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Enzymatic Properties, Tissue-Specific Expression, and Lysosomal Location of Two Highly Homologous Rat SULT1C2 Sulfotransferases

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We have isolated two highly homologous but distinct rat sulfotransferase cDNAs termed ratSULT1C2 and ratSULT1C2A encoding polypeptides of 297 amino acids each. The amino acid sequence of ratSULT1C2 is 84% identical to the human SULT1C2 and 81% identical to a rabbit SULT1C2 sulfotransferase. ratSULT1C2 and ratSULT1C2A are 92% identical but differ in 22 amino acids. The majority of these amino acid substitutions in ratSULT1C2A is not found in the human and rabbit SULT1C2, which identifies ratSULT1C2 as the orthologue of these sulfotransferases, whereas SULT1C2A is a closely related but distinct enzyme. ratSULT1C2 and 2A sulfotransferases do not sulfonate steroids, dopamine, acetaminophen, or α-naphthol, but only *p*-nitrophenol. Prokaryotically expressed ratSULT1C2A is less active than ratSULT1C2. ratSULT1C2/2A mRNAs are abundant in kidney and less abundant in stomach and liver. The enzymes are expressed as 34-kDa polypeptides in rat kidney, liver, and stomach. In addition, a 28-kDa cross-reacting polypeptide is found in kidney only. Immunohistochemistry revealed expression of ratSULT1C2/2A in the epithelial cells of the proximal tubules of the kidney, bile duct epithelia, hepatocytes, and the epithelium of the gastric mucosal glands. Although the cDNA predicted amino acid sequence identifies both sulfotransferases as cytosolic enzymes, in tissue sections, in the kidney cell line NRK 52, and in transiently transfected BHK cells a considerable fraction of the enzyme was found in a granular perinuclear compartment. Costaining with a lysosomal marker in gastric mucosa tissue sections and cultured cells identifies these structures as lysosomes. © 2000 Academic Press

Key Words: **SULT1C2 sulfotransferase; hydroxyarylamine sulfotransferase; lysosomes.**

Sulfotransferases have a variety of important biological functions (1, 2). Some sulfotransferases are membrane-bound enzymes localized in the Golgi apparatus. Substrates of this class of sulfotransferases are glycosaminoglycans, sphingolipids and tyrosine residues of proteins passing through the Golgi apparatus (3). The cytosolic enzymes play important roles in the biotransformation of various endogenous compounds such as steroids and neurotransmitters (4). They are also of major pharmacological interest, since they are able to sulfonate therapeutic drugs and other xenobiotics. In general, sulfonation of these compounds results in increased water solubility and excretion of these compounds and thus promotes biological inactivation. In some cases, however, sulfonation results in the biological activation of such compounds to be carcinogenic or pharmacologically active (5). Given the diverse functions and substrate specificities it is not surprising that sulfotransferases comprise a complex gene family (6). cDNAs of numerous members of this gene family have been isolated during the last years. Based on amino acid sequence comparisons and substrate specificities, in mammalians two families represented by phenolsulfotransferases (designated SULT1) and hydroxysteroidsulfotransferases (SULT2) can be distinguished. The phenol sulfotransferase family can be further subdivided into phenol sulfotransferases

GenBank accession numbers for sulfotransferase cDNA sequences: ratSULT1C2, AJ238391; ratSULT1C2A, AJ238392.

Abbreviations used: SULT, sulfotransferase; GST, glutathione transferase; ORF, open reading frame; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; IPTG, isopropylthiogalactoside; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; BHK, baby hamster kidney.

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(SULT1A), dopa/tyrosine sulfotransferases (SULT1B), hydroxyarylamine sulfotransferases (SULT1C) and estrogensulfotransferases (SULT1E) (2, 6, 7).

Various sulfotransferases belonging to the SULT1C family have been described. A SULT1C1 sulfotransferase is responsible for sulfonation of hydroxyarylamines in rat liver (8, 9). Two human sulfotransferases termed SULT1C2 (10, 11, 12/termed SULT1C1 in Ref. 10 and ST1C2 in Ref. 12) and SULT1C sulfotransferase 2 (7) have been characterized recently. Initially it has been suggested (10) that the human SULT1C1 sulfotransferase (termed SULT1C2 in Ref. 11) is the orthologue of the rat SULT1C1 enzyme (9), although they display only about 60% amino acid identity. The recent cloning of a rabbit and human sulfotransferase (11) of the SULT1C family and comparisons of amino acid sequences has shown that the human enzyme previously termed SULT1C1 (10) is not the rat orthologue. To distinguish this new group of sulfotransferases they have been designated SULT1C2 (11). We also use this designation in this article.

Here we present data on the rat orthologue of the previously described human and rabbit SULT1C2 sulfotransferases (10–12) and describe a new distinct but highly homologous 1C2 sulfotransferase.

MATERIALS AND METHODS

Construction of a rat kidney cDNA library. Total RNA was isolated by homogenization of a rat kidney in guanidinoisothiocyanate and subsequent centrifugation through a cesium chloride cushion (13). The RNA pellet was redissolved and subjected to two cycles of poly(A)$^+$ RNA enrichment via chromatography on an oligo dT column (12). Five micrograms of poly(A)$^+$ RNA was reverse transcribed with Super Script TM reverse transcriptase (Life Sciences) with the protocol provided by the supplier. cDNA synthesis was primed with a primer consisting of 18 T and a *Not*I restriction site at the 5′ terminus (5′-AACCCGGCTCGAGCGGCCGCT$_{18}$). After completion of cDNA synthesis following standard protocols (14) an adapter with a *Bst*XI restriction site was ligated. Subsequently, cDNA was digested with Not I, generating fragments with a *Not*I restriction site at the 3′ terminus and a *Bst*XI site at the 5′ terminus. This allowed unidirectional cloning into the pcDNA1/Amp expression plasmid (Invitrogen) digested with *Bst*XI and *Not*I. Transfection of an aliquot of the cDNA/plasmid ligation into *Escherichia coli* DH 5α yielded a non amplified library of about 120,000 independent colonies.

