Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin

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During polyamine catabolism, spermine and spermidine are first acetylated by spermidine/spermine *N*¹-acetyltransferase (SSAT) and subsequently oxidized by polyamine oxidase (PAO) to produce spermidine and putrescine, respectively. In attempting to clone the PAO involved in this back-conversion pathway, we encountered an oxidase that preferentially cleaves spermine in the absence of prior acetylation by SSAT. A BLAST search using maize PAO sequences identified homologous mammalian cDNAs derived from human hepatoma and mouse mammary carcinoma: the encoded proteins differed by 20 amino acids. When either cDNA was transiently transfected into HEK-293 cells, intracellular spermine pools decreased by 75 % while spermidine and *N*¹-acetylspermidine pools increased, suggesting that spermine was selectively and directly oxidized by the enzyme. Substrate specificity using lysates of oxidase-transfected HEK-293 cells revealed that the newly identified oxidase strongly favoured spermine over *N*¹-acetylspermine and that it failed to act on *N*¹-

acetylspermidine, spermidine or the preferred PAO substrate, *N*¹,*N*¹²-diacetylspermine. The PAO inhibitor, MDL-72,527, only partially blocked oxidation of spermine while a previously reported PAO substrate, *N*¹-(*n*-octanesulphonyl)spermine, potently inhibited the reaction. Overall, the data indicate that the enzyme represents a novel mammalian oxidase which, on the basis of substrate specificity, we have designated spermine oxidase in order to distinguish it from the PAO involved in polyamine back-conversion. The identification of an enzyme capable of directly oxidizing spermine to spermidine has important implications for understanding polyamine homeostasis and for interpreting metabolic and cellular responses to clinically relevant polyamine analogues and inhibitors.

Key words: polyamine, polyamine oxidase, spermidine, spermidine/spermine *N*¹-acetyltransferase.

INTRODUCTION

Intracellular polyamine pool homeostasis and the effect of polyamine antagonists on it can be explained by changes in rate-limiting effector molecules involved in polyamine biosynthesis, polyamine catabolism and polyamine transport inside and outside of the cell. During biosynthesis, polyamines are assembled by the sequential addition of aminopropyl units derived from decarboxylated *S*-adenosylmethionine on to a core diamine unit [putrescine (Put)] derived from ornithine. During polyamine catabolism, those same aminopropyl units are systematically removed by a consecutive two-step back-conversion pathway. 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), hydrogen peroxide and 3-acetamidopropanal [1,2]. The conversion of Spd into Put takes place by analogous reactions involving the same enzymes (Scheme 1). Although PAO is capable of oxidizing Spm directly, it is known to prefer *N*¹-acetylspermine (AcSpm) [3–5] and its reliance on SSAT for acetylated substrates represents the functional basis for the back-conversion pathway.

Compared with polyamine biosynthesis, relatively little attention has been devoted to polyamine catabolism and the

biological significance of the back-conversion pathway is only beginning to be appreciated. Recent studies indicate that conditional overexpression of SSAT lowers intracellular polyamine pools in MCF-7 cells [6]. When the acetylating enzyme is induced by polyamine analogues, it actively contributes to the polyamine pool depletion and antiproliferative effects of these compounds [6–9]. By comparison, the relationship of PAO to cell growth and analogue effects is much less clear. While changes in PAO activity are not typically associated with induction of cell growth, enzyme activity has been reported to be higher in human colon carcinoma [10] and other tumours [11] relative to normal tissue, but lower in breast carcinoma [12]. Consistent with the latter, Takenoshita et al. [13] have reported that levels of the PAO substrate *N*¹-acetylspermidine (AcSpd) is much higher in colon adenocarcinoma than in benign polyps or normal surrounding mucosa, suggesting decreased PAO activity. Others studies have also suggested that hydrogen peroxide liberated during oxidation of intracellular polyamines may mediate aspects of embryonic development [14] and apoptotic responses to polyamine analogues [15,16], and that PAO may play a protective role in Myc-driven apoptosis of transformed human haematopoietic cells [17].

PAO is found in all vertebrate tissues [18] and has been shown to be subcellularly located in both the cytoplasm and peroxisomes

Abbreviations used: AcSpd, *N*¹-acetylspermidine; AcSpm, *N*¹-acetylspermine; DAS, *N*¹,*N*¹²-diacetylspermine; DESpm, *N*¹,*N*¹²-diethylspermine; DENSpm, *N*¹,*N*¹¹-diethylhomospermine; DEHSpm, *N*¹,*N*¹⁴-diethylhomospermine; MESpm, *N*¹-monoethylspermine; *N*¹OSSpm, *N*¹-(*n*-octanesulphonyl)spermine; PAO, polyamine oxidase; ODC, ornithine decarboxylase; Put, putrescine; SAMDC, *S*-adenosylmethionine decarboxylase; SMO, spermine oxidase; Spd, spermidine; Spm, spermine; SSAT, spermidine/spermine *N*¹-acetyltransferase.

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Scheme 1 SMO and polyamine metabolism

(A) Diagram of polyamine metabolism emphasizing the roles of SSAT and PAO in the back-conversion pathway. Following acetylation by SSAT, Spd or Spm can be either exported out of the cell or degraded by PAO in a reaction that yields hydrogen peroxide and 3-acetamidopropanal. AcSpm can also be re-acetylated by SSAT to form DAS [6]. The shaded arrows indicate the prominent role that polyamine acetylation by SSAT is presumed to play in regulating the back-conversion of Spm to Spd, and Spd to Put [1]. (B) Revised diagram of polyamine metabolism taking into account the role of SMO and SSAT in Spm catabolism and export, respectively. In this scheme, the back-conversion of Spm to Spd is primarily accomplished by SMO (open arrow) in the absence of prior Spm acetylation by SSAT. Thus, polyamine acetylation by SSAT may be more involved in the export of polyamines, such as AcSpd, AcSpm and DAS, out of the cell (shaded arrows). This would serve to lower intracellular polyamines during polyamine excess. In the presence of SMO, PAO would seem to be more uniquely involved in the back-conversion of Spd to Put. These proposed roles are dependent on the relative levels and availability of these enzyme activities within the cell. SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; Spd Syn, spermidine synthase; Spm Syn, spermine synthase; MTA, 5'-methylthioadenosine; AcCoA, acetyl-CoA.

