Phosphatidylserine (PtdSer) in mammalian cells is synthesized through the action of PtdSer synthase (PSS) 1 and 2, which catalyze the conversion of phosphatidylcholine and phosphatidylethanolamine, respectively, to PtdSer. The PtdSer synthesis in intact cells and an isolated membrane fraction is inhibited by exogenous PtdSer, indicating that inhibition of PtdSer synthases by PtdSer is important for the regulation of PtdSer biosynthesis. In this study, to examine whether the inhibition occurs through the direct interaction of PtdSer with the synthases or is mediated by unidentified factor(s), we purified a FLAG and HA peptide-tagged form of Chinese hamster PSS 2 to near homogeneity. The purified enzyme, as well as the crude enzyme in a membrane fraction, was inhibited on the addition of PtdSer to the enzyme assay mixture. In contrast to PtdSer, phosphatidylcholine and phosphatidylethanolamine did not significantly inhibit the purified enzyme. Furthermore, PtdSer-resistant PtdSer synthesis was observed on cell-free assay of the membrane fraction prepared from a Chinese hamster ovary cell strain whose PtdSer synthesis in vivo is not inhibited by exogenous PtdSer. These results suggested that the interaction of PtdSer with PSS 2 or a very minor protein co-purified with PSS 2 was critical for the regulation of PSS 2 activity in intact cells.

Phosphatidylserine (PtdSer) is an essential phospholipid for the growth of mammalian cells (1), comprising ~10% of the total phospholipids in various mammalian tissues and cultured cells. PtdSer is known to interact with various proteins, such as Raf-1 protein kinase C (2), myristoylated alanine-rich C kinase substrate (3), coagulation factor V (4), synaptotagmin (5), and Masahiro Nishijima†‡§ From the Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640 and Tokyo Research Laboratories, Kyowa Hakko Co., Ltd., 3-6-6, Asahimachi, Machida-shi, Tokyo 194-0023, Japan.

PtdSer is localized almost exclusively in the inner leaflet of the lipid bilayer in various types of cells, but externalization of PtdSer to the outer leaflet is observed during apoptosis and after stimulation by various factors such as cytokines (11, 12), inflammatory reactions, and platelet activation (13–16). When externalized on the cell surface, PtdSer has been shown to act as a signal for the removal of damaged, aged, or apoptotic cells. Thus, PtdSer plays many important physiological roles, and therefore the synthesis, degradation, and intracellular localization of PtdSer appear to be strictly regulated.

PtdSer formation in mammalian cells occurs through the exchange of t-serine with the choline moiety of phosphatidylcholine (PtdCho) or the ethanolamine moiety of phosphatidylethanolamine (PtdEtn) (17, 18). The serine exchange in Chinese hamster ovary (CHO) cells is catalyzed by at least two enzymes, PtdSer synthase (PSS) 1 and 2 (17, 18), which are encoded by the pssA and pssB genes, respectively (19, 20). PSS 1 and 2 are responsible for the conversion of PtdCho and PtdEtn, respectively, to PtdSer.

The PtdSer biosynthesis in CHO-K1 cells is remarkably inhibited on the addition of PtdSer to the culture medium (21), indicating that feedback control is involved in the regulation of PtdSer biosynthesis. The activities of PtdSer synthases in the homogenates of CHO-K1 cells grown with and without exogenous PtdSer are essentially the same (21). Therefore, the cellular levels of PSS 1 and 2 appear to remain unchanged upon the addition of PtdSer. In addition, PtdSer inhibits PSS 1 and 2 in an isolated membrane fraction of CHO-K1 cells (22–24). These observations suggest that the inhibition of PtdSer synthases by PtdSer is critical for the feedback control of PtdSer biosynthesis.

