Characterization of Microsomal Glutathione S-Transferases
MGST1, MGST2, and MGST3 in Cynomolgus Macaque

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ABSTRACT

The glutathione S-transferase (GST) family comprises cytosolic, mitochondrial, and microsomal GSTs, all essential enzymes that metabolize a wide range of endogenous and exogenous substrates. Among the microsomal GSTs (MGSTs) in humans, MGST1, MGST2, and MGST3 are involved in detoxification; however, MGSTs have not been fully investigated in cynomolgus macaque, an important primate species widely used in drug metabolism and toxicology studies. In the present study, cynomolgus MGST2 and MGST3 cDNAs were isolated from liver tissue and characterized along with previously isolated cynomolgus MGST1. For comparison with the human MGSTs, MGST2 and MGST3 were expressed from rhesus macaque (closely related to cynomolgus macaque) liver. Cynomolgus MGST2 and MGST3, respectively, were highly identical (99 and 98%) to human MGST2 and MGST3 and nearly identical to the amino acid sequences of the rhesus orthologs, and they were closely clustered with human MGST2 and MGST3 by phylogenetic analysis. The analysis of genome data indicated that MGST1, MGST2, and MGST3, respectively, had similar gene structures and genomic organization in macaque and human. Therefore, cynomolgus MGSTs have molecular similarities to the corresponding human MGSTs. Cynomolgus MGST2 and MGST3 were expressed in liver, jejunum, and kidney, but at lower levels than MGST1. GST activities were measured with 1-chloro-2,4-dinitrobenzene and 1,2-epoxy-3-(p-nitrophenoxy)propane as substrates, using proteins heterologously expressed in Escherichia coli. Cynomolgus MGST1, MGST2, and MGST3 conjugated 1-chloro-2,4-dinitrobenzene and 1,2-epoxy-3-(p-nitrophenoxy)propane, indicating that cynomolgus MGST1, MGST2, and MGST3 are functional enzymes. These results suggest that these functional cynomolgus GST enzymes and the corresponding human MGSTs are molecularly similar.

Introduction

Glutathione S-transferase (GST) catalyzes the conjugation of reduced glutathione to electrophilic substrates (e.g., halogenonitrobenzenes, arene oxides, and quinones), and some GST enzymes also exhibit peroxidase and isomerase activities (Hayes et al., 2005). The GST superfamily contains at least 16 members in humans, comprising cytosolic, mitochondrial, and microsomal GSTs (MGSTs) (Hayes et al., 2005). The cytosolic and mitochondrial GSTs primarily metabolize foreign chemicals, such as drugs and environmental pollutants, and also play roles in the detoxification of reactive compounds (Hayes and Pulford, 1995).

In humans, MGSTs constitute a superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). Of the six MAPEG members, 5-lipoxygenase activating protein, leukotriene C4 synthase, and microsomal prostaglandin E2 synthase 1 are largely involved in the production of leukotriene C4 and prostaglandin E2, which are essential for fever, pain, and inflammation and do not contribute to drug metabolism or detoxification (Hayes et al., 2005). In contrast, MGST1, MGST2, and MGST3 play roles in detoxification of foreign compounds, and MGST2 and MGST3 are also capable of synthesizing leukotriene C4 (Hayes et al., 2005). Of the three human MGSTs, MGST1, MGST2, and MGST3, MGST1 is most abundant in the liver and metabolizes hydrophobic substrates, such as phospholipid hydroperoxides and halogenated hydrocarbons (Morgenstern et al., 2011).

The cynomolgus macaque (Macaca fascicularis) is an important primate species in drug metabolism studies. A number of cytochrome P450 (P450) enzymes have been identified and characterized in cynomolgus macaque, leading to the notion that the molecular properties of P450 enzymes in the cynomolgus macaque and humans are generally similar (Uno et al., 2011). The exception is CYP2C76, which is not orthologous to any human P450 and is partly responsible for species differences in drug metabolism between the cynomolgus macaque and humans (Uno et al., 2010). Therefore, characterization of drug-metabolizing enzymes corresponding to those essential for drug metabolism in humans helps to better understand the similarities and differences of drug metabolism between model animals and humans. MGST1 cDNA has been identified in cynomolgus macaque, and the gene was shown to be expressed in liver, kidney, jejunum, and adrenal gland (Uno et al., 2009); however, the enzymatic properties of MGST1 have not been investigated.