[19–21]. Although Spm and, to a lesser degree, Spd were initially thought to be the natural substrates of the enzyme [19], subsequent studies revealed that charge removal via acetylation of terminal aminopropyl moieties greatly improved their reactivity with the enzyme [3]. Thus early investigators deduced that the preferred natural substrates of PAO, AcSpm and AcSpd probably serve as intermediates in a pathway whereby Spm or Spd is first acetylated by SSAT and then oxidized by PAO to yield Spd or Put, respectively [3,4]. Studies with specific inhibitors of PAO

support the existence and functionality of this pathway in intact cells and tissues [22,23] as indicated by the accumulation of AcSpd and AcSpm following PAO inhibition. In addition, conditional overexpression of SSAT results in the predicted decreases in intracellular polyamine pools and accumulation of acetylated products [6].

In a significant recent development, Wang et al. [24] used maize PAO sequences [25] in a homology search to clone a human placental cDNA that encodes a protein with a molecular mass of ≈ 62 kDa. When expressed in an *in vitro* transcription/translation system, the protein liberated hydrogen peroxide in the presence of Spm, suggesting an oxidase activity that was presumed to be that of the PAO involved in polyamine back-conversion. It was, therefore, designated PAO-1. Importantly, the oxidase mRNA was shown to be inducible by polyamine analogues, a finding that has implications for understanding the mode of action of these clinically relevant compounds. Some time ago (in early 2001), we used a similar homology-search strategy to access the nearly identical oxidase cDNA from human hepatoma and mouse mammary carcinoma sources. When transiently transfected into HEK-293 cells, the human and murine cDNAs displayed a narrow substrate specificity for Spm over AcSpm, AcSpd or Spd. On the basis of this finding, we have designated the novel enzyme as spermine oxidase (SMO) to distinguish it from the PAO associated with the polyamine back-conversion pathway. The latter enzyme prefers AcSpm and AcSpd over Spm and Spd [3,4]. Although Spm-directed oxidases have been shown to exist in other systems [26,27], this is the first demonstration of such an enzyme in mammalian cells.

EXPERIMENTAL

Materials

The inhibitor of PAO, MDL-72,527 [28], was kindly provided by Aventis (Bridgewater, NJ, U.S.A.). N^1,N^{12} -Diacetylspermine (DAS) and the calmodulin antagonist [23] N^1 -(*n*-octanesulphonyl)spermine (N^1 OSSpm) were from Dr Nikolaus Seiler (Laboratory of Nutritional Oncology, Institut de Recherche Contre les Cancers de l'Appareil Digestif, Strasbourg, France) and various ethylated Spm analogues were from Dr Raymond Bergeron (University of Florida, Gainesville, FL, U.S.A.). Spm, Spd, Put, AcSpm and AcSpd were originally purchased from Sigma (St. Louis, MO, U.S.A.), although the latter two compounds are no longer available from that source.

Homology search and cDNA acquisition

A BLAST sequence homology search [29] of genome and cDNA databases was carried out using maize PAO sequence [25] followed by a Pfam homology search [30] for a FAD-binding domain [31]. Clones containing candidate human and mouse oxidase cDNA sequences were obtained from the Institute of Medical Sciences (University of Tokyo, Tokyo, Japan) and Incyte Genomics (St. Louis, MO, U.S.A.), respectively.

Plasmids and subcloning

Mouse cDNA (GenBank accession no. BC004831) was cloned into pCMV-Sport 6 (Invitrogen, Carlsbad, CA, U.S.A.). Human cDNAs (GenBank accession nos. AK000753 and AK025938) were cloned into pME18S-FL3 and subcloned as *XhoI/XhoI* fragments into pCMV-Sport 6. Alternatively spliced human cDNAs [GenBank accession no. AK025938 encoding NCBI

accession no. BAB15288 (389 amino acids) and GenBank accession no. BC000669 encoding NCBI accession no. AAH00669 (502 amino acids)] were cloned in pOTB 7 and subcloned as *EcoRI/XhoI* fragments into pCMV-Sport 6. All plasmid DNAs were prepared using an EndoFree Maxi-prep kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's protocol. The amount of DNA was measured spectrophotometrically (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

PCR mutagenesis

PCR megaprimer mutagenesis method was used to prepare clones encoding Arg-320 instead of Cys-320 [32]. Human SMO cDNA was mutated using the primers 5'-CGAGGAC-CGTGAGCTGAT-3' and 5'-TGGGTAGGTGAGGGTGTG-G-3' to generate a mutated megaprimer which was used with the M13-48R primer to make a mutated fragment (the bold residue represents the mutated nucleotide). The mutated fragment's *PstI/ApaI* and *ApaI/XhoI* sites were used to clone the fragment into the *PstI/XhoI* sites of the pCMV-Sport 6 vector. Mouse SMO cDNA was mutated in the same way using primers 5'-GAGGATCGCGAGGTGATC-3' and 5'-CACAGATCTTGG-GGTACCAG-3' to prepare a megaprimer that was then used with M13-48R to obtain mutated fragment cloned into the *SphI/PmlI* sites of the pCMV-Sport 6 mSMO vector.

Cells and transfections

Transformed human kidney 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% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in the presence of 5% CO₂. Cells were harvested by trypsinization and counted electronically (Coulter model ZM; Coulter Electronics, Hialeah, FL, U.S.A.). Cells were transfected with human or mouse SMO cDNA cloned into the pCMV-Sport 6 vector in the presence of Eugene 6 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) following the manufacturer's protocol. After transfection (48 h), cells were trypsinized and stored at -70 °C for polyamine pool analysis and enzyme activity assays. Control cells were transfected with an empty vector.

