Cloning and expression of murine liver phosphatidylserine synthase (PSS)-2: differential regulation of phospholipid metabolism by PSS1 and PSS2

Scot J. STONE and Jean E. VANCE[1]

Department of Medicine, 332 Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Phosphatidylserine (PtdSer) is synthesized in mammalian cells by two base-exchange enzymes: PtdSer synthase (PSS)-1 primarily uses phosphatidylcholine as a substrate for exchange with serine, whereas PSS2 uses phosphatidylethanolamine (PtdEtn). We previously expressed murine PSS1 in McArdle hepatoma cells. The activity of PSS1 *in vitro* and the synthesis of PtdSer and PtdSer-derived PtdEtn were increased, whereas PtdEtn synthesis from the CDP-ethanolamine pathway was inhibited [Stone, Cui and Vance (1998) J. Biol. Chem. **273**, 7293–7302]. We have now cloned and stably expressed a murine *PSS2* cDNA in McArdle cells and M.9.1.1 cells [which are ethanolamine-requiring mutant Chinese hamster ovary (CHO) cells defective in PSS1]. Expression of the PSS2 in M.9.1.1 cells reversed the ethanolamine auxotrophy. However, the PtdEtn content was not normalized unless the culture medium was supplemented with ethanolamine. In both M.9.1.1 and hepatoma cells transfected with *PSS2* cDNA the rate of synthesis of PtdSer and PtdSer-derived PtdEtn did not exceed that in parental CHO cells or control McArdle cells respectively, in contrast to cells expressing similar levels of murine PSS1. These observations suggest that PtdSer synthesis via murine PSS2, but not PSS1, is regulated by end-product inhibition. Moreover, expression of murine PSS2 in McArdle cells did not inhibit PtdEtn synthesis via the CDP-ethanolamine pathway, whereas expression of similar levels of PSS1 activity inhibited this pathway by approx. 50%. We conclude that murine PSS1 and PSS2, which are apparently derived from different genes, independently modulate phospholipid metabolism. In addition, mRNAs encoding the two synthases are differentially expressed in several murine tissues, supporting the idea that PSS1 and PSS2 might perform unique functions.

INTRODUCTION

Phosphatidylserine (PtdSer) is synthesized in mammalian cells by PtdSer synthase (PSS), a membrane-associated enzyme that catalyses a calcium-dependent base-exchange reaction. The existence of two forms of PSS having distinct substrate specificities was predicted when mutant Chinese hamster ovary (CHO) cell lines {designated M.9.1.1 [1] and phosphatidylserine auxotroph (PSA)3 [2]} were established that lacked choline-exchange activity and possessed only 50% of the serine- and ethanolamine-exchange activities of parental CHO-K1 cells. Neither PSS has been purified to homogeneity. However, a rat brain enzyme activity catalysing serine exchange with phosphatidylethanolamine (PtdEtn), but not phosphatidylcholine (PtdCho), has been characterized [3]. In enzymic assays of homogenates of CHO cells one enzyme, PSS1, was found to exchange serine for the phospholipid head-group of both PtdCho and PtdEtn, whereas the other, PSS2, used only PtdEtn [2]. Recent genetic studies have demonstrated that in CHO cells PSS1 uses primarily PtdCho as a substrate for PtdSer synthesis, whereas PSS2 uses only PtdEtn [4]. The substrate specificities of PSS1 and PSS2 from CHO cells were confirmed when their cDNAs were cloned and expressed [5,6]. Expression of *PSS1* cDNA from CHO cells in PSA3 cells [5], or expression of the cDNA encoding murine *PSS1* in M.9.1.1 cells [7], increased the base-exchange activity and complemented the PtdSer/ethanolamine auxotrophy of these mutant cells deficient in PSS1 activity. Moreover, expression of a cDNA encoding *PSS2* from CHO cells in PSA3 cells restored the PtdSer prototrophy and normalized the phospholipid composition [6].

PSA3 cells have been further mutagenized to generate PSB2 cells that are deficient in both PSS1 and PSS2 activities [4]. In medium that either contained no phospholipid supplement, or was supplemented with PtdEtn, PSB2 cells did not survive. However, in medium supplemented with PtdSer the growth and phospholipid composition of PSB2 cells were restored, suggesting that PtdSer is essential for the growth of CHO cells. When PSB2 cells were transfected with the cDNA encoding *PSS1*, growth and phospholipid composition were normal, even in the absence of phospholipid supplements. In contrast, expression of *PSS2* cDNA in PSB2 cells permitted growth only when the medium was supplemented with PtdSer or PtdEtn [4]. Some conclusions from these observations regarding PtdSer synthesis in CHO cells are: (i) PtdSer is required for growth, (ii) PSS1 normally supplies the majority of PtdSer, and (ii) PSS2 is dispensable for growth and PtdSer synthesis, since cells expressing PSS1, but not PSS2, survived in the absence of phospholipid supplementation.

The metabolism of PtdSer is intimately related to that of PtdEtn because PtdSer is the substrate for PtdEtn synthesis via the mitochondrial enzyme PtdSer decarboxylase [8]. Indeed, the majority of PtdEtn in several types of cultured animal cells is derived from PtdSer decarboxylation, and the supply of PtdSer

Abbreviations used: CHO, Chinese hamster ovary; CMV, cytomegalovirus; Mc/PSS2, McArdle 7777 cells expressing murine phosphatidylserine synthase-2; PSA, phosphatidylserine auxotroph; PSS, phosphatidylserine synthase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

[1] To whom correspondence should be addressed (e-mail jean.vance@ualberta.ca).

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF099053.

limits the amount of PtdEtn made by this route [9,10]. The major alternative pathway for PtdEtn synthesis, the CDP-ethanolamine pathway [11], is also active in animal cells. However, the functional importance of this biosynthetic pathway, and the relative contribution of the two pathways *in vivo*, are not yet clear. Some evidence indicates that these two PtdEtn biosynthetic pathways are co-ordinately regulated so that PtdSer and PtdEtn homoeostasis is maintained. For example, when PtdEtn production from PtdSer decarboxylation was increased by overexpression of PSS1 in hepatoma cells, synthesis of PtdEtn via the CDP-ethanolamine pathway was inhibited [7]. Moreover, in M.9.1.1 cells in which PtdEtn production from PtdSer decarboxylation was reduced, the incorporation of [^{3}H]ethanolamine into PtdEtn was approx. doubled [1]. The mechanism of coordinate regulation of the two biosynthetic routes for PtdEtn has not yet been elucidated.

In order to understand better the function and regulation of PSS1 and PSS2 we have now cloned and heterologously expressed a murine liver *PSS2* cDNA in rat hepatoma cells and M.9.1.1 cells. Our studies provide evidence that murine PSS1 and PSS2 are differently regulated and that expression of murine PSS1 and PSS2 in these cells differentially modulates phospholipid metabolism.