A CHO cell mutant, named 29, whose PtdSer biosynthesis is highly resistant to inhibition by exogenous PtdSer, has been isolated from CHO-K1 cells (22). The mutant has been shown to carry a point mutation in the pssA gene, which results in the replacement of Arg-95 of the gene product PSS 1 by Lys (23). Transfection of CHO-K1 cells with the R95K mutant pssA cDNA, but not the wild-type pssA cDNA, induces PtdSer-resistant PtdSer biosynthesis (23). PtdSer synthesis in the isolated membrane fraction of the wild-type pssA-transfected cells is inhibited by exogenous PtdSer but that of the mutant pssA-transfected cells is resistant to inhibition (23). Like mutant 29 cells, the mutant pssA-transfected cells grown without exogenous PtdSer exhibit an ~2-fold increase in the cellular PtdSer level, whereas the wild-type pssA-transfected cells do not exhibit such a significant increase (23). These observations indicate that the inhibition of PSS 1 by PtdSer is critical for the maintenance of a normal PtdSer level in CHO-K1 cells and that Arg-95 of PSS 1 is an essential residue for the normal control of PSS 1 activity.

Chinese hamster PSS 1 and 2 are similar in sequence; there
is 32% amino acid sequence identity between the two synthases (19, 20). PSS 2 has an arginine residue at position 97 that corresponds to Arg-95 of PSS 1, which was identified as a critical residue for the control of PSS 1 activity. PtdSer synthase of PSS 2 is inhibited by exogenous PtdSer in intact cells and a cell-free system (24). Like R59K mutant PSS 1, R97K mutant PSS 2 is resistant to inhibition by exogenous PtdSer (24). In medium without exogenous PtdSer, overproduction of R97K mutant PSS 2 in CHO-K1 cells induces an -4-fold elevation of the PtdSer biosynthetic rate and a 1.6-fold elevation of the cellular PtdSer level compared with those in CHO-K1 cells, although overproduction of wild-type PSS 2 does not induce such elevation (24). Thus, Arg-97 of PSS 2 is a critical residue for the regulation of PSS 2 activity.

Although the inhibition of PSS 1 and 2 by PtdSer seems to be crucial for the maintenance of a normal cellular PtdSer level, as described above, the precise mechanisms underlying the PtdSer-mediated inhibition of PtdSer synthases are currently unknown. Whether the inhibition occurs through direct interaction of PtdSer with the synthases or is mediated by unidentified factor(s) remains unresolved. To address this issue, the purification of a functional PtdSer synthase seems to be important. In this study, we purified a FLAG and HA peptide-tagged form of Chinese hamster PSS 2 to near homogeneity and then examined the effect of PtdSer on the purified enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Anti-FLAG M2 and anti-HA tag affinity gels, 3 × FLAG peptide, HA peptide, bovine brain PtdSer, egg yolk PtdCho, egg yolk PtdEtn, and bovine liver phosphatidylinositol (PtdIns) were purchased from Sigma; [L-14C]serine, [U-14C]choline, and [2-14C]ethanolamine were from Amersham Biosciences. An anti-PSS 2 N-terminal peptide antibody was raised by immunization of rabbits with a multiple antigen peptide corresponding to the 458–474 amino acid residues of Chinese hamster PSS 2. An anti-PSS 2 C-terminal peptide antibody was raised by immunization of rabbits with a multiple antigen peptide corresponding to the 458–474 amino acid residues of Chinese hamster PSS 2. Both antibodies were purified by affinity chromatography with antigen peptide-coupled matrices.

**Plasmids and CHO Cell Strains**

cDNA clones that, respectively, encoded the PSS 1 protein with a FLAG peptide tag or encoded the PSS 2 protein with a HA peptide tag were obtained by means of polymerase chain reaction (PCR). For construction of the PSS 1 fusion cDNA, a sense primer containing a Sal I site, GTCGACGCACCATGATCACAAGCGCGAGCGAGATAG, and an antisense primer containing a Not I site, GCGGCCGCTCATTTTGTTCCACTCCATGTG, and a template plasmid, pDP, SSA (19), were used for PCR. For construction of the PSS 2 fusion cDNA, a sense primer containing a Sal I site, GTCGACGCACCATGCGGGAGGC, an antisense primer containing a Not I site, GCGGCCGCTCATTTTGTTCCACTCCATGTG, and a template plasmid, pDP, SSA (19), were used for PCR. For construction of the cDNA clone encoding the FLAG and HA double-tagged PSS 1, a sense primer containing a Sal I site, ATAGTCGACGCCACCATGATCACAAGCGCGAGCGAGATAG, an antisense primer containing a Not I site, ATAGTCGACGCCACCATGATCACAAGCGCGAGCGAGATAG, and a template plasmid, pSV/F-/H9262, were used for PCR. The PCR products were cleaved with Sal I and Not I and then ligated with pSVKko1 (25) cleaved with the same restriction enzymes. The resultant plasmids were designated pSV/FH-PSSA and pSV/FH-PSSB, respectively. The nucleotide sequences of the constructs were verified with an automated DNA sequencer (ABI PRISM 310; PerkinElmer Life Sciences).