SMO activity assay

Transiently transfected cells were sonicated in a 10 mM Tris buffer (pH 8.0). Aliquots were stored at -70 °C and used as the enzyme source in the oxidase activity assay. The latter is an adaptation of an HPLC-based assay for PAO [33]. Briefly, transfected cell lysate samples were added to a reaction buffer containing 200 mM glycine (pH 9.5), 1 mM dithiothreitol and 200 µM of a candidate polyamine substrate (i.e. Put, Spd, Spm, AcSpd, AcSpm or DAS). The reaction buffer also contained 500 µM aminoguanidine as an inhibitor of endogenous diamine oxidase activity and 50 µM paraglyline as an inhibitor of endogenous monoamine oxidase activity [33]. Neither inhibitor was found to affect SMO activity or substrate specificity when deleted from or included in the assay. The assay was carried out as follows. A lysate-containing sample was inactivated with 0.6 M perchloric acid immediately following addition of a polyamine substrate; this served as the zero time 'blank'. By contrast, the assay lysate sample was incubated for 0–90 min in the presence of substrate before being inactivated with perchloric acid. Polyamine determinations by HPLC were made on all samples and expressed in nmol/mg of protein. SMO activity was evaluated by

comparing polyamine levels in the blank sample versus the assay sample. If a polyamine served as a SMO substrate (i.e. Spm), its concentration decreased during the incubation while the concentration of an oxidase reaction product (i.e. Spd) increased. A comparison of enzyme-transfected versus vector-transfected lysates indicates whether observed polyamine effects are attributable to endogenous compared with introduced enzyme activity.

HPLC

Polyamine levels were extracted from transfected cells or from lysate assay samples by treatment with 0.6 M perchloric acid followed by a 15 min centrifugation at 4 °C. Extracts were then dansylated and assayed by reversed-phase HPLC as described previously [6]. The data were collected and analysed using the Millennium 32 chromatography software (version 3.05; Waters Corporation, Milford, MA, U.S.A.). Peaks were identified and quantitated by alignment with a chromatogram of known standards including Put, Spd, Spm, N^{1,7}-diaminoheptane as an internal standard, AcSpd, AcSpm and DAS.

Radiochromatographic detection of polyamine oxidation

Oxidation of Spm was more directly confirmed by tracking radioactive shifts from radiolabelled Spm as substrate to Spd as product. Thus, 1 µCi of [¹⁴C]Spm (70 µM) per reaction (American Radiolabelled Chemicals; labelled in the central Put moiety) was included in the lysate assay and incubated for 10 min. Radioactive polyamines were separated and quantitated using a HPLC system linked to a IN/US flow-through radiodetector (Hialeah, FL, U.S.A.) as described in detail elsewhere [34,35]. Polyamines were identified on the basis of chromatographic migration relative to a cocktail of known radiolabelled standards. A shift in radioactivity from a candidate substrate polyamine to a potential product species in SMO but not vector-transfected cell lysates was taken as an indication of oxidase activity.

Polyamine enzyme assays, and SMO and SSAT mRNA

Ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and SSAT activities in transiently transfected HEK-293 cells were assayed as described previously [36].

Northern blot analysis of SMO and SSAT mRNA was conducted as reported earlier [6].

RESULTS

A BLAST search of public genome and cDNA databases was conducted using maize PAO sequences [25] and the FAD-binding domain [31]. Clones containing homologous human (GenBank accession no. AK000753) and mouse (BC004831) cDNA sequences were obtained from the Institute of Medical Sciences, University of Tokyo, and Incyte Genomics, respectively. Although identified via homology to maize PAO sequences, studies detailed below strongly indicate that the protein encoded by the mouse and human cDNAs should be designated SMO to more accurately reflect enzyme specificity and to distinguish it from the PAO involved in the two-step polyamine back-conversion pathway (i.e. the back-conversion PAO). Thus the human SMO cDNA (2291 bp) encodes a protein (NCBI accession no. BAA91360) comprised of 555 amino acids, with a molecular mass (61.8 kDa) very similar to that previously reported for semi-purified protein (62 kDa) [19–21]. The cDNA was derived

	1	11	21	31	41	51	61	71
human PAO-1	MQ	Q	S	E	S	G	S	A
human SMO	MQ	Q	S	E	S	G	S	A
mouse SMO	MQ	Q	S	E	S	G	S	A
Consensus	m	q	s	e	s	g	s	a
	81	91	101	111	121	131	141	151
human PAO-1	I	H	G	S	H	G	N	P
human SMO	I	H	G	S	H	G	N	P
mouse SMO	I	H	G	S	H	G	N	P
Consensus	i	h	g	s	h	g	n	p
	161	171	181	191	201	211	221	231
human PAO-1	N	A	E	S	Q	N	S	V
human SMO	N	A	E	S	Q	N	S	V
mouse SMO	N	A	E	S	Q	N	S	V
Consensus	n	a	e	s	q	n	s	v
	241	251	261	271	281	291	301	311
human PAO-1	R	V	V	E	L	A	E	G
human SMO	R	V	V	E	L	A	E	G
mouse SMO	R	V	V	E	L	A	E	G
Consensus	r	v	v	e	l	a	e	g
	321	331	341	351	361	371	381	391
human PAO-1	E	L	I	P	A	D	H	V
human SMO	E	L	I	P	A	D	H	V
mouse SMO	E	L	I	P	A	D	H	V
Consensus	e	l	i	p	a	d	h	v
	401	411	421	431	441	451	461	471
human PAO-1	P	E	L	W	R	K	I	C
human SMO	P	E	L	W	R	K	I	C
mouse SMO	P	E	L	W	R	K	I	C
Consensus	p	e	l	w	r	k	i	c
	481	491	501	511	521	531	541	551
human PAO-1	S	Y	S	Y	T	Q	V	G
human SMO	S	Y	S	Y	T	Q	V	G
mouse SMO	S	Y	S	Y	T	Q	V	G
Consensus	s	y	s	y	t	q	v	g

Figure 1 Amino acid sequence comparison between human placenta PAO-1, human hepatoma SMO and mouse mammary carcinoma SMO

The bottom row indicates the consensus amino acid sequence. Total identity, 95.1%; human placental PAO-1 [24] versus human SMO, 99.8%; human SMO versus murine SMO, 95.1%; human PAO-1 versus murine SMO, 95.3%. Residues that differ from human SMO are bold and underlined; the arrow indicates amino acid in human SMO mutated to be identical to that in the placental PAO-1 sequence (reported by Wang et al. [24]).

