Kappa class glutathione S-transferase (GST) cDNA sequences have been identified in rat, mouse, and human. In the present study, we determined the structure and chromosomal location of the human GST Kappa 1 (hGSTK1) gene, characterized the protein, and demonstrated its subcellular localization. The human gene spans ~5 kb, has 8 exons, and maps onto chromosome 7q34. The 5’-flanking region lacks TATA or CCAAT boxes, but there is an initiator element overlapping the transcription start site. hGSTK1 amino acid sequence showed homology to bacterial 2-hydroxychromene-2-carboxylate isomerase, an enzyme involved in naphthalene degradation pathway. hGSTK1 mRNA was expressed in all of the organs examined. Subcellular fractionation of HepG2 cells showed that the protein was located in peroxisomes and mitochondria and was not detectable in the cytoplasm. The peroxisomal localization was confirmed by transfection of HepG2 cells with a plasmid coding a green fluorescent protein fused in-frame to the N terminus of hGSTK1. The C terminus of hGSTK1 was essential for localization of the protein to peroxisomes, and the C-terminal sequence Ala-Arg-Leu represents a peroxisome targeting signal. This is the first time that a human GST has been found in peroxisomes, suggesting a new function for this family of enzymes.

Glutathione S-transferases (GSTs)1 are a family of ubiquitous intracellular enzymes that catalyze the conjugation of glutathione to many endogenous and exogenous compounds (1). These include chemical carcinogens, therapeutic drugs, and products of oxidative stress. In addition to their major role in catalyzing the conjugation of electrophilic substrates to glutathione, these enzymes have GSH-dependent peroxidase (2) and isomerase (3) activities. GSTs also have a noncatalytic role via their interaction with kinases (4–6) and their binding properties. GSTs are highly conserved between species: Alpha, Mu, Pi, Theta, Kappa, Sigma, Zeta, and Omega (1, 9–12). The GSTs of each family have short regions of high identity in common and share a common evolutionary pathway (13). Soluble GSTs have been localized mainly in cytoplasm but are also present in the nucleus and the mitochondrion where they could have functions peculiar to the integrity of these organelles (14–16). For example, GSTP1 in the nucleus might be associated with protection of DNA damage induced by doxorubicin and consequently in the acquisition of resistance to anticancer drugs by cancer cells (17), and the GSTs known to be present in the mitochondrion (i.e., GSTA1, GSTA4 and GSTM1) may play an important role in defense against chemical and oxidative stress (15).

Among the eight classes of soluble GSTs, the Kappa class has been the least studied. In 1991, Harris et al. (18) reported the purification of a GST isolated from mitochondrial matrix of rat liver, GST13-13. Although this subunit was initially classified as a class Theta GST on the basis of N-terminal analysis, elucidation of its complete amino acid sequence by Pembble et al. (9) showed the absence of sequence similarity to any other class of GST. Pembble et al. also identified human sequences in expressed sequence tag database that showed a high nucleotide similarity (77%) to that of rat GST subunit 13. Therefore, these GSTs were assigned to the Kappa class, with the rat GST subunit 13 and the human gene being rGSTK1 and hGSTK1, respectively. More recently, Jowsey et al. (19) identified and characterized a murine class Kappa subunit: mGSTK1. This protein has been reported to be present at substantially higher levels in the mitochondrial fraction than the cytosolic fraction of rodent liver.

The aim of the present study was to characterize the human GST Kappa at the gene and protein levels. Our results demonstrate that the hGSTK1 is encoded by a gene present on chromosome 7q34 and is expressed in all tissues studied, and its amino acid sequence presents similarities with a bacterial enzyme, 2-hydroxychromene-2-carboxylate isomerase. Furthermore, we showed for the first time the localization of a GST in the peroxisome, because hGSTK1 is present in this organelle.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit anti-catalase antibody was a generous gift of Professor Vollkl (Heidelberg, Germany). Rabbit anti-cytochrome c and caspase 3 were from Santa Cruz Biotechnology (Santa Cruz, CA). pDsRed2-peroxi and pEGFP-C1 plasmids were from Clontech Laboratories (Palo Alto, CA). Iodixanol was purchased as Optiprep from AxisShield (Oslo, Norway).

