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Amplification and increased expression of alpha class glutathione *S*-transferase-encoding genes associated with resistance to nitrogen mustards

(drug resistance/glutathione/preneoplasia/cancer chemotherapy)

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ABSTRACT **Glutathione-dependent enzymes play a central role in the protection of cells from cytotoxic chemicals and have been implicated in the intrinsic and acquired resistance of tumors to cytotoxic drugs. We have generated a Chinese hamster ovary line resistant to bifunctional nitrogen mustards and in this report have characterized and isolated the protein that represents the major observable phenotypic difference between the drug-sensitive and drug-resistant cell lines. This purified protein is shown to be an alpha class glutathione *S*-transferase comprising Y_cY_c subunits and possessing a pI value of ≈8.0. The intracellular level of the Y_c subunit is elevated >40-fold in the drug-resistant cell line, which could account for the increase in glutathione *S*-transferase (RX:glutathione R-transferase; EC 2.5.1.18) activity toward both 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide. Other glutathione *S*-transferase subunits within this gene family are also elevated. These changes are accompanied by a significant elevation in alpha class mRNA levels. Southern analysis indicates that the genes coding for these proteins are amplified 4- to 8-fold in the drug-resistant cell line. In addition, γ-glutamyl transpeptidase [(5-glutamyl)-peptide:amino acid 5-glutamyltransferase; EC 2.3.2.2] activity is increased 3.6-fold in the drug-resistant Chinese hamster ovary cell line, which may explain the increase in cellular glutathione level. In this case no gene amplification was seen. These data indicate that gene amplification may be important in drug resistance toward alkylating agents and also that other enzymes in glutathione homeostasis are involved.**

The ability to resist chemical stresses imposed by our environment represents a major evolutionary driving force. It is clear that a wide variety of defense systems have evolved to protect against such cytotoxic insults. Studies into tumor cell drug resistance and carcinogenesis have greatly increased our understanding of these systems, and various defense mechanisms appear to be important, including membrane permeability (1), DNA repair (2), gene amplification (3), and drug detoxification (4).

For several decades glutathione (GSH) and glutathione-dependent enzymes have been implicated in drug-detoxification reactions (5). Increased glutathione content, resulting in increased drug detoxification, was proposed as a mechanism of drug resistance in tumor cells over 20 years ago (6, 7). However, only recently have these observations become a major topic of study. There is now substantial evidence indicating that the overexpression of glutathione and glutathione-dependent enzymes is an important mechanism of acquired drug resistance in both normal and tumor cells (4–13).

In a previous report we described a chlorambucil-resistant Chinese hamster ovary (CHO) cell line (CHO-Chl^r), also cross-resistant to other bifunctional nitrogen mustards (14). These cells have elevated GSH and glutathione *S*-transferase (GST; RX:glutathione R-transferase; EC 2.5.1.18) activity but do not exhibit differences in drug accumulation or DNA repair (15). The major observable phenotypic difference between the sensitive and resistant cell line is in the expression of a protein of molecular mass ≈25 kDa. Here we report the isolation and characterization of this protein, which is shown to be a GST that is overexpressed, apparently due to gene amplification. In addition, we also show that other GST subunits and γ-glutamyl transpeptidase (γGT) are elevated in the resistant line.

The cytosolic GSTs are dimeric proteins. Three structurally distinct gene families have been identified and have been termed alpha (basic), mu (neutral), and pi (acidic) (16). Within the alpha and mu groups several proteins (subunits) that can form either homo- or heterodimers and are the products of separate genes have been identified. Y_a and Y_c subunits are from the alpha family and Y_b and Y_f subunits are from the mu and pi families, respectively.

MATERIALS AND METHODS

All chemicals were purchased from commercial sources and were of the highest grade available.