MATERIALS AND METHODS

Materials

CHO-K1 cells and McArdle 7777 rat hepatoma cells were obtained from the American Type Tissue Culture Collection (Rockville, MD, U.S.A.). Fetal bovine serum, horse serum, tissue culture media and DNA-modifying enzymes were purchased from Gibco BRL. The radiochemicals [3-^{3}H]serine, [1-^{3}H]ethanolamine and [*methyl*-^{3}H]choline were from Amersham (Oakville, Ontario, Canada). TLC plates were purchased from BDH Chemicals. Authentic phospholipid standards were from Avanti Polar Lipids (Birmingham, AL, U.S.A.). All other chemicals were from Sigma or Fisher Biochemicals (Itasca, IL, U.S.A.).

Cell culture

M.9.1.1 cells [1] (a gift from Dr. D. R. Voelker, National Jewish Research Centre, Denver, CO, U.S.A.) and McArdle 7777 rat hepatoma cells were maintained as described previously [7].

Cloning and expression of murine *PSS2*

Oligonucleotides were synthesized as primers for PCR. Two gene-specific primers were designed that were based on the CHO-K1 *PSS2* cDNA sequence and contained the start and stop codons. The oligonucleotide 85ATGs was complementary to the sense strand of *PSS2* and 1516TAGas was complementary to the anti-sense strand. 85ATGs: ATGCGGAGGGCCGAGCGCAGAGTC; 1516TAGas: ATCATGAGGCGGCTGAGGCCCCCT. cDNA from a λgt11 mouse liver expression library (Clontech, Palo Alto, CA, U.S.A.) was isolated [7] and used as a template for PCR. The 50 μl PCR reaction mixture contained 5 μl of 10-fold concentrated reaction buffer (250 mM Taps, pH 9.3, 500 mM KCl, 10 mM β-mercaptoethanol; supplied by Panvera, Madison, WI, U.S.A.), 2.5 mM $MgCl_2$, 0.25 mM of each nucleoside triphosphate, 200 ng of λ phage cDNA, 10 pmol each of 85ATGs and 1516TAGas gene-specific primers, and 2.5 units of TaKaRa Ex Taq DNA polymerase (Panvera). The PCR reaction was performed for 30 cycles at 94 °C for 1 min, 60 °C for 2 min, 72 °C for 1.5 min and a final 10 min extension at 72 °C. Agarose gel electrophoresis [1.5 % (w/v) gel] was used to separate the PCR products. A 1.4 kb band was excised from the gel and the DNA fragment was eluted using a gel extraction kit (Qiagen, Santa Clarita, CA, U.S.A.), then cloned into the pCRII vector (Invitrogen, San Diego, CA, U.S.A.). Clones containing murine *PSS2* cDNA were identified by Southern blotting using an oligonucleotide corresponding to an internal sequence from CHO-K1 *PSS2* cDNA as a probe. Inserts were sequenced by the University of Alberta DNA Core Facility (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada) using the dideoxy termination/polyacrylamide gel method [12]. The murine *PSS2* clone was sequenced in both directions. The cDNA encoding murine *PSS2* was inserted into the pRc/CMV (cytomegalovirus) mammalian expression vector (Invitrogen). M.9.1.1 and McArdle rat hepatoma cells were transfected with 10 μg of cDNA by the calcium phosphate precipitation method [13]. Stable transfectants were selected by culturing cells in medium containing 400 μg/ml G418, and individual colonies were isolated. Once the cell lines were established, the concentration of G418 was reduced to 200 μg/ml. M.9.1.1 and McArdle 7777 cells were also transfected with the pRc/CMV expression vector lacking the insert and these cells served as controls.

Northern blot analysis

mRNA was isolated from cultured cells using a MicroPoly (A) Pure mRNA Isolation Kit (Ambion, Austin, TX, U.S.A.). Briefly, cells from 5 dishes (150 mm diameter) were harvested and disrupted in the lysis buffer supplied. Once purified, the mRNA was separated by electrophoresis on a 1.2 % (w/v) agarose gel containing formaldehyde. mRNAs were transferred to a Hybond-N membrane (Amersham Corporation) and probed with the cDNAs encoding murine *PSS1*, murine *PSS2* and rat protein disulphide isomerase. The protein disulphide isomerase cDNA was provided by Dr. M. Michalak (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada). Quantification was performed by densitometric scanning.

A mouse multi-tissue Northern blot containing approx. 2 μg of polyadenylated [poly(A)$^+$] RNA was purchased from Clontech Laboratories and probed with murine liver *PSS1* and *PSS2* cDNAs. The blot was also probed with β-actin cDNA as a loading control.

PSS assay

PSS activity was measured as described previously [14]. Cells were scraped from 100 mm (diameter) dishes and disrupted by sonication with a probe sonicator (2×10 s) in 10 mM Hepes buffer (pH 7.5) containing 0.25 M sucrose. Lysates were centrifuged for 2 min at 600 ***g*** to pellet cellular debris, and base-exchange activity was measured in the supernatant in the presence of 10 mM calcium chloride using [3-^{3}H]serine (50 μCi/μmol, 0.4 mM), [1-^{3}H]ethanolamine (20 μCi/μmol, 0.2 mM), [*methyl*-^{3}H]choline (10 μCi/μmol, 0.2 mM), or [*methyl*-^{14}C]choline (10 μCi/μmol, 0.2 mM) as substrates.

Radiolabelling of cells

Cellular PtdSer and PtdEtn were radiolabelled by incubation with [3-^{3}H]serine (5 μCi/ml) in serine-free modified Eagle's medium. For experiments in which cells were labelled with [1-^{3}H]ethanolamine (0.5 μCi/ml), radioactivity in PtdEtn was determined. For transfected M.9.1.1 cells, unless otherwise indicated, the culture medium contained no supplemental ethanolamine for 24 h prior to the start of the labelling period.

Other analyses

Phospholipids were extracted from cells by the method of Bligh and Dyer [15] and separated by TLC on silica gel G60 plates in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, by vol.). Phospholipids were made visible by exposure to iodine vapours and identified by comparison with authentic standards. The phosphorus content of each phospholipid was determined as described previously [16]. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce).

RESULTS

Isolation of murine liver *PSS2* cDNA

A murine liver *PSS2* cDNA was isolated by PCR using a λgt11 mouse liver cDNA library as a template and primers based on the sequence of *PSS2* cDNA from CHO-K1 cells (GenBank accession no. AB004109) [6]. A 1.4 kb product was generated and sequenced (Figure 1). The sequence predicts a 473 amino acid polypeptide with a molecular mass of 55 kDa and six potential membrane-spanning regions. Alignment of the murine and CHO-K1 sequences of PSS2 demonstrates that PSS2 is highly conserved across species (Figure 2). However, the predicted amino acid sequences of PSS2 and PSS1 from mouse [7] (Figure 2) possess only approx. 30% identity and there are no long, continuous stretches of sequence similarity, suggesting that PSS1 and PSS2 from murine liver are encoded by different genes.

