Evidence that human class Theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse

Comparison of the tissue distribution of GST T1-1 with that of classes Alpha, Mu and Pi GST in human

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The cDNA encoding human glutathione S-transferase (GST) T1 has been expressed as two recombinant forms in *Escherichia coli* that could be purified by affinity chromatography on either IgG-Sepharose or nickel-agarose; one form of the transferase was synthesized from the pALP 1 expression vector as a *Staphylococcus aureus* protein A fusion, whereas the other form was synthesized from the pET-20b expression vector as a C-terminal polyhistidine-tagged recombinant. The yields of the two purified recombinant proteins from *E. coli* cultures were approx. 15 mg/l for the protein A fusion and 25 mg/l for the C-terminal polyhistidine-tagged GST T1-1. The purified recombinant proteins were catalytically active, although the protein A fusion was typically only 5–30 % as active as the histidine-tagged GST. Both recombinant forms could catalyse the conjugation of glutathione with the model substrates 1,2-epoxy-3-(4'-

nitrophenoxy)propane, 4-nitrobenzyl chloride and 4-nitrophenethyl bromide but were inactive towards 1-chloro-2,4-dinitrobenzene, ethacrynic acid and 1-menaphthyl sulphate. Recombinant human GST T1-1 was found to exhibit glutathione peroxidase activity and could catalyse the reduction of cumene hydroperoxide. In addition, recombinant human GST T1-1 was found to conjugate glutathione with dichloromethane, a pulmonary and hepatic carcinogen in the mouse. Immunoblotting with antibodies raised against different transferase isoenzymes showed that GST T1-1 is expressed in a large number of human organs in a tissue-specific fashion that differs from the pattern of expression of classes Alpha, Mu and Pi GST. Most significantly, GST T1-1 was found in only low levels in human pulmonary soluble extract of cells, suggesting that in man the lung has little capacity to activate the volatile dichloromethane.

INTRODUCTION

The glutathione S-transferases (GSTs) are a supergene family of drug-metabolizing enzymes comprising both membrane-bound and soluble proteins [1]. In mammals, at least six classes of soluble enzyme, designated Theta, Kappa, Alpha, Mu, Pi and Sigma, have been described [1–4]. Three membrane-bound forms that have evolved separately from the cytosolic transferases are referred to as microsomal GST, leukotriene C₄ synthase and microsomal GST II [5–8].

The catalytic activity of GST is associated almost entirely with the detoxification of carcinogens, anti-cancer drugs, pesticides and herbicides [1]. They also detoxify reactive products generated by oxidative stress such as α,β -unsaturated carbonyls, quinones and hydroperoxides [1,9,10]. GST isoenzymes are also involved in the biosynthesis of leukotriene C₄ and prostaglandins D₂, E₂ and F_{2 α} [3,6,7,11,12]. The conjugation of reduced glutathione (GSH) with foreign compounds usually results in the generation of relatively inactive products that are readily eliminated from the cell by a number of efflux pumps [13]. In certain instances, however, the glutathione conjugate formed is more reactive than the parent compound. Most chemicals so far known to undergo bioactivation by GST are small bifunctional electrophiles [1]. Such compounds are typically halide- or epoxide-containing methanes, ethanes or butanes, for example the dihaloalkanes dichloromethane (DCM), dibromomethane and dibromoethane

[14–17], and the epoxide-containing 1,2-epoxy-4-bromobutane and butadiene diepoxide [18,19].

Certain of the chemicals that are activated by GST are widely distributed in the environment; there is therefore justified concern about the risk they pose to human health. For example, DCM, which is a potent hepatic and pulmonary carcinogen in mouse [20,21], is found in paint strippers, aerosol preparations and is used extensively as an industrial solvent [22].

The activation of DCM is catalysed by a unique member of the class Theta transferases, namely GST T1-1 [14,23]. Unlike the class Alpha, Mu and Pi GSTs, the class Theta enzymes are not abundant, stable or readily purified by affinity chromatography [2,24–27]. Whereas the rat and mouse GST T1-1 enzymes have been studied by several research groups, little progress has been made in characterizing the human transferase at the protein level. Although Meyer et al. [2] purified sufficient GST T1-1 from human liver for amino acid sequencing, insufficient amounts were obtained to allow either its catalytic properties to be established or antibodies to be raised against it.

In the present study we have employed the human GSTT1 cDNA clone described by Pemble et al. [28] to direct the synthesis of a recombinant protein that could be isolated in a single chromatographic step. We now report the design of two forms of recombinant human GST T1-1, which can be simply purified after heterologous expression in *Escherichia coli*. Isolation of large amounts of recombinant human GST T1-1 has enabled the

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCM, dichloromethane; ENPP, 1,2-epoxy-3-(4'-nitrophenoxy)propane; GSH, reduced glutathione; GST, glutathione S-transferase; His-tagged, polyhistidine-tagged; Ni-NTA, nickel-agarose; 4-NBC, 4-nitrobenzyl chloride; 4-NPB, 4-nitrophenethyl bromide.

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catalytic properties of this protein to be described for the first time. In addition, antibodies raised against recombinant GST T1-1 have been used in Western blot experiments to demonstrate the tissue distribution of the enzyme in human. This information is of value in helping to assess which organs have the capacity to activate dihaloalkanes and might be particularly sensitive to exposure to these compounds.