Table 1 Effects of transiently transfected SMO on polyamine metabolism in HEK-293 cells

Transformed HEK-293 cells were transiently transfected for 48 h with empty vector or murine, human, wild-type or mutated SMO (SMO_{320R}; called PAO-1 by Wang et al. [24]), and assayed for polyamine enzyme activities or intracellular polyamine pools. An unusual feature of the HEK-293 cells is that basal SAMDC activity was much higher (>16-fold) than basal ODC activity. Data represent means $\pm$ S.E.M. where $n = 3$ experiments. Note that AcSpm and DAS were undetectable in these cells. * $P < 0.01$ based on Student's t test comparison of vector- versus SMO-transfected cells.

Transfectant	Enzyme activity			Polyamine pools (nmol/mg of protein)			
	ODC (pmol/h per mg)	SAMDC (pmol/h per mg)	SSAT (pmol/min per mg)	Put	AcSpd	Spd	Spm
Vector	10.0 $\pm$ 4.8	169 $\pm$ 20	59 $\pm$ 17	4.3 $\pm$ 0.8	< 0.2	6.8 $\pm$ 0.2	16.6 $\pm$ 0.6
Human SMO	103 $\pm$ 18*	185 $\pm$ 20	62 $\pm$ 8	26.6 $\pm$ 0.7*	1.5 $\pm$ 0.1*	26.4 $\pm$ 0.6*	4.3 $\pm$ 0.1*
Human SMO _{320R}	121 $\pm$ 22*	208 $\pm$ 29	76 $\pm$ 17	27.5 $\pm$ 0.2*	1.7 $\pm$ 0.1*	25.4 $\pm$ 0.5*	4.3 $\pm$ 0.1*
Murine SMO	116 $\pm$ 23*	225 $\pm$ 29	90 $\pm$ 12	29.7 $\pm$ 0.6*	1.7 $\pm$ 0.1*	27.2 $\pm$ 0.7*	4.3 $\pm$ 0.1*
Murine SMO _{320R}	141 $\pm$ 21*	222 $\pm$ 18	80 $\pm$ 13	26.9 $\pm$ 1.0*	1.8 $\pm$ 0.8*	23.8 $\pm$ 1.4*	4.0 $\pm$ 0.3*

from HepG2 hepatoma cells and differed from the recently reported placental oxidase (PAO-1; NCBI accession nos. BAA91360 and AAK55763) [24] by a single amino acid (i.e. Cys-320 instead of Arg-320; Figure 1). The mouse SMO cDNA (2096 bp) was derived from murine mammary carcinoma and encoded a protein (NCBI accession no. AAH04831) of the same size as human protein but differing by 20 amino acids (Figure 1). The human SMO gene (NCBI code C20orf16) is located on chromosome 20 and the mouse gene is located on chromosome

2. The human gene contains eight exons and the cDNA (GenBank accession no. AK000753) is complementary to an mRNA containing exons 1–6 and 8. The mouse gene contains seven exons and the cDNA (GenBank accession no. BC004831) is complementary to an mRNA containing seven exons.

In order to gain insight into protein function, the newly identified human SMO cDNA was transiently transfected into HEK-293 cells. Spm pools decreased by 75 % while Spd and Put pools increased $\approx$ 4- and 6-fold, respectively (Table 1). In

Figure 2 HPLC of oxidase lysate assay showing conversion of Spm into Spd in the lysate assay

(A) Chromatographic separation of a cocktail of polyamine standards (Cad, cadaverine). Note that the peak height representing endogenous Spm in the sample lacking substrate (B) is only a fraction of that representing the sample blank containing 200 μ M Spm (C). Note also that > 90% of the Spm peak in the assay sample (C) is converted into Spd product during the 30 min incubation (i.e. D). The identities of peak 1 in (C) and peak 2 in (D) are uncertain. DENSpm, N^6,N^1 -diethylinspermine.

addition, there was also a significant increase in AcSpd pools that was inconsistent with the role of AcSpd as a preferred substrate in the PAO reaction. Pool changes were accompanied by a 10-fold increase in ODC activity while SAMDC and SSAT remained similar to the vector control (Table 1). Despite a

predicted 20 amino acid difference with the human protein, the transfected mouse enzyme exerted very similar effects on polyamine pools in HEK-293 cells (Table 1). To confirm that the hepatoma SMO behaved identically to the previously described placental enzyme (PAO-1) [24], the single differing amino acid at position 320 of SMO was mutated to identity with the latter protein. The effects of SMO_{320R} on polyamine pools of transfected cells were the same as SMO (Table 1). Similarly, mutating the murine enzyme produced the same effects on polyamine pools as the parent enzyme (Table 1).

To determine the substrate specificity of the oxidase, we utilized a previously described acellular system for assaying oxidase activity [33]. Cells were transfected with SMO or empty vector, lysed and aliquots of the lysate incubated in the presence of candidate polyamine substrates. HPLC analysis of lysate polyamine levels was compared before and after incubation with each substrate for 30 min. Oxidase activity was assessed by a comparison of polyamine pools in the blank (perchloric acid at zero time) and assay (substrate-incubated for 30 min) samples. As shown in Figure 2, the assay simultaneously detected major changes in substrate versus product. More particularly, activity was measured by a decline in the candidate substrate (i.e. Spm) and a concomitant rise in predicted product (i.e. Spd) during the incubation and by the absence of similar shifts in lysates of vector-transfected cells. As shown in Table 2, the most actively oxidized substrate, Spm, decreased by 90% (a difference of ≈ 2600 nmol/mg of protein) during the 30 min incubation while the apparent product, Spd, increased by an equivalent amount (≈ 2500 nmol/mg of protein). We were also able to chromatographically detect, but not reliably quantify, the by-product 3-aminopropanal during Spm incubations. The aldehyde did not appear in a stoichiometric relationship with Spd, presumably because it is reactive and therefore highly unstable. Characterization of the pH preference of SMO using Spm as the substrate over a pH range of 6.5–11.5 revealed optimal activity at pH 9.5 (results not shown), the same as reported previously for semi-purified PAO [19,20]. Transfected murine SMO was similarly effective to human SMO in terms of reducing Spm pools (Table 2). Inclusion of the PAO inhibitor MDL-72,527 [28] at 200 μ M in the Spm substrate assay partially reduced Spd product formation, by $\approx 55\%$.