Expression of murine *PSS2* cDNA reverses the growth defect of M.9.1.1 cells

M.9.1.1 cells are mutant CHO cells that are deficient in PSS1 activity, have a reduced content of PtdSer and PtdEtn, and require one of ethanolamine, PtdSer, PtdEtn or lysoPtdEtn for normal growth and survival [1]. The serine- and ethanolamine-exchange activities of M.9.1.1 cells are approx. 50%, and the choline-exchange activity is $< 5\%$, of those of wild-type CHO-K1 cells [1,7]. Data in Figure 3 (insert) confirm that the serine-exchange activity of M.9.1.1 cells is approx. 50% of that of CHO-K1 cells. As evidence that the cDNA that we had isolated encoded *PSS2*, we stably expressed the putative murine *PSS2*

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ATGCGGAGGGCCGAGCGCAGGGTCGCCGGGGGCTCGGGATCTGAGTCTCCGCTGCTCAAGGGCCGCCGCAGCACC    75
M  R  R  A  E  R  R  V  A  G  G  S  G  S  E  S  P  L  L  K  G  R  R  S  T     25
GAGTCTGAAGTCTACGACGATGGCACTAACACCTTCTTTTGGCGTGCCCACACTTTAACTGTGCTGTTCATCCTC    150
E  S  E  V  Y  D  D  G  T  N  T  F  F  W  R  A  H  T  L  T  V  L  F  I  L     50
ACCTGTGCGCTGGGCTACGTGACTCTCCTAGAAGAAACCCCTCAGGATACAGCCTACAACACCAAGAGAGGTATT    225
T  C  A  L  G  Y  V  T  L  L  E  E  T  P  Q  D  T  A  Y  N  T  K  R  G  I     75
GTGGCCAGTATTTTGGTTTTCTTATGTTTTGGAGTCACACAAGCTAAAGACGGGCCATTTTCCAGACCTCATCCA    300
V  A  S  I  L  V  F  L  C  F  G  V  T  Q  A  K  D  G  P  F  S  R  P  H  P     100
GCTTACTGGCGGTTTTGGCTGTGTGTTAGTGTGGTCTACGAATTGTTTCTCATCTTCATCCTTTTCCAGACAGTC    375
A  Y  W  R  F  W  L  C  V  S  V  V  Y  E  L  F  L  I  F  I  L  F  Q  T  V     125
CAGGATGGCCGACAGTTTCTGAAGTATGTGGATCCCAGGCTGGGAGTCCCATTGCCAGAGAGGGACTACGGGGGC    450
Q  D  G  R  Q  F  L  K  Y  V  D  P  R  L  G  V  P  L  P  E  R  D  Y  G  G     150
AACTGCCTCATCTATGATGCTGACAACAAGACTGACCCTTTCCACAACATCTGGGACAAGCTGGATGGCTTTGTT    525
N  C  L  I  Y  D  A  D  N  K  T  D  P  F  H  N  I  W  D  K  L  D  G  F  V     175
CCTGCACACTTCATTGGCTGGTATCTGAAGACGCTCATGATCCGTGACTGGTGGATGTGCATGATCATCAGTGTG    600
P  A  H  F  I  G  W  Y  L  K  T  L  M  I  R  D  W  W  M  C  M  I  I  S  V     200
ATGTTCGAGTTCCTGGAGTACAGCCTGGAGCACCAGCTGCCCAACTTCAGCGAGTGCTGGTGGGACCATTGGATC    675
M  F  E  F  L  E  Y  S  L  E  H  Q  L  P  N  F  S  E  C  W  W  D  H  W  I     225
ATGGACGTCCTCGTCTGCAACGGGCTGGGCATCTACTGTGGCATGAAGACCCTCGAGTGGCTGTCCCTGAAGACA    750
M  D  V  L  V  C  N  G  L  G  I  Y  C  G  M  K  T  L  E  W  L  S  L  K  T     250
TATAAGTGGCAGGGCCTCTGGAACATTCCAACCTACAAGGGCAAGATGAAGAGGATTGCCTTTCAGTTCACGCCT    825
Y  K  W  Q  G  L  W  N  I  P  T  Y  K  G  K  M  K  R  I  A  F  Q  F  T  P     275
TACAGCTGGGTACGCTTTGAGTGGAAGCCAGCCTCCAGCCTGCACCGCTGGCTGGCCGTGTGTGGCATCATCCTG    900
Y  S  W  V  R  F  E  W  K  P  A  S  S  L  H  R  W  L  A  V  C  G  I  I  L     300
GTGTTCCTGCTGGCAGAGCTGAACACCTTCTACCTGAAGTTTGTGCTATGGATGCCCCCTGAACACTACTTGGTC    975
V  F  L  L  A  E  L  N  T  F  Y  L  K  F  V  L  W  M  P  P  E  H  Y  L  V     325
CTTCTGAGGCTGGTCTTCTTCGTGAACGTGGGTGGTGTGGCCATGCGTGAGATCTACGACTTCATGGATGAATTG    1050
L  L  R  L  V  F  F  V  N  V  G  G  V  A  M  R  E  I  Y  D  F  M  D  E  L     350
AAGCCCCACAGGAAGCTGGGCCAGCAGGCCTGGCTGGTGGCAGCCATCACAGTCACAGAGCTTCTCATCGTGGTG    1125
K  P  H  R  K  L  G  Q  Q  A  W  L  V  A  A  I  T  V  T  E  L  L  I  V  V     375
AAGTATGACCCGCACACACTCACCCTGTCACTGCCCTTCTACATCTCCCAGTGCTGGACTCTTGGCTCCATCCTG    1200
K  Y  D  P  H  T  L  T  L  S  L  P  F  Y  I  S  Q  C  W  T  L  G  S  I  L     400
GTGCTTACATGGACTGTCTGGCGCTTCTTCCTGCGGGACATCACCATGAGGTACAAGGAGACCCGGCGACAGAAG    1275
V  L  T  W  T  V  W  R  F  F  L  R  D  I  T  M  R  Y  K  E  T  R  R  Q  K     425
CAGCAGAGTCACCAGGCCAGAGCCGTCAACAACCGGGATGGGCACCCTGGGCCAGATGATGACCTGCTAGGGACT    1350
Q  Q  S  H  Q  A  R  A  V  N  N  R  D  G  H  P  G  P  D  D  D  L  L  G  T     450
GGAACTGCAGAAGAAGAGGGGACCACCAATGACGGTGTGACTGCTGAGGAGGGGGCCTCAGCCGCCTCATGA    1419
G  T  A  E  E  E  G  T  T  N  D  G  V  T  A  E  E  G  A  S  A  A  S           473
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Figure 1 Nucleotide and predicted amino acid sequences of murine liver *PSS2* cDNA

The translational start codon is numbered +1. The start codon (ATG) and the stop codon (TGA) of the largest open reading frame are shown in boldface. Six putative transmembrane regions are underlined.

