Phosphatidylserine (PS) in mammalian cells is synthesized through the exchange of free l-serine with the base moiety of phosphatidylcholine or phosphatidylethanolamine (PE). The serine base exchange in Chinese hamster ovary (CHO) cells is catalyzed by at least two enzymes, PS synthase (PSS) I and II. A PSS I-lacking mutant of CHO-K1 cells, PSA-3, which exhibits ~2-fold lower serine base exchange activity than CHO-K1, is defective in the conversion of phosphatidylcholine to PS but has the ability to convert PE to PS. The PSA-3 mutant requires exogenous PS or PE for cell growth. In the present study, from PSA-3 mutant cells, we isolated a mutant, named PSB-2, with a further decrease in the serine base exchange activity. The activity in the homogenate of PSB-2 mutant cells was ~10% that of PSA-3 mutant cells and ~5% that of CHO-K1 cells. The PSB-2 mutant exhibited an ~80% reduction in the PSS II mRNA level relative to that in PSA-3 mutant and CHO-K1 cells. These results showed that the PSB-2 mutant is defective in PSS II. Like the PSA-3 mutant, the PSB-2 mutant grew well in medium supplemented with PS. However, in the medium supplemented with PE, the PSB-2 mutant was incapable of growth, in contrast to the PSA-3 mutant. In the medium with exogenous PE, the PSB-2 mutant was defective in PS biosynthesis, whereas the PSA-3 mutant synthesized a normal amount of PS. A metabolic labeling experiment with exogenous [32P]PE revealed that the PSB-2 mutant was defective in the conversion of exogenous PE to PS. This defect and the growth and PS biosynthetic defects of the PSB-2 mutant cultivated with exogenous PE were complemented by the PSS II cDNA. In addition, the cDNA of the other PS synthase, PSS I, was not shown to complement the defect in the conversion of exogenous PE to PS of the PSB-2 mutant, implying that PSS I negligibly contributes to the conversion of PE to PS in CHO-K1 cells. These results indicated that PSS II is critical for the growth and PS biosynthesis of PSA-3 mutant cells cultivated with exogenous PE and suggested that most of the PS formation from PE in CHO-K1 cells is catalyzed by PSS II.

Phosphatidylserine (PS) is one of the major phospholipids in mammalian cells, comprising about 10% of the total membrane phospholipids. To elucidate the mechanism underlying PS biosynthesis in mammalian cells, we have isolated Chinese hamster ovary (CHO) cell mutants defective in PS metabolism (1–4). One of the mutants, PSA-3 (3), isolated through screening for PS auxotrophic mutants, has been successfully utilized for studying the PS biosynthetic pathway, as described below. The availability of PSA-3 mutant cells has made it possible to isolate the CHO cDNA of a PS synthase (PSS) gene, pssa, which is able to confer PS prototrophy on the mutant (5, 7). Recently, the CHO cDNA of another PS synthase gene, pssB, was isolated through a computer search for sequences similar to those of the pssa gene product (6).

Both the pssa gene product, PSS I, and pssB gene product, PSS II, catalyze the exchange of free l-serine with the polar head group (base) of preexisting phospholipid for PS formation (3, 5, 6). In addition to serine base exchange, PSS I can catalyze the exchange of the base moiety of phospholipid with choline and ethanolamine, whereas PSS II can catalyze serine and ethanolamine base exchange, but not choline base exchange (3, 5, 6). The serine and ethanolamine base exchange activities in a homogenate of PS auxotrophic PSA-3 mutant cells are decreased to ~50% of those in the parental strain, CHO-K1, and choline base exchange activity is virtually absent in the mutant cell homogenate (3). The PSA-3 mutant has no detectable amounts of pssa mRNA and its product, PSS I (5, 7). These observations indicate that the PSA-3 mutant is defective in PSS I. The PSS I-defective PSA-3 mutant is incapable of converting exogenous phosphatidylcholine (PC) to PS, suggesting that PSS I catalyzes the exchange of the choline moiety of PC with serine for PS formation in CHO-K1 cells (8). When the PSA-3 mutant is cultivated without exogenous phospholipids for 2 days, the cellular levels of PS and phosphatidylethanolamine (PE), the majority of which in CHO-K1 cells is synthesized through the decarboxylation of PS (9, 10), are reduced by ~70 and ~50%, respectively (3). The defects in the PS and PE biosynthesis of the PSA-3 mutant are complemented by the pssa cDNA (5). This cDNA also complements the defect in the conversion of PC to PS of the PSA-3 mutant.2 Thus, PSS I is responsible for the PS formation from PC in CHO-K1 cells, and it functions as an essential enzyme for the production of normal amounts of PS and PE in CHO-K1 cells cultivated without phospholipid supplementation.

