

Identification of novel hydroxysteroid-sulfating cytosolic SULTs, SULT2 ST2 and SULT2 ST3, from zebrafish: Cloning, expression, characterization, and developmental expression

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Abstract

By searching the expressed sequence tag database, two zebrafish cDNAs encoding putative cytosolic sulfotransferases (SULTs) were identified. Sequence analysis indicated that these two zebrafish SULTs belong to the cytosolic SULT2 gene family. The recombinant form of these two novel zebrafish SULTs, designated SULT2 ST2 and SULT2 ST3, were expressed using the pGEX-2TK glutathione *S*-transferase (GST) gene fusion system and purified from transformed BL21 (DE3) *Escherichia coli* cells. Purified GST-fusion protein form of SULT2 ST2 and SULT2 ST3 exhibited strong sulfating activities toward dehydroepiandrosterone (DHEA) and corticosterone, respectively, among various endogenous compounds tested as substrates. Both enzymes displayed pH optima at ~6.5. Kinetic constants of the two enzymes, as well as the GST-fusion protein form of the previously identified SULT2 ST1, with DHEA and corticosterone as substrates were determined. Developmental stage-dependent expression experiments revealed distinct patterns of expression of SULT2 ST2 and SULT2 ST3, as well as the previously identified SULT2 ST1, during embryonic development and throughout the larval stage onto maturity.

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The cytosolic sulfotransferases (SULTs) in mammals and other vertebrates constitute a group of enzymes that catalyze the transfer of a sulfonate group from the active sulfate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) [1], to substrate compounds containing hydroxyl or amino groups [2–5]. Such sulfation reactions are generally thought to serve for detoxification of xenobiotics, as well as biotransformation of endogenous compounds such as steroid and thyroid hormones, catecholamines, cholesterol, and bile acids [2–5]. Based on amino acid sequence homology,

all cytosolic SULTs from vertebrate animals are proposed to constitute a gene superfamily, and distinct gene families have been further categorized [6]. Two major gene families among them are the phenol SULT¹ family (designated SULT1) and hydroxysteroid SULT family (designated SULT2) [6–8]. The hydroxysteroid SULT (SULT2) family presently comprises two sub-families, dehydroepiandrosterone (DHEA) SULT (SULT2A) and cholesterol/pregnenolone

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¹ Abbreviations used: SULT, sulfotransferase; PAPS, 3'-phosphoadenosine 5' phosphosulfate; RT-PCR, reverse transcription-polymerase chain reaction; 3'-RACE, 3'-rapid amplification of cDNA ends; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SULT (SULT2B). Members of the hydroxysteroid SULT family are involved in the sulfation of both endogenous and exogenous steroids, which has been suggested to be an important mechanism for the homeostasis, bioactivation/inactivation, or transport of these compounds *in vivo* [9]. How the hydroxysteroid-sulfating cytosolic SULT enzymes function to regulate the activity of steroids, as well as their metabolism and homeostasis, however, remains to be fully elucidated. Moreover, only fragmentary information is available concerning the cell type/tissue/organ-specific expression of these hydroxysteroid-sulfating SULTs, and very little is known with regard to the ontogeny of these enzymes.

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies [10,11]. Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size, availability of a relatively large number of eggs, rapid development externally of virtually transparent embryos, and short generation time. These unique characteristics make the zebrafish an excellent model for a systematic investigation of the ontogeny, cell type/tissue/organ-specific expression, and physiological involvement of individual cytosolic SULTs. A prerequisite for using the zebrafish in these studies, however, is the identification of the various cytosolic SULTs and their functional characterization. We have recently embarked on the molecular cloning of zebrafish cytosolic SULTs [12–18]. Sequence analysis via BLAST search revealed that the zebrafish cytosolic SULTs we have cloned [12–18] display sequence homology to mammalian cytosolic SULTs. Of the eight zebrafish cytosolic SULTs that have been cloned, six fall within the SULT1 gene family [12,13,16–18], one belongs to the SULT2 gene family [14], and one appears to be independent from all known SULT gene families [15]. The zebrafish SULT2 enzyme (now designated SULT2 ST1) previously cloned and expressed displayed sulfating activities toward several steroids including DHEA, pregnenolone, allopregnanolone, 4-androstene 3,17-dione, and 17 α -hydroxypregnenolone [14]. Whether additional SULT2 enzymes, dedicated to the sulfation of other hydroxysteroids, exist in zebrafish remained an open question.

We report here the identification of two novel zebrafish cytosolic SULT2 enzymes, designated SULT2 ST2 and SULT2 ST3. Their enzymatic activities toward a variety of endogenous compounds and xenobiotics were examined. Kinetic parameters of the two enzymes in catalyzing the sulfation of DHEA and corticosterone were determined. Moreover, their developmental stage-dependent expression during embryogenesis onto maturity was investigated.