**In Situ Analysis**—Nucleotide sequences of human GST Kappa 1 (GSTK1; GenBank™ accession number NM_015917) were compared with the High Throughput Genome Sequences subset of GenBank™ using BLASTN program with default parameters (NCBI; www.ncbi.nlm.nih.gov.BLAST). Comparative sequence alignments were generated using PipMaker (bio.cse.psu.edu). Repeat elements were charac-

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3 The abbreviations used are: GST, glutathione S-transferase; CBP, calmodulin-binding protein; DSBA, protein disulfide bond isomerase; EGFP, enhanced green fluorescent protein; HCCA, 2-hydroxychromene-2-carboxylate; PTS, peroxisomal targeting signal; 5’-RACE, rapid amplification of 5’ cDNA ends.
Nucleotide positions for the human sequence are shown on the x-axis. The gene orientation is shown by the arrow. The constructs were verified by DNA sequencing. pDeRe2-Peroxi is a mammalian expression vector that encodes a red fluorescent protein containing peroxisomal targeting signal 1 (PTS1).

Expression and Purification of hGSTK1 Proteins—The hGSTK1 open reading frame was cloned by ligation-independent cloning using a Affinity LIC cloning and protein purification kit (pCAL-n-FLAG, Stratagene) to yield full-length hGSTK1 protein containing an N-terminal 27-aminio acid calmodulin-binding peptide (CBP) tag. CBP-hGSTK1 was overexpressed in the Escherichia coli strain BL21(DE3) by 1 mM isopropyl-β-D-thiogalactopyranoside induction for 3.5 h at 37 °C. The purification procedure followed the instructions of the manufacturer with some modification. The frozen cells were thawed and resuspended in lysis buffer containing 150 mM NaCl, 0.5 mM Tris-HCl, pH 8.0, 2 mM CaCl₂, 1 mM imidazole, 10 mM magnesium acetate, 10 mM β-mercaptoethanol. Lysosome (200 µg/ml) was added, and the suspension was incubated with gentle shaking on ice for 15 min. The cells were disrupted by sonication and centrifuged at 16,000 × g for 15 min at 4 °C. The lysate was applied on a calmodulin-resin column equilibrated in lysis buffer. The column was washed with five volumes of lysis buffer and five volumes of buffer (500 mM NaCl, 0.5 mM Tris-HCl, pH 8.0, 2 mM CaCl₂, 1 mM imidazole, 100 mM magnesium acetate, 10 mM β-mercaptoethanol). Elation fractions of 1 ml were collected and analyzed by SDS-PAGE. The native hGSTK1 was obtained after digestion with 1 unit of enterokinase/20 µl of fusion protein. The protein was dialyzed for 24 h at 4 °C against two changes, each of 2 liters, of 100 mM sodium phosphate buffer, pH 8.0, 2 mM EDTA, 1 mM dithiothreitol and 1 mM DTT. Asepsis against purified hGSTK1 protein was raised in rabbits using a standard immunization protocol (Eurogentec, Liege, Belgium).

Sequence Analysis—Dye terminator DNA sequencing was performed from both the 5′ and 3′ ends of the PCR-amplified genomic products using BigDye terminators (Applied Biosystems, Foster City, CA) and an
Applied Biosystems Prism 3100 genetic analyzer.

RNA Sources and Preparation—A Human Total RNA Master Panel was purchased from Clontech Laboratories (Palo Alto) containing total RNA isolated from 24 different human tissues. The RNA from these tissues had been pooled from multiple individuals of each gender, with the exception of heart (25-year-old male) and brain (28-year-old male).

Reverse Transcription-Quantitative PCR Analysis—RNAs were reverse-transcribed into cDNA using Superscript II (Invitrogen) following the manufacturer’s protocols with the following exceptions: 1/2 of total RNA and random hexamer primers (500 ng) were used in each reverse transcription reaction. cDNAs were stored at $-20^\circ C$ until use as template in subsequent PCRs.

