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Advances in Brief

Purification of an α Class Glutathione *S*-Transferase from Melphalan-resistant Chinese Hamster Ovary Cells and Demonstration of Its Ability to Catalyze Melphalan-Glutathione Adduct Formation[1]

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Abstract

We have shown previously that a Chinese hamster ovary cell line (designated CHO-Chl^r), generated by exposure to chlorambucil and demonstrating a greater than 20-fold collateral resistance to melphalan, showed increased expression of an α form of glutathione *S*-transferase (GST) associated with amplification of GST genes. Here, we demonstrate that GST purified from CHO-Chl^r cells contains a form with a pI of 9, not present in CHO-K1 cells or Chinese hamster liver, which has the ability to accelerate the formation of glutathione-melphalan adducts. This result provides evidence that overexpression of the α class GST may be directly responsible for the development of resistance to bifunctional alkylating agents.

Introduction

The development of drug resistance is a major obstacle to the successful treatment of many forms of cancer. Studies of the mechanisms which may underlie this process have suggested that increased expression of the cytosolic GSTs[3] may be responsible for resistance to a range of cytotoxic drugs, including bifunctional alkylating agents (1), anthracyclines (2), and BCNU (3). The cytosolic forms of GST are a multigene family of dimeric enzymes which have the ability to conjugate glutathione via the formation of a thioether bond and may be divided into four classes, π, α, μ and θ, on the basis of amino acid sequence homology and immunoreactivity (4). Each class of enzyme includes a variety of monomers with a relative molecular weight of 25,000–30,000, which may form either hetero- or homodimers only with members of the same class. Using step-wise increases in drug concentration, we isolated a chlorambucil-resistant CHO cell line with collateral resistance to melphalan and mechlorethamine but little or no cross-resistance to a wide range of other structurally unrelated agents (5). Characterization of this line, designated CHO-Chl^r, demonstrated a 3-fold increase in GST activity associated with a marked increase in the level of a cytosolic protein which cross-reacted with antibodies produced against rat Yc subunits or human α class GST (6–8). Genetic analysis demonstrated a 4–8-fold increase in genes coding for α class GST (7). Subsequently, it was demonstrated that exposure of cells to 500 μM indomethacin, a known inhibitor of GST activity, potentiated the cytotoxic effect of chlorambucil 5.5-fold in CHO-Chl^r cells but only 2.5-fold in CHO-K1 cells (8). This provided indirect evidence that, in CHO-Chl^r cells, there is a causal relationship between elevation of α class GST and resistance to bifunctional alkylating agents. In this paper, we provide direct evidence that the Yc form of GST expressed in CHO-Chl^r cells has the ability to accelerate the formation of melphalan-glutathione adducts.

Materials and Methods

Cell Culture. CHO-K1 (parental) and CHO-Chl^r (drug-resistant) cells were cultured at 37°C in Ham's F-10 medium supplemented with 1.2 g/liter sodium bicarbonate, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 4 mM L-glutamine using a humidified incubator delivering 5% CO_2. When confluent, cells were removed using 0.05% trypsin-0.2% EDTA in Dulbecco's phosphate-buffered saline without calcium or magnesium (0.2 g/liter KCl, 0.2 g/l KH_2PO_4, 8 g/liter NaCl, and 2.16 g/liter Na_2HPO_4).

Purification of GST. CHO-K1 (1.4×10^9) and CHO-Chl^r (2.5×10^8) cells were washed in phosphate-buffered saline and pelleted. All subsequent procedures were performed at 4°C. CHO cells and Chinese hamster livers were homogenized in 10 mM Tris-HCl (pH 7.8) containing 1 mM EDTA, 0.25 M sucrose, and 1 mM dithiothreitol using a Dounce glass-glass homogenizer with a close-fitting pestle. Following centrifugation at 1,400 × *g* for 20 min, the supernatant was centrifuged at 43,000 × *g* for 40 min. GST from the resulting cytosolic preparation was purified using a glutathione affinity column prepared according to the method of Simons and Vander Jagt (9). Samples were applied to a 5-ml column at a rate of 2.5 ml/h. Unbound proteins were eluted using 50 ml of 10 mM Tris-HCl (pH 7.8) containing 1 mM EDTA, 0.2 M NaCl, and 1.4 mM *b*-mercaptoethanol, followed by 10 ml of the same buffer without NaCl. GST was eluted using 50 mM glutathione, 1 mM EDTA, and 1 mM dithiothreitol adjusted to pH 8 with 1 M Tris.

