Mu-Class GSTs Are Responsible for Aflatoxin B₁-8,9-Epoxide-Conjugating Activity in the Nonhuman Primate Macaca fascicularis Liver

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Mice are resistant to the carcinogenic effects of the mycotoxin aflatoxin B₁ (AFB₁) because they constitutively express an alpha-class glutathione S-transferase (mGSTA3-3) that has high (~200,000 pmol/min/mg) activity toward aflatoxin B₁-8,9-epoxide (AFBO). Rats do not constitutively express a GST with high AFBO-conjugating activity and are sensitive to AFB₁-induced hepatocarcinogenesis. Constitutively expressed human hepatic alpha-class GSTs (hGSTA1-1 and hGSTA2-2) possess little or no AFBO-detoxifying activity (<2 pmol/min/mg). Recently, we found that the nonhuman primate, Macaca fascicularis (Mf), exhibits significant (~300 pmol/min/mg) constitutive hepatic GST activity towards AFBO. To determine which specific GST isoenzyme(s) is (are) responsible for this activity, Mf GSTs were purified from liver tissue and characterized and, Mf mu-class GST cDNAs were cloned by reverse transcriptase-coupled polymerase chain reaction (RT-PCR). Purification by glutathione agarose (GSHA) affinity chromatography yielded a protein, GSHA-GST, that exhibited relatively high AFBO-conjugating activity (239 pmol/min/mg) compared to other GST-containing peaks. Western blotting and enzymatic activity analyses revealed that GSHA-GST belongs to the mu class. Two distinct mu-class GST cDNAs, mfaGSTM1 (GenBank accession # AF200709) and mfaGSTM2 (GenBank accession # AF200710), were generated by RT-PCR. CDNA-derived amino acid sequence analysis revealed that mfaGSTM1 and mfaGSTM2 share 97% and 96% homology with the human mu-class GSTs hGSTM4 and hGSTM2, respectively. In contrast to recombinant mfaGSTM1-1, which had no detectable AFBO-conjugating activity, mfaGSTM2-2 exhibited this activity at 333 pmol/min/mg. Activity profiles for the stereoisomers exo- and endo-AFBO, and of 1-chloro-2,4-dinitrobenzene of the purified protein GSHA-GST and recombinant mfaGSTM2-2, suggested that they are two distinct enzymes. Our results indicate that, in contrast to rodents, mu-class GSTs are responsible for the majority of AFBO-conjugating activity in the liver of Macaca fascicularis.

Key Words: aflatoxin; glutathione S-transferase; nonhuman primate; biotransformation; cDNA; mu-class; liver.

AFB₁, requires activation by cytochromes P450 (CYP) to form AFB₁-8,9 epoxy (AFBO), the ultimate carcinogen that binds to DNA (reviewed in Eaton and Gallagher, 1994). CYP-mediated oxidation of AFB₁ to AFBO can produce two stereoisomers, exo- and endo-AFBO (Raney et al., 1992a,b), but only exo-AFBO binds to DNA at the N⁷ position of guanine (Iyer et al., 1994; Johnson and Guengerich, 1997). The metabolic activation of AFB₁ by human or rat microsomes produces a mixture of endo- and exo-epoxides, although human microsomes generate predominantly exo-epoxide in vitro at relatively high AFB₁ concentrations (Raney et al., 1992a,b).

Remarkable species differences in susceptibility to AFB₁-induced liver cancer have been demonstrated (Eaton and Gallagher, 1994; Monroe and Eaton, 1988; Ramsdell and Eaton, 1990). Rats are very sensitive, whereas mice are highly resistant, to the hepatocarcinogenic effects of AFB₁ (Wogan and Payne, 1967), even though mouse liver microsomes form exo-AFBO at a rate slightly higher than rat liver microsomes (Monroe and Eaton, 1987). The resistance of mice is due largely, if not exclusively, to the constitutive expression of an alpha-class GST isozyme (mGSTA3-3) that has extraordinarily high conjugating activity (~200,000 pmol/min/mg) toward the reactive intermediate AFBO (Buettler and Eaton, 1992; Hayes et al., 1992). In contrast, rats do not constitutively express a GST isoform with high AFBO-conjugating activity and are thus sensitive to AFB₁-induced hepatocarcinogenesis.

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However, an inducible, \textit{alpha}-class GST enzyme (rGSTA5-5) with high AFBO conjugating activity (~50,000 pmol/min/mg) can confer resistance to AFB1-induced hepatocarcinogenesis in rats when treated with certain chemicals such as oltipraz, ethoxyquin, and butylated hydroxyanisole (Hayes et al., 1991, 1994).

In contrast to rodents, the cytosolic fraction from human liver possesses little or no detectable AFBO-conjugating activity (Eaton and Gallagher, 1994; Eaton and Ramsdell, 1992; Moss and Neal, 1985; Slone et al., 1995). In addition, purified recombinant human \textit{alpha}-class GSTs hGSTA1-1 and hGSTA2-2 also lack significant activity toward AFBO (Buetler et al., 1996; Johnson et al., 1997; Raney et al., 1992b). However, human hepatic \textit{mu}-class GSTs M1a-1a and M2-2 exhibit measurable activity toward AFBO. This activity is directed nearly exclusively toward the \textit{endo}-AFBO stereoisomer (Raney et al., 1992b).

Recent studies from this laboratory found that liver cytosol from the nonhuman primate \textit{Macaca fascicularis} has significant constitutive hepatic cytosolic GST activity toward the exo-AFBO stereoisomer, although this activity is approximately 100-fold lower than that seen in mouse liver (Eaton and Ramsdell, 1992). It is not known which specific GST(s) is (are) responsible for this activity. The objective of this study was to identify the GST(s) in \textit{Macaca fascicularis} liver that is (are) responsible for this AFBO detoxification activity.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Aflatoxin B1 (AFB1), reduced glutathione (GSH), NADPH, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ECA), S-hexylglutathione (SHG), glutathione agarose (GSHA), and S-hexylglutathione agaroose (SHGA) were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC solvents were of analytical grade and were obtained from J. T. Baker, Inc. (Phillipsburg, NJ). Nytran-supported nylon transfer and immobilization membranes were obtained from Schleicher & Schuell (Keene, NH). Oligomers were synthesized by Oligos Etc., Inc. (Wilsonville, OR). All other reagents were of analytical reagent grade and were obtained from various commercial sources. The plasmid pBlueprint II SK(+) and PET 17b were purchased from Stratagene (La Jolla, CA) and Novagen (Madison, WI), respectively.