Screening of the rat kidney cDNA library. Replica filters of the rat kidney cDNA library were hybridized for 24 h at 37°C in 6× SSC, 0.05% sodium pyrophosphate, 1% Denhardt's solution and 100 μg/ml of tRNA to a mixture of two degenerate oligonucleotides complementary to region IV conserved among various sulfotransferases. The oligonucleotides had the sequence 5′ GA(C/T) TGGAA(A/G)AA(C/T)CA(C/T) 3′ and 5′ GA(C/T)TGGAA(AG)AA(CT)AC(ACGT) 3′. They were labeled separately with polynucleotide kinase and γ-[^{32}P]ATP following standard protocols (13). For hybridization, equal amounts of the radioactively labeled oligonucleotides of comparable specific activity were mixed. Filters were washed twice for 15 min at 43°C in 2× SSC. More than 100 double-positive signals were obtained. 60 clones were replated, subjected to a second round of hybridization and isolated. Restriction enzyme digestion demonstrated that these plasmids could be divided into several groups most likely containing inserts of the same cDNA species. Among these plasmids we focussed on two, which gave the strongest hybridization signal at highest stringency. Inserts were sequenced by the dye terminator method and subsequently analyzed on a ABI 310 capillary electrophoreses DNA sequence analyzer.

Western blot analysis. After SDS–PAGE proteins were blotted onto a PVDF membrane using the semidry method (15). After blotting the gels were stained with Coomassie blue to control transfer efficiencies. Membranes were washed with 10 mM Tris/Cl, pH 7.4, 150 mM NaCl (TBS) and nonspecific binding sites were blocked for 60 min by incubation with TBS, 2% (w/v) dry milk. After additional washing with TBS, membranes were incubated with the primary antibody for 1.5 h at room temperature in TBS/1% dry milk (1: 2500 dilution). After removal of the primary antibody and additional washing, membranes were incubated for 1.5 h in TBS/1% dry milk with an peroxidase coupled α rabbit IgG antibody raised in goats (Dianova, dilution 1:1000). Blots were developed with an enhanced chemiluminescence kit (Amersham) following the protocols supplied by the manufacturer.

Tissues to be analyzed by Western blot were homogenized in ice-cold 10 mM triethanolamine, 20% glycerol, 1.5 mM dithiothreitol, 20 μg/ml phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin by several strokes with an ultra turrax homogenizer (16). Debris was removed by two centrifugations at 5000 rpm for 30 min and 45,000 rpm for 1 h. Supernatants were stored at −20°C.

Transient transfection of BHK cells. BHK (Baby hamster kidney) cells were maintained in DMEM supplemented with 2 mM glutamine, penicillin, streptomycin and 5% fetal calf serum. For transfection 2×10^5 cells were plated onto a 3.5-cm cell culture dish. Next day medium was removed, cells were washed with DMEM devoid of supplements. Two micrograms of plasmid DNA was mixed with 750 μl of DMEM containing 5 μl of Lipofectamine reagent (Life Sciences). After 30 min of incubation DNA/Lipofectamine complexes were added to the cells in a total volume of 1.5 ml. After 5 h of incubation Lipofectamine containing medium was removed. Since the rat kidney cDNA library was generated in the eukaryotic expression vector pcDNA1/Amp (Invitrogen) the plasmid containing the ratSULT1C2 cDNA insert isolated from the library was used for transient transfections. The pcDNAI/Amp plasmid containing the insert of ratSULT1C2A was not used directly in transfection experiments, because it contained extensive 5′ and 3′ untranslated sequences. The ORF of ratSULT1C2A was amplified from a oligo(dT) primed rat kidney cDNA with two oligonucleotides located immediately upstream and downstream the ORF. The sequence of these oligonucleotides are 5′-CTGAATTCAGGCTTGCAGAC-3′ and 5′-GGACTAGATCTTCTTCCCTG-3′. The PCR fragment was digested with restriction endonucleases *Eco*RI and *Bgl*II and was ligated into a *Eco*RI and *Bam*HI cut pcDNA3 expression vector (Invitrogen). This vector was used in transient transfection experiments.

Measurements of sulfotransferase activity. In general, sulfotransferase activity was measured in 50 mM Tris/Cl, pH 7.4, 7 mM $MgCl_2$, and 20 μM [^{35}S]PAPS (3 Ci/mmol) in a total volume of 20 μl. Incubation with various substrates was for 1 h at 37°C. The reaction was terminated by the addition of 10 μl acetonitrile. Three microliters of the reaction was applied to a cellulose thin layer chromatography which was developed in propanol/ammonia/water (6:1:3) (9, 17). Subsequently, the plate was dried and analyzed with a Fuji Bioimager.

Synthesis of [^{35}S]PAPS. Radioactively labeled PAPS was synthesized with yeast extracts as described (18). Briefly, 200 μl of yeast extract (1 mg/ml) was incubated with 200 μl of [^{35}S]H_2SO_4 (Hartmann Analytics/1400 Ci/mmol) in 100 mM Tris/Cl, pH 8.5, 20 mM ATP, 8 mM $MgCl_2$ for 90 min at 37°C in a total volume of 500 μl. The reaction was terminated by heating for 3 min at 95°C. The reaction was diluted fivefold with water and applied to a 5 ml DEAE Sephacel column equilibrated with 14 mM $(NH_4)_2CO_3$. [^{35}S]PAPS was eluted with a gradient of 14 to 700 mM $(NH_4)_2CO_3$. Fractions containing radioactivity were pooled, freeze dried and redissolved in H_2O. This final preparation was adjusted to a specific activity 3 Ci/mmol by the addition of unlabeled PAPS.