Table 2 Substrate specificity of SMO in lysates of transiently transfected HEK-293 cells

HEK-293 cells were transiently transfected with human SMO, lysed and aliquoted. In the blank sample the lysate was inactivated with 0.6 M perchloric acid immediately following addition of a polyamine substrate. In the assay sample the lysate was inactivated by perchloric acid 30 min after addition of a polyamine substrate. The difference between endogenous polyamine pools in blank samples shown below and those shown in Table 1 is due to low-level oxidation of Spm during on-ice incubations and to minor polyamine contaminants in exogenously added substrates. Data represent means $\pm$ S.E.M. with $n = 3$ experiments. * $P < 0.01$ based on Student's t test comparison of blank and assay polyamines for a candidate substrate.

Substrate (200 μ M)	Lysate polyamine levels after SMO assay (nmol/mg of protein)					
	Put	AcSpd	Spd	AcSpm	DAS	Spm
AcSpd (blank)	46.9 $\pm$ 1.6	3135 $\pm$ 18	45.7 $\pm$ 1.4	< 0.2	< 0.2	< 0.2
AcSpd (assay)	49.2 $\pm$ 0.2	3149 $\pm$ 28	46.8 $\pm$ 0.6	< 0.2	< 0.2	< 0.2
Spd (blank)	30.6 $\pm$ 0.4	< 0.2	2697 $\pm$ 99	< 0.2	< 0.2	< 0.2
Spd (assay)	30.8 $\pm$ 0.4	< 0.2	2692 $\pm$ 22	< 0.2	< 0.2	< 0.2
AcSpm (blank)	24.9 $\pm$ 1.1	< 0.2	40.3 $\pm$ 1.4	2776 $\pm$ 75	< 0.2	< 0.2
AcSpm (assay)	23.6 $\pm$ 0.2	< 0.2	183.9 $\pm$ 0.6*	2583 $\pm$ 32	< 0.2	< 0.2
DAS (blank)	24.8 $\pm$ 0.8	< 0.2	27.7 $\pm$ 0.4	246.4 $\pm$ 0.6	3578 $\pm$ 112	< 0.2
DAS (assay)	24.9 $\pm$ 0.2	< 0.2	89.1 $\pm$ 2.0	167.2 $\pm$ 0.4	3665 $\pm$ 78	< 0.2
Spm (blank)	19.2 $\pm$ 1.8	< 0.2	35.3 $\pm$ 1.9	< 0.2	< 0.2	2904 $\pm$ 72
Spm (assay)	24.6 $\pm$ 1.1	< 0.2	2557 $\pm$ 14*	< 0.2	< 0.2	304 $\pm$ 16*
Spm + MDL-72,527 (assay)	21.4 $\pm$ 1.8	< 0.2	1104 $\pm$ 13*	< 0.2	< 0.2	1301 $\pm$ 63*

Figure 3 Radiochromatographic detection of Spm to Spd conversion by SMO

Representative radiochromatographic tracings of SMO lysates following incubation in 1 μ Ci (70 μ M) [14 C]Spm for 10 min. (A) Radiolabelled cocktail of Put, Spd and Spm standards; (B) SMO-transfected cell lysate blank; (C) is SMO-transfected cell lysate assay. Note that following the assay incubation (C), most of the radiolabelled Spm peak seen in the blank (B) was converted into radiolabelled Spd. Incubations with vector-transfected cells showed no conversion of radiolabelled Spm into Spd (results not shown). The small peak to the right of residual Spm peak in the SMO-transfected assay sample (C) was seen in vector-transfected lysate assays and its identity is uncertain. Also note that there is no evidence in (C) to indicate that Spd is oxidized to Put.

To confirm that the observed decrease in Spm and the concomitant increase in Spd during the lysate assay represented an oxidase product–substrate relationship, we incubated lysates with [14 C]Spm and examined the resulting radioactive products. This was achieved by radiochromatographic analysis of SMO assay extracts [34,35]. As shown in representative chromatograms (Figure 3), the decrease in radiolabelled Spm was offset by the appearance of radiolabelled Spd, thus confirming that the former is a substrate and the latter a product of the oxidase reaction. Despite the accumulation of high amounts of radiolabelled Spd, there was no indication of further oxidation to Put, indicating the specificity of the enzyme for Spm over Spd (Figure 3C).

In comparison with Spm, which decreased by 90% in the lysate assay, AcSpm decreased by only 10% (Table 2) and thus was regarded as a very poor substrate. None of the remaining polyamines, including DAS, Spd and AcSpd, served as substrates for the enzyme (Table 2). The greater preference of SMO for Spm over AcSpm is clearly depicted by the time-course kinetic

comparison of SMO effects on these two substrates shown in Figure 4. Spm levels decreased rapidly during the 60 min incubation, falling from 2300 to < 50 nmol/mg of protein while Spd increased by a similar rate and amount. By comparison, AcSpm fell from 3100 to 2700 nmol/mg of protein and Spd rose only slightly. The mutated human SMO_{320R} demonstrated identical substrate specificity to hepatoma enzyme (Tables 2 and 3). Namely, Spm was vigorously oxidized to Spd while DAS and AcSpm were only marginally acted upon by the enzyme.

Because the substrate activity of SMO was much different from expectations based on earlier studies with PAO [3–5], we considered the possibility that alternatively spliced variants may exhibit activity profiles more typical of the back-conversion PAO. To explore this possibility on a limited basis, we obtained two splice variant cDNAs of SMO [GenBank accession no. AK025938 encoding NCBI accession no. BAB15288 (389 amino acids) and GenBank accession no. BC000669 encoding NCBI accession no. AAH00669 (502 amino acids)]. When transiently transfected into HEK-293 cells, neither cDNA displayed oxidase activity in the lysate assay, using either Spm or AcSpm as candidate substrates (Table 4).