Animals and tissues. \textit{Macaca fascicularis} liver tissues were obtained from castrated adult males through the tissue acquisition program of the Regional Primate Research Center at the University of Washington. The livers were removed immediately following terminal anesthesia and exsanguination, and were rinsed in ice-cold saline, snap-frozen in liquid nitrogen, and stored at −80°C until homogenization. Hepatic cytosolic fractions were prepared and stored according to methods described previously (Gallagher et al., 1991; Ramsdell and Eaton, 1988).

Affinity purification of hepatic GSTs from \textit{Macaca fascicularis}. Hepatic GST proteins were purified, using a previously described method with the following modifications (Gallagher et al., 1996). Liver cytosols (30 ml, 38 mg/ml) were loaded onto an S-hexylglutathione agarose (SHGA) affinity column which was connected to a glutathione agarose (GSHA) affinity column such that the flow-through (FT) from the SHGA column passed directly to the GSHA column. Prior to sample loading, both columns were equilibrated with buffer A [10 mM Tris, pH 7.8; 200 mM NaCl; 1 mM EDTA; 0.5 mM dithiothreitol (DTT)] at 4°C. An initial 100 ml of eluate from the GSHA column was collected to determine the non-retained enzymatic activities. Both SHGA and GSHA columns were washed overnight with >400 ml of buffer A (more than 20 times column volume), and were disconnected before being eluted separately. The GSTs bound to the SHGA column were eluted in buffer A containing 5 mM S-hexylglutathione and 2.5 mM glutathione. The GSTs bound to the GSHA column were eluted with 200 mM Tris, pH 9.0, containing 50 mM GSH. GSTs eluted from the SHGA and the GSHA columns were dialyzed separately in 2 liters of buffer B (25 mM sodium phosphate, pH 7.4; 0.5 mM DTT), changed 4× over 48 h. All of the above procedures were carried out at 4°C. After dialysis, GST samples were aliquoted and stored at −80°C.

**GST subunit purification.** Both SHGA and GSHA column eluates were further resolved by reverse-phase HPLC to examine GST subunit composition as described by Rowe et al. (1997). An Alttech Microsphere 300 C4, 4.6 mm × 15 mm, 5 μm bore column was used with a Rainin HPLC LD-200 pumps controlled by a Rainin Dynamax HPLC method manager. Solvent A consisted of water containing 0.08% trifluoroacetic acid (TFA). Solvent B consisted of acetonitrile containing 0.1% TFA. The gradient conditions were as follows. Injection started at 20% solvent B. At 10 min, solvent B was increased to 40%, held at 40% for 10 min, and then over a period of 40 min was increased to 60%. The flow rate was 1 ml/min. Protein peaks were monitored for absorbance at 214 nm using a Waters LC 480 spectrophotometer. HPLC fractions were collected manually and dried in a Speed-Vac. Dried samples were re-suspended in water immediately prior to SDS/PAGE and Western blotting.

**Chromatofocusing of hepatic GSTs from \textit{Macaca fascicularis}.** As multiple GSTs were present in the SHGA affinity fraction, further GST protein purification was done by chromatofocusing, as described previously with certain modifications (Gallagher et al., 1996; Ramsdell and Eaton, 1990). Aliquots of the affinity-purified GSTs from the SHGA columns were equilibrated in 25 mM triethylamine (TEA), and then applied to a Mono-P chromatofocusing column (Pharmacia–LKB, Piscataway, NJ) previously equilibrated with 25 mM TEA, pH 10. The column was eluted using a mixture of low molecular weight buffers adapted from a focusing buffer described previously (Ramsdell and Eaton, 1990). It was found that a pH range from 8 to 10 provided optimal resolution of \textit{alpha} and \textit{mu} class GSTs. The pH gradient was generated with the chromatofocusing buffer groups as follows: 4 min with 25 mM TEA, pH 10, 60 min with a group of buffers consisting of a mixture of low-molecular-weight organic chemicals followed with 30 min of 1 N NaCl. The absorbance of the effluent was monitored at 280 nm and the CDNB activity of the fractions was determined using a standard method (Habig and Jakoby, 1981) adapted for 96-well micro-plate reader (Molecular Devices Corp., Menlo Park, CA) in the kinetic mode at a wavelength of 340 nm. Individual peaks were collected and were further evaluated for AFBO-conjugating activity by HPLC as previously described (Monroe and Eaton, 1987). All collected peaks were further analyzed by SDS–PAGE and Western blots.

**SDS–PAGE and western blotting.** Hepatic cytosolic fractions, SHGA, and GSHA affinity purified GSTs, as well as fractions purified by reverse-phase HPLC and chromatofocusing, were resolved on SDS–polyacrylamide gel (16% acrylamide: 0.09% BN-bis acrylamide) and gels were either stained with Coomassie brilliant blue or transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA) for Western blot analyses. Non-specific binding was blocked with 3% (w/v) nonfat-milk powder dissolved in phosphate buffered saline (PBS), pH 7.4. Primary antibodies were incubated in 1% (w/v) nonfat milk for 1 h in PBS containing 0.1% Tween 20. Blots were then incubated with a goat anti-rabbit alkaline phosphatase-conjugated antibody (Bio-Rad, Richmond, CA) for 30 min. Blots were developed using the chemiluminescent substrate CSPD (Tropix, Bedford, MA) according to the manufacturer’s recommendations.