Expression of ratSULT1C2 and ratSULT1C2A in prokaryotic cells. To allow the examination of the ratSULT1C2 and ratSULT1C2A enzymes as nonfused polypeptides both cDNAs were amplified with proof reading Pfu polymerase (Stratagene) from the original pcDNA1/Amp expression vector using the primers SULT-*Nco*I (5′-CCCTGAGACACCATGGCCCTG-3′) and SULT1C2C (5′-CTGAATTCAGGCTTGCAGAC-3′). The amplified fragments were digested with restriction endonucleases *Nco*I and *Eco*RI and ligated into the *Eco*RI and *Nco*I digested expression vector pTrc99A (Pharmacia Biotech). The position of the *Nco*I restriction site provides the initiation codon in the correct position. *E. coli* DH5α were transformed with both vectors. The bacteria were grown to an optical density of 1.0. Synthesis of sulfotransferases was induced by the addition of 0.5 mM IPTG for 2 h. The bacteria were collected by centrifugation at 600*g* for 10 min. The pellet was resuspended in 25 mM Tris/Cl, pH 7.5/5 mM EDTA and sonicated three times for 20 s at maximal output. Debris was removed by centrifugation for 10 min at 14,000*g* and the supernatants were assayed for sulfotransferase activity.

To allow the expression as a glutathione transferase (GST) fusion protein the open reading frame of ratSULT1C2 was amplified with two primers (5′-CCCTGAGAGATCTTGGCCCTG-3′ and 5′-CTGAATTCAGGCTTGCAGAC-3′) located at the 5′ and 3′ end of the ORF of the ratSULT1C2 cDNA. The amplified fragment was digested with *Bgl*II and *Eco*RI and ligated into the expression vector pGEX3X (Pharmacia Biotech). Transformation of *E. coli* DH5α allowed the expression of the GST-ratSULT1C2 fusion protein. Expression was induced by the addition of IPTG, cells were disrupted by sonication and the fusion protein was purified on a glutathione–Sepharose affinity column (Pharmacia Biotech) following the instructions provided by the manufacturer.

Generation of a polyclonal antiserum. Three hundred micrograms of purified GST-ratSULT1C2 fusion protein was used to immunize a rabbit. The antiserum obtained was absorbed on a GST affinity column to remove antibodies directed against the GST part of the fusion protein. For that purpose bacteria were transformed with a plasmid pGEX3X expressing only GST. GST synthesis was induced and GST was purified from the bacterial homogenates via glutathione–Sepharose affinity chromatography. The purified GST protein was coupled to a solid matrix (Affigel 10/Bio-Rad) following the instructions provided by the manufacturer. Aliquots of the α GST-ratSULT1C2 antiserum were passed over that column. Removal of α GST antibodies was monitored via Western blots of GST protein with the breakthrough of the column. The majority of α GST antibodies could be removed, but a low reactivity toward GST was still detectable in the breakthrough.

Immunohistochemistry. The antiserum was diluted in 0.75% BSA–PBS at a titer of 1:100 and applied to acetone-fixed cryosections for 1 h at RT after previous blocking of unspecific protein binding sites with 0.75% BSA–PBS without antiserum. Detection of bound primary antibody was performed by using biotinylated anti-rabbit IgG (Vector, Burlingame, U.S.A.) followed by avidin-biotin complexes tagged with horseradish peroxidase. Alternatively, the tyramide signal amplification technique using coumarin tyramide for fluorescence visualization was employed (reagents from NEN-DuPont). For double labeling of tissue sections antibodies to ratSULT1C2 were detected by Cy3-labeled anti rabbit IgG and antibodies to the lysosomal marker antigen lamp-1 by using biotinylated anti-mouse IgG followed by FITC-labeled avidin. The α Lamp-1 and anti-lysosomal acid phosphatase antiserum were kindly provided by P. Saftig (Göttingen).

RESULTS

Isolation of ratSULT1C2 and ratSULT1C2A Sulfotransferase cDNAs

Cytosolic sulfotransferases contain four highly conserved amino acid sequences (6, 19). We have designed two oligonucleotides corresponding to region IV containing the highly conserved amino acid sequence RKGxxGDWKNxFT. The radioactively labeled oligonucleotides where hybridized to a nonamplified rat kidney cDNA library (see Materials and Methods). Among the double positive clones we identified a plasmid containing an insert of 1271 bp strongly hybridizing to the oligonucleotides mentioned above. Sequencing of the insert revealed an ORF of 891 bp flanked by 28 bp of 5′ UTR and 352 bp of 3′ UTR (Fig. 1, top). A polyadenylation signal was found at position 1235 followed by a brief poly A tail further downstream. This cDNA was termed ratSULT1C2. Because the ratSULT1C2 cDNA probe hybridized also to a 3.5-kb mRNA species in Northern blots (see below) we screened 50 double-positive clones remaining from the initial hybridization experiment for plasmids hybridizing to the ratSULT1C2 cDNA and having inserts larger than 3 kb (data not shown). One such plasmid with an insert of 3.5 kb was isolated. Sequencing revealed an ORF of 891 bp, a 5′ UTR of 1189 bp and a 3′ UTR of 1545 bp. This cDNA was termed ratSULT1C2A. The 5′ UTR, however, contained a sequence duplication and inversion: The 1098 bp most 5′ prime sequences are identical to the 3′ terminal 1098 bp in reversed orientation (Fig. 1, top). This represents most likely a cloning artifact.

The cDNA predicted amino acid sequence of the ORF of ratSULT1C2 cDNA is different from ratSULT1C2A. Compared to the ratSULT1C2 sequence the ratSULT1C2A ORF contains 33 nucleotide exchanges predicting 22 amino acid substitutions (see Fig. 1, bottom). The first 28 bp of the 5′ UTRs of both sulfotransferases are also similar, whereas except for the first 7 nucleotides the 3′ UTRs display little sequence similarity. Since the ratSULT1C2A plasmid obviously contains a cloning artifact we amplified the ratSULT1C2A ORF from rat kidney cDNA to obtain independent evidence for the authenticity of the ratSULT1C2A ORF sequence. The DNA sequence of the amplified fragment was identical to the ORF sequence of ratSULT1C2A in the plasmid isolated from the library, proving that the nucleotide exchanges are not due to cloning artifacts (not shown).

