Identification of a new subfamily of sulphotransferases: cloning and characterization of canine SULT1D1

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Sulphation is an important conjugation pathway in drug metabolism that has been studied in several species including humans. However, few studies have been performed using the dog as a subject. In this report we describe the cloning and characterization of a canine cytosolic sulphotransferase (SULT). The overall primary structure of this enzyme is very similar to that of a rat phenol-sulphating enzyme found in the EMBL Database and to a mouse SULT termed amine-N-sulphotransferase (81 % identity). The expressed canine SULT conjugates small phenols and aromatic amines such as dopamine, minoxidil, *p*-nitrophenol and 5-hydroxytryptamine, but not dehydroepiandrosterone or β-oestradiol. These results are in agreement with the results reported for the mouse SULT. In contrast with the mouse enzyme, the canine SULT does not conjugate eicosanoid compounds, i.e. prostaglandins, thromboxane B_2 or leukotriene E_4. The canine SULT is expressed at high levels in the colon of both genders; it is also expressed in the small intestine, kidney and liver. Furthermore, because the canine, mouse and rat SULT forms exhibit significant sequence identity (more than 80 %), they seem to represent a distinct group in the SULT family tree. This suggestion is strengthened by the low identity with other SULTs. The subfamily that is most similar to this new group is SULT1A, with approx. 60 % similarity. However, the mouse and canine enzymes are not characterized by the efficient sulphation of *p*-nitrophenol, dopamine, β-oestradiol or oestrone. Thus these results seem to exclude them from the SULT1A subfamily. We therefore propose a new subfamily in the phenol SULT family, designated SULT1D, and consequently the canine enzyme is termed SULT1D1.

Key words: cytosol, dog, sulphation.

INTRODUCTION

Sulphation is an important phase II conjugation pathway for xenobiotics, endogenous compounds and drugs, generally leading to an increase in hydrophilicity and thereby facilitating increased excretion of the conjugated molecules. In addition, sulphation has an important role in the synthesis and metabolism of steroid hormones as well as in the generation of reactive electrophilic forms of mutagenic heterocyclic amines and hydroxymethyl polyaromatic hydrocarbons [1]. Characterization of the mammalian cytosolic sulphotransferases (SULTs) has led to their classification into two subfamilies, the phenol SULTs and the hydroxysteroid SULTs [2]. In general, the phenol SULTs are responsible for the sulphation of small phenolic compounds such as naphth-1-ol, acetaminophen, dopamine and *p*-nitrophenol (PNP), whereas the hydroxysteroid SULTs conjugate steroids such as dehydroepiandrosterone (DHEA) and pregnenolone. However, the SULTs in general have broad overlapping substrate reactivities and many substrates such as β-oestradiol (E_2) and genistein are sulphated by members of both families. Also, almost all of the SULTs are capable of bioactivating promutagens to electrophilic metabolites capable of binding DNA or proteins [1]. In humans, seven members of the phenol SULT family [2–5] and three members of the hydroxysteroid SULT family have been identified [6,7]. Recently, Falany et al. [8] have cloned and expressed novel SULT-like cDNA species from human and rat brain that are highly similar between species and selectively expressed in brain. Characterization and analysis of the roles of the individual SULT isoforms in drug metabolism require that the individual isoforms in a species be identified, isolated and characterized.

Unfortunately there is no common nomenclature for the SULTs and the same enzyme can have several names, for example human phenol-sulphating form of phenol-sulphotransferase (PST) (hP-PST-1) is the same as SULT1A1 and thermostable PST. However, the phenol SULT subfamily is also called SULT1, whereas the hydroxysteroid SULT subfamily is called SULT2. A further subdivision can be made, mainly depending on amino acid identity. Thus, the SULT1 family is subdivided into SULT1A, mainly sulphating small phenols such as PNP; SULT1B, mainly conjugating thyroid hormones; SULT1C, which activates *N*-hydroxy-2-acetylaminofluorene to a reactive species, and SULT1E, mainly sulphating oestrogens. The brain-specific SULTs are termed SULT4A [8].

Sulphation has been studied in many species such as rat, mouse and humans. However, information is lacking concerning the dog. There have been few attempts to identify individual SULTs in dog; for example, Oddy et al. [9] purified and characterized a cytosolic liver SULT that sulphates simple phenolic substrates such as naphth-1-ol and PNP. Human antibodies identified this enzyme as a form closely related to the human SULT1A1 (hP-PST-1) and this similarity was supported

Abbreviations used: SULT, sulphotransferase; E_2, β-oestradiol; DHEA, dehydroepiandrosterone; hDHEA-ST, human DHEA-sulphotransferase; hEST, human oestrogen SULT; PST, phenol sulphotransferase; hM-PST, human monoamine-sulphating form of PST; hP-PST, human phenol-sulphating form of PST; HRP, horseradish peroxidase; hST1B2, human sulphotransferase-1B2; hSULT1C1, human SULT1C1; 5HT, 5-hydroxytryptamine; LE_4, leukotriene E_4; MBP, maltose-binding protein; Mx, minoxidil; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; PG, prostaglandin; PNP, *p*-nitrophenol; TBB_2, thromboxane B_2.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number AY004331.

by an amino acid analysis of four peptides derived from the purified enzyme. In another study by Christ and Walle [10], dog liver cytosol was partly purified, identifying three different fractions that were eluted from DEAE-cellulose in which naphth-1-ol sulphation activity was found. However, no attempt was made to identify individual sulphating enzymes. Romain et al. [11] partly purified two canine SULTs. These two enzymes were capable of conjugating substrates such as dopamine, PNP and 2-OH-oestradiol; however, the two SULT activities seemed to be kinetically different. These enzymes were not purified or cloned; nor was any attempt made to deduce their amino acid sequences.

