ACCELERATED PUBLICATION

Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion

Slavoljub VUJCIC*, Ping LIANG†, Paula DIEGELMAN*, Debora L. KRAMER* and Carl W. PORTER*[1]

*Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, U.S.A., and †Department of Genetics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, U.S.A.

In the polyamine back-conversion pathway, spermine and spermidine are first acetylated by spermidine/spermine N^1-acetyltransferase (SSAT) and then oxidized by polyamine oxidase (PAO) to produce spermidine and putrescine respectively. Although PAO was first purified more than two decades ago, the protein has not yet been linked to genomic sequences. In the present study, we apply a BLAST search strategy to identify novel oxidase sequences located on human chromosome 10 and mouse chromosome 7. Homologous mammalian cDNAs derived from human brain and mouse mammary tumour were deduced to encode proteins of approx. 55 kDa having 82 % sequence identity. When either cDNA was transiently transfected into HEK-293 cells, intracellular spermine pools decreased by approx. 30 %, whereas spermidine increased 2–4-fold. Lysates of human PAO cDNA-transfected HEK-293 cells, but not vector-transfected cells, rapidly oxidized N^1-acetylspermine to spermidine. Substrate specificity determinations with the lysate assay revealed a preference ranking of N^1-acetylspermine = N^1-acetylspermidine > N^1,N^{12}-diacetylspermine ≫ spermine; spermidine was not acted upon. This ranking is identical to that reported for purified PAO and distinctly different from the recently identified spermine oxidase (SMO), which prefers spermine over N^1-acetylspermine. Monoethyl- and diethylspermine analogues also served as substrates for PAO, and were internally cleaved adjacent to a secondary amine. We deduce that the present oxidase sequences are those of the FAD-dependent PAO involved in the polyamine back-conversion pathway. In Northern blot analysis, PAO mRNA was much less abundant in HEK-293 cells than SMO or SSAT mRNA, and all three were differentially induced in a similar manner by selected polyamine analogues. The identification of PAO sequences, together with the recently identified SMO sequences, provides new opportunities for understanding the dynamics of polyamine homoeostasis and for interpreting metabolic and cellular responses to clinically-relevant polyamine analogues and inhibitors.

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

INTRODUCTION

In the polyamine biosynthetic pathway, polyamines are assembled by the sequential transferral of aminopropyl units derived from decarboxylated S-adenosylmethionine on to a core diamine unit (putrescine, Put) derived from ornithine via the ornithine decarboxylase (ODC) reaction. 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 stoichometric amounts of spermidine (Spd), 3-acetamidopropanal and hydrogen peroxide [1,2]. The conversion of Spd to Put takes place by analogous reactions involving the same two enzymes. Unlike the four major biosynthetic enzymes and SSAT, PAO has not yet been cloned due to difficulties in purifying the protein on the basis of enzyme activity and the lack of a specific antibody. The availability of human genomic databases, however, provides new opportunities to relate unique genomic sequences to specific enzyme activities.

We recently used a functional genomics approach to identify sequences belonging to a previously unrecognized spermine-directed oxidase [3]. A BLAST search of genomic data using maize PAO sequences [4] revealed what at first appeared to be the homologous mammalian enzyme [5]. Subsequent characterization of the substrate specificity revealed that the enzyme was actually a novel mammalian FAD-dependent spermine oxidase (SMO). Our success in this regard relied on the use of a defining assay system [6] whereby HEK-293 cells were transfected with the candidate cDNA, ruptured, and the lysate incubated for short periods in the presence of candidate polyamine substrates. HPLC analysis of lysate polyamine levels before and after incubation with each substrate revealed the effects of the transfected sequences on both candidate polyamine substrates and predicted polyamine products. Thus oxidase activity was simultaneously validated by direct measurement of substrate loss and product gain, endpoints that are much more defining than measuring liberation of hydrogen peroxide [5]. In applying this assay to the newly identified SMO sequences, we observed that the enzyme greatly preferred Spm over N^1-acetylspermine (AcSpm). Since PAO has been repeatedly reported to prefer acetylated polyamines over Spm [7–9], we deduced that the new oxidase was actually a previously unidentified mammalian SMO involved in the direct conversion of Spm to Spd [3].

Abbreviations used: AcSpd, N^1-acetylspermidine; AcSpm, N^1-acetylspermine; DASpm, N^1,N^{12}-diacetylspermine; DEHSpm, N^1,N^{14}-diethylhomospermine; DENSpm, N^1,N^{11}-diethylnorspermine; DESpm, N^1,N^{12}-diethylspermine; EST, expressed sequence tag; MESpd, N^1-monoethylspermidine; MESpm, N^1-monoethylspermine; N^1OSSpm, N^1-(n-octanesulphonyl)spermine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; Put, putrescine; SAMDC, S-adenosylmethionine decarboxylase; SMO, spermine oxidase; Spd, spermidine; Spm, spermine; SSAT, spermidine/spermine N^1-acetyltransferase.

[1] To whom correspondence should be addressed (e-mail carl.porter@roswellpark.org).

In the present report, we return our attention to the characterization of the PAO involved in acetylated polyamine oxidation. PAO is a FAD-containing amine oxidase found in all vertebrate tissues [10] and it has been shown to be subcellularly located in both the cytoplasm and peroxisomes [11–13]. It was first purified from rat and porcine liver by chromatographic methods in 1977 [11]. As noted above, it is thought to favour acetylated polyamines over non-acetylated species and has therefore been paired with the acetylating enzyme SSAT in the back-conversion pathway [9]. The significance of the enzyme response to changes in cell growth, or its role as a determinant of drug action for polyamine-directed compounds, has not yet been established. It is thought to be constitutively expressed in most tissues because of its high activity and extremely slow turnover rate [14]. Thus the more labile and inducible SSAT enzyme, rather than PAO, is considered rate-limiting to polyamine back-conversion. While changes in PAO activity are not typically associated with induction of cell growth, activity has been reported to be lower in human colon carcinoma [15], breast carcinoma [16] and other tumours [17], relative to normal tissue. Consistent with the latter, Takenoshita et al. [18] have reported that levels of the preferred PAO substrate, N^1-acetylspermidine (AcSpd), are much higher in colon adenocarcinoma than in benign polyps or normal surrounding muscosa, suggesting that PAO activity may decrease during tumorigenesis. Other studies have also suggested that hydrogen peroxide liberated during oxidation of intracellular polyamines may mediate aspects of embryonic development [19] and apoptotic responses to polyamine analogues [20,21]. PAO may also play a protective role in *myc*-driven apoptosis of transformed human haematopoietic cells [22].

