ACCELERATED PUBLICATION

Genomic identification and biochemical characterization of a second spermidine/spermine N^1-acetyltransferase

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In the polyamine back-conversion pathway, spermine and spermidine are first acetylated by spermidine/spermine N^1-acetyltransferase (SSAT-1) and then oxidized by polyamine oxidase to produce spermidine and putrescine respectively. Herein we apply homology-search methods to identify novel sequences belonging to a second SSAT, SSAT-2, with a chromosomal location at 17p13.1, which is distinct from SSAT-1 at Xp22. Human SSAT-2 cDNA derived from small-cell lung carcinoma was deduced to encode a 170-amino-acid protein having 46 % sequence identity and 64 % sequence similarity with SSAT-1. When transiently transfected into HEK-293 cells, SSAT-1 decreased spermidine and spermine pools by ≈ 30 %, while, at the same time, significantly increasing putrescine, N^1-acetylspermidine, N^1-acetylspermine and N^1,N^{12}-diacetylspermine pools. By contrast, transfected SSAT-2 had no effect on intracellular polyamine or acetylated polyamine pools. When enzyme activity was assayed on enzyme extracts from transfected cells, both SSAT-1 and SSAT-2 demonstrated much higher acetylating activity than vector-transfected cells. The data suggest that, in intact cells, SSAT-2 may be compartmentalized or it may be inefficient at low intracellular polyamine concentrations. By substituting candidate substrates in the enzyme assay, we determined that SSAT-1 shows the substrate preference norspermidine = spermidine $\gg$ spermine > N^1-acetylspermine > putrescine, whereas SSAT-2 shows the preference norspermidine > spermidine = spermine $\gg N^1$-acetylspermine = putrescine. Analysis of mRNA levels in cell lines and ESTs (expressed sequence tags) from various tissues by digiNorthern (a web-based tool for virtually displaying expression profiles of query genes based on EST sequences) indicated that SSAT-1 tends to be more widely and highly expressed than SSAT-2. While SSAT-1 mRNA was inducible by polyamine analogues in a variety of cell lines, SSAT-2 was not. The existence of an active, but possibly sequestered, SSAT-2 enzyme suggests that, under certain conditions, it may be recruited into basal or perturbed polyamine metabolism.

Key words: genomics, polyamine oxidase, polyamine analogue, spermidine, spermidine/spermine N^1-acetyltransferase, spermine oxidase.

INTRODUCTION

In the polyamine-biosynthetic pathway, polyamines are assembled via the sequential transfer of aminopropyl units derived from decarboxylated S-adenosylmethionine on to a core diamine unit putrescine (Put) derived by decarboxylation of ornithine. In the polyamine back-conversion pathway, these same aminopropyl units are systematically removed via two sequential enzyme reactions. Spermine (Spm) is first acetylated by spermidine/spermine N^1-acetyltransferase (SSAT) and then oxidized by polyamine oxidase (PAO) to yield stoichiometric amounts of spermidine (Spd), 3-acetamidopropanal and H_2O_2 [1,2]. The conversion of Spd into Put takes place by analogous reactions involving the same two enzymes. Recently, a new route for Spm to Spd back-conversion was realized with the discovery of Spm oxidase (SMO), an enzyme capable of directly oxidizing Spm without prior acetylation [3].

Since being cloned in 1991, SSAT has been regarded as a singular enzyme system [2] with demonstrated ability to markedly alter polyamine pools in cells [4] and animal tissues [5,6]. SSAT is inducible by a broad range of cytotoxic agents [1,7] and, in this regard, it may be part of a generalized cellular stress response. Enzyme induction by polyamine analogues is distinctive in both the heterogeneity of the response among cell lines and in the magnitude of the response, where, in certain cell lines, increases in activity of > 1000-fold have been noted [2,8,9]. These characteristics have been used to establish correlative relationships between the induction of SSAT-1 and the inhibition of cell growth. More definitive evidence for this relationship has been provided with analogue-resistant mutant CHO (Chinese-hamster ovary) cells [10,11], conditional expression systems [4] and, more recently, the use of siRNA (small inhibitory RNA) to silence SSAT induction [12]. N^1,N^{11}-diethylnorspermine (DENSpm) induction of SSAT activity has also been tightly linked to activation of the p53/p21/Rb-dependent checkpoint in some cells [13], to apoptotic signalling in others [14] and to anti-apoptotic survival responses such as MAPK (mitogen-activated protein kinase) induction in others [15]. Thus the enzyme clearly represents an important determinant of drug action for polyamine analogues, and, as such, it may be exploitable in combination with anticancer drugs that also potently induce SSAT expression [1,7].