MATERIALS AND METHODS

Plasmids and bacterial strains

The human GSTT1 cDNA clone pHGSTT1 [28] was generously supplied by Dr. Sally E. Pemble (CRC Molecular Toxicology Group, University College London, London, U.K.). The vector pTZ 19r was obtained from Pharmacia Biotech (Uppsala, Sweden). The expression vector pET-20b was from Novagen (Madison, WI, U.S.A.), and pALP1 was kindly provided by Dr. Mike J. R. Stark (Department of Biochemistry, University of Dundee, Dundee, U.K.) [29] along with *E. coli* strain 159 (*uvrA*, *gal*, *str^r*, *sup^o*). Other strains that were employed in this study are commonly available, including NM 522 (*supE*, *thi-1*, $\Delta(lac-proAB)$, $\Delta(mcrB-hsdSM)5$, ($r_K^- m_K^-$), [F' , *proAB*, *lac I^qZ*, $\Delta M15$]] and BL21(DE3) [F^- , *ompT*, *hsdS_B*, ($r_B^- m_B^-$), *dem*, *gal* (DE3)].

Enzymes and chemicals

Native *pfu* Taq polymerase was obtained from Stratagene (Cambridge, Cambs., U.K.). All restriction endonucleases were purchased from Gibco BRL Life Sciences (Paisley, Renfrewshire, U.K.). The fmol[®] DNA Sequencing System was supplied by Promega (Southampton, Hants., U.K.). Two affinity chromatography matrices were utilized: IgG-Sepharose 6 Fast Flow was obtained from Pharmacia Biotech, and nickel-agarose (Ni-NTA) resin was obtained from Qiagen (Dorking, Surrey, U.K.). All the chemicals examined as GST substrates were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.) except DCM, which was from Fisons (Loughborough, Leics., U.K.), and 1-menaphthyl sulphate, which was custom-synthesized by Ultrafine Chemicals (Manchester, Greater Manchester, U.K.). Pefabloc protease inhibitor was obtained from Boehringer Mannheim (Lewes, East Sussex, U.K.). All other chemicals used were from commonly available commercial sources.

Protein determination

Protein concentrations were determined by the method of Bradford [30] with reagent obtained from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.).

Enzyme assays

All enzyme activity assays were conducted at 37 °C. GST activity towards 1,2-epoxy-3-(4'-nitrophenoxy)propane (ENPP), 4-

nitrobenzyl chloride (4-NBC), 4-nitrophenethyl bromide (4-NPB), ethacrynic acid and 1-menaphthyl sulphate were measured manually on a Shimadzu UV 3000 spectrophotometer (Haverhill, Suffolk, U.K.) and were monitored at 360, 310, 310, 270 and 298 nm respectively, with GSH and substrate concentrations as described by Habig and Jakoby [31]. GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione peroxidase activity with cumene hydroperoxide were measured at 340 nm with the Cobas Fara II Chemistry System (Hoffmann-La Roche Ltd, Basel, Switzerland) [32,33]. In all enzyme activity assays, the non-enzymic reaction rate was measured and subtracted from the overall reaction rate.

Enzyme activity towards DCM was determined by monitoring the production of formaldehyde colorimetrically [34] (Scheme 1). The enzyme-catalysed reaction was performed in a final volume of 2 ml with between 50 and 100 µg of recombinant protein in a solution of 100 mM Tris/HCl (pH 8)/10 mM GSH. DCM was added to 5 mM and the reaction mixtures were then incubated at 37 °C in sealed vials. Portions (0.75 ml) were removed from these mixtures after 10 and 20 min, and the reaction was stopped by addition of trichloroacetic acid to a final concentration of 20 % (v/v) to precipitate the protein. The supernatant was assayed for formaldehyde by mixing it with an equal volume of a solution containing 2 M ammonium acetate, 0.05 M acetic acid and 0.02 M acetylacetone. The absorbance of the resulting mixtures at 412 nm was compared with that of formaldehyde standards (0–8 µg/ml formaldehyde); the addition of formaldehyde to a solution containing acetylacetone and ammonium salt results in the initiation of a Hantzsch synthesis reaction that yields diacetyldihydrolutidine, which has a maximum absorbance at 412 nm. The Hantzsch reactions were allowed to go to completion (42 °C, 30 min) before measuring the absorbance at 412 nm; under these conditions the amount of product formed was directly proportional to the amount of formaldehyde present.

The apparent K_m and k_{cat} values for glutathione were determined at pH 6.5 and 7.5. The K_m and k_{cat} values for the model GST T1-1 substrate, ENPP, were determined at pH 6.5. The initial rate of reaction was measured with a variety of substrate concentrations. For glutathione values, the GSH concentration was varied between 0 and 20 mM while the ENPP concentration was held constant at 1 mM. For ENPP values, the concentration of epoxide was varied between 0 and 1.2 mM while the GSH concentration was held at 20 mM. The initial reaction rates were then used to calculate K_m and k_{cat} with the non-linear curve-fitting package Ultrafit from Biosoft (Cambridge, Cambs., U.K.), employing simple weighting on the Marquardt algorithm.