Real time quantitative PCR was performed by the fluorescent dye SYBR Green methodology using the qPCRTM Core Kit for SybrTM Green I from Eurogentec and the ABI Prism 7000 (PerkinElmer Life Sciences). Primer pairs for hGSTK1 were chosen with the Primers 3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi): forward, 5’-GAAGAGCAAGCTCAGGGAGA-3’, and reverse, 5’-CGGTCAGACCCAAATAGCAT-3’. Briefly, cDNA was mixed with 22 μl of 10 mM Tris-MgCl₂ buffer, 3 μM of each primer, 0.025 unit/μl Hot GoldStar enzyme, and 0.75 μl of 1/2000 diluted SYBR Green stock in a final volume of 25 μl. A first step of 10 min at $95^\circ C$ was followed by 40 cycles of amplification ($95^\circ C$ for 15 s and $60^\circ C$ for 60 s). For each sample, the ABI Prism 7000 software provided an amplification curve constructed by relating the fluorescence signal intensity (normalized to the fluorescence of ROX internal passive reference) to the cycle number. Cycle threshold ($C_T$) was defined as the cycle number at which a significant increase in the fluorescence signal was first detected. The relative quantification of the steady state of the target mRNA levels was calculated after normalization of the total amount of cDNA tested by an active reference, 18 S.

Subcellular Fractionation—Human hepatoblastoma (HepG2) cells were homogenized in isotonic (0.25 M) sucrose buffered with 10 mM triethanolamine, 10 mM acetic acid, pH 7.8 (homogenization buffer) using a Teflon and glass Potter-Elvehjem homogenizer. The nuclei, cell debris, and heavy mitochondria were removed by a 10-min centrifugation at 3,000 g. A "light mitochondrial fraction" containing lysosomes, mitochondria, peroxisomes, and some microsomes was obtained by centrifugation of the supernatants at 16,000 g for 20 min. The peroxisomes were purified from the light mitochondrial fraction by ultracentrifugation (180,000 g for 4.5 h) in a self-forming density gradient of iodixanol following the manufacturer’s protocol (Axis-shield). Immediately after centrifugation, eight fractions from the gradient were collected with a syringe by upward displacement.

Western Blot Analysis—HepG2 proteins were dissolved in 1% deoxycholate, 1% β-mercaptoethanol, 10 mM Tris-HCl, pH 6.8, and 20% glycerol. 25

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**Human Glutathione Transferase Kappa Characterization**

**FIG. 2.** DNA sequence of the hGSTK1 gene. The underlined sequences show putative recognition sites for transcription factors. The initiator sequence (Inr) overlaps the putative transcription start site marked as +1. Coding nucleotides are in capital letters, and intronic sequences are in italics. Amino acid sequences are shown using the one-letter code. +, polyadenylation signal.
μg of protein were electrophoresed on a 12.5% polyacrylamide slab gel and electroblotted overnight onto Hybond ECL nitrocellulose membranes (Amersham Biosciences). After the filters were blocked in 3% bovine serum albumin, Tris-buffered saline, they were incubated with rabbit anti-hGSTK1, anti-catalase, anti-cytochrome c or anti-caspase 3 antibodies. The filters were then washed in wash buffer (Tris-buffered saline, 0.01% Tween 20) and Tris-buffered saline and incubated with peroxidase conjugated rabbit anti-rabbit IgG (DAKO SA). All of the incubations were done at room temperature for 2 h. Peroxidase activity was detected using ECL Western blotting detection system (Amersham Biosciences).

Biochemical Assays—All of the GST enzyme activity assays were conducted at 30 °C and measured with a Beckmann spectrophotometer. In all cases, the nonenzymatic reaction was measured and subtracted from the overall reaction rate. Activity toward 1-chloro-2,4-dinitrobenzene and glutathione-peroxidase activity (cumene hydroperoxide, tert-butyl hydroperoxide, and glutathione peroxidase conjugated rabbit anti-rabbit IgG) were measured at 340 nm. For glutathione-peroxidase activities, product formation was determined using the coupled NADPH/glutathione reductase-dependent reduction of the oxidized glutathione product under conditions where the nonenzymatic reaction was limiting. Other activities were measured as followed: 1,2-dichloro-4-nitrobenzene (345 nm, pH 7.5), ethacrynic acid (270 nm, pH 6.5), trans-4-phenylbut-3-en-2-one (290 nm, pH 6.5), trans-3-nonenal (225 nm, pH 6.5), and 1,2-epoxy-3-(4-nitrophenox) propane (360 nm, pH 6.5). The protein concentrations were measured by the method of Bradford (21).