PFA-3 cells (1), which are a PSS1-lacking mutant cell of CHO-K1, were transfected with pSV/FH-PSSA or pSV/FH-PSSB using Lipofectamine Plus reagent (Invitrogen). After selection with G418 (400 μg/ml) (Invitrogen), several colonies resistant to the drug were purified, propagated, and assayed for PtdSer synthase activity. Among pSV/FH-PSSA-transfected PFA-3 clones showing an increase in PtdSer synthase activity, one clone designated as PFA/FH-PSS1 was chosen for further biochemical analyses. Among pSV/FH-PSSB-transfected PFA-3 clones showing an increase in PtdSer synthase activity, one clone designated as PFA/FH-PSS2 was chosen for further biochemical analyses.

**Preparation of Membranes from CHO Cells**

CHO cells (PFA/FH-PSS1 or PSS2 cells) were cultivated in a spinner bottle containing 5 liters of Ham’s F12 medium supplemented with 5% fetal calf serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and NaHCO3 (1.14 g/liter) at 37 °C. After 5 days, cells were harvested by centrifugation at 300 × g for 15 min, washed twice with 80 ml of phosphate-buffered saline, and then solubilized in a buffer consisting of 70% CHAPS, 20% glycerol, 2 mg/ml asolectin, 150 mM NaCl, 0.1% Triton X-100, and 1 mM EDTA. The solubilized membranes were centrifuged at 100,000 × g for 1 h. The resultant precipitate, as the intact membranes, was resuspended in a buffer consisting of 30% glycerol, 2 mg/ml asolectin, 150 mM NaCl, 0.1% Triton X-100, and 1 mM EDTA. The solubilized membranes were used as the enzyme source. For the assay of PSS 1 activity, the membranes were solubilized in a buffer consisting of 30% glycerol, 2 mg/ml asolectin, 150 mM NaCl, 0.1% Triton X-100, and 1 mM EDTA.

**Purification of PFA-2 Having FLAG and HA Double Tags**

A stock suspension of aseologic (50 mg/ml in water) was prepared by sonication at room temperature. Hereafter, all manipulations were performed at 4 °C or on ice. The membrane suspension was centrifuged (100,000 × g for 1 h). The precipitated membranes were suspended in a buffer consisting of 3.5 mM Hepes/NaOH (pH 7.5), 0.1 mM t-serine, 15% (w/v) glycerol, 2 mg/ml asolectin, 150 mM NaCl, 1.4% sucrose monolaurate, incubated for 15 min, and then centrifuged at 100,000 × g for 30 min. The supernatant was recovered as the solubilized membrane fraction. The solubilized membrane fraction was washed three times with 80 ml of phosphate-buffered saline, suspended in 22.5 ml of 0.25 × sucrose containing 10 mM Hepes/NaOH (pH 7.5), and 1 mM EDTA, and then homogenized with a Potter-Elvehjem-type homogenizer. The homogenate was centrifuged at 700 × g for 5 min, followed by centrifugation of the supernatant at 100,000 × g for 1 h. The resultant precipitate, as the intact membranes, was suspended in 0.25 × sucrose containing 10 mM Hepes/NaOH (pH 7.5) and 1 mM EDTA at −10 mg of protein/ml and then stored at −70 °C until use.