Electrophoresis and Western blot analyses

Discontinuous SDS/PAGE was performed with the buffer system of Laemmli [35] in 12 % (w/v) polyacrylamide resolving gels. After electrophoresis, proteins were transferred to Immobilon-P obtained from Millipore (Watford, Herts., U.K.) by previously

Scheme 1 Biotransformation of DCM by the GST-mediated conjugation of glutathione

described techniques [36]. Antibodies against recombinant human GST T1-1 were raised in female New Zealand White rabbits by methods described previously [37]. The antibodies used to study class Alpha, Mu and Pi transferases have been described elsewhere [37–39]. Horseradish peroxidase-labelled secondary antibodies against goat anti-(rabbit IgG) obtained from Bio-Rad Laboratories were used, and detection was achieved by enhanced chemiluminescence with reagents from Amersham Life Science (Little Chalfont, Bucks., U.K.).

Molecular cloning

Two cloning strategies were devised for the production of recombinant human GST T1-1 (see Scheme 2 below).

Two sets of PCR were designed to amplify the coding region of the human GSTT1 subunit. Reactions (100 μ l) were performed in buffer containing 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl, pH 8.0, 2 mM MgSO_4 , 0.1% Triton X-100, 10 μ g/ml nuclease-free BSA, each dNTP at 0.2 mM, 5 ng of template DNA, 2.5 units of native *pfu* DNA polymerase and 0.3 μ M of each primer. One forward primer, PJS 1 (5'-GGA ATT CCA TAT GGG TCT GGA GCT CTA CCT GGA CCT GC-3'), and two reverse primers, PJS 2 (5'-CGG GAT CCC GCT CAC CGG ATC ATG GCC AGC ACC CA-3') and PJS 6 (5'-CGG GAT CCC GGA TCA TGG CCA GCA CCC AGG GCA-3'), were employed. Reactions were performed in a Hybaid omnigene thermal cycler with the following programme: two cycles each comprising 1 min at 94 °C, 1 min at 53 °C and 2 min at 72 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 58 °C and 2 min at 72 °C; the reaction was completed within 10 min at 72 °C.

After PCR amplification, products of approx. 750 bp were ligated into the *Eco*RI and *Bam*HI restriction sites of pTZ 19r. The resulting constructs (pJSHT1-2, which contained the PCR product from primers PJS 1 and 2, and pJSHT1-3, which contained the PCR product from primers PJS 1 and 6) were transformed into *E. coli* NM 522. The fidelity of these amplification reactions was examined by sequence analysis of the constructs with the fmol[®] DNA Sequencing System.

The insert from pJSHT1-2 was cloned into the *Eco*RI and *Bam*HI sites of pALP1 downstream to a portion of the *spa* gene of *Staphylococcus aureus*, encoding part of the immunoglobulin-binding portion of protein A, to form the expression vector pJSPA-T1. The insert from pJSHT1-3 was cloned into the *Nde*I and *Bam*HI sites of pET-20b upstream of a polyhistidine-encoding sequence to form the expression vector pJST1-20.

Expression of recombinant human GST T1-1

E. coli strain 159 transformed with pJSPA-T1 was routinely grown overnight in Luria–Bertani medium containing 50 μ g/ml kanamycin at 37 °C in a rotary shaker. Cells, from 200 ml of culture, were harvested by centrifugation and pellets were stored at –70 °C for between half an hour and 7 days. BL21 (DE3) transformed with pJST1-20 were routinely grown and stored in a similar manner, except that the antibiotic selection used was 50 μ g/ml ampicillin.

Purification of protein A–GSTT1 fusion

Cell pellets were thawed and resuspended in 10 ml of Tris/saline/Tween-20 [TST; 50 mM Tris/HCl (pH 7.6)/150 mM NaCl/0.05% Tween-20]. Cell lysis was achieved with 8 μ g/ml of lysozyme at 4 °C for 20 min before the cells were finally disrupted by sonication. The insoluble material from the lysates was removed by centrifugation (17000 *g* for 35 min at 4 °C) and the

resulting supernatant was applied at 4 °C to an IgG-Sepharose 6 Fast Flow column (bed volume 1.5 ml) that had been pre-equilibrated by twice washing alternately with 5 ml of 0.5 M acetic acid (adjusted to pH 3.4 with ammonium acetate) and 8 ml of TST. After the supernatant had been applied, the column was washed with 45 ml of TST and then with 6 ml of 5 mM ammonium acetate, pH 5.0, before the fusion protein was eluted with 10 ml of 0.5 M acetic acid, pH 3.4. Immediately after collection, the eluate was adjusted to neutral pH with unbuffered 2 M Tris base.

Purification of C-terminal polyhistidine-tagged GSTT1

Cell pellets were thawed and resuspended in 10 ml of lysis buffer [50 mM sodium phosphate (pH 8.0)/300 mM NaCl]. Both cell lysis and preparation of the soluble fraction were achieved by the method described above for the purification of the protein A–GSTT1 fusion. Imidazole was added to the soluble fraction of *E. coli* cell lysate to a final concentration of 5 mM. A portion (3.0 ml) of a 50% Ni-NTA resin slurry was pre-equilibrated with lysis buffer. The equilibrated resin was then added to the soluble fraction containing 5 mM imidazole and mixed at 4 °C for 60 min on a rotary mixer. The remainder of the purification procedure was performed at room temperature. The mixture of lysate and Ni-NTA resin was poured into a 15 ml column and allowed to pack under gravity. The Ni-NTA resin was treated with lysis buffer, pH 6.0 (5 bed vol.) before being washed extensively with lysis buffer containing stepwise increases of imidazole (20, 30, 40 and 60 mM) at pH 8.0. Finally, when no protein was detected in the eluate by absorbance at 280 nm, the recombinant protein was eluted with 8 bed vol. of lysis buffer containing 300 mM imidazole, pH 8.0.