Materials and methods

Materials

DHEA, 17 β -estradiol, estrone, acetaminophen, bisphenol A, butylated hydroxyanisole, butylated hydroxytoluene, caffeic acid, catechin, chlorogenic acid, daidzein, diethylstilbestrol, L-3,4-dihydroxyphenylalanine (L-Dopa), D-Dopa, dopamine, epicatechin, epigallocatechin gallate, 17 α -eth-

nylestradiol, gallic acid, genistein, hydrocortisone, minoxidil, myricetin, β -naphthylamine, β -naphthol, *p*-nitrophenol, *n*-nonylphenol, *n*-octylphenol, *n*-propyl gallate, quercetin, L-thyronine, 3,3',5-triiodo-L-thyronine, aprotinin, thrombin, adenosine 5'-triphosphate (ATP), sodium dodecyl sulfate (SDS), sodium acetate, 2-morpholinoethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid (TAPS), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), Trizma base, dithiothreitol (DTT), and isopropyl β -D-thiogalactopyranoside (IPTG) were products of Sigma Chemical Company. TRI Reagent was from Molecular Research Center, Inc. Unfertilized zebrafish eggs, embryos and larvae at different developmental stages were prepared by Scientific Hatcheries. Total RNA from a 3-month-old zebrafish was prepared as described previously [13]. *Taq* DNA polymerase was a product of Promega Corporation. Takara *Ex Taq* DNA polymerase was purchased from PanVera Corporation. T₄ DNA ligase and *Bam*HI restriction endonuclease were from New England Biolabs. Oligonucleotide primers were synthesized by MWG Biotech. pSTBlue-1 AccepTor Vector Kit and BL21 (DE3) competent cells were from Novagen. Prestained protein molecular weight standard were from Life Technologies. pGEX-2TK glutathione *S*-transferase (GST) gene fusion vector, GEX-5' and GEX-3' sequencing primers, and glutathione-Sepharose 4B were products of Amersham Biosciences. GST-fusion protein form of the previously identified zebrafish SULT2 ST1 [14] was expressed and purified based on the same procedure described below for SULT2 ST2 and ST3. Recombinant human bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate kinase was prepared as described previously [19]. Cellulose thin-layer chromatography (TLC) plates were products of EM Science. Carrier-free sodium [³⁵S]sulfate, EcoLume scintillation cocktail, corticosterone, pregnenolone, 4-androstene-3,17-dione, hydrocortisone, progesterone, 17 α -hydroxyprogesterone, and 17 α -hydroxypregnenolone were from ICN Biomedicals. Allopregnanolone was from Calbiochem. All other reagents were of the highest grades commercially available.

Cloning, bacterial expression, and purification of recombinant zebrafish cytosolic SULT2 ST2 and ST3

By searching the expressed sequence tag database, two zebrafish cDNAs (GenBank Accession Nos. CD014163 (SULT2 ST2) and BQ132464 (SULT2 ST3)) encoding putative cytosolic SULTs were identified. These two cDNAs, obtained from Open Biosystems, were subjected to nucleotide sequencing [20]. To subclone the two cDNAs for expression, sense and antisense oligonucleotide primers designed based on 5'- and 3'-coding regions of the nucleotide sequences determined were synthesized with *Bam*HI restriction site incorporated at the end (see Table 1). Using these primer sets, PCRs were carried out under the action of *EX Taq* DNA polymerase, with the two commercially obtained cDNAs as templates. Amplification conditions were 2 min at 94 °C and 20 cycles of 94 °C for 35 s, 60 °C for 40 s, 72 °C for 1 min. The final reaction mixtures were applied onto a 1.2% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The PCR product bands detected were excised from the gel, and the DNAs therein were isolated by spin filtration. Purified PCR products were subjected to *Bam*HI restriction and cloned into *Bam*HI-restricted pGEX-2TK vector, and verified for authenticity by nucleotide sequencing [20]. To express the recombinant zebrafish SULT2 ST2 and ST3, competent *Escherichia coli* BL21 (DE3) cells, transformed with pGEX-2TK harboring the cDNA encoding SULT2 ST2 or ST3, were grown in 1 L LB medium supplemented with 60 μ g/ml ampicillin. After the cell density reached 0.6 OD_{600 nm}, IPTG at a final concentration of 0.1 mM was added to induce the production of recombinant zebrafish SULT. After an overnight induction at room temperature, the cells were collected by centrifugation and homogenized in 25 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) using an Aminco French Press. Twenty microliters of 10 mg/ml aprotinin (a protease inhibitor) was added to the crude homogenate. The crude homogenate was subjected to centrifugation at 10,000g for 15 min at 4 °C. The supernatant collected was fractionated using 2.5 ml of glutathione-Sepharose, and the

bound GST-fusion protein was either eluted by an elution buffer (50 mM Tris–HCl, pH 8.0, plus 10 mM reduced glutathione) at 4 °C or treated with 3 ml of a thrombin digestion buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 2.5 mM CaCl₂) containing 5 U/ml bovine thrombin at room temperature. Following a 10 to 15-min incubation with constant agitation, the preparation was subjected to centrifugation. The recombinant zebrafish SULT (in GST-fusion protein form or free (thrombin-cleaved) form) present in the supernatant collected was analyzed for purity by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to enzymatic characterization.