The PSS I-defective PSA-3 mutant, as well as CHO-K1, has the ability to convert exogenous PE to PS (8). On the addition of exogenous PE, PSS I catalyzes the exchange of the choline moiety of PE with serine for the formation of PS (1). The availability of PSA-3 mutant cells has made it possible to isolate the CHO cDNA of a PS synthase (PSS) gene, pssa, which is able to confer PS prototrophy on the mutant (5, 7). Recently, the CHO cDNA of another PS synthase gene, pssB, was isolated through a computer search for sequences similar to those of the pssa gene product (6).
of PE to the medium, PSA-3 mutant cells recover the normal abilities to synthesize PS and to grow (8). Because two PS synthases, PSS I and II, exist in CHO-K1 cells, it is possible that PSS II is responsible for the PE-dependent restoration of PS biosynthesis in the PSA-3 mutant. However, the existence of more than two PS synthases in CHO-K1 cells has not been ruled out. In addition, it has not been resolved whether PSS I exclusively catalyzes the conversion of PC to PS or also catalyzes the conversion of PE to PS. Thus, the question of what enzyme or enzymes are responsible for the PS formation from PE in CHO-K1 cells remains to be elucidated. In the present study, we isolated CHO cell mutants defective in PSS II from PSA-3 cells to address the function of this enzyme. The results presented here suggest that the pssB gene product, PSS II, functions as the principal enzyme in the PS formation from PE in CHO-K1 cells.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—CHO-K1 cells were obtained from the American Type Culture Collection and routinely maintained in Ham's F-12 medium (ICN Biomedicals) supplemented with 10% (v/v) newborn calf serum (Biological Industries), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1.176 g/liter NaHCO₃ at 37 °C under a 5% CO₂ atmosphere and 100% humidity. PSA-3 cells (3) and PSB-1, PSB-2, and PSB-2/pssB cells obtained in this study were maintained under the same culture conditions except that the medium was supplemented with 30 μg/PS from (bovine brain; Sigma) liposomes prepared as described (11). PSB-2/pssA cells obtained in this study were maintained under the same growth conditions as those for CHO-K1 cells. J774.1 cells were obtained from the American Type Culture Collection and maintained under the same culture conditions as those for CHO-K1 cells, except that the medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum (Atlanta Biologicals) instead of newborn calf serum. The suspension of PE (from egg yolk; Sigma) added to the growth medium was prepared as described (11). The cells were obtained in this study were maintained in 500 ml of growth medium and incubated at 33 °C for 2 days. After additional culture in F-12 medium (ICN Biomedicals) supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate and then labeled with 400 μCi of [32P] orthophosphate (Amersham Pharmacia Biotech), the cells were overlaid with a polyester disc containing 2 ml of an assay mixture consisting of 50 mM HEPES (pH 7.5), 5 mM CaCl₂, cycloheximide (100 μM), and 40 μM l-[U-14C]serine (20 μCi/μmol; Amersham Pharmacia Biotech) for 20 min at 30 °C and then washed with PBS and then grown in Ham's F-12 medium supplemented with 300 colonies/dish. On day 7, the cells were divided into a polystyrene disc (Whatman No. 50), and glass beads (12), in that order. The cells were further cultivated with a medium change on days 10, and 15, and 20. On day 20, the polystyrene disc bearing immobilized colonies was transferred to a new dish containing 7 ml of the same growth medium and incubated at 33 °C for 2 days. After additional cultivation for 3 days, the polystyrene disc was subjected to two-dimensional thin-layer chromatography (11). The radioactive phospholipids separated by two-dimensional thin-layer chromatography (11). After the isolation of the spots of [32P]PE and [32P]PC on silica gel plates had been located by autoradiography, these [32P]-labeled phospholipids were scraped off and then extracted from the silica gel as described (16). The labeled lipids were dried up under a nitrogen stream and then sonicated in the growth medium using a bath-type sonicator.

Isolation of the PSA-2 Mutant Transfected with the pssB or pssA cDNAs—The plasmids pSVpsBNeo and pSVpsAneo (15), which carry 10% (v/v) fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate and then labeled with 400 μCi of [32P] (the Japan Atomic Energy Institute, Ibaragi, Japan) per dish at 37 °C for 3 days. The labeled cells were washed with phosphate-buffered saline and then harvested. Phospholipids were extracted from the labeled cells and then separated by two-dimensional thin-layer chromatography (11). After the separation of the spots of [32P]PE and [32P]PC on silica gel plates had been located by autoradiography, these [32P]-labeled phospholipids were scraped off and then extracted from the silica gel as described (16). The labeled lipids were dried up under a nitrogen stream and then sonicated in the growth medium using a bath-type sonicator.