Preparation and analysis of human tissue extracts for immunoblot analysis

Human organs were available from four male individuals who were the subjects of routine autopsy to establish the cause of death: patient 386 was 73 years old with bronchopneumonia and atherosclerosis; patient 387 was 52 years old with cerebral infarct; patient 388 was 65 years old with fractured pelvis, alcoholic cirrhosis and fibrosing alveolitis; patient 396 was 38 years old with aspiration pneumonia, bleeding oesophageal varices and alcoholic liver disease. The materials were obtained between 6 and 12 h post mortem and stored at –70 °C until use. Tissues available for analyses included cerebrum, heart, kidney, liver, lung, pancreas, prostate, skeletal muscle, small intestine, spleen and testes. Whereas GST in livers from all four patients were examined (to exclude *GSTT1* null individuals), tissues were selected from patient 386 for the organ survey.

Portions (approx. 50 mg) of tissue were allowed to thaw in 4 vol. of ice-cold 0.1 M sodium phosphate buffer, pH 6.9, containing 0.05% Nonidet P40 and 2.5 mg/ml Pefabloc protease inhibitors. Soluble extracts of human tissue were prepared by homogenization with an Omni EZ Connect Homogenizer (Omni International, Gainesville, VA, U.S.A.) which was followed by centrifugation to remove cellular debris (15000 *g* for 10 min at 4 °C).

Aliquots (10 μ g of protein) of each soluble extract were subjected to SDS/PAGE. After transfer to Immobilon-P the resolved proteins were probed with antibodies raised against classes Theta (1:10000 dilution for GST T1-1), Alpha (1:20000 dilution for GST A1-2 and 1:1000 for GST A4-4), Mu (1:1000 for GST M1a-1a and 1:3000 for GST M3-3) and Pi (1:1000 dilution for GST P1-1) transferases.

RESULTS

Heterologous expression of recombinant human GSTT1

Sequence analysis of the cDNA clone for the human GSTT1 subunit (phGSTT1) showed that it differed from the human GSTT1 clone described previously [28] at three positions. At nt

102, 165 and 377 from the first A of the ATG initiation codon, phGSTT1 contained C, A and A respectively rather than T, G and G. Of these differences, only the latter G → A substitution at codon 126 translates into a protein difference, with the replacement of a Gly residue with Glu. It is assumed that these nucleotide substitutions between the human GSTT1 clone studied

Scheme 2 Cloning strategy for the construction of plasmids for recombinant human GSTT1 expression

The coding region of human GSTT1 was amplified by PCR and ligated into pTZ 19r to yield either pJSHT1-2 or pJSHT1-3, before sub-cloning into pALP1 and pET-20b expression vectors respectively.

Figure 1 Purification of recombinant human GSTT1 from *E. coli*

The expression of recombinant human GSTT1 in bacterial extracts was examined by SDS/PAGE as described in the text. **(a)** Purification of the protein A-GSTT1 fusion, of approx. 43 kDa, by IgG affinity chromatography. The samples were loaded as follows: lane 1, molecular mass markers; lane 2, whole cell lysate; lane 3, insoluble cell extract; lane 4, soluble cell extract; lane 5, material that did not bind to the IgG-Sepharose affinity matrix; lane 6, purified protein A-GSTT1. **(b)** Purification to homogeneity of the C-terminal His-tagged GSTT1 recombinant, of approx. 30 kDa, on Ni-NTA resin. The samples were applied as follows: lane 1, molecular mass markers; lane 2, whole cell lysate; lane 3, insoluble cell extract; lane 4, soluble cell extract; lane 5, material that did not bind to the Ni-NTA resin; lane 6, purified recombinant GSTT1.

in the present paper and that described previously represent allelic variations in the population. It should be noted that a cDNA clone that encodes an identical primary sequence to that of phGSTT1 has been isolated separately from ourselves by P. Jemth and B. Mannervik (personal communication), a result that supports the authenticity of the protein difference noted. These workers also confirm the sequence difference at nt 165, which does not translate to a protein difference.

The coding region of phGSTT1 was amplified by PCR and inserted into pTZ 19r, to yield either pJSHT1-2 or pJSHT1-3, before sub-cloning the inserts into the pALP1 and pET-20b expression vectors respectively (Scheme 2). The PCR products were sequenced and over the coding region were found to be identical with that of phGSTT1, indicating that *pfu* Taq polymerase had not introduced any errors during amplification.

The pALP1 expression vector, which is under the control of the *lac* promoter, was chosen for the production of a fusion

Table 1 Specific activities for the two recombinant forms of human GST T1-1 determined as described in the Materials and methods section

Abbreviation: n.d., no detectable activity.

