A novel amphibian Pi-class glutathione transferase isoenzyme from *Xenopus laevis*: importance of phenylalanine 111 in the H-site

Antonella DE LUCA, Bartolo FAVALORO, Erminia CARLETTI, Paolo SACCHETTA and Carmine DI ILIO¹

Dipartimento di Scienze Biomediche, Università "G.D'Annunzio", Via dei Vestini, Chieti, CH 66100, Italy

Screening of a liver tumour cDNA library from *Xenopus laevis* resulted in the isolation of a full-length cDNA clone encoding a novel Pi-class amphibian glutathione transferase (GST) isoenzyme (designated as XIGSTP1-1). The gene encodes a protein of 212 amino acids with a calculated molecular mass of 24 428 Da. The product of the gene has been overexpressed in *Escherichia coli* and characterized. XIGSTP1-1 has one of the highest specific activities towards 1-chloro-2,4-dinitrobenzene (1310 $\mu\text{mol/min}$ per mg of protein) obtained with any GST. A notable feature of XIGSTP1-1 is the presence in the H-site

of Phe¹¹¹ and Pro²⁰⁸ in place of tyrosine and glycine residues respectively, present in other mammalian Pi-class GSTs. Site-directed mutagenesis indicate that Phe¹¹¹ is involved in substrate specificity of XIGSTP1-1. We provide evidence showing that XIGSTP1-1 is present only in the embryo and its expression might be associated with cellular proliferation.

Key words: amphibian, cellular proliferation, glutathione transferase (GST), phenylalanine residue, proline residue, site-directed mutagenesis.

INTRODUCTION

Glutathione transferase (GST; EC 2.5.1.18) refers to a large group of ubiquitous proteins, which catalyse the nucleophilic attack of GSH on different species of electrophilic compounds [1–3]. Recent studies [4,5] have also suggested that certain GST isoenzymes may be involved in the regulation of stress-activated cell signalling pathways through direct physical association with c-Jun N-terminal kinase ('JNK') and apoptosis signal-regulating kinase-1 ('APAF-1'). The cytosolic GST activity in mammalian tissues is often due to multiple isoenzymes that are dimers of either identical or structurally different subunits [2,6]. By analysis of their structural and functional properties, soluble GSTs can be included into at least 12 different classes: Alpha, Beta, Delta, Kappa, Mu, Omega, Phi, Pi, Sigma, Tau, Theta and Zeta (for a review, see [7]). In comparison with mammalian GSTs, relatively little information is available on amphibian GSTs. Two distinct Pi-class GSTs, named BbGSTP1-1 [8,9] and BbGSTP2-2 [10,11] respectively, have been purified and characterized from the common toad (*Bufo bufo*) in our laboratory. BbGSTP1-1 is expressed at high level in embryo tissues during its early developmental stages [12,13]. On the contrary, BbGSTP2-2 is essentially expressed in adult toad tissues, including liver and kidney [10–13]. We have also investigated the expression of GSTs in *Xenopus laevis* [14]. A new Mu-class GST has been cloned, expressed and characterized previously from this amphibian species [15]. Unlike *B. bufo*, *X. laevis* does not apparently express Pi-class GSTs [14]; however, analysis of the expressed sequence tag (EST) database suggests that a previously uncharacterized Pi-class GST isoform might be encoded by this amphibian species. Screening of a cDNA liver tumour library from *X. laevis* resulted in the isolation of a cDNA encoding Pi-class GST and its nucleotide sequence has been

determined. The predicted amino acid sequence of this putative protein is closely related to the amphibian BbGSTP2-2 (57 % identity) and, according to the recommended nomenclature [16], is designated XIGSTP1-1. The recombinant XIGSTP1-1 has also been characterized and shown to exhibit structural and catalytic properties distinct from those of other amphibian and mammalian Pi-class GSTs. Reverse transcription (RT)–PCR and Western-blot analysis indicate that XIGSTP1-1 is expressed in embryo, but not adult, tissues.

MATERIALS AND METHODS

Identification of a *X. laevis* partial GST cDNA

X. laevis cDNA encoding a partial protein with amino acid sequences related to Pi-class GSTs was identified by a BLAST search of the EST (generated by partial sequencing of random cDNA clones and assignment to gene families based on sequence similarities) database (<http://www.ncbi.nlm.nih.gov/BLAST/>). The EST clone (GenBank® accession number BG554378) was isolated from a *X. laevis* embryo (stage 31–32) cDNA library constructed by Life Technologies.

Library screening and cDNA clone isolation

The UNI-ZAP *X. laevis* liver tumour cDNA library (obtained from Dr G. Bernardini and Dr D. Vigetti, Dipartimento di Biologia Strutturale e Funzionale, Università degli Studi dell'Insubria, Varese, Italy) was screened in two steps, as described previously [17]. Briefly, the first step of the screening was performed by PCR using the following sense and antisense primers on the basis of the identified EST clone: FEst (5'-ATGCTGGCTACGTCCTC-3'; sense) and REst (5'-GCATTTTGTAGAAAGGATCCTC-3';

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione transferase; EST, expressed sequence tag; IPTG, isopropyl β -D-thiogalactoside; LB, Luria–Bertani; RT-PCR, reverse transcription–PCR; tPBO, *trans*-4-phenyl-3-buten-2-one.

¹ To whom correspondence should be addressed (e-mail enzymol@dsb.unich.it).

