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Human Cytosolic Sulphotransferase SULT1C3: genomic analysis and functional characterization of splice variant SULT1C3a and SULT1C3d

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The cytosolic sulphotransferase SULT1C3 remained the most poorly understood human SULT. The *SULT1C3* gene has been shown to contain alternative exons 7 and 8, raising the question concerning their evolutionary origin and implying the generation of multiple SULT1C3 variants. Two SULT1C3 splice variants, SULT1C3a and SULT1C3d, were investigated to verify the impact of alternative C-terminal sequences on their sulphating activity. Sequence homology and gene location analyses were performed to verify the orthology of the *SULT1C3* gene. The *SULT1C3* gene appears to be present only in humans and other primates, but alternative exons 7b and 8b share high degrees of homology with corresponding regions of rodent *SULT1C1* genes, implying their evolutionary origin being from a defunct human *SULT1C1* gene. Purified recombinant SULT1C3a and SULT1C3d were analyzed for sulphating activities toward a variety of endogenous and xenobiotic compounds. While SULT1C3a displayed weaker activities and strict substrate specificity toward hydroxyl-chlorinated biphenyls, SULT1C3d exhibited broader substrate specificity toward bile acids and thyroid hormones as well as hydroxyl-chlorinated biphenyls. Molecular docking simulation suggested that Tyr249 and Met257 may play an important role in substrate recognition by SULT1C3d. Alternative splicing of exons 7 and 8 sequences resulted in differential catalytic properties of SULT1C3 variants.

Keywords: cytosolic sulphotransferase; PCB; sulphation; SULT; SULT1C3.

Abbreviations: MBP, maltose-binding protein; OH-PCB, hydroxyl chlorinated biphenyl (OH-PCB); PAPS, 3'-phosphoadenosine 5'-phosphosulphate; SULT, cytosolic sulphotransferase.

Sulphate conjugation is known to be involved in the biotransformation of endogenous steroid and thyroid hormones, catecholamines, cholesterol and bile acids, as well as the detoxification of a wide array of dietary, industrial and environmental xenobiotics (*1*–*3*). The conjugation reaction is catalyzed by the cytosolic sulphotransferases (SULTs) that transfer the sulphonate group from 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to acceptor substrate compounds containing hydroxyl or amino group(s) (*4*, *5*). It is generally accepted that the SULTs constitute a gene superfamily, and based on their amino acid sequences, several gene families have been categorized within the SULT gene superfamily (*6*). In humans, 13 SULT isoforms that fall into four families have been identified (*6*, *7*). Eight of them that belong to the SULT1 gene family are SULT1A1 and SULT1A2 (both believed to be general detoxifying enzymes), SULT1A3 (a dopamine/catecholamine sulphotransferase), SULT1B1 (a thyroid hormone sulphotransferase), SULT1C2, SULT1C3 and SULT1C4 (previously dubbed hydroxyarylamine sulphotransferases), and SULT1E1 (an oestrogen sulphotransferase). Three that belong to the SULT2 gene family are SULT2A1 (the dehydroepiandrosterone sulphotransferase), SULT2B1a (a pregnenolone sulphotransferase) and SULT2B1b (a cholesterol sulphotransferase). In contrast to SULT1 and SULT2 families, SULT4A1 (a neuronal/brain sulphotransferase) and SULT6B1 represent sole members of their respective SULT families. Of the 13 human SULTs, the molecular identity and functional relevance of SULT1C3 has remained controversial.

Members of the SULT1C subfamily have been reported to be more highly expressed in foetal tissues than in adult tissues (*8*). Although recent studies have shown that SULT1C2 and SULT1C4 are capable of catalyzing the sulphation of procarcinogenic hydroxyarylamines such as *N*-hydroxy-2-acetylaminofluorene, leading to the activation of their carcinogenic activity (*9*–*11*), the physiological functions of SULT1C enzymes are yet to be fully elucidated. Moreover, although SULT1C2 and SULT1C4 have been cloned, expressed and characterized to some extent (*10*, *11*), the molecular identity of SULT1C3 remains obscure. A partial sequence encoding the C-terminal region of human *SULT1C3* gene was first detected in an effort to search for the human ortholog of rat *SULT1C1* gene (*12*, *13*). A full-length human ortholog of rat *SULT1C1* gene, however, could not be found. Instead of a human '*SULT1C1*' gene, a human *SULT1C3* gene which contains the previously identified partial '*SULT1C1*-like' sequence (*12*, *13*) was

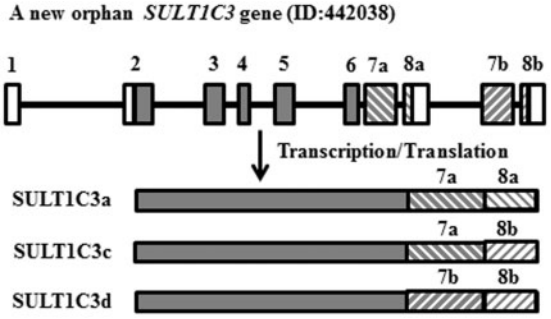

Fig. 1 Gene structure of a novel human sulphotransferase SULT1C3. Alternative exons 7 and 8 may generate three possible splice variants containing sequences corresponding to, respectively, exons 7a and 8a (SULT1C3a), exons 7a and 8b (SULT1C3c), and exons 7b and 8b (SULT1C3d). Generation of the other variant containing sequence corresponding to exons 7b and 8a is unlikely.

later identified in a database mining study (*7*). Interestingly, the *SULT1C3* gene contains alternative exons 7 and 8, which may likely generate three potential splice variants coding for distinct SULT1C3 isoforms (*7*) (cf. Fig. 1). To date, little information regarding the molecular identity and enzymatic characteristics of potential SULT1C3 isoforms is available. In a study using Ame's test, a recombinant SULT1C3d was shown to be capable of mediating the activation of promutagenic compounds including 1-hydroxymethylpyrene and 6-hydroxymethylanthanthrene, indicating SULT1C3d may in fact be an active enzyme (*14*). Moreover, in a chemical profiling study, recombinant SULT1C3d was shown to display sulphating activity toward lithocholic acid and 2-ethylphenol (*15*). Whether SULT1C3d is expressed *in vivo*, however, remains unknown. In contrast to SULT1C3d, SULT1C3a has been shown to be expressed in human colon and intestine (*16*). Unlike SULT1C3d, however, no enzymatic data concerning SULT1C3a is available to date.

We report here the cloning and expression of two splice variants of the human SULT1C3, SULT1C3a and SULT1C3d, using the pMAL-c5x bacterial expression system. The sulphating activity of recombinant SULT1C3a and SULT1C3d, affinity-purified using amylase-resin, toward a variety of endogenous compounds and xenobiotics was examined. The pH-dependence and kinetic parameters of the enzyme in catalyzing the sulphation of representative endogenous and xenobiotic substrates were determined. To gain insights into the structure–activity relationship for the sulphation of hydroxyl-chlorinated biphenyls (OH-PCBs) by the two SULT1C3 variants, enzymatic assays and molecular docking simulations were carried out using a panel of 20 OH-PCBs.