Sequence comparisons of the cDNA predicted amino acid sequence of ratSULT1C2 and ratSULT1C2A revealed homologies to several previously described cytosolic sulfotransferases (Table 1). Comparison of the cDNA predicted amino acid sequence revealed 84% amino acid identity to human sulfotransferases SULT1C2 sulfotransferase 1 (10, 11, 12/termed SULT1C1 in Ref. 10 and ST1C2 in Ref. 12) and 81% to a rabbit SULT1C2 sulfotransferase (10).

Northern Blot Analysis

The ratSULT1C2 cDNA insert was used as a probe in Northern blot analysis of RNA isolated from several

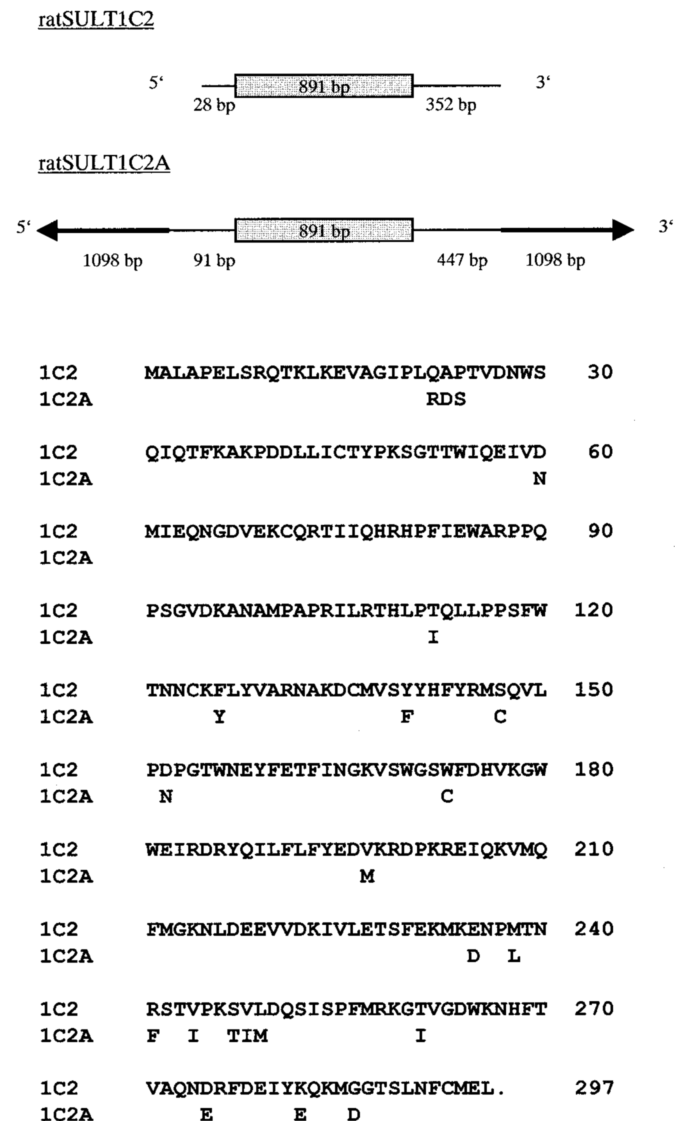

FIG. 1. Structure and predicted amino acid sequence of the ratSULT1C2 and ratSULT1C2A sulfotransferase cDNAs. On top the ratSULT1C2 and ratSULT1C2A cDNAs are shown schematically. The 5′ and 3′ UTRs are indicated by lines, the open reading frame as a gray bar. The sequence duplication in ratSULT1C2A is depicted by an arrow. Size of fragments is indicated in bp. Sequences are available under Accession Nos. AJ 238391 and AJ 238392. At the bottom the cDNA predicted amino acid sequence of ratSULT1C2 and ratSULT1C2A are shown. The ratSULT1C2 sequence is shown on top and the amino acid substitutions found in ratSULT1C2A are indicated in the bottom line.

rat tissues (Fig. 2). Two mRNA species of 1.3 and 3.5 kb hybridized even under high stringency washing conditions. In all tissues the 1.3 kb mRNA species is less frequent than the 3.5 kb mRNA species. For normalization the blot was rehybridized with an actin cDNA probe. Quantification of the signal with a Fuji Bioimager revealed that the ratSULT1C2/2A mRNA is highly abundant in kidney and that lower levels are found in liver and stomach. The mRNAs are not expressed in brain, testis, muscle, lung, intestine, and heart.

Enzymatic Properties of ratSULT1C2 and ratSULT1C2A Sulfotransferases

We have examined some enzymatic properties of the sulfotransferases encoded by the ratSULT1C2 and ratSULT1C2A cDNAs. For that purpose the enzymes have been expressed in prokaryotic and eukaryotic cells. In initial experiments we have expressed the ratSULT1C2 cDNA as a GST fusion protein (GST-ratSULT1C2) in bacteria and have purified the GST-ratSULT1C2 fusion protein by affinity chromatography. 0.5 μg of purified fusion protein was incubated with various substrates and [^{35}S]PAPS for 60 min at 37°C. Reaction products were analyzed by thin-layer chromatography. To ensure that assay conditions allow the detection of sulfonation of the substrates, a cytosolic rat liver homogenate was prepared, which was used as a positive control with all substrates tested.