RESULTS

Characterization of the Human GSTK1 Gene and Its 5’ Flanking Region—Comparison of human GST Kappa cDNA sequence (GenBank accession number NM_015917) to the High Throughput Genome Sequences subset of GenBank using the BLASTN program identified one BAC clone RP11–556113 (GenBank accession number AC073342) located on chromosome 7q34. The sequence of this clone was established as part of a mapping and sequencing collaboration between the NHGRI Chromosome 7 Mapping Project and the Washington University Genome Sequencing Center. Alignment of cDNA with genomic sequences revealed that the hGSTK1 gene spans 5.68 kb and contains eight exons separated by seven introns (Fig. 1, A and B). This genomic structure was confirmed by using human genomic DNA and amplification of each intron by PCR with primers localized in adjacent exons. Each DNA fragment was then sequenced. The nucleotide sequence data appear in the GenBank nucleotide sequence data bases under the accession number AB486465. The rules for the intron/exon junctions are fulfilled for all exons, and an open reading frame is present starting in exon 1. Exon 8 encompasses the 3’ untranslated region of the corresponding mRNA. The polyadenylation site, which begins at 250 bp downstream of the stop codon, is of a commonly found type: AATAAA (Fig. 2). To compare hGSTK1 gene structure with the recently described mouse and rat GST Kappa genes (19), we analyzed the three sequences by the MULTIPIPemaker program. This program transformed the alignment into percent positional identity plots in the three sequences. Fig. 1C shows the positions of aligned regions over the entire gene sequence including 2 kb upstream from exon 1. This figure demonstrates a high conservation of genomic organization between the three genes. The degree of conservation in these three genes (exons and introns) is remarkably similar, except that the human sequence is longer than the rat and mouse in intron 5, which contains additional repeat sequences. In contrast, there is no homology in the 5’ flanking regions between human and rodent sequences.

5’-RACE PCR was performed to determine the transcription start site(s) of the human hGSTK1 gene. By using heart cDNA, amplification of a single fragment was obtained, indicating that the adenine residue marked +1 in Fig. 2 and localized 23 bp upstream of the ATG codon could be the transcription start site. The putative promoter region upstream from the start site possesses neither CCAAT nor TATA boxes but has a sequence similar to the initiator sequence (YYANT/AYY) overlapping the transcription initiation site (22). STAT and Sp1 transcription factors and a GC-rich region were also found in the proximal promoter (Fig. 2).

Quantitative Analysis of the GSTK1 Transcripts in Human Tissues by Real Time PCR—Tissue distribution and expression levels of hGSTK1 transcripts were investigated by real time PCR method. hGSTK1 mRNA was expressed ubiquitously, but at variable levels, in all tissues examined and being most abundant in kidney, liver, and the adrenal gland (Fig. 3).

hGSTK1 Amino Acid Sequence Analysis—Comparison of hGSTK1 amino acid sequence to the Swissprot subset of GenBank using the BLASTp program showed similarity to rat and mouse GST Kappa, a Caenorhabditis elegans GST and bacterial HCCA isomerase enzymes. Alignment of these sequences by using the CLUSTALW and BOXSHADE programs is shown in Fig. 4 (A and B), respectively. The amino acid sequence of hGSTK1 is similar to that of rat and mouse GSTK1...
(69.5 and 71.2% identity, respectively). Regarding alignment with other proteins, identity was 33% with C. elegans GST (YS21_CAEEL) and 24% with HCCA isomerases (NahD, PhnD, and NagD). Furthermore, the protein family data base of alignments (Pfam) analysis suggested that the hGSTK1 amino acid sequence shares a putative conserved domain with DSBA, an oxidoreductase involved in disulfide bond formation in the periplasm of Gram-negative bacteria. However, similarities between amino acid sequences of hGSTK1 and E. coli DSBA or V. cholerae DSBA is less than 20% (Fig. 4 C).

