Cloning of a Chinese Hamster Ovary (CHO) cDNA Encoding Phosphatidylserine Synthase (PSS) II, Overexpression of Which Suppresses the Phosphatidylserine Biosynthetic Defect of a PSS I-lacking Mutant of CHO-K1 Cells*

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Phosphatidylserine (PtdSer) in mammalian cells is synthesized through the exchange of free L-serine for the polar head group (base) of preexisting phospholipid. We previously showed the presence of two different enzymes catalyzing the serine base exchange in Chinese hamster ovary (CHO) cells and isolated the cDNA of one of the enzymes, PtdSer synthase (PSS) I, which also catalyzes the exchange of the base moiety of phospholipid(s) for ethanolamine and choline. In this study, we cloned a CHO cDNA, designated as pssB, which encodes a protein exhibiting 32% amino acid sequence identity with CHO PSS I. Introduction of the pssB cDNA into CHO-K1 cells resulted in striking increases in both the serine and ethanolamine base exchange activities. In contrast to the PSS I cDNA, the pssB cDNA was incapable of increasing the choline base exchange activity. The expression of the pssB gene in Sf9 insect cells also results in striking increases in both serine and ethanolamine base exchange activities. The pssB cDNA was found to transform a PtdSer-auxotrophic PSS I-lacking mutant of CHO-K1 cells to PtdSer prototroph. The PtdSer content of the resultant transformant grown without exogenous PtdSer for 2 days was 4-fold that of the mutant and similar to that of CHO-K1 cells, indicating out exogenous PtdSer for 2 days was 4-fold that of the mutant PSA-3, grown with-
described (1). For the ethanalamine supplementation experiment, 100 ml of newborn calf serum was dialyzed three times against 2 liters of phosphate-buffered saline for about 12 h and filter sterilized. Spodoptera frugiperda (SB) cells were provided by Dr. Yoshiharu Matsuura (National Institute of Health, Tokyo, Japan) and maintained in TC-100 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum, 10 μg/ml gentamycin, and 0.26% (w/v) tryptose phosphate broth (Life Technologies, Inc.) at 27 °C.

Construction of a cDNA Library—Poly(A) + RNA was prepared from CHO-K1 cells as described (7) and used for cDNA synthesis. The cDNA synthesis and construction of a cDNA library in a plasmid vector, pSPORT1, were performed with a SuperScript reverse transcriptase system (Life Technologies, Inc.) according to the manufacturer's instructions. Cloning of a pssB cDNA—Oligonucleotides corresponding to parts of a human expressed sequence tag (EST) (GenBank number F11951) were used to amplify a pssB cDNA fragment from the CHO cDNA library by means of a two-stage polymerase chain reaction. The primers used for the first round of amplification were TCCAGACTGTCAG-CAGGGC (sense) and AGGAACTGCAACTGAGGCT (antisense). The fragments were used for the second round of amplification. The amplification reactions were performed for 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 90 s, with Taq polymerase (Perkin-Elmer) according to the manufacturer's instructions. For the second round of amplification, the first round reaction mixtures was diluted 5,000-fold and used as the template. The 0.3-kilobase pair product of the second round of amplification was cloned into a plasmid, pBluescript II SK+ (Stragtenge), sequenced, and used as a hybridization probe for screening of the CHO cDNA library.

RESULTS

Isolation of a cDNA Clone Encoding a Protein Similar in Sequence to PSS II—To identify cDNA clones encoding PSS II, the amino acid sequence of PSS I predicted from the cDNA sequence (4) was compared with the ESTs in DNA data bases using the TBLASTN search protocol at the National Center for Biotechnology Information. A human EST (GenBank number F11951) was found to encode a peptide that exhibited 30% sequence identity, in a stretch of 98 amino acids, with both CHO PSS I and a putative human PSS I (GenBank number D14694). A cDNA fragment of the CHO counterpart for human EST F11951 was generated from a CHO cDNA library, using a polymerase chain reaction and primers corresponding to parts of the EST sequence. The resulting cDNA fragment was used as a hybridization probe to screen the CHO cDNA library, after enrichment of hybridizing clones using a GeneTrapper cDNA-positive selection system (Life Technologies, Inc.). The enrichment was performed with a biotinylated oligonucleotide, GAGGAGTGGTGATGTGCATGCATC, corresponding to a part of the 0.3-kilobase pair polymerase chain reaction product, according to the manufacturer's instructions except for omission of the repair reaction. Colony filter hybridization with the 32P-labeled probe was performed as described (8); hybridization was performed for 22 h at 42 °C in 5 × SSPE, 0.5% sodium dodecyl sulfate, 0.5% sodium dodecyl sulfate, 50% formamide, and 100 μg/ml denatured salmon sperm DNA; the final wash was performed in 0.2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), and 0.1% sodium dodecyl sulfate at 50 °C for 1 h.