Herein, we apply a functional genomics approach to identify murine and human sequences belonging to the PAO involved in the polyamine back-conversion pathway. The specificity of the enzyme for AcSpm and AcSpd over Spm or Spd is identical to the activity previously described with semi-purified enzyme preparations, and is clearly different from that of SMO. In addition, we demonstrate that, like SMO, PAO expression is unexpectedly inducible by polyamine analogues and thus may contribute to the inhibition of cell growth. With this discovery, all of the major polyamine metabolic enzymes have been cloned, with the exception of spermidine N^8-acetyltransferase [23]. It should now be possible to gain a more complete understanding of polyamine metabolism and its role in normal and neoplastic cell growth, as well as a better appreciation of the specific metabolic roles of SMO and PAO.

EXPERIMENTAL

Materials

The inhibitor of PAO, MDL-72,527 [24,25], was kindly provided by Aventis (Bridgewater, NJ, U.S.A.). N^1,N^{12}-diacetylspermine (DASpm) and the calmodulin antagonist [26] N^1-(n-octanesulphonyl)spermine (N^1OSSpm) were a gift from Dr Nikolaus Seiler (Laboratory of Nutritional Oncology, Strasbourg, France) and various ethylated spermine 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 [27] of genome and cDNA databases was carried out using human SMO sequences [2,3] followed by a Pfam homology search [28] for FAD-binding domains [29]. Clones containing candidate human cDNA (IMAGE, 4932036; GenBank® accession no. BC032778 from human brain tissue) and mouse oxidase cDNA sequences (IMAGE, 5059720; GenBank® accession no. B1526976 from mouse mammary tissue) were obtained from A.T.T.C. (Rockville, MD, U.S.A.).

Plasmids

cDNAs were cloned into a *Sal*1/*Not*1 site in a pCMV-Sport 6 vector (Invitrogen, Carlsbad, CA, U.S.A.). 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.).

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% CO_2. 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 PAO or SMO cDNA in the presence of Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) following the manufacturer's protocol. Following transfection (48 h), cells were trypsinized, washed with PBS and stored at −70 °C for polyamine pool analysis, or used in the lysate activity assay described below. Control cells were transfected with an empty vector and used to detect endogenous oxidase activities.

PAO activity assay

The lysate assay for defining enzyme activity and substrate specificity was used as described recently [3]. Briefly, transiently cDNA- or vector-transfected cells were sonicated in 10 mM Tris buffer (pH 8.0). Aliquots were stored at −70 °C and used as the enzyme source in the oxidase activity assay [6]. 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 DASpm). The reaction buffer also contained 500 μM aminoguanidine as an inhibitor of endogenous diamine oxidase activity and 50 μM paragyline as an inhibitor of endogenous monoamine oxidase activity [6]. 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 'blank' lysate sample. In contrast, the assay lysate sample was incubated for 0–60 min in the presence of substrate before being inactivated with perchloric acid. Polyamine determinations by HPLC were made on all samples and expressed in nmoles/mg of protein. PAO activity was evaluated by comparing polyamine levels in the blank sample versus the assay sample. If a polyamine serves as a PAO substrate (i.e. AcSpm), 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 versus introduced enzyme activity.

HPLC

Polyamine levels were extracted from transfected cells or from blank or assay lysate 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 reverse-phase HPLC as described previously [30]. The data was collected and analysed using the Millennium 32 chromatography software (version 3.05, Waters Corp, Milford, MA, U.S.A.). Peaks were identified and quantitated by alignment with a chromatogram of known standards including Put, Spd, Spm, AcSpd, AcSpm, DASpm and $N^{1,7}$-diaminoheptane as an internal standard.

Polyamine enzyme assays

ODC, S-adenosylmethionine decarboxylase (SAMDC) and SSAT activities in transiently transfected 293 cells were assayed as described previously [31].

Northern blot analysis

Total RNA was isolated using RNeasy minikit (Qiagen, Valencia, CA, U.S.A.) and Northern blot analysis of PAO, SMO and SSAT mRNA from untreated and analogue-treated HEK-293 cells was conducted as reported previously [30].

DigiNorthern analysis of PAO versus SMO expressed sequence tag (EST) expression

A virtual analysis of the relative gene expression levels in different tissues for SMO and PAO genes was performed using our in-house bioinformatics tool, digiNorthern [32]. The method analyses indexed EST data and applies a dynamic approach to collect the most recently updated EST data. It also allows for a side-by-side comparison of two genes, such as PAO and SMO. Since PAO is a new gene, the virtual Northern data is not available from the National Cancer Institute's Cancer Genome Anatomy Project site (www.ncbi.nlm.nih.gov/ncicgap/) and could only be obtained through digiNorthern.