The nucleotide sequence reported has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ489617, and the amino acid sequence has been submitted to the National Center for Biotechnology Information under the accession number CAD33920.

antisense). A PCR product (420 bp) was recovered from the agarose gel using the GeneClean II Kit (BIO 101 Inc.) as described by the manufacturer. The DNA fragment recovered was subjected to a PCR amplification by using as primers FPst (5'-AA-CTGCAGATGCCTGGCTACGTC-3'; sense) and REco (5'-CGGAATTCGCATTTTGTAGAAAGGATCC-3' antisense). Both primers had 5'-recognition sites for the restriction enzymes *Pst*I and *Eco*RI (underlined). In addition to the template and 200 ng of each primer, the reaction mixture (50 μ l) contained 200 μ M dNTP mix and 1 unit of *Taq* Gold polymerase (PerkinElmer Biosystems) in 100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂ and 0.01 % (w/v) gelatin. Reaction mixture was subjected to 1 cycle at 95 °C for 10 min, 6 cycles of amplification (60 s at 95 °C, 60 s at 50 °C and 60 s at 72 °C), followed by 30 cycles of amplification (60 s at 95 °C, 60 s at 55 °C and 60 s at 72 °C). The PCR product was recovered from the agarose gel by using GeneClean II Kit, digested with restriction enzymes *Eco*RI/*Pst*I and cloned into the *Eco*RI/*Pst*I sites of the *Escherichia coli* vector pBluescript SK; the resulting plasmid was named pXIGSTPi. The PCR product was confirmed by DNA sequencing. A DNA probe was prepared from pXIGSTPi with fluorescein using the PCR Fluorescein Labelling Mix (Roche) according to the manufacturer's instructions.

The contents of the tube from which the PCR product was generated were plated at approx. 50 000, 5000 and 500 plaque forming units of viral encapsulated DNA for each 150-mm culture plate. Hybridization using the probe, prepared as described above, was performed using Chemiluminescent Detection System (Southernstar™; PerkinElmer Biosystems), according to the manufacturer's protocol. Single positive plaques were picked and pBluescript phagemids were excised by the *in vivo* excision protocol using ExAssist helper phage and *E. coli* SORL strain (Stratagene). The resulting plasmid, containing the full-length cDNA clone, was named XIGSTPi-pBSK. The DNA insert in pBluescript was confirmed by sequencing. The nucleotide sequence was translated into amino acid sequence and similarities between the primary structures of GSTs were searched using the Swiss-Prot Protein Sequence Data Bank, and protein analyses were carried out using the proteomics tools of ExPASy Data Bank.

Construction of an expression plasmid containing the XIGSTP1 gene

To obtain the direct insertion of the *Taq* polymerase-amplified PCR product into a plasmid vector for expression in *E. coli*, we used pTrcHis2TOPO®TA Expression Kits (Invitrogen). Since there are two more amino acids encoded in the DNA between the initiation codon and the TOPO® cloning site to obtain the expression of truly native protein, two oligonucleotide primers were designed as follows: fPi (5'-TACGTCCTCACCTATTTCC-3'; sense) and rPi (5'-TCACTTTTGTGTTTGGGGG-3'; antisense). PCR was performed using XIGSTPi-pBSK as the template, fPi and rPi as primers at a concentration of 200 ng, 200 μ M dNTP mix and 1 unit of *Taq* Gold polymerase in 100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂ and 0.01 % (w/v) gelatin. The reaction mixture (50 μ l) was subjected to 1 cycle at 95 °C for 10 min and 30 cycles of amplification (60 s at 95 °C, 60 s at 52 °C and 90 s at 72 °C), followed by 15 min at 72 °C. The PCR product was used immediately in a TOPO® cloning reaction and after 5 min of incubation, as described by the manufacturer, it was used to transform *E. coli* TOP10 cells. The reliability of PCR amplification was verified by DNA sequencing and the resulting plasmid was named XIGSTPi-TOPO.

To obtain the native protein, the plasmid XIGSTPi-TOPO was used as a template for mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The double mutation (shown in bold) Ala²Leu³ → Pro²Gly³ was made with the following oligonucleotides: fPro2Gly3 5'-GAGGAATAAAC-CATGCCTGGCTACGTCCTCACCTAT-3'; rPro2Gly3 5'-AT-AGGTGAGGACGTAGCCAGGCATGGTTTATTCCTC-3'. The fidelity of PCR amplification was verified by DNA sequencing and the plasmid was designed as XIGSTPi-pTrcHis2TOPO.

