Expression of human glutathione *S*-transferase 2 in *Escherichia coli*

Immunological comparison with the basic glutathione *S*-transferases isoenzymes from human liver

P. G. BOARD and K. PIERCE

Department of Human Genetics, John Curtin School of Medical Research, Australian National University, G.P.O. Box 334, Canberra, A.C.T. 2601, Australia

A plasmid, termed pTacGST2, which contains the complete coding sequence of a GST2 (glutathione *S*-transferase 2) subunit and permits the expression of the protein in *Escherichia coli* was constructed. The expressed protein had the same subunit M_r as the enzyme from normal human liver and retained its catalytic function with both GST and glutathione peroxidase activity. Antiserum raised against the bacterially synthesized protein cross-reacted with all the basic GST isoenzymes in human liver. The electrophoretic mobility in agarose of the bacterially expressed isoenzyme suggested that its pI is identical with that of the cationic isoenzyme from human liver previously termed GST2 type 1. The available evidence suggests that the three common cationic isoenzymes found in human liver are the products of two very similar gene loci.

INTRODUCTION

The glutathione *S*-transferases (GST, EC 2.5.1.8) are a family of isoenzymes with broad substrate specificity and appear to be of significance in the detoxification and metabolism of many xenobiotics and organic peroxides. Previous studies have shown that a variety of GST enzymes are expressed in human tissues and that the genetic, developmental and environmental factors regulating their expression are complex (Kamisaka *et al.*, 1975; Marcus *et al.*, 1978; Board, 1981; Laisney *et al.*, 1984; Strange *et al.*, 1984, 1985; Suzuki *et al.*, 1987). The various enzymes can be classified largely according to their pI values and their subunit size. The basic group have been extensively investigated. Kamisaka *et al.* (1975) chromatographically identified five basic isoenzymes, termed α - ϵ , that were all of identical size and showed immunological identity. Those authors suggested that the α - ϵ isozymes were the post-translationally modified forms of a single gene product. Board (1981) used an electrophoretic technique to study the occurrence of the basic GST isoenzymes in a large number of liver samples and identified three basic isoenzymes that occurred in three different phenotypes in different individuals. Board (1981) interpreted the different phenotypes as evidence that these isoenzymes were the products of a single gene locus, termed GST2, with two alleles that encoded subunits of type 1 and type 2. It was proposed that the most basic isoenzyme was a homodimer of the GST2 type 1 subunits, the least basic was a homodimer of the GST2 type 2 subunits, and the intermediate isoenzyme was a heterodimer composed of the type 1 and type 2 subunits. Further investigations by Strange *et al.* (1984) found that the apparent GST2 phenotype varied between tissues within the same individual and therefore concluded that the multiple GST2 isoenzymes were the result of post-translational modification rather than allelic variation. Recently, Suzuki *et al.* (1987) purified GST2 type 1 and GST2 type 2 and have shown that each

isoenzyme has an identical subunit molecular size, both express glutathione peroxidase activity, both are blocked at the *N*-terminus and both share immunological identity. In contrast, Stockman *et al.* (1985) have also purified three basic GST isoenzymes from human liver that appear to be composed of two otherwise similar, but immunologically distinct, subunits termed B₁ and B₂. On the basis of a comparison of their apparent pI values, it seems likely that the B₁B₁, B₁B₂ and B₂B₂ isoenzymes described by Stockman *et al.* (1985) correspond to the GST2 type 1, GST2 type 2-1 and GST type 2 described by Board (1981) and characterized by Suzuki *et al.* (1987). Interpretation of the immunological differences noted by Stockman *et al.* (1985) suggests that the different subunits may be the product of different gene loci rather than alleles or post-translationally modified products of a single locus.

Singh *et al.* (1985) have reported that the basic GST isoenzymes from human liver are heterodimers of two distinct subunits with different M_r values. This observation has not been confirmed by others. Vander Jagt *et al.* (1985), Soma *et al.* (1986) and Tu *et al.* (1986) have also identified several basic GST isoenzymes from human liver. It is evident that all of these studies must be characterizing the same isoenzymes and the differences in the interpretation of the available data that have occurred result largely from the different analytical techniques used and from apparent differences in the cross-reactivity of the antisera raised against different isoenzymes. These differences could result from the cross-contamination of apparently pure homodimer antigen preparations with heterodimer, which may only differ slightly in its pI.