Enzymatic assay

The sulfating activity of the recombinant zebrafish cytosolic SULTs was assayed using radioactive PAP[³⁵S] as the sulfate donor. The standard assay mixture, with a final volume of 25 μl, contained 50 mM Mops buffer at pH 7.0 (for SULT2 ST2 and ST3) or Ches at pH 9.5 (for SULT2 ST1), 14 μM PAP[³⁵S] (15 Ci/mmol), 1 mM DTT, and 50 μM substrate. Controls with DMSO or water, in place of substrate, were also prepared. The reaction was started by the addition of the enzyme, allowed to proceed for 5 min at 28 °C, and terminated by heating at 100 °C for 2 min. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to the analysis of [³⁵S]sulfated product using the previously developed TLC procedure [21], with *n*-butanol/isopropanol/88% formic acid/water (3:1:1:1; by volume) as the solvent system. To examine the pH-dependence of the sulfation of DHEA or corticosterone, different buffers (50 mM sodium acetate at 4.5, 5.0, or 5.5; Mes at 5.5, 6.0, or 6.5; Mops at 6.5, 7.0, or 7.5; Hepes at 7.0, 7.5 or 8.0; Taps at 8.0, 8.5 or 9.0; Ches at 9.0, 9.5, or 10.0; and Caps at 10.0, 10.5, 11.0 or 11.5), instead of 50 mM Mops (pH 7.0), were used in the reactions. For the kinetic studies on the sulfation of DHEA and corticosterone, varying concentrations of these substrate compounds and 50 mM Mops buffer at pH 7.0 (for SULT2 ST2 and ST3) or Ches at pH 9.5 (for SULT2 ST1) were used.

Analysis of the developmental stage-dependent expression of the zebrafish cytosolic SULT2 STs

RT-PCR was employed to investigate the developmental stage-dependent expression of the zebrafish cytosolic SULT2 ST2 and ST3, as well as the previously identified SULT2 ST1. Total RNAs from zebrafish embryos, larvae, and adult (male or female) fish at different developmental stages were isolated using TRI Reagent, based on manufacturer's instruc-

tions. Aliquots containing 5 μg each of the total RNA preparations were used for the synthesis of the first-strand cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences). One microliter aliquots of the 33 μl first-strand cDNA solutions prepared were used as the template for the subsequent PCR amplification. PCRs were carried out in 25 μl reaction mixtures using *EX Taq* DNA polymerase, in conjunction with gene-specific sense and antisense oligonucleotide primers (see Table 1). Amplification conditions were 2 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 40 s at 60 °C, and 1 min at 72 °C. The final reaction mixtures were applied onto a 1.2% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. As a control, PCR amplification of the sequence encoding zebrafish β-actin was concomitantly performed using the above-mentioned first-strand cDNAs as templates, in conjunction with gene-specific sense and antisense oligonucleotide primers (Table 1) designed based on reported zebrafish β-actin nucleotide sequence (GenBank Accession No. AF057040).

Miscellaneous methods

PAP[³⁵S] was synthesized from ATP and carrier-free [³⁵S]sulfate using the bifunctional human ATP sulfurylase/APS kinase and its purity determined as previously described [22,23]. The PAP[³⁵S] synthesized was adjusted to the required concentration and specific activity by the addition of cold PAPS. SDS–PAGE was performed on 12% polyacrylamide gels using the method of Laemmli [24]. Protein determination was based on the method of Bradford [25] with bovine serum albumin as the standard.

Results and discussion

In vertebrates, the sulfation of steroids by the cytosolic hydroxysteroid SULTs, belonging to the SULT2 gene family [6], is recognized as an important regulatory pathway for the homeostasis as well as bioactivation/inactivation of these compounds [9]. As a part of an effort to develop a zebrafish model for investigating in greater detail the functional involvement of the hydroxysteroid SULTs, we had previously cloned, expressed and characterized a zebrafish DHEA-sulfating SULT2 (now designated SULT2 ST1) [14]. In view of the diversity of SULT2 enzymes in other vertebrates including human and mouse [6,26], we specu-

Table 1

Oligonucleotide primers used for the cDNA cloning of zebrafish SULT2 ST2 and ST3 and for the RT-PCR analysis of the developmental stage-dependent expression of the SULT2 STs

Target sequence	Sense and antisense oligonucleotide primers used	
I. For cDNA cloning ^a :		
SULT2 ST2	Sense:	5'-CGCGGATCCATGACTGAATCGGAGCTGTA-3'
	Antisense:	5'-CGCGGATCCCTCAGTCCCATGGAACTTGAA-3'
SULT2 ST3	Sense:	5'-CGCGGATCCATGGAGGTCAGCGAATTCAAT-3'
	Antisense:	5'-CGCGGATCCCTTATTCTCATCCAGGGGAATTT-3'
II. For RT-PCR analysis ^b :		
SULT2 ST1	Sense:	5'-ACAAAACCGCAGTCGCTGCACAGAACCGGT-3'
	Antisense:	5'-TGATACAGATAAACATCACTATTACTGTGG-3'
SULT2 ST2	Sense:	5'-TGCAGCTGCTCTCTAGATTAATTCTTCATT-3'
	Antisense:	5'-TTTTTGGCTGTGCACCAAATGTTTATTAGAA-3'
SULT2 ST3	Sense:	5'-GAACTCTTTGCACAAAACACTACCTAGTTTTCCCA-3'
	Antisense:	5'-GGACAGACTGAAGAACTCCACCTTAACCAA-3'
β-Actin	Sense:	5'-ATGGATGAGGAAATCGCTGCCCTGGTC-3'
	Antisense:	5'-TTAGAAGCACTTCTGTGAACGATGGA-3'

^a Recognition sites of *Bam*HI restriction endonuclease in the oligonucleotides are underlined. Initiation and termination codons for translation are in bold type.