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CPSS2   MRRAERRVAGGSGSGSPLLEGRRSTESEVYDDGTNTFFWRAHTLTVLFILTCSLGYVTLL    60
MPSS2   MRRAERRVAGGSGS·SPLL·GRRSTESEVYDDGTNTFFWRAHTLTVLFILTC·LGYVTLL    60
MPSS1   M·····························E··V··D·T··FF·R·HT·T·L·····L·Y····    59

CPSS2   EETPQDTAYNTKRGIVASILVFLCFGVTQAKDGPFSRPHPAYWRFWLCVSVVYELFLIFI    120
MPSS2   EETPQDTAYNTKRGIVASILVFLCFGVTQAKDGPFSRPHPAYWRFWLCVSVVYELFLIFI    120
MPSS1   ·····-N··RGI···I··FL···V·····GPF·RPHPA·W·····SV·YFLFL·FL      118

CPSS2   LFQTVQDGRQFLKYVDPRLGVPLPERDYGGNCLIYDADNKTDPFHNIWDKLDGFVPAHFI    180
MPSS2   LFQTVQDGRQFLKYVDPRLGVPLPERDYGGNCLIYDADNKTDPFHNIWDKLDGFVPAHFI    180
MPSS1   LF·····------DP·L--·····R·--·····Y··········I·····F··AHF·      174

CPSS2   GWYLKTLMIRDWWMCMIISVMFEPLEYSLEHQLPNFSECWWDHWIMDVLICNGLGIYCGM    240
MPSS2   GWYLKTLMIRDWWMCMIISVMFEPLEYSLEHQLPNFSECWWDHWIMDVL·CNGLGIYCGM    240
MPSS1   GW··K·L·IR····C··IS···EL·E····H·LPNF·ECWWD··I·D·L··NG·GI··GM    234

CPSS2   KTLEWLSLKTYKWQGLWNIPTYKGKMKRIAFQFTPYSWVRFEW-KPASSLHRWLAVCGII    299
MPSS2   KTLEWLSLKTYKWQGLWNIPTYKGKMKRIAFQFTPYSWVRFEW-KPASSLHRWLAVCGII    299
MPSS1   ·····L···TY·W·····I····GK·KR···Q·TP··W····W··P·SS···········    294

CPSS2   LVFLLAELNTFYLKFVLWMPPEHYLVLLRLVFFVNVGGVAMREIYDFMDELKPHRKLGQQ    359
MPSS2   LVFLLAELNTFYLKFVLWMPPEHYLVLLRLVFFVNVGGVAMREIYDFMDELKPHRKLGQQ    359
MPSS1   ····L·ELNT··LK·····H·L···R··F···········Y·····-·····G·Q      353

CPSS2   AWLVAAITVTELLIVVKYDPHTLTLSLPFYIS---QCWTLGSILVLTWTVW-RFFLRDIT    415
MPSS2   AWLVAAITVTELLIVVKYDPHTLTLSLPFYIS---QCWTLGSILVLTWTVW-RFFLRDIT    415
MPSS1   ·W····I···E·····K···········Y·····C·····L·L···VW·········      413

CPSS2   MRYKETRRQKQQSHQG-RAINNGDGHPGPDDDLLGTGTAEEEGSTNDSVPAEKEGASAAS    474
MPSS2   MRYKETRRQKQQSHQ··RA·NN·DGHPGPDDDLLGTGTAEEEG·TND·V·AE-EGASAAS    473
MPSS1   ··Y·E·····················G·····················N·············    473
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Figure 2 Comparison of predicted amino acid sequences of murine liver PSS1 (MPSS1) and PSS2 (MPSS2) with the PSS2 sequence from CHO-K1 cells (CPSS2)

The sequence of CPSS2 is from Kuge et al. [6] and that of MPSS1 is from Stone et al. [7]. Non-identical amino acids are indicated by dots. Gaps in the sequences are indicated by dashes.

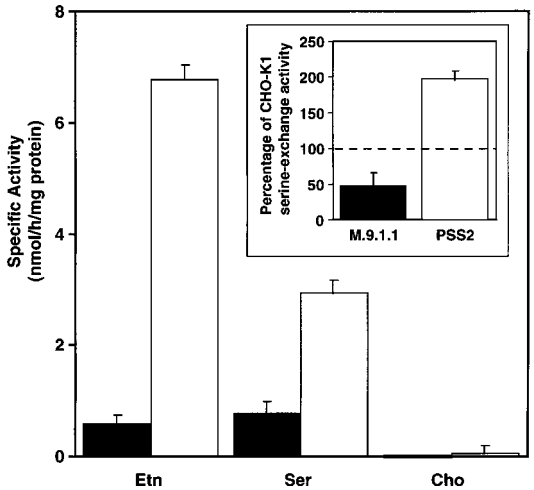

Figure 3 Base-exchange activities of control M.9.1.1 cells and M.9.1.1 cells expressing murine PSS2

Base-exchange activities were measured using [3-^{3}H]serine (Ser), [1-^{3}H]ethanolamine (Etn) and [*methyl*-^{3}H]choline (Cho) with cellular lysates from control M.9.1.1 cells (solid bars) and M.9.1.1. cells transfected with murine *PSS2* (open bars). Inset shows the serine-exchange activity of M.9.1.1 cells and M.9.1.1. cells expressing murine *PSS2* (PSS2) relative to that in wild-type CHO-K1 cells (100%, dashed line). Data are means ± S.D. for triplicate analyses from three independent experiments.

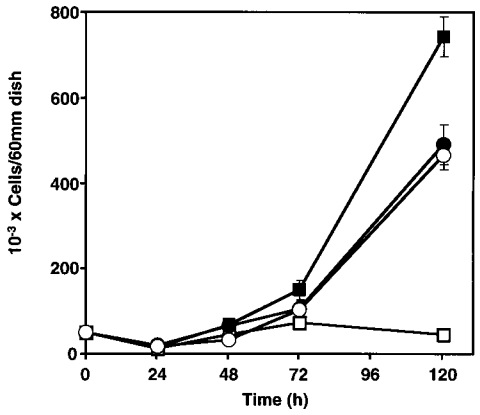

Figure 4 Growth of control M.9.1.1 cells and M.9.1.1 cells expressing murine PSS2

Control M.9.1.1 cells (squares) and M.9.1.1 cells stably transfected with murine *PSS2* cDNA (circles) were plated at a density of 50×10^3 cells/60 mm (diameter) dish and cultured in Ham's F-12 medium supplemented with 10% (v/v) delipidated fetal bovine serum without (open symbols) or with (solid symbols) 20 μM ethanolamine. Cells were harvested at 24 h intervals by trypsinization then counted. Data are means $\pm$ S.D. from triplicate dishes from one experiment which was repeated twice with similar results. Some error bars are hidden by symbols.