Since there have been reports indicating that PAO is capable of N-dealkylating certain polyamine analogues [37–39], we examined whether mono- and diethylspermine analogues might serve as substrates for the SMO identified here. The assay was identical to that described above except that 200 μ M analogue was substituted for Spm during the 10 min incubation. Based on production of Spd, the candidate substrate *N*¹-monoethylspermine (MESpm) was nearly as efficient as Spm in the substrate assay (Table 5). HPLC mobility confirmed that the product was exclusively Spd, as opposed to the alternative possibility, *N*¹-ethylspermidine, indicating a preferred cleavage site within the analogue. Two diethyl analogues were also tested. The first, *N*¹,*N*¹²-diethylspermine (DESp₁₂), is a diethyl derivative of Spm in which the intra-amine distances are comprised of 3, 4 and 3 carbon bridges and the second, *N*¹,*N*¹⁴-diethylhomospermine (DEHSp₁₄), is a diethyl derivative of homospermine in which the intra-amine distances are all 4 carbon bridges. Neither analogue was oxidized by the enzyme, as indicated by the absence of Spd product formation or by the apparent removal of terminal ethyl groups to yield monoethyl analogues of Spm, homospermine or norspermine. Despite a previous report [39] indicating that the calmodulin modulator *N*¹OSSpm could serve as a substrate of PAO, there was no evidence for either oxidative dealkylation of *N*¹OSSpm to Spm or internal oxidation to Spd. In fact, as noted below, the compound was a potent inhibitor of SMO.

The above compounds were also examined for their ability to serve as oxidase inhibitors (Table 4). They were compared at 200 μ M against 200 μ M Spm as substrate. Inhibitory activity was based on the ability of these compounds to prevent production of Spd from Spm relative to Spd production in the absence of inhibitors. As shown in Table 5, the specific PAO inhibitor MDL-72,527 inhibited Spd production by only 56% whereas *N*¹OSSpm blocked Spd production by 86%. DEHSp₁₄ was the most effective of the diethyl polyamine analogues with an inhibitory activity of 49% compared with 15% for DESp₁₂ and 21% for *N*¹,*N*¹¹-diethylnorspermine (DENSp₁₁). Because MESpm served as a substrate for the enzyme, it was not tested as an inhibitor. The potent enzyme inhibition by Spd is presumably due to described previously product inhibition [19].

Having identified the enzyme we next examined whether it was endogenously expressed in HEK-293 cells and whether it is inducible by polyamine analogues. As shown by Northern blot (Figure 5), SMO mRNA is basally expressed at levels that are less than one-third those for SSAT mRNA (note radiographic

Figure 4 Time-course comparison of SMO oxidation of Spm and AcSpm

Transiently transfected HEK-293 cell lysates were incubated with 200 μ M Spm (**A**) or AcSpm (**B**) as substrates for up to 60 min. Note that the substrate Spm decreases as rapidly as the product Spd increases in (**A**) and that the substrate AcSpm decreases and the product Spd increases very slowly in (**B**).

Table 3 Substrate specificity of human SMO_{320R} in lysate of transiently transfected HEK-293 cells

Assay conditions were identical to those described in Table 2 except that the lysate was derived from HEK-293 cells transiently transfected with human SMO_{320R}. Data represent means $\pm$ S.E.M. where $n = 3$ experiments. * $P < 0.01$ based on Student's t test comparison of blank and assay polyamine levels for a candidate substrate.

Substrate (200 μ M)	Lysate polyamine levels after SMO assay (nmol/mg of protein)					
	Put	AcSpd	Spd	AcSpm	DAS	Spm
AcSpd (blank)	48.5 $\pm$ 1.2	3231.8 $\pm$ 83.8	46.3 $\pm$ 1.5	< 0.2	< 0.2	< 0.2
AcSpd (assay)	48.6 $\pm$ 2.0	3208.9 $\pm$ 50.8	45.5 $\pm$ 1.3	< 0.2	< 0.2	< 0.2
Spd (blank)	26.3 $\pm$ 2.1	< 0.2	2822.1 $\pm$ 62.7	< 0.2	< 0.2	< 0.2
Spd (assay)	26.6 $\pm$ 1.7	< 0.2	2806.8 $\pm$ 73.3	< 0.2	< 0.2	< 0.2
AcSpm (blank)	27.7 $\pm$ 2.7	< 0.2	44.9 $\pm$ 0.6	2869.1 $\pm$ 62.5	< 0.2	< 0.2
AcSpm (assay)	28.0 $\pm$ 2.0	< 0.2	198.0 $\pm$ 12.5*	2580.8 $\pm$ 30.3	< 0.2	< 0.2
DAS (blank)	32.3 $\pm$ 2.5	< 0.2	31.7 $\pm$ 2.4	248.2 $\pm$ 5.4	3603.8 $\pm$ 48.8	< 0.2
DAS (assay)	29.5 $\pm$ 4.9	< 0.2	100.6 $\pm$ 6.5	157.6 $\pm$ 5.6	3634.4 $\pm$ 76.5	< 0.2
Spm (blank)	30.1 $\pm$ 0.9	< 0.2	32.8 $\pm$ 1.4*	< 0.2	< 0.2	2867.6 $\pm$ 27.5*
Spm (assay)	28.7 $\pm$ 1.6	< 0.2	2552.1 $\pm$ 32.2*	< 0.2	< 0.2	307.5 $\pm$ 11.8*

exposure times). There was no evidence of major mRNA isoforms in these cells, as might be expected to occur if alternative splicing were involved. Treatment with 10 μ M DENSpM for 48 h resulted in a 5-fold induction in SMO mRNA and a 20-fold induction of SSAT mRNA.

DISCUSSION

The polyamine catabolic enzyme PAO is one of several known eukaryotic oxidases capable of degrading higher polyamines [18]. In general, copper-containing amine oxidases such as diamine oxidase (also known as serum oxidase) metabolize polyamines by selectively removing primary N-termini [40], an event that can

lead to subsequent spontaneous cleavage at the secondary amine of Spm [41]. By contrast, flavin-dependent oxidases such as PAO and SMO cleave Spd or Spm at an amine internal to a terminal aminopropyl unit [18]. As repeatedly shown by others, PAO prefers acetylated polyamines over non-acetylated polyamines and thus collaborates with the acetylating enzyme SSAT in the sequential back-conversion of Spm to Spd and Spd to Put. Studies with specific inhibitors of PAO support the existence and functionality of this pathway in intact cells and tissues [22,23]. It is relevant to note, however, that PAO is also capable of directly oxidizing Spm and Spd, a reaction that is known to take place in red blood cells that lack both SSAT and acetylated polyamines [42]. In a major development, Wang et al. [24] recently cloned the

Table 4 Substrate activity of alternatively spliced SMO isoforms in HEK-293 cell lysate assay

Assay conditions were identical to those described in Table 2. Data represent mean values where $n = 2$. SMO data were taken from Table 2 for reference purposes; thus data showing the lack of SMO effects on AcSpm also appear in Table 2. GenBank accession nos. are shown in the first column.