Cell Culture. The two cell lines used in this study have been termed CHO-K1 (wild type) and CHO-Chl^r. The latter cell line exhibits a 24-, 34-, and 14-fold higher resistance to chlorambucil, mechlorethamine, and melphalan, respectively (14). Cells were maintained in Ham's F10 medium supplemented with 5% fetal calf (and in some cases 5% newborn calf) serum (vol/vol), streptomycin (100 μg/ml), and penicillin (100 international units/ml) and cultured in 100% humidity and 5% CO_2 as monolayers at 37°C.

Isolation of Subcellular Fractions. Cells were harvested from confluent cultures with 0.1% (wt/vol) trypsin and 0.001% (wt/vol) versene, washed three times in phosphate-buffered saline (PBS; 140 mM NaCl/2.7 mM KCl/8 mM $NaPO_4$, pH 7.4) and resuspended in 400 μl of this buffer.

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Abbreviations: CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; GSH, reduced glutathione; γGT, γ-glutamyl transpeptidase; CHO-Chl^r, chlorambucil-resistant CHO cell line.

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Samples were then sonicated with three 5-sec pulses at maximal power with a 5-sec cooling period at 4°C between each step. The resulting sonicate was centrifuged at 18,000 × *g* for 20 min, and the supernatant was decanted. Protein was estimated in freshly prepared cell fractions by the method of Lowry *et al.* (17) with bovine serum albumin used as the standard. Cell fractions were stored in PBS at −70°C before further biochemical analyses were undertaken; samples were stable under the storage conditions used.

Enzymic Assays. GST activity toward the substrates 1-chloro-2,4-dinitrobenzene, ethacrynic acid, and cumene hydroperoxide were determined in the supernatant fractions by the methods of Habig *et al.* (18) and Stockman *et al.* (19). Glutathione reductase was assayed fluorometrically, as described by Weiss *et al.* (20), and glutathione peroxidase activity was determined spectrophotometrically (21). The activity of γ-glutamylcysteine synthetase, the rate-determining enzyme of GSH biosynthesis, was determined using a coupled reaction by measuring the rate of oxidation of NADH spectrophotometrically (22). The particulate cell fraction of the cell preparation was taken for the assay of γGT using γ-L-glutamyl-7-amino-4-methylcoumarin as substrate (Universal Biologicals, Cambridge) (23).

Western (Immunologic) Blot Analysis. Sodium dodecyl sulfate (SDS)/PAGE was performed according to the method of Laemmli (24). Western blots were done using essentially the method described by Towbin *et al.* (25) as modified by Adams *et al.* (26). The antibodies used were raised against the human pi class GST (λ, Y_fY_f subunits), human alpha class GST (B_1B_1, Y_aY_a subunits), and human mu class GST (μ, Y_bY_b subunits). In addition, antisera to the rat alpha class GST, Y_aY_a or Y_cY_c subunits, were also used. Antisera were prepared as described (27, 28).

Two-dimensional electrophoresis was carried out according to the method of O'Farrell (29) as modified by Robson *et al.* (14). A pH 5–8 gradient was employed in the isoelectric focusing step, which represented the first dimension. Either 100 μg of total cellular protein or 5 μg of purified protein was loaded for each experiment.

Purification of GST. Cells from the CHO-Chl^r (5×10^9 cells) were harvested, frozen at −70°C, and lysed as described by Soma *et al.* (30). Briefly, the pellets were thawed and suspended at 4°C in 40 mM Tris·HCl buffer, pH 7.4, containing 160 mM KCl, 4 mM EDTA, and 5 mM dithiothreitol. The final volume was 5 ml. The suspension was homogenized with a Teflon/glass homogenizer (25 strokes), centrifuged for 30 sec in an Eppendorf Microfuge, and then centrifuged for a further 45 min at 35,000 rpm (Kontron TST 50.13 rotor). The resulting supernatant was applied to a column of *S*-hexylglutathione (Sigma) (4-ml bed volume) equilibrated with 10 mM Tris·HCl, pH 7.8, containing 0.2 M NaCl and 3 mM 2-mercaptoethanol. The column was washed with the above buffer, and the GST was eluted by the addition of 5 mM *S*-hexylglutathione to the running buffer. With this procedure the majority of the GST in the cell lysate was retarded by the affinity gel but eluted in the wash fraction from the column. The enzyme-containing fractions were combined, dialyzed against 20 mM Tris·HCl, pH 7.8/1 mM EDTA/5 mM mercaptoethanol, and reapplied to the *S*-hexylglutathione-Sepharose column. The GST, which was now retained by the affinity matrix after this second chromatographic step, was then eluted with a solution of 5 mM *S*-hexylglutathione in the 20 mM Tris·HCl running buffer, pH 7.8. Two proteins were detected in the eluate, a minor component that eluted immediately and a major protein with GST activity. This protein was of high purity as judged by SDS/PAGE (see *Results*).