**FIG. 4. Amino acid sequence alignment of hGSTK1.** The identical residues are depicted in a black background, whereas similar residues are shown in gray shading. The sequence alignments were done using CLUSTALW, and BOX-SHADE was used to obtain the shaded schematic representation. The aligned sequences are listed below, followed by the species names and GenBank™ accession numbers in parentheses. A, hGSTK1 (Homo sapiens, Q9Y2Q3), rGSTK1 (Rattus norvegicus, S83436), mGSTK1 (Mus musculus, AA299096), and YS21_CAEEL (C. elegans, Q09652). B, hGSTK1 (H. sapiens, Q9Y2Q3), NAHD_PSEPU (Pseudomonas putida, Q51948), NAGD_RALSP (Ralstonia sp., Q92555), PHNDA_ALCFA (Alcaligenes faecalis, Q94747), and PHNDB_BURSP (Burkholderia sp., Q92555). C, hGSTK1, residues 6–210 (H. sapiens, Q9Y2Q3), DSBA-VIBCH, residues 40–190 (V. cholerae, P25557), and DSBA-ECOLI, residues 40–198 (E. coli, P24991).

**Heterologous Expression and Catalytic Properties of hGSTK1**—To purify hGSTK1, a recombinant protein containing an N-terminal CBP tag (CBP-hGSTK1) was overexpressed in E. coli. After affinity purification, hGSTK1 was produced by digestion of CBP-hGSTK1 with enterokinase. The apparent molecular masses of recombinant and native hGSTK1 were respectively 33.5 and 29.5 kDa as judged from SDS-PAGE (Fig. 5). Recombinant GSTK1 had low activities toward 1-chloro-2,4-dinitrobenzene, tert-butyl-hydroperoxide, cumene hydroperoxide, and 15-S-hydroperoxy-5,8,11,13-eicosatetraenoic acid. In contrast, activities toward 1,2-dichloro-4-nitrobenzene, ethacrynic acid, trans-nonenal, trans-4-phenylbut-3-en-2-one, and 1,2-epoxy-3-(4′-nitrophenoxyl)propane were not detectable (Table 1).
Subcellular Localization of hGSTK1—Analysis of hGSTK1 by the PSORT II program predicted a peroxisomal targeting signal at the C terminus, the tripeptide ARL, and a putative cleavage site for a mitochondrial presequence at the N terminus. To confirm the presence of hGSTK1 in peroxisomes, we prepared subcellular fractions of HepG2 cells. Because differential centrifugation does not yield totally pure organelle fractions, we used equilibrium density gradient centrifugation of the light mitochondrial fraction. This method separates cellular components according to their density, allowing the separation of mitochondria (median density of approximately 1.145 g/ml) and peroxisomes (the densest of the major subcellular organelles with \( \rho = 1.18-1.20 \) g/ml). Fig. 6 shows Western blot analysis of subcellular fractions of HepG2 cells using antibodies specific for hGSTK1, catalase, cytochrome c, and caspase 3. hGSTK1 was detected in peroxisomal and mitochondrial fractions but not in cytosol, whereas catalase, cytochrome c, and caspase 3 were detected, mainly, in peroxisomes, mitochondria and cytosol, respectively. To confirm the subcellular localization of hGSTK1 and the functionality of the peroxisomal targeting signal at its C-terminal end, plasmid constructs were prepared with the cDNA encoding the GFP protein fused in-frame to the N terminus of hGSTK1 (pEGFP-hGSTK1). This construct was co-transfected with pD-Red2-Peroxi (a mammalian expression vector that encodes a fusion of Discosoma sp. red fluorescent protein and the peroxisomal targeting signal 1) into HepG2 cells. Both the tagged protein EGFP-hGSTK1 and the red fluorescent peroxisomal protein were co-localized in peroxisomes (Fig. 7, A and B). By contrast, transfection of a construct lacking the tripeptide ARL (pEGFP-hGSTK1\(^{\text{ARL}}\)) showed a diffuse distribution of fluorescence in both nucleus and cytoplasm (Fig. 7, C and D).

