Journal of Neurochemistry, 2003, **87**, 809–819 doi:10.1046/j.1471-4159.2003.02027.x

Human catecholamine sulfotransferase (SULT1A3) pharmacogenetics: functional genetic polymorphism

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Abstract

Sulfotransferase (SULT) 1A3 catalyzes the sulfate conjugation of catecholamines and structurally related drugs. As a step toward studies of the possible contribution of inherited variation in SULT1A3 to the pathophysiology of human disease and/or variation in response to drugs related to catecholamines, we have resequenced all seven coding exons, three upstream non-coding exons, exon–intron splice junctions and the 5′-flanking region of *SULT1A3* using DNA samples from 60 African-American (AA) and 60 Caucasian-American (CA) subjects. Eight single nucleotide polymorphisms (SNPs) were observed in AA and five in CA subjects, including one non-synonymous cSNP (Lys234Asn) that was observed only in AA subjects with an allele frequency of 4.2%. This change in amino acid sequence resulted in only 28 ± 4.5% (mean ± SEM) of the enzyme activity of the wild-type (WT) sequence after transient expression in COS-1 cells, with a parallel decrease (54 ± 2.2% of WT) in level of SULT1A3 immunoreactive protein. Substrate kinetic studies failed to show significant differences in apparent K_m values of the two allozymes for either dopamine (10.5 versus 10.2 μM for WT and variant, respectively) or the cosubstrate 3′-phosphoadenosine 5′-phosphosulfate (0.114 versus 0.122 μM, respectively). The decrease in level of immunoreactive protein in response to this single change in amino acid sequence was due, at least in part, to accelerated SULT1A3 degradation through a proteasome-mediated process. These observations raise the possibility of ethnic-specific inherited alterations in catecholamine sulfation in humans.

Keywords: catecholamines, genetic polymorphism, pharmacogenetics, sulfation, sulfotransferase, SULT1A3.

J. Neurochem. (2003) **87,** 809–819.

SULT1A3 catalyzes the sulfate conjugation of dopamine (DA) and other catecholamines, as well as structurally related drugs (Wood *et al*. 1994) (Fig. 1). Sulfation is an important pathway in catecholamine biotransformation, and more than 95% of circulating dopamine and approximately 70% of the norepinephrine in human blood are sulfate conjugated (Johnson *et al*. 1980). SULT1A3 is expressed in the human intestine, brain, platelet and other tissues (Glatt *et al*. 2001). Therefore, not only endogenous catecholamines but also orally administered drugs would be expected to undergo SULT1A3-catalyzed biotransformation. Many other human SULT isoforms display common, functionally significant genetic polymorphisms (Raftogianis *et al*. 1999; Freimuth *et al*. 2001; Thomae *et al*. 2002; Adjei *et al*. 2003). We set out to determine whether that might also be true for SULT1A3.

SULT1A3 is one of three genes that encode highly homologous ‘phenol SULT’ isoforms. All three of these genes map to the short arm of chromosome 16, and the

Received June 4, 2003; revised manuscript received July 11, 2003; accepted July 11, 2003.

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Abbreviations used: AA, African-American; CA, Caucasian-American; DA, dopamine; ORF, open reading frame; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RRL; rabbit reticulocyte lysate; SNP, single nucleotide polymorphism; SULT, sulfotransferase; TPMT, thiopurine S-methyltransferase; WT, wild-type.

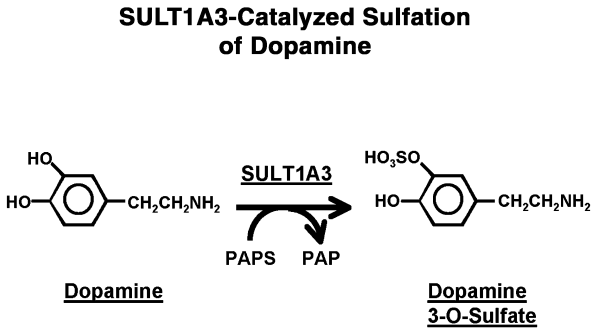

Fig. 1 SULT1A3-catalyzed dopamine sulfation.

proteins which they encode are more than 93% identical in amino acid sequence (Her *et al*. 1996; Raftogianis *et al*. 1996, 1997, 1999; Falany 1997). Cloning and characterization of the SULT1A3 cDNA and gene (Zhu *et al*. 1993; Wood *et al*. 1994; Aksoy and Weinshilboum 1995) set the stage for the present studies. The SULT1A3 cDNA open reading frame encodes a 295 amino acid protein (Wood *et al*. 1994). The gene is approximately 8.4 kb in length and consists of seven coding and at least three upstream non-coding exons (Wood *et al*. 1994). *SULT1A3*, like other members of the SULT1A gene subfamily, displays alternative transcriptional initiation and alternative splicing of non-coding exons (Wood *et al*. 1994; Aksoy *et al*. 1995; Weinshilboum *et al*. 1997). In contrast to many other human SULT genes, no SULT1A3 orthologs have been reported in other species. That fact may explain the relative importance of sulfation, or more correctly sulfonation, as a metabolic pathway for catecholamines in humans as compared with other mammalian species (Eisenhofer *et al*. 1998). Levels of SULT1A3 activity in at least one human tissue, the blood platelet, have been shown to be regulated by inheritance (Reveley *et al*. 1982/83; Price *et al*. 1988), but those studies were performed prior to the cloning and characterization of the SULT1A3 cDNA and gene. Therefore, in the present series of experiments we set out to determine whether any common, functionally significant genetic polymorphisms might be present in *SULT1A3*. We began by resequencing the gene, including all seven coding exons, the three upstream non-coding exons and a portion of the 5′-flanking region (5′-FR), using DNA samples from 60 Caucasian-American (CA) and 60 African-American (AA) subjects. We identified nine single nucleotide polymorphisms (SNPs), including one novel coding SNP (cSNP) that resulted in a Lys234Asn change in encoded amino acid. That non-synonymous cSNP was observed only in AA subjects, with an allele frequency of 4.2%. We then created expression constructs for both the wild-type (WT) and variant sequences and performed functional genomic studies of the encoded allozymes. After transient expression in COS-1 cells, the SULT1A3*2 (Asn234) variant allozyme displayed significant decreases in levels of both enzyme activity and protein as a result of rapid degradation by a proteasome-mediated process. These observations raise the possibility of ethnic-specific genetic variation in catecholamine sulfation.

