We report the subcellular localization of enzymes involved in phosphatidylserine biosynthesis in mammalian cells. Several lines of evidence suggest that phosphatidylserine synthase-1 (PSS1) is highly enriched in mitochondria-associated membranes (MAM) and is largely excluded from the bulk of the endoplasmic reticulum (ER). Taking advantage of the substrate specificity of PSS1, we showed that (i) MAM contain choline exchange activity, whereas this activity is very low in the bulk of the ER, (ii) serine exchange activity is inhibited by choline to a much greater extent in MAM than in ER, and (iii) MAM use phosphatidylethanolamine and phosphatidylethanolamine as substrates for phosphatidylserine biosynthesis, whereas the ER utilizes only phosphatidylethanolamine. According to immunoblotting of proteins from both CHO-K1 cells and murine liver, PSS1 is localized to MAM, and in hepatoma cells stably expressing PSS1 this protein is highly enriched in MAM. Since the ER contains serine and ethanolamine exchange activities, we had predicted that PSS2 would account for the serine exchange activity in the ER. Unexpectedly, using immunoblotting experiments, we found that (i) PSS2 of CHO-K1 cells is present only in MAM and (ii) PSS2 is restricted to MAM of McArdle cells expressing recombinant PSS2. These data leave open the question of which enzyme imparts PSS activity to the ER and suggest that a third isoform of PSS might be located in the ER.

Many enzymes involved in the final stages of lipid biosynthesis are integral membrane proteins of the endoplasmic reticulum (ER). Examples include cholinephosphotransferase (1, 2), ethanalaminephosphotransferase (1, 2), phosphatidylethanolamine (PtdEtn) N-methyltransferase (1, 2), diacylglycerol acyltransferase (2), glycerol-3-phosphate acyltransferase (2), 3-hydroxy-3-methylglutaryl-CoA reductase (2), and phosphatidylserine synthase (PSS).

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Received for publication, April 5, 2000, and in revised form, August 9, 2000
Published, JBC Papers in Press, August 10, 2000, DOI 10.1074/jbc.M002865200

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Phosphatidylserine Synthase-1 and -2 Are Localized to Mitochondria-associated Membranes

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* This work was supported by an operating grant (to J. E. V.) and a studentship (to S. J. S.) from the Medical Research Council of Canada.

The abbreviations used are: ER, endoplasmic reticulum; CHO, Chinese hamster ovary; MAM, mitochondria-associated membranes; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PSS, phosphatidylserine synthase.

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forms might be present in spatially distinct locations in the cell, with one isoform being located in MAM and the other in the ER. We demonstrate that PSS1 protein is found exclusively in MAM and is not detectable in the ER. In addition, and contrary to our predictions, we found that PSS2 is also restricted to MAM. These findings leave open the question of which PtdSer biosynthetic enzyme is responsible for serine exchange activity in the ER and suggest that this activity might be due to a third, presently unknown, PtdSer synthase isoform.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO-K1 cells and McArdle rat hepatoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). Fetal bovine serum, horse serum, tissue culture media, and DNA modifying enzymes were purchased from Life Technologies, Inc. The radiochemicals [3-3H]serine, [1-3H]ethanolamine, and [methyl-3H]adenine were from Amerham Pharmacia Biotech. Bovine pancreatic tryps- in and soybean trypsin inhibitor were from Sigma. All other chemicals were from Sigma or Fisher.

**Cell Culture**—M.9.1.1 cells (a gift from Dr. D. R. Voelker, National Jewish Research Center, Denver, CO), CHO-K1 cells, and McArdle 7777 rat hepatoma cells were maintained as described previously (14).