This report decribes the first cloning, expression, kinetic and immunological characterization of a canine hepatic SULT. These results have led to the identification of a unique canine sulphotransferase, designated SULT1D1, which is apparently an orthologue of the mouse amine-N-sulphotransferase enzyme and suggests the existence of a new SULT subfamily.

EXPERIMENTAL

Materials

The λgt11 male dog liver library was obtained from Clontech (Palo Alto, CA, U.S.A). DEAE-Sepharose CL-6B, oligolabelling kit, ECL® enhanced chemiluminescence substrate kit and donkey anti-rabbit IgG horseradish peroxidase (HRP) conjugate were purchased from Amersham Pharmacia Biotech. Oligonucleotide primers were synthesized by Cybergene (Huddinge, Sweden). Adenosine 3′-phosphate 5′-phosphosulphate (PAPS) was purchased from Dr Sanford Singer (University of Dayton, Dayton, OH, U.S.A.). PNP, naphth-1-ol, minoxidil (Mx), dopamine, E_2, 5-hydroxytryptamine (5HT) and DHEA were purchased from Sigma-Aldrich AB (Stockholm, Sweden). Prostaglandin $(PG)E_2$, PGD_2, $PGF_{2\alpha}$, thromboxane B_2 (TBB_2) and leukotriene E_4 (LE_4) were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). [6,7-^{3}H(N)]E_2, 3,4-[2,5,6,^{3}H]DHEA and [^{35}S]PAPS were purchased from NEN Life Science Products (Brussels, Belgium). Elongase enzyme mix was purchased from Life Technologies (Rockville, MD, U.S.A.). QIAquick gel extraction kit and QIAprep minipreps were obtained from Qiagen GmbH (Hilden, Germany). TA-cloning kit was bought from Invitrogen (Leek, The Netherlands). Big Dye Terminator Cycle sequencing Ready Reaction kit was purchased from Perkin Elmer (Boston, MA, U.S.A.). The pMal protein fusion and purification system was obtained from New England Biolabs (Beverly, MA, U.S.A.). Fast link DNA ligation kit was purchased from Epicentre Technologies (Madison, WI, U.S.A.). LK6D silica gel (60 Å pore size) TLC plates were purchased from Whatman (Clifton, NJ, U.S.A.). All other chemicals were of reagent-grade quality.

Screening of cDNA Library

Human oestrogen SULT (hEST) was used as a probe to isolate λgt11 phage containing a SULT1D1 cDNA insert from a λgt11 male dog liver cDNA library. *Escherichia coli* strain Y1090r− cells were infected with aliquots of the λgt11 phage, plated on agarose plates and blotted on nitrocellulose membranes. The membranes were denatured (1.5 M NaCl/0.5 M NaOH) and then neutralized [1.5 M NaCl/0.5 M Tris/HCl (pH 8.0)] as described previously [3]. The filters were cross-linked with a Genelinker (Bio-Rad), prehybridized in 6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/5 × Denhardt's (1 × Denhardt's is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.002 % BSA)/0.5 % SDS/0.2 mg/ml denatured salmon sperm DNA at 55 °C for 0.5 h and hybridized overnight with ^{32}P-labelled hEST probe (5×10^5 c.p.m./ml) at 55 °C. Positive plaques were purified by cycles of dilution and rescreening until single plaque-forming units could be isolated.

Subcloning into pCR-2.1 vector

To isolate and subclone the phage cDNA inserts, an aliquot of the purified phages was used as the template in a PCR reaction. λgt11 primers were used (5′ primer, 5′-GGT GGC GAC GAC TCC TGG AGC CCG-3′; 3′ primer, 5′-TTG ACA CCA GAC CAA CTG GTA ATG-3′) in a PCR procedure (100 μl reactions containing each of the 5′ and 3′ primers at 0.2 μM, each of four dNTPs at 0.2 mM, 1.1 mM $MgCl_2$ and 2 μl of Elongase enzyme mix) to amplify the cDNA inserts. The PCR programme included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a final extension period of 7 min at 72 °C. The amplification products were resolved on a 1 % (w/v) agarose gel, purified with a QIAquick gel extraction kit in accordance with the manual, then subcloned into pCR-2.1 for characterization and sequence analysis.

Sequencing and sequence analysis

The SULT1D1 cDNA species were sequenced from both strands with a BigDye Terminator Cycle sequencing Ready Reaction kit employing M13reverse and T7 primers. Additional sequencing primers were synthesized to the internal sequence of SULT1D1 to sequence specific regions. Sequence comparisons were run with the program GAP in Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, WI, U.S.A.).