RESULTS

A BLAST search of public genome and cDNA databases using human SMO sequences [3] and the FAD-binding domain [29] yielded new putative oxidase sequences which, on the basis of substrate specificity data described below, we have designated PAO. Thus the human PAO gene (NCBI accession no. XM_113592) is located on chromosome 10 and the mouse gene (NCBI accession no. XM_133921) on chromosome 7. The human PAO cDNA (1850 bp) encodes a protein (NCBI accession no. XP_113592) comprising 511 amino acids and a deduced molecular mass of 55.5 kDa, whereas the mouse cDNA (1749 bp) encodes a protein (NCBI accession no. XP_133921) comprising 504 amino acids and a deduced molecular mass of approx. 55 kDa. A sequence comparison indicated 39 % identity between human and SMO and PAO proteins. As shown in Figure 1, conservation between PAO and SMO spans almost the entire two proteins. A major noticeable difference is that PAO lacks a 31-amino-acid fragment in the middle of the protein. The human and mouse PAO share 81 % sequence identity compared with the 95 % identity between human and mouse SMO proteins, suggesting that PAO gene is less conserved than SMO. Mouse PAO has a slightly shorter N-terminus than human PAO. Although human and mouse PAO have the same general gene structure, PAO differs distinctly from SMO. There are, however, three identical exon/intron borders between the two proteins, suggesting that they share common distant ancestral origins. In addition, orthologous genes for PAO and SMO have been identified in fugu fish by searching the draft genome (results not shown). Thus it seems that the divergence of these two paralogous genes occurred at, or before, the evolution of all vertebrates.

The newly identified human and mouse PAO cDNA-containing vectors were transiently transfected with high efficiency into HEK-293 human kidney cells. In order to gain insight into the nature of the oxidase activity, we first examined enzyme effects on intracellular polyamine pools (Table 1). Spm pools decreased by approx. 30 %, whereas Spd pools increased by approx. 2-fold in cells transfected with human PAO and by approx. 4-fold in cells transfected with mouse PAO. Put pools increased by varying amounts, depending on whether human or mouse cDNA was involved. The effects of the transfected PAO on acetylated polyamines could not be easily assessed in HEK-293 cells, since neither AcSpm or AcSpd are detectable under basal conditions. As shown in Table 1, the effects of transfected SMO on intracellular Spm and Spd pools were more dramatic than with transfected PAO, a finding that is consistent with previous findings, and with the greater substrate preference of SMO for Spm [3]. In contrast with SMO [3], PAO failed to increase ODC activity, SSAT activity or AcSpd pools (Table 1), most probably because it had much less of an effect on the polyamine pools that regulate these enzymes.

We next examined the relative preference of PAO and SMO for Spm versus AcSpm in the cell lysate assay. For these studies, HEK-293 cells were transfected with human PAO, human SMO or empty vector, lysed, and aliquots of the lysate were incubated in the presence of candidate polyamine substrates. HPLC chromatograms of lysate polyamine levels were compared for each substrate before and following the 30 min incubation. Oxidase activity was assessed on the basis of substrate decline (i.e. AcSpm or Spm) relative to product gain (i.e. Spd). As shown in Table 2, the substrate AcSpm decreased by 83 % (a difference of approx. 13100 nmol/mg of protein) during the 30 min incubation with PAO-transfected lysates, whereas the Spd product increased by an equivalent amount (approx. 12400 nmol/mg of protein). By comparison, the alternative substrate, Spm, was reduced by only 13 % in the same lysate assay, giving rise to a similar amount of Spd. The preference of the new oxidase for AcSpm over Spm is consistent with the expected substrate specificity of PAO [11–13], and opposite to that of SMO [3]. As shown in Table 2, the substrate Spm was reduced by 92 % during incubations with SMO-transfected cell lysates, whereas the substrate AcSpm was reduced by only 9 %. The two enzymes were almost totally inhibited by the mechanism-based PAO inhibitor, MDL-72,527 [24,25], when it was added 10 min prior to the addition of the preferred substrate (i.e. AcSpm for PAO and Spm for SMO). However, when MDL-72,527 was added at the same time as the substrate, PAO activity was inhibited by 67 % (i.e. 83 % minus 16 %) while SMO activity was reduced by 53 % (i.e. 92 % minus 39 %), indicating slightly greater sensitivity of the former enzyme. The possible contribution of endogenous enzymes was examined using empty-vector-transfected cell lysates in the presence of AcSpm or Spm and found to be of no consequence during the 30 min incubation.

The substrate specificity of PAO was more obviously defined by a kinetic comparison of substrate loss versus product gain during a 60 min incubation. As shown in Figure 2, AcSpm and AcSpd were oxidized at rates similar to those of Spd and Put respectively. In both cases, the reaction had proceeded to completion by 30 min. There was no suggestion that the newly generated product, Spd, was acted upon by the enzyme, as indicated by the absence of Put generation during the AcSpm