Materials and methods

DNA samples

DNA samples from 60 CA and 60 AA subjects were obtained from the Coriell Cell Repository (Camden, NJ, USA) (Sample Sets HD100CAU and HD100AA). These samples had been collected and anonymized by the National Institute of General Medical Sciences. All subjects had provided written informed consent for the use of their DNA for research purposes, and the present studies were reviewed and approved by the Mayo Clinic Institutional Review Board.

SULT1A3 resequencing

The PCR was used to amplify each of the 10 SULT1A3 exons. The three non-coding exons were amplified by performing a single reaction for each exon. The coding exons were initially amplified as a group by the use of two overlapping 'long PCR' amplifications to ensure SULT1A3 gene specificity. This precaution was taken because of the high degree of sequence homology among the three genes encoding members of the SULT1A subfamily (Aksoy *et al*. 1995; Her *et al*. 1996; Falany 1997; Raftogianis *et al*. 1997). SULT1A3-specific primers that did not hybridize with the sequences of other SULT1A subfamily members or with known repeat sequences were used to perform the long PCR. Shorter, 'nested' PCR amplifications of the seven coding exons were then performed using the long PCR amplicons as template. The primers for those reactions were designed to amplify each of the exons plus a portion of the introns which flanked that exon. All primers for amplifications intended for use in DNA sequencing included M13 'tags' at their 5′-ends to make it possible to use dye primer sequencing chemistry. Dye primer sequencing was used to enhance our ability to identify heterozygous bases (Chadwick *et al*. 1996). The sequences of PCR primers used to perform these experiments are listed in Table 1.

Amplification reactions were performed with 0.5 units of AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA, USA), 5 μL of template DNA, 12.5 pmol of each primer, 2.5 mM dNTPs (Roche Diagnostics, Indianapolis, IN, USA) and 5 μL of 10× buffer with 15 mM $MgCl_2$ (Perkin Elmer), in a final reaction volume of 50 μL. The long PCR amplifications were initiated with a 12-min 'hot start' at 94°, followed by 35 cycles of 94° for 30 s, a primer-specific annealing temperature (see Table 1) for 30 s and 72° for 2 min, followed by a final 10 minute extension at 72°. Long PCR products were diluted 1 : 10 000 for use as template during the nested reactions. The nested exon-specific PCR reactions were also initiated with a 12-min 'hot start' at 94°, followed by 35 cycles at 94° for 30 s, a primer-specific annealing temperature for 30 s and 72° for 30 s, with a final 3 min extension at 72°. The only exception was the reaction used to amplify exons 5 and 6, which began with a 12-min 'hot start' at 94°, followed by 35 cycles at 94° for 30 s and 72° for 90 s, with a final 3 min extension at 72°. All reactions were performed either in a Perkin Elmer Model 9700 thermal cycler (Foster City, CA, USA) or a Biometra Tgradient thermal cycler

Table 1 Human SULT1A3 gene resequencing and site-directed mutagenesis primers.

Primer location	Primer name	Primer sequence	Annealing temperature, °C
Resequencing 'long PCR'			
Long A			
Intron 1A	UF(-1174)	GCACGAGGCTCTAGCCCCTGGTACCAGCTT	60
Intron 4	I4R1074	GCACTGTAGAGACTCACTATCCTTTGCATCAGAC	60
Long B			
Intron 4	I4F104	GTCTGATGCAAAGGATAGTGAGTCTCTACAGTGC	62
3′-UTR	DR1405	GATATGGTGACCTGGGGAGCTGAAGGCTGG	62
Resequencing 'nested PCR'			
5′-Flanking region	UF(-4118)	TGTAAAACGACGGCCAGTTAATGGACGATGGGTGCCTTCTACT	60
5′-Flanking region	UR(-3699)	CAGGAAACAGCTATGACCATGGGCCCTTAGCAGG	60
5′-Flanking region	UF(-3833)	TGTAAAACGACGGCCAGTTGTAATGCCCGCAACAGTGC	60
Intron 1B	UR(-3532)	CAGGAAACAGCTATGACCTAGGAGGGGGTAAGGACACTGAGGATCA	60
Intron 1B	UF(-1705)	TGTAAAACGACGGCCAGTCAATACCAATGTTGGCCCCTTTTG	60
Intron 1A	UR(-1212)	CAGGAAACAGCTATGACCCACCCTGTCTCAAAAATACACAAAGG	60
Intron 1A	I1F(-391)	TGTAAAACGACGGCCAGTAGCAAAAACTCTGCAAAGGGGC	60
Intron 2	I2R (52)	CAGGAAACAGCTATGACCACCAAGGTGGGGACTGCCG	60
Intron 2	X2F135	TGTAAAACGACGGCCAGTACACCTACCCCAAGTCT	60
Intron 4	I4R120	CAGGAAACAGCTATGACCTGGGATGTCATCTCTACAGCA	60
Intron 4	I4F(-151)	TGTAAAACGACGGCCAGTCGAGCAGGGTTCAGATCCCAG	72
Intron 6	I6R116	CAGGAAACAGCTATGACCGAATCCGGGCTTGCTGTGGGAG	72
Intron 6	16F(-34)	TGTAAAACGACGGCCAGTGTTTTGCTCCACTGAGGAGCCC	67
3′-UTR	DR984	CAGGAAACAGCTATGACCGAGCCACTGTGCCTGACTCA	67
Site-directed mutagenesis			
cDNA	F(-28)	GTAGAAGACTCAGAATTAGAAGAGGAACATG	64
cDNA	R982	GCCACTGTGCCTGACTCAAAAATCATAC	64
Exon 7	F702m	CAAGGAGATGAAGAATAACCCTATGACCAAC	64
Exon 7	R702m	GTTGGTCATAGGGTTATTCTTCATCTCCTTG	64

The underlined portions of the resequencing primers are M13 'tags' that were added at the 5' ends to make it possible to use dye-primer DNA sequencing chemistry. The underlined nucleotide in the primers used to perform site-directed mutagenesis is the 'mutated' base used to create the *2 allozyme. 'F' represents forward; 'R', reverse; 'U' upstream; 'D', downstream, 'I', intron and "X' for exon. 'Long A' and 'Long B' refer to two overlapping long PCR amplifications. The numbering scheme for primer locations is described in the text. Annealing temperatures for the amplifications are also listed.