Substrate	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	
	His-tagged GST T1-1	Protein A-GST T1-1
ENPP	18.0 ± 1.00	3.25 ± 0.23
4-NBC	2.02 ± 0.35	0.117 ± 0.006
4-NPB	0.223 ± 0.021	0.075 ± 0.004
Cumene hydroperoxide	2.77 ± 0.15	0.104 ± 0.007
CDNB	n.d.	n.d.
1-Menaphthyl sulphate	n.d.	n.d.
Ethacrynic acid	n.d.	n.d.
DCM	0.402 ± 0.028	0.0876 ± 0.0077

protein comprising part of the immunoglobulin-binding portion of *S. aureus* protein A and the GSTT1 subunit. The expression construct for the N-terminal tagged protein A-GSTT1 fusion, designated pJSPA-T1, was transformed into *E. coli* strain 159. After growth overnight at 37 °C, the cells were harvested and analysed by SDS/PAGE. This demonstrated the presence of a very intense band of approx. 43 kDa that was apparent in extracts from the transformed and untransformed *E. coli*. The predicted size of the fusion protein was 44.8 kDa. It was thought that this 43 kDa polypeptide represented a constitutive *E. coli* protein that co-migrated with the GST of interest. Fractionation of *E. coli* extracts into a 17000 g pellet and a supernatant revealed a 43 kDa polypeptide present only in the soluble fraction of the transformed *E. coli*. The intense co-migratory band was localized entirely in the insoluble material of the untransformed *E. coli*. Immunoblotting with antibodies raised against rat GST T2-2 estimated that more than 50% of the putative N-terminal tagged protein A-GSTT1 fusion in the pJSPA-T1-transformed *E. coli* strain 159 was recovered in the 17000 g supernatant fraction; henceforth this transferase is referred to as protein A-GSTT1.

The pET-20b expression vector, which constitutively expresses protein under the T7 polymerase promoter, was chosen for production of a recombinant GSTT1 subunit fused at the C-terminus to a polyhistidine-containing peptide (DPNSSVDKLAALAEHHHHH); henceforth this transferase is referred to as His-tagged GSTT1. The expression construct for the His-tagged protein, designated pJST1-20, was used to transform *E. coli* BL21(DE3). After growth overnight at 37 °C a major protein of 30 kDa was observed in the transformed BL21(DE3) cells. As this polypeptide was absent from control BL21(DE3) cells and the calculated mass of the His-tagged transferase subunit is 29.7 kDa, the polypeptide of 30 kDa was assumed to represent His-tagged GSTT1. Fractionation of transformed BL21(DE3) extracts showed that although a small amount of the His-tagged GSTT1 was found in the pellet from lysed bacteria, most of this protein was recovered in the soluble extract.

Purification of recombinant GSTT1

The protein A-GSTT1 fusion was purified from *E. coli* strain 159 by affinity chromatography on IgG-Sepharose as described in the Materials and methods section. Examination of the affinity-purified material by SDS/PAGE showed that it comprised a

Table 2 Apparent K_m and k_{cat} values for His-tagged human GST T1-1 determined as described in the Materials and methods section

Substrate	pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
GSH	6.5	5.75 ± 0.87	21.3 ± 1.0	3.70
	7.5	1.93 ± 0.14	13.3 ± 0.8	6.89
ENPP	6.5	1.02 ± 0.08	14.3 ± 0.3	14.02

major band of 43 kDa (Figure 1a) with the presence of a minor 60 kDa polypeptide that typically represented less than 10 % of the total protein. A significant amount of this additional 60 kDa species could be removed from the preparation by anion-exchange chromatography on a mono Q FPLC column equilibrated with 10 mM Tris/HCl, pH 8.0, and developed with a linear 0–200 mM NaCl gradient. The best preparations of protein A–GSTT1 were estimated by SDS/PAGE to be at least 95 % pure. From this method, a yield of approx. 15 mg of recombinant protein A–GSTT1 per litre of bacterial culture was obtained.

The His-tagged GSTT1 was purified from *E. coli* BL21(DE3) by a batch-type adsorption chromatography procedure with Ni-NTA resin as described in the Materials and methods section. Electrophoretic analysis of the adsorbed material that was eluted from the resin by an imidazole buffer showed the presence of a single 30 kDa protein band, indicating that the preparation was homogeneous (Figure 1b). From this method a yield of approx. 25 mg of highly purified His-tagged GSTT1 per litre of bacterial culture was obtained.

Characterization of catalytic properties of recombinant GST T1-1

The specific activities of both recombinant enzymes towards a range of substrates are shown in Table 1. Significantly, human GST T1-1 can catalyse the conjugation of DCM with GSH; it therefore has the capacity to activate this carcinogen in man. Among the other potential substrates studied, the highest activity was seen with ENPP, a compound that was first identified by Fjellstedt et al. [40] to be a good substrate for transferase E (now called rat GST T1-1 [1]). The recombinant human GST T1-1 proteins were found to catalyse the conjugation of 4-NBC and 4-NPB, both of which have been reported by Meyer [41] to be metabolized by rat GST T1-1. Neither protein A–GST T1-1 nor the His-tagged GST T1-1 proteins were found to be active towards CDNB, ethacrynic acid or 1-menaphthyl sulphate, compounds that are used as GST model substrates [1]. Human GST T1-1, however, was shown to exhibit glutathione peroxidase activity and is able to catalyse the reduction of cumene hydroperoxide.

The activity of the protein A–GST T1-1 fusion was found to be considerably lower with all substrates than was the His-tagged GST T1-1. This difference in specific activity varied from substrate to substrate; it was least obvious with 4-NPB, and was greatest with cumene hydroperoxide. Because it was thought that the differences in activity of the two recombinant proteins were probably attributable to interference with the catalytic capacity of protein A–GST T1-1 by its large N-terminal extension, it was decided that only the C-terminal His-tagged protein should be subjected to kinetic characterization. The K_m and k_{cat} values of this transferase were determined for GSH at pH 6.5 and 7.5 and were found to correlate with the ionization state of GSH (Table

Figure 2 Analysis of human tissue extracts by SDS/PAGE

Portions (10 μ g) of soluble cell extracts from human tissue were resolved in a 12% (w/v) polyacrylamide gel before transfer to Immobilon-P for immunoblotting. The gel, which was stained with Coomassie R250, was loaded as follows: lane 1, molecular mass markers; lane 2, pooled GST isolated from rat liver used as standards (loading was increased to ensure detection by protein stain); lane 3, cerebrum; lane 4, heart; lane 5, kidney; lane 6, liver; lane 7, lung; lane 8, pancreas; lane 9, prostate; lane 10, skeletal muscle; lane 11, small intestine; lane 12, spleen; lane 13, testes.