**Assaying of PtdSer Synthase (Serine Base Exchange), Ethanolamine Base Exchange, and Choline Base Exchange Activities**

**Method I**—The enzyme source was incubated in 100 μl of a standard assay buffer (50 mM Hepes/NaOH (pH 7.5), 5 mM dGlucose, and 5 mM CaCl2) containing either 0.2 mM [l-14C]serine (10 μCi/μl), 0.2 mM [2-14C]ethanolamine (10 μCi/μl), or 0.2 mM [methyl-3H]choline (20 μCi/μl), at 37 °C for 20 min. The reaction was started by adding the enzyme source. After stopping the reaction by adding 50 μl of 20 mM EDTA containing 0.2 mM t-serine, ethanolamine, or choline, lipids were extracted as described above, and the radioactivity incorporated into lipids (the CHCl3 phase) was measured. The chloroform-soluble materials generated by the exchange with [l-14C]serine, [2-14C]ethanolamine, and [methyl-3H]choline consist of ~80% PtdSer plus 20% PtdEtn, the decarboxylation product of PtdSer, more than 95% PtdEtn, and 0.1% PtdCho.
and more than 95% PtdCho, respectively (27).

Method 2—When inhibition by exogenous phospholipid of the activity was examined, a lipid suspension stock (5 mg/ml in water) was prepared by sonication, and the enzyme source was preincubated with the lipid suspension in 90 μl of 50 mM Hepes/NaOH (pH 7.5) containing 5.6 mg/ml asolectin on ice for about 40 min. After the preincubation, the reaction was started by adding 10 μl of 50 mM CaCl2 containing 2 mM 1-[3H]serine (10 μCi/μmol) or 2 mM [2-14C]ethanolamine (10 μCi/μmol). After stopping the reaction by adding 50 μl of 20 mM EDTA containing either 0.2 M thiosulfate or ethanolamine, lipids were extracted as described (26) and the radioactivity incorporated into lipids (the CHCl3 phase) was measured.

SDS-PAGE, Silver Staining, and Western Blotting

SDS-PAGE was carried out according to a modification of the method of Laemmli (28). Samples for SDS-PAGE were incubated in a SDS-sample buffer (0.1 M Tris/Cl (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, and 13 μg/ml bromophenol blue) at 37 °C for 1 h. Proteins separated on gels were stained with a silver staining kit (Amersham Biosciences). For Western blot analysis, proteins separated by SDS-PAGE were transferred to a cellulose nitrate membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated with the antibodies were detected using a horseradish peroxidase-conjugated secondary antibody. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated with the antibodies were detected using a horseradish peroxidase-conjugated secondary antibody. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated with the antibodies were detected using a horseradish peroxidase-conjugated secondary antibody.

Preparation of Membranes from K1/R97K-pSSB Cells

K1/R97K-pSSB cells were centrifuged in four 150-mm-diameter dishes containing 35 ml of Ham’s F12 medium supplemented with 10% newborn calf serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and NaHCO3 (1.176 g/liter) under a 5% CO2 atmosphere of 7.5% (Fig. 1). However, when the elution buffer used for the FLAG-affinity purification step caused an ~2-fold decrease in the specific activity (Table I), the solubilized membrane fraction was incubated with anti-FLAG antibody-coupled agarose beads. After extensive washing, the proteins bound to the beads were eluted with a buffer including 3 × FLAG peptide. This step was very effective for purification, leading to ~1,000-fold enrichment of PtdSer synthase activity with a ~20% recovery (Table I). The eluent was incubated further with anti-HA antibody-coupled agarose beads, and the proteins bound to the beads were eluted with a buffer including HA peptide. The fraction eluted from the anti-HA antibody beads was used as the purified FH-PSS2 membranes for further characterization. The HA affinity purification step caused an ~2-fold decrease in the specific activity (Table I), probably due to partial inactivation of the enzyme, but was effective for the elimination of contaminating proteins as described below.