^b The sense and antisense oligonucleotide primer sets listed were verified by BLAST Search to be specific for the target zebrafish SULT2 or β-actin nucleotide sequence.

lated that additional SULT2 enzymes may be present in zebrafish. Here, we report the identification, characterization and ontogeny of two novel zebrafish cytosolic SULT2 enzymes, designated SULT2 ST2 and ST3.

Molecular cloning of the zebrafish cytosolic SULT2 ST2 and ST3

By searching the expressed sequence tag database, two zebrafish cDNAs (GenBank Accession No. CD014163 (SULT2 ST2) and BQ132464 (SULT2 ST3)) encoding putative cytosolic SULTs were identified. These two cDNAs, obtained commercially, were subjected to nucleotide sequencing [20]. The nucleotide sequences obtained were submitted to the GenBank database under the Accession No. DQ640387 for SULT2 ST2 and DQ640388 for SULT2 ST3. Fig. 1 shows the alignment of the deduced amino acid sequence of the two newly cloned zebrafish SULT2 STs. The open reading frame of the SULT2 ST2 encompasses 864 nucleotides and codes for a 287-amino acid polypeptide, and that of the SULT2 ST3 contains 867 nucleotides and encodes a 288-amino acid polypeptide. Similar to other cytosolic SULTs, these two new zebrafish SULTs contains sequences resembling the so-called “signature sequences” (YPKSGTxW in the N-terminal region and RKGxxGDWKNxFT in the C-terminal region; as underlined) characteristic of SULT enzymes [8]. Of these two sequences, YPKSGTxW has been demonstrated by X-ray crystallography to be responsible for binding to the 5'-phosphosulfate group of PAPS, a co-substrate for SULT-catalyzed sulfation reactions [27], and thus designated the “5'-phosphosulfate binding (5'-PSB) motif” [28]. The cloned zebrafish SULT also contains the “3'-phosphate binding (3'-PB) motif” (amino acid residues 187–197; as underlined) responsible for the binding to the 3'-phosphate group of PAPS [28]. Sequence analysis based on a BLAST Search revealed that the deduced amino acid sequence of the zebrafish SULT2 ST2 displays 43 and 40%

identity to human SULT2B1a and SULT2A1, and lower % identity to other known SULTs. The deduced amino acid sequence of the zebrafish SULT2 ST3 displays 49% identity to human SULT2B1a and SULT2B1b, and lower % identity to other known SULTs. It is generally accepted that members of the same SULT gene family share at least 45% amino acid sequence identity, and members of subfamilies further divided in each SULT gene family are greater than 60% identical in amino acid sequence [6–8]. Based on these criteria, both the zebrafish SULT2 STs appear to belong to the SULT2 gene family, and are designated the zebrafish SULT2 ST2 and ST3 in accordance with the nomenclature used in ZFIN database (cf. the dendrogram shown in Fig. 2). It is interesting to note that the newly cloned zebrafish SULT2 ST2 and ST3 displays, respectively, 87.5 and 52.6% amino acid sequence identity to the previously identified zebrafish SULT2 ST1 [14].

Expression, purification, and characterization of recombinant zebrafish cytosolic SULT2 ST2 and ST3

The coding region of the zebrafish SULT2 ST2 or ST3 cDNA was subcloned into pGEX-2TK, a prokaryotic expression vector, for the expression of recombinant enzyme in *E. coli*. As shown in Fig. 3, the GST-fusion protein form of the recombinant zebrafish SULT2 ST2 (lane 1) and ST3 (lane 3), purified from the *E. coli* extract, migrated at ~58 kDa position upon SDS-PAGE. Upon thrombin digestion, the free form of SULT ST2 (lane 2) and ST3 (lane 4) migrated as ~33 kDa proteins (Fig. 3). This is in agreement with the molecular weight (32,000–35,000) generally found for cytosolic SULTs [1–3]. It was subsequently noted that the thrombin-digested zebrafish SULT2 ST2 and ST3 exhibited lower and unstable sulfating activity in comparison with the GST-fusion protein form of these two enzymes (data not shown). The GST-fusion protein form of both zebrafish SULT2 STs, therefore, was used for the enzymatic characterization. (The specific activities deter-

