BIOCHEMICAL PHARMACOLOGY 71 (2006) 1619–1628

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Several glutathione S-transferase isozymes that protect against oxidative injury are expressed in human liver mitochondria

Evan P. Gallagher [a,*], *James L. Gardner* [b,1], *David S. Barber* [b]

[a] *Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, United States*
[b] *Department of Physiological Sciences, University of Florida, Gainesville, FL, United States*

ARTICLE INFO

Article history:
Received 15 January 2006
Accepted 23 February 2006

Keywords:
Glutathione
Glutathione S-transferase
Mitochondria
Human liver

Abbreviations:
4HNE, 4-hydroxynonenal
δ^5-ADI, δ^5-androstene-3,17-dione
CDNB, 1-chloro-2,4-dinitrobenzene
CuOOH, cumene hydroperoxide
DCNB, 1,2-dichloro-4-nitrobenzene
DTT, dithiothrietol
ECA, ethacrynic acid
ECL, enhanced chemiluminescence
EDTA, ethylenediaminetetraacetic acid
ESI-MS, electrospray ionization-mass spectrometry
GPX, glutathione peroxidase
GSH, glutathione
GST, glutathione S-transferase
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ABSTRACT

The mitochondrial environment is rich in reactive oxygen species (ROS) that may ultimately peroxidize membrane proteins and generate unsaturated aldehydes such as 4-hydroxy-2-nonenal (4HNE). We had previously demonstrated the presence of hGSTA4-4, an efficient catalyst of 4HNE detoxification, in human liver mitochondria to the exclusion of the cytosol. In the present study, GSH-affinity chromatography was used in conjunction with biochemical and proteomic analysis to determine the presence of additional cytosolic glutathione S-transferases (GSTs) in human hepatic mitochondria. HPLC-subunit analysis of GSH affinity-purified liver mitochondrial proteins indicated the presence of several potential mitochondrial GST isoforms. Electrospray ionization-mass spectrometry analysis of eluted mitochondrial GST subunits yielded molecular masses similar to those of hGSTP1, hGSTA1 and hGSTA2. Octagonal matrix-assisted laser desorption/ionization time of flight mass spectrometry and proteomics analysis using MS-FIT confirmed the presence of these three GST subunits in mitochondria, and HPLC analysis indicated that the relative contents of the mitochondrial GST subunits were hGSTA1 > hGSTA2 > hGSTP1. The mitochondrial localization of the alpha and pi class GST subunits was consistent with immunoblotting analysis of purified mitochondrial GST. Enzymatic studies using GSH-purified mitochondrial GST fractions demonstrated the presence of significant GST activity using the nonspecific GST substrate 1-chloro-2,4-dinitrobenzene (CDNB), as well as 4HNE, δ^5-androstene-3,17-dione (ADI), and cumene hydroperoxide (CuOOH). Interestingly, the specific mitochondrial GST activities toward 4HNE, a highly toxic α,β-unsaturated aldehyde produced during the breakdown of membrane lipids, exceeded that observed in liver cytosol. These observations are suggestive of a role of GST in protecting against mitochondrial injury during the secondary phase of oxidative stress, or modulation of 4HNE-mediated mitochondrial signaling pathways. However, other properties of mitochondrial GST, such as conjugation of environmental chemicals and binding of lipophilic non-substrate xenobiotics and endogenous compounds, remain to be investigated.

* *Corresponding author at*: Department of Environmental and Occupational Health Sciences, 4225 Roosevelt Way Northeast Suite 100, Seattle, WA 98105-6099, United States. Tel.: +1 206 616 4739; fax: +1 206 685 4696.
E-mail address: evang3@u.washington.edu (E.P. Gallagher).
[1] KineMed Inc., 5980 Horton Street, Suite 400, Emeryville, CA 94608, United States.
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.02.018

HPLC, high performance liquid chromatography
LDH, lactate dehydrogenase
NADPH, nicotinamide adenine dinucleotidephosphate, reduced
NBC, nitrobutyl chloride
oMALDI, octagonal matrix-assisted laser desorption/ionization
oMALDITOF, octagonal matrix-assisted laser desorption/ionization time of flight mass spectrometry
PBS, phosphate buffered saline
TFA, trifluoroacetic acid

1. Introduction

The glutathione S-transferases (GSTs) are a large and diverse group of class II biotransformation enzymes that function in the detoxification of xenobiotics and endogenous toxicants (reviewed in [1,2]). First discovered as ligandin proteins due to their ability to bind lipophilic non-substrate xenobiotics and endogenous compounds, the primary catalytic activity of GSTs is the conjugation of electrophilic compounds which is accomplished through nucleophilic attack by reduced glutathione (GSH). The compounds detoxified by GSTs include a broad array of carcinogens, anticancer drugs, metabolic byproducts, as well as environmental chemicals [1,2]. Additionally, some GST isozymes are capable of using GSH as an electron donor in peroxidative reactions, thus, providing protection from oxidative byproducts. Three major families of widely distributed proteins that exhibit GST activity have been described and include cytosolic, mitochondrial, and microsomal GST (now referred to as membrane-associated proteins in eicosanoid and glutathione metabolism, or MAPEG enzymes [1]). Collectively, the GSTs currently comprise eight separate GST gene subfamilies (α, θ, κ, μ, π, σ, ω, and MAPEG) and 24 distinct GST human proteins have been identified [1].

Although the cytosolic GST represent the largest family of transferases, the presence of mitochondrial GST isoforms has also been described, with most studies directed towards hepatic mitochondrial GST expression in rats [3–6] and mice [4,7]. GST isoforms identified in mammalian species have included members of several GST subfamilies, including alpha [4], mu [8], kappa [3], and theta class GST [3]. The exclusively mitochondrial kappa GST appears to consist of a single GST isozyme in human liver [9]. Because the mitochondrial electron transport accounts for the majority of the molecular oxygen consumed by cells and tissues, these organelles are susceptible to peroxidative damage from reactive oxygen species liberated during oxidative metabolism. Based on the available information to date, a distinct commonality to the mitochondrial GST appear to be their ability to participate in the detoxification of byproducts of oxidative stress, primarily via the reduction of cellular peroxides and α,β-unsaturated aldehydes generated during lipid peroxidation [1].