**Preparation of myc Epitope-tagged PSS1 and PSS2 cDNA**—The myc epitope (EQKLISEEDL) was appended to the 5’-end of murine PSS1 cDNA (14) and either the 5’- or 3’-end of murine PSS2 cDNA (16) by the polymerase chain reaction. A myc-PSS1 mutant was generated by deleting the last two codons (encoding two lysine residues) of murine PSS1 cDNA. The cDNA fragments were sequenced by the DNA core facility at the University of Alberta to confirm the modifications. Each of the cDNAs encoding myc-PSS1, myc-PSS2 with two C-terminal lysine residues deleted (myc-PSS1.CKK), and myc-PSS2 with the myc tag appended to either the N terminus (N-myc-PSS2) or the C terminus (C-myc-PSS2) was inserted into the eukaryotic expression vector pcDNA 3.1 (Invitrogen). McArdle rat hepatoma cell lines were transfected with 10 μg of the cDNAs using the calcium phosphate precipitation method (17). Stable transfectants were selected by culturing the cells in medium containing 600 μg/ml G418. Individual colonies were isolated. When cell lines had been established, the concentration of G418 was reduced to 200 μg/ml. Control cells for experiments with McArdle cells were transfected with the expression vector lacking a cDNA insert.

**Double Immunofluorescence**—McArdle cells (80% confluent) expressing myc-PSS1 and C-myc-PSS2 were diluted 1:10 with fresh medium and plated on coverslips in 100-mm dishes and then allowed to attach overnight. Cells were fixed with methanol/acetone (1:1) for 2 min at room temperature and then preincubated with phosphate-buffered saline containing 3% bovine serum albumin (w/v) and 0.2% Triton X-100 (v/v) for 1 h. The cells were incubated with a mixture of anti-myc (1:1 dilution) and anti-rat calnexin (1:250 dilution) antibodies in phosphate-buffered saline containing 3% bovine serum albumin (w/v) and 0.02% Triton X-100 (v/v) for 1 h. After five washes with phosphate-buffered saline containing 0.02% Triton X-100 (v/v), cells were incubated with secondary antibodies (fluorescein isothiocyanate-conjugated anti-mouse IgG (1:1000 dilution) and Texas Red-conjugated anti-rabbit IgG (1:100 dilution)) in phosphate-buffered saline containing 0.02% Triton X-100 (v/v) for 30 min. The cells were washed five times with phosphate-buffered saline containing 0.02% Triton X-100 (v/v) and then mounted on microscope slides in phenylene diamine mounting medium and processed for confocal microscopy (Zeiss LSM 510 confocal microscope).

**Subcellular Fractionation of Cultured Cells**—Microsomes, MAM, and mitochondria were isolated from cultured cells by slight modifications of a method previously described (17). Cells were scraped into phosphate-buffered saline, pelleted by centrifugation at 500 × g for 5 min, and resuspended in 8 ml of homogenization buffer (0.25 M sucrose and 10 mM HEPES (pH 7.4)). The cells were gently disrupted by 15 up-and-down strokes in a Potter-Elvehjem motor-driven homogenizer. The homogemate was centrifuged twice at 600 × g for 5 min to remove cellular debris and nuclei, and the supernatant was centrifuged at 10,500 × g for 10 min to pellet crude mitochondria. The resultant supernatant was centrifuged at 100,000 × g for 1 h in a Beckman Ti 70.1 rotor at 4 °C to pellet microsomes, which were resuspended in homogenization buffer. The mitochondrial pellet was resuspended in 300 μl of isolation medium (250 mM mannitol, 5 mM HEPES (pH 7.4), and 0.5 mM EGTA) and layered on top of 8 ml of Percoll medium (225 mM mannitol, 25 mM HEPES (pH 7.4), 1 mM EGTA, and 30% Percoll (v/v)) in a 10-ml polycarbonate ultracentrifuge tube and then centrifuged for 30 min at 95,000 × g. A dense band containing purified mitochondria was recovered from approximately ¾ down the tube. The mitochondrial band was removed, diluted with isolation medium, and washed twice by centrifugation at 6300 × g for 10 min to remove the Percoll, after which the mitochondria were resuspended in isolation medium. MAM were removed from the Percoll gradient as the diffuse white band located above the mitochondria. Isolation medium was added, and the suspension was centrifuged at 6300 × g for 10 min. The supernatant containing MAM was centrifuged at 100,000 × g for 1 h in a Beckman Ti 70.1 rotor, and the resulting MAM pellet was resuspended in homogenization buffer.