DNA Sequencing—Both strands of the pssB cDNA were determined by the dyeoxy chain termination method with Sequenase (U.S. Biochemicals Corp., Cleveland, OH) according to the manufacturer's instructions, using a series of deletion mutants generated by exonIII/mung bean nuclease treatment (9), in combination with walking primers.

Transient Transfection of CHO-K1 Cells with the pssB and pssA cDNAs—A plasmid, pSPORTI/pssB, carrying the pssB cDNA, was cleaved at the SalI and NotI sites, and the resulting pssB cDNA fragment was inserted into these restriction enzyme sites of a mammalian expression plasmid vector, pSV-SPORT1 (Life Technologies, Inc.). The resulting construct, pSVpssB, and pCDPSSA encoding the pssA protein (CHO PSS I) (4) were introduced into CHO-K1 cells using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions.

Heterologous Expression of the pssB Gene—The plasmid, pSPORTI/pssB, was cleaved at the SalI and HindIII sites, and the resulting pssB cDNA fragment was inserted into the XhoI and HindIII sites of a baculovirus transfer vector, pBlueBac4.5 (Invitrogen). A monolayer of SB9 cells (35-mm diameter dish) was cotransfected with the resulting construct, pBlueBac4.5pssB and Bac-N-Blue® Autographa californica DNA (Invitrogen), using Lipofectin reagent (Life Technologies, Inc.). For production of control virus, another monolayer of SB9 cells was cotransfected with the transfer vector pBlueBac4.5 and Bac-N-Blue® DNA. After 4 days of culture, the transfection supernatants containing pssB recombinant virus or control virus were collected. Fresh monolayers of SB9 cells (75-cm² flask) were infected with the transfection supernatant containing pssB recombinant virus or control virus and cultured in TC-100 insect medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal bovine serum. After 4 days at 27 °C, cells were harvested twice with 1 ml of phosphate-buffered saline, pH 7.4, resuspended in 0.25 ml sucrose containing 1 ml EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotonin, 1 mM (p-amidinophenyl)mercuriethyl fluoride, and 10 mM HEPES, pH 7.5, and disrupted by two 15-s sonications on a Branson Sonifier. Phospholipid base exchange activity in the preparations was assayed as described (1).

Isolation of Transformant PSA-3/pssB—Mutant PSA-3 cells were transfected with pSVpssB by the calcium phosphate precipitation method (10), and the resultant transformant, designated as PSA-3/pssB, which was able to grow in the growth medium without exogenous phospholipids, was purified by limited dilution of the transfected cells. Other Methods—Radioactive labeling, extraction, separation, and quantitation of phospholipids were performed as described in Tables III and IV and in the legends to Figs. 5, 6, and 7. [32P]PtdCho was prepared from CHO-K1 cells metabolically labeled with [32P] orthophosphate, as described (2). Protein was measured according to Lowry et al. (11), using bovine serum albumin as a standard.

Phospholipid Base Exchange Activities in pssB-transfected Cells—To determine whether the pssB gene product is relevant to serine base exchange for PtdSer formation, the pssB cDNA was placed downstream of the mammalian expression promoter of a plasmid, pSV-SPORT1, and the resulting construct, pSVpssB, was introduced into CHO-K1 cells. The transient transfectant with pSVpssB exhibited a 6-fold higher specific activity of serine base exchange for PtdSer formation than CHO-K1 cells transfected with the control vector (Table I). The ethanalamine base exchange activity also increased 10-fold upon transfection with the pssB cDNA (Table I). On the other hand, there was no significant difference in the choline base

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exchange activity between the transfectant with pSVpssB and the control CHO-K1 cells. In contrast to pSVpssB, a plasmid pcDPSSA (4) encoding CHO PSS I was capable of increasing the choline base exchange activity, as well as the serine and ethanolamine base exchange activities, upon transient transfection (Table I). These results, together with the sequence similarity of the pssB gene product to PSS I, suggested that pssB encoded an enzyme catalyzing both the serine and ethanolamine base exchange but not the choline base exchange in cell extracts.