With the exception of the kappa GST, relatively little is known regarding the expression or catalytic function of other human liver mitochondrial GSTs. We have previously characterized the expression of an important aldehyde metabolizing GST isoform (hGSTA4-4) in human liver mitochondria [10]. The hGSTA4-4 isoform and related A4-4 subclass of GST found in other species (mGSTA4-4, rGSTA4-4) exhibit an efficient catalytic activity profile directed towards the detoxification of α,β-unsaturated aldehydes such as 4HNE, a reactive aldehyde known to be involved in a number of cellular pathologies [11–14] as well as intracellular signaling [15]. Over expression of mGSTA4-4 in HL-60 cells inhibits JNK-mediated cell signaling and protects against 4HNE apoptosis [15]. The functionality of hGSTA4-4 in protecting against oxidative damage has been also demonstrated in cell transfection experiments which indicate that over-expression of hGSTA4-4 protects cells against oxidative stress and 4HNE-mediated apoptosis [15,16]. However, the expression level of hGSTA4-4 in cells is relatively low, and other more abundant GSTs with catalytic activity towards α,β-unsaturated aldehydes are important contributors towards 4HNE detoxification [17].

In addition to hGSTA4-4, we have reported the presence of at least one additional human liver mitochondrial GST protein with immunological cross-reactivity toward hGSTA1-1 [17]. However, we did not identify the hGSTA1-1-reactive protein(s), examine the mitochondria for other GST isozymes, or analyze the enzymatic properties of human liver mitochondria. In the current report, we describe the isolation and characterization of three human hepatic mitochondrial alpha class GST isoforms and their role in protecting against oxidative injury. Our approach was to use high resolution analytical methods that combine the resolving power of HPLC with the conclusive identification of subunits using electrospray ionization–mass spectrometry (ESI/MS) and octagonal matrix-assisted laser description/ionization time of flight mass spectrometry (oMALDI-TOF MS). Catalytic activity assays were also performed and compared to enzymatic reactions of cytosolic GST to better understand the functions of mitochondrial GST. Our results indicate a substantial ability of the human liver mitochondrial GST to metabolize cytotoxic breakdown products of oxidative injury, thus, suggesting that GST may comprise a pathway protection in human liver mitochondria against the secondary phase of oxidative stress.

2. Materials and methods

2.1. Chemicals and biochemicals

δ^5-Androstene-3,17-dione (δ^5-ADI), 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), cumene hydroperoxide (CuOOH), ethacrynic acid (ECA), 4-hydroxynonenal (4HNE), nitrobutyl chloride (NBC), secondary antibodies, cyanogen bromide-activated agarose, protein A, horseradish peroxidase (HRP)-linked rabbit anti-IgY, glutathione (GSH), buffer salts, HPLC solvents and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilon-PSQ™ polyvinylidene difluoride (PVDF) membranes were purchased from Millipore, and Immun-blot™ PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA). ECL™ and ECL Plus™ detection reagents, as well as Hyperfilm MP™ autoradiographic film were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Polyclonal antibodies rabbit anti-hGSTA1-1 and rabbit anti-hGSTP1-1 were purchased from Oxford Biochemical Inc. (Oxford, MI).

2.2. Subcellular fractionation and isolation of hepatic mitochondria

All use of human tissues was approved by Institutional Review Boards at the University of Florida and the University of Washington. Human hepatic tissue was obtained through Vitron Inc. (Tucson, AZ). The anonymous donor was a 40-year old male Caucasian who died from a cerebral hemorrhage. Isolation of subcellular components from liver tissue was performed as previously described [10] with all steps performed at 4 °C. Briefly, approximately 100 g of liver tissue was minced, placed in 250 mM sucrose, 1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, pH 7.5 buffer and homogenized with a Potter–Elvehjem mortar and Teflon pestle. After centrifugation and washing to remove unbroken cells and nuclei, mitochondria were isolated by centrifugation at 10,000 × *g* for 10 min. Mitochondrial fractions were then washed twice in isotonic buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.125 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothrietol (DTT), pH 7.4) prior to further purification.

2.3. Purification of mitochondrial fractions and GSH affinity isolation of mitochondrial GST

The rinsed mitochondria isolated above were further purified using ficoll gradient centrifugation [18]. Briefly, the mitochondrial fractions were loaded into ultracentrifuge tubes that contained 20 ml 30% (w/v) ficoll in 225 mM mannitol, 1 mM EDTA, 25 mM HEPES, pH 7.4, 0.1% (w/v) bovine serum albumin and centrifuged for 30 min at 95,000 × *g*. Mitochondria were collected from the bottom of the dense yellow/brown band, recentrifuged, washed in IM buffer, resuspended in 50 ml aliquots in IM buffer, and stored at −80 °C until lysis. Purified intact mitochondria were treated with proteinase K (10 mg/ml) for 20 min at 0 °C to remove any contaminating enzymes from the cytoplasm or nuclei. Mitochondrial cytochrome *c* oxidase, microsomal cytochrome *c* reductase, and cytosolic lactate dehydrogenase activities were measured as indicators of mitochondrial purity [19] with all assays optimized for human liver fractions. Protein content of samples was determined using the bicinchoninic acid method and a commercial kit. After verification of mitochondrial purity, the gradient-purified mitochondria were lysed with 0.5% Triton X-100 with stirring for 10 min on ice. After centrifugation at 106,000 × *g* for 1 h, the supernatant was passed over a GSH-sepharose affinity column. Bound protein was eluted with 150 mM glutathione, 50 mM Tris–HCl, and 1.4 mM β-mercaptoethanol, pH 9.6. Eluate and flow-through material from the GSH affinity purification were tested for GST activity using CDNB and subjected to electrophoresis. Catalytically active GST fractions were pooled and dialyzed against 150 volumes of 1× PBS containing 0.5 mM DTT, with changes every 3 h for a total of 15 h for further purification [20].

2.4. GST subunit analysis using reverse phase HPLC

Reverse-phase HPLC [21] was used to characterize the GST subunits from GSH affinity-purified mitochondria using a 150 mm × 4.6 mm Vydac 214TP C4 column (Grace Vydac, Hesperia, CA) equilibrated with 37% (v/v) acetonitrile containing 0.075% trifluoroacetic acid (TFA) in water. The column flow rate was 1.5 ml/min with 37–43% (v/v) gradient of acetonitrile containing 0.075% TFA over 25 min, followed by a 43–55% (v/v) gradient of acetonitrile and 0.075% TFA over 20 min. The polypeptide peaks were detected by UV absorption at 214 nm. Peak area integration was performed using Turbochrom software (Perkin Elmer, Norwalk, CT). The HPLC fraction peaks were collected and dried under vacuum to remove the TFA. Desiccated fractions were resuspended in water immediately prior to SDS page or other analytical procedures.