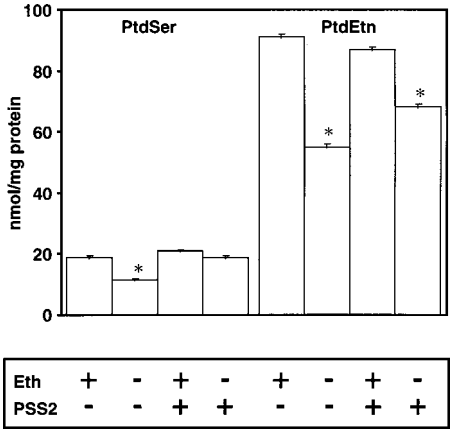

Figure 5 PtdSer and PtdEtn content of control M.9.1.1 cells and M.9.1.1 cells expressing murine PSS2

Cells were grown in medium supplemented with 20 μM ethanolamine for 24 h. Medium with (+) or without (−) 20 μM ethanolamine (Eth) was then added for an additional 72 h, after which the cells were harvested and the PtdSer and PtdEtn content was measured. PSS2 (+) denotes M.9.1.1 cells transfected with murine *PSS2* cDNA; PSS2 (−) denotes control cells transfected with empty vector. Data are means $\pm$ S.D. for triplicate analyses from three independent experiments. *, Statistical significance ($P < 0.05$) of differences between cells cultured in the presence or absence of 20 μM ethanolamine was evaluated by the Student's *t* test.

cDNA in M.9.1.1 cells. The serine-exchange activity in cell lysates of the PSS2-expressing cells was 4-fold higher than in lysates from control M.9.1.1 cells transfected with empty vector (Figure 3), and twice as high as that in CHO-K1 cells (Figure 3, insert). In addition, the ethanolamine-exchange activity was 12-fold higher in the PSS2-transfected cells than in control M.9.1.1 cells (Figure 3). As expected, since M.9.1.1 cells are deficient in choline-exchange activity compared with parental CHO-K1 cells, and since PSS2 has been reported to lack this activity [6], expression of murine PSS2 in M.9.1.1 cells did not increase the choline-exchange activity above that in control M.9.1.1 cells (Figure 3). These results confirm that the isolated cDNA encodes PSS2 activity.

M.9.1.1 cells are ethanolamine auxotrophs [1] presumably because they are deficient in PtdEtn production from PtdSer and ethanolamine is required for PtdEtn production via the CDP-ethanolamine pathway. Therefore, supplementation of the culture medium with ethanolamine would be expected to stimulate this pathway as compensation for the reduced ability of M.9.1.1 cells to generate PtdEtn from PtdSer decarboxylation. As shown in Figure 4, control M.9.1.1 cells did not grow without ethanolamine supplementation, but resumed normal growth when supplemented with 20 μM ethanolamine. Expression of murine PSS2 in M.9.1.1 cells restored the majority of normal growth, and the growth rate was the same regardless of the presence or absence of supplementary ethanolamine (Figure 4). Thus, over-expression of PSS2 activity reversed the ethanolamine auxotrophy of M.9.1.1 cells. As we reported previously, expression of murine PSS1 in M.9.1.1 cells also by-passes the growth requirement for ethanolamine [7].

The PtdSer and PtdEtn content of M.9.1.1 [1] and PSA3 [10] cells, both of which are deficient in PSS1 activity, have been reported to be reduced compared with that of CHO-K1 cells. In agreement with previous observations [1], Figure 5 shows that the PtdSer and PtdEtn content of M.9.1.1 cells grown in the absence of ethanolamine was significantly lower (40% and 41%, respectively) than that of M.9.1.1 cells grown in the presence of ethanolamine. When PSS1 activity was over-expressed in M.9.1.1 cells the quantities of these phospholipids were restored to normal even in the absence of ethanolamine supplementation [5,7]. In contrast, although the PtdSer content of M.9.1.1 cells expressing murine PSS2 was normal, and independent of ethanolamine supplementation, the PtdEtn content was normalized only in the presence of supplementary ethanolamine (Figure 5). Therefore, expression of murine PSS2 rescued the ethanolamine auxotrophy of M.9.1.1 cells although the PtdEtn level was not normalized. In contrast, expression of murine PSS1 in M.9.1.1 cells both rescued the growth defect and normalized the phospholipid composition in the absence of ethanolamine supplementation [7].

Metabolic labelling of M.9.1.1 cells expressing murine PSS2

We compared the incorporation of [^{3}H]serine into PtdSer and PtdEtn in parental CHO-K1 cells, control M.9.1.1 cells, and M.9.1.1 cells expressing murine PSS1 or PSS2, as an indication of the rates of synthesis of these two lipids. The uptake of [^{3}H]serine by all cell types was the same. After 6 h of incubation with [^{3}H]serine, radiolabelling of PtdSer and PtdEtn in M.9.1.1 cells was 44% and 50% less respectively, than in wild-type CHO-K1 cells (Figure 6). In the M.9.1.1 cells stably expressing PSS1, [^{3}H]serine incorporation into PtdSer and PtdEtn was approx. 3-fold higher than in M.9.1.1 cells, and almost twice as high as in wild-type CHO-K1 cells (Figure 6). However, in M.9.1.1 cells expressing murine PSS2, the incorporation of [^{3}H]serine into PtdSer and PtdEtn was higher than in M.9.1.1 cells but did not exceed that in wild-type cells (Figure 6) in spite of the *in vitro* serine-exchange activity being approx. 4-fold higher than in control M.9.1.1 cells and approx. twice that in CHO cells (Figure 3). To reduce the possibility that over-expression of PSS2 had depleted the ethanolamine pool and limited the amount of PtdEtn available as substrate for PSS2, the PSS2-transfected cells were incubated with 100 μM ethanolamine for 24 h prior to, as well as during, the labelling period, and

similar results were obtained. Therefore, expression of similar levels of PSS1 and PSS2 activities differently modulates PtdSer synthesis: expression of PSS2 in M.9.1.1 cells increased PtdSer and PtdEtn synthesis from [^{3}H]serine to the level in wild-type CHO cells, but no higher, whereas PSS1 expression increased PtdSer synthesis above that in parental CHO cells.

In agreement with our finding that expression of murine PSS1 in McArdle cells inhibited the CDP-ethanolamine pathway for PtdEtn synthesis (as measured by the incorporation of [^{3}H]ethanolamine into PtdEtn) [7], expression of murine PSS2 in M.9.1.1 cells reduced the incorporation of [^{3}H]ethanolamine into PtdEtn by 44% after 6 h (results not shown). In addition, in pulse–chase experiments in M.9.1.1 cells, the degradation of [^{3}H]serine-labelled PtdSer and PtdEtn, and of [^{3}H]ethanolamine-derived PtdEtn, was not altered significantly by the expression of murine PSS2 (results not shown).