Characterization of Purified Enzymes. Discontinuous SDS-PAGE using 12% monomer was performed using the apparatus supplied by Bio-Rad (Minigel). Proteins were detected with Coomassie blue. Isoelectric focusing was performed in 0.4-mm thick polyacrylamide gels with an ampholyte pH range of 3–10. Gels were cast and run using a Bio-Rad model 111 mini-IEF cell according to the manufacturer's instructions.

GST enzymes were separated by HPLC by the method of Ostlund Farrants *et al.* (10) using a Waters model 600E system with 5-μm C_4 reverse phase column (Vydac) and were detected at 214 nm. The flow rate was 1 ml/min; the mobile phase had a linear gradient of 40–50% acetonitrile with 1% TFA.

For amino acid sequencing, proteins were separated by SDS-PAGE as described above and transferred onto polyvinylidene membranes. Proteins were sequenced using a gas-phase microsequencer (Beckman; model LF 3000) according to the manufacturer's instructions.

Assay of Melphalan Adduct Formation. GST preparations were passed down a desalting column (Econopac 10DG; Bio-Rad) equilibrated in 140 mM potassium phosphate (pH 6.5), and 25 μl of a 20 mM solution of glutathione was added to 450-μl aliquots, followed by 5 μl of a 2 mg/ml solution of melphalan in acidified ethanol (1% 12 M HCl v/v). At 5-min intervals, 7 μl of TFA was added to a 70-μl aliquot of the reaction mixture. Control incubations were performed in which water was substituted for glutathione and/or buffer for the enzyme solution.

Following centrifugation at 12,000 rpm, 50-μl samples were analyzed by reverse phase chromatography using a 3-μm ODS cartridge column (Beck-

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[3] The abbreviations used are: GST, glutathione *S*-transferase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CHO, Chinese hamster ovary; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid.

man). Peaks were detected at 263 nm. The flow rate was 1 ml/min, and the mobile phase had a linear gradient of 10–50% acetonitrile with 0.1% TFA. In the presence of glutathione, a peak was detected with a retention time of 8 min from the onset of a 15-min linear gradient. This was identified as the mono-adduct of glutathione and melphalan by a combination of radioisotope studies and nuclear magnetic resonance (data not shown). Using this separation technique, there was no evidence of di-adduct formation. The amount of adduct formed was calculated in terms of "melphalan equivalents" from a standard curve of peak area *versus* melphalan concentration prepared using pure melphalan.

Results

Purification of GST. SDS-PAGE of GST purified from CHO-Chl^r cells demonstrated the presence of a predominant band with a molecular weight of approximately 30,000, which was also present in Chinese hamster liver but not CHO-K1 cells (Fig. 1). Two minor species were also detected with molecular weights of approximately 29,000. These proteins were also present in Chinese hamster liver but not CHO-K1 cells. The predominant band seen in the sample from CHO-K1 cells had a molecular weight of 27,000. This form was detected in extracts from CHO-Chl^r cells but not Chinese hamster liver. Isoelectric focusing demonstrated the presence of one major protein from CHO-Chl^r cells with a pI of approximately 9, which did not correspond to any of the isoforms present in the sample from CHO-K1 cells. The sample from Chinese hamster liver contained multiple isoforms, suggesting some microheterogenity of these GSTs, but none of these comigrated with the protein purified from CHO-Chl^r cells (Fig. 2). Reverse phase chromatography of GSTs purified from CHO-Chl^r revealed the presence of a protein with a retention time of 21 min which was not present in CHO-K1 cells (Fig. 3). A species with a retention time of 18 min was present in both samples.

Sequencing of the NH_2-terminus of the band with a molecular weight of 27,000 gave the sequence PPYTIVYFPVRGRCE. Other bands present in the sample from CHO-Chl^r cells were found to be blocked at the NH_2-terminus.

Effect of Purified GST on the Rate of Melphalan-Glutathione Adduct Formation. The effect of GST purified from CHO-K1 and CHO-Chl^r cells and from Chinese hamster liver on the rate of melphalan-glutathione adduct formation is shown in Fig. 4. The amount of enzyme present in the incubation mixture was 0.12 mg/ml, 0.05 mg/ml, and 0.5 mg/ml for CHO-Chl^r, CHO-K1, and Chinese hamster liver, respectively.