SULT2 ST1 (100%)*	1	<u>MTEAE</u> LYSS- <u>YKGV</u> LVPSYVHPAESLKYYEDFTFRPDDILIL <u>IFPKSGTTW</u> MOEIVPLVW	59
SULT2 ST2 (87.5%)	1	MTESELYSV- <u>HKGV</u> FVPTHLHPAESLKYYEDFTFRPDDILIVTYPKSGT <u>IFW</u> MOEIVPLVW	59
SULT2 ST3 (52.6%)	1	MEVSEF-NLLYKGF <u>ILPKLA</u> HTEESLQMLEN <u>HKVKDD</u> DFVAVTYPKSGT <u>IFW</u> MQNIILPPL	59
SULT2 ST1	60	SEGDLT <u>PVLT</u> VPNWDRVPWLEEHRALLSLEQRASPRIFATHFHQMMNPSYFKIKPRV <u>LI</u>	119
SULT2 ST2	60	SEGDLT <u>ILML</u> TVPNWDRVPWLEEHRALLSLEQRASPRIFATHFHQMMNPSYFK <u>IF</u> PRV <u>LI</u>	119
SULT2 ST3	60	NGD <u>LT</u> VPQTVPNWDRAPWLEETRAAVV-LDE <u>RPSPRA</u> IVSHMPYRLMP <u>SFYK</u> SRAK <u>MI</u>	118
SULT2 ST1	120	YVMRNP <u>KDVF</u> ISSYHHEMASFLVSPGTQDEFMEKFL <u>NDG</u> TFMFGSWFDHVKSWLNAGE <u>QEQE</u>	179
SULT2 ST2	120	YVMRNP <u>KDVF</u> ISSYHGYMASFLVNPGTQDEFMEKFL <u>NGNI</u> MFGSWFDHVK <u>GWLN</u> AGE <u>QEQE</u>	179
SULT2 ST3	119	YVMRNP <u>KDVF</u> ISSYHFKMASFL <u>EDP</u> GT <u>FD</u> HDHVNKFL <u>SGED</u> VF <u>GKWS</u> DHTK <u>SRN</u> PEL <u>KD</u>	178
SULT2 ST1	180	HILYL <u>CYED</u> MTISDLKASVEKIATFLGKLSLSSEVVEKIADHCVFENMKQNKMSNYSLVPEE	239
SULT2 ST2	180	HILYISYEEMINDLRASVEKIATFLGKLSLSSEVVEKIADHCVFENMKQNKMSNYSLVPEE	239
SULT2 ST3	179	RILYVTYEEMLDLRGVL <u>CRLMK</u> FLGRELST <u>EAL</u> DRVVXNSTFKNMKTNKMSNY <u>TMV</u> PEE	238
SULT2 ST1	240	FMDQKKSEFLRKGIVGDWKNHFS <u>TAQEQK</u> FNAVYQEKMKDVKFSFPWD--	287
SULT2 ST2	240	FMDQKKSEFLRKGIVGDWKNHFS <u>AAQER</u> FNAVYDDKMKDVKFKFPWD--	287
SULT2 ST3	239	IMDNNKSAFLRKGIVAGWKNHFS <u>PELDAK</u> HTAVIREEMKGTNIKFPWDEE	288

Fig. 1. Alignment of the deduced amino acid sequences of the zebrafish SULT2 ST1, ST2, and ST3. Residues conserved between the two enzymes are boxed. Two “signature sequences,” respectively, located in the N- and C-terminal regions, as well as a conserved sequence in the middle region are underlined. The percent numbers in parentheses refer to the percent amino acid identities to the SULT2 ST1 sequence.

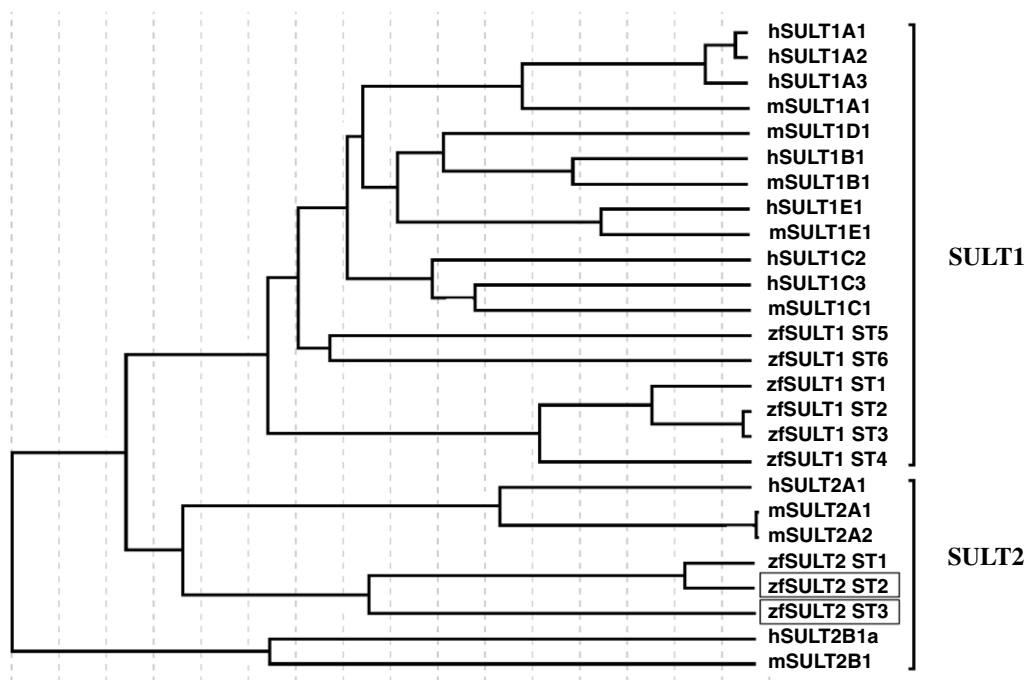


Fig. 2. Classification of the zebrafish SULT2 ST2 and ST3 on the basis of their amino acid sequences. The dendrogram shows the degree of amino acid sequence homology among cytosolic SULTs. For references for individual SULTs, see the review by Blanchard et al. [6]. h, human; m, mouse; and zf, zebrafish. The dendrogram was generated based on Greedy algorithm [42,43].