Isolation of Mutants with Decreased Serine Base Exchange Activity by Means of In Situ Colony Assaying—For mutagenesis, exponentially growing cells were treated with 300 μg/ml ethyl methanesulfonate (Sigma) at 33 °C for 18 h, followed by cultivation without ethyl methanesulfonate at 33 °C for 3 days. The mutagenized cells were seeded to a 100-mm-diameter dish containing 10 ml of growth medium supplemented with 300 colonies/dish and then grown for 300 colonies/dish. On day 7, the cells were overlaid with a polystyrene disc (12), a filter paper (Whatman No. 50) (13), and glass beads (12), in that order. The cells were further cultivated with a medium change on days 10, and 15, and 20. On day 20, the polystyrene disc bearing immobilized colonies was transferred to a new dish containing 7 ml of the same growth medium and incubated at 33 °C for 2 days. After additional cultivation for 3 days, the polystyrene disc was subjected to two-dimensional thin-layer chromatography (11). The radioactive phospholipids separated by two-dimensional thin-layer chromatography (11). After the isolation of the spots of [32P]PE and [32P]PC on silica gel plates had been located by autoradiography, these [32P]-labeled phospholipids were scraped off and then extracted from the silica gel as described (16). The labeled lipids were dried up under a nitrogen stream and then sonicated in the growth medium using a bath-type sonicator.

Isolation of Mutants with Decreased Serine Base Exchange Activity by Means of In Situ Colony Assaying—For mutagenesis, exponentially growing cells were treated with 300 μg/ml ethyl methanesulfonate (Sigma) at 33 °C for 18 h, followed by cultivation without ethyl methanesulfonate at 33 °C for 3 days. The mutagenized cells were seeded to a 100-mm-diameter dish containing 10 ml of growth medium supplemented with 300 colonies/dish and then grown for 300 colonies/dish. On day 7, the cells were overlaid with a polystyrene disc (12), a filter paper (Whatman No. 50) (13), and glass beads (12), in that order. The cells were further cultivated with a medium change on days 10, and 15, and 20. On day 20, the polystyrene disc bearing immobilized colonies was transferred to a new dish containing 7 ml of the same growth medium and incubated at 33 °C for 2 days. After additional cultivation for 3 days, the polystyrene disc was subjected to two-dimensional thin-layer chromatography (11). The radioactive phospholipids separated by two-dimensional thin-layer chromatography (11). After the isolation of the spots of [32P]PE and [32P]PC on silica gel plates had been located by autoradiography, these [32P]-labeled phospholipids were scraped off and then extracted from the silica gel as described (16). The labeled lipids were dried up under a nitrogen stream and then sonicated in the growth medium using a bath-type sonicator.

RESULTS

Isolation of PSS II-Defective Mutants from PSA-3 Mutant Cells—A PSS I-defective mutant of CHO-K1 cells, PSA-3, exhibited a decrease in serine base exchange activity below that of CHO-K1 cells when the activity was determined using cell homogenates (3). To isolate mutants with a further decrease in the serine base exchange activity, PSA-3 mutant cells were mutagenized and screened by means of an in situ assay for serine base exchange. Among approximately 50,000 colonies of mutagenized cells, a mutant clone, named PSB-1, with signifi-
serine base exchange activity was found. The serine base exchange activity in the homogenate of the PSB-1 mutant cells was ~50% of that in the PSA-3 homogenate (Table I). The ethanolamine base exchange activity in the PSB-1 homogenate was also decreased to ~50% of that in the PSA-3 homogenate (Table I). Regardless of the decreases in the serine and ethanolamine base exchange activities, the PSB-1 mutant showed no remarkable alteration in cell growth, PS biosynthesis, or phospholipid composition compared with the parental strain, PSA-3 (data not shown).

To obtain mutants with further decreased serine base exchange activity, PSB-1 mutant cells were subjected to second mutagenesis and successive screening by means of the in situ serine base exchange assay. Among approximately 10,000 colonies of mutagenized cells, a mutant clone, named PSB-2, with further decreased serine base exchange activity was found. The serine base exchange activity in the homogenate of the PSB-2 mutant cells was ~20% of that in the PSB-1 homogenate, ~10% of that in the PSA-3 homogenate, and ~5% of that in the CHO-K1 homogenate (Table I). The ethanolamine base exchange activity in the PSB-2 homogenate was also decreased, in proportion to the decrease in the serine base exchange activity (Table I). The choline base exchange activity in the PSB-2 homogenate was negligible, as observed in the PSA-3 homogenate (Table I), indicating that the PSB-2 mutant remained defective in PSS I activity.

CHO-K1, PSA-3, PSB-1, and PSB-2 cells were examined as to the level of the mRNA of pssB-encoded PSS II, which catalyzes serine and ethanolamine base exchange, but not choline base exchange (6). Poly (A)+ RNAs from these cells cultivated with exogenous PS were probed with a 32P-labeled pssB cDNA fragment. As shown in Fig. 1A, a 2.4-kilobase pssB-specific mRNA was detected among the RNAs from all the cell lines. The levels of the pssB mRNA in CHO-K1 and PSA-3 cells were similar to each other (Fig. 1A). However, the pssB mRNA levels in PSB-1 and PSB-2 cells were decreased by ~40 and ~80% relative to those in CHO-K1 and PSA-3 cells, respectively (Fig. 1A), when the radioactivity of the 2.4-kilobase pssB-specific band was determined using a bioimage analyzer (FUJIX BAS2000). The levels of an internal standard, β-actin mRNA, in all the cell lines were similar, as determined by reprobing with the β-actin cDNA fragment (Fig. 1B). These results, obtained through Northern blot analysis and enzyme assay for phospholipid base exchange, indicated that the PSB-2 mutant is defective in PSS II.