**DISCUSSION**

The first Kappa class GST was described by Harris et al. (18), who isolated homodimeric GST13-13 (now named rGSTK1) from the rat mitochondrial matrix. Subsequently, a human cDNA was identified that exhibited a high level of identity with rGSTK1 (9). However, the hGSTK1 gene and protein have not been extensively characterized.

The gene encoding hGSTK1 is located on chromosome 7q34. Typically, the individual genes for each GST class are clustered together on the same chromosome (23–25). The fact that no other related sequences were found neither in this chromosomal region nor in other part of the genome seems to indicate that hGSTK1 is the only representative in this GST family in humans. The gene comprises eight exons and spans 5.68 kilobases. The size of the gene, its introns and exons lie within the range of other human GST genes studied to date. The coding region of the hGSTK1 gene shows 77% homology with mouse and rat Kappa class subunit cDNAs, and the deduced amino acid sequence shares 69 and 71% identity with mGSTK1 and rGSTK1, respectively. In addition, there is a high degree of nucleotide sequence identity not only in exons but also in introns between the human, rat, and mouse genes. The human cDNA sequence is also highly similar to expressed sequence tags identified in other species: Bos taurus, Sus scrofa, Gallus gallus, Xenopus laevis, and C. elegans (data not shown). Interestingly, hGSTK1 mRNA was found to be ubiquitously expressed, being found in all 24 human tissues (organs) analyzed, confirming and extending the recent observations of Jowsey et al. (19) showing expression of mGSTK1 and rGSTK1 in eight different tissues. The gene sequence conservation and the broad range of expression of hGSTK1, contrasting with many other GSTs that show distinct tissue-specific patterns of expression, suggest that hGSTK1 plays a fundamental role in cellular metabolism.

Analysis of the 5’-flanking region of the hGSTK1 gene showed that there are no significant similarities between the 5’-flanking sequences of human and rodent (rat and mouse) GSTK1, suggesting different regulatory mechanisms between human and rodent GSTK1 expressions. Human GSTK1 gene was further analyzed by determining the transcription start site. By using human heart total RNA and 5’-RACE PCR, we localized the transcription start site 23 bp upstream the methionine codon. Sequence analysis of the 5’-flanking region upstream of this site revealed that the hGSTK1 gene lacks both...

**TABLE I**

Activity of hGSTK1 towards various substrates

| Substrates                          | Substrate concentration | Specific activity (\( \mu \text{mole/min/mg} \)) |
|-------------------------------------|-------------------------|-----------------------------------------------|
| 1-Chloro-2,4-dinitrobenzene         | 1 mm                    | 1.05 ± 0.29                                   |
| 1,2-Dichloro-4-nitrobenzene         | 1 mm                    | ND                                            |
| 1,2-Epoxy-3-(4-nitrophenoxy)propane | 0.5 mm                  | ND                                            |
| 4-Phenyl-but-3-en-2-one             | 0.2 mm                  | ND                                            |
| Ethacrinic acid                     | 0.2 mm                  | ND                                            |
| t-Butyl-hydroperoxide               | 0.2 mm                  | 0.015 ± 0.001                                 |
| Cumene hydroperoxide                | 1.5 mm                  | 0.173 ± 0.06                                  |
| trans-Nonenal                       | 25 \( \mu \)m           | ND                                            |
| 15-S-Hydroperoxy-5,8,11,13-eicosatetraenoic acid | 20 \( \mu \)m           | 0.109 ± 0.07                                  |

The values are the means ± S.D. of at least three determinations. ND, not detectable (limit of detection, 0.01).
a TATA box and a CAAT sequence at the usual positions; nevertheless, a sequence present around the putative transcription start site (+1) has some similarity to an initiator element. This initiator sequence has been proposed to function as an alternate TFIIH-binding site in genes lacking a TATA box (22). An Sp1 site, which is important for the initiator activity, is present upstream (−163 to −172) of the transcription start site. Previously, the hGSTK4 gene has been shown to contain such an initiator element involved in its transcriptional activity (26).