**RNA isolation.** Frozen liver tissue (0.1 g) was homogenized in 2 ml Trizol reagent (GIBCO BRL, Gaithersburg, MD) with a Polytron tissue mixer. Homogenates were centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was precipitated with isopropanol and collected by centrifugation at 12,000 × g for 10 min. RNA pellets were washed with 75% ethanol, air dried, and re-suspended in 50 μl of Formazol (GIBCO BRL, Gaithersburg, MD), and
stored at –80°C. RNAs were precipitated with 2.5 volumes of isopropanol and washed with 75% ethanol, air dried, and resuspended in water prior to RT-PCR reaction.

**Prime design and complementary DNA cloning strategy.** All human mu-class GST cDNAs (M1–M5) were aligned using the Clustal software sequence-alignment program. Two sets of primers were designed based on the multiple sequence alignment of human mu-class GST cDNAs at certain conserved regions covering all coding sequences. The primers were synthesized by Oligos Etc. Inc. (Wilsonville, OR). The 5′ forward primers were identical for both sets of primers. Primer 1 (5′ forward): gcaccaacagcatctgcaatgct. Primer 2 (3′ reverse): gtaagctgtcatgacggaag. Primer 3 (3′ reverse): acagctccgggtgagttca. In primer 1, a Ndel (catatg) restriction site was introduced to allow the subsequent PCR products to be cloned directly into the expression vector, pET 17b. Primers 2 and 3 were designed so that they contained a Spe I and Sac I sites respectively to facilitate subsequent cloning of the resulting PCR products.

Initially, cDNAs were synthesized using 50 units of Moloney murine leukemia virus (MMLV) reverse transcriptase, 1 μg total RNA template, 20 units RNase inhibitor (Stratagene, La Jolla, CA), 1 mM dNTP, 1 μg oligo d(T)20 primer and a buffer containing 50 mM Tris–HCl, pH 8.3; 75 mM KCl, and 3 mM MgCl2. The RNA was heated to 65°C for 10 min and immediately cooled on ice before MMLV-RT was added into the reaction mixture. The reverse transcription reaction was performed with a thermal cycler (DNA Engine, MJ Research, Inc., Watertown, MA), using the following temperature profile: (1) 37°C for 60 min; (2) 99°C for 5 min; (3) 5°C for 5 min. Five μl of RT mix was used for the PCR reaction in the presence of either primers 1 and 2 or primers 1 and 3 (1 μM each) in a 100 μl volume containing 1 × Pfu buffer, 200 μM of dNTP, 2.5 units of cloned Pfu polymerase (Stratagene, La Jolla, CA). PCR reactions were done with the same thermal cycler using the following temperature profile: (1) 94°C for 30 s; (2) 55°C (primers 1 and 2) or 60°C (primers 1 and 3) for 15 s; (3) 72°C for 2 min; with 30 cycles from (1) to (3); and (4) 72°C for 10 min.

**Isolation of Macaca fascicularis mu-class GST cDNA clones.** To ensure an adequate amount of cDNAs for the subsequent cDNA cloning and to ensure representative amplification of all mRNAs, 3 rounds of independent PCR reactions were completed for each set of primers. All cDNAs were pooled from 3 rounds of reactions for each set of primers, respectively. PCR amplified cDNAs were resolved on 1% agarose gel containing ethidium bromide and expected bands (~1 kb) were cut from the gel and extracted, using the Qiagen II gel extraction kit (Qiagen, Valencia, CA). The cDNAs synthesized with primers 1 and 2 were cloned into the pET17b, digested with both Nde I and Spe I restriction enzymes. The pET 17b vectors containing the GST cDNA inserts were then transfected into E.coli BL21 for GST recombinant protein expression.

cDNAs synthesized using primers 1 and 3 were initially cloned into pBluescript II SK(–) (Stratagene, La Jolla, CA) digested with Sna I (Stratagene), producing blunt ends. The plasmids were prepared on the white colonies and the vectors hosting cDNA inserts were confirmed by DNA sequencing as well as digestions with both Nde I and Sna I restriction enzymes. The pET 17b vectors containing the GST cDNA inserts were then transfected into E.coli BL21 for GST recombinant protein expression.

**Sequencing.** cDNA clones were sequenced using T3, T7, and nested primers on both strands. Sequencing reactions were done on a thermal cycler using 0.5 μl of plasmid DNA and 8 μl of dye-terminator mix purchased from Perkin-Elmer (Foster City, CA). Sequences were obtained using an ABI377 automatic sequencer in the Biomarker Lab of the Center for Ecogenetics and Environmental Health at the University of Washington. All sequences were analyzed with DNAide (Laboratoire de Biochimie, 91128 Palaiseau cedex, France) and DNA Strider (Service de Biochimie, Cedex, France) software and compared to the human mu-class GST cDNA sequences M1 through M5.

**Northern blotting.** Twenty μg of hepatic RNA samples were separated on a 1.25% agarose/formaldehyde gel, blotted onto Nytran membranes, and hybridized with 32P-end-labeled oligonucleotide or cDNA probes labeled by random priming. The mfaGSTM1-specific oligo (ggagaactaataactgtaggat) and mfaGSTM2-specific oligo (actgttgctctcaaggg) were synthesized by Oligos Etc. Inc. (Wilsonville, OR). The specificity of mfaGSTM1-specific and mfaGSTM2-specific oligos was examined both by Northern blotting for 4 human mu-class cDNA expressed mRNAs (M1, M2, M3 and M4) and Southern blotting for 5 different primate cDNAs (hGSTM1, hGSTM3, hGSTM4, mfaGSTM1 [Genbank accession # AF200709] and mfaGSTM2 [Genbank accession # AF200710]). Northern blots were hybridized in QuickHyb (Stratagene) solution for 1 h at 68°C with cDNA probes or at 6°C below the Tm of the oligonucleotide probes. After hybridization, blots probed with cDNA probes (mfaGSTA1 and mfaGSTM2) were washed twice at room temperature with 2× SSC buffer and 0.1% (w/v) SDS wash solution followed by a high stringency wash at 60°C with a 0.1× SSC buffer and 0.1% (w/v) SDS solution for 30 min in a hybridization oven (Robbins Scientific Corp., Sunnyvale, CA). Blots probed with oligonucleotides (mfaGSTM1- and mfaGSTM2- specific probes) were washed 2 times with 2× SSC buffer and 0.1% SDS for 20 min at 42°C. Followed by an additional wash with the same buffer at 45°C. Autoradiography utilized Kodak X-OMAT AR film.