**Growth and PS Biosynthesis of the PSB-2 Mutant—**The PSS I-defective PSA-3 mutant and the PSS I- and PSS II-defective PSB-2 mutant were incapable of growth in the medium without phospholipid supplementation, whereas CHO-K1 cells with normal PSS I and II activities were able to grow well in this medium (Fig. 2). Although the growth defect of the PSA-3 mutant was suppressed by the addition of either PS or PE to the medium, exogenous PS, but not exogenous PE, suppressed the growth defect of the PSB-2 mutant (Fig. 2). These results implied that PSS II is required for the exogenous PE-dependent restoration of cell growth of the PSA-3 mutant.

The phospholipid compositions of CHO-K1, PSA-3, and PSB-2 cells cultivated in the medium without phospholipid supplementation or in the medium supplemented with exogenous PS or PE were determined. Both the PSB-2 and PSA-3 mutant cells cultivated without phospholipid supplementation exhibited significant decreases in the levels of PS and its metabolite, PE, as compared with those in CHO-K1 cells (Table II). On cultivation with exogenous PS, the levels of PS and PE

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### Table I

The serine, ethanolamine, and choline base exchange activities in homogenates of CHO-K1, PSA-3, PSB-1, and PSB-2 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Serine</th>
<th>Ethanolamine</th>
<th>Choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td></td>
<td>4.2</td>
<td>5.2</td>
<td>1.6</td>
</tr>
<tr>
<td>PSA-3</td>
<td></td>
<td>2.0</td>
<td>4.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PSB-1</td>
<td></td>
<td>1.0</td>
<td>2.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PSB-2</td>
<td></td>
<td>0.2</td>
<td>0.6</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Northern blot analysis of CHO-K1, PSA-3, PSB-1, and PSB-2 cells with pssB (A) and β-actin (B) probes. An equal amount (2.5 μg) of poly (A)+ RNA prepared from cells grown in the medium supplemented with 30 μM PS at 39.5 °C was applied to each lane. Lane 1, PSB-2; lane 2, PSB-1; lane 3, PSA-3; lane 4, CHO-K1.

**Fig. 2.** Growth of CHO-K1, PSA-3, and PSB-2 cells. Cells were seeded at 2.5 × 10⁴ cells per 60-mm-diameter dish in growth medium supplemented with 30 μM PS (○), 30 μM PE (●), or without phospholipid supplementation (△) and then grown at 39.5 °C. At the times indicated, cells were dispersed with 0.25% (w/v) trypsin and then counted with a Coulter model ZB1 counter.
TABLE II

Phospholipid compositions of CHO-K1, PSA-3, and PSB-2 cells

Cells were grown with 30 μM exogenously added PS or PE, or without phospholipid supplementation at 39.5 °C for 3 days. Phospholipids were extracted from the cells and separated by two-dimensional thin-layer chromatography as described under “Experimental Procedures.” The amount of each phospholipid was determined chemically as described (19). The data are for one of two experiments with similar results. PI, phosphatidylinositol; SM, sphingomyelin.

<table>
<thead>
<tr>
<th>Phospholipid supplementation</th>
<th>Strain</th>
<th>PS</th>
<th>PE</th>
<th>PC</th>
<th>PI</th>
<th>SM</th>
<th>Other*</th>
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<tbody>
<tr>
<td>None</td>
<td>CHO-K1</td>
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<td>16.6</td>
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<td>7.2</td>
<td>8.4</td>
<td>9.7</td>
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<tr>
<td></td>
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<td>11.7</td>
<td>64.3</td>
<td>8.4</td>
<td>7.3</td>
<td>4.6</td>
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<tr>
<td></td>
<td>PSB-2</td>
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<td>71.4</td>
<td>8.4</td>
<td>6.2</td>
<td>4.3</td>
</tr>
<tr>
<td>PS</td>
<td>CHO-K1</td>
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<td>58.1</td>
<td>6.4</td>
<td>8.7</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>PSA-3</td>
<td>6.2</td>
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<td>54.8</td>
<td>7.2</td>
<td>8.8</td>
<td>4.7</td>
</tr>
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<td>51.7</td>
<td>8.2</td>
<td>12.6</td>
<td>4.9</td>
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<td>PE</td>
<td>CHO-K1</td>
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<td>45.9</td>
<td>5.8</td>
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<td>46.0</td>
<td>8.2</td>
<td>8.7</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Other lipids include lyso phosphatidylcholine, phosphatidylglycerol, phosphatidic acid, and cardiolipin.