Using a functional genomics approach, we recently identified and characterized sequences belonging to a previously unrecognized SMO and to the PAO involved in polyamine back-conversion [16]. cDNA sequences identified by homology

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BLAST search [17] of genomic and cDNA databases were transfected into HEK-293 cells and were functionally identified, according to their unique substrate specificities, by a cell-lysate assay. Given the potential biological and pharmacological significance of SSAT, we have undertaken a similar search for SSAT-related enzymes. Of particular interest are acetyltransferases that have been shown to acetylate the aminobutyl (as opposed to aminopropyl) segment of Spd to yield N^8-acetylspermidine, since evidence exists to suggest that it may be able to acetylate histones [18]. In the search for this enzyme, we have encountered a second and independent SSAT, designated SSAT-2, in order to distinguish it from the originally described polyamine acetyltransferase, now designated SSAT-1. Although SSAT-2 did not obviously acetylate aminobutyl moieties of Spd, it did display subtle differences in substrate specificity relative to SSAT-1. Perhaps more interestingly, this enzyme appeared to be sequestered in cells and, unlike SSAT-1, it was not inducible by polyamine analogues. The functional significance of SSAT-2 with respect to polyamine metabolism or various cellular responses to polyamine perturbations remains to be determined.

EXPERIMENTAL

Materials

Spm, Spd, Put, N^1-acetylspermine (N^1-AcSpm) and N^1-acetyl-spermidine (N^1-AcSpd) were originally purchased from Sigma (St. Louis, MO, U.S.A.), but we note that the latter two compounds are no longer available from that source. Human SSAT-1 cDNA was obtained from Dr Robert Casero, Jr (Johns Hopkins Oncology Center, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.). Norspermidine (norSpd) was generously supplied by Dr. Raymond Bergeron (Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, FL, U.S.A.), and N^1,N^{12}-diacetylspermine (DASpm) was a gift from Dr Nikolaus Seiler (Laboratory of Nutritional Oncology, Institut de Recherche Contre les Cancers, Strasbourg, France).

Homology search and cDNA acquisition

A BLAST sequence homology search [17] of genome and cDNA databases was carried out using human SSAT-1 sequences as previously described [3,16]. SSAT-2 cDNA [IMAGE: 3534187; NCBI (National Center for Biotechnology Information) accession no. BC011751 from human lung small-cell carcinoma] was obtained from the American Tissue Type Collection (Rockville, MD, U.S.A., now at Manassas, VA, U.S.A.).

Plasmids

SSAT-1cDNA was cloned into an *Eco*RI site in a pCMV-Sport 6 vector (Invitrogen, Carlsbad, CA, U.S.A.). Human SSAT-2 cDNA was cloned into pOTB 7 and subcloned into an *Eco*RI/*Xho*I site in pCMV-Sport 6. All plasmid DNAs were prepared using an EndoFree Maxi-prep kit (Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's protocol. The amount of DNA was measured spectrophotometrically using a SmartSpec™ 3000 spectrometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Cells and transfections

Transformed HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, U.S.A.), supplemented with 2 mM glutamine, 10 % (v/v) fetal-bovine serum, penicillin at 100 units/ml and streptomycin at 100 μg/ml, at 37 °C in the presence of 5 % CO_2. Cells were harvested by trypsinization and were counted electronically (Coulter Model ZM counter; Coulter Electronics, Hialeah, FL, U.S.A.). Cells were transfected with pCMV-Sport 6 vector, human SSAT-1 or SSAT-2 in the presence of LIPOFECTAMINE™ 2000 (Invitrogen, Grand Island, NY, U.S.A.) following the manufacturer's protocol. Following a 24 h transfection period, cells were trypsinized, washed with PBS and stored at -70 °C for polyamine pool analysis or for use in the SSAT enzyme assay described below.

SSAT and ODC (ornithine decarboxylase) enzyme assays

Assays were performed using transiently transfected HEK-293 cell extracts. ODC activity was assayed as previously described and expressed as nmol/h per mg of protein [8]. The SSAT activity assay was determined as previously described [8] and expressed as pmol/min per mg of protein. Briefly, cells were washed with cold PBS and resuspended in 5 mM Hepes buffer, pH 7.2, at 250 μl/5 × 10⁶ cells. Cells were sonicated with 2 × 5 s bursts using a Branson Sonifier 250 (VWR, Rochester, NY, U.S.A.) at a power setting of 2.5, and centrifuged at 22000 *g* for 10 min at 4 °C. The supernatants were used for assay. The reaction mixture for SSAT determinations (final volume 50 μl) included 10 μl of 5.5 M Bicine buffer, pH 8.0, 5 μl of 30 mM Spd or other candidate substrate, 10 μl of doubly distilled water, 5 μl of 0.1 mM [¹⁴C]acetyl-CoA (60 mCi/mmol; PerkinElmer Life Sciences Inc., Boston, MA, U.S.A.) and 20 μl of each sample. The mixture was incubated for 5 min at 37 °C. The enzyme reaction was stopped by the addition of 20 μl 0.5 M hydroxylamine hydrochloride, and the mixture was heated in boiling water for 3 min. The resulting samples were centrifuged (22000 *g*), and an aliquot of 50 μl was spotted on to Whatman P81 phosphocellulose discs and counted for radioactivity in a liquid-scintillation counter.