Expression and purification of recombinant XIGSTP1-1

A single colony of *E. coli* XL1Blue transformed with XIGSTPi-pTrcHis2TOPO was removed and grown overnight in Luria-Bertani (LB) broth containing ampicillin (100 μ g/ml) and glucose (0.5 %). After overnight incubation, the culture was diluted 1:40 in LB broth supplemented with glucose (0.5 %) and ampicillin (100 μ g/ml) and grown until *D*₆₀₀ reached 0.6. To induce expression, the cells were harvested by centrifugation (2000 *g* for 12 min) and resuspended in LB broth without glucose. Isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and the incubation was continued for further 5 h at 37 °C. Cells were harvested by centrifugation (15 000 *g* for 30 min) and cell pellet was frozen at -20 °C overnight. The frozen bacterial pellet was allowed to thaw at room temperature before being resuspended in 10 mM potassium phosphate buffer (pH 7) containing 1 mM EDTA (buffer A) or 1 mM dithiothreitol (buffer B). The resuspended cells were disrupted by sonication at 4 °C. The insoluble bacterial debris was removed by centrifugation (15 000 *g* for 20 min at 4 °C) and the resulting supernatant was loaded on to a GSH-Sepharose 6B (Sigma) column equilibrated previously with buffer A. The column was washed overnight with 500 ml of buffer A containing 200 mM KCl (more than 30 times the column volume). The overexpressed protein, which bound to the affinity column, was eluted at 0.5 ml/min with 200 mM Tris/HCl buffer (pH 7.8) containing 50 mM GSH. The protein eluted by GSH was collected, dialysed against buffer B, concentrated and glycerol was added to a final concentration of 15 % (v/v) before being stored at -20 °C.

Enzyme assay

GST activity with 1-chloro-2,4-dinitrobenzene (CDNB; Aldrich), 1,2-dichloro-4-nitrobenzene (Fluka), *p*-nitrobenzyl chloride (Aldrich), ethacrynic acid (Sigma), 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (Eastman Kodak), Δ^5 -androstene-3,17-dione (Sigma), *trans*-4-phenyl-3-buten-2-one (tPBO; Aldrich), *p*-nitrophenyl acetate (Serva) and bromosulphthalein (Serva) was measured as described by Habig and Jakoby [18]. The selenium-independent GSH peroxidase activity of GST was measured with cumene hydroperoxide (Sigma) as reported previously [19]. GST activity toward 4-nitroquinoline-1-oxide (Fluka) was measured as described by Stanley and Benson [20]. Activity with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Sigma) was determined as reported previously [21]. GST activity with allylthiocyanate, benzylthiocyanate (Fluka) and phenethylthiocyanate (Fluka) was measured as described by Kolm et al. [22]. For kinetic determination, CDNB and GSH were held constant at 1 and 2 mM respectively, whereas the other substrates varied (from 0.05–8.50 mM for GSH, and from 0.02–2.00 mM for CDNB). Protein concentrations were calculated by the method of Bradford [23] using γ -globulin as a standard.

Preparation of anti-XIGSTP1-1 antibodies

Approx. 300 µg of recombinant XIGSTP1-1 was injected subcutaneously into a New Zealand White rabbit. After primary immunization, the animal was given additional injections on days 21 and 42, it was then bled 14 days later and the antiserum was used for immunoblotting.

Electrophoresis and Western-blot analyses

For electrophoresis and Western-blot analyses, the cytosolic extracts were prepared as described previously [13]. Total protein extracts (100 µg) of liver, lung, heart, kidney and ovary from adult *X. laevis* and from embryo stages 11–15 and stages 24–25 were separated on SDS/PAGE [12 % (w/v) gel] and then subjected to Western-blot analysis as described previously [12].

RNA isolation

X. laevis were anaesthetized by hypothermia and killed by head concussion. Tissues were excised and frozen immediately in liquid nitrogen before being stored at –80 °C until use. Total RNAs were isolated from liver, lung, heart, kidney and ovary by using the High pure RNA tissue kit (Roche). Samples were loaded on to 1.3 % (w/v) formaldehyde–agarose gel to verify the integrity of total RNAs purified.

RT-PCR

First-strand cDNAs were synthesized using 1 µg of total RNA and the First-Strand Synthesis Kit for RT-PCR (RETROscript™; Ambion). For each sample, we used 100 units of Moloney murine leukaemia virus reverse transcriptase and gene-specific reverse primer, according to the manufacturer's instructions. The reaction was carried out in 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 5 mM dithiothreitol, 500 µM dNTP mix and 10 units of placental RNase inhibitor at 37 °C for 50 min, followed by 10 min at 90 °C. The following primers were used: Pirev (5'-TCACTTTTTGTGTTTGGGGG-3') for XIGSTP1-1; and Murev (5'-TTATTTTTTGTGTTGCCCCAGG-3') for XIGSTM1-1 [15].

PCR amplifications of the resulting cDNAs were performed by using the following sense and antisense oligonucleotides as primers: FEst and REst for Pi-class GST cDNAs, and Fmu (5'-ATGGTGATAGTACTAGGATACTGG-3') and Rmu (5'-AAATTCTGAAGCCAAGTGGGATC-3') for XIGSTM1-1. Reverse primers, REst for XIGSTP1-1 and Rmu for XIGSTM1-1, were annealed to internal sequence of cDNAs. PCR amplifications were performed using 1–4 µl of synthesis mix (from a total volume of 20 µl), 200 ng of each primer, 200 µM dNTP mix and 1 unit of *Taq* Gold polymerase in 100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂ and 0.01 % (w/v) gelatin. The reaction mixture was subjected to a cycle at 95 °C for 10 min, followed by 30 cycles of amplification (60 s at 95 °C, 60 s at 50 °C and 60 s at 72 °C), as described by the manufacturer of the *Taq* polymerase. PCR amplification products were verified on an agarose gel.