The protein patterns of the purified fractions were analyzed by SDS-PAGE followed by silver staining. As shown in Fig. 1, the eluted fraction at the FLAG-affinity purification step gave ~15 visible protein bands, including a band of a major protein with an apparent molecular mass of ~55 kDa, similar to the calculated molecular mass of FH-PSS2, 57,082 Da. The final purified fraction gave the major 55-kDa protein band, three faintly visible protein bands at the positions of molecular masses of 100, 90, and 45 kDa, and two faintly visible protein bands at the positions of molecular masses of 40 and 23 kDa (Fig. 1). However, when the elution buffer used for the affinity purification steps was analyzed by SDS-PAGE, the 40- and 23-kDa doublet bands were observed (Fig. 1, lane 6). Therefore, the doublet bands appeared to originate from the elution buffer; asolectin that was included in the elution buffer for enzyme stabilization was probably contaminated by the 40- and 23-kDa proteins. Thus, the successive FLAG and HA affinity purification yielded the major 55-kDa protein and three

| Table I | Purification of PSS 2 having FLAG and HA peptide tags |
|-----------------|-----------------|-----------------|-----------------|
| Fraction | Protein | Total activity | Specific activity |
| | | ng | mmol/h | μmol/h mg protein | Fold |
| Detergent-treated membranes | 4,300 | 2,700 | 0.63 | 1.0 |
| Solubilized membranes | 3,400 | 2,600 | 0.76 | 1.2 |
| Elution of anti-FLAG antibody beads | 1.0a | 600 | 0.10 | 950 |
| Elution of anti-HA antibody beads | 0.4a | 100 | 0.60 | 700 |

* Protein concentrations were estimated by densitometric comparison of silver-stained proteins in the fraction with stained calibration bands of bovine serum albumin at known concentrations on SDS-PAGE.
minor proteins of 100, 90, and 45 kDa.

Next, we analyzed the purified enzyme by Western blotting with polyclonal antibodies raised against peptides that, respectively, corresponded to the N- and C-terminal 17-amino acid residues of Chinese hamster PSS 2. As shown in Fig. 2, the major 55-kDa protein cross-reacted with both antibodies. This, together with its apparent molecular mass, suggested that the 55-kDa protein was the FH-PSS2 protein. In addition to the 55-kDa protein, the minor 100-, 90-, and 45-kDa proteins also cross-reacted with the anti-N-terminal peptide antibody (Fig. 2), suggesting that the minor proteins were derivatives of the PSS 2 protein, such as degradation products, aggregates, or modified forms of the FH-PSS2 protein. In addition, it was shown that the 100-kDa protein, but not the 90- and 45-kDa proteins, cross-reacted with the anti-C-terminal peptide antibody (Fig. 2) (see “Discussion”).

Enzymatic Characterization of Purified FH-PSS2—The time course of PtdSer formation by the purified enzyme was almost linear for 60 min (Fig. 3A), and this PtdSer formation activity was proportional to the amount of the purified enzyme up to ~2 ng (Fig. 3B). Double reciprocal plots of PtdSer formation versus L-serine showed that the apparent \( K_m \) for L-serine was 89 \( \mu \)M and that the apparent \( V_{\text{max}} \) was 0.29 nmol of PtdSer/ng protein (Fig. 4). Under standard assay conditions, we used asolectin, a heterogeneous phospholipid mixture, as the phospholipid substrate source. When the activity of the purified enzyme was measured in the absence of exogenous asolectin, the activity was very low, as shown in Fig. 5. To examine which phospholipid is used as a substrate, the PtdSer formation was measured as a function of the asolectin, PtdEtn, PtdCho, PtdSer, and PtdIns concentrations (Fig. 5). Among the phospholipids examined, only PtdEtn and asolectin that contained PtdEtn functioned as effective phospholipid substrates (Fig. 5).

Our previous experiments involving CHO-K1 cells overproducing PSS 2 (20) suggested that, in a cell homogenate, PSS 2 was able to catalyze ethanolamine base exchange for PtdEtn formation as well as serine base exchange for PtdSer formation but unable to catalyze choline base exchange for PtdCho formation. To confirm this, the serine, ethanolamine, and choline base exchange activities of the purified enzyme were examined. As shown in Table II, the purified enzyme was able to effectively catalyze serine and ethanolamine base exchange but not choline base exchange, as was expected.