Transfected cDNA	Substrate (200 μ M)	Lysate polyamine levels after SMO assay (nmol/mg of protein)				
		Put	AcSpd	Spd	AcSpm	Spm
SMO	Spm (blank)	19	< 0.2	35	< 0.2	2904
	Spm (assay)	25	< 0.2	2557	< 0.2	304
BC00069	Spm (blank)	37	< 0.2	91	< 0.2	2616
	Spm (assay)	36	< 0.2	90	< 0.2	2498
BC00069	AcSpm (blank)	35	< 0.2	89	2758	190
	AcSpm (assay)	33	< 0.2	101	2805	170
AK025938	Spm (blank)	45	< 0.2	89	< 0.2	2402
	Spm (assay)	46	< 0.2	97	< 0.2	2382
AK025938	AcSpm (blank)	43	< 0.2	88	2629	200
	AcSpm (assay)	44	< 0.2	123	2505	215

Table 5 Relative substrate and inhibitory activities of polyamines, PAO inhibitors and polyamine analogues

Substrate activity is based on nmol of Spd generated during a 10 min incubation relative to nmol of Spd generated in human SMO-transfected HEK-293 cell lysates containing 200 μ M Spm; data represent the percentage change of mean values derived from three experiments with S.E.M. < 2%. Inhibitory activity is based on nmol of Spd generated during a 10 min incubation with inhibitor plus 200 μ M Spm relative to nmol of Spd generated in Spm alone; data represent the percentage change of mean values subtracted from 100 as derived from three experiments with S.E.M. < 2%. * $P < 0.01$ based on Student's t test comparison of enzyme activity in the presence and absence of polyamine or analogue. ND, not determined; DENSpm, N^1,N^2 -diethylnorspermine.

Polyamine or analogue (200 μ M)	As substrate (% activity)	As inhibitor (% inhibition)
Spm	100%	0%
Spd	< 1%	40.8%*
MDL-72,527	< 1%	44.2%*
N^1 OSpm	< 1%	85.8%*
MESpm	98%*	ND
DENSpm	< 1%	21.1%*
DESpm	< 1%	15.2%*
DEHSpm	< 1%	48.8%*

first mammalian oxidase from human placenta. When expressed in an *in vitro* transcription and translation system, the enzyme generated hydrogen peroxide in the presence of Spm, indicating an oxidation reaction. Although the authors designated the enzyme PAO-1, substrate specificity towards Spd or the acetylated polyamines AcSpd and AcSpm was not evaluated. This report demonstrates the functionality of this oxidase in transfected mammalian cells by more specifically defining the unexpected substrate specificity. As discussed below, our findings indicate that the enzyme is actually a SMO not previously recognized in mammalian cells.

The present oxidase was also derived by BLAST search with maize PAO [25] and found to differ from PAO-1 [24] by a single amino acid. Polyamine pool perturbations following transient transfection provided the first insights into the unexpected substrate specificity of the enzyme. While the 75% decline in Spm pools and the 4-fold increase in Spd are consistent with the back-conversion of Spm by PAO, the significant rise in one of the preferred PAO substrates, AcSpd, is not. Likewise, the increase in Put is also in agreement with PAO back-conversion of Spd to

Figure 5 Northern blot analysis of SMO and SSAT mRNA expression and induction in HEK-293 cells

Cells were treated in the presence and absence of 10 μ M polyamine analogue DENSpm (DEN) for 48 h before being harvested for mRNA extraction. RNA was loaded at 20 μ g/well. Note that radiographic exposure for membranes probed for SMO was 72 h compared with 24 h for membranes probed for SSAT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For quantitation, SMO and SSAT mRNA bands were scanned densitometrically, normalized to GAPDH and expressed as fold increase for control (CON) versus DENSpm treatment. There was no evidence of major bands corresponding to possible SMO mRNA isoforms on the full-scale Northern blot (results not shown).

Put; however, the 10-fold compensatory rise in ODC activity could also account for Put accumulation. In fact, the inability of the oxidase to convert AcSpd or Spd into Put in the lysate assay indicates that ODC activity is almost certainly responsible for the Put accumulation by transient transfection. At the very least these findings demonstrate that the transfected enzyme is functional in intact cells and that it has access to intracellular Spm pools. More importantly they suggest that the enzyme is preferentially directed towards Spm over acetylated polyamines and Spd. This indication is more clearly defined by substrate specificity studies (discussed below).

As a less complex but cell-relevant alternative to the whole cell assays, we used transfected cell lysates to evaluate substrate specificity. Such an acellular system is unaffected by issues

relating to regulatory enzyme responses, cellular uptake of candidate substrates and possible subcellular sequestration of enzyme or substrate. The assay also allows for direct quantification of changes in both substrate and product as opposed to measuring the less specific by-product hydrogen peroxide. The robust nature of the assay is indicated by the 90 % conversion (3000 nmol/mg of protein) of Spm to Spd (Figure 2). Standard errors of typically < 5 % (Tables 2 and 3) attest to the reliability and reproducibility of the assay.

Findings obtained with the lysate assay revealed that Spm was clearly oxidized directly by the enzyme, as indicated by a rapid decline in Spm levels and a concomitant increase in the Spd product. Substrate preference was apparent by the fact that AcSpm was much less actively converted into Spd in the lysate system, indicating a greater preference of the oxidase for Spm over AcSpm. Thus in agreement with the whole cell polyamine pool findings, the new enzyme preferentially oxidized Spm over AcSpd. We note that the same unexpected substrate specificity was also observed with lysates of SMO-transfected MCF-7 breast carcinoma cells (results not shown), indicating that the results are not cell-type specific. Because the transfection efficiency of SMO of HEK-293 cells was much higher than in MCF-7 cells, the former were selected for further study and assay development.