Bacterial expression of canine SULT1D1

To generate native protein for kinetic characterization, SULT1D1 was expressed in *E. coli*. An oligonucleotide with an *Nco*I restriction site incorporated to contain the initial methionine (underlined) residue was synthesized (5′ primer, 5′-TTT CTG AAC **CAT GG**A CAA GAA GCC-3′; the *Nco*I site is shown in bold type). M13reverse was used as the 3′ primer and pCR-2.1/SULT1D1 as the template in a PCR reaction to generate the cDNA to subclone into pKK233-2. The PCR included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 45 s at 94 °C, 45 s at 50 °C and 2 min at 72 °C, with a final extension period of 7 min at 72 °C. The PCR product was digested with *Nco*I and *Hin*dIII, resolved by agarose-gel electrophoresis and purified with a QIAquick gel extraction kit. The SULT1D1 fragment was ligated into pKK233-2 and the SULT1D1 enzyme was expressed in *E. coli* XL1-Blue cells as described previously [12].

Subcloning and expression in pMal-c2x

The pMal protein fusion and purification system was used for generating pure protein to raise antibodies against SULT1D1. An oligonucleotide was synthesized to the 5′ end of the coding region of the SULT1D1 fragment (5′-ATG GAC AAG AAG CCG GAT ATC TAC-3′). This oligonucleotide and the M13-reverse primer were used with pCR-2.1/SULT1D1 as template in a PCR reaction to amplify the SULT1D1 cDNA. The PCR programme was as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min 10 s at 72 °C, with a final extension period of 5 min at 72 °C. The resulting PCR product was purified with a Wizard DNA CleanUp Kit and digested with *Hin*dIII. The SULT1D1 fragment was ligated into the *Xmn*I and *Hin*dIII sites of pMal-c2x. The pMal-c2x/SULT1D1 was expressed in *E. coli* XL1-Blue

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                                                GA ATT CCG GGG GAA ATT TCC AGT AAC CAA   -60
TTC CTG TAG AAA CAA AAT CTG GAC CTC TAG TTC TTC AAG ACA GGC ATC TGT TTC TGA AAA     0

ATG GAC AAG AAG CCG GAT ATC TAC CGG AGG GAG TTA GTG GAT GTT CAG GGT GTC CCC CTC    60
Met Asp Lys Lys Pro Asp Ile Tyr Arg Arg Glu Leu Val Asp Val Gln Gly Val Pro Leu    20

TTC TGG AGC ATT GCT GAG GAG TGG TCC CAG GTG GAG TCA TTT GAG GCC CGA CCT GAT GAC   120
Phe Trp Ser Ile Ala Glu Glu Trp Ser Gln Val Glu Ser Phe Glu Ala Arg Pro Asp Asp    40

CTT CTG ATC TCA ACC TAC CCC AAA TCT GGG ACA ACC TGG GTC AGC GAA ATA TTG GAT TTG   180
Leu Leu Ile Ser Thr Tyr Pro Lys Ser Gly Thr Thr Trp Val Ser Glu Ile Leu Asp Leu    60

ATC TAT AAC AAT GGA GAT GTG GAG AAA TGT AAG CGG GAT GCA ATA TAC AAA CGA GTG CCG   240
Ile Tyr Asn Asn Gly Asp Val Glu Lys Cys Lys Arg Asp Ala Ile Tyr Lys Arg Val Pro    80

TTC ATG GAA TTG ATA ATT CCT GGA TTC GAG AAT GGT ATA GAG GAT TTG AAA AAA ATG CAG   300
Phe Met Glu Leu Ile Ile Pro Gly Phe Glu Asn Gly Ile Glu Asp Leu Lys Lys Met Gln   100

CCT CCT AGA TTA GTG AAA ACA CAC CTA CCT GTT CAA CTT CTC CCT TCT TCA TTT TGG AAG   360
Pro Pro Arg Leu Val Lys Thr His Leu Pro Val Gln Leu Leu Pro Ser Ser Phe Trp Lys   120

AAT AAC TGC AAG ATG GTG TAT GTT GCA CGA AAT GCT AAA GAT GTG GCT GTA TCT TAC TAT   420
Asn Asn Cys Lys Met Val Tyr Val Ala Arg Asn Ala Lys Asp Val Ala Val Ser Tyr Tyr   140

TAT TTC TAC CAG ATG GCA AAA ATA CAC CCA AAG GCT GGT ACC TGG GAG GAA TTC CTG GAT   480
Tyr Phe Tyr Gln Met Ala Lys Ile His Pro Lys Ala Gly Thr Trp Glu Glu Phe Leu Asp   160

AAA TTC ATG ACT GGG AAG GTG GCT TTT GGT TCT TGG TAT GAC CAT GTG AAG GGC TGG TGG   540
Lys Phe Met Thr Gly Lys Val Ala Phe Gly Ser Trp Tyr Asp His Val Lys Gly Trp Trp   180