We have examined *p*-nitrophenol, α- naphthol, dehydroepiandrosterone, β-estradiol, androsterone, acetaminophen, cholecalciferol, and dopamine, each at 10 μM and 1 mM substrate concentration (data not shown). Of these substrates only p-nitrophenol was sulfonated by the GST-ratSULT1C2 fusion enzyme. The K_m of the GST-ratSULT1C2 fusion protein toward *p*-nitrophenol was determined to be 4.2 mM (data not shown). To exclude that the restricted substrate specificity was not an effect of structural alterations due to the GST fusion process, we have expressed ratSULT1C2 and ratSULT1C2A as non-fused enzymes in bacteria and transiently in eukaryotic BHK cells. Enzyme assays with all substrates mentioned above have been performed with whole cell homogenates. Mock transformed or transfected controls were run in parallel. Again, prokaryotically expressed enzymes ratSULT1C2 and ratSULT1C2A sulfated *p*-nitrophenol only. In homogenates containing prokaryotically expressed ratSULT1C2A sulfonation activity toward *p*-nitrophenol was diminished compared to homogenates containing ratSULT1C2 (data not shown).

In homogenates of eukaryotic BHK cells transiently transfected with a plasmid expressing the ratSULT1C2 cDNA, sulfonation of p-nitrophenol, but not with any of the other substrates mentioned above could be detected. Cells transfected with the ratSULT1C2A plasmid always expressed little cross reacting material, although transfection efficiencies were comparable to ratSULT1C2 transfections. The amounts were insufficient to demonstrate sulfonation activity (Fig. 3, bottom left).

Characterization of SULT1C2/2A Sulfotransferase Expression by Western Blot Analysis

The fusion protein GST-ratSULT1C2 has been used to generate a polyclonal antiserum in rabbits. An ali-

TABLE 1

Comparison of cDNA Predicted Amino Acid Sequences of ratSULT1C2 and 2A and Various Sulfotransferases

	RatSULT1C2	RatSULT1C2A	Rabbit SULT1C2	Human SULT1C2	RatSULT1C1	Human SULT1C#2	Mouse EST	Human HSST
RatSULT1C2	—	92	81	84	61	60	46	35
RatSULT1C2A	92	—	78	80	59	61	47	35

Note. ratSULT1C2 and 2A amino acid sequences were aligned with the cDNA predicted amino acid sequence of human SULT1C2 sulfotransferase (11), human SULT1C sulfotransferase 2 (SULT1C#2) (7), rabbit SULT1C2 sulfotransferase (11), ratSULT1C1 sulfotransferase (9), murine estrogensulfotransferase (EST, 20), and a human hydroxysteroidsulfotransferase (HSST, 21). Numbers give the percentages of identical amino acid residues.

quot of this antiserum was absorbed on an affinity column containing GST only, to remove antibodies directed against the GST portion of the fusion protein. This absorbed antiserum was used in the following experiments.

Homogenates of different organs of rat and mouse were subjected to SDS–PAGE and subsequent Western blot analysis (Fig. 3, top). This revealed a polypeptide of 34 kDa in liver, kidney and stomach homogenates of rat and mouse, except that no signal was obtained in mouse liver homogenates. Specifically in kidney of both species an additional 28-kDa polypeptide was detectable. Cross reacting material other than these polypeptides was not detected demonstrating the specificity of the antiserum. To exclude that the 28-kDa protein detected in kidney homogenates was due to proteolysis during preparation, the homogenates were prepared in the absence and presence of protease inhibitors. Aliquots of these homogenates were incubated for up to 14 h at 37°C and analyzed by Western blot analysis. The absolute and relative amounts of the 28- and 34-kDa polypeptide remained unchanged independent of the absence or presence of protease inhibitors and incubation at 37°C (data not shown). This excludes proteolytic generation of the 28-kDa polypeptide after homogenization of kidney tissue.

Western blot analysis of homogenates of bacteria expressing ratSULT1C2 and ratSULT1C2A cDNAs revealed a protein of 34 kDa in case of 1C2 and 28 kDa in case of 1C2A, whereas Western blots of transiently transfected BHK cells revealed a 34-kDa polypeptide in both cases (Fig. 3).

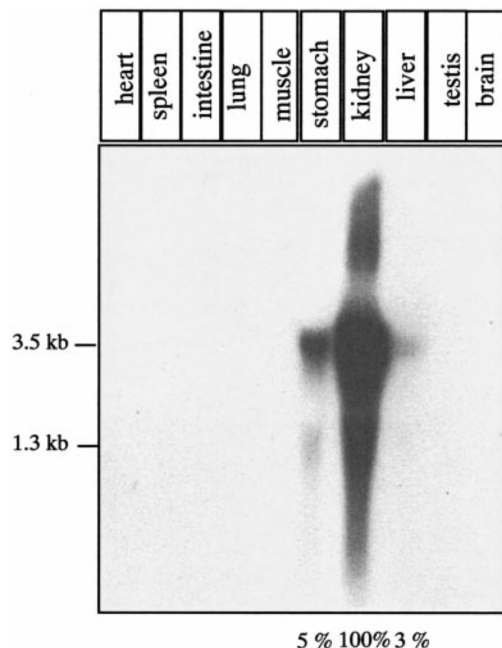

FIG. 2. Northern blot analysis of various rat tissues. RNA was isolated from various rat tissues and subjected to Northern blot analysis using the entire ratSULT1C2 cDNA as a probe. Subsequently, the blot was hybridized with an actin probe cDNA and all signals were quantified with a Fuji Bioimager. Numbers below the lanes give the actin normalized signals with kidney taken as 100%.

Immunohistochemistry of ratSULT1C2/SULT1C2A Sulfotransferases

Cryosections of rat kidney, stomach and liver were incubated with the α ratSULT1C2 polyclonal antiserum (Figs. 4A, 4B, and 4D). Within the kidney, the strongest immunoreactivity was present in epithelial cells of the proximal tubules and faintly also in distal tubules. In contrast, kidney glomerula were completely devoid of ratSULT1C2/2A. In the stomach, ratSULT1C2/2A immunoreactivity was seen in epithelial cells of mucosal glands, the parietal cells displaying the highest intensity. No immunoreactivity was detected in other parts of the gastrointestinal tract. In liver, bile duct epithelia labeled strongly and hepatocytes weakly. In addition to the restriction of ratSULT1C2/2A immunoreactivity to the epithelial cell types in all three tissues, a further common denominator was the punctate staining pattern within the cells indicative of a vesicular localization of ratSULT1C2/2A (see Fig. 4B).