In this study, cDNAs encoding MGST2 and MGST3 were isolated using total RNA extracted from cynomolgus macaque liver. These MGSTs, and previously identified MGST1, were characterized by sequence and phylogenetic analyses, quantitative polymerase chain reaction (qPCR) analysis for tissue expression patterns, and enzymatic analysis using proteins heterologously expressed in Escherichia coli.

ABBREVIATIONS: bp, base pairs; CDNB, 1-chloro-2,4-dinitrobenzene; EPNPP, 1,2-epoxy-3-(p-nitrophenoxy)-propane; GST, glutathione S-transferase; MAPEG, membrane-associated proteins in eicosanoid and glutathione metabolism; MGST, microsomal glutathione S-transferase; P450, cytochrome P450; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RT, reverse transcription.

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Materials and Methods

1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-epoxy-3-μ-nitrohexylpropane (EPNNP) were purchased from Wako Pure Chemicals (Osaka, Japan). Oligonucleotides were synthesized by Greiner (Tokyo, Japan), and TaqMan probes were synthesized by BioSearch Technology Japan (Tokyo, Japan). Anti-histidine tag antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from BioDynamics Laboratory (Tokyo, Japan) and BD Gentest (Woburn, MA), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Wako Pure Chemicals, unless otherwise specified.

Tissues and Preparation of RNA and Microsomes. Tissue samples, including brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus, were collected from six cynomolgus macaques (three males and three females from Indochina, 4–5 years of age, 3–5 kg), from which total RNA was extracted, as previously described (Uno et al., 2006). A pool of total RNA samples was used for qPCR analysis of each tissue type. Total RNA extracted from liver samples of six cynomolgus macaques (three males and three females from Cambodia, 4–5 years of age, 3–5 kg) and three rhesus macaques (three males from China, 7 years of age, 3–5 kg) were used to isolate MGST cDNAs. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd.

Molecular Cloning. Reverse transcription (RT)-PCR was performed as previously described (Uno et al., 2006) using total RNA extracted from cynomolgus and rhesus macaque livers. Briefly, RT was performed in a reaction containing 1 μg of total RNA, oligo (dT), and SuperScript III RT reverse transcriptase (Invitrogen, Tokyo, Japan) at 50°C for 1 hour. The synthesized cDNAs were subsequently used as the templates for PCR reactions, which were performed using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) with a thermal cycler (Applied Biosystems, Tokyo, Japan) according to the manufacturer’s protocols. Thermal cycler conditions included an initial denaturation at 94°C for 2 minutes and 35 cycles at 94°C for 10 seconds, 65°C for 30 seconds, and 68°C for 30 seconds, followed by a final extension at 68°C for 5 minutes. The primers used were 5'-CGTCTCTAAAGATTCTGCGA-GAAAGATG-3' and 5'-TTAAGTTGCGCCTATGTTCTTGC-3' for MGST2; 5'-GCAAAGATGGCTGTCCTTCTCAAAGG-3' and 5'-CTAAAATTCTTTAATGGCAGCATTTGGA-3' for MGST3. The amplified PCR products were cloned into pCR4 vectors using Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen) according to the manufacturer’s instructions. The inserts were sequenced using ABI PRISM BigDye Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

Sequence Analysis. Sequence data were analyzed with DNASIS Pro (Hitachi Software, Tokyo, Japan), the Genetyx system (Software Development, Tokyo, Japan), and Sequencher (Gene Codes Corporation, Ann Arbor, MI). A phylogenetic tree was created by the neighbor-joining method. The nucleotide search tool CLUSTAL (National Center for Biotechnology Information) was used for the homology search, and the CLUSTAL alignment tool (University of California Santa Cruz Genome Bioinformatics) was used for the analysis of the human and macaque (rhesus) genome data. Amino acid sequences found in GenBank were used in this analysis, including human GSTA1 (NP_665683), MGST1 (NP_001247440), MGST2 (NP_002404), and MGST3 (NP_000451); cynomolgus MGST1 (AB021637); rat GST1 (NP_599176), MGST2 (NP_001099900), and MGST3 (NP_00178523); and mouse Gst1 (NP_064330), Gst2 (NP_778160), and Gst3 (NP_079845). The amino acid sequence of rhesus MGST1 was deduced from a cDNA sequence (C058354) highly identical to cynomolgus MGST1 cDNA. The amino acid sequences of cynomolgus and rhesus MGST2 and MGST3 were deduced from the cDNAs identified in this study. For analysis of gene structure and genomic organization, the cynomolgus MGST2 and MGST3 cDNAs identified in this study were used. The MGST cDNA sequences found in GenBank were also used, including human MGST1 (NM_020300), MGST2 (NM_002443), and MGST3 (NM_004528); and cynomolgus MGST1 (EF104260).