GAG AAG AGG AAT GAT TAT CCT ATC TTT TAT CTA TTT TAT GAA GAC ATG AAA GAG AAT CCA   600
Glu Lys Arg Asn Asp Tyr Arg Ile Phe Tyr Leu Phe Tyr Glu Asp Met Lys Glu Asn Pro   200

AAA CAT GAA ATT CAG AAG TTG TTA CAG TTT CTA GAG AAA GAT TTG TCA GAA GAA ACT GTG   660
Lys His Glu Ile Gln Lys Leu Leu Gln Phe Leu Glu Lys Asp Leu Ser Glu Glu Thr Val   220

GAT AAA ATC CTC TAT CAC AGC TCT TTC AAT GTG ATG AAA CAG AAT CCA AGT ACA AAT TAT   720
Asp Lys Ile Leu Tyr His Ser Ser Phe Asn Val Met Lys Gln Asn Pro Ser Thr Asn Tyr   240

ACC ACT ATA CCA GAT TTT GAT ATG GAT CAT TCT GTA TCT CCT TTC ATG AGA AAG GGT ATT   780
Thr Thr Ile Pro Asp Phe Asp Met Asp His Ser Val Ser Pro Phe Met Arg Lys Gly Ile   260

TCA GGA GAT TGG AAA AAC CAA TTC ACC GTA GCC CAG TAT GAA AGA TTT GAA AGA GAT TAT   840
Ser Gly Asp Trp Lys Asn Gln Phe Thr Val Ala Gln Tyr Glu Arg Phe Glu Arg Asp Tyr   280

GAA AAG AAA ATG AAA GGT TCT ACA CTG CGG TTT CGT TCA GAG ATT GAA GTC CTG CTC TTT   900
Glu Lys Lys Met Lys Gly Ser Thr Leu Arg Phe Arg Ser Glu Ile                       295

CTC CGA CTT GTT TAA CTG ACA CTT AAA TTA AAA AAA AAA AAA CCG GAA TT               950
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Figure 1 Nucleotide sequence and derived translation of the coding region of canine SULT1D1

cells. The resulting maltose-binding protein (MBP)–SULT1D1 fusion protein was purified from the bacterial cytosol by amylose-affinity chromatography, digested with Factor Xa to separate the MBP and repurified with a DEAE-Sepharose CL-6B column followed by amylose-affinity chromatography [13].

Immunoblot analysis

The pure SULT1D1 generated by the pMal-c2x system was used to raise polyclonal antibodies in rabbits by Antibody AB (Sodra Sandby, Sweden). Dog liver, kidney, lung, skin, duodenum, jejunum, ileum and colon were analysed for their content of immunoreactive SULT1D1 protein by immunoblot analysis. The above-mentioned organs from one male and one female beagle were cut into small pieces and homogenized in 3 vol. of buffer [10 mM Tris/HCl (pH 7.4)/1 mM dithiothreitol/0.57 mM PMSF/10 % (v/v) glycerol]. No dog was killed for the sole purpose of preparing the tissues. The homogenate was then centrifuged at 40000 ***g*** for 60 min at 4 °C. Protein concentration was determined by the method of Bradford [14]. After resolution by SDS/PAGE [12.5 % (w/v) gel], proteins were electro-transferred to nitrocellulose membranes. The membranes were blocked in 5 % (w/v) non-fat dried milk for 1 h and incubated with a 1/10000 dilution of rabbit anti-SULT1D1 serum as primary antibody for 1 h. Donkey anti-rabbit IgG HRP conjugate was used as the secondary antibody. Detection of immuno-conjugates was performed with a substrate kit based on the chemiluminescence resulting from the HRP-catalysed breakdown of luminol.

Bacterial cytosol preparation

Cultures of *E. coli* XL1-Blue containing pKK233-2/SULT1D1 or pMalc2x/SULT1D1 were induced for 2 h with 0.3 mM isopropyl β-D-thiogalactoside. The bacterial cultures were harvested by centrifugation at 3200 ***g*** for 15 min and resuspended in 10 mM Tris/HCl (pH 7.4)/0.1 mM dithiothreitol/1 mM EDTA. After sonication on ice with short bursts for 30 min, a final centrifugation was performed at 100000 ***g*** at 4 °C for 45 min. The cytosolic fraction containing SULT1D1 (from the pKK233-2/SULT1D1 vector) was partly purified by DEAE-cellulose chromatography as described previously [15]. Cytosol containing the MBP–SULT1D1 fusion protein was purified and cleaved in accordance with the instructions for the pMal protein fusion and purification system.