DNA and RNA Analysis. A full-length cDNA clone alpha class GST [pMP 37, 942 base pairs (bp)] was isolated from a human liver λgt11 library, which was a gift from U. Meyer (Biocenter, Basel), using an oligonucleotide coding for the six NH_2-terminal amino acids of the B_1B_1 protein. Over the coding region this cDNA had an identical sequence to that published by Board and Webb (31). A rat liver γGT cDNA clone containing the full coding sequence was isolated from a liver cDNA library in λGT10 from an ethoxyquin-treated male Fischer rat using a cDNA to the rat kidney enzyme (32). *Eco*RI digest of this clone yielded two fragments. The 5′ fragment of 1230 bp (pEGL1.1) was used as a probe.

DNA Isolation. DNA isolation was done essentially as described by Maniatis *et al.* (33). Cells (10^7) were washed twice with PBS and then harvested by scraping into PBS. Cells were then lysed in 0.5% SDS/150 mM NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.5 (2 ml) and treated with RNase A (100 μg/ml) and then proteinase K (250 μg/ml) at 37°C for 1 hr and 4 hr, respectively. Samples were extracted with phenol followed by phenol/chloroform (1:1 vol/vol), and the DNA was precipitated in 60% EtOH containing 1 M ammonium acetate. The precipitated DNA was wound onto a glass rod, air dried, and resuspended in 10 mM Tris·HCl, pH 7.5/1 mM EDTA. RNA was isolated using the method described by Birnboim (34). DNA and RNA concentrations were determined from the absorption at 260 nm. Southern and Northern (RNA) analysis were done as described by Hill *et al.* (35).

RESULTS AND DISCUSSION

The levels of a variety of glutathione-dependent enzymes in the drug-sensitive and resistant CHO cell lines are shown in Table 1. In agreement with a previous report (15), both the GSH level and the GST activity (toward 1-chloro-2,4-dinitrobenzene) were significantly higher in the chlorambucil-resistant cell line (1.8- and 2.7-fold, respectively). In addition, the peroxidase activity toward cumene hydroperoxide was also elevated 5.1-fold. This increase appeared to be completely due to differences in GST expression, as the selenium-dependent activity (determined using H_2O_2 as substrate) was similar in both cell lines (Table 1). The majority of the peroxidase activity toward cumene hydroperoxide in the CHO-K1 cells was mediated by the selenium-dependent enzyme. When this contribution was subtracted from the values obtained, CHO-Chl^r had ≈50-fold higher GST-mediated peroxidase activity than the wild-type cell line.

A variety of enzymes are involved in maintaining the levels of reduced glutathione within cells (36). Three of these—

Table 1. Glutathione-dependent enzyme expression in CHO-K1 and CHO-Chl^r cell lines

	Enzyme activity							
		GST						
Cell line	GSH	CDNB	EA	CHP	GPX, H_2O_2	γGT	γGCS	GRD
CHO-K1	18.4 ± 2.2	239 ± 36	19.7 ± 3.2	16.4 ± 2.0	8.9 ± 4.8	0.82 ± 0.26	70.0 ± 16.2	96.5 ± 23
CHO-Chl^r	33.0 ± 5.6	638 ± 115†	38.0 ± 12.1‡	83.4 ± 18.3*	10.0 ± 2.7	2.85 ± 0.48*	97.7 ± 51.8	129.5 ± 61.5