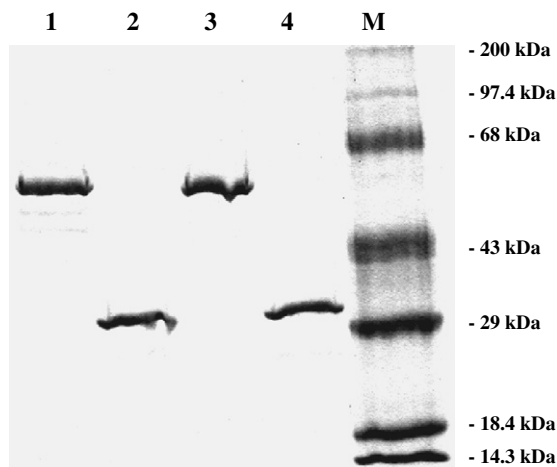


Fig. 3. SDS gel electrophoretic pattern of the purified recombinant zebrafish SULT2 ST2 and ST3. Purified zebrafish SULT samples were subjected to SDS-PAGE on a 12% gel, followed by Coomassie blue staining. Samples analyzed in lanes 1 and 2 were, respectively, GST-fusion protein and thrombin-digested forms of SULT2 ST2. Samples in lanes 3 and 4 were GST-fusion protein and thrombin-digested forms of SULT2 ST3. Protein molecular weight markers co-electrophoresed are: lysozyme ($M_r=14,300$), β -lactoglobulin ($M_r=18,400$), carbonic anhydrase ($M_r=29,000$), ovalbumin ($M_r=43,000$), bovine serum albumin ($M_r=68,000$), phosphorylase b ($M_r=97,400$), and myosin (H-chain) ($M_r=200,000$).

mined in the following studies were corrected for the molecular mass of the GST moiety in the fusion protein form of the enzymes.) A pilot experiment first revealed that the SULT2 ST2 and ST3 exhibited strong activity toward DHEA and corticosterone, respectively. A pH-dependence

experiment subsequently performed showed that the SULT2 ST2 with DHEA as substrate (Fig. 4A) and ST3 with corticosterone as substrate (Fig. 4B) both exhibited optimum activity at pH 6.5. It is interesting to note that the previously identified SULT2 ST1, while sharing a high degree of sequence homology (87.5% amino acid identity) with the SULT2 ST2, displayed a pH optimum at 9.5 [14]. Representative endogenous and xenobiotic compounds were tested as substrates for these enzymes, as well as the GST-fusion protein form of the previously identified SULT2 ST1. The activity data obtained are compiled in Table 2. Among the compounds we tested, the zebrafish SULT2 ST2 and ST3 displayed strongest sulfating activities toward DHEA (at 579 pmol/min/mg enzyme) and corticosterone (at 510 pmol/min/mg enzyme), respectively. In addition, SULT2 ST2 also showed sulfating activities toward pregnenolone, 17 β -estradiol and estrone; and SULT2 ST3 displayed activities toward pregnenolone, 17 β -estradiol, DHEA and allopregnanolone. Neither enzyme, however, exhibited detectable activities toward other endogenous compounds including L-Dopa, dopamine, 4-androstene-3,17-dione, hydrocortisone, 17 α -hydroxypregnenolone, 17 α -hydroxyprogesterone, progesterone, L-thyroxine, and L-triiodothyronine, and exogenous compounds including acetaminophen, bisphenol A, *n*-nonylphenol, *n*-octylphenol, *p*-nitrophenol, β -naphthol, β -naphthylamine, butylated hydroxyanisole, caffeic acid, catechin, epicatechin, gallic acid, chlorogenic acid, daidzein, genistein, myricetin, quercetin, *n*-propyl gallate, and minoxidil. That both SULT2 ST2 and ST3 exhibited sulfating activities toward exclusively hydroxysteroids is in line with these enzymes being

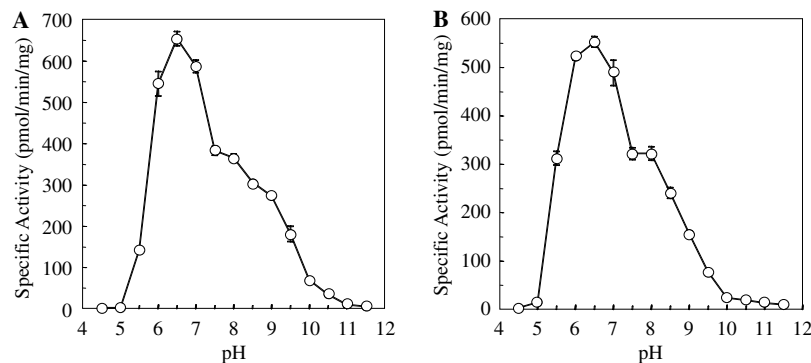


Fig. 4. pH dependency of the sulfating activity of the zebrafish SULT2 ST2 with DHEA (A) and ST3 with corticosterone (B) as a substrate. The enzymatic assays were carried out under standard assay conditions as described under Materials and methods, using different buffer systems as indicated. The data represent calculated mean \pm standard deviation derived from three experiments.

members of the SULT2 gene family. Compared with the highly homologous SULT2 ST2, the previously identified SULT2 ST1 exhibited sulfating activities toward DHEA, pregnenolone, 17β -estradiol, allopregnanolone, 4-androstene-3,17-dione and 17α -hydroxypregnenolone, but not estrone (Table 2). The three zebrafish SULT2 enzymes identified to date therefore appeared to sulfate distinct, albeit overlapping, groups of hydroxysteroids. Whether their distinct substrate specificity may reflect the differential functional involvement of the three enzymes *in vivo* remains to be clarified.