Mutagenesis of the XIGSTP1 gene

The plasmid XIGSTPi–pTrcHis2TOPO, carrying the *XIGSTP1* gene, was used as a template for mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The single mutation (shown in bold type) Phe¹¹¹ → Tyr (Phe¹¹¹Tyr) was

made with the following oligonucleotides: fF111Y (5'-TATGGGCGGCTTATCTATTTTGAATATGAGAC-3') and rF111Y (5'-GTCTCATATTCAAAATAGATAAGCCGCCCAT-3'). The single mutation (shown in bold type) Pro²⁰⁸ → Gly (Pro²⁰⁸Gly) was made with the following oligonucleotides: fP208G (5'-AAACGGCCTATCACAGGGAAACACAAAAAGTGA-3') and rP208G (5'-TCACTTTTTGTGTTTCCCTGTGATAGGCCGT-TT-3'), and the single mutation Pro²⁰⁸ → His (Pro²⁰⁸His) was made with fP208H (5'-AAACGGCCTATCACACAAACACAAAAAGTGA-3') and rP208H (5'-TCACTTTTTGTGTTTGTGGGTGATAGGCCGTTT-3'). The clones used for the production of all mutants were confirmed by DNA sequencing. *E. coli* XL1Blue cells transformed by the XIGSTPi–pTrcHis2TOPO mutants were induced by IPTG under the same conditions as for cells transformed by XIGSTPi–pTrcHis2TOPO wild-type. The crude extracts were loaded on to GSH–Sepharose 6B (Sigma) columns.

RESULTS AND DISCUSSION

Molecular cloning, heterologous expression and tissue distribution of XIGSTP1-1

Analysis of *X. laevis* EST database revealed the presence of a partial cDNA from embryo (GenBank® accession number BG554378) encoding a Pi-class GST fragment. In order to identify the complete cDNA encoding this Pi-class GST, two oligonucleotides were designed and a partial XIGSTP1 cDNA was obtained by PCR amplification of a liver tumour cDNA library. The PCR product was cloned in pBluescript, sequenced and found to encode a partial protein closely related to the GSTs within the Pi-class. This PCR product was then used to screen the liver tumour cDNA library. Several cDNA clones were isolated and an insert of 918 bp was sequenced. The nucleotide sequence of this clone, including the 5'- and 3'-untranslated regions, is shown in Figure 1. The translation termination codon TGA is followed downstream by two conventional polyadenylation signals AATAAA (Figure 1). The sequence contains an open reading frame that encodes a protein of 212 amino acids with a calculated molecular mass of 24 428 Da. A comparison of the deduced amino acid sequence of *X. laevis* GST with other mammalian and amphibian Pi-class GSTs is shown in Figure 2. The highest identity (57 %) was found with the amphibian BbGSTP2-2 [11] and, according to the guidelines adopted for GST nomenclature [16], the protein was designated as XIGSTP1-1. RT-PCR was performed to investigate the presence of the XIGSTP1 transcript during embryo development as well as in different adult tissues. XIGSTM1-1 [15] was used as a positive control to evaluate the sensitivity of the method (results not shown). Figure 3 shows that a 420 bp product corresponding to XIGSTP1-1 was present in embryos, but not in the unfertilized eggs, liver, lung, heart, kidney and ovary. Moreover, the data obtained by Western-blot analysis were in agreement with those obtained by RT-PCR (results not shown). In fact, the protein was detected only in the crude extracts obtained from the embryo at stages 11–15. These findings indicate that XIGSTP1-1 is not expressed in amphibian adult life. These results, in addition to the fact that the cDNA coding for XIGSTP1-1 was obtained from a liver tumour cDNA library from *X. laevis*, support further the notion that the isoenzymes belonging to Pi-class in amphibia, as in mammals [24], might be associated with cellular proliferation.

Biochemical characterization of XIGSTP1-1

To investigate the physico-chemical properties of this previously uncharacterized protein and to confirm its GST activity, the

```

1      GCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCGGGCTGCAGGAATTCGGCACGAGATCCA

78  CTGAAATAGTCGCCGTTTCCAGCCCATCCTGACA ATG CCT GGC TAC GTC CTC ACC TAT TTC CCC GTC
1      M P G Y V L T Y F P V

146 AGA GGA AGG GCT GAA CCT ATC CGG CTG CTG TTG GCA GAT CAG GGC ATT TCC TGG AAA GAG
12  R G R A E P I R L L L A D Q G I S W K E

206 GAT GAA GTG CAG ATT CCA GAC TGG TTT TCA GGA AAA GAT GCC AGA AAG AAA GAG GCG GTA
32  D E V Q I P D W F S G K D A R K K E A V

266 TTC GGT CAG CTG CCT CAG TTT CAG GAT GGA GAC TAT GTC CTT TAT CAG AGT AAC AGT ATT
52  F G Q L P Q F Q D G D Y V L Y Q S N S I

326 CTA AGG TAT CTG GGT AAT AAA CAT GGA CTG ACT GGA GCC AAT GAT GAG GAA CGT GGT CAC
72  L R Y L G N K H G L T G A N D E E R G H

386 ATA GAC ATG GTG AAC GAT GGC GTG GAA GAT CTG AGA CAG AAA TAT GGG CGG CTT ATC TTT
92  I D M V N D G V E D L R Q K Y G R L I F

446 TTT GAA TAT GAG ACA GGT AAA GAT AAA TAT CTC AAG GAA CTT CCC AGT CAG TTG GAT TTC
112 F E Y E T G K D K Y L K E L P S Q L D F

506 TTC GAG AGG ATC CTT TCT AAA AAT GCT AAT GGA TCT AAA TTT GTT GTG GGA CAG AAG ATT
132 F E R I L S K N A N G S K F V V G Q K I

566 TCC TTT GCT GAT TAT AAC CTC CTG GAT ATC CTT CAG TGT CAC CTT GAT TTG TGT TCA AAG
152 S F A D Y N L L D I L Q C H L D L C S K

626 TCC CTT TCA GCC TAC CCT TTG CTC ACT GCC TAT GTT GAA AGA CTT GTT GCC CGG CCA AAA
172 S L S A Y P L L T A Y V E R L V A R P K

686 ATC AGC GAA TAC TTA AAA TCA GAT GCC CGC AAT AAA CGG CCT ATC ACC CCC AAA CAC AAA
192 I S E Y L K S D A R N K R P I T P K H K

746 AAG TGA CCAAAACAAATGACACCCCAAATGGGAAGTGATTTTATTTCATTATTTTACCTGTAGGGTCTCAGCATCT
212 K Stop

823 AATAATAGCTACATGAGTAACCTTAATACCATATGCATTTAATAAATACAATTTATATACAAATAAATCCATCTATTTC

902 AAAAAAAAAAAAAAAA