Inhibition of the Purified Enzyme by Exogenous PtdSer—PtdSer formation by the purified enzyme in the presence of asolectin was remarkably inhibited by exogenous PtdSer, as shown in Fig. 6. PtdEtn formation (ethanolamine base exchange) by the purified enzyme was also inhibited by exogenous PtdSer in a similar dose-dependent manner (Fig. 6). In contrast to PtdSer, PtdCho and PtdEtn did not significantly inhibit PtdSer formation by the purified enzyme, and PtdIns inhibited it partially (Fig. 7). Because the inhibition of the purified enzyme by exogenous PtdSer was examined under non-physiological conditions involving a detergent that was contained in the enzyme source, there was the possibility that the inhibition was not related to the inhibition of PtdSer synthesis in intact cells by exogenous PtdSer. To address this point, we took advantage of R97K-mutant PSS 2 (24). The R97K mutation renders PSS 2 resistant to inhibition by exogenous PtdSer (24), and the PtdSer synthesis in a CHO-K1 cell transformant, K1/R97K-pssB, that produces R97K-mutant PSS 2 is not inhibited by exogenous PtdSer (24). As shown in Fig. 8, the enzymatic formation of PtdSer by the solubilized membrane fraction prepared from K1/R97K-pssB cells was highly resistant to inhibition by exogenous PtdSer, although the formation by the crude solubilized membrane fraction prepared from PSA/FH-PSS2 cells producing FH-PSS2 was remarkably inhibited by exogenous PtdSer. These results supported that the inhibition of the purified enzyme by exogenous PtdSer was related to the PtdSer-mediated inhibition of PtdSer synthesis in intact cells.

**Fig. 1.** SDS-PAGE analysis of proteins obtained at different steps of FH-PSS2 purification. Proteins were separated on 5–20% gradient acrylamide gels and then stained with a silver staining kit. Lane 1, molecular mass standards; lane 2, solubilized membrane fraction (0.5 \( \mu \)l); lanes 3 and 4, flow-through fraction (0.5 \( \mu \)l) and eluted fraction (11 \( \mu \)l), respectively, on anti-FLAG M2 affinity chromatography; lane 5, eluted fraction on anti-HA tag affinity chromatography (18 \( \mu \)l); lane 6, elution buffer used for anti-HA tag affinity chromatography (18 \( \mu \)l).

**Fig. 2.** Western blot analysis of purified FH-PSS2 with anti-PSS 2 N- and C-terminal peptide antibodies. Proteins in the purified FH-PSS2 fraction were separated by SDS-PAGE (5–20% gradient acrylamide gel) and analyzed by Western blotting with an anti-PSS 2 N-terminal peptide antibody (N) or an anti-PSS 2 C-terminal peptide antibody (C) as described under “Experimental Procedures.”
DISCUSSION

Purification of enzymes is a crucial step for elucidating their catalytic and regulatory mechanisms. Suzuki and Kanfer (29) purified a PtdSer synthase, which is also known as the serine base exchange enzyme, from rat brain. The purified enzyme also catalyzes ethanolamine base exchange for PtdEtn formation and utilizes PtdEtn, but not PtdCho, as a phospholipid substrate. This substrate specificity of the purified enzyme implies that the purified enzyme is a rat ortholog of Chinese hamster PSS 2. However, the apparent molecular mass (100 kDa) of the purified enzyme determined by SDS-PAGE is about 2-fold larger than the calculated molecular mass of Chinese hamster PSS 2. Thus, it is uncertain whether the purified enzyme is rat PSS 2 or not.

In this study, we purified Chinese hamster PSS 2 tagged with FLAG and HA peptides (FH-PSS2) by successive affinity chromatography. The purification procedures were very effective for enrichment of PtdSer synthase activity and yielded a major 55-kDa protein and minor 100-, 90-, and 45-kDa proteins, all of which cross-reacted with the antibody raised against a PSS 2 N-terminal peptide. Furthermore, the major 55-kDa protein and minor 100-kDa protein also cross-reacted with the antibody raised against a PSS 2 C-terminal peptide. These results, together with the observation that the apparent molecular mass (55 kDa) of the major protein was similar to the

| Substrate      | Specific activity (nmol/h/ng protein) |
|----------------|--------------------------------------|
| Serine         | 0.26 ± 0.04                          |
| Ethanolamine   | 0.51 ± 0.02                          |
| Choline        | <0.01                                |

The activities were measured according to “Method 1” described under “Experimental Procedures.” Values are mean ± S.D. of five (serine) and three (ethanolamine and choline) independent experiments with duplicate determinations.