(Göttingen, Germany). Amplicons from these reactions were sequenced with an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) in the Mayo Molecular Biology Sequencing Core Facility using Big Dye™ (Perkin Elmer) dye primer chemistry. All samples were sequenced on both strands and those with ambiguous chromatograms, as well as samples with SNPs that had been observed in only a single sample, were subjected to a second, independent round of amplification, followed by DNA sequencing.

SULT1A3 COS-1 cell expression

An expression construct for the variant SULT1A3*2 sequence (Asn234) was created by site-directed mutagenesis of the WT cDNA sequence (SULT1A3*1, Lys234) using overlap extension (Ho *et al*. 1989). Sequences of primers used to create this construct are listed in Table 1. Expression constructs were then created by cloning the WT and variant sequences into the expression vector pCR3.1 (Invitrogen, Carlsbad, CA, USA). Sequences of the inserts were verified by completely sequencing both strands. These expression constructs were used to transfect COS-1 cells using the TransFast reagent (Promega, Madison, WI, USA) at a charge ratio of 1 : 1 in serum-free Delbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD, USA) with a 2-h transfection time. An 'empty' pCR3.1 vector, one that did not contain insert, was used as a control to make it possible to correct for possible endogenous SULT1A3 enzyme activity in the COS-1 cells. Endogenous activity was found to be negligible, averaging less than 1% of the activity present after transfection with the WT construct. During transfection, 7 μg of construct DNA was cotransfected with 7 μg of pSV-β-galactosidase DNA (Promega) to make it possible to correct for transfection efficiency. The COS-1 cells were then incubated at 37° for 48 h,

washed with phosphate-buffered saline (PBS) and resuspended in 2 mL of 5 mM potassium phosphate buffer, pH 6.5. The cells were lysed with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA), and the homogenates were centrifuged at 100 000 × *g* for 1 h at 4°. The resulting cytosol preparations were stored at −80° prior to assay. Nine separate plates were transfected with each construct.

Enzyme assays

Recombinant allozymes were assayed for SULT1A3 activity with the radiochemical enzymatic assay of Foldes and Meek (1973) as modified by Anderson and Weinshiboum (1980) and Anderson *et al*. (1981). Specifically, 40 μM dopamine (DA) was the sulfate acceptor substrate and 0.4 μM ^{35}S-3′-phosphoadenosine 5′-phosphosulfate (PAPS) was the sulfate donor. Blanks were samples that contained no sulfate acceptor substrate. Enzyme activity was corrected for transfection efficiency by measuring β-galactosidase activity spectrophotometrically with the β-Galactosidase Enzyme Assay system (Promega) as suggested by the manufacturer. The same SULT1A3 assay was used to determine apparent K_m values for the two cosubstrates for the reaction. Specifically, six DA concentrations that varied from 2.5 to 80 μM were tested in the presence of 0.4 μM ^{35}S-PAPS. Activity was also measured in the presence of 40 μM DA with five concentrations of PAPS that varied from 0.035 to 0.55 μM.

Western blot analysis

Levels of immunoreactive SULT1A3 were determined for each recombinant allozyme by performing western blot analysis. A rabbit polyclonal antibody (1 : 1000 dilution) directed against SULT1A3 amino acids 85–101, with an additional C-terminus cysteine, was used to perform these studies. This portion of the protein was chosen to generate the antibody because it included an area within which SULT1A3 differed maximally from SULT1A1 but, even so, there were only five differences between the two enzymes in amino acid sequence at this location. This peptide, linked to keyhole limpit hemocyanin, was used by Cocalico (Reamstown, PA, USA) to generate rabbit polyclonal antibody. The antibody was tested for specificity by performing western blot analysis with both recombinant human SULT1A1 and SULT1A3. Furthermore, no cross-reacting protein was present in the cytosol of COS-1 cells that had not been transfected with SULT1A3 expression constructs. No binding to human SULT1A1 was observed, but the antibody detected human SULT1A3. During western blot analysis, COS-1 cytosol was loaded on 12% SDS mini-gels (Bio-Rad, Hercules, CA, USA) in quantities that resulted in equal β-galactosidase activity in order to correct for transfection efficiency. Electrophoresis was performed for 1 h at 150 V, and proteins were transferred to nitrocellulose membranes. The membranes were then blocked overnight with 5% milk in Tris-buffered saline with Tween-20 (TBST). The following day, they were incubated with primary antibody diluted 1 : 1000 with 5% milk in TBST, followed by three washes. The secondary antibody was a 1 : 10 000 dilution of goat anti-rabbit horseradish peroxidase (Bio-Rad) applied for 1 h in 5% milk in TBST, also followed by three washes. Bound antibody was detected by enhanced chemiluminescence performed with the ECL western Blotting System (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously (Aksoy *et al*. 1993). The data were analyzed with the AMBIS Radioanalytic Imaging System, Quant Probe version 4.31 (AMBIS, San Diego, CA, USA). Multiple blots were performed for each allozyme, and immunoreactive protein levels were expressed as a percentage of the intensity of the WT construct on that same gel.