BHK cells were transiently transfected with pcDNA1/Amp or pcDNA3 expression plasmids containing the ratSULT1C2 and ratSULT1C2A cDNA. 48 hours after transfection cells were fixed and incubated with the α

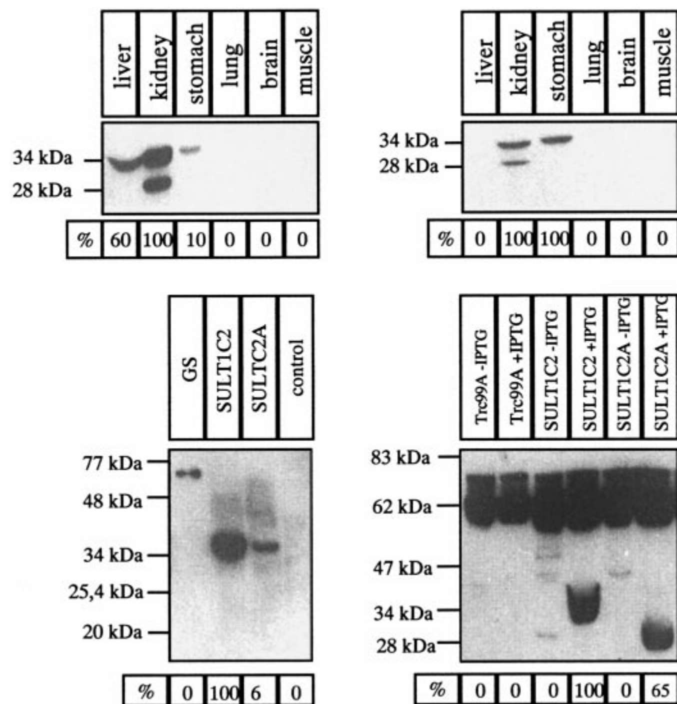

FIG. 3. Western blot analysis. (Top left) Twenty micrograms of homogenates of the tissues indicated were subjected to SDS–PAGE and Western blot analysis. The membranes were incubated with the α ratSULT1C2 antiserum preabsorbed on a GST affinity column. The primary antibody was visualized with an α rabbit peroxidase coupled antibody and the chemiluminescence kit from Amersham. Numbers below the lanes give the relative amounts of cross reacting material. Signals were quantified by densitometry and the signal of kidney was taken as 100%. (Top right) Same as top left but homogenates were prepared from mouse tissues. (Bottom left) BHK cells were transiently transfected with the pcDNA1/Amp-ratSULT1C2 and pcDNA3-ratSULT1C2A plasmid. In the control lane, cells were transfected with pcDNAI/Amp without insert. Forty-eight hours after transfection cells were harvested and homogenates were subjected to Western blot analysis. The signals were quantified by densitometry. Numbers on the left give the molecular weight standards. Lane GS contains 20 ng of purified GST-ratSULT1C2 fusion protein. (Bottom right) DH5α *E. coli* were transformed with Trc99A expression plasmid without insert or with the ratSULT1C2 and ratSULT1C2A insert. In lanes −IPTG protein expression was not induced, in lanes +IPTG expression was induced. Ten micrograms of bacterial extracts was subjected to Western blot analysis as described earlier. Aliquots of these extracts were used in the experiments shown in Fig. 3. Numbers on the left give the molecular weight standards.

ratSULT1C2 antiserum. The primary antibody was visualized with a fluorescently labeled secondary antibody. A fine reticular staining pattern throughout the entire cells was visible and in the majority but not all cells a perinuclear granular staining was detected (Fig. 4K). NRK 52 rat kidney cells showed the same granular staining pattern (Fig. 4H). Since this staining pattern strongly suggested a lysosomal localization, cells were also stained with an antiserum directed against lysosomal acid phosphatase (Fig. 4I). These double labeling experiments revealed an almost complete overlap in the distribution of ratSULT1C2/2A and the lysosomal acid phosphatase (Figs. 4H, 4I, and 4J).

To confirm that the sulfotransferase also colocalizes with lysosomes in tissues sections of the stomach were also stained with a ratSULT1C2 antiserum and an antiserum against the lysosomal membrane protein Lamp-1 (Figs. 4E and 4F). For both antigens the most intense immunoreactivity is found in the epithelial cells of the gastric glands. Digital image subtraction of ratSULT1C2 and Lamp-1 immunoreactivity revealed a substantial overlap of the distribution of both antigens and proves the lysosomal localization of the sulfotransferase *in vivo.*

DISCUSSION

We have isolated the cDNAs of two highly homologous sulfotransferases from a rat kidney cDNA library. Both cDNAs display an ORF of 891-bp predicting polypeptides of 297 amino acids. Comparison of the cDNA predicted amino acid sequences with those of previously described sulfotransferases revealed a high degree of amino acid residue identity (84%) with rabbit and human sulfotransferase termed humSULT1C2 (10–12). Thus, we have isolated the rat orthologue of these previously reported cDNAs.

So far it has been unknown that at least in rats there are two sulfotransferases of this group, which are 92% identical but differ in 22 amino acids. If previously published cDNA predicted amino acid sequences of human SULT1C2, ST1C2 and rabbit SULT1C2 sulfotransferase are compared with respect to the amino acid residues different between ratSULT1C2 and ratSULT1C2A, it becomes obvious that these previously described sulfotransferases are the orthologs of ratSULT1C2 but not of ratSULT1C2A. At these positions human SULT1C2 displays 13 amino acids identical to ratSULT1C2, but only one identical to ratSULT1C2A. Comparisons with the rabbit SULT1C2 sulfotransferase give similar results.