in the two mutant cells were restored to normal levels, similar to those in CHO-K1 cells (Table II). In the medium supplemented with PE, PSA-3 and CHO-K1 cells exhibited similar phospholipid compositions, as reported previously (8), but PSB-2 cells exhibited a remarkably lower PS level than that in PSA-3 and CHO-K1 cells (Table II). The levels of other major phospholipids such as PE, PC, phosphatidylinositol, and sphingomyelin in PSB-2 cells were similar to those in PSA-3 and CHO-K1 cells on cultivation with exogenous PE (Table II). To determine the biosynthetic rate for PS, PSA-3, and CHO-K1 cells were metabolically labeled with L-[14C]serine, and then the incorporation of L-[14C]serine into PS was determined. In the absence of phospholipid supplementation, the PS biosynthetic rates in PSB-2 and PSA-3 mutant cells were less than 10% of that in CHO-K1 cells (Fig. 3A). In the presence of exogenous PE, the PS biosynthetic rate in PSB-3 mutant cells was similar to that in CHO-K1 cells, as reported previously (8), but the rate in PSB-2 mutant cells was 2–3-fold lower than that in CHO-K1 cells (Fig. 3B). These results implied that PSS II is required for the exogenous PE-dependent restoration of PS biosynthesis in the PSA-3 mutant.

The PSB-2 Mutant Is Defective in the Conversion of Exogenous PE to PS—The PSS-3 mutant is defective in the conversion of exogenous PE to PE, because of the defect in PSS I. To determine whether or not the PSB-2 mutant is defective in the conversion of exogenous PE to PS, PSB-2 and PSA-3 mutant cells were cultivated with [32P]PE (Fig. 3A) or growth medium supplemented with 30 μM PE at 39.5 °C for 24 h. At zero time, the medium was replaced with the same growth medium except that it contained 0.2 μCi/ml L-[U-14C]serine (2 μCi/μmol), and then the cells were incubated at 39.5 °C for the times indicated. Phospholipids were extracted from the cells and separated by one-dimensional thin-layer chromatography as described under “Experimental Procedures.” The radioactivity of L-[U-14C]serine incorporated into PS was determined using a bioimage analyzer (FUJIX BAS2000) and then normalized as to the cell number at zero time. The data are the means for duplicate determinations.

Incorporation of L-[U-14C]serine into PS. Cells were seeded in growth medium supplemented with 30 μM PS at 2–4×10^7 cells per 60-mm-diameter dish and then grown at 33 °C for 2 days. Subsequently, the cells were preincubated in growth medium without phospholipid supplementation (A) or growth medium supplemented with 30 μM PE (B) at 39.5 °C for 24 h. At zero time, the medium was replaced with the same growth medium except that it contained 0.2 μCi/ml L-[U-14C]serine (2 μCi/μmol), and then the cells were incubated at 39.5 °C for the times indicated. Phospholipids were extracted from the cells and separated by one-dimensional thin-layer chromatography as described under “Experimental Procedures.” The radioactivity of L-[U-14C]serine incorporated into PS was determined using a bioimage analyzer (FUJIX BAS2000) and then normalized as to the cell number at zero time. The data are the means for duplicate determinations.

The PS-2 Mutant Transfected with the pssB cDNA Recovers the Exogenous PE-dependent Growth and PS Biosynthesis—In contrast to the PSS I-defective PSA-3 mutant, the PSS I- and PSS II-defective PSB-2 mutant was incapable of growth and defective in PS biosynthesis in the medium with exogenous PE, as described above. To verify that these growth and PS biosynthetic defects of the PSB-2 mutant are due to the deficiency in the pssB gene product, PSS II, we transfected PSB-2 mutant cells with a plasmid, pSVpssB1/neo, which carries the pssB cDNA from CHO-K1 cells and a G418-resistant gene. From the resultant G418-resistant stable transfectants, a clone that exhibited an increase of serine base exchange activity but did not have much higher serine base exchange activity than the PSA-3 mutant was selected for further characterization, because the overproduction of PSS II might have secondary effects on growth and PS biosynthesis (6). The serine and ethanolamine base exchange activities in the homogenate of the selected transfectant, PSB-2/pssB, were both ~8-fold those in the PSB-2 homogenate and similar to those in the PSA-3 homogenate (Table III), indicating that the PSS II activity in this transfectant was comparable to that in the PSA-3 mutant. The choline base exchange activity in the PSB-2/pssB homogenate was negligible, indicating that the PSB-2/pssB transfectant remained defective in PSS I activity (Table III). In the medium without phospholipid supplementation, the PSB-2/pssB transfectant remained incapable of growth (Fig. 5A), and the PS and PE levels in this transfectant remained low (Table IV). In the medium with exogenous PE, the PSB-2/pssB transfectant, in contrast to the PSB-2 mutant, grew well (Fig. 5A) and exhibited a normal phospholipid composition, similar to that in PSA-3 and CHO-K1 cells (Table IV). Thus, it is very likely that the deficiency in PSS II is the cause of the growth and PS biosynthetic defects of the PSB-2 mutant cultivated with exogenous PE.