qPCR Analysis. Expression of cynomolgus MGST2 and MGST3 was quantified by real-time RT-PCR in brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus by using gene-specific primers and probes, as described previously. Briefly, RT reactions were carried out using random primers (Invitrogen), and one-twenty-five of the reaction was used for the subsequent PCR. PCR amplification was conducted in a total volume of 20 μl using TaqMan Universal PCR Master Mix (Applied Biosystems) with the ABI PRISM 7500 sequence detection system (Applied Biosystems), according to the manufacturer’s protocols. The primers and the probe were used at a final concentration of 300 and 200 nM, respectively. The primers used were 5'-TGGAAAGCAGAATGTTATAATAAACAGG-3' and 5'-AGAAGTAATGACGAA- AAACCTTGGTGA-3' for MGST2; 5'-CACCCAGAACGTGGAAGT-3' and 5'-TAGCTTTGTCGGTGCTCCT-3' for MGST3. The probes were used as 5'-FAM-TCCGGCACAACAAAAGCTTGCGNT-BHQ1-3' for MGST2 and 5'-FAM-AGGTTTCTACCCCGCTATGCTCC-BHQ1-3' for MGST3. To determine the relative expression levels, the raw data were normalized to the 18S ribosomal RNA level. For each tissue type, value is expressed as the average ± S.D. of three independent amplifications. MGST1 expression was also measured, as previously described (Uno et al., 2009), for comparison with MGST2 and MGST3.

Heterologous Expression of Proteins in E. coli. Expression plasmids were generated and cynomolgus MGST1, MGST2, and MGST3 proteins were expressed in E. coli. To prepare expression plasmids, PCR was performed as described earlier using each MGST cDNA as a template. To facilitate subcloning of the PCR products into vectors, reverse primers containing the restriction enzyme site sequence for XhoI were used. The forward primers used were 5'-ATGTTTGACCTACCCAGATAGGA-3' for MGST1, 5'-ATGTTCTTCAGGCGGCAAGA-3' for MGST2, and 5'-ATGTTGACCTACCCAGATAGGA-3' for MGST3. The reverse primers used were 5'-CCGCTCGATGTCGAGTCC-3' for MGST2, and 5'-CCGCTCGATGTCGAGTCC-3' for MGST3. PCR products were digested using restriction enzyme (XhoI) and were subcloned into a pET30a vector (Novagen, Madison, WI), which provides an N terminus 6×His-tag. Proteins were expressed in E. coli using the generated expression plasmids according to the manufacturer’s instructions.

Proteins were expressed in E. coli using the expression plasmids according to the manufacturer’s instructions. Briefly, starter Luria-Bertani medium/kanamycin (30 μg/ml) cultures inoculated with single transformed colonies of E. coli strain BL21 were incubated, with continuous shaking at 180 rpm, at 37°C for 12 hours, and then diluted (1:100) into Terrific-Broth/kanamycin (30 μg/ml) medium. After incubation for 4 hours 0.4 mM isopropyl-β-D-thiogalactoside was added to the cultures, and incubation was continued at 25°C for 16 hours. After harvesting the bacterial cells, bacterial microsomal fractions were prepared by a series of fractionation and high-speed centrifugation steps (Guanerichi et al., 1996). Expressed MGST proteins were electrophoresed and visualized on sodium dodeyl sulfate-polyacrylamide gels and were quantified by immunoblotting using antihistidine tag antibody.