2.5. oMALDI-TOF analysis and electrospray mass spectrometry

The molecular masses of the putative GST subunits were determined by HPLC coupled to ESI/MS (LC/MS). Affinity purified GST protein (approximately 60 μg) was diluted in equal volume of water containing 0.075% TFA and separated on the C_4 column as described above. Positive ion ESI/MS were acquired using a ThermoFinnigan LCQ Classic ion trap mass spectrometer (Austin, TX). The ESI source was operated at 4.2 kV with the heated capillary at 220 °C and a relative nitrogen flow of 80%. Spectra were scanned from *m/z* 200–2000 and deconvoluted using ThermoFinnigan Navigator 1.2 software (Austin, TX).

For positive identification, HPLC purified subunits were reduced with DTT and alkylated with iodoacetamide followed by digestion with trypsin overnight at 37 °C. Resulting peptides were desalted with a C_{18} ZipTip (Millipore Inc., Billerica, MA) and analyzed by oMALDI-TOF using an API QSTAR Pulsar LC/MS/MS system (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an oMALDI source. Mass spectrometry data were subjected to peptide mass fingerprinting using MS-FIT (University of California, San Francisco, CA) to confirm the identity of the unknown proteins after trypsin digestion.

2.6. Prediction of GST subunit subcellular localization

Prediction of subcellular localization of putative mitochondrial GST subunits was accomplished using pTARGET (University of Albany Center for Cancer Genomics, Albany, NY). pTARGET is a computational method to predict the subcellular localization of eukaryotic proteins based on the occurrence patterns of protein functional domains and the amino acid compositional differences in proteins from different subcellular locations [22].

2.7. GST catalytic activity assays

Mitochondrial and cytosolic GST initial rate activities toward ADI, CDNB, CuOOH, DCNB, ECA, and NBC was measured using a 96-well microplate reader [17]. GST catalytic activity toward 4HNE was determined by the spectrophotometric method of Singhal et al. [23] with minor modifications [24]. The reaction mixtures (final volume 1 ml) contained 100 mM KPO_4, pH 6.5, 0.5 mM GSH, 25 μl of lysed mitochondrial GST or cytosol, and 0.1 mM 4HNE. The reactions were initiated by the addition of 4HNE, and its utilization was monitored at 224 nm in a Cary dual beam spectrophotometer against a blank that contained all of the reactants except 4HNE. Assays were conducted at 30 °C and were corrected for non-enzymatic activity and non-specific binding of 4HNE using heat-inactivated subcellular fractions.

2.8. SDS-PAGE and Western blotting analysis

One microgram of recombinant GST or GSH affinity purified mitochondrial GST, or 60 μg of human liver cytosol were subjected to 14% SDS-PAGE and visualized by Coomassie blue staining. Western blot analysis using polyclonal antibodies against hGSTA1-1 and hGSTP1-1 was carried out as previously described [10,25]. Imaging quantification was performed with a Fluor-S imager (BioRad Laboratories Inc., Hercules, CA) as previously described [10].

3. Results

As reported in Table 1, mitochondria purified from an adult human liver using gradient purification exhibited little cytosolic protein contamination. For example, cytosolic LDH activities in the rinsed human liver mitochondrial fractions were 6% that observed in the cytosol. However, further purification of liver mitochondria using ficoll reduced mitochondrial LDH activity to a non-detectable rate (<1% than that observed in the cytosol). Accordingly, the 4.7-fold higher specific GST–CDNB activity in the rinsed mitochondrial lysate as compared to the gradient purified mitochondria may have reflected the presence of some minor adsorbed cytosolic protein present in the rinsed mitochondrial fraction. GSH-affinity purification of hepatic mitochondrial GST yielded a 807-fold enrichment of GST (11.3 μmol/min/mg in the GSH-affinity purified gradient mitochondrial GST versus 0.014 μmol/min/mg in gradient purified mitochondria, Table 2) and a 60% yield of total GST activity from the gradient purified mitochondria (2.1 versus 3.5 total units of GST–CDNB activity in the GSH-affinity purified gradient mitochondria, and gradient mitochondrial fractions, respectively, Table 2). All of the eluted GST–CDNB activity was found in three GSH-affinity fractions which were pooled for the analyses, with no activity detected in the flow-through material. Based upon protein recoveries, GST accounted for 0.14% of the total mitochondrial protein content (Table 2.)

Table 1 – Lactate dehydrogenase (LDH) and cytochrome oxidase activities in human liver subcellular fractions

Fraction	LDH activity (U/mg)	Cytochrome oxidase (U/mg)
Beginning homogenate	1645	0.6
Cytosol	2641	BDL[a]
Rinsed mitochondria lysate	157	5.6
Gradient-purified mitochondria	BDL	5.8

[a] Not detected.

In addition to catalyzing CDNB conjugation, the GSH affinity-purified mitochondrial fractions exhibited substantial GST catalytic activity toward CuOOH, 4HNE, NBC, and ADI (Table 3). In contrast, we did not detect mitochondrial GST activity towards ECA or DCNB (Table 3). The specific mitochondrial GST catalytic activities were CDNB > CuOOH > 4HNE > NBC > ECA. Interestingly, the catalytic activities of affinity-purified GST from human hepatic cytosol and mitochondria when normalized on a per milligram protein basis towards CDNB and CuOOH were relatively similar, which was not the case for 4HNE (Table 4). In particular, mitochondrial GST exhibited at least two-fold higher 4HNE activity compared

Table 2 – Protein content and GST–CDNB activities during mitochondrial GST purification

Fraction	Total protein protein (mg)	Total GST–CDNB activity (μmol/min)	Specific GST–CDNB activity (μmol/min/mg)
Beginning homogenate	10000	2760	0.276
Cytosol	1900	1024	0.539
Rinsed mitochondria	250	8.2	0.066
Gradient-purified mitochondria	140	3.5	0.014
Gradient-purified mitochondrial GSH-affinity eluate	0.19	2.1	11.3
Gradient-purified mitochondrial GSH-affinity flow-through	130	BDL*	ND

* Not detected.