Growth and PS Biosynthesis of pssA-transfected PSB-2 Mutant Cells—The PSB-2 mutant was defective not only in PSS II

FIG. 3. Incorporation of L-[U-14C]serine into PS. Cells were seeded in growth medium supplemented with 30 μM PS at 2–4×10^7 cells per 60-mm-diameter dish and then grown at 33 °C for 2 days. Subsequently, the cells were preincubated in growth medium without phospholipid supplementation (A) or growth medium supplemented with 30 μM PE (B) at 39.5 °C for 24 h. At zero time, the medium was replaced with the same growth medium except that it contained 0.2 μCi/ml L-[U-14C]serine (2 μCi/μmol), and then the cells were incubated at 39.5 °C for the times indicated. Phospholipids were extracted from the cells and separated by one-dimensional thin-layer chromatography as described under “Experimental Procedures.” The radioactivity of L-[U-14C]serine incorporated into PS was determined using a bioimage analyzer (FUJIX BAS2000) and then normalized as to the cell number at zero time. The data are the means for duplicate determinations.
FIG. 4. The PSB-2 mutant is defective in the conversion of exogenous PE to PS. Cells were seeded in growth medium supplemented with 30 μM PS at 5–10 \times 10^5 cells per 100-mm-diameter dish and then grown at 39.5 °C for 3 days. After incubation in the growth medium without phospholipid supplementation for 2 h, the cells were cultivated in growth medium containing 10^6 cpm/ml of [32P]PE for the times indicated at 39.5 °C. Phospholipids were extracted from the cells and separated by two-dimensional thin-layer chromatography as described under “Experimental Procedures.” The radioactivities of PS and PE on the chromatogram were determined using a bioimage analyzer (FUJIX BAS2000) and then normalized as to the cell numbers of parallel unlabeled cultures at the times indicated. The data are for one of two experiments with similar results.

TABLE III

The serine, ethanolamine, and choline base exchange activities in homogenates of the PSB-2/pssB and PSB-2/pssA transfectants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Serine (nmol/1 mg protein)</th>
<th>Ethanolamine</th>
<th>Choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSB-2/pssB</td>
<td>2.0</td>
<td>4.5</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>PSB-2/pssA</td>
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<td>PS-2</td>
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</tr>
<tr>
<td>PS-3</td>
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<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>CHO-K1</td>
<td>4.2</td>
<td>5.2</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

The preparation of cell homogenates and assaying of the serine, ethanolamine, and choline base exchange activities were performed as described in Table I. The values indicate specific activities and are the means for duplicate determinations varying by <10%.

but also in the pssA-encoded PSS I. To examine the effects of expression of pssA in the PSB-2 mutant on growth and PS biosynthesis, we transfected this mutant with a plasmid, pSSA, and a G418-resistant gene. The resultant G418-resistant transfectant seemed to be 50% of that in CHO-K1 cells. The PSB-2/pssA transfectant grew normally in the medium supplemented with PS or PE (data not shown), and even in the medium without phospholipid supplementation (Fig. 5B). The PSB-2/pssA transfectant grown in the medium without phospholipid supplementation exhibited a normal phospholipid composition, similar to that of CHO-K1 cells (Table IV).

The Defect in the Conversion of PE to PS in the PSB-2 Mutant Is Complemented by the pssB cDNA, But Not by the pssA cDNA—To determine whether or not the defect in the conversion of exogenous PE to PS of the PSB-2 mutant was complemented by each of the pssB and pssA cDNAs, the PSB-2/pssB and PSB-2/pssA transfectants, together with PSA-3, PSB-2, and CHO-K1 cells, were cultivated with [32P]PE, and then the radioactivity of cellular PS was determined. As shown in Fig. 6, the activity of conversion of exogenous PE to PS of the PSB-2/pssB transfectant was <5% of that of PSB-2 cells. The PSB-2/pssA transfectant was defective in the conversion of exogenous PE to PS, as shown in Fig. 7. To determine whether or not this defect of the PSB-2 mutant is complemented by each of the pssB and pssA cDNAs, the PSB-2/pssB and PSB-2/pssA transfectants were cultured with [32P]PC, and then the radioactivity of cellular PS was determined. As shown in Fig. 8, the activity of conversion of exogenous PE to PS of the PSB-2/pssB transfectant was <5% of that of PSB-2 cells. The PSB-2/pssA transfectant was defective in the conversion of exogenous PE to PS, as shown in Fig. 9. To determine whether or not this defect of the PSB-2 mutant is complemented by each of the pssB and pssA cDNAs, the PSB-2/pssB and PSB-2/pssA transfectants were cultivated with [32P]PC, and then the radioactivity of cellular PS was determined. The PSB-2/pssB transfectant showed negligible activity of conversion of exogenous PE to PS, but the PSB-2/pssA transfectant exhibited activity comparable to that of CHO-K1 cells. These results indicated that the defect in the...
The serine base exchange in CHO-K1 cells is catalyzed by at least two enzymes, the pssA gene product, PSS I, and the pssB gene product, PSS II (Refs. 3 and 5–7; for a review, see Ref. 22). On the characterization of a PSS I-defective mutant of CHO-K1 cells, PSA-3, PSS I was shown to function as a critical enzyme in the production of PS and PE in CHO-K1 cells (3, 5). In the present study, we tried to isolate PSS II-defective mutants to address the function of PSS II in CHO-K1 cells. A CHO cell mutant, named PSB-2, isolated from PSA-3 mutant cells was shown to be defective in PSS II by several lines of evidence. First, the serine base exchange activity of the PSB-2 mutant was decreased to ~10% of that of the PSA-3 mutant and ~5% of that of CHO-K1 cells. Second, the activity of ethanolamine base exchange, which PSS II is capable of catalyzing (6), of the PSB-2 mutant was also decreased, being less than 15% of that of PSA-3 and CHO-K1 cells. Third, the defects of the PSB-2 mutant in the serine and ethanolamine base exchange activities were complemented by the pssB cDNA. Finally, the level of pssB mRNA in PSB-2 cells was ~20% of that in PSA-3 and CHO-K1 cells.