Values are expressed as nmol per min/mg of protein for 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide (CHP), ethacrynic acid (EA), H_2O_2, γ-glutamylcysteine, synthetase (γGCS), and glutathione reductase (GRD) or nmol per min/10^6 cells for γGT. GSH is expressed as nmol/mg of protein. GPX, glutathione peroxidase. *$P < 0.005$, †$P < 0.01$, ‡$P < 0.07$.

glutathione reductase, γGT, and γ-glutamylcysteine synthetase—were measured. Only γGT was elevated significantly in the drug-resistant cell line, although some change in γ-glutamylcysteine synthetase and glutathione reductase was observed. This suggests that these proteins, in particular γGT, may be responsible for maintaining the elevated cellular GSH level. Elevated γGT concentrations have been seen in cell lines resistant to cytotoxic drugs (37, 38). In addition, the fact that preneoplastic foci in rat liver, which have elevated GSH content, also have elevated γGT levels (39) supports the hypothesis that this enzyme is involved in maintaining increased cellular GSH levels. It is interesting that in the models for drug resistance induced by alkylating agents that we have studied, γGT activity is consistently elevated (ref. 38, and C.R.W. and A.D.L. unpublished data).

Our initial studies indicated that altered expression of the alpha class GST represented the major change in GST expression in the CHO-Chl^r cell line (15). In support of this, the staining pattern of cytosolic samples clearly showed an elevation in a protein with a mobility similar to that of the rat Y_c GST subunit (Fig. 1 arrow). This subunit could, therefore, be the polypeptide with an estimated molecular size of 25 kDa, which was previously shown to be dramatically overexpressed in the CHO-Chl^r cell line (15); more recent work has estimated the molecular size of the Y_c subunit to be 27–27.5 kDa (40).

To establish whether this was the case, the overexpressed protein was purified. In addition, Western blots of CHO cytosol with antibodies to known rat or human GST subunits (Y_a, Y_b, Y_c, and Y_f) were done. A two-step purification procedure (Fig. 2) yielded a pure protein of similar mobility to the rat Y_c (molecular mass, 27.5 kDa) standard. This protein was shown to be a GST based on its activity towards 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide, and ethacrynic acid, the turnover numbers for these substrates being 6031, 964, and 550 nmol per min/mg of protein, respectively. The relative activities of the Chinese hamster GST toward these three substrates are similar to that exhibited by the rat Y_c subunit (41). Western blots showed that the purified protein and the protein overexpressed in CHO-Chl^r reacted strongly with antibodies to the alpha class rat Y_c GST subunit (Fig. 3). This blot also demonstrated a 40-fold difference in the expression of this protein between the drug-sensitive and resistant cell lines. This result would agree with the 50-fold elevation in GST-mediated peroxidase activity. To confirm these findings the mobility of the purified protein after electrophoresis in two-dimensional polyacrylamide gels was compared with the overexpressed protein in the CHO-Chl^r cells after two-dimensional electrophoresis. The two proteins were found to have identical mobilities in this system (Fig. 4). The isoelectric point of the purified protein was ≈pH 8.0.

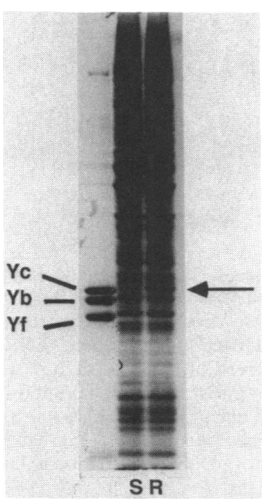

FIG. 1. Protein staining pattern of CHO-K1 and CHO-Chl^r. Samples were separated by SDS/PAGE. Fifty micrograms of soluble protein was taken per lane. Mobilities of the rat Y_c, Y_b, and Y_f standards are also shown. CHO-K1 and CHO-Chl^r are the wild type (S) and resistant (R) cell lines, respectively.