Table 3 – Catalytic profile of GSH affinity-purified human liver mitochondrial GST

Substrate (class)	GST activity[a] (μmol/min/mg)	Ratio to CDNB activity
1-Chloro-2,4-dinitrobenzene (CDNB)	11.3 (0.3)	1
Cumene hydroperoxide (CuOOH)	9.7 (0.2)	0.85
4-Hydroxynonenal (4HNE)	6.5 (1.7)	0.58
Nitrobutyl chloride (NBC)	6.3 (2.7)	0.56
δ^5-Androstene-3,17-dione[b] (ADI)	2.3 (0.2)	0.2
Ethacrynic acid (ECA)	<0.1	<0.01
1,2-Dichloro-nitrobenzene (DCNB)	<0.1	<0.01

[a] Data represents mean (S.D.) of assays that were conducted in triplicate and therefore no statistical analyses were performed. Enzyme activities for all substrates (with the exception of ADI) reflects nmol substrate conjugated/min/mg protein.
[b] GST–ADI activity reflects GST-mediated keto-steroid isomerization of ADI.

Table 4 – Comparative GST catalytic activities in GSH-affinity purified cytosol and mitochondrial fractions from human liver[a]

Substrate (class)	Cytosolic GST activity (μmol/min/mg)	Mitochondrial GST activity (μmol/min/mg)
CDNB	14.3 ± 1.4	11.3 ± 0.3
CuOOH	8.8 ± 2.4	9.7 ± 0.2
4HNE	2.9 ± 0.3	6.5 ± 1.7

[a] Data represents mean (S.D.) of assay conducted in triplicate.

to cytosol (Table 4). Furthermore, a comparison of the specific mitochondrial GST–4HNE catalytic activities in Table 4 shows a substantially high proportion of mitochondrial GST activity directed towards 4HNE relative to cytosol.

Reversed phase HPLC provided additional information regarding the identity and relative amounts of individual mitochondrial GST subunits. Of the three distinct HPLC peaks present on chromatograms obtained from GST subunit analysis (Fig. 1), peaks eluting at 19.2 (peak 2) and 21.5 min (peak 3) were the most prevalent. Collectively, the area under the curve (AUC) values for the HPLC peaks were as follows: peak 1 (14.7 min), 24,379 μV; peak 2 (19.2 min), 230,508 μV; and peak 3 (21.5 min), 167,293 μV. As observed in Fig. 1, two minor peaks (17.9 and 20.3 min, both shouldering peak 2) were also eluted by reverse phased HPLC. Subsequently, the molecular masses of these five peaks were determined by LC–MS. These data were compared to the molecular masses of human GST based upon deduced amino acid sequences [26]. These peaks were collected and subjected to trypsin digestion followed by oMALDI and MS-FIT analysis. LC–MS analysis of GST peak 1 (peak 1) eluting at 14.7 min yielded a molecular weight of 23,225 Da, closely matching the 23,224 Da molecular mass reported for the hGSTP1 subunit reported by Rowe et al. [26]. Trypsin digestion of the purified protein eluting at 14.7 min followed by oMALDI and MS-FIT analysis of the peptide fragments revealed 23% match of the peptide fragments covering 35% of the hGSTP1 subunit protein (SwissPro accession number P09211, Tables 5 and 6).

On the basis of its deduced amino acid sequence, the molecular mass of the hGSTA1 subunit should be 25,500 Da. Rowe et al. [26] reported a molecular mass of 25,544 Da for hGSTA1 purified from human testes that was analyzed by LC/MS. Our HPLC/MS analysis of the major mitochondrial GST peak eluting at 19.2 min (peak 2) revealed a molecular weight of 25,541 Da. Trypsin digestion of the purified protein corresponding to this HPLC peak followed by oMALDI and MS-FIT analysis of the peptide fragments revealed a 66% match of the peptide fragments covering 24% of the hGSTA1 subunit protein (SwissPro accession number P08263, Tables 5 and 6). In addition, the two minor peaks eluting at 17.9 and 20.3 min, which appeared to be shoulders of the hGSTA1 peak, were confirmed by LC/MS to also have molecular masses of

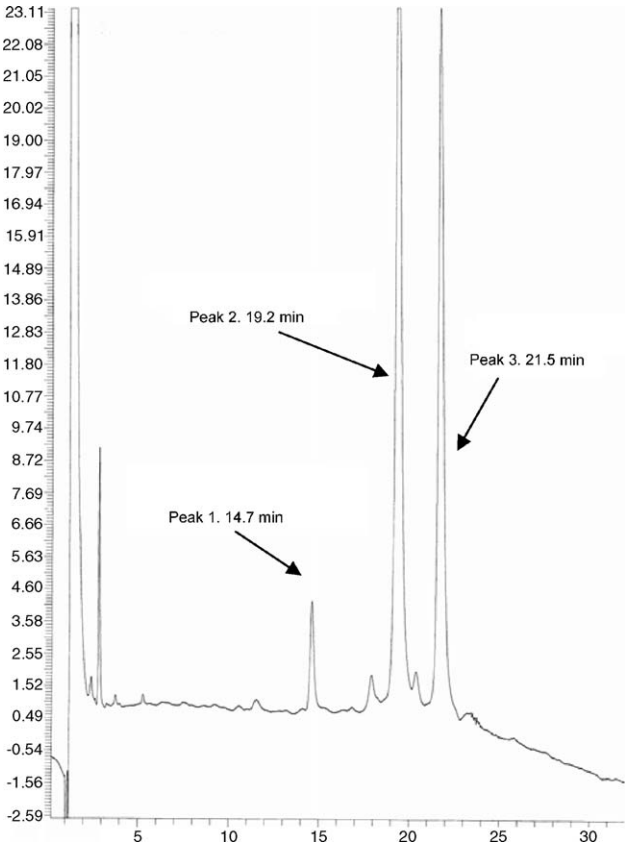

Fig. 1 – GST subunit analysis using reverse phase HPLC and GSH affinity-purified human liver mitochondria proteins yields three distinct peaks. Chromatographic data are represented in absorbance units at 214 nm as a function of time. Elution times are indicated with each peak.