PSS I activity appears to account for ~50% of the total serine base exchange activity in the homogenate of CHO-K1 cells, because the serine base exchange activity in the homogenate of PSA-3 mutant cells, which has no detectable amounts of pssA mRNA and its product, PSS I (5, 7), is ~50% of that of CHO-K1 cells (3). The serine base exchange activity in PSB-2 mutant cells is ~10% of that in PSA-3 mutant cells, indicating that PSS II accounts for at least ~90% of the total serine base exchange activity of the PSS I-lacking PSA-3 mutant. The remaining ~10% of the total serine base exchange activity of the PSA-3 mutant might also be attributed to PSS II, because the decrease in the serine base exchange activity of the PSB-2 mutant is proportional to the decrease in the cellular level of pssB mRNA of this mutant; both the activity and mRNA level in the PSB-2 mutant are 10–20% of those in the PSA-3 mutant. It is therefore likely that the serine base exchange in the homogenate of CHO-K1 cells is catalyzed almost exclusively by PSS I and PSS II, each of which accounts for approximately 50% of the total serine base exchange activity in the cell homogenate.

Both the PSB-2 and PSA-3 mutants are incapable of growth and producing a normal amount of PS when cultivated in a medium without phospholipid supplementation. Although the PSS I-defective PSA-3 mutant is rescued from the growth and PS biosynthetic defects by the addition of exogenous PE to the medium, the PSB-2 mutant deficient in both PSS I and II remains incapable of growth and defective in PS biosynthesis in the medium with exogenous PE. Labeling experiments with [32P]PE revealed that the PSB-2 mutant, but not the PSA-3 mutant, is defective in the conversion of exogenous PE to PS. This defect and the growth and PS biosynthetic defects of the PSB-2 mutant cultivated with exogenous PE are complemented by the pssB cDNA for PSS II. These results indicate that PSS II catalyzes the conversion of PE to PS and functions as a principal enzyme in PS synthesis in PSA-3 mutant cells, which has no detectable amounts of pssA mRNA. Therefore, PSS II is indispensable for the growth of PSA-3 mutant cells in the medium supplemented with PE.

PSS I can catalyze the PS formation from PC (8), but it remained to be elucidated whether PSS I catalyzes exclusively the conversion of PC to PS or can also catalyze the conversion of PE to PS. In the present study, it was shown that the pssA cDNA for PSS I is incapable of complementing the defect of the PSB-2 mutant in the conversion of PE to PS, although the pssB cDNA for PSS II complements this defect. On the other hand, the defect in the conversion of PC to PS of the PSB-2 mutant was shown to be complemented by the pssA cDNA but not by the pssB cDNA.

DISCUSSION

PS formation in mammalian cells occurs through the exchange of l-serine with the base moiety of PC or PE (8, 20–22). The serine base exchange in CHO-K1 cells is catalyzed by at least two enzymes, the pssA gene product, PSS I, and the pssB gene product, PSS II (Refs. 3 and 5–7; for a review, see Ref. 22). On the characterization of a PSS I-defective mutant of CHO-K1 cells, PSA-3, PSS I was shown to function as a critical enzyme in the production of PS and PE in CHO-K1 cells (3, 5). In the present study, we tried to isolate PSS II-defective mutants to address the function of PSS II in CHO-K1 cells. A CHO cell mutant, named PSB-2, isolated from PSA-3 mutant cells was shown to be defective in PSS II by several lines of evidence. First, the serine base exchange activity of the PSB-2 mutant was decreased to ~10% of that of the PSA-3 mutant and ~5% of that of CHO-K1 cells. Second, the activity of ethanolamine base exchange, which PSS II is capable of catalyzing (6), of the PSB-2 mutant was also decreased, being less than 15% of that of PSA-3 and CHO-K1 cells. Third, the defects of the PSB-2 mutant in the serine and ethanolamine base exchange activities were complemented by the pssB cDNA. Finally, the level of pssB mRNA in PSB-2 cells was ~20% of that in PSA-3 and CHO-K1 cells.