HPLC

Polyamines were extracted from transfected cell pellets by treatment with 0.6 M perchloric acid (100 μl/1 × 10⁶ cells), followed by a 15 min centrifugation at 4 °C. Extracts were then dansylated and assayed by reverse-phase HPLC as previously described [14]. The data were collected and analysed using the Millennium 32 chromatography software (version 3.05; Waters Corp., Milford, MA, U.S.A.). Peaks were identified and quantified by alignment with a chromatogram of known standards, including Put, Spd, norSpd Spm, N^1-AcSpm and N^1- and N^8-AcSpd, DASpm and $N^{1,7}$-diaminoheptane as an internal standard.

Northern-blot analysis

Total RNA was isolated using RNeasy minikit (Qiagen) and Northern-blot analysis of PAO, SMO, SSAT-1 and SSAT-2 mRNA from untreated and analogue-treated HEK-293 cells was conducted as reported previously [19].

digiNorthern analysis of SSAT-1 vs SSAT-2 EST (expressed-sequence-tag) expression

A virtual analysis of the relative gene expression levels in different tissues for *SSAT-1* and *SSAT-2* genes was performed using our in-house bioinformatics tool digiNorthern [20]. The method analyses indexed EST data and applies a dynamic approach to collect the most updated EST data. It also allows for a side-by-side comparison of two genes such as *SSAT-1* and *SSAT-2*. Since *SSAT-2* is a new gene, the virtual Northern data is not available from the
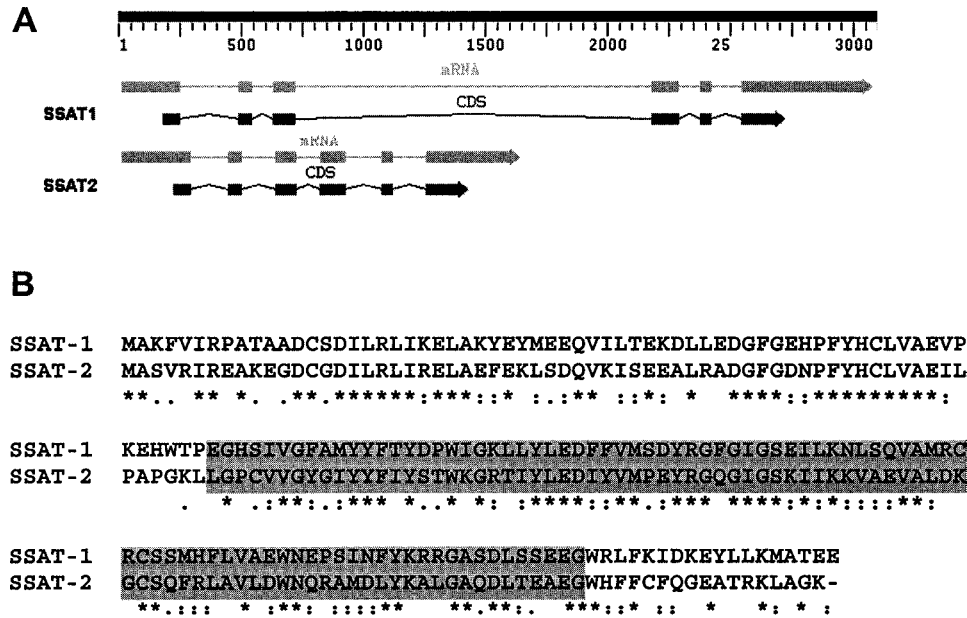
A

B

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SSAT-1   MAKFVIRPATAADCSDILRLIKELAKYEYMEEQVILTEKDLLEDGFGEHPFYHCLVAEVP
SSAT-2   MASVRIREAKEGDCGDILRLIRELAEFEKLSDQVKISEEALRADGFGDNPFYHCLVAEIL
         **.. ** *. .**.******:***:* :..:** ::*: *  ****::*********:

SSAT-1   KEHWTPEGHSIVGFAMYYFTYDPWIGKLLYLEDFFVMSDYRGFGIGSEILKNLSQVAMRC
SSAT-2   PAPGKLLGPCVVGYGIYYFIYSTWKGRTTYLEDIYVMPEYRGQGIGSKIIKKVAEVALDK
         .  * .:**:.:*** *..* *: :****:.**.:*** ****:*:*::::**:

SSAT-1   RCSSMHFLVAEWNEPSINFYKRRGASDLSSEEGWRLFKIDKEYLLKMATEE
SSAT-2   GCSQFRLAVLDWNQRAMDLYKALGAQDLTEAEGWHFFCFQGEATRKLAGK-
         **.::: * :**: :::::** **.**:. ***::* ::  *  *: * :
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Figure 1   Comparison of gene structure (A) and peptide sequence (B) for human SSAT-1 and SSAT-2

(**A**) Shows similarity in gene structure for *SSAT-1* and *SSAT-2* (exons are indicated by the thick bars, whereas while introns are indicated by thin lines) obtained using the NCBI SEQUIN program. The scale indicates the nucleotide base position. CDS, coding sequence. (**B**) compares multiple peptide sequence alignment for human SSAT-1 (NP_002961.1) and SSAT-2 (NP_597998) performed using the CLUSTAL_X alignment tool [21]. '*' Indicates an identical residue, ':' indicates a similar residue, and '.' indicates less similar residues. The grey shaded area is the GNAT domain.