Mannervik *et al.* (1985) have proposed that there are at least three evolutionary classes of GST (termed α , μ and π) that appear to be conserved between rat and man. Considerably more is known about the GSTs of the rat, and molecular-genetic studies have shown that there are multiple α -class subunits (Lai *et al.*, 1984; Mannervik *et al.*, 1985). Studies by Board & Webb (1987) and Suzuki

et al. (1987) have indicated that, in man, the basic isoenzymes termed GST-2 by Board (1981) are equivalent to the α -class subunits. Tu & Qian (1986) and Board & Webb (1987) have cloned and sequenced identical α -class cDNA clones from human liver. Southern-blot hybridizations carried out by Board & Webb (1987) suggested that there may be multiple human α -class genes. The recent isolation by Rhoads *et al.* (1987) of an additional cDNA clone that encodes a different low-abundance α -class subunit confirms that suggestion. It is not yet clear which of the basic isoenzymes these clones represent. The range of nomenclature systems and the questions regarding immunological cross-reactivity of different subunits makes interpretation of the available data difficult. In an attempt to resolve some of the confusion over the relationships between the different cationic GST isoenzymes, and to provide an unlimited source of human α -class subunit for further study, we have constructed a plasmid that promotes the expression of the GST subunit cloned by Board & Webb (1987). The GST product is functionally active and has been electrophoretically and immunologically characterized.

MATERIALS AND METHODS

Liver samples

Human liver specimens were obtained within 24 h of death from individuals without obvious liver pathology. Samples were homogenized in 50 mM-Tris/HCl/150 mM-NaCl (pH 7.5) and centrifuged for 5 min in an Eppendorf Microfuge.

Isoenzyme purification

Human GST2 type 1 and type 2 were purified from human liver by ion-exchange and affinity chromatography (Suzuki *et al.*, 1987). Human GST 2 synthesized in *Escherichia coli* was purified by affinity chromatography. An overnight culture containing plasmid pTacGST2 was subcultured at a dilution of 1:100 and grown at 37 °C with vigorous shaking to an A_{600} of 0.2. IPTG was added to a final concentration of 1 mM, and the culture was grown on for a further 3 h to allow expression of GST2. The bacteria were then pelleted by centrifugation (1500 g; 10 min) and washed in 150 mM-NaCl/50 mM-Tris/HCl, pH 7.5. The cells were re-pelleted then suspended in distilled water and sonicated (Branson B12 instrument; 50W). The cell debris was pelleted by centrifugation at 10000 g for 15 min, and the supernatant was dialysed against 50 mM-sodium phosphate, pH 7.2. The cell extract was then passed through a 5 ml column of *S*-hexylglutathione-agarose (Sigma) equilibrated in 50 mM-phosphate buffer, pH 7.2. The column was washed sequentially in 50 mM-Tris/HCl, pH 7.2, and 50 mM-Tris/HCl, pH 9.6. The enzyme was eluted with 5 mM-GSH in 50 mM-Tris/HCl, pH 9.6.

Enzyme activity

GST activity was determined at 30 °C by the spectrophotometric method of Habig *et al.* (1974), with GSH and CDNB as substrates. Glutathione peroxidase activity was determined spectrophotometrically by the method of Beutler (1974). In addition, glutathione peroxidase activity was detected by specific staining after starch-gel electrophoresis as previously described by Board (1983).

Electrophoresis and blotting

Denaturing SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970). Non-denaturing electrophoresis was carried out at pH 8.6 in 1.2%-(w/v)-agarose gels containing 1% hydrolysed starch. The electrode buffer was a 1:7 dilution and the gel buffer a 1:4 dilution of a stock buffer containing 0.9 M-Tris, 0.5 M-boric acid and 0.02 M-EDTA. Specific staining of GST activity was carried out by the two-stage procedure described by Board (1980). Electrophoretic transfer of proteins to nitrocellulose sheets from acrylamide and agarose gels was carried out by the method of Towbin *et al.* (1979). Blotted proteins were detected with primary antiserum raised in rabbits and a secondary anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) as described by Board (1984).