**Mass spectrometry analysis.** Mass spectra of GST subunits were acquired using a delayed extraction matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Voyager DE, Perseptive Biosystems, Inc., Framingham, MA) at the Department of Biochemistry of the University of Washington. Protein samples were dissolved in 40% (v/v) acetonitrile containing 0.1% trifluoroacetic acid (TFA), and 1 μl of solution was deposited on MALDI plate along with 1 μl of sinapinic acid matrix, and were air dried and analyzed.

**Enzymatic assays.** AFBO-conjugating activity was determined using a method described previously (van Ness et al., 1998), in which mouse microsomes were used to generate a racemic mixture of endo and exo AFBO with an initial AFB1 substrate concentration of 128 μM. This concentration of AFB1 has been found to provide adequate substrate to the microsomal P450 system used to generate the AFBO substrate for the GST assay, such that production of AFBO is not rate-limiting under the conditions of the assay (Monroe and Eaton, 1987). AFBO endo- and exo-activities were determined using the method by Raney et al. (1992a), with the following modifications: (1) mouse microsomes were used to generate AFBO with an initial AFB1, substrate concentration of 128 μM and final GSH concentration of 5 mM. (2) 1 mM NADPH was used instead of using an NADPH regenerating system. (3) The gradient was changed, as follows: times 0, 90% Solvent A, 10% Solvent B; 45 min, 20% Solvent B; 47 min, 50% Solvent B; and 56 min, 100% Solvent B. Solvent A was 20 mM ammonium acetate, pH 4.0; solvent B was 1:1 acetonitrile:methanol. A 250 × 4.6 mm Econosphere C18 column, heated to 40°C, and a flow rate of 2.0 ml/min was used. Base line resolution of the exo and endo AFBO-GSH conjugates were obtained with these conditions. Both UV (365 nm) and fluorescence (excitation 365, emission 425 nm) detection were used for all assays.

With this AFBO generating system, it is difficult to know what is the actual concentration of substrate (AFBO) is because of the very rapid hydrolysis of AFBO in an aqueous environment. However, each assay is repeated with at least 2 different dilutions of GST enzyme to ensure that substrate (AFBO) availability is not rate limiting. If the apparent specific activity of the GST toward endo- or exo-AFBO is significantly higher in the more dilute enzyme solution, the assay is repeated at a lower enzyme concentration. Because mouse liver microsomes form the exo isomer predominately, it is technically difficult to accurately determine AFBO-conjugating activity of a GST which predominantly conjugates the endo isomer. However, because the endo isomer is approximately 15 times more fluorescent than the exo isomer, fluorescence detection provides adequate sensitivity to measure even relatively low levels of GST activity toward the endo-AFBO. All AFBO-conjugating-activity data were repeated at least 4 times under identical conditions, and, therefore, provide reliable estimates of relative AFBO-conjugating activity of these GSTs. Specific activities for GST conjugating activity of both endo and exo
AFBO were determined in triplicate, and ratios of the average of the 3 determinations were used to express the relative enantioselectivity of the GST. Other general enzymatic glutathione S-transferase activities were assayed using 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and ethacrynic acid (ECA) as substrates, according to standard procedures (Habig and Jakoby, 1981).

Biohazards and safety precautions. All primate tissues were handled according to University of Washington biohazard safety protocols for primate tissues. Wastes were treated with bleach prior to disposal. Aflatoxin-contaminated wastes and glassware were also treated with bleach, according to established procedures.

RESULTS

GST Affinity Purification and Activities Recovery

Table 1 shows the AFBO and CDNB conjugating activities, distribution and recovery from affinity columns. There was 58 pmol/min/mg of AFBO conjugating activity in GSTs eluted from the SHGA column, whereas GSTs eluted from the GSHA column had approximately 4 times this activity (239 pmol/min/mg). GST protein was monitored and selected at OD280. GSTs retained on the SHGA column accounted for 26% of total cytosolic AFBO conjugating activity, whereas GSTs retained on the GSHA column accounted for almost 40% of total AFBO conjugating activity. However, GSTs eluted from the SHGA column accounted for 76% of CDNB activity, whereas GSTs eluted from the GSHA column contained only 11% of total CDNB activity (Table 1). AFBO-conjugating activity not retained on either affinity column (initial flow-through; FT) was just above the detection limit of AFBO-conjugating activity (3.3 pmol/min/mg). However, because of the relatively large volume of FT, this accounted for 23% of total cytosolic AFBO-conjugating activity loaded onto the columns. This is likely an over-estimate, as the measured AFBO conjugating activity was close to the detection limit (2 pmol/min/mg), and the FT fraction contained only 1% of total CDNB activity. Overall, there was an 88% recovery in both AFBO and CDNB activities. Interestingly, the AFBO conjugating activity seen in all fractions, including the cytosolic GSTs and the GSTs purified from the SHGA and GSHA columns, was almost exclusively (>95%) directed toward the exo-AFBO stereoisomer (Table 1).

Subunit Analysis by HPLC

GST subunit analysis by HPLC was completed for GSTs eluted from the SHGA (Fig. 1A) and GSHA (Fig. 1B). Four different peaks (P1–P4) were identified in the GST fraction eluted from the SHGA column, whereas only a single GST subunit peak was found in the eluate from the GSHA column. Therefore, only the SHGA eluate was further subjected to chromatofocusing.