SULT assays

SULT activities of SULT1D1 were determined by several different assay procedures. The SULT reactions were run at pH 7.4 and final PAPS concentration was 25 μM except for that containing Mx, where the pH was 7.3 and the PAPS concentration was 29 μM. In all reactions the amounts of SULT1D1 and reaction times were adjusted so that the production of sulphated products was less than 30 %. Each measurement was repeated three times and V_{max} and K_m parameters were determined with a computer program (GraFit 4; Erithacus Software) and non-linear regression. Mx (0.2–4.0 mM) was assayed with an extraction procedure described by Johnson and Baker [16]. PNP (5.0–600 μM), α-naphthol (3.0–300 μM) and dopamine (0.5–20 μM) were assayed with the barium precipitation procedure by Foldes and Meek [17]. DHEA (1.0–25 μM) and E_2 (20 nM–25 μM) were assayed by the method of Falany et al. [15]. 5HT (0.25–6 mM), PGE_2 (50 nM–1 mM), PGD_2 (50 nM–1 mM), $PGF_{2\alpha}$ (50 nM–1 mM), TBB_2 (50 nM–1 mM) and LE_4 (50 nM–11 μM) were assayed as described by Wang et al. [3] except that no $MgCl_2$ was added to the reaction. The ^{35}S-sulphated product was analysed by the method of Liu et al. [18].

RESULTS

Isolation, bacterial expression and DNA sequence analysis of SULT1D1

With the hEST cDNA as a probe, a novel SULT from dog was isolated after screening of a λgt11 male dog liver cDNA library under relatively low stringency conditions. PCR amplification of two independent purified λgt11 cDNA inserts generated products of approx. 1200 bp. One of these inserts contained the entire coding region of SULT1D1; the other insert lacked 85 bp at the 5′ end. The full-length SULT1D1 cDNA was ligated into pCR-2.1 for sequence analysis.

The SULT1D1 cDNA cloned in pCR-2.1 had an 885 bp open reading frame encoding a 295-residue protein with a calculated molecular mass of 35206 Da (Figure 1). The amino acid sequence was 80.7 % and 81.0 % identical with the amino acid sequences of mouse amine-N-sulphotransferase [19] and rat tyrosine-ester-sulphotransferase (EMBL U32372) (Figure 2) respectively. In comparison with known human SULTs, the amino acid similarity was much lower, being 61.0 %, 59.3 %, 56.1 %, 58.3 %, 53.2 %