Eight of the 22 amino acid substitutions are not conservative. In comparison, charged amino acid residues are introduced at position Q22R, A23D, and G286D, charge is lost at positions D60N, D152N, and R241F and a reversion of charge is found at position K282E. This suggest that the two enzymes may *in vivo* serve different functions.

Comparison of amino-acid sequences with rat SULT1C1 (9), estrogen (20), and hydroxysteroid sulfotransferases (21) revealed that ratSULT1C2 and ratSULT1C2A have about 60% amino acid identity to rat SULT1C1 hydroxylaminesulfotransferase and a murine phenolsulfotransferase, 46% amino acid identity to a murine estrogensulfotransferase and only about 35% identity

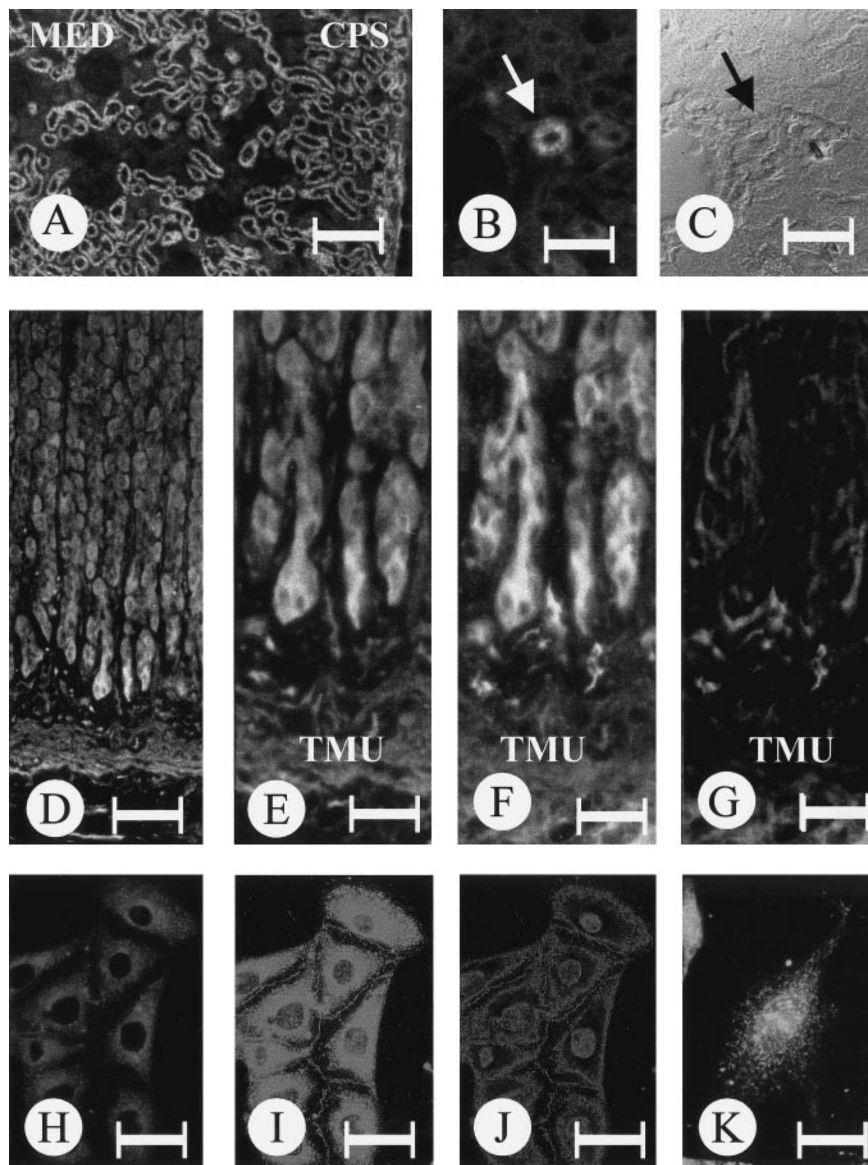

FIG. 4. Immunohistochemistry of ratSULT1C2/2A. The figure shows immunohistochemical staining for sulfotransferase cross-reacting material of rat kidney (A), rat liver (B), rat stomach (D), NRK 52 cells (I) and BHK cells transfected with the ratSULT1C2 cDNA (K). C shows a phase contrast picture of B, the arrow depicts a bile duct. F and G demonstrate the distribution of ratSULT1C2 (E) and Lamp-1 (F) in the adult rat mucosa at higher resolution. Digital image subtraction (E minus F) is shown in G. H shows rat kidney NRK 52 cells stained with the α ratSULT1C2 antiserum and in part I these cells were stained with an antiserum against the lysosomal acid phosphatase. Digital subtraction (H minus I) is shown in J. K shows BHK cells transfected with the ratSULT1C2 cDNA and stained with the α ratSULT1C2 antiserum. MED, medulla; CPS, capsule; TMU, tunica muscularis. Bars: A, 400 μm; D, 200 μm; B, C, E, F, G, 50 μm; H to K, 5 μm.

to a human hydroxysteroidsulfotransferase. Thus, based only on amino acid comparisons ratSULT1C2 and 2A cannot be grouped unambiguously into one of the existent subfamilies (6, 10, 11).

It has been suggested that the human SULT1C1 sulfotransferase (10, 11, 12/termed SULT1C2 in Ref. 11 and ST1C2 in Ref. 12) is the orthologue of rat SULT1C1 sulfotransferase (9). The ratSULT1C2 shares more amino acids with the human SULT1C1 sulfotransferase (10) than with the rat SULT1C1 sulfotransferase, which indicates that the previously described human enzyme is the orthologue of ratSULT1C2 and not of ratSULT1C1. This in accordance with conclusions drawn from the characterization of the rabbit and human SULT1C2 sulfotransferases described recently (11).