```

Figure 1 cDNA sequence and deduced amino acid sequence of XIGSTP1-1

The start and stop codons are shown in bold; polyadenylation sites are underlined.

entire open reading frame was subcloned into an expression vector. A culture of *E. coli* XL1Blue containing the plasmid was induced by IPTG. The supernatant from this cell extract overexpressed a protein having a molecular mass of approx. 24 000 Da (Figure 4). Subsequently, the overexpressed protein was purified by using GSH-affinity chromatography [25]. The yield of cloned enzyme was approx. 11 mg/litre of culture, sufficient to allow for its characterization. The recombinant purified protein had an apparent molecular mass of 24 000 Da as measured by SDS/PAGE, consistent with the predicted molecular mass (24 428 Da) calculated on the basis of its amino acid sequence. The purified enzyme was unstable when stored at room temperature and at concentration of less than 0.6 mg/ml. Under these conditions, a rapid loss of its enzymic activity occurred. To avoid enzyme inactivation during kinetic measurements, 80 µg of BSA was added to the standard assay mixture and no loss of enzymic activity was observed. The substrate specificity of recombinant XIGSTP1-1 towards several commonly used electrophilic substrates was determined and the results are summarized in Table 1. Recombinant XIGSTP1-1 was very active towards CDNB. In fact, it appears to be

the most active GST described to date with this electrophilic substrate (1310 µmol/min per mg of protein). The enzyme also had potent activity towards organic isothiocyanates, but was unable to catalyse the isomerization of Δ^5 -androstene-3,17-dione. Unlike other Pi-class GSTs [2,26,27], recombinant XIGSTP1-1 lacked detectable activity towards 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzylchloride, tPBO and ethacrynic acid, but possessed the ability to catalyse the reduction of cumene hydroperoxide. The reaction of the Pi-class GST with ethacrynic acid is considered a characteristic feature of this family of isoenzymes [1,2]. It has been demonstrated that the conserved residue Tyr¹¹¹ (the amino acid residue numbering is assigned on the basis of XIGSTP1-1 primary structure), located in the H-site of Pi-class GST, plays a key role in the conjugation of GSH to ethacrynic acid [28,29]. In XIGSTP1-1, Phe¹¹¹ is present instead of Tyr¹¹¹ and this can explain its inability to catalyse the addition of ethacrynic acid to GSH. As shown in Table 1, mutation of Phe¹¹¹ to Tyr¹¹¹ allowed XIGSTP1-1 to acquire the capacity to conjugate ethacrynic acid as well as tPBO to GSH but, at the same time, significantly compromised the catalytic feature of the amphibian enzyme. It is interesting to note that this point mutation produces an

Figure 2 Alignment of XIGSTP1-1 and other Pi-class GSTs

Pi-class GST sequences were obtained for the following: human (P09211), mouse (P19157), rat (P04906), pig (P80031) and *B. bufo* (P83325). Accession numbers are from GenBank®.

Figure 3 Distribution of the XIGSTP1 in different adult tissues and during embryo development by RT-PCR analysis

Lane 1, DNA ladder; lane 2, liver; lane 3, lung; lane 4, heart; lane 5, kidney; lane 6, ovary; lane 7, unfertilized eggs; lane 8, embryo stages 11–15; lane 9, embryo stages 24–25.

enzyme which has totally lost the capacity to conjugate the carcinogenic compound 4-nitroquinoline-1-oxide. Thus the single point mutation of Phe¹¹¹ to Tyr¹¹¹, resulting in only a change in hydrophobicity of the residue, seems to play a role in altering the substrate specificity of amphibian XIGSTP1-1 compared with mammalian Pi-class GSTs. On the other hand, there are two other non-conserved H-site-defining residues in XIGSTP1-1, Thr²⁰⁷ and Pro²⁰⁸, that could also play an important role in determining the specificity of this enzyme. It has been suggested [30] that the flexibility of the C-terminal tail, which is an important factor in determining the CDNB-conjugation capacity of mammalian Pi-class GSTs, is partly controlled by a hydrogen bond formed between the amide nitrogen of Gly²⁰⁸ and the hydroxy group of Tyr¹¹¹. In XIGSTP1-1, this hydrogen bond cannot be formed, due to the presence of Phe¹¹¹ and Pro²⁰⁸. Thus the relatively high CDNB-conjugating activity measured for XIGSTP1-1 (Table 1) may be due to the high flexibility of its C-terminal tail. On the other hand, Pro²⁰⁸ as well as Phe¹¹¹ are important residues for