2). The K_m and k_{cat} values were also determined at pH 6.5 for ENPP, and as had been noted by Meyer [41] in previous studies with rat GST T1-1 they were in the low millimolar range.

Tissue distribution of the GSTT1 subunit

Human organs were probed with antibodies raised against recombinant GST T1-1 to establish which tissues express this class Theta transferase. Livers from four male cadavers were screened by Western blotting to ensure that the survey was not conducted with organs from a *GSTT1*-null individual; it has been estimated that approx. 18 % of the Caucasian population is *GSTT1* *0/0, whereas as many as 60 % of the Chinese and Korean population possess this genotype [42,43]. All four liver specimens gave a single strongly positive immunoreactive band that, in comparison with protein standards (including recombinant His-tagged GSTT1), migrated with an apparent molecular mass of 28 kDa during SDS/PAGE. Electrophoretic analysis showed that the organs from patient 386 were not subject to obvious proteolysis (Figure 2). Immunoblotting of organs from this individual showed that, of the tissues studied, the GSTT1 subunit is highly expressed in kidney and liver, with substantial levels also present in the prostate and small intestine (Figure 3A). The GSTT1 subunit was observed in cerebrum, pancreas and skeletal muscle, although the levels of this protein seemed to be only approx. 10 % of that in the liver. Prolonged exposure of the film to the blot allowed the detection of GSTT1 in lung, spleen, heart and testis, but these organs contained less than 5 % of the amount of GSTT1 protein seen in the liver. It is important to note the low levels of GSTT1 protein in lung. Carcinogens activated by this transferase are volatile compounds that would primarily be expected to pose a risk of lung toxicity and carcinogenesis.

The tissue distribution of GSTT1 was compared with those of classes Alpha, Mu and Pi GST. This demonstrated that the tissue specificity of GSTT1 did not coincide with that of other transferases. The class Alpha GSTA1 and/or A2 subunits were expressed at high levels in kidney, liver and testis (Figure 3B). Trace amounts of these subunits were observed in pancreas,

Figure 3 Tissue-specific expression pattern of class Theta GSTT1 in human organs

Extracts prepared from human organs were subjected to immunoblotting as described in the Materials and methods section. The SDS/PAGE gel was loaded as follows: lane 1 contained either C-terminal His-tagged human GSTT1 as the standard [(A) only] or affinity-purified GST isolated from rat spleen (B–F); lane 2, cerebrum; lane 3, heart; lane 4, kidney; lane 5, liver; lane 6, lung; lane 7, pancreas; lane 8, prostate; lane 9, skeletal muscle; lane 10, small intestine; lane 11, spleen; lane 12, testes. The transfers were probed with the following antibodies: antibody raised against recombinant His-tagged human GSTT1 (A); antibody raised against native human GST A1-2 (B); antibody raised against rat GST A4-4 (previously called YkYk) (C); antibody raised against native human GST M1a-1a (D); antibody raised against native human GST M3-3 (E); antibody raised against native rat GST P1-1 (F).

prostate and small intestine, but were essentially absent from cerebrum, heart, lung, skeletal muscle and spleen. In contrast, the GSTA4 subunit described by Awasthi and co-workers [44] was observed in all organs, with the highest levels seen in the small intestine (Figure 3C); a faster-migrating electrophoretic form of GSTA4 was observed in liver and testes, the appearance of which might be due to thiol modification as observed with rat GSTA4 [24,45]. Antibodies raised against native class Mu GST M1a-1a identified three polypeptides that were resolved by SDS/PAGE (Figure 3D). These had apparent molecular masses of 26.7, 26.6 and 26.0 kDa and represent M1a, M1b and M2 subunits

respectively [46,47]. The fact that both M1a and M1b polypeptides were observed indicates that patient 386 was heterozygous at the GSTM1 locus. The class Mu GSTM1a/1b subunits were expressed at highest levels in liver and skeletal muscle, with lower levels in cerebrum, prostate and testis. Trace amounts of GSTM1a/1b were also detected in heart, pancreas, small intestine and spleen. The 26.0 kDa GSTM2 subunit was observed only in heart and skeletal muscle. The class Mu GSTM3 subunit was expressed strongly in testis but was also observed to a smaller extent in cerebrum and small intestine (Figure 3E). Trace amounts of GSTM3 were detected in heart, kidney, prostate and skeletal muscle. The class Pi GSTP1 subunit was observed in all organs except liver (Figure 3F). The GSTP1 polypeptide expressed in cerebrum, heart, kidney, lung, prostate, small intestine, spleen and testis was the 24.8 kDa form, whereas that in skeletal muscle had an apparent molecular mass of 25.5 kDa during SDS/PAGE. The appearance of this apparently larger GSTP1 form in the skeletal muscle might be due to the formation of intra-subunit disulphide bonds between cysteine residues, as has been demonstrated for rat GST P1-1 [48]. The electrophoretically slower-migrating band persisted even when the sample was preincubated in the presence of 5 mM dithiothreitol.