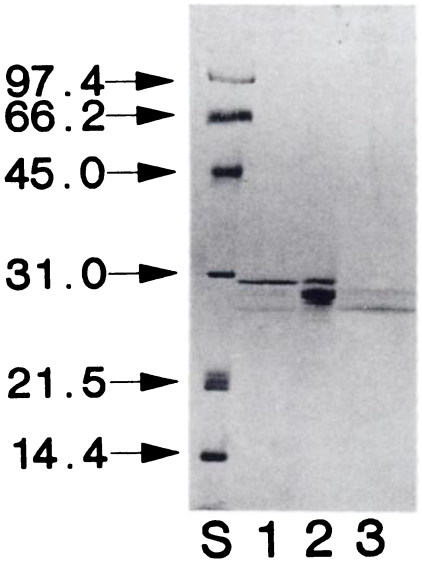

Fig. 1. SDS-PAGE of GST purified from CHO-Chl^r cells (*track 1*), Chinese hamster liver (*track 2*), and CHO-K1 cells (*track 3*). The track marked *S* contained molecular weight markers. The figures in the *left margin* refer to molecular weights in kilodaltons.

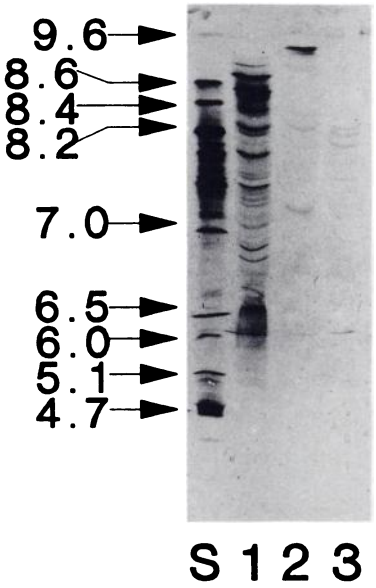

Fig. 2. IEF of GST purified from Chinese hamster liver (*track 1*), CHO-Chl^r cells (*track 2*), and CHO-K1 cells (*track 3*). The track marked *S* contained IEF markers. The figures in the *left margin* refer to the pI of the marker proteins.

Discussion

GSTs have been purified from a wide variety of plant and animal species (11) and appear to form an essential component of defense mechanisms which have evolved to protect cells against toxic xenobiotics (12). Resistance to a range of cytotoxic drugs including BCNU (3), Adriamycin (2), and the bifunctional alkylating agents (1) has been associated with increased levels of the cytosolic forms of the enzyme in drug-resistant cell lines. Attempts have been made to provide evidence that such elevations are directly involved in the development of drug resistance. However, GST gene transfection experiments have produced conflicting results, perhaps reflecting differences in the isoform used, the level of enzyme expressed, or the lineage of the cells used as targets. For example, Puchalski and Fahl (13), using COS cells as targets for transient transfection, demonstrated modest increases in resistance to chlorambucil and melphalan in cells transfected with α class GST, to cisplatin in cells transfected with μ class GST, and to doxorubicin in cells transfected with π class GST. In contrast, transfection of either human π or α class GST genes into MCF-7 cells failed to produce any demonstrable increase in resistance to doxorubicin, melphalan, or cisplatin (14, 15).

For some cytotoxic drugs, there is biochemical evidence that GSTs may provide protection from cytotoxicity via direct detoxification. For example, μ class GST purified from rat liver has been shown to detoxify BCNU by denitrosation (3). Glutathione has been shown to react with either melphalan (16) or chlorambucil (17) to form mono and subsequently di-adducts, and using a crude preparation, Dulik *et al.* (16) demonstrated that the formation of melphalan-glutathione conjugates was increased in the presence of liver enzymes. Subsequently, Bolton *et al.* (18) reported that α class GST purified from mouse liver enhanced the rate of glutathione-melphalan conjugation. Recently, Meyer *et al.* (19) showed that α class GST purified from human liver has the ability to sequester chlorambucil-glutathione adducts, providing an alternative mechanism whereby enhanced expression of GST may confer resistance. These results emphasize that GST isoforms differ greatly in their ability to detoxify cytotoxic drugs