On the basis of its amino acid sequence, hGSTK1 is recognized as part of the Pfam DSBA family, which is a subfamily of the thioredoxin family (27). The efficient and correct folding of bacterial disulfide-bonded proteins in vivo is dependent upon a class of periplasmic oxidoreductase proteins called DsbA, which catalyze disulfide bond formation during the folding of secreted proteins (27). This family contains a diverse set of proteins with a thioredoxin-like structure and is defined by an active site containing a CXXC motif (cysteines separated by two amino acids) and by a thioredoxin fold seen in the three-dimensional structure of the prototypical thioredoxin 1 of E. coli. The DSBA subfamily also includes HCCA isomerase enzymes, which catalyze one step in prokaryotic polycyclic aromatic hydrocarbon catabolic pathways (28–30). Interestingly, BLAST search showed that HCCA isomerases share the highest sequence similarity to Kappa class GSTs. HCCA isomerases are involved in naphthalene degradation and have been purified from *Pseudomonas sp.*, *Burkholderia sp.*, and *Sphingomonas yanoikuyae*, and it is noteworthy that HCCA isomerase needs glutathione as a co-factor to metabolize 2-hydroxychromene-2-carboxilate to trans-o-hydroxy-benzilidenepyrurate (31). Further analysis of amino acid sequence set hGSTK1 apart from the other members of the GST superfamily. Indeed, prediction of the secondary structure demonstrated distinct N-terminal and C-terminal motifs having βαβ and ββα arrangement, respectively (data not shown), which is similar to the DSBA fold but differing from the characteristic GST fold with an N-terminal αβ GSH-binding domain and a C-terminal domain composed entirely of α-helices (13). Furthermore, the sequence lacks the SNAIL/TRAiL (Ser-Asn-Ala-Ile-Leu/Thr-Arg-Ala-Ile-Leu) motif located 60–80 amino acids from the N terminus and found in all other classes of soluble GSTs. Finally, hGSTK1 does not bind to agarose-coupled glutathione (data not shown). Even so, some similarities are found between hGSTK1 and other GSTs. Although the secondary structure differs from other GST families, structural modeling using DSBA as a model demonstrates that hGSTK1 adopts a topology similar to the thioredoxin fold (data not shown). This structural modeling of hGSTK1 is confirmed by the recently published crystal structure of the rat GST Kappa in complex with glutathione (32). The structures of rGSTK1–1 and the canonical superfamily members indicate that the protein folds have diverged from a common thioredoxin/glutaredoxin progenitor but did so by different mechanisms (32). The authors also suggest that the Kappa class represents a fourth protein superfAMILY that supports GST activity. Similarities also concern its molecular mass (29.5 kDa) and, although it is low, activity toward substrates such as 1-chloro-2,4-dinitrobenzene, tert-butyl-hydroperoxide, cumene hydroperoxide, and 15-S-hydroperoxy-5,8,11,13-eicosatetraenoic acid.

Because rGSTK1 was originally isolated from the matrix of liver mitochondria (18), we investigated the subcellular distribution of the human class Kappa GST. Analysis of amino acid sequence of hGSTK1 by the PSORT II program predicts a PTS at the C-terminal end. To confirm this prediction, we used two

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**Fig. 6. Distribution of hGSTK1 during the density gradient centrifugation of a light mitochondrial fraction from HepG2 cells.** Peroxisomes and mitochondria were obtained from the light mitochondrial fraction by ultracentrifugation (180,000 × g for 3 h) in a self-forming density gradient of iodixanol. After centrifugation, eight fractions were collected, and an equal amount of protein from each fraction was prepared for SDS-PAGE and immunoblotting. Lane C represents the cytosolic fraction. Lane 1 represents the top (lightest fraction), and lane 8 represents the bottom (densest fraction) of the gradient. The blots were probed with antibodies for hGSTK1, catalase, cytochrome c, and caspase 3.