The kinetics of the sulfation of DHEA by SULT2 ST1 and ST2 and the sulfation of corticosterone by SULT2 ST3 were further examined. Data obtained were processed using the Excel program to generate the best fitting trendlines for the Lineweaver–Burk double-reciprocal plots. Table 3 shows the kinetic constants determined for the sulfation of DHEA by SULT2 ST1 and SULT2 ST2, as

well as the sulfation of corticosterone by ST3. The K_m values of SULT2 ST1 and SULT2 ST2 with DHEA and ST3 with corticosterone were 102, 177, and 101 μM , respectively. These K_m values are considerably higher than those previously determined for the sulfation of DHEA, cholesterol or pregnenolone by human SULT2A1, SULT2B1a or SULT2B1b [29–31]. It is possible that the high K_m values of SULT2 ST1, ST2, and ST3 may represent adaptation to high steroid concentrations that might be present systematically or locally in zebrafish. Previous studies have revealed circulating levels of some hydroxysteroids in fish to be in μM concentration ranges [32,33], which are considerably higher than those found in humans (sub-nM to nM) [34]. Moreover, it has been demonstrated that physiological levels of hydroxysteroids may increase dramatically in response to aquatic environmental stimuli [32,35,36]. Another possibility is that certain yet-unidentified co-factors or mechanisms may exist for the regulation

Table 2
Specific activities of the zebrafish SULT2 ST1, ST2, and ST3 with endogenous compounds as substrates^a

	Specific activity (pmol/min/mg)		
	SULT2 ST1	SULT2 ST2	SULT2 ST3
Dehydroepiandrosterone (DHEA)	554 \pm 48	579 \pm 24	40.9 \pm 2.5
Corticosterone	ND ^b	ND	510 \pm 23
Pregnenolone	628 \pm 59	47.4 \pm 5.9	68.8 \pm 4.0
17β -Estradiol	63.2 \pm 0.5	23.6 \pm 2.7	51.7 \pm 3.3
Allopregnanolone	245 \pm 18	ND	37.8 \pm 2.1
Estrone	ND	17.9 \pm 0.4	ND
4-Androstene-3,17-dione	122 \pm 10	ND	ND
17α -Hydroxypregnenolone	44.8 \pm 9.7	ND	ND

^a Specific activity refers to pmol substrate sulfated/min/mg purified enzyme. Data represent means \pm SD derived from three experiments.

^b Specific activity determined is lower than the detection limit (estimated to be \sim 10 pmol/min/mg protein).

Table 3
Kinetic constants of the zebrafish SULT2 ST1 and ST2 with DHEA, and SULT2 ST3 with corticosterone as substrate^a

	V_{\max} (nmol/min/mg)	K_m (μM)	V_{\max}/K_m
SULT2 ST1 (with DHEA)	2.10 \pm 0.20	102 \pm 13	0.021
SULT2 ST2 (with DHEA)	2.64 \pm 0.21	177 \pm 26	0.015
SULT2 ST3 (with corticosterone)	1.63 \pm 0.25	101 \pm 18	0.016

^a Data shown represent means \pm SD derived from three experiments.

of the kinetic properties of SULT2 ST2 and ST3 for their functioning in the homeostasis of hydroxysteroids in zebrafish. Based on calculated V_{\max}/K_m shown in Table 3 the catalytic efficiency of SULT2 ST1 or ST2 with DHEA as substrate appeared to be comparable to that of ST3 with corticosterone as substrate.

Developmental stage-dependent expression of the zebrafish cytosolic SULT2 STs

In vertebrates, steroid hormones, including mineralocorticoids, glucocorticoids, and sex steroids, are known to play critical roles in the regulation of mineral balance, glucose homeostasis, and sexual differentiation [37]. In view of their hydroxysteroid-sulfating activity, an important question is whether the expression of the newly identified SULT2 ST2 and ST3 correlates with the development of endocrine system of the zebrafish. To gain insight into this issue, RT-PCR was employed to examine the expression of mRNAs encoding SULT2 ST2 and ST3, as well as the previously identified SULT2 ST1, at different developmental stages. As shown in Fig. 5A, the mRNA encoding SULT2 ST1 was not present in unfertilized eggs and was not expressed until the embryos reached the neurula/segmentation period (12-hour pf). Thereafter, the expression of the SULT2 ST1 mRNA continued into

the larval stage onto maturity. For SULT2 ST2, a significant level of its coding mRNA was detected in unfertilized eggs, indicating clearly its maternal origin. Upon fertilization, however, no SULT2 ST2 mRNA was detected until the neurula/segmentation period (12-h pf), which then increased dramatically when reaching the larval stage (1-week pf) onto maturity. Both SULT2 ST1 and ST2 mRNAs were detected in adult male and female zebrafish. It is worthwhile pointing out that DHEA, a major substrate for both SULT2 ST1 and ST2, occupies an important position as the biosynthetic precursor for testosterone and 17β -estradiol, as well as other sex steroids [38]. It has been reported that DHEA and some other steroids are also produced *de novo* in the nervous system where these so-called “neurosteroids” may play important roles in the regulation of the activity of genes and protein synthesis, cellular development, and the functioning of neuroendocrine system and behavioral pattern [39]. That the initiation of the expression of both SULT2 ST1 and ST2 coincided with the beginning of the development of the nervous system (at the neurula/segmentation stage) poses an interesting question whether these two enzymes are involved in the regulation and/or homeostasis of DHEA and other neurosteroids. Moreover, since the expression of SULT2 ST1 and ST2 continues into the larval stage onto maturity, it is also possible that they may