Materials and Methods

Materials

Butylated hydroxyanisole, chenodeoxycholic acid, dehydroepiandrosterone (DHEA), deoxycholic acid, diethylstilbestrol, 5,7-dihydroxy-4'-methoxyisoflavone, 17α-ethynylestradiol, 17β-estradiol, estrone, glycochenodeoxycholic acid, 1-hydroxymethylpyrene, hyodeoxycholic acid, lithocholic acid, *p*-nitrophenol, 2-phenylphenol, 4-phenylphenol, pentachlorophenol, taurochenodeoxycholic acid, L-thyroxine (L-T_4), 3,3',5-triiodo-L-thyronine (L-T_3), ursodeoxycholic acid, adenosine 5'-triphosphate (ATP), sodium dodecyl sulphate (SDS), sodium acetate, 2-morpholinoethanesulphonic acid (MES), 3-(*N*-morpholino)propanesulphonic acid (MOPS), *N*-2-hydroxylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulphonic acid (TAPS), 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid (CAPSO), Trizma base, dithiothreitol (DTT) and isopropyl β-D-thiogalactopyranoside (IPTG) were products of Sigma Chemical Company. 3-Chloro-4-biphenylol and 3,3′,5,5′-tetrachloro-4,4′-biphenyldiol (4,4′-OH-3,3′,5,5′-TCB) were from Ultra Scientific Corporation. 1-Hydroxypyrene and all other hydroxylated polychlorinated biphenyls were obtained from AccuStandard. [^{35}S]-PAPS was synthesized from ATP and carrier-free [^{35}S]sulphate using the bifunctional human ATP sulphurylase/APS kinase and its purity determined as previously described (*17*). Human total RNA Master Panel II, PrimeSTAR Max DNA Polymerase, and Takara *Ex Taq* DNA polymerase were purchased from Clontech Laboratories, Inc. Human total RNAs isolated from foetal heart, liver, lung and kidney were obtained from Agilent Technologies. First-strand cDNA synthesis kit was from GE Healthcare Life Sciences. *Taq* DNA polymerase was a product of Promega Corporation. *Nde*I, *Eco*RI and *Dpn*I restriction endonucleases, pMAL-c5x vector, NEB 5-alpha competent cells, amylose resin and unstained protein markers were from New England Biolabs. Oligonucleotide primers were synthesized by MWG Biotech. BL21 (DE3) competent cells were from Novagen. Cellulose thin-layer chromatography (TLC) plates were products of EM Science. Carrier-free sodium [^{35}S]sulphate was a product of American Radiolabeled Chemicals, Inc. Ecolume scintillation cocktail was from MP Biomedicals. All other reagents were of the highest grades commercially available.

Molecular cloning of recombinant human SULT1C3a and SULT1C3d

SULT1C3a cDNA was synthesized from small intestine total RNA by RT-PCR. First-strand cDNA was synthesized using small intestine total RNA as the template, with oligo (dT) as the primer. Using sense (5′-CGCGAACATATGGCGAAGATTGAGAAAAACGCT CCCACG-3′) and antisense (5′-CGCGAACATATGTCAGATCT CCAGGCAGAAGTTCAGTGTGGACCC-3′) oligonucleotide primers with *Nde*I restriction site incorporated at the end, PCR was carried out under the action of PrimeSTAR Max DNA Polymerase. Amplification conditions were 1 min at 98 °C and 35 cycles of 98 °C for 10 s, 55 °C for 5 s and 72 °C for 10 s. The final reaction mixture was applied onto a 0.8% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The PCR product band detected was excised from the gel, and the DNA therein was isolated by spin filtration. Purified PCR product was restricted using *Nde*I, subcloned into pMAL-c5x prokaryotic expression vector, and transformed into NEB 5-alpha competent *E. coli* cells. As to be described in the Results section, nucleotide sequencing revealed that the cloned SULT1C3a cDNA contained three mismatched nucleotides (C at +117, A at +535, C at +580), compared with that of the 'wild-type' SULT1C3a predicted based on the *SULT1C3* gene. This cDNA was, therefore, designated the 'mutated' SULT1C3a. A SULT1C3d cDNA (GenBank accession no. BC146362.1), packaged in pENTR223.1, was purchased from Thermo Scientific (Clone ID: 100015215). To clone the SULT1C3d cDNA into the pMAL-c5x vector, sense (5′-CGCGAATTCATGGCGAAGATTGAGAAAA ACGCTCCCACG-3′) and antisense (5′-CGCGAATTCTCAGAT CTCTGTGCGGAAGGTTAGGGTGCT-3′) oligonucleotide primers designed based on 5′- and 3′- regions of the coding sequence were synthesized with *Eco*RI restriction site incorporated at the end. Using these primers, PCR was carried out under the action of EX *Taq* DNA polymerase, with the original cDNA packaged in pENTR223.1 as the template. Amplification conditions were 2 min at 94 °C and 20 cycles of 94 °C for 35 s, 60 °C for 40 s and 72 °C for 1 min. The final reaction mixture was separated by agarose electrophoresis and the PCR product was purified based on the procedure described above. Purified PCR product was restricted using *Eco*RI, subcloned into pMAL-c5x vector, and verified for authenticity by nucleotide sequencing.

Generation of the 'wild-type' SULT1C3a

Site-directed mutagenesis was performed to generate 'wild-type' SULT1C3a cDNA using the 'mutated' SULT1C3a cDNA (see above) as the template, in conjunction with mutagenic primers (sense: 5′-GGAAAAGTTGTTGGCGGGTCCTGGTTTGACCAT GTGAAAGGATGGTGGGCTGCAAAAGACATGCACCGGAT CCTCTAC-3′, antisense: 5′-GTAGAGGATCCGGTGCATGTCT TTTGCAGCCCACCATCCTTTCACATGGTCAAACCAGGA CCCGCCAACAACTTTTCC-3′) that correspond to 'wild-type' sequence. Amplification conditions were 1 min at 98 °C and 18 cycles of 98 °C for 20 s, 40 °C for 2 min and 72 °C for 5 min using PrimeSTAR Max DNA Polymerase. The PCR product was treated with *Dpn*I for 5 h at 37 °C, followed by transformation into NEB 5-alpha *Escherichia coli* competent cells. The authenticity of the 'wild-type' SULT1C3a cDNA sequence was verified by nucleotide sequencing.

Bacterial expression and purification of human SULT1C3a and SULT1C3d

To express recombinant human SULT1C3a and SULT1C3d, competent *E. coli* BL21 (DE3) cells transformed with pMAL-c5x expression vector harbouring the SULT1C3a or SULT1C3d cDNA were grown in 1 L LB medium supplemented with 60 mg/ml ampicillin. After the cell density reached 0.6 OD at 600 nm, isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce the production of recombinant maltose-binding protein (MBP)-SULT fusion protein. After a 6-h induction at room temperature, the cells were collected, homogenized in lysis buffer, and the crude homogenate was subjected to centrifugation as previously described (*18–20*). The supernatant collected was fractionated using 2.5 ml of amylose resin, and the bound MBP-SULT1C3a or MBP-SULT1C3d fusion protein. Upon washing with lysis buffer supplemented with protease inhibitor cocktail to remove unbound proteins, the MBP-SULT1C3a or MBP-SULT1C3d fusion protein was eluted from amylose resin using a stepwise gradient of maltose (1–10 mM) in 50 mM Tris-HCl, pH 8.0. The MBP-SULT1C3 fusion protein present in eluted fractions was analyzed for purity by SDS–PAGE based on the method of Laemmli (*21*) and the protein concentration of purified enzymes was determination based on the method of Bradford (*22*) with bovine serum albumin as the standard.

Enzymatic assay

The sulphating activity of purified recombinant human MBP-SULT1C3a and MBP-SULT1C3d was assayed using radioactive [^{35}S]-PAPS as the sulphonate donor. The standard assay mixture, with a final volume of 20 µl, contained 50 mM HEPES buffer at pH 7.0, 14 µM [^{35}S]-PAPS (15 Ci/mmol), 1 mM DTT and 50 µM substrate. Control with DMSO, in place of substrate, was also prepared. The reaction was started by the addition of 1.0 µg enzyme, allowed to proceed for 10 min at 37 °C. After the reaction was terminated, the reaction product was analyzed as previously described (*18–20*). To examine the pH-dependence of the sulphation of lithocholic acid and 3,3′,5,5′-tetrachloro-4,4′-biphenyldiol (4,4′-OH-3,3′,5,5′-TCB), different buffers (50 mM MES at pH 5.5, 6.0 or 6.5; MOPS at pH 6.5, 7.0 or 7.5; HEPES at pH 7.0, 7.5 or 8.0; TAPS at pH 8.0, 8.5 or 9.0; and CAPSO at pH 9.0, 9.5, 10.0 or 10.5), instead of 50 mM HEPES (pH 7.0), were used in the reactions with 50 µM of each substrate. For the kinetic studies on the sulphation of lithocholic acid and 4,4′-OH-3,3′,5,5′-TCB, varying concentrations of each of these two substrates and 50 mM HEPES buffer at pH 7.0 were used, and the reactions were carried out under the same conditions as described above. Data obtained were analyzed by Eadie–Hofstee plots and the subsequent non-linear regression analysis was carried out based on Michaelis–Menten kinetics, substrate inhibition kinetics or sigmoidal kinetics using GraphPad Prism5 software.