Rabbit reticulocyte lysate protein degradation studies

Transcription and translation of the two SULT1A3 allozymes (Lys234 and Asn234) were performed using the TNT® coupled rabbit reticulocyte lysate (RRL) System (Promega, Madison, WI, USA). Specifically, 25 μL of RRL that had been 'treated' to inhibit protein degradation, plus 2 μL T7 buffer, 1 μL T7 polymerase, 1 μL of a mixture of amino acids that lacked methionine, 1 μL RNasin, and 2 μL ^{35}S-methionine (1000 Ci/mmol, 10 mCi/mL, 0.4 μM final concentration) were used to perform these studies. With the exception of the RNasin (Promega), and the ^{35}S-methionine (Amersham Pharmacia Biotech), all reagents were included in the Promega kit. One microgram of the pCR3.1 expression construct DNA was then added to the mixture; the reaction volume was increased to 50 μL with nuclease-free water (Promega); and the mixture was incubated at 30° for 90 min. A 5-μL aliquot was then used to perform electrophoresis with a 12% SDS-PAGE gel that was subsequently dried and exposed to X-ray film (Kodak, Rochester, NY, USA).

For the protein degradation experiments, 60 μL of an adenosine 5′-triphosphate (ATP) generating system, 60 μL of 'untreated' RRL and 12 μL of ^{35}S-methionine radioactively labeled protein were combined. The ATP generating system consisted of 100 μL each of: 1 M Tris-HCl, pH 7.8, 160 mM $MgCl_2$, 120 mM KCl, 100 mM dithiothreitol, 100 mM ATP, 200 mM creatine phosphate and 2 mg/mL kinase (all from Sigma, St. Louis, MO, USA), plus 300 μL of nuclease-free water (Promega). This mixture was incubated at 37°, and aliquots were removed every 3 h for 24 h. A variant allozyme for a genetically polymorphic drug metabolizing enzyme that is known to be degraded rapidly, thiopurine *S*-methyltransferase (TPMT)*3A (Tai *et al*. 1999), was used as a positive control for these experiments. Specifically, the TPMT ORF sequence with GenBank accession number U30512 that had been cloned into pCR3.1 was used to perform *in vitro* transcription and translation. Aliquots removed at different times were subjected to electrophoresis on a 12% SDS–PAGE gels. The gels were dried and exposed to X-ray film, and ^{35}S-methionine radioactively labeled proteins were quantified using the AMBIS System. Similar experiments were also performed in the presence of proteasome inhibitors. Specifically, 80 μM MG132 (Calbiochem, La Jolla, CA, USA) or 100 μM hemin (Calbiochem) were incubated at 37° for 10 min in the protein degradation mixtures. Control samples that contained no inhibitor were also studied. Five microliters of ^{35}S-methionine radioactively labeled translated protein was then added, the mixture was incubated at 37° for 24 h, and aliquots of each sample were taken at 0 and 24 h. These samples were also subjected to electrophoresis, followed by autoradiography.

Data analysis

DNA sequence for the gene resequencing studies was analyzed using the PolyPhred 4.0 (Nickerson *et al*. 1997) and Consed 8.0 (Gordon *et al*. 1998) programs. The Wisconsin Genetics Computer Group (GCG) package, version 10, was also used to analyze nucleotide sequence and to confirm that the sequence obtained was specific for SULT1A3 rather than SULT1A1 or SULT1A2. The

SULT1A3 cDNA and genomic consensus sequences against which the resequencing data were compared were those with GenBank Accessions numbers L19956 and U20499, respectively. The SULT1A1 cDNA and genomic consensus sequences used were those with GenBank Accession numbers U28170 and U52852, respectively, while the SULT1A2 cDNA and genomic consensus sequences were those with GenBank Accession numbers U28169 and U34804, respectively. Human SNP databases, including the public Human Genome Project and Celera databases, were searched to determine whether polymorphisms that we observed had been reported previously. The RepeatMasker (University of Washington) program was used to screen for repeat sequences. Values for π and θ were calculated using the Arlequin program (Schneider *et al*. 2000). D′ values for linkage analysis of polymorphism pairs were calculated as described by Hartl and Clark (2000) and Hedrick (2000). Haplotype analysis was performed as described by Schaid *et al*. (2002). Apparent K_m values for enzyme kinetic studies were calculated with the method of Wilkinson (1961) and a computer program developed by Cleland (1963). Points that deviated from linearity on double inverse plots as a result of substrate inhibition were not included in these calculations. Specifically, four concentrations of each substrate were used for all K_m calculations within the following ranges: 2.5–20 μM for dopamine and 0.035 and 0.273 μM for PAPS. Differences between mean values were determined by use of student's *t*-test with the Statview 4.5 program (Abacus Concepts, Berkeley, CA, USA). Half-life values for protein degradation were calculated by using the Excel program (Microsoft, Redman, WA, USA) to analyze semilogarithmic plots of the quantity of protein remaining at various time points.

Results

Human *SULT1A3* resequencing

SULT1A3 was resequenced using DNA samples from 60 AA and 60 CA subjects. Seven PCR amplifications were performed for each sample, and a total of over 900 000 bp of DNA sequence was analyzed on both strands. Resequencing resulted in the identification of nine single nucleotide polymorphisms (SNPs), eight in DNA from AA and five in DNA from CA subjects. SULT1A3 SNP locations and frequencies are listed in Table 2 and are depicted graphically in Fig. 2. One coding region SNP was non-synonymous and changed the amino acid at position 234 from Lys → Asn. This polymorphism was observed only in AA subjects, with an allele frequency of 4.2%. Seven of the nine SNPs were novel (i.e. not present in any publicly available database), and six of the nine had frequencies of greater than 1% and would therefore be considered 'common' in these populations. Polymorphism locations were numbered based on their position in the cDNA, with the 'A' in the translation initiation codon assigned the number (+ 1). Negative numbers were located 5′-upstream of this location, positive numbers were located 3′-downstream and there was no 'zero'. SNPs in introns have been numbered from intron-exon splice junction, with (+ 1) located at the initial position in the 5′ splice donor site. The average number of SULT1A3 polymorphisms per kb of sequence in the 120 samples studied, 2.4 polymorphisms/kb, was smaller than that observed in similar studies of a large number of human genes, 4.6 polymorphisms/kb (Halushka *et al*. 1999). We also determined 'nucleotide diversity', a quantitative measure of genetic variation, adjusted for the number of alleles studied. Two standard measures of nucleotide diversity are π, average heterozygosity per site, and θ, a population mutation measure which is theoretically equal to the neutral mutation parameter (Fullerton *et al*. 2000). In the samples that we studied, $\pi = 0.94 \pm 0.65 \times 10^{-4}$ for SULT1A3 in AA and $0.89 \pm 0.63 \times 10^{-4}$ for CA subjects, while θ was $1.49 \pm 0.62 \times 10^{-4}$ for AA and $0.93 \pm 0.45 \times 10^{-4}$ for CA subjects. These figures were both lower than average values reported by Stephens *et al*. (2001) for 292 human autosomal genes (average $\pi = 5.8 \times 10^{-4}$ and average $\theta = 9.6 \times 10^{-4}$).