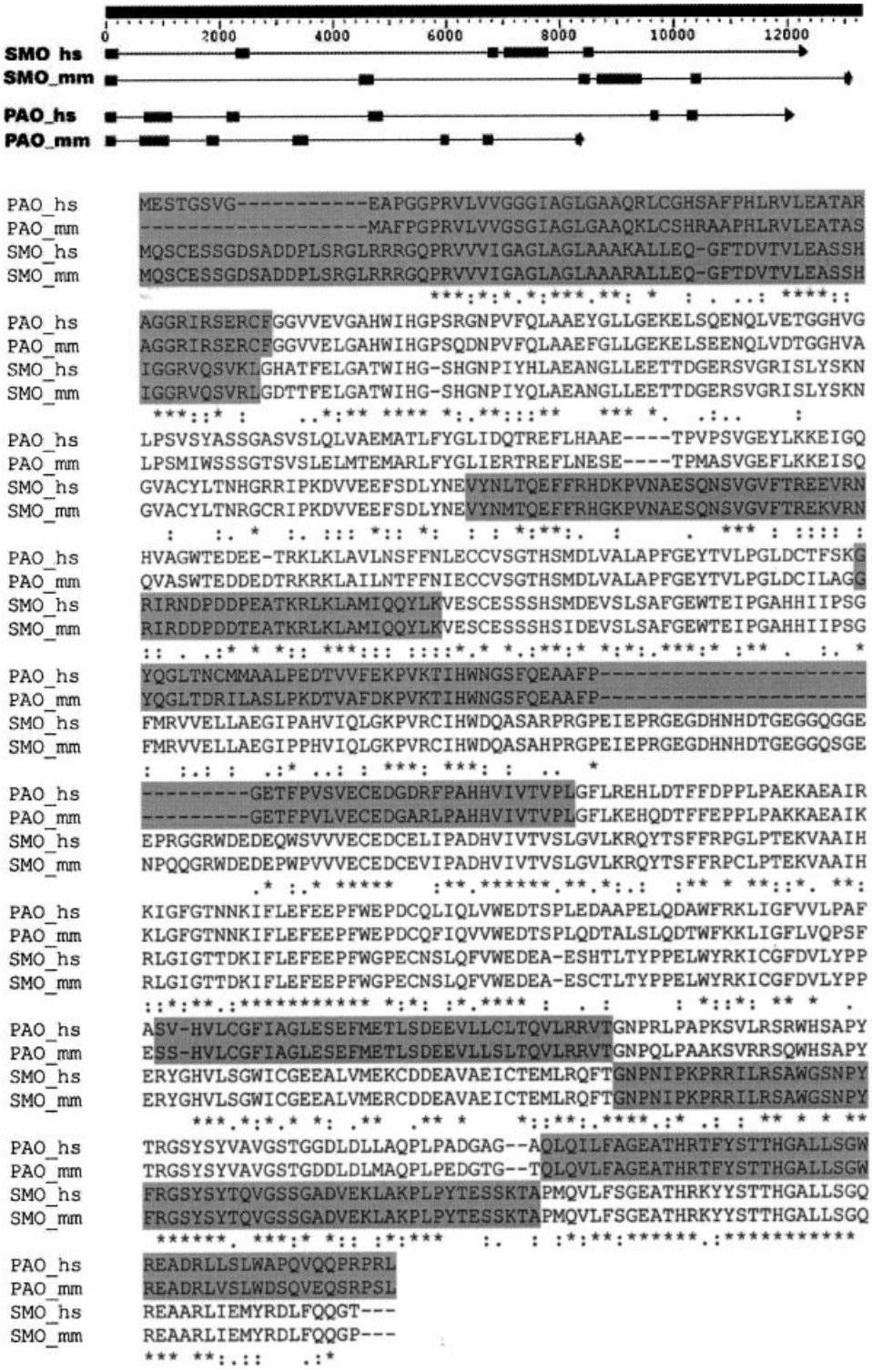

Figure 1 Comparison of gene structure and peptide sequence for human (hs) and mouse (mm) PAO and SMO

The top panel shows the similarity in gene structure for human and mouse PAO and for human and mouse SMO (exons are indicated with thick bars and introns with thin lines). The scale indicates the nucleotide base position. The lower panel compares multiple peptide sequence alignment performed using CLUSTAL X. Identical residues (*) and similar residues (:) are indicated. The boundaries of exons in peptide sequences are indicated by the alternating grey and white backgrounds. Note that there are three shared exon/intron boundaries.

incubation. The SSAT metabolite, DASpm [30], was also a preferred substrate of the PAO, as indicated by the fact that it was rapidly oxidized first to AcSpd and then to Put. Of the compounds tested, Spm was the least favoured substrate of PAO, decreasing by only 30 % during the 60 min incubation. Thus the kinetic studies show that the substrate priority ranking for this

Table 1 Comparison of PAO and SMO effects on polyamine metabolism in transfected HEK-293 cells

Transformed human kidney HEK-293 cells were transiently transfected for 48 h with empty vector, human SMO, human PAO or mouse PAO and assayed for polyamine enzyme activities or intracellular polyamine pools. Data represents means ± S.E.M., where n is 3 experiments. We have shown previously [3] that empty vector transfection has no effect on polyamines in HEK-293 cells. *Statistical significance of $P < 0.01$ based on Student's t test comparison of vector versus PAO transfected cells; **AcSpm and DASpm were undetectable in these cells.

	Enzyme activity			Intracellular polyamine pools** (nmol/mg protein)			
Transfection (48 h)	ODC (pmol/min/mg)	SAMDC (pmol/hr/mg)	SSAT (pmol/hr/mg)	Put	AcSpd	Spd	Spm
Vector	239 ± 33	728 ± 114	15.5 ± 0.7	2.9 ± 0.1	< 0.2	12.9 ± 0.3	46.3 ± 1.0
Human PAO	220 ± 15	616 ± 92	12.8 ± 2.9	6.9 ± 0.1*	< 0.2	25.8 ± 0.3*	34.8 ± 0.5*
Mouse PAO	281 ± 30*	683 ± 17	14.4 ± 0.6	17.9 ± 0.7*	< 0.2	46.6 ± 1.5*	31.3 ± 0.1*
Human SMO	661 ± 20*	751 ± 37	32.5 ± 2.2*	28.3 ± 1.2*	3.4 ± 0.2*	59.2 ± 2.4*	14.8 ± 0.6*

Table 2 Comparison of PAO and SMO substrate specificity in HEK-293 cell lysate assay

HEK-293 cells were transiently transfected with human PAO or SMO, or empty vector, lysed and aliquoted. In the blank sample, the lysate was inactivated with 0.6 M perchloric acid immediately after the addition of a polyamine substrate. In the assay sample, the polyamine substrate was added and incubated 30 min before the addition of perchloric acid. MDL-72,527 (MDL) is a mechanism-based inhibitor of PAO [24,25]. Data represents means ± S.E.M., where $n = 3$ experiments. The percentage oxidized substrate was obtained by dividing blank substrate levels by assay substrate levels, multiplying by 100 and then subtracting from 100. Bold letters indicate newly formed product. *Statistical significance of $P < 0.01$ based on Student's t test comparison of blank versus assay polyamines for a candidate substrate; **MDL-72,527 was added 10 min prior to addition of substrate as opposed to being added simultaneously (i.e. AcSpm + MDL).