Substrate-binding simulation analysis

The molecular simulations for the docking of OH-PCBs into the substrate-binding sites were carried out with the crystal structure of SULT1C3d in complex with PAP (Protein Data Bank code: 2H8K (*15*)) as a template. PAP in the complex was removed and replaced with PAPS. The structure of PAPS was obtained from the SULT1E1–PAPS structure (PDB: 1HY3 (*23*)) and docked into the PAPS-binding site with a binding energy −9.8 kcal/mol using AutoDock Vina (*24*). The structure of substrate ligands, OH-PCBs, were prepared using ACD/ChemSketch software, converted into PDB format by USCF Chimera software and assigned Gasteiger partial charges (*25*) by AutoDock Tool. Ligands were docked into the putative active site of the structure of SULT1C3d–PAPS complex using AutoDock 4.2 based on the Lamarckian algorithm (*26*) with a hundred times of GA run.

Results

Molecular cloning of human SULT1C3a and SULT1C3d

In a preliminary RT-PCR experiment, the expression of mRNA(s) encoding possible SULT1C3 variants was examined using a common 5′-primer paired individually with different 3′-primers corresponding to, respectively, exons 8a and 8b. Of the 19 adult and five foetal organ samples analyzed, only adult small intestine was found to express SULT1C3a mRNA, whereas no other SULT1C3 variants were detected in any of the organ samples tested (data not shown). The nucleotide sequence determined for the SULT1C3a cDNA amplified using small intestine total RNA as the template, however, contained three mismatched nucleotides (C at +117, A at +535, C at +580), compared with that of the 'wild-type' SULT1C3a mRNA (Accession no. NM_001320878) deposited in the GenBank database. Of those three alterations, A117C was synonymous and G535A and T580C were nonsynonymous which produce a mutant enzyme with two amino acid changes, Gly179Arg and Met194Thr. The wild-type SULT1C3a cDNA was re-generated by site-directed mutagenesis with the cloned SULT1C3a as a template. The cDNA coding for another SULT1C3 variant, SULT1C3d, was amplified by PCR using a synthetic cDNA clone (Clone ID: 100015215) as the template since mRNA of SULT1C3d was not detected in this study. The open reading frames of both SULT1C3 isoforms encompass 915 nucleotides and code for 304-amino acid polypeptides (Fig. 2). Similar to other cytosolic SULTs, both SULT1C3 isoforms contain sequences resembling the 5′-phosphosulphate binding (5′-PSB) loop (YPKSGTxW in the N-terminal region), 3′-phosphate binding (3′-PB) motif (YxYRNP KDVLISxFH in the central region) and p-loop-related motif (RKGxxGDWKNxFT in the C-terminal region; as underlined), which are important in the binding of the sulphonate donor, PAPS (*27*, *28*).

Sequence alignment analysis of human SULT1C3 variants with other SULT1C subfamily

Sequence analysis revealed that the deduced amino acid sequence of the human SULT1C3a displays 53.7, 56.3, 58.2 and 52.4% identity to human SULT1C2, human SULT1C4, mouse SULT1C1 and mouse SULT1C2, respectively. In the case of human SULT1C3d, sequence analysis yielded 53.7, 56.0, 65.1 and 51.7% identity to human SULT1C2, human SULT1C4, mouse SULT1C1, and mouse SULT1C2, respectively. As shown in Fig. 2 and Table I, the amino acid sequences coded by exons 7b and 8b, as found in SULT1C3d, are highly homologous to the corresponding regions of mouse SULT1C1. An 88.7% amino acid sequence identities between exons 7b and 8b (as found in SULT1C3d), in contrast to the 67% found between exons 7a and 8a (as found in SULT1C3a), and the

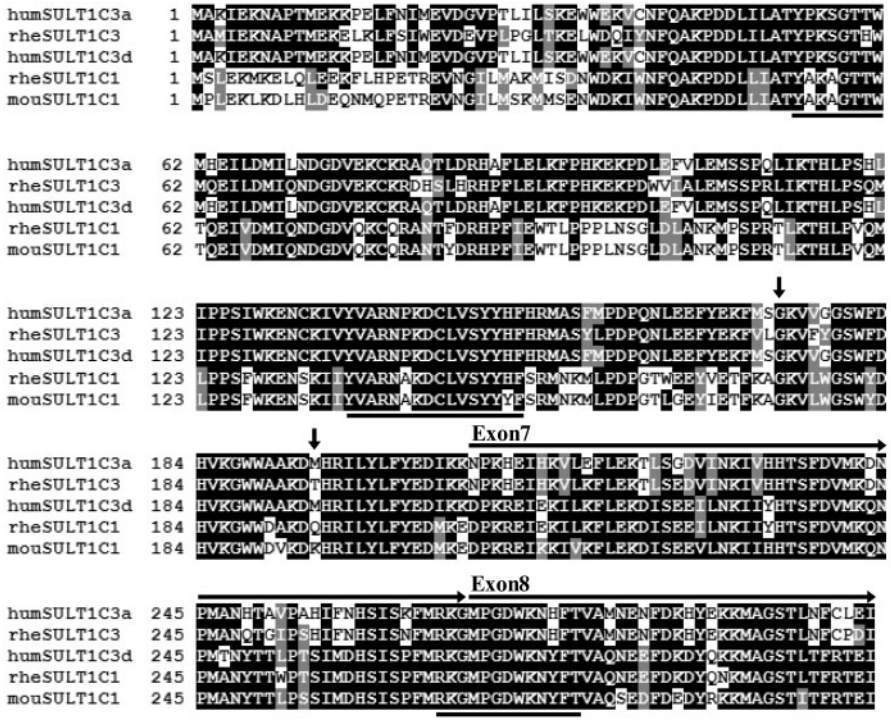

Fig. 2 Sequence alignment of amino acid sequences of human, rhesus monkey, and mouse SULT1C1 and SULT1C3. The deduced amino acid sequences of SULTs were referred to the following NCBI Reference Sequence number; hum SULT1C3a (NM_001320878), hum SULT1C3d (BC146362.1), rhe SULT1C1 (XM_002799370), rhe SULT1C3 (XM_001082103) and mou SULT1C1 (AF033653). The alignment was demonstrated using Clustal Omega program in EMBL-EBI. The underlines represent the signature sequences, 5′-PSB, 3′-PB and p-loop. Two arrowheads indicate the nonsynonymous SNPs of SULT1C3a cloned in this study.

Table I. Amino acid sequence homology of human, rhesus monkey, and mouse SULT1C1 and SULT1C3

	1	2	3	4	5
1: mouSULT1C1	100	(87.6)[a]	(69.1)	(67.0)	(88.7)
2: rheSULT1C1	89.8	100	(69.1)	(68.0)	(96.9)
3: rheSULT1C3	59.9	60.5	100	(90.7)	(69.1)
4: humSULT1C3a	58.2	59.5	84.5	100	(68.0)
5: humSULT1C3d	65.1	68.8	77.6	89.8	100

[a]The percent identities in parentheses represent the homology of amino acid sequence encoded by exons 7 and 8.

corresponding regions of mouse SULT1C1was found (Table I). By searching the GenBank database, putative rhesus monkey SULT1C1 (GeneBank accession no. XM_002799370) and SULT1C3 (XM_001082103) were found. Sequence analysis revealed that the amino acid sequence of rhesus monkey SULT1C3 displays 59.9, 84.5 and 77.6% identity to mouse SULT1C1, human SULT1C3a and SULT1C3d (Table I). In contrast, the amino acid sequence of rhesus monkey SULT1C1 displays 89.8, 59.5 and 68.8% identity to mouse SULT1C1, human SULT1C3a and SULT1C3d, respectively. Interestingly, the C-terminal sequence of rhesus monkey SULT1C1 displays the highest, 96.9%, amino acid sequence identity to that of human SULT1C3d. In view of the proximity of the chromosomal localizations of rhesus monkey *SULT1C1* and *SULT1C3* genes (Fig. 3A), it is possible that the alternative exons 7b and 8b of the human *SULT1C3* gene might have been derived from a defunct human *SULT1C1* gene (Fig. 3B).