Antibody preparation

Approx. 50 μ g of GST2, affinity-purified from extracts of *E. coli* carrying pTacGST2, was injected intramuscularly into rabbits at weekly intervals. The first injection was mixed with Freund's complete adjuvant, and subsequent injections were mixed with Freund's incomplete adjuvant. Antiserum was collected after 4 weeks.

GST2 expression

A cDNA containing the entire coding sequence for a GST2 subunit was subcloned from the λ GST2-3 clone (Board & Webb, 1987) into the endonuclease-*Sma*I site of plasmid pUC18. The resulting plasmid, pGST2, was used as a source of GST2 cDNA for the construction of a plasmid capable of expressing GST2. The steps taken in the total process are summarized in Fig. 1. The plasmid pGST2 was digested with *Eco*RI and then subjected to *Bal*31 digestion for various lengths of time to remove increasing amounts of 5' non-coding sequence from the cDNA. The samples were pooled, phenol-extracted, and the DNA precipitated with ethanol. The plasmid was then digested with *Bam*HI to excise the shortened cDNA, and the recessed ends of the *Bam*HI restriction site were filled in with the appropriate deoxynucleotide triphosphates and the Klenow fragment of DNA polymerase 1. The DNA was fractionated on a low-melting-point agarose gel (Sea Plaque), and the cDNA fragments were recovered by phenol extraction and ethanol precipitation. The cDNA fragments were ligated with phage-T₄ ligase into the *Eco*RI site of the plasmid pTac-11 (Amann, *et al.*, 1983). The plasmid had previously been digested with *Eco*RI, and the recessed ends were filled in by the Klenow fragment of DNA polymerase 1. The resulting blunt ends of pTac-11 were treated with phosphatase to prevent self-religation. The recombinant plasmids containing GST2 cDNA were transfected into *E. coli* JM103 and screened by the colony-blot method of Stanley & Luzio (1984) on dry nitrocellulose filters previously saturated with 1 mM-IPTG. Colonies expressing cross-reacting antigen were detected by using affinity-purified antibody directed against GST2 (Board & Webb, 1987).

Nucleotide sequence analysis

Nucleotide sequence analysis was carried out by the dideoxy chain-termination method of Sanger *et al.* (1977), after subcloning the appropriate fragment in M13mp19 (Norranders *et al.*, 1983).

Fig. 1. Construction of plasmid pTacGST2

The black heavy segments represent GST2 cDNA sequences, and the heavy black arrow represents the Tac promoter. The ampicillin (*amp*) and tetracycline (*tet*) resistance genes are indicated by open boxes.

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                10
      A G G A A A C A G A
                20
      A T T A G G A G A C
                30
      T G A T C T C A T G
                Met
  
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Fig. 2. Nucleotide sequence of the junction between pTac-11 and the GST2 cDNA

The underlined nucleotides are derived from pTac-11. The initiating methionine residue is shown (Met).

RESULTS

The steps undertaken to gain expression of GST2 in *E. coli* are summarized in Fig. 1. Because there were no suitable restriction-enzyme sites close to the ATG initiation codon of the GST2 coding sequence, progressive *Bal31* digestion was undertaken to remove 5' non-coding sequence. Colonies containing plasmids with the cDNA positioned in the correct orientation and with the ATG an appropriate distance from the Tac promoter could be detected by their ability to synthesize protein recognized by antiserum to GST2. Numerous antigen-positive colonies were detected; however, only one clone,

termed pTacGST2, was characterized in detail. Sequence analysis of pTacGST2 has shown that this clone retains 14 5' non-coding nucleotides (Fig. 2).

Direct spectrophotometric assay of GST activity in extracts of JM103 cells containing pTacGST2 showed significant levels of activity which was not present in JM103 cells containing the pTac-11 plasmid. It is evident from SDS/polyacrylamide-gel electrophoresis shown in Fig. 3 that the GST2 expressed by pTacGST2 in *E. coli* cultures forms a significant component of the total protein synthesized by the cells. The GST produced by pTacGST2 was affinity-purified and, as shown in Fig. 3, has a subunit M_r identical with that of GST2 purified from human liver.

Electrophoresis of purified cell extracts on agarose/starch gels, followed by specific staining for GST activity, shows an isoenzyme with an electrophoretic mobility similar to that of GST2 type 1 from human liver (Fig. 4). Similar gels were stained for glutathione peroxidase with *t*-butyl hydroperoxide as a substrate and show activity at the same site (results not shown). Gel filtration of bacterial extracts on Sephadex G-100 showed that bacterially synthesized GST2 was eluted with an apparent M_r of 50 100, which clearly indicates that the subunit assembles into the normal dimeric form.