		Lysate polyamine levels (nmol/mg of protein)				
Transfected enzyme	Lysate incubation	Put	Spd	AcSpm	Spm	% Substrate oxidized
Empty vector	200 μM AcSpm, blank	10	< 0.2	14081 ± 190	28 ± 2	–
	200 μM AcSpm, assay	18	< 0.2	14988 ± 199	17 ± 1	0%
	200 μM Spm, blank	5	< 0.2	< 0.2	10338 ± 254	–
	200 μM Spm, assay	21	< 0.2	< 0.2	10780 ± 201	0%
Human PAO	200 μM AcSpm, blank	< 0.2	< 0.2	15695 ± 220*	28 ± 2	–
	200 μM AcSpm, assay	< 0.2	**12387 ± 243***	2599 ± 159*	17 ± 1	83%
	MDL/AcSpm, assay**	< 0.2	**< 0.2**	14554 ± 540*	29 ± 6	0%
	AcSpm + MDL, assay	< 0.2	**2468 ± 303***	12128 ± 137*	37 ± 2	16%
	200 μM Spm, blank	36 ± 3	44 ± 9*	< 0.2	11091 ± 254*	–
	200 μM Spm, assay	48 ± 22	1488 ± 48*	< 0.2	984 ± 252*	13%
Human SMO	200 μM AcSpm, blank	26 ± 1	103 ± 43	2982 ± 23	< 0.2	–
	200 μM AcSpm, assay	26 ± 1	266 ± 53	2655 ± 19	< 0.2	9%
	200 μM Spm, blank	45 ± 11	**35 ± 2***	< 0.2	2505 ± 159*	–
	200 μM Spm, assay	25 ± 1	**2312 ± 38***	< 0.2	173 ± 6*	92%
	MDL/Spm, assay**	36 ± 4	**35 ± 2***	< 0.2	2266 ± 118*	0%
	Spm + MDL, assay	33 ± 3	**977 ± 46***	< 0.2	1391 ± 60*	39%

oxidase is AcSpm = AcSpd > DASpm ≫ Spm; Spd was not acted upon at all. This high concordance with previously published findings based on semi-purified enzyme preparations [11–13] was taken as evidence that the oxidase is actually PAO. Mouse PAO was found to have the same substrate specificity as human PAO (results not shown).

Since there have been reports that PAO is capable of N-dealkylating certain polyamine analogues [33–35], we compared the substrate potential of four ethylated spermine analogues (Table 3). The assay was identical to that described above, except that 200 μM analogue was substituted for Spm during the 30 min incubation. Substrate activity was assessed by analogue decline and by analogue product accumulation. N^1-Monoethylspermine (MESpm) was actively oxidized by PAO to yield Spd. Thus instead of the expected N-dealkylation reaction [32–34], N-ethylated 5-aminopropanal was removed from the analogue. Three diethyl analogues were also tested. The first, N^1,N^{11}-diethylnorspermine (DENSpm), is a diethyl derivative of norSpm in which the intra-amine distances are all composed of three carbon bridges. The second, N^1,N^{12}-diethylspermine (DESpm), is a diethyl derivative of Spm in which the intra-amine distances are composed of three, four and three carbon bridges. The third, N^1,N^{14}-diethylhomospermine (DEHSpm) is a diethyl derivative of homospermine in which the intra-amine distances are all four carbon bridges. All three of these compounds were previously found to be competitive inhibitors [3] and poor substrates (S. Vujcic, P. Diegelman and C. W. Porter, unpublished work) of the SMO reaction. When incubated in the PAO lysate assay, these analogues appeared to undergo internal intra-amine cleavage by the enzyme. In the case of DESpm, a N^1-monoethylspermidine (MESpd) standard was used to chromatographically identify the product of the reaction. In the case of DENSpm and DEHSpm, the reaction products were determined by retention times relative to the MESpd standard. Thus DESpm analogues were oxidized to MESpd analogues (i.e. DENSpm was oxidized to N^1-monoethylnorspermine, DESpm to MESpd and DEHSpm to DEHSpd). There was no chromatographic evidence to suggest N-dealkylation of the terminal ethyl group (i.e. DESpm analogues were not converted to a MESpm analogues). None of the newly formed Spd analogues appeared to be oxidized further, as indicated by the absence of new products, such as Put or Put analogues. The diethyl analogues