Chromatofocusing

GSTs eluted from the SHGA column were further purified by chromatofocusing with a Mono P column. Figure 2 shows

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**TABLE 1**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Immunoreactivities</th>
<th>AFBO* pmol/min/mg</th>
<th>Exo b%</th>
<th>Total AFBO pmol/min (%)</th>
<th>CDNB** μmol/min/mg</th>
<th>Total CDNB μmol/min (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>alpha/mu</td>
<td>6.7 ± 1.2</td>
<td>&gt;95</td>
<td>7638</td>
<td>2.0 ± 0.03</td>
<td>2220</td>
</tr>
<tr>
<td>SHGA</td>
<td>alpha/mu</td>
<td>57.8 ± 6</td>
<td>&gt;95</td>
<td>1998 (26)</td>
<td>60.3 ± 3.9</td>
<td>1668 (76)</td>
</tr>
<tr>
<td>GSHA</td>
<td>mu</td>
<td>239 ± 12</td>
<td>&gt;95</td>
<td>2940 (39)</td>
<td>20.7 ± 1.8</td>
<td>249 (11)</td>
</tr>
<tr>
<td>FT</td>
<td>–</td>
<td>3.3 ± 1.0</td>
<td>&gt;95</td>
<td>1749 (23)</td>
<td>0.05 ± 0.03</td>
<td>26 (1)</td>
</tr>
</tbody>
</table>

Note. SHGA: GSTs eluted from the SHGA column; GSHA: GST eluted from the GSHA column; FT: Flow-through fraction.

*Western blotting results showing immunoreactivities of purified fraction with polyclonal antibodies against rat alpha- or mu-class GSTs.

**AFBO conjugating activity was measured using mouse liver microsome-generated AFBO at an initial substrate concentration of 128 μM of AFBO. Each value represents the mean and SE of 3 independent determinations.

**CDNB was measured using an assay described by Habig and Jakoby (1981). Each value represents the mean and SE of 3–4 independent determinations.

AFBO were determined in triplicate, and ratios of the average of the 3 determinations were used to express the relative enantioselectivity of the GST. Other general enzymatic glutathione S-transferase activities were assayed using 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and ethacrynic acid (ECA) as substrates, according to standard procedures (Habig and Jakoby, 1981).

FIG. 1. Affinity purification of Mf hepatic GSTs. (A) GSTs eluted from the SHGA column were further purified by reverse-phase HPLC using a C4 column to determine subunit composition. (B) GST eluted from the GSHA column was further purified by reverse-phase HPLC using a C4 column. GST protein peaks were monitored at OD 214 nm.
the pH gradient curve from pH 10 to 8.4 (A) and isofocusing chromatogram (B). Five peaks were observed at OD 280 nm, two major peaks (P1 and P3) and three minor peaks (P2, P4, and P5). Peak 3 was split up in 2 fractions named P3-1 and P3-2, whereas only one fraction was collected from each of the other peaks (P1, P2, P4, and P5). To remove any tightly bound protein, the column was eluted with a high salt buffer, resulting in one peak at 78 min. P3-1 and P3-2 shared a pI of 9.4 and had highest AFBO-conjugating activity (Fig. 3) among all fractions collected. The GST fraction eluted from the GSHA column was not subject to chromatofocusing as only a single GST peak was identified by reverse-phase HPLC, and Western blotting analyses revealed that it had immunoreactivity exclusively against a mu-class GST polyclonal antibody (see below).

**SDS–PAGE and Immunoreactivities of Macaca fascicularis Hepatic GSTs Purified by Chromatography and Chromatofocusing**

Figure 3 shows SDS–PAGE followed by Coomassie brilliant blue staining of affinity purified GSTs and the fractions collected from the chromatofocusing column, along with two recombinant primate GSTs, mfaGSTM1 (see below) and hGSTA1 (panel A). Immunoreactivities of each protein toward antibodies selective for alpha, mu, or pi class GSTs are shown in panel B. AFBO-conjugating activity for each peak is included in panel C. Only a single 25.6 kD band was observed from the GSHA affinity-purified fraction, which cross-reacted only with anti-mu-class antibody. However, there were 2 protein bands from the GSTs purified by the SHGA affinity column (lower band, 25.3 kD; top band, 25.6 kD). The top band reacted with mu-class-specific antibodies, whereas the lower band reacted with alpha-class-specific antibodies, based on Western blotting analyses. There were no peaks that reacted with pi class antibodies.

![Fig. 2. Chromatofocusing of affinity-purified Mf hepatic GSTs. (A) pH gradient curve (from pH 10 to 8). (B) Chromatofocusing of GSTs eluted from the SHGA column by Mono P column. GST protein peaks were monitored at OD 280 nm.](image)

![Fig. 3. Characteristics of purified Mf hepatic GSTs. (A) SDS–PAGE and Coomassie brilliant blue staining of affinity purified GSTs (eluates from SHGA and GSHA columns) and fractions collected from chromatofocusing column (P1, P2, P3-1, P3-2, P4, and P5), along with 2 primate cDNA-expressed GSTs (mfaGSTM1-1 and hGSTA1-1). (B) Immunoreactivities against 3 different classes of GSTs such as alpha (A), mu (M), and pi (P). (C) AFBO-conjugating activities of each GST as labeled above the top panel. GSHA, GST eluted from the GSHA column; SHGA, GSTs eluted from the SHGA column; P1, P2, P3-1, P3-2, P4 and P5 are peaks collected from Mono P column; mfaM1, recombinant mfaGSTM1-1; hA1, recombinant hGSTA1-1.](image)
Only a single band was seen with both P1 and P2 fractions purified by the chromatofocusing column, and it cross-reacted only with anti-alpha-class antibody. These bands had the same mobility as the lower band of the SHGA-column purified GSTs. The P1 and P2 proteins had no GST activity toward AFBO. Both fractions of P3 from the chromatofocusing column contained 2 different protein bands. Western-blot analysis revealed that the top band was a mu-class protein while the lower band belonged to the alpha-class GST family. AFBO-conjugating activity appeared on inspection to be correlated with the intensity of the mu-class band. Similarly, there were 2 different bands on the P4 and P5 peaks eluted from the chromatofocusing column; the upper band was a mu-class GST and the lower band was an alpha-class GST, based on Western-blotting analyses. Substantial AFBO-conjugating activity was also associated with fractions P4 and P5, which contained both alpha- and mu-class immunoreactive proteins.