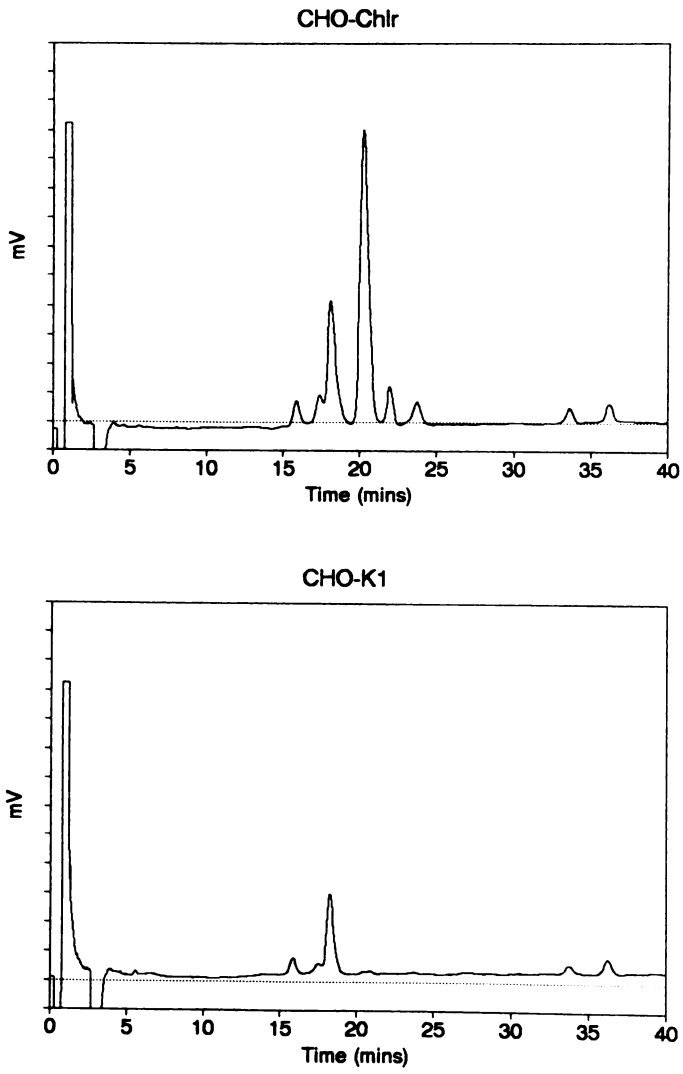

Fig. 3. HPLC of GST purified from CHO-Chl^r (*upper trace*) and CHO-K1 cells (*lower trace*). Absorbance was measured at 214 nm. Full-scale deflection represents 0.2 absorbance units.

and that detailed knowledge of the catalytic potential of enzymes with enhanced expression in resistant cell lines or tumors is important before they can be directly implicated in the development of the resistance phenotype.

In this paper, we demonstrate that α class GST purified from a well-characterized cell line with increased resistance to bifunctional alkylating agents has the ability to catalyze the formation of melphalan-glutathione conjugates to a greater extent than α class GST purified from either the parental CHO-K1 cells or Chinese hamster liver.

GSTs were purified from CHO-Chl^r and CHO-K1 cells and from Chinese hamster liver by affinity chromatography and characterized by SDS-PAGE, isoelectric focusing, reverse phase HPLC, and amino acid sequencing. A comparison of the results obtained demonstrated the presence of one predominant form in the sample from CHO-Chl^r cells which was not present in CHO-K1 cells, as determined by SDS-PAGE, isoelectric focusing, and reverse phase HPLC analysis. Amino acid sequencing of this protein was unsuccessful due to blockage at the NH_2-terminus, in common with other α class GSTs from hamster tissues (20). Sequencing of a minor band in the sample with a molecular weight of 27,000 showed it to be homologous at the NH_2-terminus to the P_1 form purified from Syrian golden hamster tissues and to rat the Yf monomer (20). Characterization of the GST from CHO-K1 cells demonstrated one predominant form, which comigrated with the Yf form in the sample from CHO-Chl^r cells.