Bacterial expression, purification and substrate specificity of recombinant 'mutated' SULT1C3a, 'wild-type' SULT1C3a and SULT1C3d

pMAL-c5x harbouring 'mutated' SULT1C3a, 'wild-type' SULT1C3a or SULT1C3d cDNA was transformed into BL21 (DE3) *E. coli* cells for the expression of recombinant enzymes. Recombinant 'mutated' SULT1C3a, 'wild-type' SULT1C3a or SULT1C3d was purified from the *E. coli* cell extract in N-terminal MBP-fusion protein form. As shown in Fig. 4, purified 'mutated' SULT1C3a, 'wild-type' SULT1C3a and SULT1C3d fusion proteins all migrated at approximately 75 kDa positions upon SDS-PAGE. Taking into consideration the 40 kDa molecular mass of the MBP portion in the MBP fusion proteins, these results were in agreement with the predicted molecular weight (35,674, 35,619 and 35,889, respectively) of 'mutated' SULT1C3a, 'wild-type' SULT1C3a and SULT1C3d. It should be pointed out that attempts had been made to remove the MBP moiety by digestion with Factor Xa. Repeated experiments, however, showed that Factor Xa also cleaved both SULT1C3a and SULT1C3d. The MBP-fusion protein form of these two SULT1C3s therefore was used in the enzymatic characterizations. To examine the substrate specificity of purified 'mutated' SULT1C3a, 'wild-type' SULT1 C3a and SULT1C3d, a total of 127 endogenous and xenobiotic compounds was tested as substrates. No

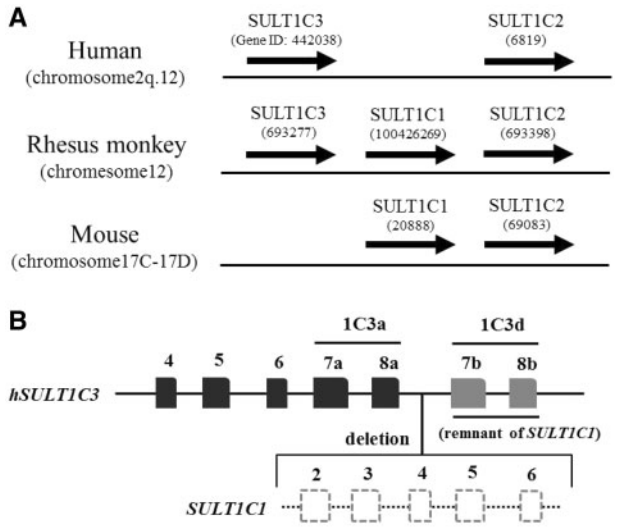

Fig. 3 Evolution of sulphotransferase SULT1C3 gene. (A) Chromosomal location of human, rhesus monkey, and mouse SULT1C1, SULT1C2 and SULT1C3 genes. (B) Possible origin of alternative exons 7b and 8b of human SULT1C3 *gene*. Alternative exons 7b and 8b may be derived from a truncated *SULT1C1* gene.

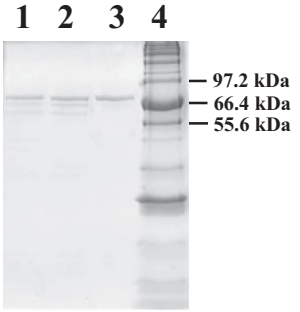

Fig. 4 SDS gel electrophoretic image of the purified human SULT1C3 variants. SDS-PAGE was performed on a 12% gel, followed by Coomassie blue staining. Samples analyzed in lanes 1, 2 and 3 were SULT1C3a-Gly179Arg/Met194Thr, SULT1C3a-WT and SULT1C3d. Protein molecular weight markers were co-electrophoresed as shown in lane 4.

Table II. Specific activities of hSULT1C3 isoforms with endogenous and xenobiotic compounds as substrate[a]

	Specific activity (nmol/min/mg)	
Substrate compounds	**SULT1C3a**	**SULT1C3d**
Endogenous substrates		
Chenodeoxycholic acid	N.D.	0.11 ± 0.01
Deoxycholic acid	N.D.	0.01 ± 0.01
Hyodeoxycholic acid	N.D.	0.01 ± 0.01
Lithocholic acid	N.D.	0.38 ± 0.01
Ursodeoxycholic acid	N.D.	0.13 ± 0.01
Glycochenodeoxycholic acid	N.D.	0.08 ± 0.01
Taurochenodeoxycholic acid	N.D.	0.09 ± 0.02
L-T3	N.D.	0.19 ± 0.02
L-T4	N.D.	0.12 ± 0.02
Pregnenolone	N.D.	0.02 ± 0.01
DHEA	N.D.	0.05 ± 0.01
E2	N.D.	0.02 ± 0.01
Xenobiotic substrates		
Butylated hydroxyanisole	N.D.	0.07 ± 0.01
4,4′-OH-3,3′,5,5′-TCB	0.06 ± 0.01	2.45 ± 0.02
Ethinyl estradiol	N.D.	0.03 ± 0.01
2,6-Dichloro-4-nitrophenol	N.D.	0.02 ± 0.01
Diethylstilbesterol	N.D.	0.04 ± 0.01
5,7-Dihydroxy-4'-methoxyisoflavone	N.D.	0.09 ± 0.01
1-Hydroxypyrene	N.D.	0.46 ± 0.01
1-Hydroxymethylpyrene	N.D.	0.09 ± 0.01
p-Nitrophenol	N.D.	0.03 ± 0.01
2-Phenylphenol	N.D.	0.23 ± 0.01
4-Phenylphenol	N.D.	0.01 ± 0.01
Pentachlorophenol	0.04 ± 0.01	1.31 ± 0.05

[a]Specific activity refers to nmol substrate sulphated/min/mg purified enzyme. Data present mean ± S.D. derived from three determinations. The concentration of substrates used in reaction was 50 μM.

sulphating activity was detected for 'mutated' SULT1C3a toward any of the compounds tested. As shown in Table II, 'wild-type' SULT1C3a displayed weak activities toward only two compounds, 3,3′,5,5′-tetrachloro-4,4′-biphenyldiol (4,4′-OH-3,3′,5,5′-TCB) and pentachlorophenol. In contrast, SULT1C3d displayed much stronger sulphating activities toward a variety of compounds tested. Among them, lithocholic acid appeared to be the best substrate among the endogenous compounds tested, and 4,4′-OH-3,3′,5,5′-TCB showed the highest specific activity among the xenobiotic compounds tested. These results indicated that compared with SULT1C3a, SULT1C3d showed not only stronger sulphating activity but also broader substrate spectrum.

Characterization of recombinant 'wild-type' SULT1C3a and SULT1C3d

To characterize further the enzymatic properties of 'wild-type' SULT1C3a, and SULT1C3d, the pH-dependence of their sulphating activity was first examined. As shown in Fig. 5, with 4,4′-OH-3,3′,5,5′-TCB as the substrate, both 'wild-type' SULT1C3a and SULT1C3d showed comparable pH-profile and were both active over pH 6.5–10, with a pH optimum at pH 7.0 for 'wild-type' SULT1C3a and pH 7.5 for SULT1C3d. With lithocholic acid as the substrate, SULT1C3d appeared to be active over a broader pH range spanning pH 6.6–10. The kinetics of the sulphation of 4,4′-OH-3,3′,5,5′-TCB and lithocholic acid were examined. As shown in Fig. 6A, the sulphation of 4,4′-OH-3,3′,5,5′-TCB by SULT1C3a appeared to follow Michaelis–Menten saturation kinetics. On the other hand, SULT1C3d appeared to follow Michaelis–Menten saturation kinetics at low-substrate concentrations. At high-substrate concentrations, however, there was a significant substrate inhibition. When lithocholic acid was used as the substrate, a sigmoidal curve was observed, indicating the occurrence of positive cooperativity at concentrations below 20 μM. Based on the results from these experiments, the kinetic constants, K_m and V_{max}, were calculated. Table III shows the kinetic constants determined for the sulphation of 4,4'-OH-3,3',5,5'-TCB by both 'wild-type' SULT1C3a and SULT1C3d and lithocholic acid by SULT1C3d. In agreement with the specific activity data shown in Table II, SULT1C3d displayed a V_{max} with 4,4′-OH-3,3′,5,5′-TCB which is approximately 10 times that