NCI CGAP (National Cancer Institute Cancer Genome Anatomy Project) site and could only be obtained through digiNorthern technology [20].

Identification of SSAT-2 orthologous sequences and sequence alignments

Complete coding sequences for SSAT-2 orthologous genes from non-human species were assembled from the ESTs collected by BLAST searching the NCBI dbEST database using the predicted human SSAT2 protein sequence. Sequences corresponding to SSAT-1 in the same species were also identified to ensure that the claimed SSAT-2 sequences are distinguishable from SSAT-1. All new SSAT-2, and SSAT-1 sequences were submitted into the NCBI TPA (Third Party Annotation) database (http://www.ncbi.nlm.nih.gov/Genbank/tpa.html). Multiple sequence alignments were performed using CLUSTAL_X [21].

RESULTS

A BLAST search of public genome and cDNA databases using human SSAT-1 sequences yielded sequences which, on the basis of functional domain and enzyme-activity data discussed below, we have designated SSAT-2. While the human *SSAT-1* gene (NM_002970) is known to be located at chromosome Xp22.1 [22], the newly identified *SSAT-2* gene (NM_133491) was found at chromosome 17p13.1. Human SSAT-1 cDNA (1069 bp) encodes a protein (NCBI accession no. NP_002961) comprising 171 amino acids and had a deduced molecular mass of 19 kDa, whereas the newly identified human SSAT-2 cDNA (962 bp) was deduced to encode a protein (NCBI accession no. NP_597998) comprising 170 amino acids and a nearly identical molecular mass. The two enzymes have the same gene structure and share 46 % amino acid sequence identity and 64 % sequence similarity (Figure 1). Conservation between SSAT-1 and SSAT-2 transverses the entire length of the two proteins and both contain a general N^1-acetyltransferase (GNAT) domain spanning 87 amino acids [23].

Homologous genes for SSAT-2 were detectable in a variety of species from bacteria to eukaryotes in all major lineages, indicating the ubiquitous distribution of this enzyme. In addition to the protein sequences already archived in the NCBI non-redundant protein database (mostly from the complete genome sequences), we also assembled several additional homologous sequences from higher organisms, including sequences that are orthologous with SSAT-2, by making use of the in-progress genome sequences and ESTs. While all species below vertebrate, including the sea squirt *Ciona intestinalis*, a basal chordate, contain only one copy of SSAT (results not shown), all vertebrate species examined seem to possess two copies of functional SSAT genes (i.e. SSAT-1 and SSAT-2) with the exception of chicken and the frog *Xenopus*, which show no detectable SSAT-2 expression based on ESTs. As shown in Figure 2, mammalian SSAT-2 genes are highly conserved at the protein sequence level (identity 85 % for all pairwise comparisons), but this is significantly lower than the conservation level among mammalian SSAT-1 orthologues (identity 95 %; results not shown), suggesting that SSAT-1 may be under higher functional selection pressure than SSAT-2. A lower overall expression of *SSAT-2* gene than *SSAT-1* gene in humans (discussed below) seems to be supportive of this possibility.

The SSAT-1- and SSAT-2-cDNA-containing plasmids were transiently transfected with high efficiency into HEK-293 cells. As shown by the Northern blots presented in Figure 3, transfection increased the mRNA of each acetyltransferase without affecting the expression of the alternative acetyltransferase. SSAT-1 transcripts appeared as a major band with at least two minor bands that are due to multiple polyadenylation sites in the transfected plasmid [24]. As shown by RNA levels, the two plasmids were transfected and transcribed to similar levels in the cells. Importantly, the blots also demonstrate that the cDNAs hybridize specifically to their respective messages and there was no indication of cross-hybridization. To gain insight into the nature of *SSAT-2* and whether it was translated into active protein in cells, we first examined whether the transfected gene altered

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SSAT-2 hs     1 MASVRIREAKEGDCGDILRLIRELAEFEKLSDQVKISEEALRADGFGDNPFYHCLVAEIL
SSAT-2 bt     1 MAFVMIREAKEGDCGNILRLIRELAEYEKLSDQVKISEEALRADGFGETPFYHCLVAEIL
SSAT-2 ss     1 MASVLIREAKEGDCGNILRMIRELAEYEKLSDQVKISEEALRADGFGENPFFHCLVAEIL
SSAT-2 mm     1 MASTRIREARESDCGDIMRMIRELAEFEKLSHQVKISEEALRADGFGENPFFHCLVAEII
SSAT-2 rn     1 MASTRILEAKESDCGDIMTMIRELAEFEKLSHQVKISEEALRADGSGENPFFHCLVAEII
                ** . * **:*.***:*: :******:****.*************  *:.**:*******:

SSAT-2 hs    61 PAPGKLLGPCVVGYGIYYFIYSTWKGRTIYLEDIYVMPEYRGQGIGSKIIKKVAEVALDK
SSAT-2 bt    61 SAPGEPQGPCVVGYGLYYFSYSTWKGRNIYLEDIYVKPEYRGQGIGSKIIKKVAEVALDK
SSAT-2 ss    61 PAPGEPQGSCMVGYGLYYFIYSTWTGRNIYLEDIYVKPEYRGQGIGSKIIKKVAEVALDK
SSAT-2 mm    61 PAPGESQGSLVVGYGLYYFIYSTWTGRNVYLEDIYVMPQYRGQGIGTKIIKKVAEVALNK
SSAT-2 rn    61 PAPGEPQGSLVVGYGLYYFIYSTWTGRNIYLEDIYVMPKYRGQGIGTKIINKVAEVALRK
                .***: *. :****:*** ****.**.:******* *:*******:***:******* *

SSAT-2 hs   121 GCSQFRLAVLDWNQRAMDLYKALGAQDLTEAEGWHFFCFQGEATRKLAGK
SSAT-2 bt   121 GCSQLRLAVLDWNKRAMDLYKALGAQDLTEAEGWHCFRFEGEAMRELAGK
SSAT-2 ss   121 GCSQFRLAVLDWNKKAVDLYKTLGARDLTEAEGWHSFRFEGEAMRELAGK
SSAT-2 mm   121 GCSQFRLAVLDWNKKAVNLYKFLGAQDLTESEGWLSFRFEGEAMRELAGR
SSAT-2 rn   121 GCSQFRLAVLNWNKKAVNLYKFLGAQDLTESEGWLSFRFEGEAMRELAGR
                ****:*****:**::*::*** ***:****:***  * *:*** *:***:
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Figure 2   Comparison of amino acid sequence for human SSAT-2

Multiple peptide sequence alignment performed using the CLUSTAL_X alignment tool [21]. Protein sequences used in the alignment include NP_597998 from human (SSAT2 hs), XP_181304 from mouse (SSAT2 mm), XP_220605 from rat (SSAT2_rn), BK_001359 from ox (bovine) (SSAT2 bt) and BK_001358 from pig (SSAT2 ss).
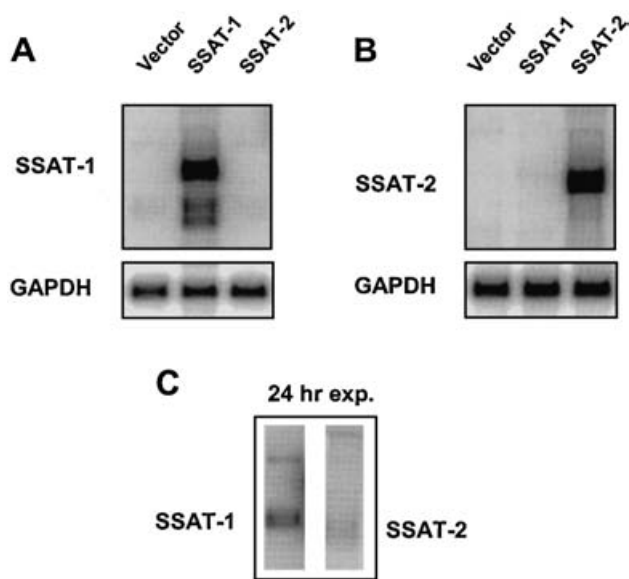

Figure 3   Northern-blot analysis of HEK-293 cells transfected with SSAT-1 or SSAT-2 and probed with either SSAT-1 (A) or SSAT-2 (B)

Note that both probes are specific for their respective mRNAs and that transfected SSAT-1 appears as two transcripts that differ according to polyadenylation sites in the plasmid. At the 4 h exposure (**A** and **B**), endogenous SSAT-1 and SSAT-2 transcripts were not discernible, but, when exposure was extended to 24 h (**C**), endogenous SSAT-1 levels in vector-transfected cells are seen to be significantly higher those of SSAT-2. As indicated by electrophoretic migration, SSAT-1 mRNA is ≈ 100 bp larger than that of SSAT-2.

intracellular polyamine pools (Table 1). In SSAT-1-transfected cells, Spm and Spd pools decreased by $\approx 30\%$, whereas Put pools increased by > 10-fold. Acetylated polyamines, including N^1-AcSpd, N^1-AcSpm and DASpm, all increased markedly. In SSAT-2-transfected cells, polyamine pools, including those of the acetylated polyamines, remained within the range of vector-transfected cells.