Table 2 Human *SULT1A3* genetic polymorphisms

		Frequency of Variant allele			
Location	**Nucleotide**	**Sequence Change**	**Amino Acid Change**	**African-American**	**Caucasian-American**
Intron 1C	I1C(96)	C → T		0.000	0.008
Intron 1C	I1C(99)	G → C		0.660	0.500
Intron 1C	I1C(105)	C → T		0.017	0.050
Intron 1A	I1A(1325)	A → G		0.075	0.000
Exon 2*	**105**	**A → G**		**0.033**	**0.108**
Intron 4	I4(1369)	G → A		0.008	0.000
Exon 7	**702**	**G → T**	**Lys234Asn**	**0.042**	**0.000**
Intron 7*	I7(113)	C → T		0.008	0.042
Exon 8	**843**	**G → A**		**0.083**	**0.000**

The table lists the nine polymorphisms observed during the SULT1A3 resequencing experiments. Frequencies for each population are listed. Exon sequencies are shown in bold. The numbering scheme for polymorphism locations is described in the text. *Reported previously

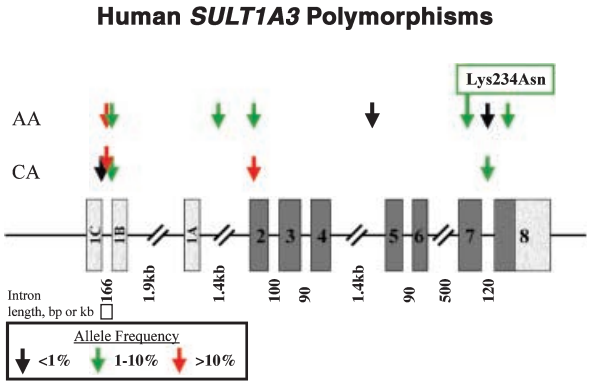

Fig. 2 Human *SULT1A3* polymorphisms. The figure shows a schematic representation of the human SULT1A3 gene with the locations of SNPs indicated by arrows. Dark rectangles represent exons encoding the open reading frame, while light rectangles represent exons or portions of exons that encode untranslated region (UTR) sequence. 'AA' represents data obtained with DNA from African-American subjects, and 'CA' represents data obtained with DNA from Caucasian-American subjects. Arrows show polymorphism locations, and allele frequencies are indicated by the colors of the arrows. The amino acid change as a result of the non-synonymous cSNP is also indicated.

Table 3 Human *SULT1A3* Linkage Analysis

AA Polymorphism pair		D′ value	*p* value
I1C(99)	I1A(1325)	−1.0	0.031
IC1(99)	843	0.8	0.017
CA Polymorphism Pair			
IC1(99)	105	−1.0	0.003

Only polymorphism pairs with a D′ value ≥ (0.8) or ≤ (−0.8) and with *p* values less than 0.05 have been listed.

Linkage and haplotype analysis

There is increasing appreciation of the fact that haplotype can often be even more useful than individual polymorphisms for the prediction of function (Drysdale *et al*. 2000). Therefore, we performed linkage and haplotype analyses for the nine SNPs that we observed in *SULT1A3*. For the linkage analysis, D′ values were calculated for all pairwise combinations of SNPs. D′ values can range from (+ 1.0) when two polymorphisms are maximally positively associated to (−1.0) when two polymorphisms never occur together. As already mentioned, there were relatively fewer SNPs in *SULT1A3* than in other genes – including SULT genes – that we have resequenced (Freimuth *et al*. 2001; Thomae *et al*. 2002; Adjei *et al*. 2003). Therefore, only two pairs of SNPs for DNA samples from AA subjects and only one pair for CA samples had D′ values of ≥ (0.8) or ≤ (− 0.8) with *p*-values < 0.05 (Table 3). Haplotypes can be determined unequivocally if not more than one location is heterozygous, but it is also possible to 'infer' haplotypes computationally (Schaid *et al*. 2002). Haplotype analysis for *SULT1A3* showed the presence of nine unequivocal *SULT1A3* haplotypes for AA subjects and six for CA subjects as well as three inferred haplotypes for AA subjects (Table 4).

SULT1A3 allozyme activity and western blot analysis

Functional genomic studies were performed after transient expression in COS-1 cells of the WT (SULT1A3*1) and Asn234 (SULT1A3*2) allozymes. A mammalian cell line was used to perform these experiments to ensure that appropriate post-translational modification and protein degradation systems would be present. There have already been several reports that the alteration of only one or two amino acid as a result of common genetic polymorphisms can result in decreased levels of protein (Szumlanski *et al*. 1996; Preuss *et al*. 1998; Freimuth *et al*. 2001; Thomae *et al*. 2002; Adjei *et al*. 2003), often as a result of rapid variant allozyme degradation through a proteasome-mediated process (Tai *et al*. 1999; Siegel *et al*. 2001). After transient expression in COS-1 cells and correction for transfection efficiency, there was a 72% decrease in level of SULT1A3 enzyme activity for the *2(Asn234) variant when compared with *1(WT, Lys234) allozyme (Fig. 3a and Table 5).

One way in which alterations in encoded amino acid might lead to decreased enzyme activity is as a result of changes in substrate kinetics. Therefore, we determined apparent K_m values for the WT SULT1A3*1 (Lys234) and the variant *2 (Asn234) allozymes. Since SULTs can display profound substrate inhibition (Weinshilboum and Otterness 1994), a range of substrate concentrations that did not display deviations from Michaelis–Menten behavior were used to perform these calculations. Apparent K_m values for SULT1A3*1 and SULT1A3*2 were nearly identical for both cosubstrates (Table 5). Therefore, we next turned our attention to the possibility, as has been observed for many other genetically polymorphic enzymes (Szumlanski *et al*. 1996; Preuss *et al*. 1998; Freimuth *et al*. 2001; Thomae *et al*. 2002; Adjei *et al*. 2003), that the common SULT1A3 non-synonymous cSNP might result in a decrease in the quantity of enzyme protein.