A distinct advantage of assaying the enzyme by measuring substrate and product, as opposed to the by-product hydrogen peroxide [24], is that it provides a clearer indication of the actual reaction being measured. Thus as illustrated in Figure 2, substrate (i.e. Spm) oxidation and product (i.e. Spd) liberation can be simultaneously tracked. Conversion of Spm into Spd is strong evidence that oxidation involved internal cleavage of the Spm molecule, as is typical for flavin-dependent oxidases. As noted above, the selective removal of Spm N-termini by copper-containing amine oxidases such as diamine oxidase or monoamine oxidase [40] can lead to subsequent spontaneous cleavage at the secondary amine of Spm [41]. Such reactions were precluded in the present assays by the inclusion of aminoguanidine, an inhibitor of diamine oxidase and paraglyline, an inhibitor of monoamine oxidase [33], in the assay buffer. Additionally, there was no chromatographic evidence for an oxidized intermediate. Thus the enzyme does not represent a diamine oxidase. Finally, this conclusion was further confirmed by the radiochromatographic data indicating that Spd generated in the lysate assay derives directly from radiolabelled Spm.

Analogue studies provided insight into the mode of enzyme action. Since SMO acts on Spm but not Spd and only slightly on AcSpm, the substrate-binding requirement would seem to specify four positively charged amines. At least one of the terminal amines must be non-alkylated since the enzyme actively oxidized the monoethylated analogue MESpm but only weakly affected the diethylated analogue DESpm. In addition, the data suggest that SMO preferentially cleaves nearest to the alkylated terminal amine, as demonstrated by the fact that the oxidation of MESpm yields Spd but not *N*¹-ethylspermidine in the lysate assay.

Various differences and similarities between SMO and the back-conversion PAO are apparent. The two have similar molecular masses (≈ 62 kDa) and pH optima (≈ 9.5) [3–5] and both liberate hydrogen peroxide as part of the reaction. Due to differences in substrate specificity, PAO yields acetamidopropanal as a by-product and SMO yields amidopropanal. They differ distinctly in properties related to enzyme activity. The present enzyme prefers Spm over AcSpm and shows no activity towards Spd, AcSpd or DAS while PAO prefers acetylated polyamines, including DAS, over nonacetylated polyamines [3–5]. More particularly, Bolkenius and Seiler [3] reported K_m

values of $0.6 \mu\text{M}$ for AcSpm and $14 \mu\text{M}$ for Spm using semi-purified PAO preparation. The specific PAO inhibitor MDL-72,527 [28] only partially blocked Spm oxidation by SMO and a previously reported substrate of PAO, *N*¹OSSpm [39], was found to be a potent inhibitor of SMO. On the basis of the above findings, we have designated the novel oxidase SMO to distinguish it from the back-conversion PAO.

The SMO derived from hepatoma differed from the oxidase reported earlier as PAO-1 [24] by a single amino acid at position 320. This is probably due to a polymorphism of minimal functional significance since it did not occur in the putative FAD- or polyamine-binding site domains [25,35]. However, to establish that the currently observed enzyme behaviour reported here was similar for both oxidases, the differing amino acid in the hepatoma protein was mutated to be identical to the placenta protein (i.e. Cys-320 to Arg-320), transfected into cells and found to have a similar effect on polyamine pools (Table 1) and identical substrate specificity (Table 3). A murine oxidase identified in this study differed from the human hepatoma protein by 20 amino acids and was also found to behave like SMO when transfected into HEK-293 cells. Alternative pre-mRNA splicing is an important mechanism for modulation of gene expression in eukaryotes and a large proportion ($\approx 40\%$) of human genes are affected in this manner [43]. We also examined the possibility that the alternative spliced isoforms of the enzyme may exhibit more PAO-like substrate specificities; however, neither of the two candidates examined here showed oxidase activity in the lysate assay system. This does not exclude the possibility that other active isoforms might exist [44,45], although Northern blot analysis of HEK-293 cells failed to reveal any obvious alternatively spliced mRNA species.

The current findings strongly support the idea that in addition to the traditional back-conversion pathway in which Spm is first acetylated by SSAT and then oxidized by PAO, mammalian cells contain an enzyme capable of directly oxidizing Spm to Spd. Northern blot analysis revealed basal levels of SMO mRNA in HEK-293 cells, an indication that enzyme expression is not confined to highly metabolic organs such as the liver. Although a flavin-containing PAO that oxidizes Spm and Spd but not acetylated polyamines has been identified in *Ascaris suum* [26] and a Spm amine oxidase encoded by the FMS1 gene has been reported in yeast [27], this is the first example of such an enzyme being present in mammalian cells. The importance of this capability is further enhanced by the unexpected observation that SMO expression is inducible by polyamine analogues at the level of mRNA, as shown here and elsewhere [24,45]. Although the area of amine oxidases is fraught with problems, these difficulties are now surmountable via functional genomics based on specific enzyme assays such as the one reported here and by the related development of specific molecular reagents. Regarding the back-conversion PAO, it is possible that sequences belonging to this oxidase are among those bearing relatively low sequence homology to either maize PAO [25] or SMO.

Taken together, these findings have implications for polyamine homeostasis. Although the role for SSAT in this process is most often associated with regulating flux through the back-conversion pathway (Scheme 1A) the enzyme may contribute in another way. It has long been thought that because acetylated polyamines are more commonly found outside of cells [1], acetylation by SSAT may signal export of polyamines. Given the ability of SMO to back-convert Spm to Spd, SSAT may be more critically involved in facilitating Spd and Spm excretion out of the cell. These hypothetical roles for SSAT, PAO and SMO are depicted in Scheme 1(B) and are highly dependent on the relative enzyme activity levels within the cell. A final possibility not presented in

Scheme 1 is that SMO may contribute to β -alanine production via aldehyde dehydrogenase conversion of 3-aminopropanal, a role proposed recently for yeast spermine amine oxidase [27]. At the very least, this novel enzyme represents an important additional component in our understanding of polyamine homeostasis. Recognizing its existence, its enzymic capabilities and its inducibility will better enable us to interpret how clinically relevant inhibitors, analogues and other agents impact on this complicated process as well as cell growth inhibition. The enzyme also provides a gene reagent for genetically manipulating polyamine pools in cells and tissues, as has been done with SSAT [6,46,47]. Such systems provide a novel means for addressing issues of physiological and pathophysiological relevance.

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