ODC and SSAT activities were measured in transfected cells using the standard enzyme assays based on enzyme extracts

(Table 1). When Spd was the substrate, acetylating activity in SSAT-1-transfected cells was approx. 37-fold higher than in vector-transfected cells. Perhaps due to the decrease in Spm pools, ODC activity increased 7-fold and this probably contributed to a sharp rise in Put pools. Unexpectedly, enzyme-containing extracts from SSAT-2-transfected cells contained 10-fold higher acetylating activity than that in vector-transfected cell extracts, despite the fact that polyamine pools were not affected in intact cells. ODC activity remained unchanged, consistent with the fact that Spm pools were not lowered as in SSAT-1 transfected cells. Detection of SSAT-2 enzyme activity in extracts, but not in intact cells, suggests that the protein might be sequestered in cellular organelles.

We next examined the relative substrate specificity of the two acetyltransferases using the enzyme-activity assay. For these studies, HEK-293 cells were transfected with human SSAT-1, human SSAT-2 or empty vector for 24 h. Cell extracts were assayed in the presence of 3 mM candidate polyamine substrates. The contribution of endogenous enzymes obtained from vector-transfected cell extracts was found to be minor under conditions of the enzyme incubation. Substrate preferences were compared relative to Spd, the most preferred natural substrate for both enzymes. As shown in Table 2, a rank order of substrate preference was established for each enzyme. SSAT-1 showed the preference: $\text{norSpd} = \text{Spd} \gg \text{Spm} > N^1\text{-AcSpm} > \text{Put}$; SSAT-2, on the other hand, showed the preference: $\text{norSpd} > \text{Spd} = \text{Spm} \gg N^1\text{-AcSpm} = \text{Put}$.

We next examined the relative levels of endogenously expressed SSAT-1 and SSAT-2 in HEK-293 cells and their inducibility by three Spm analogues known to differentially induce SSAT-1 [13,24]. Under basal conditions, endogenous SSAT-1 was expressed at higher levels than SSAT-2 (Figure 3). As previously reported [16], SSAT-1 is differentially induced by Spm analogues according to their intra-amine carbon-chain lengths – DENSpm being the most potent inducer of all three enzymes, followed by DESpm (N^1,N^2-diethylspermine), followed by DEHSpm (N^1,N^{14}-diethylhomospermine). As shown in Figure 4, we repeated these findings and, in addition, observed that SSAT-2 mRNA was not inducible by any of these analogues. Finally, we examined whether SSAT-2 protein might be induced by

Table 1   Comparison of SSAT-1 and SSAT-2 effects on polyamine metabolism in transfected HEK-293 cells

HEK-293 cells were transiently transfected with empty vector, human SSAT-1 or SSAT-2 and assayed 24 h later for polyamine enzyme activities and intracellular polyamine pools. Results are means $\pm$ S.D. ($n = 4$).

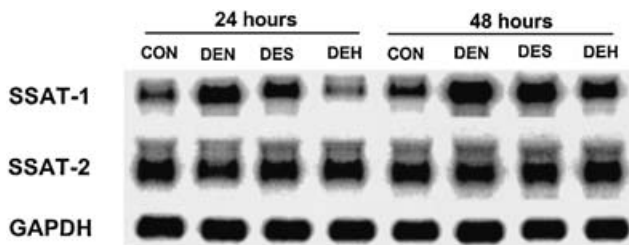| Transfection (24 h)	Enzyme activity in cell extracts		Intracellular polyamine pools (pmol/10^6 cells)					
	ODC (nmol/h per mg)	SSAT (pmol/min per mg)	Put	N^1-AcSpd	Spd	N^1-AcSpm	DASpm	Spm
Vector	0.24 ± 0.07	27.7 ± 2.3	< 10	< 10	1102 ± 128	< 10	< 10	3594 ± 228
SSAT-1	$1.67 \pm 0.13^*$	$1031 \pm 103^*$	$1252 \pm 167^*$	$2473 \pm 249^*$	$887 \pm 135^*$	$135 \pm 21^*$	$731 \pm 107^*$	$2569 \pm 392^*$
SSAT-2	0.42 ± 0.01	$282 \pm 63.5^*$	< 10	< 10	1044 ± 197	< 10	< 10	3969 ± 503

  * Indicates statistical significance of $P < 0.01$ based on Student's t test comparison of vector- versus SSAT-transfected cells.

Table 2   Comparison of SSAT-1 and SSAT-2 substrate specificity in HEK-293-cell-lysate assay

HEK-293 cells were transiently transfected with human SSAT-1 or SSAT-2. After 24 h, cells were harvested for SSAT enzyme assay using candidate substrates at 3 mM. The percentage Spd activity was obtained by dividing the acetylated product of various substrates by that generated in the presence of Spd. Endogenous activity seen in vector-transfected controls was subtracted for each substrate from the SSAT-transfected activity. Typically it accounted for < 10 % enzyme activity in SSAT-1 transfected cells and for < 10 % enzyme activity in SSAT-2 transfected cells.