Quantitative western blot analysis, corrected for transfection efficiency, showed a significant decrease in the level of immunoreactive protein for the variant SULT1A3*2 allozyme as compared with SULT1A3*1. Specifically, the level of immunoreactive protein during nine independent transfections showed a decrease to 53.6% of the WT value (Fig. 3b and Table 5). The antibody used to detect SULT1A3 in these experiments was directed against peptides 85–101, located some distance from the codon 234 SNP. Therefore, this series of experiments demonstrated that the alteration of a single SULT1A3 amino acid, without a significant change in K_m values, resulted in a striking decrease in both levels of activity and quantity of immunoreactive enzyme protein. Similar observations have been made in the cases of several other genetically polymorphic enzymes (Szumlanski *et al*. 1996; Preuss *et al*. 1998;

Table 4 Human *SULT1A3* haplotype analysis

Allele	Frequency		Type	I1C(96)	I1C(99)	I1C(105)	I1A(1325)	105	I4(1369)	702	17(113)	843
	AA	CA										
**1A*	0.52	0.40	O	C	G	C	A	A	G	G	C	G
**1B*	0.23	0.39	O	C	**C**	C	A	A	G	G	C	G
**1C*	0.07	–	O	C	G	C	**G**	A	G	G	C	G
**1D*	0.07	–	O	C	**C**	C	A	A	G	G	C	**A**
**1E*	0.03	0.11	O	C	G	C	A	**G**	G	G	C	G
**1F*	0.02	0.05	O	C	**C**	**T**	A	A	G	G	C	G
**1G*	0.01	–	O	C	G	C	A	A	G	G	C	**A**
**1H*	0.01	–	O	C	G	C	A	A	**A**	G	C	G
**1I*	0.01	–	I	C	**C**	C	A	**G**	G	G	C	**A**
**1J*	–	0.04	O	C	**C**	C	A	A	G	G	**T**	G
**1K*	–	0.01	O	**T**	G	C	A	A	G	G	C	G
**2A*	0.03	–	O	C	G	C	A	A	G	**T**	C	G
**2B*	0.01	–	I	C	**C**	C	A	A	G	**T**	**T**	G
**2C*	0	–	I	C	G	C	**G**	A	G	**T**	C	G

The 11 unambiguous haplotypes ('O'= observed), as well as three inferred haplotypes ('I' = inferred) in the 120 DNA samples studied are listed. Sequence within each of the alleles that differed from the *SULT1A3* consensus sequence (**1A*) has been shown in bold type. Initial haplotype designations were made on the basis of the encoded amino acid sequence, with the WT (Lys234) sequence designated as *1* and* the variant (Asn234) designated as **2*. 'Letter' designations were then added based on descending allele frequencies, starting with haplotypes present in both ethnic groups and then making assignments based on haplotypes observed in samples from African–American subjects.

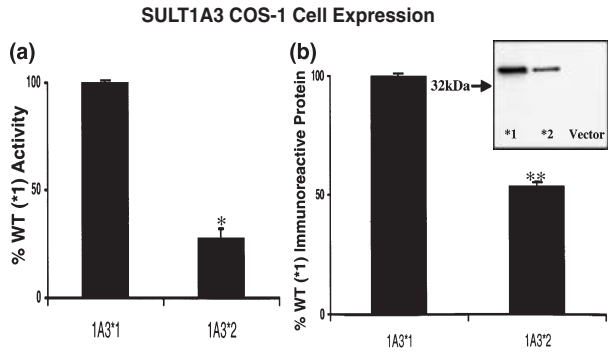

Fig. 3 SULT1A3 COS-1 cell expression. Results of the expression of SULT1A3*1 (Lys234) and *2 (Asn234) in COS-1 cells are shown. (a) Average levels of SULT1A3 enzyme activity for the *1 and *2 allozymes with DA as the sulfate acceptor substrate are shown. Each bar represents the average of nine independent transfections which have been corrected for transfection efficiency (mean ± SEM). * = $p < 0.01$ when compared to the *1 construct. (b) Average levels of SULT1A3 immunoreactive protein are shown for the *1 and *2 allozymes using a SULT1A3-specific antibody to perform western blot analyses. Each bar represents the average of 9 independent transfections (mean ± SEM). **$p < 0.001$. The gels were loaded on the basis of cotransfected β-galactosidase activity to correct for transfection efficiency. The 'insert' is a representative western blot used to obtain these data. The 32 kDa marker was soybean trypsin inhibitor (Bio-Rad).

Freimuth *et al*. 2001; Thomae *et al*. 2002; Adjei *et al*. 2003). In those situations that have been studied in detail, this decrease has resulted from accelerated degradation of the genetically variant allozyme (Tai *et al*. 1999; Siegel *et al*. 2001). Therefore, in the next series of experiments, we attempted to determine whether rapid degradation might also contribute to the decrease in level of SULT1A3*2 protein that we had observed.

SULT1A3 degradation

The degradation of SULT1A3*1 and *2 was studied in the rabbit reticulocyte lysate (RRL), an experimental system that has been used commonly to perform this type of experiment (Tai *et al*. 1999; Siegel *et al*. 2001). As a first step, we translated each allozyme, plus the human TPMT*3A variant allozyme as a positive control for rapid degradation, using the TNT® RRL system (Fig. 4). The same expression constructs that had been used to transfect COS-1 cells were used to perform these experiments. The ^{35}S-methionine radioactively labeled proteins produced using the TNT® RRL system were then added to untreated RRL that included an ATP generating system. These mixtures were incubated for 24 h, and aliquots were removed at regular intervals to perform SDS-PAGE. During the 24 h observation period, the level of the WT *1 allozyme protein decreased approximately 15%, while 71% of the variant protein was lost during the same time (Fig. 5). The figure shows average data for four independent experiments. The half-life of the SULT1A3*1 allozyme was estimated to be approximately 53 h, while that for the SULT1A3*2 variant allozyme was approximately 14 h ($p < 0.001$) (Table 5).