We have investigated some enzymatic properties of the SULT1C2/2A sulfotransferases. P-nitrophenol was the only substrate accepted by ratSULT1C2 and 2A. This in accordance with investigations performed with the human and rabbit SULT1C2 sulfotransferases. ratSULT1C2 and 2A differed with respect to the turn-

over of p-nitrophenol. Homogenates of bacteria expressing ratSULT1C2A had a lower sulfonation activity than homogenates of bacteria expressing ratSULT1C2. Normalization of the sulfonation activities in the bacterial homogenates for ratSULT1C2 and 2A cross reacting material suggests, that the specific activity of ratSULT1C2 is about 20 times higher than that of ratSULT1C2A. This may be due to either proteolytic processing of ratSULT1C2A in bacteria to a 28-kDa polypeptide (Fig. 3, bottom right) or to the amino acid differences between the two sulfotransferases.

In homogenates of BHK cells expressing ratSULT1C2A sulfonation activity could not be detected, which is most likely due to the low amounts of cross reacting material (Fig. 3). The amount of cross reacting material of ratSULT1C2 and 2A in these extracts differed by a factor of about 20. We have not been able to express ratSULT1C2A in BHK cells to an extent, which would allow reliable examination of enzymatic properties. Thus, it needs further investigation whether the eukaryotically expressed ratSULT1C2 and 2A enzymes also differ in their specific activities.

Northern and Western blot analysis of rat tissues revealed expression of ratSULT1C2/2A in kidney, liver and stomach. In murine tissues SULT1C2/2A cross-reacting material was only detected in kidney and stomach. The human homologous SULT1C2 (10, 11) is expressed predominantly in kidney and in stomach and thyroid gland, the latter has not been examined in this study. Human SULT1C2 is not expressed in adult liver but in fetal liver (11). Thus, the lack of expression in adult human liver may resemble the expression pattern in murine tissues. In rabbits SULT1C2 immunoreactive polypeptides were shown in liver, kidney and throughout the intestinal tract (10), whereas in rats expression in the gastrointestinal tract is restricted to the stomach.

In Northern blots two mRNA species of 1.3 and 3.5 kb can be detected, the amount of the large species exceeds by far that of the small mRNA species. These mRNAs are particularly abundant in kidney. When normalized for actin, liver and stomach show only 3 and 5% of the signal compared to kidney, respectively. The size of the ratSULT1C2 cDNA corresponds to the size of the small 1.3 kb mRNA species. The ratSULT1C2A cDNA isolated from the library has a size of 3.5 kb, which is close to the size of the large mRNA species. However, the most 3′ 1098 bp of this cDNA were duplicated and occurred in reverse orientation at the 5′ end. This most likely represents a cloning artifact, so that the size of the cDNA cannot be correlated with the 3.5 kb mRNA species. The 3′ end of this cDNA seems to be authentic, since it contains a polyadenylation signal, but part of the 5′ untranslated sequence may have been replaced by the duplication. However, even if the 5′ prime duplicated sequence is not included in the calculation, the size of the SULT1C2a mRNA exceeds that of the SULT1C2 mRNA, which suggests that the 3.5-kb mRNA species represents the ratSULT1C2A mRNA.

Specifically in kidney a 28-kDa polypeptide in addition to the 32-kDa was detectable. We have ruled out that this polypeptide is due to proteolytic cleavage after tissue homogenization. Interestingly, when ratSULT1C2A is expressed in prokaryotic cells it also appears as a 28 kDa polypeptide. Since a 34-kDa peptide is predicted by the cDNA sequence and is detected when ratSULT1C2A is expressed in BHK cells the loss of 6 kDa may be explained by limited proteolysis. Perhaps proteases present in bacteria and specifically in kidney process ratSULT1C2A to the 28-kDa polypeptide. The alteration of charges at several amino acid positions in comparison to ratSULT1C2 may render ratSULT1C2A susceptible to limited proteolytic cleavage.

Immunohistochemical examination of rat tissue cryosections revealed expression of ratSULT1C2/2A in the proximal tubules, bile duct epithelia, hepatocytes and mucosal gland cells in the stomach. Except for kidney, liver and stomach, in rabbits SULT1C2 immunoreactivity could also be demonstrated in the jejunum, colon, cecum and rectum. Thus, there seem to be species specific differences in the SULT1C2 expression pattern. As yet, the functional role of the enzymes in these cells remains enigmatic. The implication of the respective cells in absorptive or secretive processes could hypothetically lend support to a role in the modification of the respective metabolites.

Immunohistochemical examination of the distribution of ratSULT1C2 and 2A yielded the most interesting results. ratSULT1C2 and 2A do not seem to be primarily located in the cytoplasma but in large perinuclear structures. This applies for transfected BHK cells, the rat kidney cell line NRK 52 and tissue sections. Due to the low resolution of cryosections the granular staining pattern was less clearly seen in tissue sections (see Fig. 4B).

Since the staining pattern was reminiscent for lysosomes, we performed costaining experiments in tissue sections with an antibody directed against the lysosomal membrane protein Lamp-1. This revealed substantial overlap of the Lamp-1 and ratSULT1C2 staining pattern identifying these structures as lysosomes. Digital subtraction of the distribution pattern of ratSULT1C2 and Lamp-1 cross-reacting material in gastric gland parietal cells demonstrated a considerable overlap, but clearly the Lamp-1 distribution extends farther into the periphery of the cells. This, demonstrates that ratSULT1C2 is not present in all, but only in a fraction of lysosomes. The same applies for costaining of cultured rat kidney NRK 52 cells with lysosomal acid phosphatase. This enzyme can be detected even in the periphery of the cells, whereas the distribution of ratSULT1C2 is more perinuclear.

Further studies will have to focus on a detailed description of the subcellular localization of these enzymes and the search for their physiologic substrates.

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