*Proc. Natl. Acad. Sci. USA*
Vol. 95, pp. 4199–4203, April 1998
Biochemistry

# Control of phosphatidylserine biosynthesis through phosphatidylserine-mediated inhibition of phosphatidylserine synthase I in Chinese hamster ovary cells

OSAMU KUGE*, KAZUHIDE HASEGAWA†, KYOKO SAITO, AND MASAHIRO NISHIJIMA

Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162, Japan

*Communicated by Eugene P. Kennedy, Harvard Medical School, Boston, MA, February 6, 1998 (received for review December 23, 1997)*

**ABSTRACT** **Phosphatidylserine (PtdSer) synthesis in Chinese hamster ovary (CHO) cells occurs through the exchange of L-serine with the base moiety of phosphatidylcholine or phosphatidylethanolamine. The synthesis is depressed on the addition of PtdSer to the culture medium. A CHO cell mutant named mutant 29, whose PtdSer biosynthesis is highly resistant to this depression by exogenous PtdSer, has been isolated from CHO-K1 cells. In the present study, the PtdSer-resistant PtdSer biosynthesis in the mutant was traced to a point mutation in the PtdSer synthase I gene, *pssA*, resulting in the replacement of Arg-95 of the synthase by lysine. Introduction of the mutant *pssA* cDNA, but not the wild-type *pssA* cDNA, into CHO-K1 cells induced the PtdSer-resistant PtdSer biosynthesis. In a cell-free system, the serine base-exchange activity of the wild-type *pssA*-transfected cells was inhibited by PtdSer, but that of the mutant *pssA*-transfected cells was resistant to the inhibition. Like the mutant 29 cells, the mutant *pssA*-transfected cells grown without exogenous PtdSer exhibited an ≈2-fold increase in the cellular PtdSer level compared with that in CHO-K1 cells, although the wild-type *pssA*-transfected cells did not exhibit such a significant increase. These results indicated that the inhibition of PtdSer synthase I by PtdSer is essential for the maintenance of a normal PtdSer level in CHO-K1 cells and that Arg-95 of the synthase is a crucial residue for the inhibition.**

Phosphatidylserine (PtdSer) is an essential phospholipid for the growth of mammalian cells (1), comprising ≈10% of the total membrane phospholipids of various mammalian tissues and cultured cells. PtdSer formation in mammalian cells occurs through the exchange of L-serine with the base moiety of phosphatidylcholine or phosphatidylethanolamine (PtdEtn) (2–4). The serine base-exchange activity in Chinese hamster ovary (CHO) cells is catalyzed by at least two different enzymes named PtdSer synthase (PSS) I and II, which are encoded by the *pssA* and *pssB* genes, respectively (1, 4–9). The PtdSer biosynthesis in CHO-K1 cells is depressed on the addition of PtdSer to the growth medium (10), implying that feedback control is involved in the regulation of PtdSer biosynthesis. A CHO cell mutant named mutant 29, whose PtdSer biosynthesis is highly resistant to this depression by exogenous PtdSer, has been isolated from CHO-K1 cells (11). In medium without exogenous PtdSer, the mutant 29 cells produce PtdSer at a 2- to 3-fold higher rate and have an ≈2-fold higher amount of PtdSer compared with those in CHO-K1 cells (11). Therefore, even in cells growing without exogenous PtdSer, the feedback control may operate to maintain a normal level of PtdSer. The serine base-exchange activities in homogenates of CHO-K1 cells grown with and without exogenous PtdSer are essentially the same (10), implying that the cellular levels of PSS I and II remain unchanged on the supplementation of PtdSer. In addition, the serine base-exchange activity in a membrane fraction prepared from CHO-K1 cells, but not the activity from the mutant 29 cells, is inhibited by PtdSer (11). These results suggest that the inhibition of serine base-exchange activity by PtdSer is involved in the control of PtdSer biosynthesis.

For further understanding of the molecular mechanisms underlying the control of PtdSer biosynthesis by PtdSer, identification of the mutation responsible for the PtdSer-resistant PtdSer biosynthesis in mutant 29 cells may be helpful. Because one of the possible explanations for the PtdSer-resistant PtdSer biosynthesis is that the PtdSer synthase gene is mutated, we have examined whether or not a PtdSer synthase gene, *pssA* or *pssB*, of mutant 29 has a mutation relevant to the PtdSer-resistant PtdSer biosynthesis. The results presented here show that the *pssA* gene for PSS I of mutant 29 has a missense mutation changing codon 95 from arginine to lysine, which renders PSS I resistant to the inhibition by PtdSer. This finding indicates that the inhibition of PtdSer synthase I by PtdSer is important for the control of PtdSer biosynthesis in CHO cells.

## MATERIALS AND METHODS

**Strains and Culture Conditions.** Strain CHO-K1 was obtained from the American Type Culture Collection. CHO-K1, mutant 29 (11), and the CHO strains constructed in this study were maintained in Ham's F-12 medium supplemented with 10% newborn calf serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and $NaHCO_3$ (1.176 g/liter) under a 5% $CO_2$ atmosphere of 100% humidity at 37°C. An ouabain-resistant subclone of CHO-K1 cells was selected in the growth medium containing 1 mM ouabain, after mutagenesis with 400 μg of ethyl methanesulfonate per ml of growth medium at 37°C for 16 hr. For the isolation of an ouabain and 6-thioguanine-resistant clone, the ouabain-resistant cells were subjected to a second round of mutagenesis and then cultivated in the growth medium containing 30 μM 6-thioguanine and 1 mM ouabain. A hybrid clone of the resultant thioguanine/ouabain-resistant CHO-K1 and mutant 29 cells was selected and purified in the growth medium containing 5 μg/ml hypoxanthine, 0.02 μg/ml aminopterin, 5 μg/ml thymidine, and 1 mM ouabain after exposure of a mixed cell monolayer to 50% PEG 4000 in Ham's F-12 medium for 1 min.