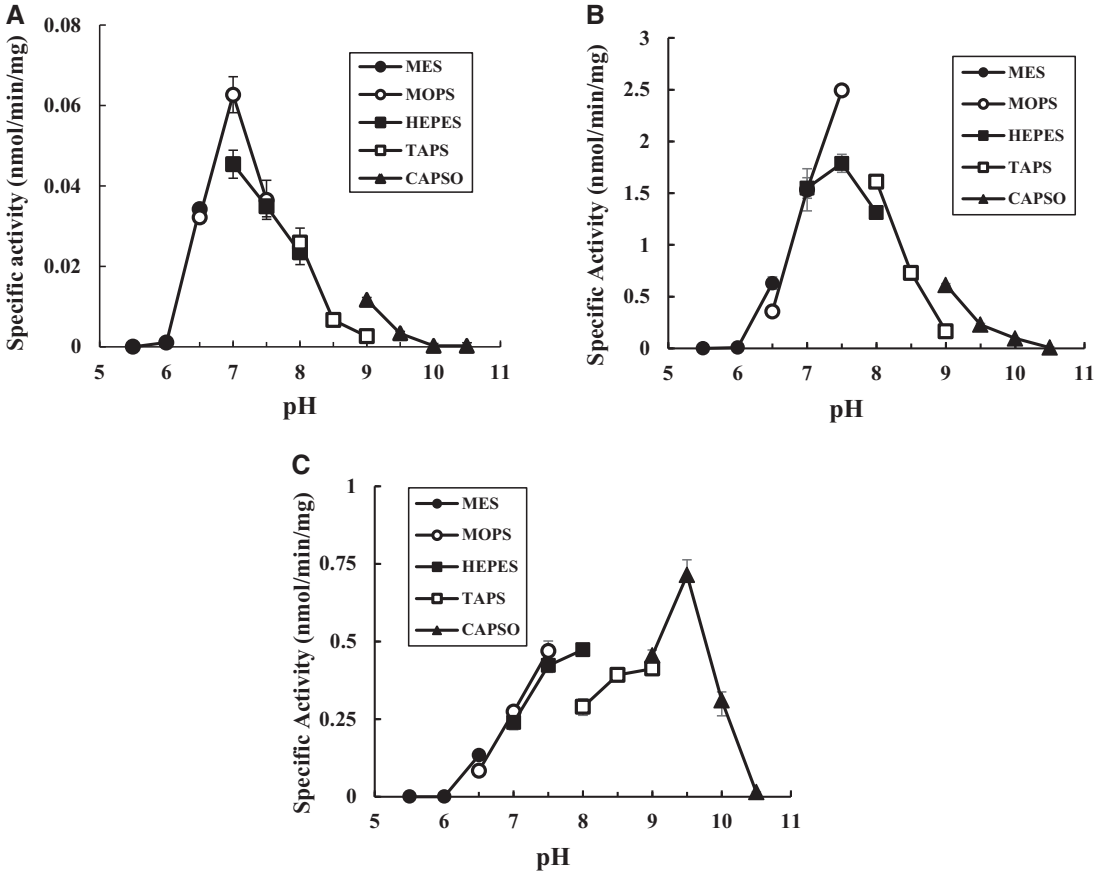

Fig. 5 pH-dependency of the sulphating activity of human SULT1C3a and 1C3d. The pH-profile of the sulphating activity of human SULT1C3a with 4,4′-OH-3,3′,5,5′-TCB (A), human SULT1C3d with 4,4′-OH-3,3′,5,5′-TCB (B) and lithocholic acid (C) were shown. The data represent calculated mean ± SD derived from three experiments.

with lithocholic acid. In contrast, the K_m with 4,4′-OH-3,3′,5,5′-TCB was more than three times lower than that with lithocholic acid. The catalytic efficiency of SULT1C3d, as reflected by V_{max}/K_m, therefore, was greater than 30 times higher with 4,4′-OH-3,3′,5,5′-TCB than with lithocholic acid as a substrate. Between 'wild-type' SULT1C3a and SULT1C3d, the former showed much lower V_{max} and much higher K_m values with 4,4′-OH-3,3′,5,5′-TCB as the substrate, indicating the much higher catalytic activity of SULT1C3d.

Sulphating activity of 'wild-type' SULT1C3a and SULT1C3d toward hydroxychlorobiphenyls

Twenty commercially available hydroxy polychlorobiphenyls (OH-PCBs), in addition to 4,4′-OH-3,3′,5,5′-TCB, were tested as substrates for investigating further the catalytic properties of 'wild-type' SULT1C3a and SULT1C3d (Supplementary Fig. S1 and Table SI). 'Wild-type' SULT1C3a exhibited sulphating activities toward only three OH-PCBs, 2-OH-2',5,5'-TriCB (OH-TriCB-1), 4-OH-2',3,5'-TriCB (OH-TriCB-3) and 4-OH-2',3,5,5'-TCB (OH-TCB-1) among the 20 OH-PCBs tested. Like 'wild-type' SULT1C3a, SUL T1C3d also showed considerably stronger sulphating activity toward 2-OH-2′,5,5′-TriCB (OH-TriCB-1) and 4-OH-2′,3,5,5′-TCB (OH-TCB-1) among the 20 OH-PCBS tested. These results indicated that, for both SULT1C3a and SULT1C3d, adjacent chloro group(s) to the hydroxy group may be a critical factor in the catalytic reaction as shown in Fig. 7A. In terms of the sulphation of 2-hydroxylated biphenyls, the 5-chrolo group may also play a role in the catalytic reaction of 'wild-type' SULT1C3a and SULT1C3d. Fig. 7B shows the sulphating activities of SULT1C3d toward hydroxy-2′,4′,6′-TCB derivatives. (SULT1C3a showed no detectable activities toward any of these compounds.) Much stronger activities were detected with 2- or 3-hydroxy-2′,4′,6′-TCB than with 4-hydroxy-2′,4′,6′-TCB, indicating that SULT1C3d prefers the 2- or 3-hydroxyl group rather than 4-hydroxyl group. In agreement with the activity data for the 4-hydroxy-2′,5′-DiCB derivatives (Fig. 7A), two chloro groups adjacent to the hydroxyl group may play an important role in the sulphation of 4-hydroxy PCB.

Molecular modelling of OH-PCBs in the active site of SULT1C3d

The sulphonate donor, PAPS, was first docked into the structure of SULT1C3d to generate the PAPS-bound active site of SULT1C3d, which yielded a good fit of PAPS with a binding energy −9.8 kcal/mol (Fig. 8A). 4,4′-OH-3,3′,5,5′-TCB and each of the 20 OH-PCBs