Complementary DNA Cloning and Sequencing

Eight positive clones were obtained with the first set of primers, and each had an insert of 918 base pairs. Restriction analysis revealed that these clones shared identical patterns (data not shown). DNA sequencing using T3, T7, and nested primers indicated all 8 were identical clones. This clone, referred to as mfaGSTM1, based on the nomenclature recommendations of Mannervik et al. (1992), had an open reading frame of 654 base pairs that codes for 218 amino acids (Fig. 4). A comparison of the cDNA-derived amino acid sequence with human mu-class GSTs indicated that mfaGSTM1 shares 97% homology with hGSTM4 (Table 2 and Fig. 4). Based on cDNA-derived protein sequences, mfaGSTM1 shares 85%, 84%, 69% and 84% homology with human mu-class GSTs hGSTM1a, hGSTM2, hGSTM3 and hGSTM5 respectively (GenBank accession # for mfaGSTM1 is: AF200709) (Table 2).

cDNAs generated with a second set of primers produced four positive clones. DNA sequencing indicated that all four clones were identical, yet different from mfaGSTM1. This clone, named mfaGSTM2, has a 989-base pair insert with 654 base pairs of open reading frame that also codes for 218 amino acids (Fig. 4). Sequence analysis suggests that mfaGSTM2 is a homolog of hGSTM2 (Table 2 and Fig. 4), as it shares 96% amino acid sequence identity with hGSTM2 (Table 2). In addition, cDNA-derived amino acid sequence comparison indicated that mfaGSTM2 shares 86%, 68%, 86%, and 84% similarities with human mu-class GSTs hGSTM1a, hGSTM3, hGSTM4 and hGSTM5 (Table 2). mfaGSTM2 shares 86% amino acid sequence identity with mfaGSTM1.

Characteristics of Recombinant Macaca fascicularis mu-Class GSTs

MfaGSTM1-1 and mfaGSTM2-2 were expressed in bacteria and purified by glutathione affinity chromatography (McHugh et al., 1996). Table 3 shows activity characterization of these two recombinant GST proteins. mfaGSTM1-1 had no AFBO conjugating activity, although it had detectable conjugating activity toward CDNB (0.34 µmol/min/mg) as well as some activity toward ECA (0.1 µmol/min/mg). In contrast, mfaGSTM2-2 had substantial conjugating activity toward AFBO (333 pmol/min/mg). However, in contrast to what was observed for affinity-purified GSTs from Macaca fascicularis liver, this activity was directed almost exclusively toward the endo-AFBO stereoisomer. mfaGSTM2-2 had somewhat lower CDNB (207 µmol/min/mg) but higher DCNB-conjugating activity (4.9 µmol/min/mg) than its human homologue hGSTM2-2 (295 µmol/min/mg and 2 µmol/min/mg, respectively) (Table 3).

Messenger RNA Expression in the Macaca fascicularis Liver and Other Tissues

To assess expression levels of the mfaGSTM1 and mfaGSTM2 genes, specific oligonucleotide probes were used for Northern-blot analysis. mfaGSTM2 was expressed in the 4 Macaca fascicularis livers examined (Fig. 5). In addition, mfaGSTM2 mRNA was also found at a low level in the duodenum, but was barely detectable in kidney and lung in the one animal examined (Fig. 5). The mfaGSTM2 message was approximately 1.3 kb in size.

In contrast to mfaGSTM2, mfaGSTM1 expression was not detected in any of the 4 tissues examined, including liver (data not shown). Interestingly, when the mfaGSTM2 cDNA was used as a probe in Northern-blot analysis, 2 distinct bands were detected, suggesting that at least 2 different mu-class GSTs are expressed in Mf liver and duodenum. The size of the lower-molecular-weight band was estimated to be 1.3 kb and was most likely identical to the message detected with the mfaGSTM2-specific oligonucleotide probe (Fig. 5).

Mass Analysis of mfaGSTs by Protein Mass Spectrometry

Because some of the results above suggested that the hepatic mfaGST protein with AFBO-conjugating activity, isolated by GSHA affinity chromatography, might not be the same as the recombinant mfaGSTM2-2 protein, the molecular masses of these and other GST subunits were determined by MALDI-TOF mass spectrometry, with an accuracy of 0.1%. Overall, masses obtained by protein mass spectrometry were very consistent with peptide sequence-deduced mass. The discordance was particularly good for mfaGSTM1 and mfaGSTM2 cDNA expressed subunits (mfaGSTM1: 25430 Dalton estimated by MS vs. 25429 Dalton deduced from the cDNA sequence; mfaGSTM2: 25556 Dalton determined by MS vs. 25560 Dalton deduced from the cDNA sequence). However, there was an 81-Da difference between the GSHA column purified GST and the recombinant mfaGSTM2-2 subunit, based on MW determined by MS.
DISCUSSION