In an attempt to further clarify the mechanism responsible for accelerated degradation of SULT1A3*2, we repeated the

Table 5 Human SULT1A3 Functional Genomics

Allozyme	Enzyme activity %	Immunoreactive protein %	Dopamine Km, μM	PAPS, Km, μM	Protein half-life, h
SULT1A3*1	100 ± 8.2	100 ± 3.4	10.5 ± 1.3	0.114 ± 0.003	53 ± 2.1
SULT1A3*2	27.6 ± 4.5	53.6 ± 2.2	10.2 ± 0.6	0.112 ± 0.010	14 ± 2.9

The results of the SULT1A3 functional genomic studies are listed. Average values for levels of activity and immunoreactive protein for nine independent COS-1 cell transfections, apparent K_m values for dopamine and PAPS, as well as protein half-life values in rabbit reticulocyte lysate are listed. All values are mean ± SEM.

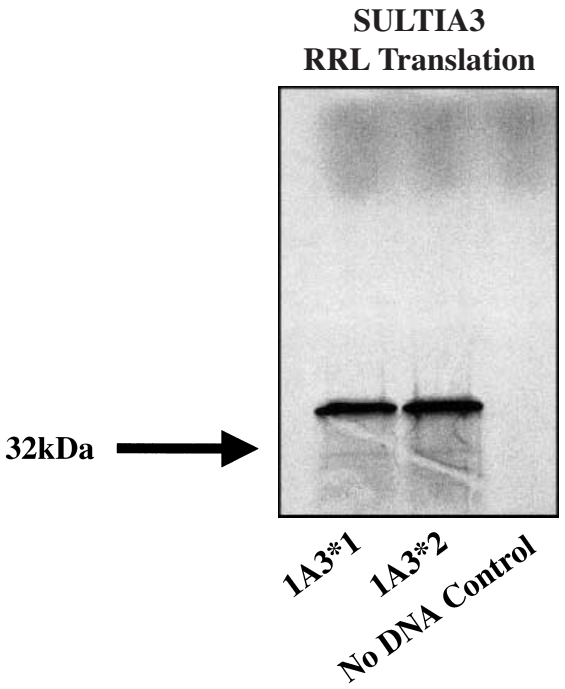

Fig. 4 SULT1A3 rabbit reticulocyte lysate (RRL) translation. SULT1A3*1 and *2 were translated using the TNT® RRL system. A representative autoradiograph of ^{35}S-methionine radioactively labeled recombinant SULT1A3 protein is shown.

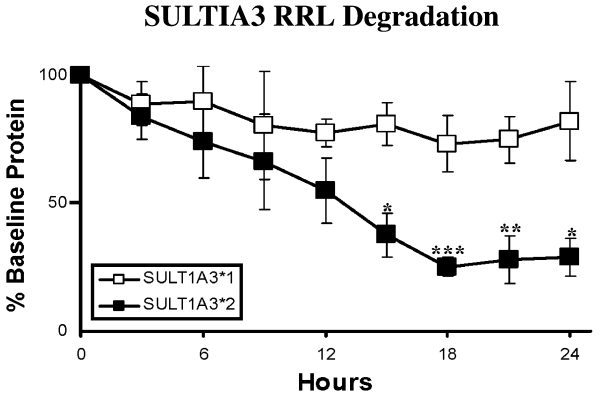

Fig. 5 SULT1A3 rabbit reticulocyte lysate (RRL) protein degradation. ^{35}S-Methionine radioactively labeled SULT1A3*1 and *2 were incubated in a RRL for 24 h, and loss of the protein was determined by SDS-PAGE. Each point represents the average of 4 independent experiments (mean ± SEM). * = $p < 0.02$, ** $p < 0.05$, *** $p < 0.005$ when compared with the *1 allozyme at the same time.

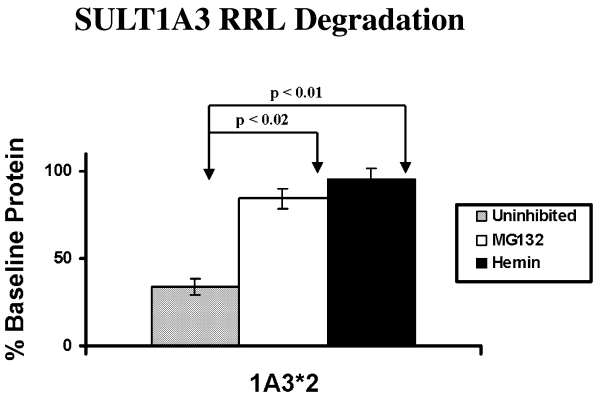

Fig. 6 SULT1A3*2 rabbit reticulocyte lysate (RRL) protein degradation. ^{35}S-Methionine radioactively labeled SULT1A3*2 was incubated in a RRL for 24 h in the presence of the proteasome inhibitors hemin (100 μM) and MG132 (80 μM). Bars represent average values for five independent experiments (mean ± SEM) expressed as a percentage of baseline.

degradation experiments for that allozyme in the presence of 2 different proteasome inhibitors, MG132 and hemin, at concentrations that have been shown to effectively inhibit the proteasome in the RRL (Tai *et al*. 1999). Each inhibitor was preincubated with the ATP generating system and untreated RRL. Radioactively labeled SULT1A3*2 protein was then added, and degradation was allowed to occur for 24 h. Inclusion of both MG132 and hemin significantly inhibited degradation of the SULT1A3*2 variant allozyme (Fig. 6), indicating that degradation was, at least in part, proteasome-dependent.