DISCUSSION

Mice exposed to DCM have a marked increase in the incidence of tumours of the lung and the liver [16,17]. The carcinogenicity of DCM requires its metabolic activation by GST, catalysing a nucleophilic substitution of chloride for the stabilized glutathione thiolate ion. This yields an *S*-chloromethylglutathione conjugate that is susceptible to a further nucleophilic substitution of chloride by an attacking group such as a DNA base. As the formation of the GSH conjugate of DCM is catalysed by class Theta GST, these enzymes are thought to have a critical role in the carcinogenic mechanism [14]. Purification of GST isoenzymes from mouse liver has demonstrated that the murine GST T1-1 enzyme has high conjugating activity towards DCM [23]. The risk posed to human health by DCM is uncertain. Human liver expresses a GST T1-1 enzyme that shares 82% amino acid identity with the murine DCM-metabolizing transferase. However, the catalytic properties of this human transferase have not been defined, partly because the native enzyme is labile during purification from human tissues [27]. Therefore the aim of the present study was to express and characterize recombinant human GST T1-1.

Class Theta GSTs do not bind to the commercially available *S*-hexylglutathione-Sepharose or glutathione-agarose affinity columns [2,24–27] and therefore the GSTT1 subunit was fused to either a polyhistidine or protein A tag to allow rapid isolation on Ni-NTA resin or IgG-Sepharose respectively. The initial strategy for the production of a recombinant GSTT1 subunit involved fusion at the N-terminus to a polyhistidine-containing peptide (MGSSHHHHHSSGLVPRGSH). This approach was abandoned because the product was predominantly insoluble and furthermore the small amount of soluble protein produced exhibited poor affinity for Ni-NTA resin (results not shown). On studying the crystal structure of a class Theta GST from *Lucilia cuprina*, the Australian blowfly [49], it became apparent that the poor affinity of the N-terminal His-tagged recombinant could possibly be attributed to the peptide-tag's being obscured, or partly hidden, within the α/β region of the N-terminal domain. It was also evident from the crystal structure that the C-terminus of class Theta GST is located on the surface of the enzyme. With

the relative accessibility of the C-terminus, it seemed logical to fuse a His-tag to this end of the subunit rather than the N-terminus. This would minimize the likelihood of the His-tag's becoming buried within the protein. In addition to the His-tagged form, an N-terminal tagged recombinant protein was designed in which part of the IgG-binding portion of protein A from *S. aureus* was fused to the GSTT1 subunit.

Both of the recombinant forms of human GSTT1 proved to be expressed at high levels in *E. coli*. Furthermore both recombinant proteins were highly soluble and readily purified in large amounts: the protein A fusion was recovered in yields of approx. 15 mg/l of culture, whereas yields of approx. 25 mg/l of culture of the His-tagged form were obtained. The recombinant GST T1-1 proteins were shown to catalyse the conjugation of DCM with GSH (Table 1). The enzyme also possessed transferase activity towards the epoxide-containing model substrate ENPP and the halide-containing aryl substrates 4-NBC and 4-NPB, but was inactive with the α,β -unsaturated carbonyl compound ethacrynic acid and the substituted aryl halide CDNB. Failure of GST T1-1 to utilize CDNB as a substrate is a common feature of the class Theta transferases; recombinant rat GST T2-2 has recently been shown to exhibit very low activity with this aryl halide [50], whereas human GST T2-2 demonstrated no activity [51]. In addition to transferase activity, recombinant GSTT1 possessed significant peroxidase activity and could catalyse the reduction of cumene hydroperoxide.

The activity values for the protein A-GSTT1 fusion represent underestimates of the contribution of the class Theta enzyme because the calculation of specific activity in terms of enzyme mass fails to take into account the presence of the protein A portion (18 kDa in size) and the small amounts of the 60 kDa contaminant. However, even when the activity data are re-calculated to allow for the contribution to total mass of the N-terminal fusion, it is apparent that protein A-GST T1-1 has between 5% and 35% of the activity towards ENPP, 4-NBC and 4-NPB exhibited by the His-tagged GST. Two possible explanations might account for this difference: first, the relatively large size of the protein A portion might interfere with either the folding or the subunit dimerization of GST T1-1; secondly, the protein A portion might sterically hinder the passage of substrate to the active centre of the enzyme. The crystal structure of a class Theta GST from *Lucilia cuprina* suggests that an addition of a tag to the N-terminus of the enzyme would place it close to the G-site of the active centre [49]. However, the crystal structure suggests that a C-terminal addition would not be in the vicinity of the active centre. Thus it seems likely that the protein A portion interferes with the access of substrates to the active centre.

The activity of human GST T1-1 has features that are similar to the orthologous transferases from rat and mouse. For example, GST T1-1 from human, rat and mouse exhibit higher activity towards DCM than class Alpha, Mu and Pi GSTs. Similarly, GST T1-1 from human and rat possess higher activity for ENPP than Alpha, Mu and Pi GSTs. The human GST T1-1 had no detectable activity for either CDNB, ethacrynic acid or 1-menaphthyl sulphate and can therefore be discriminated from the other class Theta transferase, GST T2-2 (see [51] for properties of recombinant human GST T2-2). Both class Theta enzymes, however, have glutathione peroxidase activity. From comparisons with data for rat GST T1-1, it seems that the recombinant human GST T1-1 is less active towards DCM, ENPP, 4-NBC and 4-NPB. It remains to be established whether this is due to differences in primary structure, post-translational modifications or methods of enzyme activity determination.