Table 5 – LC/MS and oMALDI analysis of GST subunits from human liver

HPLC peak	Retention time	Protein reported MW (Da) by LC/MS	Previous reported mass (Da)	Deduced mass from literature (Unipro)	# Masses matched (oMALDI)	% Coverage of protein	Putative identity (MS-FIT)	SwissProtein accession #
1	14.7	23225	23224 (hGSTP1)	23224	6/26 (23%)	35% (75/210)	hGSTP1	P09211
2	19.2	25540	25541 (hGSTA1)	25500	14/23 (61%)	24% (55/222)	hGSTA1	P08263
3	21.5	25574	25579 (hGSTA2)	25549	13/28 (46%)	41% (92/222)	hGSTA2	P09210

Table 6 – Representative tryptic peptide sequences deduced from LC/MS

	HPLC peak	Peptide sequence	Protein sequence start/end	Matched sequence (accession number)
1	14.5 min	MPPYTVVYFPVR	1–12	hGSTP1 (P09211)
		GRCAALR	13–19	
		EEVVTVETWQEGSLK	31–45	
		ASCLYGQLPK	46–55	
		TLGLYGK	76–82	
		YISLIYTNYEAGK	104–116	
		DDYVK	117–121	
		LSARPKLK	184–191	
2	19.2 min	MAEKPK	1–6	hGSTA1 (P08263)
		GRMESTR	14–20	
		SAEDLDK	37–43	
		YNLYGK	79–84	
		DIKER	85–89	
		LALIKEK	121–127	
		EKIKNR	126–131	
		ALKTR	183–187	
		SLEEAR	212–217	
		KIFRF	218–222	
3	21.5 min	MAEKPK	1–6	hGSTA2 (P09210)
		MESIR	16–20	
		LVQTR	65–69	
		DIKEK	85–89	
		LALIQEK	121–127	
		ISNLPTVK	188–195	
		SLEESRK	212–218	
		KIFRF	218–222	

25,541 Da, suggesting that these peaks were also comprised of hGSTA1. Tryptic peptide sequences deduced from LC/MS were consistent with the protein sequence of hGSTA1, and these peaks were not further analyzed. The molecular mass for hGSTA2 based upon its deduced amino acid sequence is 25,549 Da, and as determined by Rowe et al. is 25,579 Da [26]. Our LC–MS data of the peak eluting at 21.5 min (peak 3) was 25,574 Da. Trypsin digestion of the LC-purified protein followed by oMALDI and MS-FIT analysis of the peptide fragments revealed 46% match of the peptide fragments covering 41% of the hGSTA2 (SwissPro accession number P09210, Tables 5 and 6).

Western blot analysis of GSH-affinity purified mitochondria was used to determine if immunoblotting of alpha and pi class GST reflected the results from the ESI/MS and LC/MS analyses. As observed in Fig. 2, significant levels of protein cross-reacting with hGSTA1-1 antibody were present in purified mitochondria. As noted in the methods, this antibody does not discriminate between the A1 and A2 isoforms. Similarly, a protein cross-reacting with the hGSTP1-1 antibody

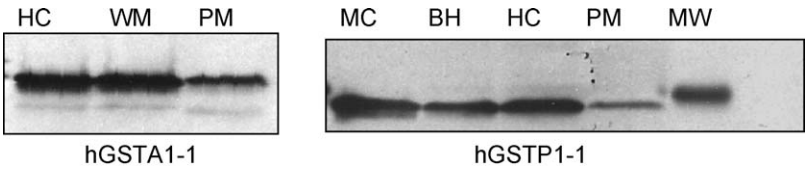

Fig. 2 – Western analysis of cytosolic and mitochondrial proteins. Panel A depicts Western blotting of hGSTA1-1 using human liver cytosol (HC, positive control 20 μg), washed mitochondrial preparation (WM, 50 μg), and purified mitochondria (PM, 25 μg). Panel B depicts Western blotting of human polyclonal hGSTP1-1 antisera against mouse liver cytosol (MC, 25 μg), beginning human liver homogenate (BH, 50 μg), human liver cytosol (HC, 50 μg), and purified human liver mitochondria (PM, 25 μg).

was present in cytosol and purified mitochondria. In addition, bioinformatics analysis based on the occurrence patterns of protein functional domains and the amino acid composition in proteins from different subcellular locations predicted with 69% confidence mitochondrial localization for hGSTP1, and 81.4% for both hGSTA1 and hGSTA2.

4. Discussion

Mitochondria are under constant oxidative challenge from the continuous production of reactive oxygen species such as $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ during oxidative phosphorylation. Accordingly, maintenance of cellular defenses against these intracellular ROS is critical for cell survival. In this regard, protection against the initial phase of ROS-mediated oxidative damage is accomplished through the scavenging of oxygen radicals by low molecular weight thiols such as GSH and thioredoxin, antioxidants such as Vitamin E, and also enzymatic systems such as the mitochondrial superoxide dismutases, catalase, and mitochondrial GSH peroxidases (reviewed in [27]). However, once these defenses are overwhelmed, a number of secondary events such as mitochondrial membrane depolarization and electrophilic attack on mitochondrial DNA and proteins may occur and lead to cell death. During this process a number of secondary toxic byproducts, including α,β-unsaturated aldehydes, which are highly reactive and relatively long-lived and substrates for aldehyde detoxifying systems are released [14,28,29]. Our previous observation of the presence of hGSTA4-4, an alpha class GST isoform with exceptionally high catalytic efficiency towards α,β-unsaturated aldehydes in the mitochondria of human liver, suggested a role for GST in maintaining favorable mitochondrial redox status during the secondary phase of oxidative stress. The aforementioned hypothesis was supported by the presence of substantial hGSTA1-1-reactive mitochondrial proteins in a human liver donor [10].

In the present study, we have discriminated and identified three additional human liver mitochondrial GST isozymes, including two additional alpha class GST subunits (hGSTA1 and hGSTA2), as well as a pi class GST (hGSTP1). Our GSH affinity-purified protein from the human liver mitochondria comprised approximately 0.1% of total mitochondrial protein content from this particular donor, as opposed to the cytosolic GST which account for approximately 3–4% of total cytosolic protein [2]. The mitochondrial GST enzymes could not have arisen from cytosolic contamination of the mitochondrial preparations for several reasons. First, our marker enzyme assays revealed that the gradient purified mitochondria contained little or no contamination from the cytosolic fraction, and thus could not have accounted for the high GST catalytic activities observed in the mitochondria. This is consistent with other mitochondrial fractions prepared in our laboratory from human liver which show little or no enzymatic or immunoreactive activity originating from enzyme markers specific for other cellular compartments [10]. In addition, our comparative analysis of GST catalytic activities in the cytosolic and mitochondrial fractions revealed different GST catalytic activity patterns, including a higher mitochondrial GST–4HNE activity relative to cytosol and also a lack of detectable mitochondrial GST–ECA and GST–DCNB activities, both of which are detectable in human liver cytosol [17].