SULT1A3 X-ray crystal structure and comparative genomics

The X-ray crystal structure of SULT1A3 has been solved by two groups (Bidwell *et al*. 1999; Dajani *et al*. 1998, 1999). Unfortunately, the structure of a 26 amino acid region of SULT1A3 could not be determined by either group of investigators, and that region included the polymorphic

codon 234. Two areas of high sequence conservation that are present in all cytosolic SULTs have been referred to as 'region I' and 'region IV' (Weinshilboum and Otterness 1994; Varin *et al*. 1995; Weinshilboum *et al*. 1997). Those areas have been shown by site-directed mutagenesis to be involved in binding the sulfate donor cosubstrate, PAPS (Komatsu *et al*. 1994; Chiba *et al*. 1995). There is also an area located just beyond region IV which is required for dimerization (Petrotchenko *et al*. 2001). The non-synonymous cSNP in SULT1A3 was not located in any of these highly conserved areas, but it was 23 amino acids upstream of region IV. The WT amino acid encoded by codon 234, Lys, was not highly conserved among human cytosolic SULTs. However, Lys was present at this location in all members of the SULT1A family, and the only human SULT that contained the variant amino acid (Asn234) at this location was the estrogen sulfotransferase, SULT1E1 (GenBank accession number U08098). When the SULT1A3 amino acid sequence was compared with those for mouse, rat and cow SULT1A1 orthologs, this particular residue was either Lys or Glu. GenBank accession numbers for the sequences used to make these comparisons were AAH05413 and BAB22294 for mouse, AAA41644 and CAA37065 for rat and AAA85510 for the cow.

Discussion

Sulfation is an important pathway in the biotransformation of many endogenous and exogenous compounds (Weinshilboum and Otterness 1994; Falany *et al*. 1997). SULT1A3 catalyzes the sulfate conjugation of dopamine, other catecholamines and structurally related drugs. Sulfation plays an important role in catecholamine biotransformation in humans, with 95–99% of circulating dopamine being sulfate conjugated (Johnson *et al*. 1980). Previous twin and family studies of Caucasian subjects indicated that inheritance is a major factor in the regulation of level of SULT1A3 activity in the human blood platelet (Reveley *et al*. 1982/83; Price *et al*. 1988). Because of the importance of this metabolic pathway in humans, we set out to determine whether SULT1A3, like the genes encoding other human SULT isoforms, might have common, functionally significant genetic polymorphisms, polymorphisms which might explain the influence of inheritance on level of this enzyme activity. A genotype-to-phenotype strategy was used to test that hypothesis. Nine polymorphisms were identified during gene resequencing, including one novel non-synonymous cSNP that changed the amino acid at codon 234 from Lys to Asn. That cSNP was observed only in AA subjects, with an allele frequency of 4.2%. A mammalian expression system, the COS-1 cell, was used to express both the SULT1A3*1 (WT) and the *2 variant proteins. Cells transfected with the variant allozyme expressed less than 30% of the activity of those transfected with the WT allozyme (Fig. 3a), a difference that could not be explained by an alteration in substrate kinetics (Table 5).

Enzymes encoded by other genes that contain common non-synonymous cSNPs which alter one or two amino acids have shown patterns of reduction in both levels of enzyme activity and protein similar to that which we observed for SULT1A3 (Szumlanski *et al*. 1996; Preuss *et al*. 1998; Freimuth *et al*. 2001; Thomae *et al*. 2002; Adjei *et al*. 2003). In those cases that have been studied in detail, accelerated degradation of the variant protein has been the most frequent mechanism responsible (Tierney *et al*. 1992; Roberts 1997; Tai *et al*. 1999; Siegel *et al*. 2001). Therefore, we tested the possibility that SULT1A3*2 might undergo more rapid degradation than the WT allozyme, SULT1A3*1. The RRL system, an experimental system that has been an important tool for studies of protein degradation, was used to test that hypothesis (Mayer *et al*. 1989; Tai *et al*. 1999). The results demonstrated that the SULT1A3*2 allozyme was degraded more rapidly than was SULT1A3*1 (Fig. 5). Furthermore, this process appeared to be proteasome-mediated – at least in part – because the addition of proteasome inhibitors to the assay significantly inhibited SULT1A3*2 degradation (Fig. 6). Obviously, we cannot eliminate the possibility that processes other than accelerated degradation might contribute to decreased levels of the SULT1A3*2 allozyme. Future experiments will be required to determine whether those mechanisms, e.g. variations in mRNA stability, might also contribute to decreased levels of SULT1A3*2 protein.

Catecholamines are important neurotransmitters and hormones, and SULT1A3-catalyzed sulfate conjugation is a major pathway in the biotransformation of catecholamines in humans (Rein *et al*. 1982; Reiter *et al*. 1983; Campbell *et al*. 1987; Sundaram *et al*. 1989; Glatt *et al*. 2001; Richard *et al*. 2001). We resequenced *SULT1A3* using DNA from two different ethnic groups, and identified a novel, common polymorphism with an allele frequency of 4.2% in AA subjects that alters the encoded amino acid and greatly reduces the activity and level of SULT1A3 protein after expression in COS-1 cells. These observations raise the possibility of ethnic-specific, genetically determined variation in catecholamine biotransformation and therefore risk for cardiovascular and/or neuropsychiatric disease as well as 'pharmacogenetic' variation in response to drugs metabolized by this pathway. These possibilities should be pursued in the course of future studies. However, the present observations do not explain previous reports that platelet SULT1A3 activity in Caucasian subjects is strongly controlled by inheritance (Reveley *et al*. 1982/83; Price *et al*. 1988). Therefore, additional studies of the possible genetic control of *SULT1A3* transcription and/or splicing will be needed to explore potential genetic variation in those processes. Finally, our results for SULT1A3 add to a growing body of knowledge which indicates that a common mechanism responsible for the functional effects of

non-synonymous cSNPs is decreased level of the encoded protein, often as a result of rapid degradation. Molecular mechanisms responsible for that process will also have to be pursued in the course of future studies.

Acknowledgements

We thank Mrs Luanne Wussow for her assistance with the preparation of this manuscript, Dr Daniel Schaid for performing the linkage and haplotype analysis and Dr Liewei Wang for her advice.

Supported in part by National Institutes of Health grants RO1 G28157 (RMW), RO1 G35720 (RMW) and UO1 G61388 (RMW and EDW).

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