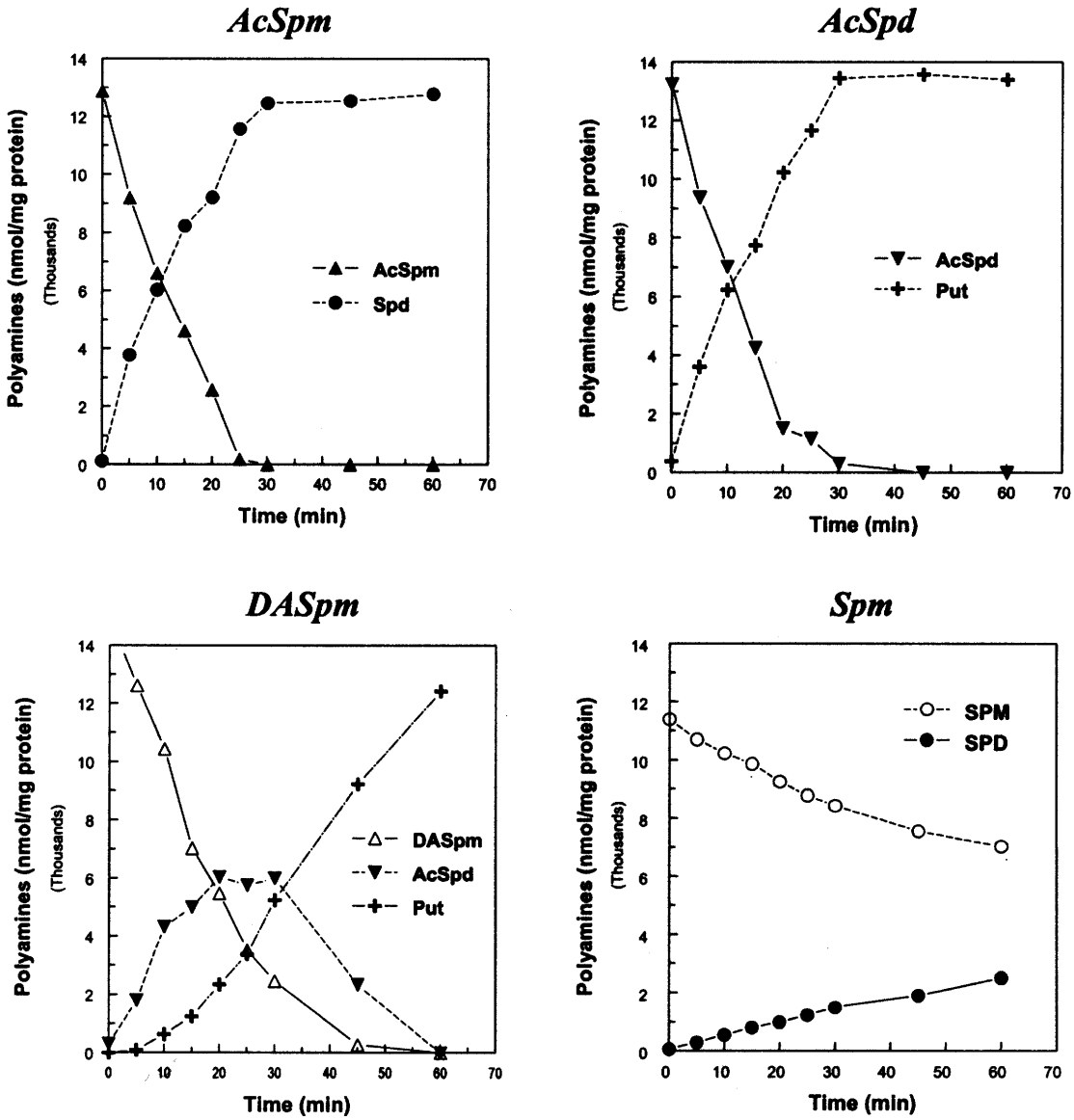

Figure 2 Time-course comparison of PAO oxidation of various substrates

Transiently transfected HEK-293 cell lysates were incubated with 200 μM AcSpm, AcSpd, DASpm or Spm as potential substrates for up to 60 min. Note that the substrates AcSpm and AcSpd decline as rapidly as the products Spd and Put accumulate and that, by comparison, the substrate Spm decreases and the product Spd increases very slowly. When DASpm is used as a substrate, AcSpd is first liberated as a product and then oxidized further to Put.

were differentially acted upon by the enzyme in a manner that correlated with the number of aminopropyl moieties contained within the molecule. Thus the rank order of substrate preference by PAO was MESpm > DENSpm > DESpm > DEHSpm. Consistent with previous reports [26,34], the calmodulin antagonist N^1OSSpm was also found to be internally oxidized to form Spd. This also contrasts our finding with SMO, in which N^1OSSpm was previously found to be a potent inhibitor [3].

We next examined whether PAO was endogenously expressed in HEK-293 cells and whether, like SMO and SSAT, PAO expression is inducible by polyamine analogues. As shown by Northern blot analysis (Figure 3), PAO mRNA is basally expressed at $< 5\%$ of SSAT mRNA and $< 25\%$ of SMO mRNA. Treatment with 10 μM DENSpm for 48 h resulted in a 2.0-fold induction in PAO mRNA and an 4.6-fold induction in SSAT mRNA, compared with a 3.4-fold induction of SMO mRNA. The differential mRNA response to the three analogues clearly demonstrates that all three enzymes are preferentially induced in correlation with the aminopropyl moieties contained within each analogue, i.e. DENSpm (which contains 3 aminopropyl moieties) was the most effective, DESpm (which contains two) was intermediately effective and DEHSpm (which contains none) was the least effective.

Finally, we compared the expression of PAO and SMO in various tissues and tumour counterparts by virtual Northern analysis using publicly available EST databases. Among the tissues represented, there were a total of 112 SMO ESTs and 60 PAO ESTs. As shown in the virtual Northern blot analysis depicted in Figure 4, the two genes showed distinctly different expression profiles from one another, and interesting tumour

Table 3 PAO substrate potential of polyamine analogues in HEK-293 cell lysate assay

HEK-293 cells were transiently transfected with human PAO for 48 h, lysed and aliquoted. In the blank sample, the lysate was inactivated with 0.6 M perchloric acid immediately after the addition of a polyamine analogue substrate. In the assay sample, the polyamine substrate or analogue was added and then incubated 30 min before addition of perchloric acid. Data represents means ± S.E.M., where $n = 3$ experiments. The percentage oxidized substrate was obtained by dividing blank substrate levels by assay substrate levels, multiplying by 100 and then subtracting from 100. Bold letters indicate newly formed product. *Statistical significance of $P < 0.01$ based on Student's t test comparison of blank versus assay polyamines for a candidate substrate or apparent product.

	Lysate polyamine levels (nmol/mg protein)				
	Possible products			Substrate	
Lysate incubation	Spd	Spd Analogue	Spm	Spm Analogue	% Oxidized
200 μM MESpm, blank	44 ± 17	< 0.2	130 ± 51	14460 ± 875*	–
200 μM MESpm, assay	**8083 ± 539***	< 0.2	219 ± 17	5528 ± 328*	62%
200 μM DENSpm, blank	< 0.2	< 0.2	< 0.2	15504 ± 589*	–
200 μM DENSpm, assay	< 0.2	**7841 ± 402***	< 0.2	9109 ± 540*	48%
200 μM DESpm, blank	< 0.2	< 0.2	17 ± 11	14431 ± 637*	–
200 μM DESpm, assay	205	**5087 ± 240***	12 ± 7	8060 ± 341*	44%
200 μM DEHSpm, blank	< 0.2	< 0.2	< 0.2	15019 ± 840*	–
200 μM DEHSpm, assay	< 0.2	**3845 ± 889***	< 0.2	11174 ± 533*	26%
200 μM OSSpm, blank	84 ± 10	< 0.2	342 ± 33	10224 ± 671*	–
200 μM OSSpm, assay	**1533 ± 75***	< 0.2	429 ± 29	8276 ± 158*	19%