FIG. 3. PtdSer formation by the purified enzyme fraction. A, time course of the activity. Purified FH-PSS2 was incubated in the standard assay buffer containing L-[14C]serine at 37 °C for various periods according to “Method 1” described under “Experimental Procedures.” The radioactivity incorporated into lipids was then measured as described under “Experimental Procedures.” Values are mean ± S.D. of three independent experiments with duplicate determinations. B, protein dependence of the activity. Various amounts of purified FH-PSS2 were incubated in the standard assay buffer containing L-[14C]serine at 37 °C for 30 min according to “Method 1” described under “Experimental Procedures.” The radioactivity incorporated into lipids was then measured as described under “Experimental Procedures.” Values are mean ± S.D. of three independent experiments with duplicate determinations.

FIG. 4. L-Serine dependence of the purified enzyme activity. Purified FH-PSS2 was incubated in the standard assay buffer containing various concentrations of L-[14C]serine at 37 °C for 20 min, according to “Method 1” described under “Experimental Procedures.” The radioactivity incorporated into lipids was then measured as described under “Experimental Procedures.” Values are mean ± S.D. of three independent experiments with duplicate determinations.

FIG. 5. Effect of various potential phospholipid substrates on PtdSer formation by purified FH-PSS2. Purified FH-PSS2 was incubated at 37 °C for 20 min, according to “Method 1” described under “Experimental Procedures,” in a buffer consisting of various phospholipids at the indicated concentrations, 50 mM Hepes/NaOH, 5 mM CaCl2, and 0.2 mM L-[14C]serine. The radioactivity incorporated into lipids was then measured as described under “Experimental Procedures.” Values are mean ± S.D. of three independent experiments with duplicate determinations.
respectively. Values are mean ± S.D. of three independent experiments with duplicate determinations.

Obtained through affinity purification using C-terminal FLAG and HA peptide tags. A possible explanation for this is that the FH-PSS2 protein exists as a multimeric form and the C-terminal-lacking protein that was associated with the full-length protein was obtained through affinity purification. Alternatively, after affinity purification small portions of purified proteins might be degraded to a C-terminal peptide-lacking form.

The purified FH-PSS2 was shown to catalyze serine and ethanolamine base exchange for PtdSer and PtdEtn formation, but not choline base exchange for PtdCho formation. In addition, the purified enzyme was shown to use PtdEtn, but not PtdCho, as a phospholipid substrate. These results were consistent with the PSS 2 substrate specificity that was suggested by experiments involving the crude enzyme or intact cells (1, 20, 30, 31). Thus, the substrate specificity of PSS 2 was directly confirmed using the purified enzyme.

The PtdSer-mediated inhibition of PSS 1 and 2 was shown to be critical for the maintenance of a normal cellular PtdSer level in CHO-K1 cells (22–24). However, it remained to be elucidated whether the inhibition occurs through direct interaction between PtdSer and PtdSer synthases or is mediated by unidentified factor(s). In this study, we showed that the purified FH-PSS2 was inhibited by exogenous PtdSer. In contrast to PtdSer, PtdCho and PtdEtn did not significantly inhibit the purified enzyme. PtdIns partially inhibited the purified enzyme. PtdIns partially inhibited the purified enzyme.

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PSS 2. PtdSer synthesis by the solubilized membrane fraction prepared from K1/R97K-pssB cells was shown to be highly resistant to inhibition by exogenous PtdSer, whereas PtdSer synthesis by the purified FH-PSS2 and the crude solubilized membrane fraction prepared from a CHO strain producing FH-PSS2 was strikingly inhibited by exogenous PtdSer. Thus, inhibition of the purified enzyme by PtdSer appeared to be related to the PtdSer-mediated inhibition of Ptdser synthesis observed in intact cells. These results, taken together, suggested that the interaction of PtdSer with PSS 2 or a very minor protein co-purified with PSS 2 was critical for the regulation of PSS 2 activity and thus for the control of PtdSer synthesis in intact cells.

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