Murine PSS1 and PSS2 activities might be regulated post-transcriptionally

Northern blot analysis was performed on mRNA samples from parental CHO cells, control M.9.1.1 cells (transfected with empty vector) and M.9.1.1 cells expressing murine PSS1 or PSS2. The PSS1 and PSS2 mRNA content was analysed by hybridization with cDNA probes encoding murine *PSS1* and *PSS2* respectively, and compared with the mRNA content of a loading control, protein disulphide isomerase. Before insertion into the pRc/CMV mammalian expression vector, the murine *PSS1* and *PSS2* cDNAs were truncated in the 3′-untranslated region so that mRNAs smaller than the endogenous PSS mRNAs were generated. Figure 7 (upper panel) shows that less of the 2.4 kb PSS1 transcript was present in control M.9.1.1 cells than in wild-type CHO-K1 cells, suggesting that the primary defect in M.9.1.1 cells is a reduced expression of the *PSS1* gene. In M.9.1.1 cells expressing murine PSS1, the amount of the murine PSS1 mRNA transcript was approx. 14-fold higher than in control cells, whereas the cells expressing murine PSS1 exhibited 5-fold higher serine-exchange activity than did control cells (Figure 3) [7].

Expression of PSS2 mRNA in the four cell types is also shown in Figure 7 (middle panel). The amount of endogenous PSS2 mRNA (2.2 kb transcript) was approx. the same in M.9.1.1 cells and parental CHO cells, and was not obviously altered upon expression of murine PSS1. However, the 1.4 kb murine PSS2 mRNA transcript was approx. 300-fold more abundant in M.9.1.1 cells expressing murine PSS2 than in control cells. The 4-fold increase in serine-exchange activity in the cells expressing murine PSS2 compared with control M.9.1.1 cells (Figure 3) was not proportional to this large increase in mRNA. These experiments suggest that expression of murine PSSs, particularly PSS2, might be regulated post-transcriptionally, although other explanations are also possible.

Differential tissue expression of murine PSS1 and PSS2 mRNAs

A multiple tissue Northern blot containing mRNAs from eight mouse tissues (testis, kidney, skeletal muscle, liver, lung, spleen, brain and heart) was purchased (Clontech) and probed with the cDNAs encoding murine *PSS1* and *PSS2* as well as β-actin cDNA as a loading control. PSS1 mRNA was detected in most of the tissues examined and was especially abundant in testis, kidney, liver, brain and heart (Figure 8). In contrast, PSS2 mRNA was very abundant only in testis, with smaller amounts in kidney and heart (Figure 8). Thus the tissue distribution of PSS1 and PSS2 mRNAs appears to be markedly different.

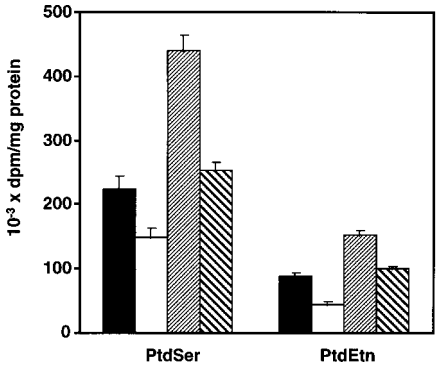

Figure 6 Incorporation of [^{3}H]serine into PtdSer and PtdEtn of CHO-K1 cells, control M.9.1.1 cells and M.9.1.1 cells expressing murine PSS1 or PSS2

Cells were incubated in medium containing [3-^{3}H]serine (15 μCi/dish) for 6 h, then harvested and PtdSer and PtdEtn were isolated. Solid bars, CHO-K1 cells; open bars, M.9.1.1 cells; grey bars, M.9.1.1 cells expressing murine PSS1; hatched bars, M.9.1.1 cells expressing murine PSS2. Data for PSS1 are taken from [7] and are included for comparison. Data are means ± S.D. for triplicate analyses from one experiment which was repeated twice with similar results.

Expression of murine PSS2 in McArdle rat hepatoma cells

We next generated several McArdle 7777 rat hepatoma cell lines that stably expressed murine PSS2 in addition to endogenous PSS1 and PSS2. The cell line expressing the highest serine- and ethanolamine-exchange activities (2.5-fold and 5-fold higher

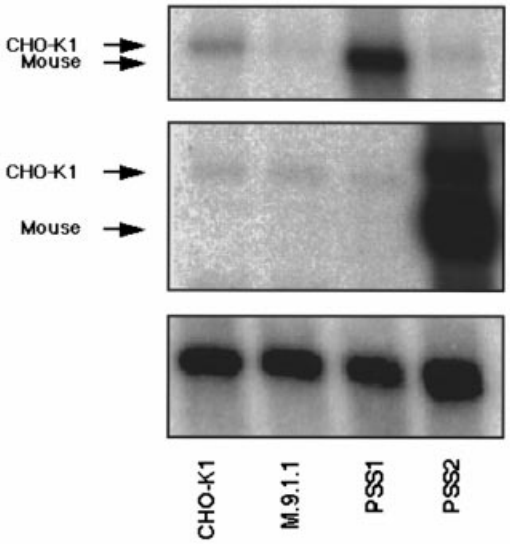

Figure 7 Northern blot analysis of PSS1 and PSS2 mRNAs

Polyadenylated [poly(A)$^+$] RNA (5 μg) was separated on a 1.2% (w/v) agarose/formaldehyde gel and transferred to a Hybond N membrane. Murine *PSS1*, *PSS2* and rat protein disulphide isomerase (*PDI*) cDNA probes were hybridized at 42 °C in the presence of 50% (v/v) formamide. Washes were performed at the following stringencies: PSS1, 1 × SSC buffer (SSC buffer: 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at 60 °C; PSS2, 0.1 × SSC buffer at 60 °C; protein disulphide isomerase, 0.1 × SSC buffer at 55 °C. Upper panel, *PSS1* cDNA probe; middle panel, *PSS2* cDNA probe; lower panel, protein disulphide isomerase (*PDI*) cDNA probe. RNA samples are indicated at the bottom and are from CHO-K1 cells, M.9.1.1 cells transfected with empty vector and M.9.1.1 cells transfected with cDNAs encoding murine *PSS1* (PSS1) or murine *PSS2* (PSS2).

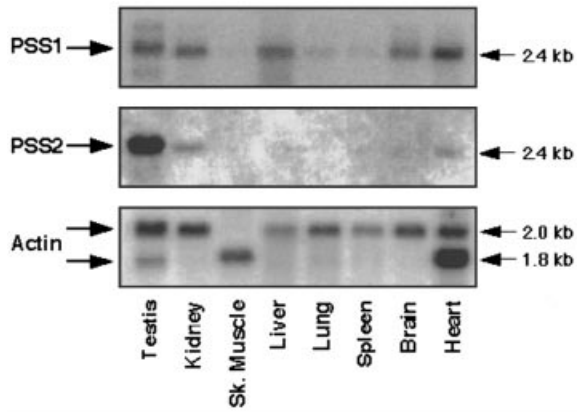

Figure 8 Multi-tissue Northern blot of PSS1 and PSS2 mRNAs in murine tissues

A mouse multiple tissue Northern blot containing approx. 2 μg of polyadenylated [poly(A)$^+$] RNA (Clontech Laboratories) was probed with mouse liver *PSS1* and *PSS2* cDNAs as well as β-actin as a loading control.