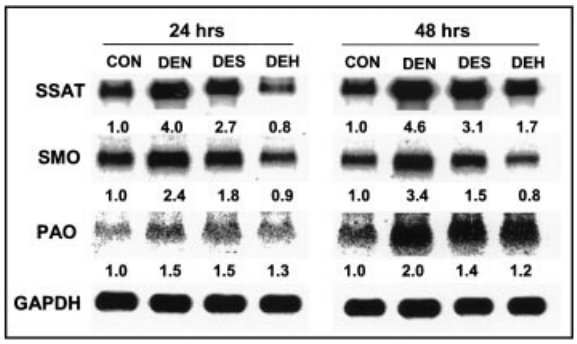

Figure 3 Northern blot analysis of PAO, SMO and SSAT mRNA expression and induction in analogue-treated HEK-293 cells

Cells were treated in the presence or absence (CON) of 10 μM polyamine analogue DENSpm (DEN), DESpm (DES), or DEHSpm (DEH) for 24 or 48 h before being harvested for mRNA extraction. Gels were loaded with 30 μg of total RNA and radiographically exposed for 72 h. For quantitation, SSAT, SMO and PAO mRNA bands were scanned densitometrically, normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as fold-increase of treated versus control (values located below respective bands). Note that SMO mRNA was higher than PAO mRNA and that the rank order of analogue induction was DENSpm > DESpm > DEHSpm.

versus normal comparisons emerged in certain cases. Of the six tissues for which EST comparisons between normal and neoplasia are available, three (kidney, ovary and prostate) showed reduced levels of PAO in neoplastic tissues. Of particular interest, PAO expression is significantly lower in neoplastic versus normal prostate, whereas SMO expression is markedly higher in this same comparison.

DISCUSSION

As a tool, functional genomics is only as meaningful as the specificity of the assay used for assigning functional activity to genomic sequences. A distinct advantage of the enzyme assay used for assigning SMO and PAO function is that it simultaneously measures substrate loss and product gain, and thereby yields direct indication of the nature of the oxidation reaction being measured. Several lines of evidence obtained from this assay indicate that the oxidase sequences identified by BLAST search using SMO sequences belong to the PAO involved in the polyamine back-conversion pathway. First, the lysate assay revealed that, in accordance with earlier studies with PAO [7,8], the present enzyme oxidized AcSpm to Spd and AcSpd to Put. In addition, our findings indicate that the priority ranking for substrate preference (i.e. AcSpm = AcSpd > DASpm ≫ Spm) is identical to earlier findings with purified enzyme preparations [11–13]. Lastly, the enzyme-activated irreversible inhibitor of PAO, MDL-72,527 [24,25] completely inhibited the present enzyme when added prior to the preferred substrate (i.e. AcSpm). We note that MDL-72,527 also completely inhibited SMO. While this reveals a lack of specificity of MDL-72,527 for PAO, dual inhibition of SMO and PAO by MDL-72,527 would be advantageous when attempting to block intracellular back-conversion of Spm to Spd by both routes. Taken together, these data indicate that the new oxidase is the SSAT-coupled oxidase involved in polyamine back-conversion. Although the predicted molecular mass of the oxidase (approx. 55 kDa) is lower than that originally reported by Holtta (approx. 61 kDa) [11], we note that Holtta first obtained a molecular mass of approx. 55 kDa by molecular sieving experiments and subsequently arrived at the value of approx. 61 kDa from calculations based on the combined data from molecular sieving and density gradient centrifugation. Unless the protein undergoes considerable post-translational modification, it would appear that the molecular sieving data are more correct.

Despite the above similarities, certain PAO properties revealed here are not in agreement with earlier biochemical findings. Although, we also found that analogues such as DENSpm, DESpm, DEHSpm, MESpm and N^1OSSpm are substrates of the present oxidase, the site of oxidative cleavage (i.e. next to an internal secondary amine) differs from previous reports by other workers [32–34]. More particularly, prior studies predicted that PAO was capable of N-dealkylation of terminally modified amines while we find that the reaction involves an internal cleavage adjacent to a secondary amine. Thus DESpm, DEHSpm or DENSpm are converted to MESpd or a corresponding

Tissue/Organ Type	SMO_hs (Hits/Total)	PAO_hs (Hits/Total)	Ratio (SMO:PAO)
brain, neoplasia	(9/191278)	(9/191278)	1:1
brain, normal	(4/330558)	(8/330558)	1:2
cervix, neoplasia	(4/26732)	(0/26732)	4:0
colon, neoplasia	(1/184285)	(0/184285)	1:0
eye, normal	(1/61118)	(1/61118)	1:1
genitourinary, neoplasia	(17/35752)	(1/35752)	17:1
germ cell, neoplasia	(0/55572)	(5/55572)	0:5
head and neck, neoplasia	(1/85490)	(1/85490)	1:1
kidney, neoplasia	(4/82357)	(0/82357)	4:0
kidney, normal	(0/73166)	(3/73166)	0:3
liver, neoplasia	(1/36764)	(0/36764)	1:0
lung, neoplasia	(6/162283)	(0/162283)	6:0
lung, normal	(2/113618)	(0/113618)	2:0
ovary, neoplasia	(0/85876)	(1/85876)	0:1
ovary, normal	(0/13634)	(2/13634)	0:2
pancreas, neoplasia	(2/55275)	(1/55275)	2:1
placenta, neoplasia	(2/43465)	(0/43465)	2:0
placenta, normal	(2/204343)	(0/204343)	2:0
prostate, neoplasia	(13/56922)	(0/56922)	13:0
prostate, normal	(2/72952)	(10/72952)	1:5
skin, neoplasia	(4/94290)	(0/94290)	4:0
spleen, normal	(1/18921)	(0/18921)	1:0
stomach, neoplasia	(2/133448)	(1/133448)	2:1
uterus, neoplasia	(3/139158)	(0/139158)	3:3
All tissues	**112**	**60**	**1.75:1**