**Subcellular Fractionation of Liver Homogenates**—MAM and mitochondria were isolated from murine liver as described previously (18). ER fractions enriched in rough ER (ER1) and smooth ER (ER2) were prepared by the method of Croze and Morre (18) as modified by Vance and Vance (19). ER1 was isolated from the final discontinuous sucrose gradient at the interface between sucrose solutions of 1.5 and 2.0 M. ER2 was isolated from the same gradient at the interface between sucrose solutions of 1.3 and 1.5 M.

**Measurement of PtdSer Synthase Activity**—PtdSer synthase activity was measured in subcellular fractions of mouse liver as described previously (19). Cultured cells were scraped from 100-mm dishes and washed twice with 2 ml of culture medium (1:10 w/w) with a program of 10 ml of 20 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 PSS activity was measured in the supernatant in the presence of 10 mM calcium using [3-3H]serine, [1-3H]ethanolamine, and [methyl-3H]choline (10 μCi/μl) or [1-3H]ethanolamine (20 μCi/μl), or [methyl-3H]choline (10 μCi/μl) as substrates (19). In some experiments, as indicated, unlabeled choline or ethanolamine was added to the reaction mixture to final concentrations of 0.5, 5, or 50 mM at pH 7.4.

**Measurement of PSS Activity Using PtdCho or PtdEtn Dispersions**—10 μl of 200 μM PtdCho or PtdEtn dissolved in chloroform were evaporated to dryness under a stream of N2. The phospholipid residue was resuspended in 100 μl of Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20) and then incubated for 30 min on ice with frequent vortexing, and then sonicated until the solution cleared. PtdCho or PtdEtn (60 μl of solution) was added to each PSS assay to a final concentration of 2 mM.

**Tryptsin Treatment of Membranes**—An aliquot of ER membranes or MAM was incubated with trypsin (ratio of membrane protein to trypsin, 2:1 (w/w)) at 37 °C for 15 min. The reaction was terminated by the addition of 20 μl of trypsin inhibitor (40 mg/ml). Treated membranes were diluted in isolation medium, and 25 μg of protein from each fraction was used for the PSS assay.

**Immunoblotting**—Proteins were separated by electrophoresis on 10% (w/v) polyacrylamide gels containing 0.1% SDS and then transferred to polycrylilene difluoride membranes in ice-cold 62.5 mM boric acid (pH 8.0) at 60 V for 1 h. Membranes were blocked by incubation overnight at 4 °C with 5% (w/v) skimmed milk in T-TBS (20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20) and then incubated for 1 h with one antibody (anti-PSS1 (1:2500 dilution), anti-PSS2 (1:2500 dilution), anti-rat protein-disulfide isomerase (1:1000 dilution), or anti-myc (1:50 dilution) antibody) in T-TBS containing 5% (w/v) milk. The membranes were subsequently washed with T-TBS containing 5% (w/v) milk and then incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG for PSS1, PSS2 or protein-disulfide isomerase (1:10,000 dilution), or goat anti-mouse IgG for myc (1:10,000 dilution). Membranes were washed with T-TBS containing 5% (w/v) milk, and bound antibody was detected by enhanced chemiluminescence (Amerham Pharmacia Biotech).

**Other Methods**—Protein concentrations were determined by the BCA method (Pierce) using bovine serum albumin as a standard.
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**RESULTS AND DISCUSSION**