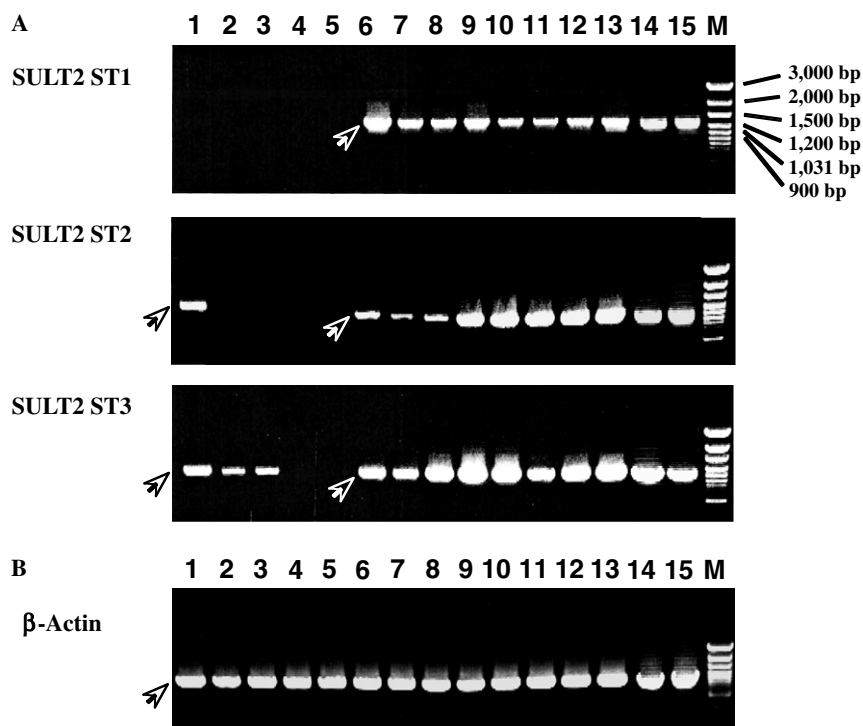


Fig. 5. Developmental stage-dependent expression of the zebrafish SULT2 STs. (A) RT-PCR analysis of the expression of mRNAs encoding SULT2 ST2 and ST3, as well as the previously identified zebrafish SULT2 ST1, at different stages during embryogenesis and larval development onto maturity. Final PCR mixtures were subjected to 2% agarose electrophoresis. Samples analyzed in lanes 1 through 15 correspond to unfertilized zebrafish eggs, zebrafish embryos during the zygote period (0-h post-fertilization (pf)), cleavage period (1-h pf), blastula period (3-h pf), gastrula period (6-h pf), neurula/segmentation period (12-h pf), pharyngula period (24-h pf), and hatching period (48- and 72-h pf), 1, 2, 3, 4-week-old zebrafish larvae, and 3-month-old male (lane 14) or female (lane 15) zebrafish. The PCR products corresponding to different zebrafish SULT2 ST cDNAs, visualized by ethidium bromide staining, are marked by arrows. (B) RT-PCR analysis of the expression of the zebrafish β -actin at the same developmental stages as those described in (A).

be involved in the regulation of sex steroids. The physiological relevance of the expression of SULT2 ST2, but not SULT2 ST1, as a maternal transcript in unfertilized zebrafish eggs remains unclear. For SULT2 ST3, a significant level of its coding message was detected in unfertilized eggs, indicating its maternal origin. During the embryonic development, there appeared to be an initial decrease in expression, followed by disappearance during the blastula (3-h pf) and gastrula period (6-h pf). Thereafter, the expression of SULT2 ST3 mRNA resumed in the neurula/segmentation period (12-h pf) and continued into the larval stage onto maturity. SULT2 ST3 mRNA was detected in both male and female zebrafish. The physiological relevance of this unique pattern of developmental stage-dependent expression of SULT2 ST3 remains to be clarified. It is to be noted, however, that corticosterone, a favorite substrate for SULT2 ST3, is a major glucocorticoid in rodents, and the precursor of aldosterone, the major mineralocorticoid in man [37]. In fish, corticosterone can also be converted to cortisol [40], which is known to play an important role in the osmoregulation [41]. It is plausible that SULT2 ST3 may be critical to the homeostasis of corticosterone and therefore the ability of fish to face osmotic challenges, as well as glucose homeostasis. The expression of SULT2 ST3 in unfertilized eggs and embryos during early developmental stages may serve to regulate the corticosteroids of maternal origin, prior to the development of endocrine organs. In contrast to the developmental stage-dependent expression of the SULT2 STs, β -actin, housekeeping protein, was found to be expressed throughout the entire developmental process (Fig. 5B).

To summarize, we have identified two novel DHEA/corticosterone-sulfating cytosolic SULT2 STs, designated as SULT2 ST2 and ST3, which may be involved in metabolism and homeostasis of DHEA and corticosterone, respectively, in zebrafish. Our goal is to obtain a complete repertoire of the cytosolic SULT enzymes present in zebrafish, and this study is part of an overall effort. As pointed out earlier, the identification of the various cytosolic SULTs and their biochemical characterization is a prerequisite for using the zebrafish as a model for a systematic investigation on fundamental issues regarding cytosolic SULTs. More works is warranted in order to achieve this goal.

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