17 units was significant. It should be noted that, since the isoelectric focusing gels were run under nondenaturing conditions, these values represent isoelectric points for the protein dimers.

Since the net difference in charge between the overproduced microsomal and cytosolic subunits was small, this difference may represent varying types or degrees of post-translational modification. Alternatively, the microsomal subunit may have been analyzed bound to a substrate, thus influencing its charge. One can speculate that translocation of the subunit from one compartment to another may require substrate binding or that transport of the substrate is contingent upon interaction with the enzyme. Although such processes have not been investigated for the GST enzyme family, an alteration in protein charge may signal or facilitate transport of the 29 kD protein to the microsomes.

The microsome-associated GST detected in Walker cells resistant to chlorambucil differs in many respects from the microsomal form previously described by Morgenstern and coworkers [22, 24]. Unlike all other proteins possessing GST activity, the form identified in rat liver microsomes had a molecular weight of 14 kD. Polyclonal antibodies raised against the 14 kD microsomal GST did not crossreact with the 29 kD microsomal GST, ruling out possible dimerization of the 14 kD in tumor cells. However, the 14 kD GST was detected in micro-

somes from both drug-sensitive and -resistant cells. Lastly, sulfhydryl reagents such as *N*-ethylmaleimide did not induce the GST activity of the 29 kD protein described in the present study.

The compartmentalization of GST in the microsomes of cells is not surprising based on present knowledge of the location of other detoxification enzymes within the cell. For example, both epoxide hydrolase and the cytochrome P-450 enzyme family reside in the microsomal compartment. Furthermore, many of the hydrophobic substrates for these enzymes are also substrates for GST [11]. Recent experimentation has demonstrated that both chlorambucil [27] and melphalan [7] are substrates for GST in hepatic microsomes. In these experiments, GSTs from rabbit microsomes were immobilized on sepharose beads and subsequently incubated with glutathione and drug. Reaction products were purified from the incubation mixtures using high pressure liquid chromatography, identifying glutathione conjugates of the parent compounds and their metabolites at levels exceeding those produced by spontaneous conjugation alone.

Results from the present study suggested that an additional microsomal GST subunit was present in the resistant cell population selected by drug exposure. Reversion of a WR subpopulation to the sensitive phenotype in the absence of biannual drug maintenance was accompanied by a loss of this microsome-associated GST subunit. Although the precise function of this 29 kD subunit is not known, this observation implied that regulation of microsomal GST levels may dictate the susceptibility of a cell to the effects of cytotoxic agents. The signal responsible for an overproduction of microsomal GST may be protein-mediated as seen with Y_a mRNA induction by polycyclic aromatic hydrocarbons [28] or it may be merely triggered by chlorambucil itself.

In summary, detection of a 29 kD GST in microsomes of WR cells implicates the potential involvement of this subunit in the establishment of cellular resistance to chlorambucil. The absence of this microsomal GST in the parent cell line as well as in a subpopulation of resistant cells which reverted to the sensitive phenotype (WR_r) suggests the induction of this subunit following selection of the WR cell line by drug exposure. The effect of chlorambucil on the regulation of the GST enzyme family is currently being investigated using cDNA probes encoding the various GST subunits.

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