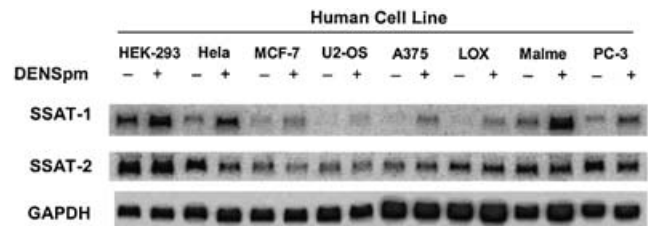| Candidate substrates (3 mM)	SSAT-1- transfected cell extracts		SSAT-2-transfected cell extracts	
	Enzyme activity (pmol/min per mg)	Spd activity (%)	Enzyme activity (pmol/min per mg)	Spd activity (%)
norSpd	1106 ± 146	110 ± 17	675 ± 10.2	266 ± 34
Spd	1003 ± 103	100 ± 10	254 ± 64	$100 \pm 21^*$
Spm	411 ± 41	$41 \pm 13^*$	228 ± 17	90 ± 15
N^1-AcSpm	181 ± 2	$18 \pm 7^*$	81 ± 40	$32 \pm 5^*$
Put	90 ± 9	$9 \pm 2^*$	73 ± 36	29 ± 10

  * Indicates statistical significance of $P < 0.01$ relative to the next higher percentage based on Student's t test.

Figure 4   Northern-blot comparison of SSAT-1 and SSAT-2 mRNA expression and induction in analogue-treated HEK-293 cells

Cells were treated in the absence (CON) or presence of 10 μM DENSpm (DEN), DESpm (DES), or DEHSpm (DEH) for 24 or 48 h before mRNA extraction. Gels were loaded with 30 μg of total RNA. Note that the films were radiographically exposed for 1 day for SSAT-1 and exposed for 3 days for SSAT-2. As previously reported [16], SSAT-1 mRNA was differentially inducible by the analogues as follows: DENSpm > DESpm > DEHSpm. SSAT-2 was not induced by any of the analogues. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as the loading control.

Figure 5   Comparative analysis of relative SSAT-1 and SSAT-2 mRNA levels among various tumour-cell lines

Cells were grown for 24 h in the absence or presence of 10 μM DENSpm before Northern-blot analysis. Gels were loaded with 20 μg of total RNA. Note that the film was radiographically exposed for 1 day for SSAT-1 and 3 days for SSAT-2.

DENSpm at the levels of protein translation or stabilization, as is the case for SSAT-1 [19,25]. For these studies, cells were transiently transfected with SSAT-1, SSAT-2 or empty vector for 24 h, incubated for another 24 h in the presence or absence of 10 μM DENSpm, and then assayed for enzyme activity. DENSpm treatment induced acetyltransferase activity 30-fold in SSAT-1-transfected cells relative to vector-transfected cells, but activity was only induced 2-fold in SSAT-2-transfected cells. Thus we deduce that SSAT-2 is not inducible by DENSpm at the level of either mRNA or enzyme activity.

We then examined the generality of the above findings. The relative levels of SSAT-1 and SSAT-2 endogenous expression and its inducibility by DENSpm were then compared in a series of randomly obtained cell lines by Northern-blot analysis to examine heterogeneity of expression. As shown in Figure 5, HEK-293 cells expressed the highest basal levels of SSAT-1, followed by HeLa cells and PC-3 prostate-melanoma cells. All of the cell lines showed some level of SSAT-1 inducibility to DENSpm with MALME-3M melanoma cells being the most responsive. By comparison, these cell lines also expressed SSAT-2 to various extents, with HEK-293 and HeLa cells giving the highest values. However, there was no indication of DENSpm induction of SSAT-2 in any of the cell lines. Finally, we compared expression of *SSAT-1* and *SSAT-2* in various normal and tumour tissue counterparts by virtual Northern analysis using publicly available EST databases. Amongst all the tissues represented, there were a total of 1036 ESTs for SSAT-1 as compared with 174 ESTs for SSAT-2. Thus SSAT-1 was typically expressed at much higher levels in more tissues than SSAT-2. There were many examples of tissues that

express only SSAT-1, several instances of tissues that express both SSAT-1 and SSAT-2, and a few instances, such as bone, cervix, ovary and pineal gland, that express only SSAT-2. There were no consistent differences between various normal and tumour tissues.

DISCUSSION

Since most genes in the human genome are redundant, it is not surprising that two similar polyamine-associated acetyl-transferase transcripts might exist. Several lines of evidence suggest that the newly discovered sequences reported here are, in fact, those encoding an acetyltransferase that is similar in function to SSAT-1. Although the coding sequences for these two enzymes are located on different chromosomes, they share similar gene structure, nearly identical molecular masses, 46% amino acid identity and 64% amino acid similarity, as well as an 86-amino-acid GNAT domain which contributed to the identification of SSAT-2. Perhaps most importantly, the two genes are expressed at the level of mRNA in various cell lines and, when transfected into cells, they can be detected on the basis of N-acetyltransferases in cell extracts.