Enzyme Assays. The ability of cynomolgus MGST proteins to catalyze GST conjugation reactions was analyzed using typical GST substrates (CDNB and EPNNP) as described previously (Habig et al., 1974). Briefly, a typical reaction mixture (1.0 ml) contained bacterial microsomes expressing recombinant MGST protein, 5.0 μM GSH, and substrate (1.0 μM CDNB or 0.50 μM EPNNP) in 0.10 mM potassium phosphate buffer (pH 6.5). The product formation rates were recorded at 340 and 360 nm for CDNB and EPNNP at 25°C for 5 minutes in a spectrophotometer (Hitachi U-3000; Tokyo, Japan) and were calculated with the coefficients of 9.6 and 0.5 mM cm⁻¹, respectively.

Results and Discussion

RT-PCR was used to successfully isolate MGST2 and MGST3 cDNAs from cynomolgus macaque liver, and for comparisons, rhesus macaque (a species closely related to cynomolgus macaque) liver. Cynomolgus MGST2 and MGST3 cDNAs contained open reading frames of 147 and 152 amino acid residues, respectively, and shared high amino acid sequence identities (98–99%) with human MGST2 and MGST3, respectively, both of which were higher than with MGST1 (Table 1). Cynomolgus MGST2 and MGST3 amino acid sequences were nearly identical to the corresponding rhesus orthologs. A phylogenetic analysis of human, cynomolgus, rhesus, rat, and mouse amino acid sequences indicated that cynomolgus MGST1, MGST2, and MGST3, together with the rhesus orthologs, were most closely clustered with the corresponding human orthologs, respectively.
Characterization of Cynomolgous MGSTs

To determine exon-intron structures and genomic organization, the human and (rhesus) macaque genomes were analyzed using BLAT. The first exon (noncoding) of macaque MGST3 was predicted from the human MGST3 sequence because the identified cynomolgus/ rhesus MGST cDNAs contained only the coding sequence. Among the macaque MGST sequences analyzed, the largest was MGST2 (>37 kilobases), followed by MGST3 and MGST1, similar to human sequences (Supplemental Fig. 1). Macaque MGST1, MGST2, and MGST3 contained 4, 5, and 6 exons, respectively, similar to humans (Supplemental Fig. 1). Other than the first and last exons, whose sizes vary due to inclusion of noncoding sequences, the sizes of the MGST1, MGST2, and MGST3 exons were the same in macaques and humans, ranging from 58 base pairs (bp) to 124 bp, except for exon 2 of MGST1 (158 bp in macaques and 148 bp in humans). This discrepancy might be attributable to the use of a splice acceptor site (AG) in macaque MGST1, 10 bp upstream of that of human MGST1. The corresponding site in human MGST1 was AA and thus would not readily serve as a splice acceptor site. Several human MGST1 transcript variants, due to alternative exon 1 sequences, were found in GenBank. Although such MGST1 transcript variants have not been identified in cynomolgus/rhesus macaque, sequences highly identical to the exon 1 sequences of those human MGST1 variants were found in the macaque genome. Therefore, the MGST1 transcripts with various exon 1 sequence sizes might also exist in macaque. Virtually all introns begin with the dinucleotide GU and end with AG, consistent with the consensus sequences for splice junctions in eukaryotic genes. Moreover, MGST1, MGST2, and MGST3 were located in corresponding regions of the macaque and human genomes in the same directions, respectively (Supplemental Fig. 2). These results suggest similarities between macaque and human MGST gene structure and genomic organization.

qPCR analysis was performed to measure expression of cynomolgus MGST2 and MGST3 in brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus. Cynomolgus MGST2 was expressed most abundantly in the jejunum, followed by adrenal gland and kidney, whereas MGST3 was expressed most abundantly in heart, followed by jejunum and kidney (Fig. 2A). Similarly, in humans, MGST2 is expressed in the liver and small intestine (Jakobsson et al., 1996), and MGST3 is expressed in heart (Jakobsson et al., 1997). Cynomolgus MGST1 is expressed in the liver, jejunum, and kidney (Uno et al., 2009), similar to human MGST1 (Estonius et al., 1999). The results indicate similarities between cynomolgus macaque and human MGST tissue expression patterns.