The primary mitochondrial GST subunits identified from this human liver donor by LC/MS consisted of hGSTA1 and hGSTA2. The molecular masses of the mitochondrial hGSTA1 and hGSTA2 subunits were relatively similar to those reported previously for their cytosolic counterparts [26]. The slight differences in masses (1 Da for hGSTA1 and 5 Da for hGSTA2) observed in the present study are within the mass error for this technique. The presence of substantially higher amounts of hGSTA1 as compared to hGSTA2 based upon HPLC and AUC analysis is consistent with other studies in human liver showing higher amounts of hGSTA1 relative to hGSTA2 in human tissues [26,30]. In this regard, AUC calculations provided a reasonable estimate of the relative amounts, but not precise abundance of mitochondrial GST from this donor. The level of cellular hGSTA1-1 protein has been shown to be influenced by a genetic polymorphism that consists of two alleles *hGSTA1*A* and *hGSTA1*B*, containing three linked base substitutions in the proximal promoter at positions −567, −69, and −52 [31]. Accordingly, the patterns of expression of the hGSTA1 and hGSTA2 units are variable and may result in differing ratios of hGSTA1/hGSTA2 among individuals [30]. Because the hGSTA1/hGSTA2 subunit ratios arise from polymorphisms in the *hGSTA1* promoter, it is possible that mitochondrial hGSTA1/hGSTA2 ratios may also be subject to inter-individual variability.

The relatively high amounts of hGSTA1 and hGSTA2 in human liver mitochondria identified in the present study using HPLC and LC/MS analyses were consistent with our enzymatic studies demonstrating that purified mitochondrial GST metabolized the alpha class GST substrate ADI [2]. The alpha class GSTs also exhibit high activity toward oxidizing toxicants such as CuOOH, and toxic byproducts of oxidative stress such as 4HNE. In particular, both hGSTA1-1 and hGSTA2-2 have high GSH peroxidase activity towards fatty acid hydroperoxides, phospholipid hydroperoxides, and cumene hydroperoxide [32]. However, the activity of hGSTA2-2 towards these substrates appears to be higher than observed for hGSTA1-1 [32]. The GSH peroxidase activity exhibited by GST provides important protection against mitochondrial membrane lipid peroxidation by scavenging damaging peroxides. Accordingly, control of ROS by mitochondrial hGSTA1-1 and hGSTA2-2 is likely an important mechanism for maintaining mitochondrial membrane integrity and ROS-induced apoptosis.

As discussed, GST alpha class proteins have been reported in the mitochondria of tissues from other species. In particular, rGSTA4-4, the rat orthologue of hGSTA4-4, is found in mitochondrial and cytosolic fractions prepared from rat lung, brain, and liver [5] and also mice [8]. Mice liver mitoplasts also express the mu class isoform mGSTM1-1 [8]. Interestingly, our initial ESI/MS studies using another liver donor also suggested the presence of a mu class GST in liver mitochondria, but we could not conclusively verify the presence of hGSTM1-1 in that donor, or in the present donor. Because the *hGSTM1* gene is polymorphic, it could be that the donor from the present study was *hGSTM1* deleted. Also as discussed, a single mammalian mitochondrial kappa GST isozyme present

in humans, rats and mice [3,7,33] has been generally recognized as a major mitochondrial GST. The mammalian kappa GST can actively reduce CuOOH and has high activity towards aryl halides [3]. Although GST kappa was originally isolated from mitochondria and is not present in cytoplasm, it has also been shown to be located in peroxisomes [33], suggesting a new function for this GST family of enzymes. Although we did not detect the kappa class GST in our preparations, the methods of purification and identification described here may have excluded one or more mitochondrial GST through the losses incurred at affinity purification or during the HPLC sample preparation and elution. Similarly, the lack of detection of the hGSTA4 subunit in this donor was probably a result of retention of hGSTA4-4 on the GSH affinity column, as it is our experience that this isoform has a relatively high affinity for GSH matrices compared to other GST (unpublished observations). Our approach positively identified what may be the most prevalent GSTs found in the mitochondria of human liver tissue. Further experimentation is required to exhaustively characterize mitochondrial GST activities and isoform profiles both under normal conditions, as well as under oxidative stress.

In general, most proteins that are targeted to the mitochondrion are translated as presequences with N-terminal targeting leaders which are typically cleaved from the mature protein after targeting is complete. Subcellular compartment analysis using pTARGET and protein sequence information predicted the mitochondrial targeting of all three GSTs identified in the present study. In the case of mGSTA4-4, mitochondrial targeting is dependent upon phosphorylation of mitochondrial targeting signal residues within the C-terminus of the GST protein by protein kinase A and protein kinase C, resulting in increased affinity for binding to the cytoplasmic Hsp70 chaperone and translocation to the mitochondria [34]. We have previously reported that hGSTA4-4 lacked a classic mitochondrial targeting leader sequence. It is likely that phosphorylation or other post-translational modification activates the mitochondrial targeting signal of cytosolic GST proteins, such as observed for mGSTA4-4 and several of the rat liver cytochrome P450s [35,36]. Ongoing work in our laboratory is directed towards the confirmation of the aforementioned hypothesis, as well to verify the precise mitochondrial membrane localization of the GST forms [4].