**Fig. 7. Intracellular localization of EGFP-hGSTK1 constructs in transfected HepG2 cells.** Forty-eight hours after co-transfection with the EGFP-hGSTK1 constructs and pDsRed2-peroxi vector, HepG2 cells were fixed and visualized directly using microscopy. Localization of hGSTK1 in fusion with EGFP in peroxisomes was confirmed by co-localization of pEGFP-hGSTK1 (A) and pDsRed-peroxi (B). HepG2 cells co-transfected with pEGFP-hGSTK1ΔARL (C) and pDsRed-peroxi (D).
complementary approaches: Western blot analysis with anti-hGSTK1 on HepG2 subcellular fractions obtained by density gradient and transfection of HepG2 cells with fluorescent recombining proteins. Western blot analysis showed the presence of hGSTK1 in peroxisomal and mitochondrial fractions, whereas no signal was observed in cytosol. Transfection of HepG2 cells with a plasmid construct containing the cDNA encoding the GFP fused to the N-terminal of hGSTK1 (pEGFP-hGSTK1) revealed an intense fluorescent signal in peroxisomes. By contrast, transfection of a construct coding for the recombinant protein lacking the N-terminal tripeptide ARL (pEGFP-hGSTK1ARL) showed a diffuse distribution of fluorescence in both nucleus and cytoplasm. This result clearly demonstrates the importance of the peroxisomal signal (ARL) for the transport of hGSTK1 from cytoplasm to peroxisomes. This is in accordance with literature describing peroxisomal proteins. These proteins are generally synthesized on free polysomes, and the post-translational targeting to peroxisomes is controlled by targeting signal sequences (33). Two types of targeting sequences have been characterized for peroxisomal matrix proteins, PTS1 and PTS2. PTS1 is the C-terminal tripeptide SKL or one of its variants such as ARL (33). Interestingly, this peroxisomal targeting sequence is found in Kappa GST from other species (i.e., rat, mouse, bovine, chicken, and C. elegans) suggesting that the presence of this GST in peroxisomes is conserved between species. The peroxisomal localization of rat and mouse GSTK1 has not been described in previous studies. However, the presence of GSTK1 in the mitochondrion has been demonstrated by using differential centrifugation, which does not allow the complete separation of peroxisomes and mitochondria. Thus, the isolation of rGSTK1 and mGSTK1 from mitochondrial isolates (34) does not preclude their presence in peroxisomes. Interestingly, two proteomic analyses performed on rat liver peroxisomes and human heart mitochondria, respectively, indicate the presence of a GSTK1 in rat peroxisomes (34), whereas a “GST13-13 homolog” was found in human heart mitochondria (35). Taken together, these data indicate that GST Kappa is present in both mitochondria and peroxisomes. The subcellular location of GSTK1 leads to question about its role(s) and substrate(s). Most peroxisomal functions are related to lipid metabolism including α- and β-oxidation of fatty acids that generate acetyl-CoA and acyl-CoA (36). A previous study has demonstrated binding properties of alpha class GSTs with fatty acyl-CoA and xenobiotic-CoA (37). Thus, it could be hypothesized that the class Kappa GSTs are also involved in such binding activities and play a role in the buffering system of acyl-CoA or xenobiotic-CoA. Another important aspect of peroxisomes is that they represent the main site in the cell where oxygen free radicals, hydroxyl radicals, and hydrogen peroxides are generated (38). Although peroxisomes contain enzymes (catalase and superoxide dismutase) involved in the detoxification of radical oxygen species, oxygen-based radicals still present a major route of lipid peroxidation and the consequent deleterious effects on membrane stability and function. Our results demonstrate a peroxidase activity of hGSTK1 toward three different substrates, tert-butyl hydroperoxide, cumene hydroperoxide, and 15-S-hydroperoxy-5,8,11,13-eicosatetraenoic acid. Therefore, GST Kappa might have an important role in detoxification of lipid peroxides generated in peroxisomes. Interestingly, these two hypothetical roles might also be found in mitochondria that are involved in lipid metabolism and produce reactive oxygen species.

In conclusion, our study describes the identification and the characterization of the human class Kappa GST. The similarities of hGSTK1 with prokaryotic enzymes suggest that the Kappa class GSTs are an early product of evolution. The conservation of gene sequence and the ubiquitous expression of the Kappa class GSTs in mammalian tissues suggest that the proteins have a critical role in eukaryotes. Furthermore, GST Kappa is the first human GST known to be localized in peroxisomes, whereas it is not detected in cytoplasm, strongly suggesting a new function for this family of enzymes.

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Gene and Protein Characterization of the Human Glutathione S-Transferase Kappa and Evidence for a Peroxisomal Localization

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