Figure 4 SDS/PAGE analysis of GST from *X. laevis*

Lane 1, molecular-mass markers (from top to bottom): phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa); lane 2, total cellular extracts of *E. coli* XL1Blue (XIGSTP1-pTrcHis2TOPO) before induction; lane 3, as lane 2, but after induction with IPTG; lane 4, affinity-purified cloned XIGSTP1-1.

the catalytic capacity of XIGSTP1-1. In fact, replacement of Phe¹¹¹ with a tyrosine residue (Phe¹¹¹Tyr) and Pro²⁰⁸ with a glycine or histidine residue (Pro²⁰⁸Gly and Pro²⁰⁸His) drastically decreases its ability to conjugate all of the electrophilic substrates *in vitro* (Table 1). The fact that these residues are important for the activity of XIGSTP1-1 is also supported by the results obtained by measuring the capacity of *E. coli*-carrying genes coding for wild-type and mutant XIGSTP1-1 to grow *in vivo* [31] in the presence of CDNB. In fact, the growth of *E. coli* carrying wild-type XIGSTP1-1 in the presence of CDNB was inhibited, whereas *E. coli* carrying Phe¹¹¹Tyr, Pro²⁰⁸His or Pro²⁰⁸Gly mutant genes were able to grow (Figure 5). A comparison of the primary sequence of XIGSTP1-1 with those of mammalian Pi-class GSTs (pig, mouse and human)

Table 1 Activities for wild-type and mutant forms of XIGSTP1-1

The data are means of quadruplicate preparations; S.E.M. never exceeded 10 % of the mean. n.d., no detectable activity.

Substrate	Specific activity ($\mu\text{mol/min per mg of protein}$)			
	Wild-type	Pro ²⁰⁸ Gly	Pro ²⁰⁸ His	Phe ¹¹¹ Tyr
CDNB	1310	151	171.8	57
Allyl isothiocyanate	100	12.5	14.75	12.12
Benzyl isothiocyanate	85	30.3	36	17
Cumene hydroperoxide	0.24	0.19	n.d.	0.27
<i>p</i> -Nitrobenzyl chloride	18.73	5	7.76	6.1
2-Phenylethyl isothiocyanate	19	12	7.5	11.3
4-Nitroquinoline-1-oxide	7.98	1.86	0.39	n.d.
<i>p</i> -Nitrophenyl acetate	n.d.	n.d.	n.d.	n.d.
Ethacrynic acid	n.d.	n.d.	n.d.	0.05
Δ^5 -Androstene-3,17-dione	n.d.	n.d.	n.d.	n.d.
1,2-Dichloro-4-nitrobenzene	n.d.	n.d.	n.d.	n.d.
1,2-Epoxy- <i>p</i> -nitro-phenoxy-propane	n.d.	n.d.	n.d.	n.d.
tPBO	n.d.	n.d.	n.d.	0.025
7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole	18.73	5	7.76	6.1

Figure 5 Activity of wild-type and mutant XIGSTP1-1 'in vivo'

XIGSTP1-1 activity was tested *in vivo* as described by Lee et al. [31]. The molecular basis of this test relies on the fact that CDNB, the common substrate for GSTs, also has antibiotic activity [37]. This activity is enhanced when *E. coli* expresses a functional GST [31]. Bacteria (*E. coli* XL1Blue) expressing wild-type or mutant enzymes are indicated as follows: 1, pTrcHis2TOPO vector alone; 2, XIGSTP1-pTrcHis2TOPO wild-type; 3, XIGSTP1-pTrcHis2TOPO Phe¹¹¹Tyr mutant; 4, XIGSTP1-pTrcHis2TOPO Pro²⁰⁸Gly mutant; and 5, XIGSTP1-pTrcHis2TOPO Pro²⁰⁸His mutant. Left-hand panel, one loopful of overnight culture was evenly streaked on to an LB agar plate containing 100 $\mu\text{g/ml}$ ampicillin alone to serve as a control for normal growth. Right-hand panel, one loopful of overnight culture was evenly streaked on to an LB agar plate containing 100 $\mu\text{g/ml}$ ampicillin and 20 $\mu\text{g/ml}$ CDNB. Plates were incubated overnight at 37 °C.

shows that XIGSTP1-1 differs in some places. In particular, in the region between residues 40 and 50, there is an insertion of four residues in *X. laevis* (serine, glycine, lysine and aspartate) when compared with the pig sequence [32] and of two residues (lysine and aspartate) when compared with the human [33] and mouse [24] sequences. This amino acid insertion encompasses the 3₁₀B region that joins the α 2 helix and β 3 sheets of domain I. Important GSH-binding residues are located within this region [34]. Thus the insertion of the four residues in the structure of XIGSTP1-1 might result in a different structural arrangement of this protein, which may consequently produce a different GSH-binding capacity. In this context, it is interesting to note that the K_m value of XIGSTP1-1 for GSH (2.17 mM) is relatively higher than that found for the mammalian Pi-class enzymes [35,36]. Furthermore, the absence of the conserved Cys⁴⁹ in XIGSTP1-1 (replaced by Ala⁴⁹) may also contribute to lower its affinity for GSH. In fact, an increase in the K_m value of hGSTP1-1 for GSH (from 0.15 mM to 0.87 mM) occurs when the conserved residue Cys⁴⁹ is replaced by Ala⁴⁹

[35]. On the other hand, as opposed to the mammalian enzymes, the K_m value of XIGSTP1-1 for CDNB (0.27 mM) is significantly lower than that reported for mammalian GST.