Our finding of the presence of the hGSTP1 subunit in human liver mitochondria is especially intriguing. In addition to detoxifying certain chemical carcinogens and anti-tumor agents, hGSTP1-1 also protects against oxidative damage by inactivating endogenous α,β-unsaturated aldehydes, base propenals and hydroperoxides formed as secondary metabolites during oxidative stress [2]. Interestingly, hGSTP1-1 is not appreciably expressed in human hepatocytes, but is present in biliary epithelium [37]. Accordingly, the significant cross-reactivity of hGSTP1-1 in cytosolic proteins in our Western blotting studies likely originated from the biliary epithelial cells and possibly other liver cell types than hepatocytes. To the best of our knowledge, GST pi expression has not been reported in the mitochondria of cells from other species. The *hGSTP1* gene is polymorphic, with allelic variants of hGSTP1-1 differing in their primary structures by the amino acids in positions 104 (isoleucine or valine) and/or 114 (alanine or valine) [38,39]. These substitutions result in polymorphic forms of the hGSTP1-1 enzyme with altered affinity for substrates, as well as reduced enzymatic activity [38]. Based upon molecular mass information, the only allelic variant detected in the present study was wild type for both amino acids (e.g. 105 isoleucine/114 alanine, see Table 6 peptide sequences for hGSTP1). The expression of hGSTP1-1 is of particular interest due to recent associations among the presence of *hGSTP1* allelic variants and susceptibility to liver disease such as cirrhosis [40], and also secondary liver disease in individuals with cystic fibrosis [39] and hereditary hemochromatosis [42]. As an example, the *hGSTP1-Ile*(105)/*Ile*(105) genotype has been associated with a eight-fold increase in the risk of liver disease in young patients with cystic fibrosis as compared to other *hGSTP1* genotypes [41]. In addition, the *hGSTP1 Val/Val* genotype is more common in hereditary hemochromatosis patients who also have cirrhosis [42]. Both of the aforementioned liver diseases have an etiology in which oxidative stress is an important contributor to disease onset. Collectively, these data suggest that the expression and catalytic activity of mitochondrial hGSTP1-1 may be a determinant of protection against oxidative stress in this organelle.

An important aspect of GST-mediated cellular defense is the fact that several GST isoforms are inducible by exposure to xenobiotics and under conditions of oxidative stress. Although not investigated here, others have demonstrated a redistribution of cytosolic and mitochondrial GST pools in simian fibroblast (COS) cells under oxidative stress, with increased levels of mGSTA4-4 occurring in the mitochondria on exposure to 4HNE and H_2O_2 [8]. The data generated by our laboratory demonstrating the presence of multiple GSTs with catalytic activity towards products of oxidative stress in human liver mitochondria provides a rationale for investigation of modulation of mitochondrial GST expression during oxidative stress.

In summary, our results indicate that several GSTs with metabolic activity toward secondary products of oxidative injury, including 4HNE and cumene hydroperoxide, are expressed in human liver mitochondria. The mitochondrial targeting of these GSTs are suggestive of a role in the metabolism of lipid peroxidation products generated in the mitochondrial respiration-linked electron transport chain, or GST-modulation of intracellular signaling through 4HNE conjugation. However, it should be noted that the ability of human liver mitochondrial GST to metabolize oxidative substrates in vitro does not necessarily predict the function of these isoforms in the intact cell or in vivo. Of particular interest in future studies will be determining if mitochondrial GSTs modulate cell signaling or bind endogenous lipophilic mitochondrial breakdown products and other GST substrates.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01-ES09427). The technical assistance of Ms. Helene' LaVire, Dr. Nancy Szabo, Carolyn Diaz, Dr. Stan Stevens and Dr. Nancy Denslow is greatly appreciated.

REFERENCES

[1] Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol 2005;45:51–88.

[2] Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. CRC Crit Rev Biochem Mol Biol 1996;30: 445–600.

[3] Harris JM, Meyer DJ, Coles B, Ketterer B. A novel glutathione transferase (13-13) isolated from the matrix of rat liver mitochondria having structural similarity to class theta enzymes. Biochem J 1991;278(Pt 1):137–41.

[4] Addya S, Mullick J, Fang JK, Avadhani NG. Purification and characterization of a hepatic mitochondrial glutathione S-transferase exhibiting immunochemical relationship to the alpha-class of cytosolic isoenzymes. Arch Biochem Biophys 1994;310(1):82–8.

[5] Bhagwat SV, Vijayasarathy C, Raza H, Mullick J, Avadhani NG. Preferential effects of nicotine and 4-(*N*-methyl-*N*-nitrosamine)-1-(3-pyridyl)-1-butanone on mitochondrial glutathione S-transferase A4-4 induction and increased oxidative stress in the rat brain. Biochem Pharmacol 1998;56(7):831–9.

[6] Kraus P, Wigand J, Ostermaier R. Mitochondrial glutathione transferases. The alkylation of mitochondrial membrane yields a catalyst with glutathione-transferase-like properties. Biol Chem Hoppe Seyler 1986;367(9):937–41.

[7] Jowsey IR, Thomson RE, Orton TC, Elcombe CR, Hayes JD. Biochemical and genetic characterization of a murine class kappa glutathione S-transferase. Biochem J 2003;373(Pt 2):559–69.

[8] Raza H, Robin MA, Fang JK, Avadhani NG. Multiple isoforms of mitochondrial glutathione S-transferases and their differential induction under oxidative stress. Biochem J 2002;366(Pt 1):45–55.

[9] Robinson A, Huttley GA, Booth HS, Board PG. Modelling and bioinformatics studies of the human kappa-class glutathione transferase predict a novel third glutathione transferase family with similarity to prokaryotic 2-hydroxychromene-2-carboxylate isomerases. Biochem J 2004;379(Pt 3):541–52.

[10] Gardner JL, Gallagher EP. Development of a peptide antibody against human glutathione S-transferase A4 (hGSTA4-4) reveals preferential localization in hepatic mitochondria. Arch Biochem Biophys 2001;390:19–27.

[11] Kirichenko A, Maria LL, Moriandi T, Holian A. 4-Hydroxynonenal-protein adducts and apoptosis in murine lung cells after acute ozone exposure. Toxicol Appl Pharmacol 1996;141:416–24.

[12] Markesbery WR, Lovell MA. Four hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. Neurobiol Ageing 1998;19:33–6.

[13] Chen Q, Cai H, Chen X. The relationship between 4-hydroxynonenal-derived epitopes on apolipoprotein B and coronary heart disease]. Chung Hua I Hsueh Tsa Chih 1995;75(10):581–3.

[14] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biology of 4-hydroxynonenal, malondialdehyde and related aldehydes. Free Radic Biol Med 1991;11:81–128.

[15] Cheng JZ, Singhal SS, Sharma A, Saini M, Yang Y, Awasthi S, et al. Transfection of mGSTA4 in HL-60 cells protects against 4-hydroxynonenal-induced apoptosis by inhibiting JNK-mediated signalling. Arch Biochem Biophys 2001;392(2):197–207.

[16] Cheng JZ, Singhal SS, Saini M, Singhal J, Piper JT, Van Kuijk FJ, et al. Effects of mGSTA4 transfection on 4-hydroxynonenal-mediated apoptosis and differentiation of K562 human erythroleukemia cells. Arch Biochem Biophys 1999;372(1):29–36.