The tissue-specific expression of GSTT1 protein in the human

is poorly described. Protein purification and measurement of transferase activity with ENPP and DCM have provided evidence that GST T1-1 is present in human liver and lung [2,52]. In the present investigation, antibodies raised against the human His-tagged GSTT1 were used to demonstrate that substantial amounts of the GSTT1 subunit are expressed in liver and kidney. Lower levels of GSTT1 were also observed in cerebrium, pancreas, prostate, skeletal muscle and small intestine. Most significantly, GSTT1 was found to be present in only trace amounts in human lung. This observation is consistent with hybridization data *in situ* of Mainwaring et al. [52], which suggested that in pulmonary tissue GSTT1 mRNA is restricted to Clara cells and to ciliated cells at the alveolar/bronchiolar junction.

In contrast with our results, Juronen et al. [27] have reported the existence of a polypeptide expressed in human lung at levels equivalent to those seen in other tissues that cross-reacts with a monoclonal antibody against GST T1-1 [53]. As these workers studied human organs from Estonian patients, it is possible that ethnic, environmental or tissue-sampling differences account for the apparent variations in expression of the T1 subunit in human lung. It is also possible that the bronchopneumonia that was the cause of death in patient 386 influenced the level of GSTT1 in human lung. However, the pulmonary polypeptide that Juronen et al. [27] found to cross-react with their GSTT1 monoclonal antibody possessed a slightly faster anodal mobility during SDS/PAGE than the hepatic and renal immunoreactive proteins. It should be further noted that the polypeptide that cross-reacted with the monoclonal antibody had an estimated molecular mass of 25.3 kDa, whereas our GSTT1 preparation comprised subunits of 28.0 kDa. It might therefore be that the protein in human lung that reacted with the monoclonal antibody was not authentic GST T1-1 but rather a related class Theta transferase. We have examined the specificity of our polyclonal antisera used for the GSTT1 tissue blots and have found that it did not cross-react significantly with heterologously expressed native GST T2-2 (P. J. Sherratt and J. D. Hayes, unpublished work). In view of these results it would be helpful to know more about the differential expression of the T1 and T2 subunits in human organs.

The present paper and that of Mainwaring et al. [52] suggest that human lung has little metabolic capacity to activate DCM. However, as liver, small intestine, kidney, pancreas, prostate and skeletal muscle express significant amounts of GSTT1 protein it is probable that these organs activate DCM. A class Theta transferase that is related to GST T1-1 is also expressed in human erythrocytes [27,54] and it has been suggested that this enzyme enables the erythrocyte to protect other organs against DCM by the sequestration of such chemicals in GSTT1-positive individuals [55]. Surprisingly this erythrocyte enzyme possesses only approx. 12% of the specific activity for DCM that is exhibited by GST T1-1 isolated from human liver [56]. As evidence suggests the erythrocyte enzyme has a blocked N-terminus, it might represent a post-translationally modified form of GST T1-1. In addition to activation of DCM, human GST T1-1 can bioactivate dibromomethane, dibromoethane and 1,2,3,4-diepoxybutane [57]. Individuals might be occupationally exposed to these compounds as they are used as fumigants, degassing agents and in the rubber industry. Expression of GST T1-1 is subject to variation between individuals. Between 15% and 20% of the Caucasian population in the U.K. and U.S.A. fail to express the GSTT1 protein as a result of gene deletion [28,42]. However, more than 60% of Chinese and Koreans are null for this enzyme [43]. As we have shown the GSTT1 protein to be expressed in a number of organs it would seem important to establish whether polymorphism at the *GSTT1* locus is associated

Figure 4 Identification by immunoblotting of a novel Mu-like GST polypeptide

The tissue samples were loaded as described in the legend to Figure 3. The blot was probed with a separate batch of anti-(GST M1a-1a-IgG) from that shown above. Similar data were obtained with antibodies from two rabbits.

with an increased risk of malignant disease other than lung cancer, particularly among individuals who are exposed to DCM, dibromomethane, dibromoethane and butadiene.

Among GSTs, the expression of the human GSTT1 subunit shows a unique tissue specificity. By comparison, the GSTA1/2, GSTM1 and GSTM3 subunits were shown to be restricted in their tissue distribution: GSTA1/2 is restricted to kidney, liver and testis; GSTM1 is restricted to cerebrum, liver, skeletal muscle and testis; GSTM3 is found primarily in testis. In common with GSTT1, the GSTA4 and GSTP1 proteins were expressed in most organs although differences in relative amounts were obvious in heart, liver, lung and small intestine. Listowsky and co-workers [58] have affinity-purified GST from various human organs and quantified the relative amounts of individual class Alpha, Mu and Pi subunits. Their data are in broad agreement with our immunoblot results. Interestingly, we carried out Western blotting for GSTM1 with at least three antisera; two of these cross-reacted strongly with a polypeptide of 25.0 kDa that did not co-segregate with known class Mu GST. For example, substantial amounts of this cross-reacting polypeptide were observed in kidney, an organ that expresses little GSTM1, M2 or M3 (Figure 4). It is therefore unlikely that this class Mu-like 25.0 kDa polypeptide represents an alternative form of either GTTM1, M2 or M3. The identity of this putative novel class Mu GST awaits further investigation.

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