Guengerich and colleagues have demonstrated previously that mu-class GSTs, rGSTM2-2 and rGSTM2-3, and hGSTM1a-1a purified from rat and human liver respectively, can conjugate both exo- and endo-AFB, epoxides, with the endo-stereoisomer being the preferred substrate (Guengerich et al., 1998; Johnson et al., 1997; Raney et al., 1992a,b). Consistent with these findings, this study clearly showed that mu-class enzyme GSHA-GST purified from liver tissue exhibited a specific activity of 239 pmol/min/mg toward AFBO (Table 1). In contrast, 2 partially purified alpha-class GSTs (peaks P1, P2, in Fig. 3) lacked AFBO-conjugating activity. In addition, the partially purified fractions P3-1, P3-2, P4, and P5 obtained by SHGA affinity chromatography followed by chromatofocusing exhibited AFBO-conjugating activities of 140, 280, 70, and 140 pmol/min/mg respectively (Fig. 3). Immunoblot analyses showed that each of these fractions contained a mixture of alpha- and mu-class GSTs. In addition, based on visual inspection of the immunoblot band intensities, AFBO-conjugating activity appeared to correlate with the intensity of the mu-class, but not the alpha-class bands. Together, these results suggest that mu-class GSTs are responsible for the majority of the AFBO-conjugating activity in Macaca fascicularis liver. However, in this study the activities of theta and zeta class GSTs were not evaluated, and it is possible that either of these forms could contribute to AFBO-conjugating activity seen in cytosolic fractions. They could not, however, contribute to the GST-conjugating activity isolated from the affinity columns as neither form is retained on GSH- or GSHA-affinity columns.

FIG. 4. Amino acid sequence alignments of primate mu-class GSTs. Asterisks indicate positions that have a single, fully conserved residue. Double dots indicate positions that have conserved substitutions. Single dots indicate positions that have less conserved substitutions.
Interestingly, in contrast to rat and human mu-class GSTs (Guengerich et al., 1998; Johnson et al., 1997; Raney et al., 1992a,b), but similar to the rodent alpha-class GSTs mGSTA3-3 and rGSTA5-5 (Buetler and Eaton, 1992; Hayes et al., 1991, 1992, 1994), it appears that mu-class GST(s) purified from Macaca fascicularis liver preferentially conjugate the exo-AFB1 epoxide (Table 1). It is very difficult to compare quantitatively the AFBO-conjugating activity between the study of Raney et al. (1992b) and this study, because the assays were carried out under very different conditions, and the results were expressed in different activity units (Raney et al., 1992b). Nevertheless, these findings suggest that the Mf hepatic mu-class GST(s) GSHA-GST present in the GSHA affinity-purified fraction may be the only known primate GST(s) to date that has (have) high and selective activity toward the exo-AFBO stereoisomer.

Identification and characterization of the homologous mu-class GST(s) in humans may have important implications in the prevention of AFB1-induced hepatocarcinogenesis, as it is known that only the exo-AFBO isomer reacts with DNA (Iyer et al., 1994; Johnson and Guengerich, 1997; Raney et al., 1992b). Although Mf mu-class GST purified by the GSHA column catalyzes the formation of the exo-AFBO-glutathione conjugate (AFBO-SG), it has to be pointed out that this activity is approximately 2 orders of magnitude lower than that of rodent alpha-class GSTs, mGSTA3-3 and rGSTA5-5 (Buetler and Eaton, 1992; Hayes et al., 1991, 1992, 1994).

To further characterize the mu-class GST(s) with high activity toward exo-AFBO, we attempted to clone the corresponding cDNA by RT-PCR from a Macaca fascicularis liver sample. Two different mu-class GST cDNAs containing complete open reading frames were obtained and named mfaGSTM1 and mfaGSTM2, based on the recommendations by Mannervik et al. (1992). Analysis of cDNA-deduced amino acid sequences revealed that mfaGSTM1 and mfaGSTM2 are most likely homologs of the human mu-class GSTs, hGSTM4 and hGSTM2, respectively, as they share 97% and 96% identities with these proteins respectively (Table 2).

TABLE 2
Amino Acid Sequence Similarities of Primate Mu-class GSTs

<table>
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<tr>
<th></th>
<th>mfaGSTM1</th>
<th>mfaGSTM2</th>
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<th>hGSTM2</th>
<th>hGSTM3</th>
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</table>

Note. The percentage of protein sequence identity was determined using an “Align” program of Fasta package downloaded from a web site (ftp://ftp.virginia.edu/pub/fasta/mac). The recommended nomenclature of Mannervik et al. (1992) was used to name 2 Macaca fascicularis novel mu-class GSTs.

Interestingly, the human GSTM2 cDNA was initially isolated from myoblasts (Vorachek et al., 1991), and it is significantly expressed in brain and heart, in addition to muscle (Rowe et al., 1997). Human GSTM2-2 displays unique and high catalytic activity toward reactive quinones of endogenous catecholamines such as dopaminochrome, produced by oxidation.
oration of dopamine, and it has been suggested that hGSTM2-2 may play some cytoprotective role against endogenous oxidative tissue damage in the brain (Baex et al., 1997). In contrast to hGSTM2, which is barely detectable in human liver (Hussey and Hayes, 1993; Rowe et al., 1997), mfaGSTM2 was significantly expressed in Macaca fascicularis liver, but similar to its human counterpart, mfaGSTM2 was barely detectable in either kidney or lung (Rowe et al., 1997). However, it should be noted that there can be large interindividual differences in expression of GST isoforms in different tissues, and only a few human livers have been evaluated for the presence of hGSTM2.

Although mfaGSTM2-2 shares 96% sequence identity with hGSTM2-2, it exhibited 30% lower activity toward CDNB and 2.5-fold higher activity toward DCNB than hGSTM2-2 (Table 3). Comparison of the amino acid sequences revealed that mfaGSTM2 and hGSTM2 differ by 8 amino acids at positions 25, 90, 94, 104, 107, 113, 129, and 130 (Fig. 4). Only one out of the 8 differences is non-conservative: at position 25, aspartic acid is found in hGSTM2, whereas a glycine occupies this position in mfaGSTM2. It is possible that the amino acid difference at position 25 or any of the remaining 7, alone or in combinations, may be responsible for the difference of CDNB activities between mfaGSTM2-2 and hGSTM2-2. Several studies have demonstrated that single-site changes in GSTs can profoundly influence the catalytic properties, substrate specificity, and stereoselectivity (Bammler et al., 1995; Bjornestedt et al., 1995; Boehlert and Armstrong, 1984; Cobb et al., 1983; Hu et al., 1997a,b,c; Nanduri et al., 1996; Shan and Armstrong, 1994; Zimniak et al., 1994).