Conclusions

The present study describes the cloning and characterization of a novel Pi-class GST from *X. laevis* designated as XIGSTP1-1. This enzyme has higher catalytic activity with CDNB than any other GSTs reported to date. Site-directed mutagenesis shows that its high activity is linked to Phe¹¹¹, which is an active-site residue based on X-ray structures of related GSTs. Tissue-specific expression of XIGSTP1-1 suggests that it is correlated with cellular proliferation.

We wish to thank G. Bernardini and D. Vigetti (Dipartimento di Biologia Strutturale e Funzionale, Università degli Studi dell'Insubria, Varese, Italy), F. Amaldi (Dipartimento di Biologia, Università di Roma 'Tor Vergata', Roma, Italy) and M. Kirschner and L. Collavin (Department of Cell Biology, Harvard Medical School, Boston, MA, U.S.A.) for kindly providing the *X. laevis* liver tumour, stages 24–25 embryo, stages 11–15 embryo and unfertilized egg cDNA libraries. We also thank M. Andreazzoli (Dipartimento di Fisiologia e Biochimica, Università di Pisa, Pisa, Italy) for kindly providing the *X. laevis* embryos.

REFERENCES

- Mannervik, B. and Danielson, U. H. (1988) Glutathione transferases: structure and catalytic activity. *CRC Crit. Rev. Biochem.* **23**, 283–337.
- Hayes, J. D. and Pulford, D. J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600.
- Armstrong, R. N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol.* **10**, 2–18.
- Wang, T., Arifoglu, P., Ronai, Z. and Tew, K. D. (2001) Glutathione S-transferase P1-P1 (GSTP1-GSTP1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. *J. Biol. Chem.* **276**, 20999–21003.
- Cho, S. G., Lee, Y. H., Park, H. S., Ryoo, K., Kang, K. W., Park, J., Eom, S. J., Kim, M. J., Chang, T. S., Choi, S. Y. et al. (2001) Glutathione S-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. *J. Biol. Chem.* **276**, 12749–12755.
- Mannervik, B. (1985) The isoenzymes of glutathione transferase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**, 357–417.
- Sheehan, D., Meade, G., Foley, V. M. and Dowd, C. A. (2001) Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* **360**, 1–16.
- Di Ilio, C., Aceto, A., Bucciarelli, T., Dragani, B., Angelucci, S., Miranda, M., Poma, A., Amicarella, F., Barra, D. and Federici, G. (1992) Glutathione transferase isoenzymes from *Bufo bufo* embryos at an early developmental stage. *Biochem. J.* **283**, 217–222.
- Sacchetta, P., Petruzzelli, R., Melino, S., Bucciarelli, T., Pennelli, A., Amicarella, F., Miranda, M. and Di Ilio, C. (1997) Amphibian embryo glutathione transferase: amino acid sequence and structural properties. *Biochem. J.* **322**, 679–680.
- Bucciarelli, T., Sacchetta, P., Pennelli, A., Cornelio, L., Romagnoli, R., Melino, S., Petruzzelli, R. and Di Ilio, C. (1999) Characterization of toad liver glutathione transferase. *Biochim. Biophys. Acta* **1431**, 189–198.
- Bucciarelli, T., Sacchetta, P., Amicarella, F., Petruzzelli, R., Melino, S., Rotilio, D., Celli, N. and Di Ilio, C. (2002) Amino acid sequence of the major form of toad liver glutathione transferase. *Int. J. Biochem. Cell Biol.* **34**, 1286–1290.
- Aceto, A., Dragani, B., Sacchetta, P., Bucciarelli, T., Angelucci, S., Miranda, M., Poma, A., Amicarella, F., Federici, G. and Di Ilio, C. (1993) Developmental aspects of *Bufo bufo* embryo glutathione transferases. *Mech. Ageing Dev.* **68**, 59–70.
- Amicarella, F., Ragnelli, A. M., Aimola, P., Cattani, F., Bonfigli, A., Zarivi, O., Miranda, M. and Di Ilio, C. (2001) Developmental expression and distribution of amphibian glutathione transferases. *Biochim. Biophys. Acta* **1526**, 77–85.
- Angelucci, S., Sacchetta, P., De Luca, A., Moio, P., Amicarella, F. and Di Ilio, C. (2002) Glutathione transferase isoenzymes from frog (*Xenopus laevis*) liver and embryo. *Biochim. Biophys. Acta* **1569**, 81–85.
- De Luca, A., Favaloro, B., Angelucci, S., Sacchetta, P. and Di Ilio, C. (2002) Mu-class glutathione transferase from *Xenopus laevis*: molecular cloning, expression and site-directed mutagenesis. *Biochem. J.* **365**, 685–691.