**PtdSer Synthase Exchange Activities in ER and MAM**—We have previously shown that serine exchange activity is present in the ER and is relatively enriched in MAM (1, 2). As a first approach to determining the subcellular locations of PSS1 and PSS2, we compared the substrate specificities for base exchange in subcellular fractions by assessing the ability of unlabeled choline or ethanolamine to inhibit serine exchange activity. Since PSS1 exchanges all three bases (serine, choline, and ethanolamine) in *in vitro* assays, unlabeled choline and ethanolamine would be expected to compete with radiolabeled serine in a serine exchange assay (11). In contrast, since PSS2 catalyzes the exchange of serine and ethanolamine, but not choline, one would expect that ethanolamine, but not choline, would compete with radiolabeled serine in a *in vitro* serine exchange assay. The addition of 5 mM ethanolamine to the serine exchange assay mixture inhibited the serine exchange activity of the ER and MAM by 92 and 85%, respectively (Fig. 1). Although 0.5 and 5 mM choline inhibited the serine exchange activity in MAM by ~40 and 70%, respectively, the serine exchange activity in the ER was not inhibited by 0.5 mM choline and inhibited only slightly, if at all, by 5 mM choline (Fig. 1). Additional data presented below demonstrate that MAM also contain PSS2; therefore, one would not expect the serine exchange activity of MAM to be completely inhibited by choline. These observations suggest that choline exchange activity (*i.e.*, PSS1) is primarily concentrated in the MAM, since the serine exchange activity was much more potently inhibited by choline in the MAM than in the ER. When the choline concentration was increased to 50 mM, inhibition of the serine exchange activity in the MAM increased to 83%, whereas the activity in the ER was inhibited by ~40%. We found that 50 mM choline modestly inhibits the serine exchange activity in M.9.1.1 cells (which lack PSS1), by ~20%. Kuge *et al.* (11) reported that in PSA3 cells (in which PSS1 is defective and choline exchange is <1% of that in parental CHO cells) 5 mM choline inhibited serine exchange activity by ~20%. The partial inhibition by 50 mM choline of the serine exchange activity in the ER probably reflects a combination of some inhibition of PSS2 serine exchange activity by the very high concentration of choline and the presence of small amounts of PSS1 in the ER.

**Sensitivity of Serine, Ethanolamine, and Choline Exchange Activity to Trypsin Treatment of ER and MAM**—In addition to using the free bases for the base exchange reaction, PSS also uses phospholipids as substrates. PSS1 uses both PtdCho and PtdEtn, whereas PSS2 uses only PtdEtn (20). Serine exchange activity was measured in the ER and MAM with exogenously added PtdEtn and PtdCho in the presence of the detergent Triton X-100. Very little serine exchange activity was detected in membranes assayed in the absence of exogenously added phospholipid (Fig. 3) because Triton X-100 inhibits the reaction (19). PtdEtn stimulated the serine exchange activity of both ER and MAM, but the enzyme-specific activity was 39% less in the ER than in MAM (Fig. 3). In addition, PtdCho stimulated the serine exchange activity in MAM (by ~18-fold) to a greater extent than in the ER (by ~6-fold).

A combination of the results presented in Figs. 1–3 suggests that the bulk of PSS1, which is responsible for choline exchange activity, resides in MAM, whereas the ER contains little PSS1. In addition, ethanolamine exchange activity, which is contributed by both PSS1 and PSS2, is present in both ER and MAM. These experiments do not, however, permit us to determine whether the ethanolamine exchange activity in the ER and MAM is contributed by PSS1 or PSS2.

**Localization of PSS1 and PSS2 by Immunofluorescence Microscopy**—The subcellular localization of PSS1 and PSS2 was further investigated using immunofluorescence confocal microscopy. A myc epitope tag was appended to the 5’-end of the
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PSS activity in murine liver MAM and ER in the presence of exogenously added phospholipid substrates. Serine exchange activity was measured in mouse liver subcellular fractions in the presence or absence of exogenous PtdCho or PtdEtn (final concentration 2 mM in 0.04% Triton X-100). No additions, 0.04% Triton X-100. Data are averages ± S.D. of triplicate analyses from two independent experiments.

Immunoblotting Analysis of Localization of PSS1—We next determined by immunoblotting experiments if PSS1 protein was localized to MAM or was distributed throughout the ER. Proteins of MAM isolated from CHO-K1 and M.9.1.1 cells (mutant CHO cells defective in PSS1 activity) (20) were immunoblotted with anti-PSS1 antibody. Fig. 5 shows that PSS1 protein was present in MAM of wild-type CHO-K1 cells but was not detectable in MAM of M.9.1.1 cells (Fig. 5A). In addition, small amounts of PSS1 were detected in microsomes isolated from CHO-K1 cells but not in microsomes from M.9.1.1 cells (not shown). These results were as expected, since PSS1 activity is greatly reduced in M.9.1.1 cells (14, 20) and these cells also lack detectable PSS1 mRNA (16). These observations are consistent with the report of Saito et al. (13), who showed that PSS1 was present in MAM and, to a lesser extent, in microsomes of CHO-K1 cells.