MfaGSTM1 (the homolog of human M4), isolated by RT-PCR from the Macaca fascicularis liver, was not detected in liver, kidney, lung, or duodenum (data not shown), suggesting that mfaGSTM1-1 is either not expressed in these tissues or is expressed at extremely low levels. The human GSTM4 cDNA was initially cloned from a human testis cDNA library (Ross and Board, 1993). Comstock et al. (1993) detected hGSTM4 mRNA at various levels in many human tissues including heart, placenta, lung, brain, liver, skeletal muscle, pancreas, testis, cerebral cortex, uterus, and ovary. In addition, a more recent study indicated that hGSTM4 subunit is actually a minor mu-class GST form that is expressed at extremely low levels in all tissues except testis, which is rich in almost all human mu-class GSTs (M1 through M3 and M5) (Rowe et al., 1997). Similar to hGSTM4-4, recombinant mfaGSTM1-1 exhibited no AFBO and low CDNB activities (Table 3) (Ross and Board, 1993).

This study raises the question of whether recombinant mfaGSTM2-2 is identical to the native GST, GSHA-GST, obtained by GSHA affinity purification from the same liver. Immunoblot analysis identified both enzymes as GSTs belonging to the mu class. However, differences in enantioselectivity toward exo- versus endo-AFBO suggest that the cloned mfaGSTM2-2 protein is not identical to that purified from the liver. Furthermore, there was an 81-Dalton difference in the molecular weight determined by MS between recombinant mfaGSTM2-2 and the native GSHA-GST. Finally, the cDNA-expressed mfaGSTM2-2 protein had 10 times higher CDNB activity than did GSHA-GST.

While it is highly unlikely, it is possible that there was a second, but highly similar GST subunit in the GSHA affinity column-purified GST(s) that could not be separated from the major subunit by reverse phase HPLC. This contamination from a second GST could have altered the molecular weight of the GSHA column purified GST. Alternatively, it is possible that a single site mutation was introduced into mfaGSTM2 during RT-PCR cloning that resulted in an alteration of the enantioselectivity toward AFBO and catalytic activity toward CDNB. This hypothesis is supported by recent studies demonstrating that single-site changes in GSTs and P450s are responsible for differences in enzyme catalytic efficiency, substrate specificity and stereoselectivity or enantioselectivity (Bammler et al., 1995; Bjornestedt et al., 1995; Boehlert and Armstrong, 1984; Cobb et al., 1983; Hu et al., 1997a,b,c; Hu et al., 1998; Lindberg and Negishi, 1989; Nanduri et al., 1996; Shan and Armstrong, 1994; Zimniak et al., 1994).

It should be pointed out that the design of forward primer (primer 1, see Materials and Methods for sequence) was based on human mu-class GST cDNA sequences and included the first 10 highly conserved nucleotides of the coding regions of hGSTM1, hGSTM2, and hGSTM5. Therefore, the theoretical possibility exists that the first 2 codons following the initiator methionine were biased in mfaGSTM2. However, this possibility seems unlikely because the first 3 codons are conserved in the human mu-class GST cDNAs hGSTM1, hGSTM2, and hGSTM5. In the unlikely event that the 2 codons following the initiator methionine in the RT-PCR-generated clone

![FIG. 5. Northern-blot analysis of mfaGST mRNA in different Mf tissues. (A) Northern blot using mfaGSTM2-specific oligomer probe. (B) Northern blot using mfaGSTM2 cDNA probe. (C) Northern blotting using mouse β-actin cDNA probe. L, liver (1 to 4); K, kidney; Lu, lung; Duo, duodenum.](image-url)
mfaGSTM2 were indeed different from the actual cDNA, it is still highly unlikely that they would account for the differences in stereoselectivity towards \( \text{exo} \)- and \( \text{endo} \)-AFBO and the approximately 10-fold difference in CDNB activity exhibited by recombinant mfaGSTM2-2 and the native GSHA-GST purified from tissue.

Together our data strongly suggest that (a) mfaGSTM2-2 and GSHA-GST are 2 distinct enzymes, and (b) the cDNA encoding GSHA-GST still remains to be cloned.

In summary, in contrast to rodents, \( \mu \)-class GSTs are responsible for the majority of AFBO-SG activity found in \( \text{Macaca fascicularis} \) liver. In addition, our data suggest that at least two distinct \( \mu \)-class GSTs exhibiting this activity are expressed constitutively in the liver of this species. One of the enzymes, GSHA-GST, preferentially conjugates \( \text{exo} \)-AFBO, whereas the other, mfaGSTM2-2, almost exclusively metabolizes the \( \text{endo} \) isomer. To date, none of the known human \( \mu \)-class GSTs exhibit predominant activity toward the ultimate genotoxic AFB\(_1\) metabolite \( \text{exo} \)-AFBO. Identification of a potential human homolog of GSHA-GST would be relevant where the other, mfaGSTM2-2, almost exclusively metabolizes the \( \text{endo} \) isomer. To date, none of the known human \( \mu \)-class GSTs exhibit predominant activity toward the ultimate genotoxic AFB\(_1\) metabolite \( \text{exo} \)-AFBO. Identification of a potential human homolog of GSHA-GST would be relevant where the other, mfaGSTM2-2, almost exclusively metabolizes the \( \text{endo} \) isomer. To date, none of the known human \( \mu \)-class GSTs exhibit predominant activity toward the ultimate genotoxic AFB\(_1\) metabolite \( \text{exo} \)-AFBO. Identification of a potential human homolog of GSHA-GST would be relevant where the other, mfaGSTM2-2, almost exclusively metabolizes the \( \text{endo} \) isomer. To date, none of the known human \( \mu \)-class GSTs exhibit predominant activity toward the ultimate genotoxic AFB\(_1\) metabolite \( \text{exo} \)-AFBO.