**Heterologous Expression of the pssB Gene—** To obtain further evidence that pssB encodes an enzyme catalyzing both the serine and ethanolamine base exchange, we used heterologous expression of the pssB gene in insect cells. The pssB cDNA was placed within the genome of baculovirus under control of the polyhedrin promoter and expressed by viral infection of Sf9 cells. The serine and ethanolamine base exchange activities in a homogenate of Sf9 cells infected with the pssB-containing baculovirus were, respectively, 5.3- and 7.5-fold higher than those of Sf9 cells infected with a control virus (Fig. 4). On the other hand, the choline base exchange activity was not elevated by the pssB virus infection (Fig. 4). Thus, in this heterologous system the pssB cDNA was capable of increasing both the serine and ethanolamine base exchange activities, suggesting

FIG. 1. Nucleotide and predicted amino acid sequences of the pssB cDNA. The putative initiation codon, stop codon, and polyadenylation signal are underlined.

![Diagram](image1.png)

**FIG. 2.** Comparison of the predicted amino acid sequence of the pssB gene product with that of the pssA gene product, PSS I.

![Diagram](image2.png)

**FIG. 3.** Hydrophobicity plots for the predicted pssB gene product (panel A) and the pssA gene product, PSS I (panel B). The average hydrophobicity (12) of a nanodecapeptide composed of amino acids \( n = 9 \) to \( n + 9 \) is plotted against \( n \), the amino acid number.

![Diagram](image3.png)
that the cDNA encodes an enzyme catalyzing these two different base exchange reactions.

**pssB cDNA Complements the Growth Defect of PSS I-lacking Mutant PSA-3—** A PSS I-lacking mutant of CHO-K1 cells, PSA-3, requires the addition of either PtdSer or PtdEtn to the medium for cell growth (1, 2). To determine whether or not the pssB cDNA compensates for the lack of PSS I activity, the pssB cDNA was able to complement the PtdSer biosynthetic defect of the PSS I-lacking mutant, PSA-3, as shown in Fig. 6. These results showed that the pssB cDNA was able to complement the PtdSer biosynthetic defect of the PSS I-lacking mutant, PSA-3.

**The pssB-transformed PSA-3 Mutant Remains Defective in Conversion of PtdCho to PtdSer—** Mutant PSA-3 is defective in the conversion of exogenous [32P]PtdCho to [32P]PtdSer because of a lack of PSS I activity (2). To determine whether or not the pssB cDNA complements this defect, cells were metabolically labeled with [32P]PtdChos. CHO-K1 cells incorporated the radioactivity into PtdSer, in an amount comprising 3.6% of the radioactivity of cellular PtdCho (Fig. 7). In contrast to CHO-K1 cells, both transformant PSA-3/pssB and mutant PSA-3 were incapable of incorporating the radioactivity into PtdSer in significant amounts, although the level of cellular [32P]PtdCho in the transformant and mutant was almost the same as that in CHO-K1 cells (Fig. 7). These results indicated that the transformant PSA-3/pssB remained defective in conversion of PtdCho to PtdSer.

Transformant PSA-3/pssB Cultivated in the Medium with Dialyzed Newborn Calf Serum Exhibits a Normal PtdSer Bio-

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**TABLE I**

**Phosphatidylserine Synthase II of CHO Cells**

CHO-K1 cells were transiently transfected with the pssB cDNA or the pssA cDNA encoding PSS I activity as described (1). Values indicate specific activities and are the averages of duplicate assays, with variation of <10% between duplicates.

| CHO-K1 cells transfected with | Substrate | (nmol/h)/mg protein |
|-----------------------------|-----------|---------------------|
|                             | Serine    | Ethanolamine | Choline |
| pSV-SPORT1                  | 2.52      | 3.83          | 1.16    |
| pSV-pssB                    | 15.18     | 37.10         | 1.07    |
| pcDPSSA                     | 13.87     | 24.05         | 17.11   |

**TABLE II**

**Phospholipid base exchange activities of CHO-K1, mutant PSA-3, and transformant PSA-3/pssB**

Cell extracts were prepared from cells growing exponentially in the growth medium supplemented with 30 μg PtdSer at 37°C and then assayed for phospholipid base exchange activities as described (1). Values indicate specific activities and are the averages of duplicate assays, with variation of <10% between duplicates.

| Strain            | Substrate | (nmol/h)/mg protein |
|-------------------|-----------|---------------------|
| CHO-K1            | Serine    | 2.42                | 1.20    |
|                   | Ethanolamine | 4.88    | 0.01    |
|                   | Choline   | 13.31               | 43.12   |
| PSA-3             | Serine    | 0.97                | 0.05    |
|                   | Ethanolamine | 2.37    | 0.01    |
| PSA-3/pssB        | Choline   | 13.31               | 43.12   |
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TABLE III
Phospholipid compositions and contents of CHO-K1, mutant PSA-3, and transformant PSA-3/pssB

| Strain    | Percentage of total phospholipids | Total |
|-----------|-----------------------------------|-------|
|           | PS PE PC SM PI Other*            |       |
| CHO-K1    | 6.1 16.8 55.8 10.7 7.0 3.7       |       |
| PSA-3     | (9.8) (27.0) (89.7) (17.2) (11.2) (5.9) (160.8) |
| PSA-3/pssB| 5.6 17.8 55.2 11.6 6.3 3.5       |       |
|           | (8.9) (28.4) (67.9) (18.5) (10.0) (5.5) (159.2) |

* Other lipids comprise phosphatidylglycerol, phosphatic acid, and cardiolipin.