Microsomes are ER-enriched membranes that in addition to ER contain other organelle membranes including MAM. Consequently, the possibility existed that the presence of PSS1 in microsomes was due to MAM rather than the bulk of the ER. We therefore isolated MAM, mitochondria, and two membrane fractions highly enriched in ER from murine liver (18): ER1, which is enriched in rough ER, and ER2, which is enriched in smooth ER. Immunoblotting with anti-PSS1 antibody showed that PSS1 is localized to MAM (Fig. 5B); neither ER1 nor ER2 contained any detectable PSS1 protein (Fig. 5B). Included in Fig. 5B are immunoblots of the same membrane proteins probed with an antibody directed against rat liver PtdEtn methyltransferase-2, a specific marker protein for MAM (5). Similar to PSS1, and in agreement with previous observations (5), PtdEtn methyltransferase-2 was present in MAM but was absent from ER1 and ER2 (Fig. 5B). To confirm that the ER1 and ER2 fractions were indeed derived from the ER, the membrane proteins were also immunoblotted with an antibody directed against protein-disulfide isomerase, a resident ER protein. Protein-disulfide isomerase immunoreactivity was observed in ER1, ER2, and MAM and was absent from mitochondria (Fig. 5B), confirming that ER1 and ER2 are derived from the ER. These immunoblotting data demonstrate that in murine liver PSS1 resides in MAM but is undetectable in the ER and suggest that the PSS1 detected in microsomes of CHO-K1 cells was due to MAM being a constituent of the microsomal preparation.

One possible explanation for the apparent absence of PSS1 from the ER is that proteolytic cleavage of the N terminus of PSS1 occurs, generating a truncated protein that would not be recognized upon immunoblotting with the antibody used (the data in Fig. 5 were generated using an antibody directed against a peptide consisting of amino acids 1–17 of PSS1 from CHO-K1 cells). Therefore, if the ER had contained an isoform of PSS1 that lacked this N-terminal epitope, no immunoreactive protein would have been detected in the ER. However, this scenario is unlikely because Saito et al. (13) previously exam-
immunoblotting with anti-
was expressed in McArdle hepatoma cells and detected by
mito
branes enriched in rough ER;
PSS1 antibody, the membrane was reprobed with anti-rat PtdEtn
addition, data presented in Fig. 6, in which
an antibody directed against the N terminus of PSS1 (amino
exceptionally high content of hydrophobic amino acids in PSS1
dicted from the cDNA sequence (55.3 kDa). This atypical be-
polyacrylamide gel electrophoresis compared with the size pre-
anomalously low apparent molecular mass of 42 kDa upon
cholesterol addition (Fig. 2) supports the conclusion that PSS1 is
highly enriched in MAM and is largely absent
little choline exchange activity (Fig. 2) supports the conclusion
that PSS1 is highly enriched in MAM but undetectable
in the ER. Moreover, the observation that the ER contains
little choline exchange activity (Fig. 2) supports the conclusion
that PSS1 is highly enriched in MAM and is largely absent
from the ER. However, the possibility that an N-terminally
truncated isoform of PSS1 is present in the ER, which would
not have been detected by our immunoblotting experiments,
cannot be discounted.