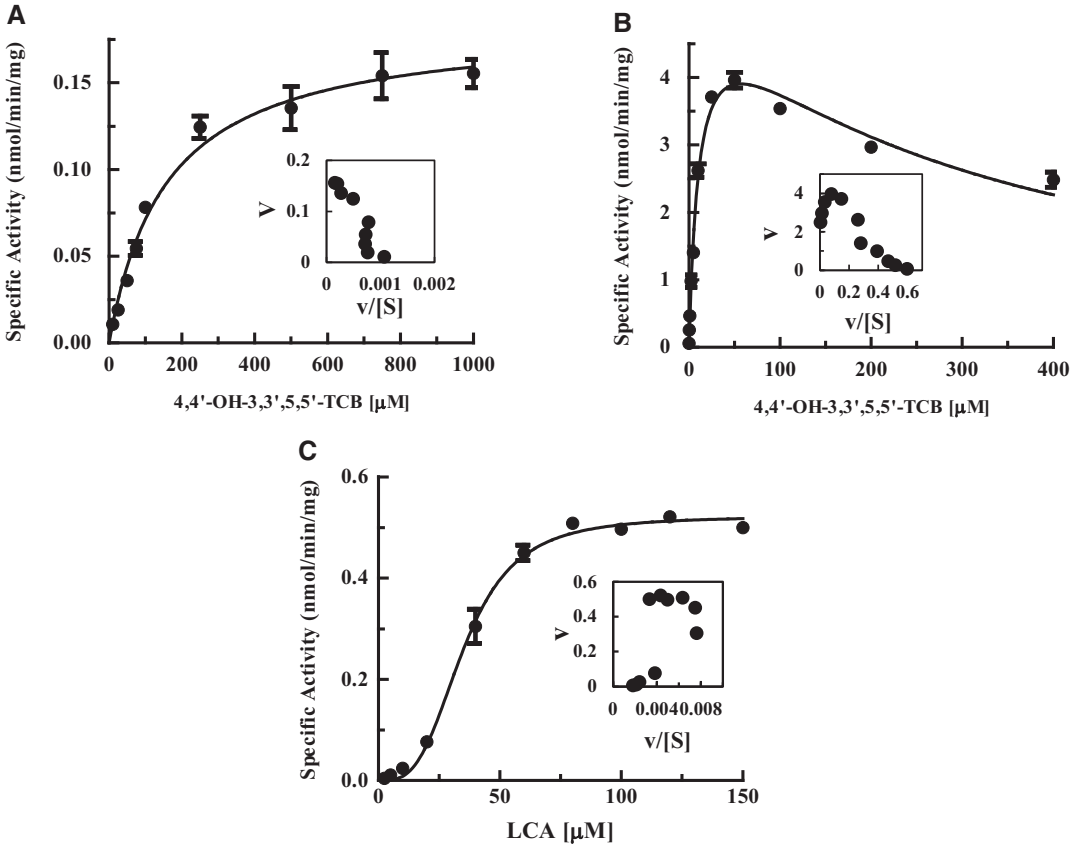

Fig. 6 Kinetic analyses for the sulphation catalyzed by human SULT1C3a and 1C3d. The fitting curves were generated using Michaelis–Menten for the sulphation of 4,4′-OH-3,3′,5,5′-TCB by SULT1C3a (A), substrate-inhibition for the sulphation of 4,4′-OH-3,3′,5,5′-TCB by SULT1C3d (B) or sigmoidal kinetics for the sulphation of lithocholic acid by SULT1C3d (C). Eadie–Hofstee plots are inserted under each fitting curve. Data shown represent calculated means ± SD derived from three experiments.

Table III. Kinetic constants of human SULT1C3 isoforms[a]

Substrate	V_{max} (nmol/min/mg)	K_m (S_{50}) (μM)	V_{max}/K_m (V_{max}/S_{50})	K_i (μM)	h
SULT1C3a					
4,4'-OH-3,3',5,5'-TCB[b]	0.18 ± 0.01	158.1 ± 13.6	0.001		
SULT1C3d					
4,4'-OH-3,3',5,5'-TCB[c]	5.45 ± 0.18	11.29 ± 0.86	0.482	289.4 ± 26.4	
Lithocholic acid[d]	0.52 ± 0.01	35.17 ± 0.69	0.015		3.27 ± 0.18

[a]Results represent means ± SD derived from three determinations.
[b]Kinetic parameters were determined based on the equation for the Michaelis–Menten kinetics.
[c]Kinetic parameters were determined based on the equation for the substrate-inhibition kinetics.
[d]Kinetic parameters were determined based on the equation for the sigmoidal kinetics. S_{50} was used instead of K_m.

were then individually docked into the putative active site of SULT1C3d bound with PAPS. Among all OH-PCBs tested, 2-OH-2′,5,5′-TriCB (OH-TriCB-1) showed the lowest minimum binding energy upon superposition into the active site (Fig. 8B). While all 2-OH- or 3-OH-PCBs tested could be appropriately docked into the active site, none of the 4-OH-PCBs, except 4,4′-OH-3,3′,5,5′-TCB, could be appropriately docked into the active site. Results indicated that 4-hydroxyl group of most 4-OH-PCBs was oriented to the substrate entry side due to the docking of chloro group rich ring into the centre of active site (data not shown). Fig. 8 shows the superposition of 2-OH-2′,5,5′-TriCB (**C**) and 4,4′-OH-3,3′,5,5′-TCB (**D**), with minimum binding energy, into the active site, respectively. In the docking of 2-OH-2′,5,5′-TriCB, the ring with the two chrolo groups was docked into the upper portion of the catalytic residue His and PAPS and the 2′-chrolo group interacted with Lys115 and Thr116, while the 5′-chrolo group interacted with Asp24 and Tyr249. The ring with the hydroxyl group was twisted and the hydroxyl group formed hydrogen bonds with the catalytic residue His117 and the sulphate group of PAPS. In contrast, 4,4′-OH-3,3′,5,5′-TCB was docked into the active site with a conformation different from that of

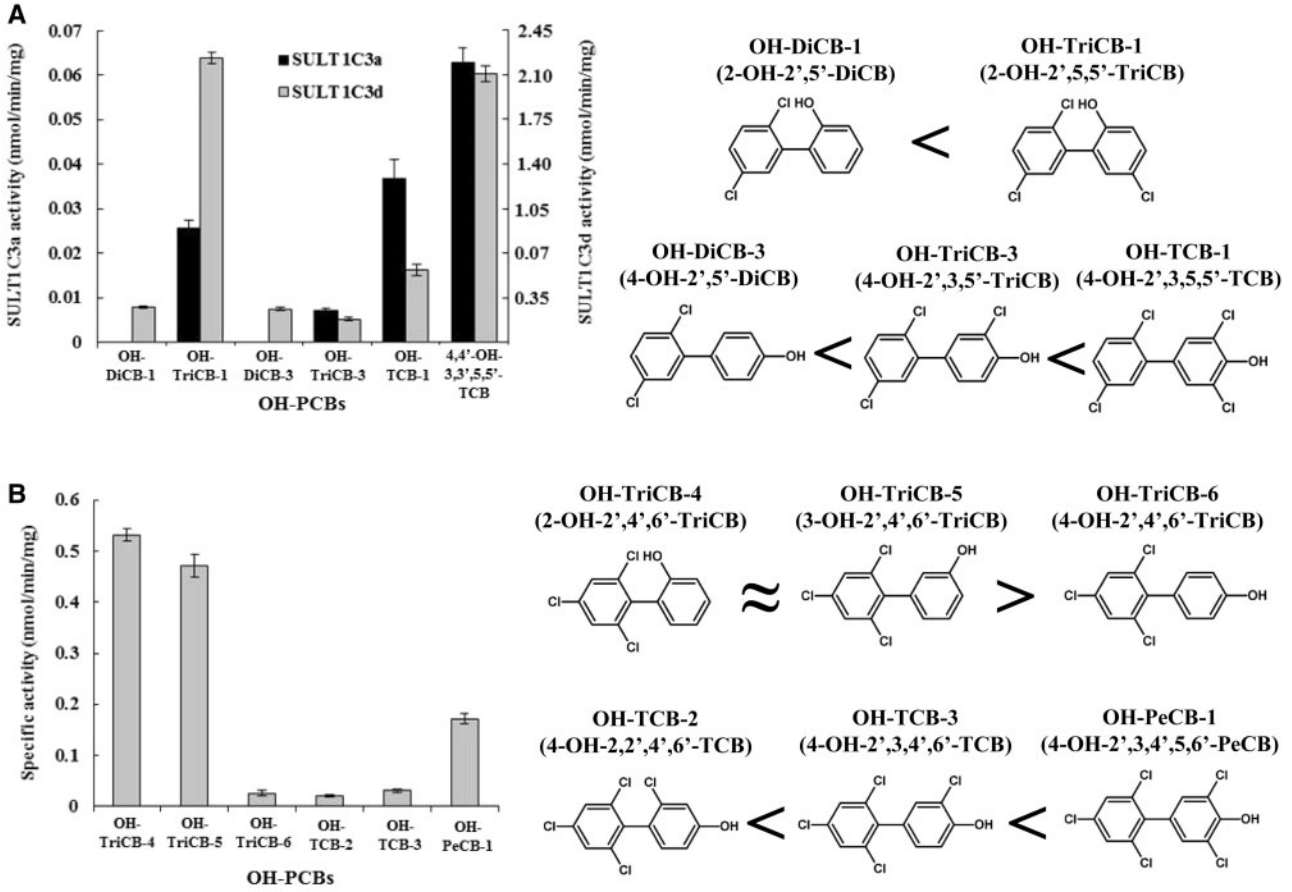

Fig. 7 Structure–activity relationship of OH-PCBs for the sulphating activity of human SULT1C3a and SULT1C3d. (A) Specific activity of human SULT1C3a and SULT1C3d toward hydroxy-2′,5′-DiCB derivatives. (SULT1C3a showed no activities toward OH-DiCB-1 and OH-DiCB-3.) Right panel represent the order in the activity of SULT1C3a and SULT1C3d toward hydroxy-2′,5′-DiCB derivatives. (B) Specific activity of human SULT1C3d toward hydroxy-2′,4′,5′-TriCB derivatives. Right panel represents the order in the activity of SULT1C3d toward hydroxy-2′,4′,5′-DiCB derivatives. Data shown represent calculated means ± SD derived from three experiments.