**DISCUSSION**

Mutant PSA-3 incorporates exogenous ethanolamine into PtdEtn which functions as a precursor phospholipid of PtdSer in the mutant (1, 2). We examined if exogenous ethanolamine derived from newborn calf serum is involved in the restoration of PtdSer biosynthesis in transformant PSA-3/pssB. By using dialyzed newborn calf serum, transformant PSA-3/pssB and CHO-K1 cells, but not mutant PSA-3, grew exponentially in the medium supplemented with 10% dialyzed newborn calf serum (Fig. 8). When ethanolamine was added to the medium at a concentration of 10 µm, mutant PSA-3 was able to grow normally for 5 days (Fig. 8). The addition of ethanolamine did not affect the cell growth of transformant PSA-3/pssB and CHO-K1 cells (Fig. 8). A labeling experiment with [32P]PtdCho for 48 h revealed that the PtdSer level of transformant PSA-3/pssB grown in the medium with the dialyzed serum was similar to that of CHO-K1 cells (Table IV). Although mutant PSA-3 cultivated in the medium with the dialyzed serum was defective in PtdSer biosynthesis, the addition of ethanolamine to the medium restored a normal level of PtdSer among all three strains (Table IV). These results indicated that the restoration of PtdSer biosynthesis in transformant PSA-3/pssB occurred without ethanolamine supplementation to the medium containing the dialyzed serum and that the addition of ethanolamine to the medium complemented the PtdSer biosynthetic defect of mutant PSA-3.

**FIG. 7. Conversion of exogenous [32P]PtdCho to [32P]PtdSer.** Approximately 2 x 10⁶ cells were seeded into 100-mm-diameter dishes containing the growth medium supplemented with 30 µM PtdSer, followed by incubation for 1 day at 37 °C. Then cells were washed twice with the growth medium without PtdSer and then incubated in the growth medium without PtdSer at 37 °C. After 2 h, the cells were metabolically labeled at 37 °C by replacing the medium with fresh growth medium containing 5.4 x 10⁶ cpm/ml [32P]PtdCho. After labeling (24 h), the cells were washed three times with the growth medium, and then the cellular phospholipids were extracted and analyzed by two-dimensional thin layer chromatography as described (20). The numbers of cells/dish of parallel unlabeled cultures were determined and used to standardize the results. Panel A, cellular [32P]PtdCho; panel B, [32P]PtdSer derived from [32P]PtdCho.

**FIG. 8. Cell growth in the medium supplemented with dialyzed newborn calf serum.** Cells were seeded at approximately 2.5 x 10⁵ cells/60-mm-diameter dishes in Ham's F-12 medium supplemented with 10% dialyzed newborn calf serum containing 0.2 µCi/ml l-[U-14C]serine (Amersham). At 1 day, at time 0, the cells were washed three times with the growth medium, and then the cellular phospholipids were extracted and analyzed by one-dimensional thin layer chromatography as described (20). The numbers of cells/dish of parallel unlabeled cultures were determined and used to standardize the results. A, CHO-K1; B, PSA-3; C, PSA-3/pssB.
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Cells were seeded at 5 × 10^5 cells/100-mm-diameter dish in Ham's F-12 medium supplemented with 10% (v/v) dialyzed newborn calf serum and 10 μM ethanolamine or the dialyzed serum only at 37 °C. After 1 day, 32Pi was added into the medium at a final concentration of 2 μCi/ml. An additional 48 h, the cellular phospholipids were extracted and analyzed by two-dimensional thin layer chromatography (19). Radioactivity of the individual phospholipids was analyzed with a bioimage analyzer (Fujix BAS 2000). The values are radioactivities of individual phospholipids, expressed as percentages of the total radioactivity of phospholipids. The abbreviations used are the same as in Table III.