The C-terminal KK Motif of PSS1 Is Not Required for Target-
ing to MAM—PSS1 contains two lysine residues at the car-boxy terminus of PSS1 (residues 472 and 473, i.e. -KK-COOH) (14, 21). This motif is similar to that of reported ER-targeting consensus sequences (-KKXCOOH or -XXKXX-COOH) (22–24). Since the results presented in Fig. 5 show that PSS1 is highly enriched in MAM, we hypothesized that the KK motif might be required for targeting PSS1 to MAM. The MAM marker protein, PtdEtN N-methyltransferase, similarly contains a highly positively charged C-terminal sequence (-RRKATRLHKRS-COOH) (5). We therefore generated a cDNA en-
coding a mutant form of murine PSS1 in which the two lysine

![Fig. 5. Subcellular distribution of PSS1 in CHO cells and mu-
rine liver. Subcellular membrane fractions were separated from
CHO-K1 and M.9.1.1 cells (A) and murine liver (B). Proteins (50 μg)
were separated by electrophoresis on 10% polyacrylamide gels contain-
ing 0.1% SDS and then transferred to polyvinylidene difluoride mem-
branes and probed with affinity-purified antibody directed against
PSS1. The band corresponding to PSS1 is indicated by the arrow at 42
kDa. A, MAM isolated from CHO-K1 and M.9.1.1 cells. B, ER1, mem-
branes enriched in rough ER; ER2, membranes enriched in smooth ER;
mito, mitochondria. After the membrane had been probed with anti-
PSS1 antibody, the membrane was reprobed with anti-rat PtdEtN N-
methyltransferase-2 (PEMT2) antibody and subsequently with anti-rat
protein-disulfide isomerase (PDI) antibody.

![Fig. 6. Expression of murine cDNAs encoding myc-PSS1 in
McArdle hepatoma cells. The myc epitope was added to the N ter-
minus of murine cDNAs encoding PSS1 and a mutant form of murine
PSS1 from which the C-terminal two lysine residues had been deleted
(designated PSS1:ΔcKK). A, serine exchange activity in cellular lysates
from McArdle cells transfected with empty expression vector (control),
myc-PSS1 (PSS1), and myc-PSS1:ΔcKK (PSS1:ΔcKK). Data are aver-
as ± S.D. of triplicate analyses from three independent experiments.
B, 50 μg of protein from cellular lysates was separated by electrophore-
sis on a 10% polyacrylamide gel containing 0.1% SDS. Proteins were
transferred to polyvinylidene difluoride membranes and incubated with
anti-myc antibody. Recombinant PSS1 proteins are indicated by the
arrow at 42 kDa on the right. C, microsomes (Micr.), MAM, and mito-
chondria (Mito.) were isolated from the same cells, and proteins (50 μg)
were separated by polyacrylamide gel electrophoresis and then immu-
nobotted with anti-myc antibody. McArdle cells were transfected with
empty expression vector (lanes 1), myc-PSS1 (lanes 2), or myc-PSS1:
ΔcKK (lanes 3).]
PS2 is abundant in MAM, little immunoreactive PS2 (Fig. 7 shows the unexpected result that although PS2 was blotted with an antibody directed against a peptide corresponding to the C-terminal sequence of PS2 from CHO cells. Proteins (50 µg) were separated by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS and then transferred to polyvinylidene difluoride membranes and incubated with affinity-purified antibody directed against a synthetic peptide corresponding to a sequence of PS2 from CHO-K1 cells. PS2 is indicated by the arrow at ~52 kDa.

anti-myc antibody. In cells expressing either myc-PS1 or myc-PS1:acKK, the majority of recombinant PS1 protein was found in MAM. Both proteins were also present at lower levels in microsomes but were absent from mitochondria (Fig. 6C). Therefore, deletion of the lysines at positions 472 and 473 did not hinder the targeting of PS1 to MAM. Moreover, the myc-tagged PS1 was predominantly located in the MAM.

PS2 Is Also Localized Specifically to MAM—Since the preceding data demonstrated that PS1 was undetectable in the ER but was highly enriched in the MAM, we hypothesized that the serine and ethanolamine exchange activities in the ER would be imparted by PS2. Support for the idea that the ER contains PS2 comes from the observations that (i) the serine exchange activity in the ER is robustly stimulated by PtdEtn (Fig. 3), (ii) the ER contains ethanolamine exchange activity (Fig. 2), and (iii) serine exchange activity of the ER is inhibited by ethanolamine (Fig. 1). Microsomes, MAM, and mitochondria were isolated from CHO-K1 cells, and the proteins were immunoblotted with an antibody directed against a peptide corresponding to the C-terminal sequence of PS2 from CHO cells. Fig. 7 shows the unexpected result that although PS2 was abundant in MAM, little immunoreactive PS2 (M, ~52 kDa) was detectable in microsomes from CHO cells.