[17] Gallagher EP, Gardner JL. Comparative expression of two alpha class glutathione S-transferases in human adult and prenatal liver tissues. Biochem Pharmacol 2002;63(11):2025–36.

[18] Hovius R, Lambrechts H, Nicolay K, de Kruijff B. Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the inner and outer membrane. Biochim Biophys Acta 1990;1021(2): 217–26.

[19] Statham CN, Szyjka SP, Menahan LA, Lech JJ. Fractionation and subcellular localization of marker enzymes in rainbow trout liver. Biochem Pharmacol 1977;26(15):1395–400.

[20] Doi AM, Pham RT, Hughes EM, Barber DS, Gallagher EP. Molecular cloning and characterization of a glutathione S-transferase from largemouth bass (*Micropterus salmoides*) liver that is involved in the detoxification of 4-hydroxynonenal. Biochem Pharmacol 2004;67(11): 2129–39.

[21] Ostlund Farrants AK, Meyer DJ, Coles B, Southan C, Aitken A, Ketterer B. The separation of glutathione S-transferase subunits using reversed phase high-performance liquid chromatography. Biochem J 1987;245:423–8.

[22] Guda C, Subramaniam S. TARGET: a new method for predicting protein subcellular localization in eukaryotes. Bioinformatics 2005;21(21):3963–9.

[23] Singhal SS, Zimniak P, Awasthi S, Piper JT, He NG, Teng JI, et al. Several closely related glutathione S-transferase isozymes catalyzing conjugation of 4-hydroxynonenal are differentially expressed in human tissues. Arch Biochem Biophys 1994;311(2):242–50.

[24] Gardner JL, Doi AM, Pham RT, Huisden CM, Gallagher EP. Ontogenic differences in human liver 4-hydroxynonenal detoxification are associated with in vitro injury to fetal hematopoietic stem cells. Toxicol Appl Pharmacol 2003;191(2):95–106.

[25] Gallagher EP, Stapleton PL, Slone DH, Schlenk D, Eaton DL. Channel catfish glutathione S-transferase isoenzyme activity toward (+)-anti-benzo[a]pyrene-trans-7,8-diol-9,10-epoxide. Aquat Toxicol 1996;34:135–50.

[26] Rowe JD, Nieves E, Listowsky I. Subunit diversity and tissue distribution of human glutathione S-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. Biochem J 1997;325(Pt 2):481–6.

[27] Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 2005;70(2):200–14.

[28] Chen JJ, Schenker S, Henderson GI. 4-Hydroxynonenal levels are enhanced in fetal liver mitochondria by in utero ethanol exposure. Hepatology 1997;25:142–7.

[29] Grune T, Siems WG, Zollner H, Esterbauer H. Metabolism of 4-hydroxynonenal, a cytotoxic lipid peroxidation product, in Ehrlich mouse ascites cells at different proliferation stages. Cancer Res 1994;54(19):5231–5.

[30] Coles BF, Morel F, Rauch C, Huber WW, Yang M, Teitel CH, et al. Effect of polymorphism in the human glutathione S-transferase A1 promoter on hepatic GSTA1 and GSTA2 expression. Pharmacogenetics 2001;11(8):663–9.

[31] Morel F, Rauch C, Coles B, Le Ferrec E, Guillouzo A. The human glutathione transferase alpha locus: genomic organization of the gene cluster and functional characterization of the genetic polymorphism in the hGSTA1 promoter. Pharmacogenetics 2002;12(4): 277–86.

[32] Zhao T, Singhal SS, Piper JT, Cheng J, Pandya U, Clark-Wronski J, et al. The role of human glutathione

S-transferases hGSTA1-1 and hGSTA2-2 in protection against oxidative tress. Arch Biochem Biophys 1999;367(2): 216–24.
[33] Morel F, Rauch C, Petit E, Piton A, Theret N, Coles B, et al. Gene and protein characterization of the human glutathione S-transferase kappa and evidence for a peroxisomal localization. J Biol Chem 2004;279(16): 16246–53.
[34] Robin MA, Prabu SK, Raza H, Anandatheerthavarada HK, Avadhani NG. Phosphorylation enhances mitochondrial targeting of GSTA4-4 through increased affinity for binding to cytoplasmic Hsp70. J Biol Chem 2003;278(21):18960–7.
[35] Anandatheerthavarada HK, Biswas G, Mullick J, Sepuri NB, Otvos L, Pain D, et al. Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at ser128. EMBO J 1999;18:5494–504.
[36] Robin MA, Anandatheerthavarada HK, Biswas G, Sepuri NB, Gordon DM, Pain D, et al. Bimodal targeting of microsomal CYP2E1 to mitochondria through activation of an N-terminal chimeric signal by cAMP-mediated phosphorylation. J Biol Chem 2002;277: 40583–9.
[37] Hayes PC, May L, Hayes JD, Harrison DJ. Glutathione S-transferases in human liver cancer. Gut 1991;32(12):1546–9.
[38] Ali-Osman F, Akande O, Antoun G, Mao JX, Buolamwini J. Molecular cloning, characterization, and expression in *Escherichia coli* of full-length cDNAs of three human glutathione S-transferase pi gene variants. J Biol Chem 1997;272(15):10004–12.
[39] Krajinovic M, Labuda D, Sinnett D. Glutathione S-transferase P1 genetic polymorphisms and susceptibility to childhood acute lymphoblastic leukaemia. Pharmacogenetics 2002;12(8):655–8.
[40] Ghobadloo SM, Yaghmaei B, Bakayev V, Goudarzi H, Noorinayer B, Rad FH, et al. GSTP1, GSTM1, and GSTT1 genetic polymorphisms in patients with cryptogenic liver cirrhosis. J Gastrointest Surg 2004;8(4):423–7.
[41] Henrion-Caude A, Flamant C, Roussey M, Housset C, Flahault A, Fryer AA, et al. Liver disease in pediatric patients with cystic fibrosis is associated with glutathione S-transferase P1 polymorphism. Hepatology 2002;36(4 Pt 1):913–7.
[42] Stickel F, Osterreicher CH, Datz C, Ferenci P, Wolfel M, Norgauer W, et al. Prediction of progression to cirrhosis by a glutathione S-transferase P1 polymorphism in subjects with hereditary hemochromatosis. Arch Intern Med 2005;165(16):1835–40.