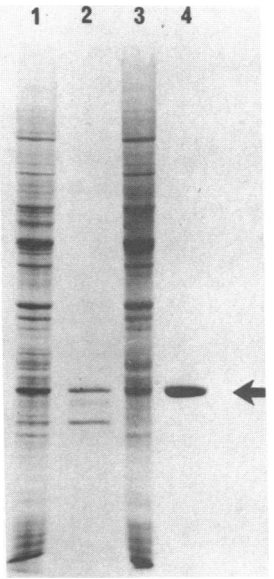

FIG. 2. Purification of the overproduced protein of molecular mass 27.5 kDa from CHO-Chl^r cells. Samples were taken at various stages during the purification and run on 11% SDS/PAGE gels and stained with Coomassie blue. Lanes 1, 2, 3, and 4 are total cell extract, peak of GST activity eluted from the *S*-hexylglutathione column, column flowthrough in the presence of 0.2 M salt, and the purified GST obtained after rechromatography of sample in lane 3, respectively.

In view of the observation that other GST subunits may be involved in drug resistance, Western blots were done with antibodies to other GST enzymes (Fig. 5). Slight differences between CHO-K1 and CHO-Chl^r in the expression of the pi and mu class proteins were seen. The pi class GST is present at much higher concentrations than the alpha class proteins in the CHO-K1 CHO cells and will account for most of the 1-chloro-2,4-dinitrobenzene activity in these cells. This subunit does not have activity toward cumene hydroperoxide, which will explain the much smaller difference in 1-chloro-2,4-dinitrobenzene relative to peroxidase activity seen between CHO-K1 and CHO-Chl^r. A significant elevation in the Y_a subunit, another alpha class protein, was seen. The antibody to this subunit also cross-reacted weakly with the CHO Y_c subunit (Fig. 5). The change in the expression of alpha class GST protein was accompanied by a much higher level of the mRNA coding for these proteins (Fig. 6).

To establish the molecular mechanism for the overexpression of alpha class GST, DNA from the cell lines was analyzed by Southern blot analysis (Fig. 7). The complexity of the banding pattern obtained together with the identification of two alpha class proteins (Y_a and Y_c) indicates that this is a multigene family; this result is consistent with the findings in other species. In Chinese hamsters this gene family appears to cover up to 100 kb of DNA. The intensity of almost all bands was much higher for CHO-Chl^r cells, and in view of the specificity of the cDNA probe for the alpha class GST gene family, the amplified bands almost certainly contain the genes for the Y_a and Y_c proteins. Based on limited dilution of CHO-Chl^r DNA, until the bands were of equal intensity to CHO-K1, it appeared that there was a 4- to 8-fold elevation in gene copy number (data not shown). Densitometric scan-

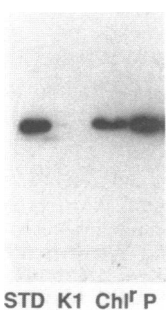

FIG. 3. Comparison of GST subunit content in CHO-K1 and CHO-Chl^r and the purified protein. Western blots were carried out as described. Y_c, standard; K1 and Chl^r, samples from the sensitive and resistant cell lines, respectively; P, purified GST protein (5 μg). The antibody used was to the rat Y_c subunit.