Unfortunately, the anti-PS2 antibody, which is directed against a peptide sequence of PS2 from CHO cells, does not cross-react with murine liver PS2, and all of our attempts to generate an anti-murine PS2 antibody have been unsuccessful. Therefore, as an alternative approach to confirm that PS2 is present primarily in MAM, we generated McArdle cells stably expressing murine PS2 containing a C-terminal myc tag. In these cells, the serine exchange activity (specific activity 5.1 nmol/h/mg of protein) was ~7-fold higher than in control cells transfected with empty vector (specific activity 0.69 nmol/h/mg of protein). Immunoblotting of cellular lysates with an antibody confirmed that C-myc-PS2 protein was expressed in the McArdle cells (Fig. 8A), as indicated by the presence of an ~52-kDa immunoreactive protein that was absent from cells transfected with empty vector alone. Subcellular fractions (microsomes, MAM, and mitochondria) were prepared from McArdle cells expressing C-myc-PS2-expressing cells and control cells, and proteins were immunoblotted with anti-myc antibody. Consistent with the results shown in Fig. 7 for CHO cells, C-myc-PS2 was abundant in MAM but was undetectable in microsomes and mitochondria (Fig. 8B).

We considered the possibility that the apparent absence of PS2 from the ER according to immunoblotting (Fig. 8) was the result of proteolytic cleavage of the myc epitope from the C terminus of the protein in the ER. This possibility was investigated by expression of a cDNA encoding PS2 with a myc tag appended to the N terminus in McArdle cells. N-myc-PS2 protein was also detected in the MAM but not in microsomes by immunoblotting using an anti-myc antibody (data not shown). Since N-myc-PS2 and C-myc-PS2 were similarly localized to the MAM, the presence of the myc tag clearly did not affect their subcellular targeting. The lack of immunoreactive PS2 in the ER was probably not due to proteolytic cleavage of either the N or C terminus to generate a truncated PS2. The data do not, however, eliminate the possibility that a truncated isoform of PS2, from which both the N and C termini have been proteolytically cleaved, is present in the ER, but to our knowledge there are no known examples of such proteins in the ER. We therefore conclude that both PS1 and PS2 are highly enriched in the MAM but are largely excluded from the ER.

Conclusion—The studies presented herein show that full-length PS1 and PS2 are localized almost exclusively to MAM and are largely excluded from the bulk of the ER. However, the possibility that truncated isoforms of these proteins are present in the ER cannot be completely eliminated. The reason why both PS1 and PS2 are so highly enriched in MAM is not clear. Our previous studies have demonstrated that nearly all mitochondrial PtdEtn is derived from imported PtdSer (7). Therefore, one possible explanation for the localization of the two PtdSer synthases in MAM is that a robust synthesis of PtdSer at this site, in close proximity to mitochondrial outer membranes, would provide an efficient mechanism for the import of newly synthesized PtdSer into mitochondria for decarboxylation to PtdEtn. The findings of the present study, however, leave open the question of which protein is responsible for the serine and ethanolamine exchange activities in the ER. Saito et al. (12) have used a CHO-K1 cell line, PSA-3, which is defective in PS1, to generate a mutant cell line, PSB-2. The PSB-2 cells are defective in both PS1 and PS2 and contain
essentially no choline exchange activity. The ethanolamine and serine exchange activities of PSB-2 cells are reduced to 4.8 and 11.5%, respectively, of those in wild-type CHO-K1 cells. It is possible that the residual base exchange activity detected in PSB-2 cells is contributed by a putative “ER isoform” of PSS, which, as indicated by our trypsin proteolysis experiments, might have a different structure and/or a different topological arrangement in the ER membranes compared with PSS1 and PSS2 in the MAM.

Acknowledgments—We thank Russ Watts and Igor Cvetkovic for excellent technical assistance.

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