Expression levels of MGSTs identified in this study were compared with the expression level of the previously identified cynomolgus MGST1. In liver tissue, cynomolgus MGST1 was abundantly expressed, whereas the expression of MGST2 and MGST3 was negligible (Fig. 2B). Similarly, in jejunum and kidney, there was much higher expression of cynomolgus MGST1 than MGST2 or MGST3 (Fig. 2B). Therefore, among the cynomolgus MGSTs, cynomolgus MGST1 was expressed most abundantly in liver, jejunum, and kidney. In human, MGST1 is also abundantly expressed in the liver (Morgenstern et al., 2011). In contrast, cynomolgus MGST3 was expressed most abundantly in brain and heart cells (Fig. 2B). Similarly, expression of human MGST3 was detected in these tissues and appears to be abundant in the heart (Jakobsson et al., 1997).

The ability of cynomolgus MGST1, MGST2, and MGST3 to catalyze conjugation reactions of typical GST substrates, CDNB and EPNPP, was analyzed using proteins heterologously expressed in E. coli. All cynomolgus MGSTs catalyzed the conjugation of CDNB and EPNPP (Table 2), indicating that all three MGSTs were functional enzymes in the cynomolgus macaque. CDNB was metabolized most efficiently by MGST3, followed by MGST1 and MGST2, and EPNPP was most efficiently metabolized by MGST1, followed by MGST3 and MGST2 (Table 2). In humans, MGST1, MGST2, and MGST3 also catalyze the conjugation of CDNB (Hayes et al., 2005), partly indicating similarities in cynomolgus macaque and human MGST1, MGST2, and MGST3 enzymatic properties, respectively. Although all three cynomolgus MGSTs are functional enzymes, abundant expression of MGST1 in the liver, kidney, and jejunum, essential organs for drug metabolism and toxicity, suggests that MGST1 likely plays the major role in detoxification (among cynomolgus MGSTs), similar to humans. In contrast, more ubiquitous expression of MGST2 and MGST3 might reflect their roles in synthesis of leukotriene C4.

In the GST superfamily, many cytosolic GSTs have evolved through recent gene duplications (Sherratt and Hayes, 2002), probably under selective pressure from oxidative stress, including the GSTA, GSTM, GSTO, and GSTT subfamily genes, which comprise highly identical paralogous genes in the subfamily. In contrast, the MAPEG family (including MGST1, MGST2, and MGST3) does not contain paralogs in the subfamily (Fig. 1), possibly reflecting their essential physiologic roles in synthesis of leukotriene C4 established early in their evolution (Sherratt and Hayes, 2002).

### TABLE 1

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<tr>
<th>Cynomolgus</th>
<th>Human</th>
<th>Sequence Identity</th>
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<tr>
<td></td>
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The cDNA and amino acid sequences of cynomolgus and human MGSTs were compared using BLAST as described in Materials and Methods.
In summary, we identified MGST2 and MGST3 cDNAs from cynomolgus macaque liver tissue. The amino acid sequences of these cynomolgus MGSTs were highly identical to those of the human orthologs. The gene structures and genomic organization of MGST1, MGST2, and MGST3 of macaques and humans are highly conserved. Moreover, tissue expression patterns of cynomolgus MGST1, MGST2, and MGST3 and the corresponding human orthologs were similar. Cynomolgus MGST1, MGST2, and MGST3 catalyzed conjugation of typical GST substrates, CDNB and EPNPP. These results suggest that cynomolgus MGST1, MGST2, and MGST3 generally have molecular and functional characteristics similar to human MGST1, MGST2, and MGST3, respectively.

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Performed data analysis: Uno, Kunori, Murayama.
Wrote or contributed to the writing of the manuscript: Uno, Yamazaki.

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<th>EPNPP</th>
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<td>μmol/min/μmol MGST</td>
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<tr>
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<td>MGST3</td>
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Fig. 2. Expression of cynomolgus MGSTs in tissues. qPCR analysis was conducted as described in Materials and Methods. The expression level of each gene was normalized to the 18S rRNA level, and the greatest expression value was adjusted to 1, to which all other expression levels were adjusted accordingly. (A) Expression levels of cynomolgus MGST2 and MGST3 were measured in brain, lung, heart, liver, kidney, adrenal gland, jejunum, testis, ovary, and uterus. Values are shown as the average ± S.D. of three independent amplifications. (B) Expression levels of cynomolgus MGST2 and MGST3 were compared with the expression level of the previously identified MGST1 in liver, jejunum, kidney, brain, and heart. Values are shown as the average of three independent amplifications.
References


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