PSS I activity appears to account for ~50% of the total serine base exchange activity in the homogenate of CHO-K1 cells, because the serine base exchange activity in the homogenate of PSA-3 mutant cells, which has no detectable amounts of pssA mRNA and its product, PSS I (5, 7), is ~50% of that of CHO-K1 cells (3). The serine base exchange activity in PSB-2 mutant cells is ~10% of that in PSA-3 mutant cells, indicating that PSS II accounts for at least ~90% of the total serine base exchange activity of the PSS I-lacking PSA-3 mutant. The remaining ~10% of the total serine base exchange activity of the PSA-3 mutant might also be attributed to PSS II, because the decrease in the serine base exchange activity of the PSB-2 mutant is proportional to the decrease in the cellular level of pssB mRNA of this mutant; both the activity and mRNA level in the PSB-2 mutant are 10–20% of those in the PSA-3 mutant. It is therefore likely that the serine base exchange in the homogenate of CHO-K1 cells is catalyzed almost exclusively by PSS I and PSS II, each of which accounts for approximately 50% of the total serine base exchange activity in the cell homogenate.

Both the PSB-2 and PSA-3 mutants are incapable of growth and producing a normal amount of PS when cultivated in a medium without phospholipid supplementation. Although the PSS I-defective PSA-3 mutant is rescued from the growth and PS biosynthetic defects by the addition of exogenous PE to the medium, the PSB-2 mutant deficient in both PSS I and II remains incapable of growth and defective in PS biosynthesis in the medium with exogenous PE. Labeling experiments with [32P]PE revealed that the PSB-2 mutant, but not the PSA-3 mutant, is defective in the conversion of exogenous PE to PS. This defect and the growth and PS biosynthetic defects of the PSB-2 mutant cultivated with exogenous PE are complemented by the pssB cDNA for PSS II. These results indicate that PSS II catalyzes the conversion of PE to PS and functions as a principal enzyme in PS synthesis in PSA-3 mutant cells, which has no detectable amounts of pssA mRNA. Therefore, PSS II is indispensable for the growth of PSA-3 mutant cells in the medium supplemented with PE.

PSS I can catalyze the PS formation from PC (8), but it remained to be elucidated whether PSS I catalyzes exclusively the conversion of PC to PS or can also catalyze the conversion of PE to PS. In the present study, it was shown that the pssA cDNA for PSS I is incapable of complementing the defect of the PSB-2 mutant in the conversion of PE to PS, although the pssB cDNA for PSS II complements this defect. On the other hand, the defect in the conversion of PC to PS of the PSB-2 mutant was shown to be complemented by the pssA cDNA but not by the pssB cDNA. These results suggested that PSS I negligibly catalyzes the conversion of PE to PS in CHO-K1 cells and confirmed our previous finding that PSS I, but not PSS II, catalyzes the conversion of PC to PS (6, 8).

As described above, the results presented here suggested the following. 1) The serine base exchange in CHO-K1 cells is catalyzed almost exclusively by PSS I and PSS II. 2) PSS II functions as the principal enzyme in PS synthesis in PSA-3.
mutant cells cultivated with exogenous PE. 3) PSS II, but not PSS I, can catalyze the conversion of PE to PS. Thus, it is very likely that most of the PS formation from PE in wild-type CHO-K1 cells is catalyzed by PSS II.

Transfection of PSS I- and PSS II-defective PSB-2 mutant cells with the pssa cDNA for PSS I rendered the PSB-2 mutant capable of growth in the medium without phospholipid supplementation. This restoration of the cell growth of the PSB-2 mutant does not require overproduction of PSS I, because one of the resultant phospholipid-prototrophic transformants, PSB-2/pssA, exhibited less PSS I activity than that of CHO-K1 cells. In the medium without phospholipid supplementation, the PSB-2/pssA transfectant exhibits a normal phospholipid composition. These results raise the possibility that PSS II is dispensable for the cell growth and PS biosynthesis of CHO-K1 cells cultivated without phospholipid supplementation. However, the PSS II mutation in the PSB-2 mutant seems to be leaky, because this mutant exhibits reduced but significant serine base exchange activity and pssB mRNA. Thus, elucidation of the significance of PSS II in cell growth and PS biosynthesis under ordinary growth conditions without phospholipid supplementation awaits the isolation of PSS II-null mutants.

The cellular levels of PS and its decarboxylation product, PE, in PSS I-defective PSA-3 mutant cells immediately decreased upon cultivation in the medium without phospholipid supplementation (23), in which this mutant is incapable of growth. The addition of either PS or PE to the medium restored the PS and PE levels of the PSA-3 mutant to normal and suppressed the growth defect of this mutant. These previous observations suggest that PS production by PSS I is critical for the growth of CHO-K1 cells in the medium without phospholipid supplementation. However, it has not been resolved whether PS, PE, or both are critical for cell growth. In the present study, we have shown that exogenous PS, but not PE, complements the growth defect of the PSB-2 mutant. The PSB-2 mutant cultivated with exogenous PE exhibited a reduction in the cellular PS level. The levels of other major phospholipids, including PE, in these mutant cells are normal upon cultivation with exogenous PE. These results suggest that PS is indispensable for the growth of CHO-K1 cells.

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Genetic Evidence That Phosphatidylserine Synthase II Catalyzes the Conversion of Phosphatidylethanolamine to Phosphatidylserine in Chinese Hamster Ovary Cells
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