Despite similarities, distinct differences exist between the two acetyltransferases. First, transfected SSAT-1 is active in intact cells, as indicated by its effects on polyamine pool profiles. This was similar to previous findings following conditional expression of SSAT-1 in cultured breast MCF-7 tumour cells [4], and in SSAT-1 transgenic mice [5]. By contrast, transfected SSAT-2 is apparently not active in intact cells, as indicated by the lack of an effect on intracellular polyamine pool profiles. This raises the possibilities that: (a) the gene is not transcribed, (b) the message is not translated, (c) the protein is inactive, or (d) the protein is sequestered into intracellular organelles. The first three possibilities can be dismissed by the findings that SSAT-2 mRNA is clearly present in transfected cells (Figure 3) and by the observation that enzymically active SSAT-2 protein is detectable in disrupted cell extracts (Tables 1 and 2). Thus we conclude that the protein is probably contained within an organelle that prevents interaction with intracellular polyamines and/or with the enzyme cofactor acetyl-CoA. Acetylated polyamines seem capable of entering peroxisomes, as indicated by the fact that transfected peroxisomal PAO alters intracellular polyamine pools [16]. It is not clear, however, that unacetylated polyamines can behave similarly. Likewise, it is known that acetyl-CoA does not penetrate the plasma membrane of cells [26] and may, therefore, be incapable of entering organelles containing SSAT-2. Apparent sequestration of SSAT-2 and the absence of activity in intact transfected cells raise interesting questions regarding the role of the enzyme in polyamine catabolism and under what circumstances it might become functional.

The two acetyltransferases were found to differ in more subtle ways involving their respective substrate specificities. In the enzyme assay, SSAT-1 showed the substrate preference: norSpd = Spd $\gg$ Spm > N^1-AcSpm > Put. Normalized to Spd, this rank order was nearly identical with that previously obtained by kinetic studies using semi-purified mammalian SSAT-1 [27]. This agreement with kinetic data lends credence to substrate preference similarities and differences seen between SSAT-1 and SSAT-2. Perhaps the most metabolically relevant differences involve the substrates Spd and Spm. Whereas SSAT-1 shows a much greater preference for Spd than for Spm, SSAT-2 has a similar preference for the two polyamines. The much greater preference for norSpd relative to Spd by SSAT-2 suggests stronger recognition of aminopropyl moieties. Overall, the lack of major differences in

substrate preferences between the two enzymes is consistent with the 87% sequence similarity in the 87-amino-acid GNAT domain contained within the two proteins.

Northern-blot findings and EST analysis indicate that SSAT-1 is more typically expressed at higher levels than SSAT-2. When compared with other catabolic enzymes [3,16], the rank order for basal mRNA level expression in HEK-293 cells is: SSAT-1 > SSAT-2 > SMO > PAO. A comparison of mRNA levels from control and analogue-treated cell lines consistently showed that SSAT-1 is inducible and SSAT-2 is not (Figure 5). It is well recognized that SSAT-1 is post-transcriptionally induced at the levels of translation and enzyme stabilization [19,24]. Although not definitive, our present data indicate that SSAT-2 is not induced at the level of enzyme activity (Figure 5), a finding that is at least partially consistent with the facts that it appears to be sequestered and that it lacks the C-terminal amino acid sequence (MATEE in one-letter code) shown by others to be involved in the stabilization of SSAT-1 by polyamine analogues [11,28].

The detection of homologous *SSAT-2* genes in bacteria and eukaryotes indicates a ubiquitous biological distribution of this enzyme. All vertebrate species examined possess two copies of functional *SSAT* genes (i.e. *SSAT-1* and *SSAT-2*) with the exception of chicken and *Xenopus*, which seem to have no detectable expression of SSAT-2 based on ESTs. This suggests that the duplication of SSAT occurred during the early evolution of vertebrates from a primitive chordate ancestor. A comparative analysis of *SSAT-1* and *SSAT-2* ESTs by digiNorthern analysis failed to implicate the latter enzyme in any obvious association with specific tissue function or with neoplasia. Basal mRNA expression, lack of inducibility and apparent enzyme sequestration all combine to suggest that SSAT-2 has clearly different cellular functions than SSAT-1. It is also significant that, in most tissues, SSAT-1 has a higher and broader tissue distribution of expression than SSAT-2. Antibody development for use in immunohistochemistry and Western-blot analysis is essential for the further elucidation of SSAT-2 function during physiological and pharmacological perturbations. At this point, it is useful to know that the plasmid cDNAs for SSAT-1 and SSAT-2 hybridize specifically to their respective mRNA.

Note added in proof (received 25 June 2003)

Since the acceptance of this manuscript, we have become aware of an abstract [29] reporting the identification, cloning and heterologous expression of SSAT-2. Their finding of K_m values for Spd in the low-millimolar range may account for the apparent inactivity of the transfected enzyme in HEK-293 cells, as observed in the present study.

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