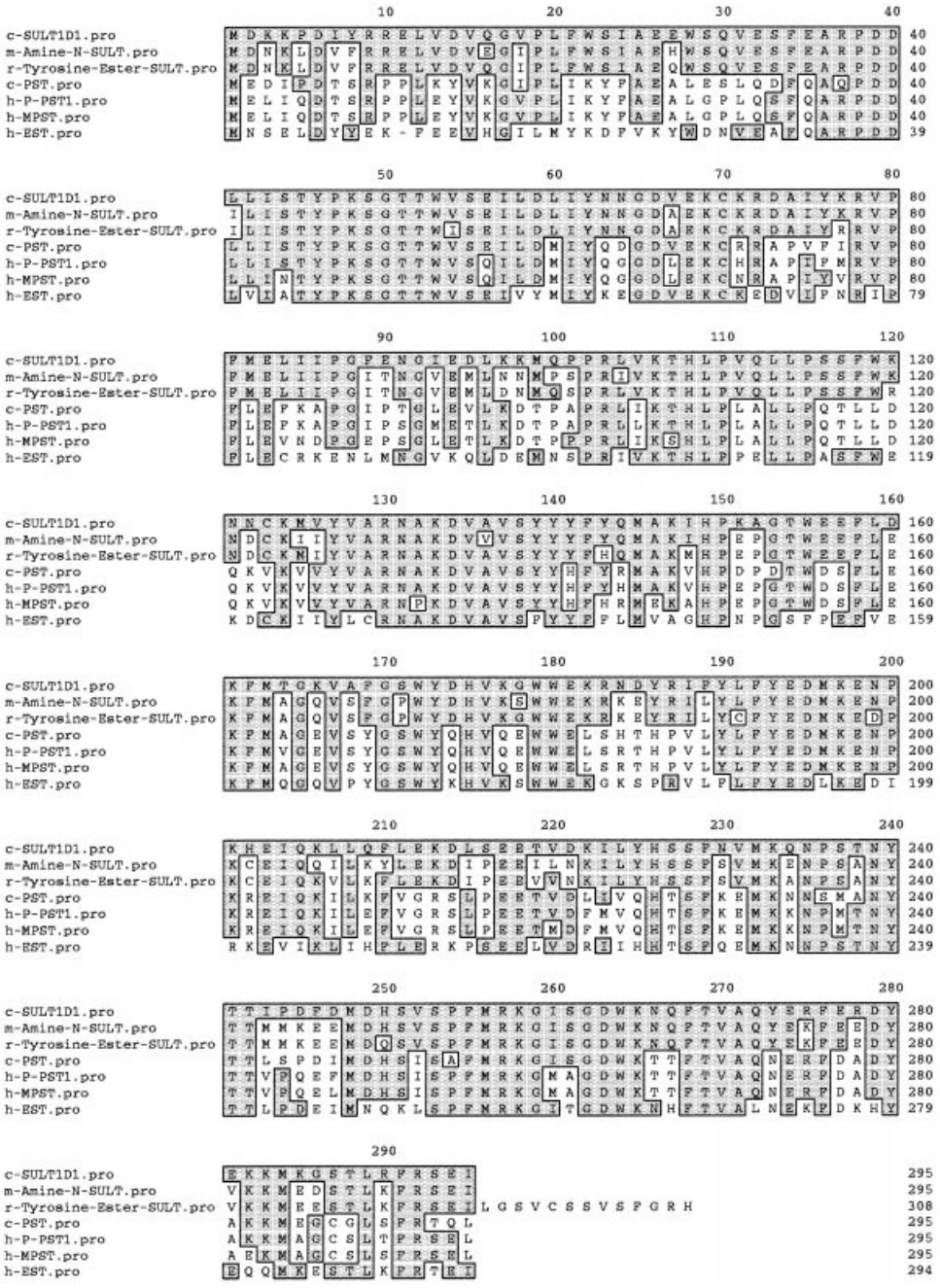

Figure 2 Comparison of amino acid sequence of canine SULT1D1 with selected SULTs

The amino acid sequence of SULT1D1 is compared with those of mouse amine-N-sulphotransferase [19], rat tyrosine-ester-sulphotransferase (EMBL U32372), dog PST (GenBank accession number D29807), hP-PST-1 [20], hM-PST [21] and hEST [13]. Sequence comparisons were run with the program GAP in Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, WI, U.S.A.).

Table 1 Amino acid sequence comparison of canine SULT1D1 with other SULTs

Percentage identities and percentage similarities of the amino acid sequence of SULT1D1 to other SULTs are shown. Sequence comparisons were run with the GAP program.

Enzyme	GenBank accession number	Identity (%)	Similarity (%)
Mouse amine-N-sulphotransferase	AF026073	80.7	86.4
Rat tyrosine-ester-sulphotransferase	U32372	81.0	86.8
hP-PST-1	L19999	61.0	68.1
hM-PST	U08032	59.3	66.8
hEST	S77383	56.1	67.3
hST1B2	U95726	58.3	70.2
hSULT1C1	U66036	53.2	63.1
hDHEA-ST	L20000	39.3	51.4
Dog PST	D29807	60.2	68.7

Table 2 Substrate specificity of canine SULT1D1

Samples were run in triplicate. Each measurement was repeated three times; V_{max} and K_m values were determined with a computer program (GraFit 4; Erithacus Software) and non-linear regression analysis. Results are means ± S.E.M. Abbreviation: n.d., no detectable activity.

Substrate	K_m (μM)	V_{max} (nmol/min per mg of protein)	V_{max}/K_m (nmol/min per mg of protein per μM)
PNP	200 ± 36	4.8 ± 0.35	0.02
Dopamine	5.2 ± 0.75	5.7 ± 0.27	1.1
Naphth-1-ol	8.9 ± 1.4	4.4 ± 0.26	0.49
Mx	1400 ± 80	3.7 ± 0.09	0.0027
5HT	1100 ± 140	1.0 ± 0.04	0.00091
DHEA (1–25 μM)	n.d.	n.d.	–
E_2 (20 nM–25 μM)	n.d.	n.d.	–
PGE_2 (50 nM–1 mM)	n.d.	n.d.	–
PGD_2 (50 nM–1 mM)	n.d.	n.d.	–
$PGF_{2\alpha}$ (50 nM–1 mM)	n.d.	n.d.	–
TBB_2 (50 nM–1 mM)	n.d.	n.d.	–
LE_4 (50 nM–11 μM)	n.d.	n.d.	–

and 39.3% identical with hP-PST-1 [20], human monoamine-sulphating form of PST (hM-PST) [21], hEST [13], human sulphotransferase-1B2 (hST1B2) [3], human SULT1C1 (hSULT1C1) [5] and human DHEA-sulphotransferase (hDHEA-ST) [6] respectively (Table 1). This novel SULT from dog was termed SULT1D1. The amino acid sequence of SULT1D1 is 60.2% identical with the amino acid sequences of the dog SULT reported previously, dog PST (GenBank accession number D29807).

Kinetic characterization of expressed SULT1D1

The native form SULT1D1 was expressed with the pKK233-2 vector in *E. coli* XL1-Blue cells and the construct was resequenced to confirm the identity of SULT1D1. Enzymically active SULT1D1 was partly purified from bacterial cytosol by DEAE-Sepharose CL-6B chromatography and used to characterize the catalytic and kinetic properties. Expressed SULT1D1 sulphated small phenols and aromatic amines such as naphth-1-ol, PNP, dopamine and 5HT (Table 2). Like most other SULTs, this enzyme also sulphates the pyrimidine N-oxide Mx [22]. However, it has no detectable activity towards DHEA, E_2, PGE_2, PGD_2, $PGF_{2\alpha}$, TBB_2 and LE_4. The apparent K_m and V_{max} values for the sulphation of PNP, dopamine, Mx, naphth-1-ol and 5HT were determined (Table 2). The apparent K_m values were 200 μM, 5.2 μM, 1.4 mM, 8.9 μM and 1.1 mM and the apparent V_{max} values were 4.8, 5.7, 3.7, 4.4 and 1.0 nmol/min per mg of protein for PNP, dopamine, Mx, naphth-1-ol and 5HT respectively. Substrate inhibition was observed in the sulphation of naphth-1-ol.