- 16 Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M. and Pearson, W. R. (1992) Nomenclature for human glutathione transferases. *Biochem. J.* **282**, 305–306
- 17 Lardelli, M. and Lendahl, U. (1994) Generating bacteriophage lambda sublibraries enriched for rare clones. *Biotechniques* **16**, 420–422
- 18 Habig, W. H. and Jakoby, W. B. (1981) Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* **77**, 398–405
- 19 Di Ilio, C., Sacchetta, P., Lo Bello, M., Caccuri, A. M. and Federici, G. (1986) Selenium independent glutathione peroxidase activity associated with cationic forms of glutathione transferase in human heart. *J. Mol. Cell. Cardiol.* **18**, 983–991
- 20 Stanley, J. S. and Benson, A. M. (1988) The conjugation of 4-nitroquinoline 1-oxide, a potent carcinogen, by mammalian glutathione transferases. 4-Nitroquinoline 1-oxide conjugation by human, rat and mouse liver cytosols, extrahepatic organs of mice and purified mouse glutathione transferase isoenzymes. *Biochem. J.* **256**, 303–306
- 21 Del Boccio, G., Pennelli, A., Whitehead, E. P., Lo Bello, M., Petruzzelli, R., Federici, G. and Ricci, G. (1991) Interaction of glutathione transferase from horse erythrocytes with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. *J. Biol. Chem.* **266**, 13777–13782
- 22 Kolm, R. H., Danielson, U. H., Zhang, Y., Talalay, P. and Mannervik, B. (1995) Isothiocyanates as substrates for human glutathione transferases: structure–activity studies. *Biochem. J.* **311**, 453–459
- 23 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254
- 24 Xu, X. and Stambrook, P. J. (1994) Two murine GSTpi genes are arranged in tandem and are differentially expressed. *J. Biol. Chem.* **269**, 30268–30273
- 25 Simons, P. C. and Vander Jagt, D. L. (1977) Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. *Anal. Biochem.* **82**, 334–341
- 26 Zimniak, P., Nanduri, B., Pikula, S., Bendorowicz-Pikula, J., Singhal, S. S., Srivastava, S. K., Awasthi, S. and Awasthi, Y. C. (1994) Naturally occurring human glutathione S-transferase GSTP1-GSTP1 isoforms with isoleucine and valine in position 104 differ in enzymic properties. *Eur. J. Biochem.* **224**, 893–899
- 27 Bammler, T. K., Driessen, H., Finnstrom, N. and Wolf, C. R. (1995) Amino acid differences at positions 10, 11, and 104 explain the profound catalytic differences between two murine pi-class glutathione S-transferases. *Biochemistry* **34**, 9000–9008
- 28 Oakley, A. J., Rossjohn, J., Lo Bello, M., Caccuri, A. M., Federici, G. and Parker, M. W. (1997) The three-dimensional structure of the human Pi class glutathione transferase P1-P1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. *Biochemistry* **36**, 576–585
- 29 Lo Bello, M., Oakley, A. J., Battistoni, A., Mazzetti, A. P., Nuccetelli, M., Mazzaresse, G., Rossjohn, J., Parker, M. W. and Ricci, G. (1997) Multifunctional role of Tyr 108 in the catalytic mechanism of human glutathione transferase P1-P1. Crystallographic and kinetic studies on the Y108F mutant enzyme. *Biochemistry* **36**, 6207–6217
- 30 Dirr, H., Reinemer, P. and Huber, R. (1994) X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *Eur. J. Biochem.* **220**, 645–661
- 31 Lee, H. C., Toung, Y. P., Tu, Y. S. and Tu, C. P. (1995) A molecular genetic approach for the identification of essential residues in human glutathione S-transferase function in *Escherichia coli*. *J. Biol. Chem.* **270**, 99–109
- 32 Dirr, H. W., Mann, K., Huber, R., Ladenstein, R. and Reinemer, P. (1991) Class pi glutathione S-transferase from pig lung. Purification, biochemical characterization, primary structure and crystallization. *Eur. J. Biochem.* **196**, 693–698
- 33 Kano, T., Sakai, M. and Muramatsu, M. (1987) Structure and expression of a human class pi glutathione S-transferase messenger RNA. *Cancer Res.* **47**, 5626–5630
- 34 Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G. and Parker, M. W. (1992) Three-dimensional structure of class pi glutathione S-transferase from human placenta in complex with S-hexylglutathione at 2.8 Å resolution. *J. Mol. Biol.* **227**, 214–226
- 35 Ricci, G., Lo Bello, M., Caccuri, A. M., Pastore, A., Nuccetelli, M., Parker, M. W. and Federici, G. (1995) Site-directed mutagenesis of human glutathione transferase P1-P1. Mutation of Cys-47 induces a positive cooperatively in glutathione transferase P1-P1. *J. Biol. Chem.* **270**, 1243–1248
- 36 Vega, M. C., Walsh, S. B., Mantle, T. J. and Coll, M. (1998) The three-dimensional structure of Cys-47-modified mouse liver glutathione S-transferase P1-P1. Carboxymethylation dramatically decreases the affinity for glutathione and is associated with a loss of electron density in the αB-310B region. *J. Biol. Chem.* **273**, 2844–2850
- 37 Bochner, B. R., Lee, P. C., Wilson, S. W., Cutler, C. W. and Ames, B. N. (1984) AppppA and related adenylated nucleotides are synthesized as a consequence of oxidation stress. *Cell (Cambridge, Mass.)* **37**, 225–232

Received 17 February 2003/16 April 2003; accepted 23 April 2003

Published as BJ Immediate Publication 23 April 2003, DOI 10.1042/BJ20030261