| Strain        | Ethanolamine supplement | Percentage of total phospholipids | Other       |
|---------------|-------------------------|----------------------------------|------------|
| CHO-K1        | –                       | 6.1 14.5 56.5 9.3 8.6 5.0        |            |
| PSA-3         | –                       | 1.0 9.2 64.3 6.4 11.6 7.5        |            |
| PSA-3/pssB    | –                       | 6.6 20.7 52.1 8.4 8.1 3.9        |            |
| CHO-K1        | +                       | 6.6 22.3 50.3 9.5 6.8 4.5        |            |
| PSA-3         | +                       | 6.1 22.1 50.5 10.1 7.2 3.9       |            |
| PSA-3/pssB    | +                       | 7.3 25.0 48.5 10.0 5.8 3.4       |            |

Enzymes, PSS I, has been isolated by means of genetic complementation with a PSS I-lacking mutant, PSA-3 (4, 5). Introduction of the pssA cDNA into the mutant leads to increases in choline and ethanolamine base exchange activities, in addition to an elevation of the serine base exchange activity (4). In this study, we have tried to isolate the cDNA of the second PSS, on the assumption that the second PSS is similar in sequence to PSS I. A CHO pssB cDNA isolated here encodes a protein showing a high (32%) amino acid sequence identity with the pssA-encoding PSS I. Transient transfection of CHO-K1 cells with the pssB cDNA results in a 6-fold increase in the serine base exchange activity. The pssB-transfected cells also exhibit a 10-fold elevated ethanolamine base exchange activity. However, the pssB cDNA is incapable of elevating the choline base exchange activity, in contrast to the pssA cDNA. The expression of the pssB gene in Sf9 insect cells also results in striking increases in both serine and ethanolamine base exchange activities. When the pssB cDNA is introduced into the mutant PSA-3, the mutant recovers a normal level of PtdSer biosynthesis. These results suggest that the pssB cDNA encodes the second PtdSer synthase PSS II, which catalyzes the ethanolamine base exchange and ethanolamine base exchange, which is supposed to be the reverse reaction of PtdSer formation from PtdEtn. These results suggest that the majority of PtdSer in the pssB-transformed PSA-3 mutant is produced through the exchange of the ethanolamine moiety of PtdEtn with l-serine.

PtdEtn can be synthesized by three pathways. First, in the CDP-ethanolamine pathway, ethanolamine is phosphorylated and converted to CDP-ethanolamine, and then the phosphoethanolamine moiety is transferred to diacylglycerol for PtdEtn formation (19). The second pathway is the decarboxylation and ethanolamine base exchange requires PtdSer. Thus, the exchange of l-serine for the ethanolamine moiety of PtdEtn made by the decarboxylation and ethanolamine base exchange does not yield a net increase in cellular PtdSer content. On the other hand, the ethanolamine-serine exchange using PtdEtn produced through the CDP-ethanolamine pathway results in a net increase in cellular PtdSer content. It is therefore likely that PtdEtn produced through the CDP-ethanolamine pathway contributes to the restoration of PtdSer biosynthesis in the pssB-transformed PSA-3 mutant. Because the restoration of PtdSer biosynthesis in the transformant occurs without ethanolamine supplementation to the medium containing diazylx newborn calf serum, the level of endogenous ethanolamine appears to be sufficient for PtdSer biosynthesis in the transformant. In contrast, in the PSA-3 mutant cultivated without ethanolamine supplementation, the level of endogenous ethanolamine appears to be insufficient for PtdSer biosynthesis because the mutant is defective in PtdSer biosynthesis unless the growth medium is supplemented with either ethanolamine or PtdEtn (2). Why is the pssB-transformed PSA-3 mutant capable of synthesizing normal amounts of PtdEtn and PtdSer in the absence of ethanolamine supplementation? If the ethanolamine-serine exchange reaction is written together with the reutilization of ethanolamine for PtdEtn synthesis via CDP-ethanolamine pathway,

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Ethanolamine + ATP → phosphoethanolamine + ADP
Phosphoethanolamine + CTP → CDP-ethanolamine + ADP
CDP-ethanolamine + diacylglycerol → PtdEtn + CMP
PtdEtn + l-serine → PtdSer + ethanolamine
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a catalytic amount of recycling ethanolamine is sufficient to achieve net synthesis of PtdSer from diacylglycerol and l-serine. Therefore, an increase in the overall rate of the ethanolamine cycle comprising the phosphoethanolamine-serine exchange and the CDP-ethanolamine pathway would lead to increased cellular levels of PtdSer and its decarboxylation product, PtdEtn, without consumption of ethanolamine. Considering the above mentioned results, we speculate that the overexpression of PSS II which catalyzes the ethanolamine-serine exchange for PtdSer formation induces an increase in the overall rate of the ethanolamine cycle, thereby complementing the defect of the PSA-3 mutant in PtdSer and PtdEtn biosyntheses.

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