Using an HPLC assay to measure the extent of melphalan-glutathione adduct formation at timed intervals, it was shown that the GSTs purified from CHO-Chl^r cells have the ability to accelerate the rate of conjugation. This activity was not demonstrated in GST purified from CHO-K1 cells which contained π class GST alone, suggesting that the small amount of π class enzyme also present in the preparation from the resistant cell line was not responsible for this activity. GST isoforms expressed in Chinese hamster liver, although not including the Yc form found in CHO-Chl^r cells, also possess the ability to accelerate the conjugation of melphalan and glutathione. This indicates that, in common with many other GST isoforms, the ability to conjugate a given substrate may be shared by other members of the same class, although the specific activity of the liver forms appeared

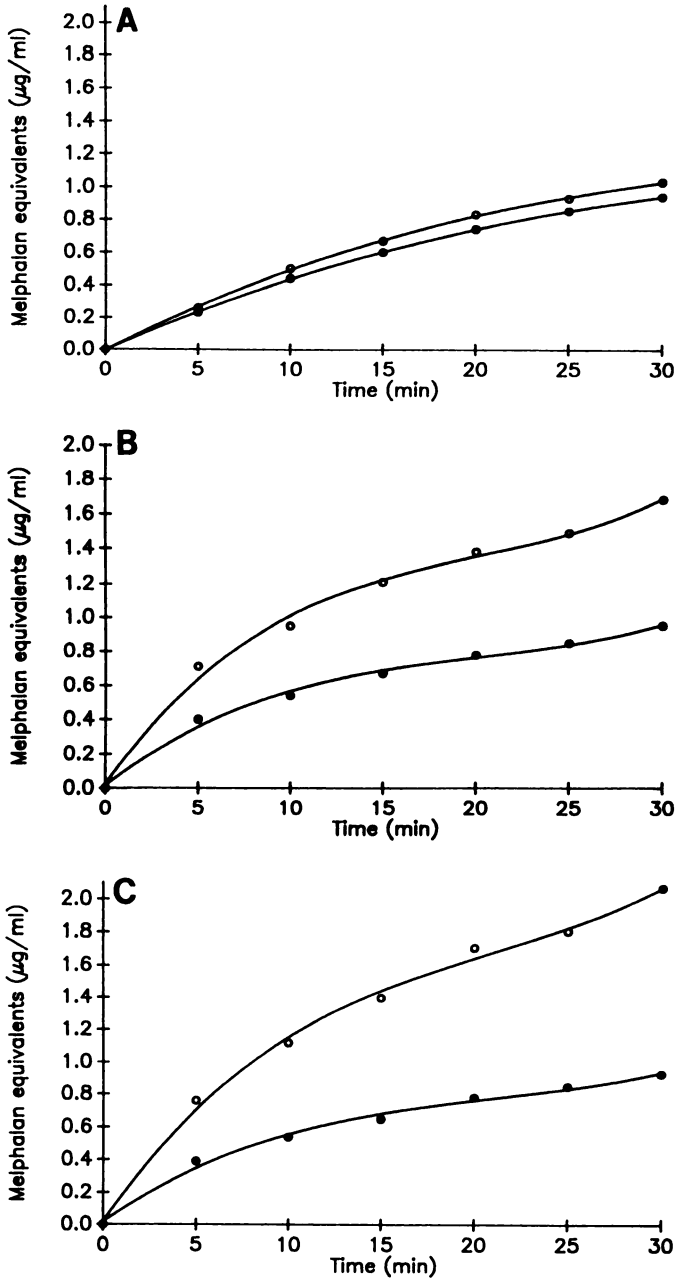

Fig. 4. Effect of GST from CHO-K1 (*A*), CHO-Chl^r (*B*) and Chinese hamster liver (*C*) on the rate of melphalan-glutathione conjugate formation. ○, with enzyme; ●, no enzyme.

to be lower than that of the enzyme purified from chlorambucil-resistant cells.

These results provide direct evidence that α class GST expressed in a cell line resistant to bifunctional alkylating agents has the ability to accelerate the formation of melphalan-glutathione conjugates but that this ability is not shared by all GST isoforms. Due to the difficulty of purifying adequate quantities of enzyme from CHO-Chl^r cells to allow the production of homogenous enzyme preparations in sufficient quantities to allow detailed kinetic studies, we intend to clone the Yc isoform overexpressed in CHO-Chl^r cells in order to produce recombinant protein. Transfection studies will also be undertaken in order to confirm that overexpression of Yc GST is the main factor responsible for resistance in the CHO-Chl^r cell line.

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