Antibody cross-reaction

Antiserum raised against the GST2 expressed in *E. coli* was used to identify cross-reacting isoenzymes in human

Fig. 3. SDS/polyacrylamide-gel electrophoresis of *E. coli* extracts and purified GST2

Lane 1, M_r standards; lane 2, extract of *E. coli* containing pTacGST2 grown in the presence of 1 mM-IPTG; lane 3, GST2 affinity-purified from the extract shown in lane 2; lane 4, GST2 type 1 purified from human liver.

Fig. 4. Electrophoresis of cell extracts on agarose containing 1% hydrolysed starch and specifically stained for GST activity

Lane 1, human liver (three zones of activity represent GST2 type 2, GST2 type 1-2, and GST2 type 1 in order of increasing cathodal mobility); lane 2, extract of *E. coli* containing pTacGST2.

liver. Liver cytosol samples were subjected to electrophoresis in agarose gels and then electroblotted on to nitrocellulose filters. As shown in Fig. 5, the antiserum to bacterially synthesized GST2 cross-reacts with all three GST2 isoenzymes that can be identified by direct activity staining. The liver sample used in Fig. 5 was different from that used in Fig. 4, and comparison of lane 1 from each Figure illustrates the differences in the intensity of each GST2 isoenzyme that occur between individuals.

Fig. 5. Electrophoresis of a human liver extract in agarose

Lane 1, agarose gel containing 1% starch and stained specifically for GST activity; lane 2, same extract as lane 1, but electrophoresed in agarose without starch, electroblotted on to nitrocellulose and isoenzymes localized by the use of antiserum to GST2 produced in *E. coli* from pTacGST2.

DISCUSSION

The recombinant pTacGST2 construct allows the expression of large amounts of human GST2 in *E. coli*. Our results have shown that the expressed product has the same subunit M_r as GST2 from liver and assembles as a dimer. In addition, the expressed enzyme is catalytically active as a GST and retains glutathione peroxidase activity, which is a distinctive feature of the GST2 isoenzymes (Suzuki *et al.*, 1987). The demonstration that recombinant constructs of this type produce a protein that can be affinity-purified and retains apparently normal functions will permit the unlimited production of human GST for experimental study. Furthermore, the selective modification of this isoenzyme by site-directed mutagenesis will permit the specific analysis of the relationships between its structure and function.

Electrophoresis of bacterial extracts in agarose has shown that the expressed isoenzyme has a pI equivalent to that of the most basic of the GST isoenzymes found in human liver. This suggests that the λ GST2-3 or pGTH1 (Tu & Qian, 1986) clones encode the GST2 type 1 (Board, 1981) or B₁ (Stockman *et al.*, 1985) subunits. The relatively low-abundance cDNA (pGTH2) reported by Rhoads *et al.* (1987) has only 11 amino acid substitutions when compared with the sequences of λ GST2-3 and pGTH1. These substitutions indicate that there are at least two genes encoding α -class subunits in man. The close similarity of the two sequences suggests that antiserum raised against either protein may cross-react. Certainly in the experiments carried out here, antiserum raised against the bacterially expressed human GST2 subunit cross-reacted with all three GST2 isoenzymes. These observations suggest that immunological cross-reactivity is not, in this case, helpful in defining the genetic relationships between the basic human GST isoenzymes.

Because the substitutions of the pGTH2 sequence (Rhoads *et al.*, 1987) would lead to a net decrease in the pI, it seems likely that the pGTH2 clone might encode

the subunit termed GST2 type 2 (Board, 1981) or B₂ (Stockman *et al.*, 1985). If this is the case, then the GST2 2-1 or B₁B₂ isoenzymes could be expected to be heterodimers of the products of the two genes represented by λ GST2-3/pGTH1 and pGTH2. Although other α -class genes may exist, they must be poorly expressed, as their cDNAs have not been detected in studies by Board & Webb (1987) and by Rhoads *et al.* (1987), and the three commonly encountered basic isoenzymes can be accounted for by the homo- and hetero-dimeric combination of subunits expressed by two gene loci (Stockman *et al.*, 1985).

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