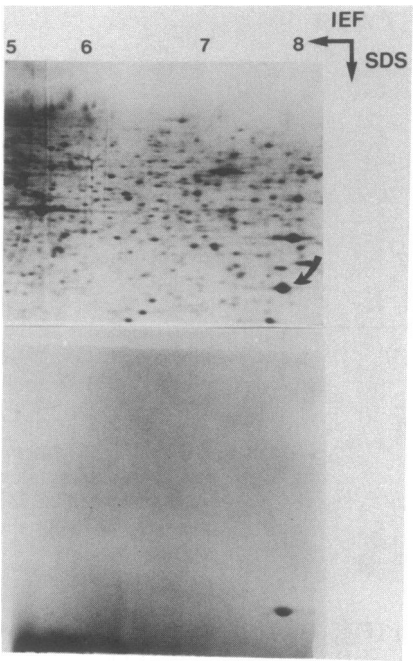

FIG. 4. Two-dimensional gel electrophoresis of CHO-Chl^r and the purified GST protein. Gels were run as described in the text and in ref. 14. One hundred micrograms of soluble protein from CHO-Chl^r (*Top*) or 5 μg of the purified GST enzyme (*Bottom*) were taken. The overexpressed protein in CHO-Chl^r is indicated by a curved arrow. IEF, isoelectric focusing.

ning of the bands indicated that the increase may be up to 20-fold. No difference between CHO-K1 and CHO-Chl^r was seen when a γGT cDNA was used as a probe (Fig. 7*B*), indicating that the change in γGT activity was not due to an amplification event.

The above data provide strong evidence that the class alpha Y_a and Y_c GST are the major proteins overexpressed in CHO cells made resistant to chlorambucil. This increase in expression appears to be a consequence of increased transcription and mRNA levels resulting from a gene amplification. A direct relationship between these effects, however, requires further study. It is worthy of note that DNA repair capacity and also drug accumulation are unchanged in the CHO-Chl^r cells (15) and that the mechanism of the drug resistance appears to be due to enhanced drug detoxification, which results in reduced DNA damage (15). The alpha class GST subunits, therefore, appear important in this mechanism. In support of this possibility GSTs have been shown to catalyze the conjugation of melphalan to GSH, leading to its detoxification (42). Melphalan is a structural homologue of chlorambucil, to which CHO-Chl^r is also resistant. Which GST subunit is responsible for this reaction, however, has not been established.

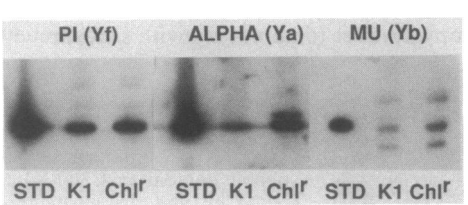

FIG. 5. GSH subunit content in CHO-K1 and CHO-Chl^r. Western blots were done as described with 50 μg of soluble protein per track. The bands were identified by the antibodies raised to the human Y_f, class PI; Y_a, class ALPHA; and Y_b, class MU GST subunits; STD, human GST standard (1 μg); K1, CHO-K1; Chl^r, CHO-Chl^r.

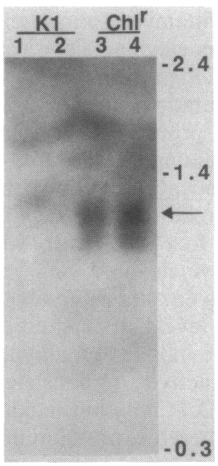

FIG. 6. Alpha class GST mRNA levels in CHO-K1 and CHO-Chl^r. Twenty micrograms of RNA from separate cultures (lanes: 1 and 2, CHO-K1; 3 and 4, CHO-Chl^r) was separated on 1.5% agarose gels containing 6% (wt/vol) formaldehyde and then transferred to Hybond-N membrane. Subsequent analysis was carried out according to Hill *et al.* (35). Molecular sizes in kilobases (kb) are given at right. Arrow, alpha GST.