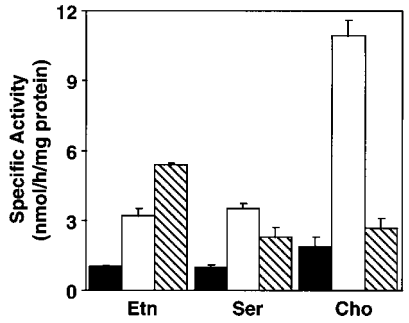

Figure 9 Base-exchange activities of control McArdle 7777 cells and McArdle 7777 cells expressing murine PSS2 and PSS1

Base-exchange activities were measured in cellular lysates using [3-^{3}H]serine (Ser), [*methyl*-^{3}H]choline (Cho) and [1-^{3}H]ethanolamine (Etn). Control McArdle cells transfected with vector alone (filled bars); cells expressing murine PSS1 (open bars); cells expressing murine PSS2 (hatched bars). Data for PSS1 are taken from Stone et al. [7] and are included for comparison. Data are means $\pm$ S.D. for triplicate analyses from three independent experiments.

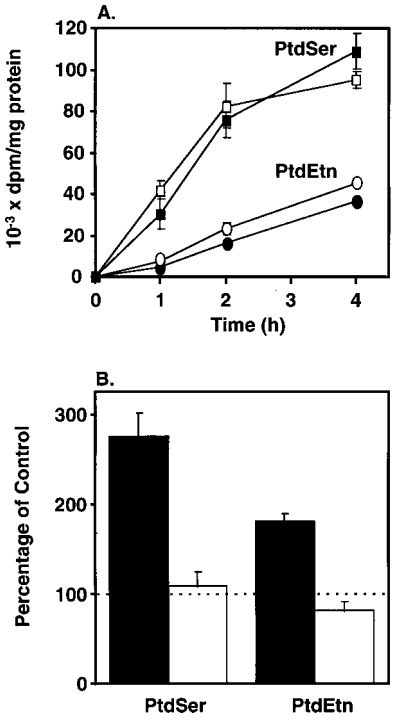

Figure 10 Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle cells expressing murine PSS1 or PSS2

(**A**) Control McArdle cells (solid symbols) and McArdle cells expressing murine PSS2 (open symbols) were incubated with [^{3}H]serine (15 μCi/dish) for 1–4 h, at which times radioactivity incorporation into PtdSer (squares) and PtdEtn (circles) was determined. (**B**) The same cell types were incubated in medium containing [3-^{3}H]serine for 4 h, harvested, and then PtdSer and PtdEtn were isolated. Data are expressed as the amount of radiolabel in PtdSer and PtdEtn relative to that in control McArdle cells (100%). McArdle cells expressing murine PSS1 (solid bars); McArdle cells expressing murine PSS2 (open bars). Data for PSS1 are taken from Stone et al. [7] and are included for comparison. Data are means $\pm$ S.D. for triplicate analyses from one experiment which was repeated twice with similar results.

respectively, than in control McArdle cells) was designated Mc/PSS2 and, as expected, exhibited little increase in choline-exchange activity (Figure 9).

The incorporation of [^{3}H]serine into PtdSer and PtdEtn was compared in control and Mc/PSS2 cells as an indication of whether or not the increased base-exchange activity was reflected in an increased rate of biosynthesis of these two phospholipids. The incorporation of [^{3}H]serine into PtdSer and PtdEtn was unaltered by the expression of murine PSS2 (Figure 10A) even though the serine- and ethanolamine-exchange activity was approx. 2.5-fold and 5-fold higher respectively, than in control cells. In contrast, expression of PSS1 at a similar level (3-fold higher serine-exchange activity) in McArdle cells resulted in the incorporation of [^{3}H]serine into PtdSer and PtdEtn being 3-fold and 2-fold higher respectively, than in control cells (Figure 10B) [7]. The total uptake of [^{3}H]serine by Mc/PSS2 and control McArdle cells was equivalent. Only small changes in PtdSer and PtdEtn mass resulted from over-expression of PSS2. In control cells transfected with empty vector, the amounts of PtdSer and PtdEtn were 19.3 ± 1.3 nmol/mg of protein and 53.8 ± 2.0 nmol/mg of protein respectively, whereas Mc/PSS2 cells contained 14.8 ± 0.7 nmol of PtdSer/mg of protein and 49.5 ± 1.9 nmol of PtdEtn/mg of protein. Therefore, as for McArdle cells over-expressing PSS1 [7], the PtdSer and PtdEtn contents of PSS2-overexpressing cell appears to be tightly regulated.

Expression of murine PSS2 in McArdle cells does not inhibit PtdEtn synthesis from CDP-ethanolamine

Our previous studies in McArdle cells showed that [^{3}H]ethanolamine incorporation into PtdEtn was dramatically inhibited when murine PSS1 was expressed [7]. We suggested that this apparent down-regulation of the CDP-ethanolamine pathway for PtdEtn biosynthesis represented compensation for the increased production of PtdEtn from PtdSer decarboxylation, and was therefore a mechanism for maintaining PtdEtn homoeostasis. In contrast to the expression of PSS1 [7], expression of murine PSS2 in McArdle cells did not significantly reduce the incorporation of [^{3}H]ethanolamine into PtdEtn. In McArdle cells expressing murine *PSS1* cDNA, the incorporation of [^{3}H]ethanolamine into PtdEtn after 1 h was only 39 ± 11 % of that in control cells, whereas in Mc/PSS2 cells the incorporation of [^{3}H]ethanolamine into PtdEtn after 1 h was 128 ± 30 % of that in control McArdle cells transfected with empty vector. These

results imply that in McArdle cells, increased expression of murine PSS2, in contrast to murine PSS1, does not inhibit PtdEtn synthesis via the CDP-ethanolamine pathway. Therefore, the PtdEtn content in Mc/PSS2 cells appears to be primarily regulated at the level of PtdSer synthesis/decarboxylation rather than by the CDP-ethanolamine pathway.

DISCUSSION

We report the cloning of a cDNA encoding murine PSS2 and its stable expression in M.9.1.1 cells and McArdle 7777 rat hepatoma cells. In M.9.1.1 cells expressing murine PSS2, the serine and ethanolamine exchange *in vitro* was increased 4-fold and 12-fold respectively, with no significant increase in choline-exchange activity. Similarly, in Mc/PSS2 cells, the serine- and ethanolamine-exchange activities were 2.5-fold and 5-fold higher respectively, than in control cells transfected with empty vector. We have compared the impact of over-expression of PSS1 and PSS2 activities on phospholipid metabolism in these cells.