Figure 4 Virtual Northern blot based on EST data comparing SMO and PAO expression in various human normal and neoplastic tissues

A virtual Northern analysis of the relative gene expression levels in different human tissues for SMO and PAO genes was performed using digiNorthern analysis [32] based on EST databases. The EST counts were normalized against the total number of ESTs available for each tissue to reflect the relative expression levels between different tissues, as well as between the two genes, as represented by the densities of virtual gel bands. Tissues that registered no ESTs for both genes are not shown (i.e. normal colon). Note that there are several normal versus neoplastic comparisons for a number of tissues.

analogue. Regarding this discrepancy, we can only suggest that the present assay allows for more precise determination of substrate to product conversion. Bergeron et al. [38] observed significant analogue N-dealkylation in the liver and kidney, and suggested that it took place via an alternative oxidation system, such as cytochrome P450. Although our present data indicate the potential for PAO to cleave analogues in the intracellular context, we do not generally observe cleavage products by HPLC analysis of analogue-treated cells (S. Vujcic, P. Diegelman and C. W. Porter, unpublished work). This may be due to the low basal levels of PAO (as suggested by mRNA) and/or to possible compartmentalized segregation of analogues from enzyme.

There are various similarities and differences between PAO and the recently identified SMO [3]. The two share 39 % sequence homology and a different molecular mass (i.e. approx. 55 kDa versus approx. 62 kDa respectively). As discussed above, PAO preferentially oxidizes AcSpm or AcSpd over Spm, whereas SMO prefers Spm over AcSpm or AcSpd. Although both liberate hydrogen peroxide, SMO generates 3-aminopropanal as a by-product, whereas PAO generates 3-acetamidopropanal. Given the substrate specificity of PAO, and the fact that AcSpm or AcSpd are uncommon in most cells, it is not surprising that, when transfected into HEK-293 cells, PAO exerts only minor effects on intracellular polyamine pools. In comparison, trans-

fected SMO markedly lowers the readily available Spm pools and causes profound up-regulation of ODC activity [3], a response not elicited by the modest pool shifts brought about by PAO transfection. We have no obvious explanation for the observation that human SMO and mouse PAO increase total intracellular polyamine pools (Table 1) except the possibility that by breaking down polyamines, they increase biosynthetic flux and/or uptake of polyamines.

Although PAO and SMO are readily inhibited by MDL-72,527, they differ distinctly in their interactions with polyamine analogues. Analogues, such as DEHSpm, DESpm, DENSpm and the calmodulin inhibitor N^1OSSpm, act as competitive inhibitors [3] and poor substrates of SMO. As shown here, these analogues serve as substrates for PAO. This difference may provide the basis for the design of selective inhibitors of these enzymes. The analogue studies provide further insights into the nature of the two enzyme reactions. Since PAO acts upon AcSpm and AcSpd, the substrate-binding requirement would seem to specify only two positively-charged amines, whereas SMO requires three. In addition, the data suggest that both SMO and PAO preferentially cleave the aminopropyl unit associated with the ethylated portion of the analogue, as indicated by the fact that oxidation of MESpm consistently yields only Spd in the lysate assay. Finally, it is interesting that the analogues are differentially oxidized by PAO, with a priority ranking of DENSpm > DESpm > DEHSpm, a finding that is consistent with preference for the aminopropyl moieties.

Northern blot comparison of PAO, SMO and SSAT mRNA from control and analogue-treated HEK-293 cells revealed several interesting pieces of information. First, it is unexpected that basal PAO mRNA levels are much lower than those of SSAT, since dogma suggests that PAO activity occurs in excess of SSAT activity, making the latter enzyme rate-limiting in the back-conversion pathway. The differences in activity could be related to protein turnover, which is reported to be quite slow for PAO [14] and very rapid for SSAT [39]. It is also interesting that the level of PAO mRNA is much lower than SMO mRNA in HEK-293 cells, as determined by Northern blot and confirmed in other tissues by virtual Northern analysis based on ESTs. If protein and enzyme activity are expressed in parallel with mRNA levels, SMO may be the more metabolically important of the two enzymes. Consistent with earlier findings based on the measurement of PAO activity [16–18], the results of virtual Northern analysis suggest that PAO mRNA is lower in most neoplastic versus normal tissue comparisons. Results from the prostate comparison were especially interesting, since the data suggest that SMO expression is markedly increased in neoplasia, whereas PAO is markedly decreased. Although these findings need to be confirmed experimentally, they are consistent with gene-profiling studies, indicating that polyamine metabolism is among the most dysregulated pathways in human prostate cancer [40].

Similarly with SMO [3,5], PAO mRNA was found to be inducible by analogues, an unexpected finding, since SSAT has traditionally been thought to be regulated and rate-limiting, whereas PAO was considered to be constitutively expressed [2]. PAO mRNA is differentially induced by polyamine analogues in a manner that parallels induction of SSAT and SMO mRNA. This suggests the existence of a common regulatory mechanism for all three genes, perhaps involving the polyamine modulating transcription cofactor reported by the Casero laboratory [41]. An important implication of this finding is that both PAO and SMO may be more significant contributors to polyamine analogue-induced growth inhibition than has been previously appreciated. The development of molecular reagents relating to both oxidases will allow for the dissection of their relative roles in both mediating anti-tumour activity and/or host tissue toxicities of polyamine analogues. Such tools will also facilitate studies on the role of these enzymes in polyamine catabolism and homoeostasis. In this regard, it would seem that, on the basis of enzyme function and relative mRNA levels, SMO may be more critically involved in the conversion of Spm to Spd, PAO in the conversion of Spd to Put, and SSAT in the export of polyamines out of the cell, but this remains to be demonstrated experimentally.

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