The molecular mechanisms responsible for the changes in the levels of GST and other glutathione-dependent enzymes have remained elusive. However, we have now obtained evidence that gene amplification may be an important part of this process and, therefore, may play an important role in drug resistance induced by alkylating agents. The time point at which this amplification occurs—i.e., whether some cells

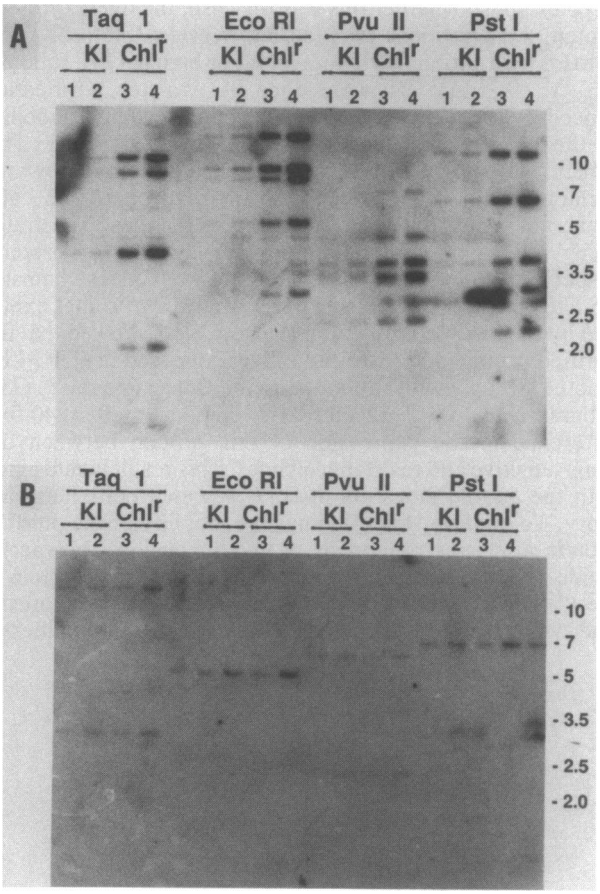

FIG. 7. DNA analysis of CHO-K1 and CHO-Chl^r using an alpha class GST and γGT cDNAs as probes. Two DNA samples from separate cultures for each cell line were digested with restriction endonucleases according to the manufacturer's instructions. (Lanes: 1 and 2, CHO-K1 (K1); 3 and 4, CHO-Chl^r). DNA (5 μg) was separated on a 1% agarose gel and transferred to Hybond-N. Fragments hybridizing the human alpha class GST cDNA (*A*) or the γGT cDNA (*B*) were detected as described by Hill *et al.* (35). On the basis of absorption at 260 nm 1.4-fold more DNA was loaded in lane 4. Molecular sizes in kb are given at right.

intrinsically contain amplified sequences or whether gene amplification is induced by chemical exposure—is unclear.

The phenotypic changes observed in protein expression in drug resistance—i.e., in glutathione-dependent enzymes, P-glycoprotein, and DNA repair enzymes—bear a remarkable similarity to those seen in chemical-induced neoplasia in rat liver (12, 43–45). These changes are often independent of the chemical reagent used and may well reflect the existence of a fundamental cellular response mechanism to combat chemical insult. Whether common genetic changes are involved in such a response is at present unknown.

The role of glutathione-dependent enzymes in this adaptive change is uncertain. However, the ubiquitous nature of the changes seen in the expression of these proteins after cytotoxic insult indicates that they are important. For example, changes in levels of these enzymes have been reported to occur during oxidative stress in both bacteria (46) and rodent lung (47) and also in chemical-induced stress in the mouse bone marrow (13, 48). With particular regard to the GST, it is known that in preneoplasia in rat liver the Y_a, Y_b, Y_c, and Y_f GST subunits are overexpressed (12, 43, 44, 49, 50). The Y_f subunit has also been shown to be overexpressed in MCF7 cell lines made resistant to doxorubicin (adriamycin) (51) or to a variety of other chemotherapeutic agents (Hill B.J., Karen, P. and C.R.W, unpublished). Our study shows that in tumor cell drug resistance the expression of other GST subunits, as well as a variety of glutathione-dependent enzymes, could be of significance in the resistance mechanism. Overexpression of these genes, for example, as a consequence of a gene amplification, implies the presence of another form of cross-resistance distinct from the multidrug resistance phenotype that involves alkylating agents and compounds that are themselves, or which generate, peroxides.

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