Reversal of the ethanolamine auxotrophy of M.9.1.1 cells

Stable expression of murine PSS2 or PSS1 in PSS1-deficient M.9.1.1 cells reversed the growth defect and ethanolamine auxotrophy. In M.9.1.1 cells, the PtdSer and PtdEtn content is approx. one-half of that in wild-type CHO-K1 cells. However, the PtdSer and PtdEtn content of M.9.1.1 cells was normalized by expression of murine PSS1 in cells grown in either the presence or absence of ethanolamine [7]. In contrast, in M.9.1.1 cells expressing murine PSS2 and cultured in the absence of ethanolamine, the amount of PtdEtn was 25% less than in M.9.1.1 cells cultured in the presence of ethanolamine. The amount of PtdSer in the *PSS2*-transfected cells was normal and independent of ethanolamine supplementation. One possible explanation for why these cells contain a reduced PtdEtn content is that PSS2 consumes PtdEtn as a substrate for PtdSer synthesis. Consequently, in the absence of supplementary ethanolamine, PtdEtn synthesis from the CDP-ethanolamine pathway might be insufficient to maintain normal PtdEtn levels, although the cells apparently were able to tolerate the deficit in PtdEtn and grew normally. These observations in M.9.1.1 cells differ somewhat from recent experiments reported by Kuge et al. [6] in which PSA-3 cells (another CHO-K1 mutant defective in PSS1 activity) were transfected with a cDNA encoding *PSS2* from CHO cells. In these cells cultured in the absence of exogenous ethanolamine and PtdEtn, over-expression of PSS2 (which increased the ethanolamine- and serine-exchange activity by 7-fold and 5-fold respectively, similar to the levels we attained) completely normalized the PtdEtn content and rescued the growth defect. The explanation for the apparent difference between our results and those of Kuge et al. [6] is not clear.

Regulation of PtdSer synthesis by PSS1 and PSS2

Cells apparently strive to maintain constant levels of PtdSer, as illustrated by experiments in which the PtdSer content remained unchanged when PSS1 and PSS2 activity in cells was increased [7]. We found that expression of murine PSS2 in M.9.1.1 cells increased the incorporation of [^{3}H]serine into PtdSer and PtdEtn to that in wild-type CHO-K1 cells, but no higher, in spite of the serine-exchange activity *in vitro* being 4-fold higher than in M.9.1.1 cells, and almost double that in CHO-K1 cells. Similarly, in Mc/PSS2 cells the serine-exchange activity was 2.5-fold higher than in McArdle cells but the incorporation of [^{3}H]serine into PtdSer was the same as that in control cells. Expression of murine PSS1 produced different results [7] and indicated that PtdSer synthesis is differently regulated by PSS1 and PSS2, since the incorporation of label into PtdSer of Mc/PSS1 cells was increased to approx. the same extent (approx. 3-fold) as the increase in serine-exchange activity [7]. In addition, when murine PSS1 was expressed in M.9.1.1 cells the serine-exchange activity was approximately 2.5-fold higher than in wild-type CHO-K1 cells and the incorporation of [^{3}H]serine into PtdSer was double that in CHO-K1 cells (results not shown).

The reason why similar levels of expression of PSS1 and PSS2 differently modulate PtdSer synthesis is not clear. One explanation might be that over-expression of PSS2 results in the rate of PtdSer synthesis being limited by a factor other than the amount of PSS2, for example, by the supply of PtdEtn, the substrate for PSS2. However, our results suggest that this is not the case because inclusion of 100 μM ethanolamine in the culture medium restored the PtdEtn content to that in CHO cells, without increasing the rate of PtdSer synthesis above that in wild-type CHO cells.

An alternative explanation for these observations is that synthesis of PtdSer via murine PSS2 is regulated by end-product inhibition, as has been suggested for both PSS1 and PSS2 from CHO-K1 cells [17,18]. End-product inhibition of PtdSer synthesis in CHO cells has been investigated by Hasegawa et al. [19], who have isolated a CHO-K1 mutant, called mutant 29, that is defective in the regulation of PtdSer synthesis by PtdSer. Their studies show that Arg^{95} of PSS1 from CHO-K1 cells is required for end-product inhibition by PtdSer, since mutation of Arg^{95} to a lysine residue abolishes this regulation [18]. However, our studies indicate that PtdSer synthesis via murine PSS1 might not be regulated by end-product inhibition since expression of murine PSS1 in both McArdle and M.9.1.1 cells resulted in an apparently increased rate of PtdSer synthesis above that in parental cells [7]. Examination of the predicted amino acid sequences of PSS1 and PSS2 from CHO-K1 cells and murine liver reveals that all four synthases contain this crucial arginine residue. Furthermore, the sequence of amino acids surrounding Arg^{95} (PNGPFT**R**PHPALWRM) of PSS1 is identical in CHO-K1 cells and mouse liver. Although the sequences in this region of the protein differ slightly between PSS1 and PSS2, they are identical in PSS2 from the two species (KDGPFS**R**PHPAYWRF, in which the arginine is located at position 97). Consequently, the apparent lack of end-product inhibition of murine PSS1 by PtdSer cannot be fully explained by a lack of this arginine residue.

Northern blot analysis of PSS1 and PSS2 mRNA levels in M.9.1.1 cells expressing murine PSS1 and PSS2 suggests an additional possible mode of regulation of PtdSer synthesis. The increase in mRNA expression was much greater than the increase in serine-exchange activity measured in cellular lysates. Although this apparent discrepancy might be an artefact of the transfection procedure, these observations might alternatively suggest that expression of murine PSS1 and, especially, PSS2 is negatively regulated post-transcriptionally. Since we do not yet have an antibody available that recognises PSS1 or PSS2 proteins we cannot determine whether regulation of enzyme activity occurs translationally or post-translationally.

Co-ordinate regulation of the PtdSer decarboxylation and CDP-ethanolamine pathways

Our data also show that PSS1 and PSS2 differ in their ability to regulate the CDP-ethanolamine pathway. Over-expression of PSS1, but not PSS2, in McArdle cells inhibited PtdEtn production from CDP-ethanolamine. However, the inability of over-

expression of PSS2 to inhibit the CDP-ethanolamine pathway is not an inherent property of the PSS2 protein itself, since the incorporation of [^{3}H]ethanolamine into PtdEtn was significantly decreased when murine *PSS2* cDNA was expressed in M.9.1.1 cells. Our current interpretation of these observations is that the CDP-ethanolamine pathway is inhibited when the rate of synthesis of PtdSer and/or PtdEtn from [^{3}H]serine is increased, but not merely when the amount of PSS2 protein/activity is increased.

In conclusion, we provide data showing that PtdSer synthesis by murine liver PSS1 and PSS2 is differentially regulated and that these two enzymes independently modulate PtdEtn metabolism. Although the function of the two PSS isoforms is not entirely clear, the combined data suggest that PSS1 normally supplies the majority of PtdSer from PtdCho, whereas PSS2 might perform dual functions. One function might be to generate ethanolamine for PtdEtn synthesis via CDP-ethanolamine. A second function of PSS2 might be to participate in PtdSer production from PtdEtn when PtdSer synthesis from PSS1 is impaired. The differential tissue-specific expression of PSS1 and PSS2 mRNAs also supports the hypothesis that PSS1 and PSS2 perform distinct functions in whole animals.

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