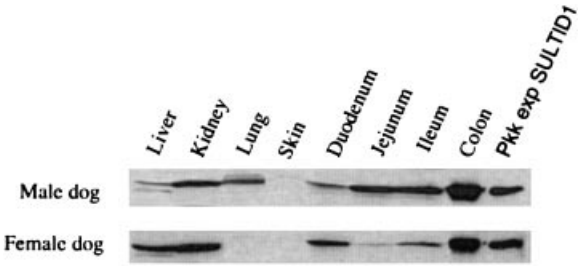

Figure 3 Tissue distribution of canine SULT1D1

An immunoblot analysis of SULT1D1 in male and female dog was performed: cytosol from liver, kidney, lung, skin, duodenum, jejunum, ileum and colon (from one male and one female dog) were analysed for their content of immunoreactive proteins. Lane 1, 300 μg of liver; lane 2, 300 μg of kidney; lane 3, 300 μg of lung; lane 4, 100 μg of skin; lane 5, 300 μg of duodenum; lane 6, 300 μg of jejunum; lane 7, 300 μg of ileum; lane 8, 30 μg of colon; lane 9, 0.1 μg of bacterially expressed canine SULT1D1.

Immunoblot analysis of the tissue distribution of SULT1D1 in male and female dog

Polyclonal antibodies raised in rabbits against pure SULT1D1 were used in immunoblot analysis of cytosol prepared from several different tissues from one male and one female dog (Figure 3). In the male dog, immunoreactive protein was detected in liver, kidney, lung, duodenum, jejunum, ileum and colon. In the female dog, immunoreactive protein was detected in the same organs as in the male dog with the exception of the lung, where

no band was visible. The highest expression of SULT1D1 was found in the colon. The colon-to-liver ratio of SULT1D1 expression was more than approx. 150-fold in the male dog and approx. 20-fold in the female dog. The kidney-to-liver ratio of SULT1D1 expression was approx. 7-fold in the male dog and approx. 2-fold in the female dog.

DISCUSSION

Here we describe the cloning and characterization of a canine SULT. This enzyme contains the specific SULT signature sequences YPKSGTXW and RKGXXGDWKNXFT, which have been proposed to be involved in PAPS binding [23]. The canine enzyme has also been found to be very similar in amino acid sequence to an enzyme cloned from mouse by Sakakibara et al. [19], displaying 81% identity. In addition, this enzyme displays strong identity with an enzyme found in the EMBL Database, a rat SULT (accession number U32372). This enzyme also displays an amino acid identity of approx. 81% with the canine SULT (Figure 2 and Table 1). Because the expression and characterization of this rat enzyme have not been reported, it is not yet possible to judge its enzymic properties. However, because closely related SULTs have been identified in three different species so far and display more than 80% identity, we propose that this group of enzymes forms a new SULT subfamily. In comparison with other SULTs, this novel canine SULT is, for example, 61.0% identical with hP-PST-1 [20] and 34.4% identical with hDHEA-ST [6] (Figure 2 and Table 1). Weinshilboum et al. [2] suggested a cut-off level at which members in a subfamily are at least 60% identical; we found a mean of 59.3% identity for the three related enzymes compared with hP-PST-1, which is the most closely related enzyme (Figure 4). In addition, the mouse and canine enzymes are not characterized by the efficient sulphation of either PNP or dopamine, and neither E_2 nor oestrone is sulphated (Table 2) [19]. Thus these results seem to exclude these enzymes from the SULT1A subfamily [22,24]. Sakakibara et al. [19] also placed the mouse enzyme clone 679153 outside the four subfamilies known hitherto, namely SULT1A, SULT1B, SULT1C and SULT1E. Thus we assign this subfamily to the PST family and suggest the designation SULT1D and the novel canine enzyme as cSULT1D1. In addition, an attempt was made to identify an orthologue to SULT1D1 in humans by searching through the public EMBL expressed sequence tag database but no such enzyme was found. Although these results seem to indicate that the dog and rodents such as mouse and rat might have evolved a specific enzyme not found in humans, the possibility cannot be excluded that such an orthologue might be identified in humans in the future.

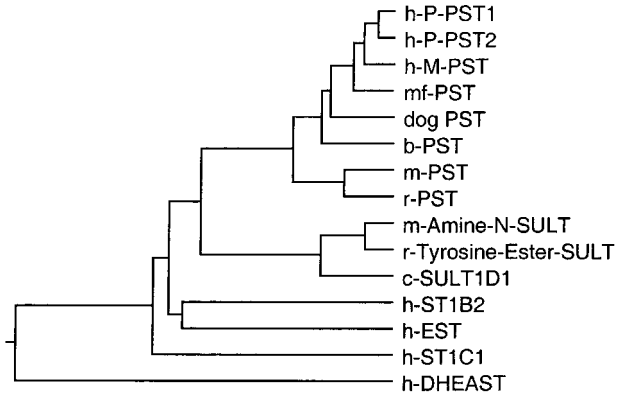

Figure 4 SULT dendrogram

The dendrogram is based on the degree of amino acid sequence similarity between the following cytosolic SULTs: hP-PST-1 (h-P-PST1) [20], hP-PST-2 (h-P-PST2) [28], hM-PST (h-M-PST) [21], *Macaca fascicularis* PST (mf-PST) (GenBank accession number D85514), dog PST (GenBank accession number D29807), bovine (b)-PST [20], mouse (m)-PST [30], rat (r)-PST [31], mouse (m)-amine-N-SULT [19], rat (r)-tyrosine-ester-SULT (EMBL accession number, U32372), hST1B2 (h-ST1B2), hEST (h-EST) [13], hSULT1C1 (h-SULT1C1) [5], and hDHEA-ST (h-DHEAST) [6].