2-OH-2′,5,5′-TriCB. The hydroxyl group formed a hydrogen bond with the sulphate group of PAPS, but not with His117. The hydroxyl group of the substrate was docked into a position closer to Tyr249 than His117, implying that Tyr249 may play a more important role than His 117 in the catalysis of the sulphation of 4,4′-OH-3,3′,5,5′-TCB.

Discussion

Of all human *SULT* genes, the gene coding for *SULT1C3* may be the most intriguing one. The human *SULT1C3* gene, revealed in a database mining study (*7*), was found to contain alternative exons 7a/7b and 8a/8b (cf. Fig. 1). Exons 7b and 8b of the human *SULT1C3* gene were, in fact, first detected in an effort to search for the human ortholog of the rat *SULT1C1* gene (*13*). It appears, however, that the bulk of the original human *SULT1C1* gene has been lost and only exons 7 and 8 have survived and are present as alternative exons 7b and 8b of the *SULT1C3* gene. While the evolutionary origin of exons 7b and 8b remains an open issue, the presence of alternative exons 7a/7b and 8a/8b potentially allows for the expression of multiple splice variants of SULT1C3 mRNA. It is noted that of the four possibilities of alternate splicing, the generation of a 7b/8a transcript is considered unlikely due to the reverse order of these exons (*7*). In a preliminary RT-PCR experiment using RNA samples prepared from 19 adult and 5 foetal tissues, we only detected a SULT1C3 transcript containing sequences corresponding to 7a and 8a, designated SULT1C3a, in small intestine. It is noted that in an earlier study, a transcript containing the sequence corresponding to exon 7b preceded by the sequence coded by exon 8a was detected in intestinal cells (*16*). However, a follow-up 3′-RACE experiment demonstrated the mature mRNA did not contain these sequences, implying that exons 7b and 8b might have been transcribed subsequent to exons 7a and 8a, but were spliced off during post-transcriptional processing of the transcript. While the other two splice variants, SULT1C3b and SULT1C3d, have not yet been detected, it is possible that they may be present in other tissues and/or at different developmental stages. Interestingly, since the alternative exon 7b and 8b are likely remnants of an original *SULT1C1* gene (Fig. 3B), the SULT1C3d splice variant, if expressed *in vivo*, would constitute a $SULT1C3^{exons1\text{-}6}/1C1^{exons7,8}$ chimera. In relation to this latter notion, it is an interesting issue how SULT1C3a

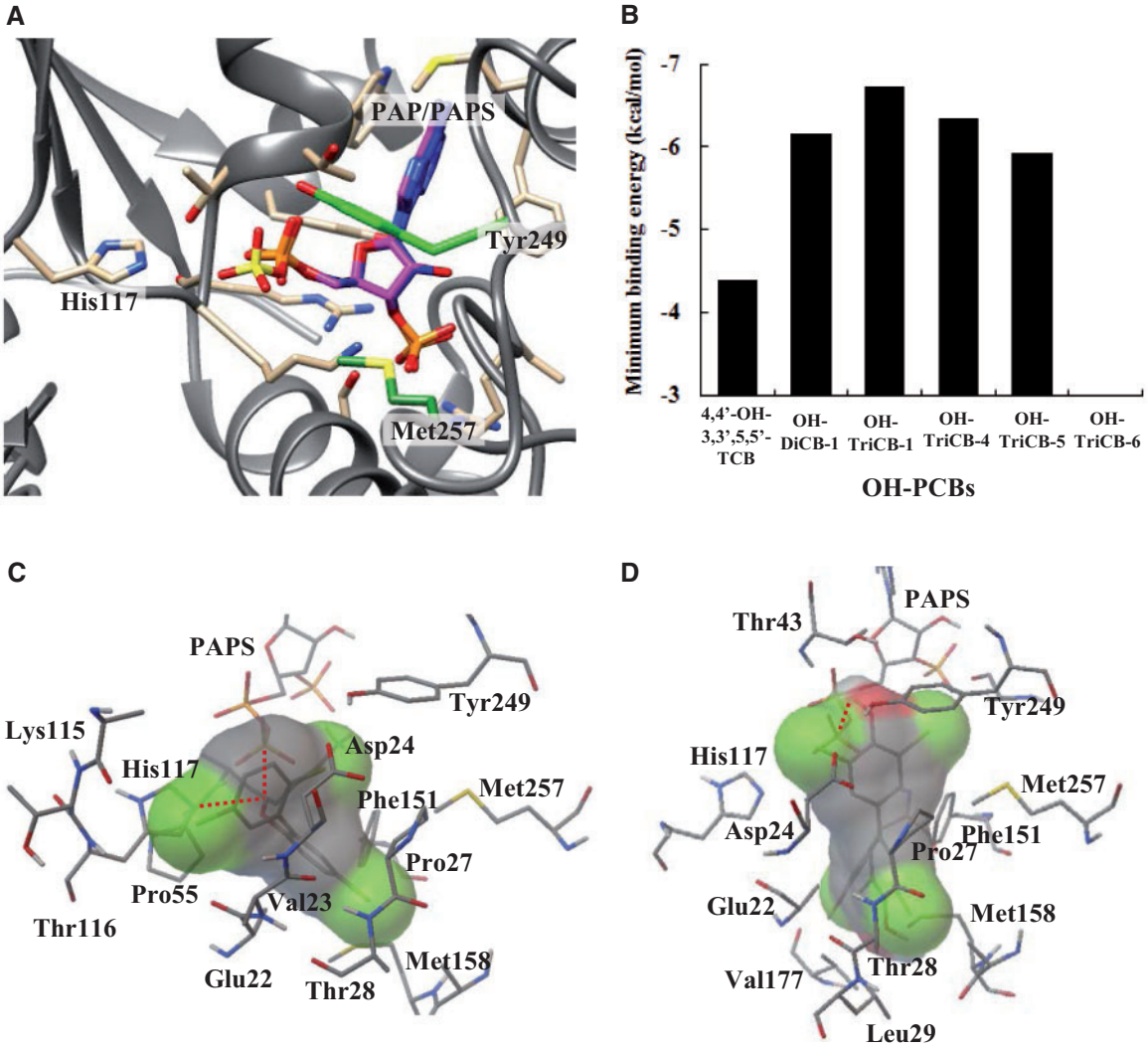

Fig. 8 Docking simulation of OH-PCBs into the active site of SULT1C3d. (A) Stereo view of the PAPS docked into the active site of SULT1C3d. (Online version: PAPS and PAP are shown in cyan and magenta, respectively.) (B) Minimum binding energy of OH-PCBs in the active site of SULT1C3d. OH-TriCB-6 (4-OH-2′,4′,6′-TriCB) was not properly docked into the active site and the binding energy was not obtained. (C) Stereo view of 2-OH-2′,5,5′-TriCB (OH-TriCB-1) in the active site of SULT1C3d. Hydrogen bonds of the hydroxyl group of substrate with His117 and sulphate were connected with dashed lines. (Online version: Chloro groups were highlighted with green colour.) (D) Stereo view of 4,4′-OH-3,3′,5,5′-TCB in the active site of SULT1C3d. Hydrogen bond of the hydroxyl group of substrate with sulphate was connected with dashed line. (Online version: Chloro groups were highlighted with green colour.)

and SULT1C3d may differ in their enzymatic characteristics.