SULT1D1 has been found to sulphate small phenols or aromatic amines such as dopamine, Mx and PNP but not DHEA or E_2. These results are in agreement with those of Sakakibara et al. [19], who found a similar metabolic pattern for the SULT called clone 679153. However, none of these substrates are sulphated very efficiently by the mouse or canine enzyme orthologues, as mentioned above (Table 2). We found a 50-fold difference in V_{max}/K_m values between dopamine and PNP, indicating a preference for catechols over small phenols. These results are in contrast with the mouse orthologue, which utilizes PNP preferentially [19]. However, the SULT1D1 enzyme does not seem to be closely related to the human SULT1A3, as might be suspected on the basis of substrate specificity, because the important residue Glu^{146} is missing from SULT1D1 [25]. Glu^{146} seems to induce selection for monoamine substrates, whereas an Ala residue is found instead in this position in SULT1D1. Dajani et al. [25] have shown that Glu^{146} selects for sulphation activity with catechols relative to small phenols in the human SULT1A3 enzyme. Mutation of this amino acid to an Ala residue, as is found in SULT1D1 (Figure 2), switched the selectivity to phenols instead. However, we see a preference for dopamine over PNP for SULT1D1 despite an Ala residue at prosition 146. In addition, the overall amino acid sequence of SULT1D1 is different from that in the human ST1A3 enzyme (59%). Furthermore, we found that pentachlorophenol, which is a potent inhibitor of many phenol SULTs of the SULT1A family, does not inhibit SULT1D1 (results not shown).

Interestingly, Liu et al. [18] demonstrated recently that the mouse enzyme named clone 679153 sulphates eicosanoids such as PGs, LE_4 and TBB_2 as well as 5HT. Because SULT1D1 is closely related to this enzyme, we investigated this possibility for SULT1D1. SULT1D1 sulphates 5HT but has no detectable activity towards PGE_2, PGD_2, $PGF_{2\alpha}$, TBB_2 or LE_4 (Table 2). It therefore does not seem that eicosanoids and 5HT are natural substrates for all SULT1D family members. Further studies are thus required to determine whether or not these arachidonic-acid-derived mediators are natural substrates.

The distribution studies of tissues from one male and one female dog show that SULT1D1 is highly expressed in the colon in both dog genders. The enzyme is also detected in high quantities in jejunum and ileum but less in duodenum in the male dog. This is in contrast with the female dog, which expresses SULT1D1 in large amounts in duodenum but less in jejunum and ileum. It therefore seems that there is a gradient of increasing expression of SULT1D1 in the intestine for the male dog in contrast with that in the female dog (Figure 3). This is an interesting observation that we cannot explain at present; however, the expression of SULT1D1 seems to focus on the lower parts of intestine, including the colon (see below). In addition, the enzyme is found in the kidney and in liver, although it is found in higher amounts in the female than the male liver; the lung also demonstrates a gender difference in that the enzyme is not expressed in the female dog. The pattern of expression of the mouse orthologue of SULT1D1 was studied with Northern blots and, in agreement with the canine SULT, it is also expressed at high levels in the kidney [19]. In contrast, the mouse orthologue

was not detected in the lung or in intestinal tissue. Thus the patterns of tissue distribution for the mouse and canine orthologues are different. This might indicate that the functions of these orthologues are different in mouse and in dog (which was also indicated by different substrate selectivities).

The highest expression of SULT1D1 is found in the colon, not in the liver (Figure 3). The colon-to-liver ratio is at least approx. 20 for the female dog and approx. 150 for the male dog. The implication of this finding is obscure but because the colon can absorb drugs it is perhaps not unreasonable that sulphation in the colon might be important for excretion in the same way as in the liver. This suggestion can be strengthened by the fact that the microflora contain sulphatase activity, perhaps necessitating a high sulphating activity in the colon [26]. A strong capacity to glucuronidate androsterone and testosterone has been found in the intestine, including the colon, by Radominska-Pandya et al. [27], also indicating the metabolic importance of the intestine. However, the sulphation of steroid hormones seems not to be the function of the SULT1D1 enzyme because it does not sulphate steroids such as DHEA.

In summary, we have isolated a unique canine SULT that is an orthologue of one mouse and one rat enzyme, exhibiting more than 80 % identity for all three enzymes, that we believe constitute a separate SULT subfamily based on amino acid identity as well as substrate preferences. We have therefore designated the family SULT1D and the canine enzyme SULT1D1.

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