In our initial experiments, the nucleotide sequence of SULT1C3a cDNA, cloned using small intestine total RNA as the template, showed differences from the reference sequence (GenBank accession no. NM_001 320878). Interestingly, the two nonsynonymous mismatches (G535A and T580C) detected in our experiments had previously been deposited in NCBI dbSNP database as natural variants (allele ID: rs2219078 and rs6722745, respectively). The SULT1C3a cDNA cloned in the present study, therefore, may code for an allozyme of SULT1C3a, with Gly179Arg/Met194Thr amino acid alterations. Enzymatic assays indicated that this SULT1C3a allozyme, unlike the 'wild-type' SULT1 C3a, displayed no sulphating activity toward 4,4′-OH-3,3′,5,5′-TCB and pentachlorophenols (Table II), implicating that Gly179 and Met194 may be important for the enzymatic activity of SULT1C3a. Since codons for these two amino acid residues are present in exon 6, this SNP allele may also appear in other SULT1C3 variants. Exploring the locations of Gly179 and Met194 in the crystal structure of SULT1C3d (2H8K), it was found that Gly179 and Met194 are located in a loop between α-helices 9 and 10 and a loop between α-helix 11 and β-sheet F, respectively (cf. (*29*)). Gly179 is relatively close to the substrate binding pocket and is conserved in all human SULTs, while Met194 is located on the outer surface and is not conserved. Intriguingly, in our docking experiments with 4,4′-OH-3,3′,5,5′-TCB, Val177 was found to be present in the substrate-binding pocket (Fig. 8D). The change of Gly179 to Arg, therefore, may affect the positioning of Val117 and change the conformation of substrate-binding pocket. Further studies are

warranted in order to clarify the impact of these natural variants on the enzyme activity of SULT1C3 allozymes.

In contrast to SULT1C3a, SULT1C3d was found to be capable of sulphating a wide range of substrates including bile acids, steroid hormones, thyroid hormones, pyrenes and hydroxyl biphenyls (Table II). It has been proposed that SULT1C3d may be capable of sulphating large molecule compounds like pyrenes (*14*). In our study, SULT1C3d showed indeed sulphating activity toward 1-hydroxypyrene (0.46 ± 0.01) and 1-hydroxymethylpyrene (0.09 ± 0.01), indicating that phenolic hydroxyl groups may be preferred substrates than benzylic alcohols. While our data showed that lithocholic acid was the best endogenous substrate among endogenous compounds tested, SULT1C3d showed stronger sulphating activity toward chlorinated phenols like 4,4′-OH-3,3′,5,5′-TCB and pentachlorophenol among the 100-plus compounds tested in this study. Interestingly, 4,4′-OH-3,3′,5,5′-TCB and pentachlorophenol were the only two compounds with which SULT1C3a displayed sulphating activity. To compare the enzymatic characteristics of SULT1C3a and SULT1C3d, the pH-dependence and kinetic analysis with 4,4′-OH-3,3′,5,5′-TCB as a substrate was investigated. While the pH-dependence analysis showed similar pH profiles of the sulphating activity of SULT1C3a and SULT1C3d, kinetic analysis revealed that SULT1C3a and SULT1C3d followed distinct kinetic mechanism and catalytic action for the sulphation of 4,4′-OH-3,3′,5,5′-TCB (Fig. 5). Those results indicated that the alternative C-terminal amino acid sequences from exons 7a/7b and 8a/8b may significantly affect the substrate recognition of SULT1C3 variants. Major differences were found in the C-terminal sequences of SULT1C3a and SULT1C3d, particularly amino acid residues 245–258 in a loop-3 structure (amino acid residues 245–269) (*29*). Previous molecular simulations analyses have demonstrated that the loop-3 structure may shift from an open state to a close state in response to the PAPS binding, which generates a substrate entry gate and becomes itself an outer portion of substrate-binding pocket in the SULT molecule (*29*, *30*). It has been reported that the alteration of amino acids in the N-terminal portion (the substrate entry site) of loop-3 of SULT1C3d may change the shape and charge distribution of the substrate-binding site of loop-3 (*16*). In accord to this proposition, the effect of the alteration of exons 7 and 8 on the activity of SULT1C3a versus SULT1C3d was clearly observed in this study, with the decreased sulphating capacity of SULT1C3a. In the docking simulation data with 4,4′-OH-3,3′,5,5′-TCB, Tyr249 and Met257 has emerged as critical residues in the substrate-binding site and Tyr249 may form hydrogen bonding with the hydroxyl group of the substrate (Fig. 8D), indicating that the alteration of Tyr249 and Met257 to His and Phe, respectively, may affect the substrate recognition of SULT1C3a. Members of the SULT1C subfamily have been shown to preferably catalyze the sulphation of large molecules including methoxyestrogens, thyroid hormones, naphthols, and *N*-hydroxy-2-acetylaminofluorene (*8*, *15*, *30–32*). Like other SULT1C isoforms, SULT1C3d preferably catalyzed the sulphation of large molecules, especially chlorinated phenols (Table II). Using a panel of 20 hydroxyl-polychlorobiphenyls (OH-PCBs), it was found that while the sulphating activity of SULT1C3a was much weaker than that of SULT1C3d, the activity spectra were comparable between the two (Fig. 7). Interestingly, both SULT1C3a and SULT1C3d preferably catalyzed the sulphation of 2-hydroxyl (or 3-hydroxyl) PCBs rather than 4-hydroxy PCBs. For the sulphation of 4-hydroxy PCBs, 3,5-dichloro groups seemed to be important in the interaction with the active site of SULT1C3. Results from the docking simulation analysis were relatively consistent with the activity data. 2-Hydroxy PCBs were docked into the active site with the lowest binding energy and the chloro group rich ring was preferably docked into the catalytic centre with sulphate group. It therefore appears that 3,5-dichloro groups are important for the proper orientation of the 4-hydroxy group, unless the other ring is chloro group rich. The docking simulation analysis with 4-hydroxy PCBs showed that chloro rich ring tended to fit well into the active site. Previous studies have indicated that OH-PCBs may serve as substrates and inhibitors for many SULT enzymes, including SULT1A1, SULT1B1, SULT1E1 and SULT2A1 (*33–36*), and may affect the homeostasis of thyroid hormones and steroid hormones through sulphation. Moreover, OH-PCBs have been known to disrupt the endocrine system such as thyroid hormones and steroid hormones (*37*, *38*). In this regard, SULT1C3a and/or SULT1C3d may play a role in the detoxification of the OH-PCBs as well as other large xenobiotic molecules like hydroxypyrenes.

To summarize, genomic analysis indicated that the human *SULT1C3* gene appears to be a hybrid gene with alternative exons 7b and 8b being originated from a defunct *SULT1C1* gene, raising the question concerning their evolutionary origin and implying the generation of multiple SULT1C3 variants. Two splice variants, SULT1C3a and SULT1C3d, were cloned, purified and characterized. Enzymatic characterization revealed that, while SULT1C3a exhibited weak sulphating activity toward chloro phenols, SULT1C3d was capable of sulphating a range of compounds, including bile acids, thyroid hormones, chloro phenols and hydroxypyrenes. Our results indicated that the alternative exons 7a/7b and 8a/8b may dramatically affect the catalysis and/or substrate recognition. It remains to be verified whether in contrast to the limited substrate spectrum found in the present study, SULT1C3a may have unique substrate specificity toward endogenous compounds.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest
None declared.

Authors' Contribution

Participated in research design: K. Kurogi, H. Kouriki-Nagatomo, Y. Sakakibara, M. Suiko and M.-C. Liu.
Conducted experiments: K. Kurogi, T. Shimohira, G. Zhang and E. Miller.
Performed data analysis: K. Kurogi and M.-C. Liu.
Wrote or contributed to the writing of the manuscript: K. Kurogi and M.-C. Liu.

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