**Metabolic Labeling of PtdSer with [$^{14}$C]Serine.** Approximately $5 \times 10^5$ cells were seeded into 60-mm-diameter dishes or the wells of a 24-well plate in Ham's F-12 medium supple-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



Abbreviations: CHO, Chinese hamster ovary; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PSS, PtdSer synthase.

*To whom reprint requests should be addressed. e-mail: kuge@nih.go.jp.
†Present address: Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6, Asahi-machi, Machida-shi, Tokyo 194, Japan.

mented with 10% newborn calf serum, followed by incubation at 37°C. After 1 day, the cells were incubated in fresh growth medium with or without exogenous 80 μM PtdSer at 37°C for 2 hr, and then labeled with L-[U-$^{14}$C]serine (0.5 μCi/ml; 1 Ci = 37 GBq; Amersham) for 3 hr at 37°C in the corresponding growth medium with or without exogenous PtdSer. PtdSer liposomes added to the medium were prepared as described (10). Phospholipids in the labeled cells were extracted by the method of Bligh and Dyer (12), and then the radioactivity of PtdSer was determined as described (10). The amount of protein per dish or number of cells per well of parallel unlabeled cultures was determined and used to standardize the results.

**Isolation of Stable *pssA* and *pssB* Transfectants of Mutant 29 Cells.** A plasmid, pcDPSSA (7), carrying the *pssA* cDNA from CHO-K1 cells was cleaved at the *Pst*I and *Xho*I sites, and the resulting *pssA* cDNA fragment was inserted into the multiple-cloning site of a mammalian expression plasmid, pSVOK*neo* (13), carrying a G418-resistance determinant. The resultant plasmid was designated as pSV*pssA*/*neo*. A plasmid, pSPORT/*pssB* (9), carrying the *pssB* cDNA from CHO-K1 cells was cleaved at the *Sal*I and *Not*I sites, and the resulting *pssB* cDNA fragment was inserted into these restriction enzyme sites of pSVOK*neo*. The resultant plasmid was designated as pSV*pssB*/*neo*. Mutant 29 cells were transfected with each of pSV*pssA*/*neo* and pSV*pssB*/*neo* by the calcium phosphate precipitation method (14), and G418-resistant transformants were selected in the growth medium containing 400 μg/ml of G418. From the transformants, a pSV*pssA*/*neo*-transformed clone (designated as M29/*pssA*) and a pSV*pssB*/*neo*-transformed clone (designated as M29/*pssB*) were purified by limited dilution.

**Isolation of *pssA* and *pssB* cDNAs from Mutant 29 Cells.** Poly(A)$^+$ RNAs were prepared from mutant 29 cells with a FastTrack mRNA isolation kit (Invitrogen) and used for reverse transcription–PCR for amplification of *pssA* and *pssB* cDNAs. First-strand cDNA was synthesized by using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and an oligo(dT) primer. PCR was performed with AmpliTaq Gold DNA polymerase (Perkin–Elmer) according to the manufacturer's instructions. The *pssA*-specific PCR primers, ATTGTCGACAAGGGCCATGGCGTCG (sense) and ATTAGCGGCCGCTCATTTCTTTCCAACTCC (antisense), and the *pssB*-specific PCR primes, ATTGTCGACAGGCTGGGCGCCATGCGG (sense) and ATTAGCGGCCGCTCATGAGGCGGCTGAGGCC (antisense), were used to introduce *Sal*I and *Not*I sites into the resulting PCR products containing the *pssA* or *pssB* full-length ORF. After digestion with *Sal*I and *Not*I, the PCR products were inserted into these restriction sites of pSVOK*neo*. The resultant plasmids were designated as pSVm29-*pssA*/*neo* and pSVm29-*pssB*/*neo*, respectively.

**Isolation of Stable pSVm29-*pssA*/*neo* and Wild-Type *pssA* Transfectants of CHO-K1 Cells.** Each of pSVm29-*pssA*/*neo* and pSV*pssA*/*neo2*, which were constructed through essentially the same procedure as that for the construction of pSVm29-*pssA*/*neo* except for the use of pcDPSSA (7) as a PCR template, was introduced into CHO-K1 cells by the calcium phosphate precipitation method (14), and G418-resistant transformants were selected in the growth medium containing 400 μg/ml of G418. From the transformants, a pSVm29-*pssA*/*neo*-transformed clone (designated as K1/m29-*pssA*) and a pSV*pssA*/*neo2*-transformed clone (designated as K1/wt-*pssA*) were purified by limited dilution.

**Construction of the R95K Mutant *pssA* cDNA.** R95K mutation was introduced into the wild-type *pssA* sequence by using PCR-based site-directed mutagenesis (15). pcDPSSA (7) was used as a PCR template. The following oligonucleotides were used as PCR primers: a wild-type sense primer containing *Sal*I site, ATTGTCGACAAGGGCCATGGCGTCG; a wild-type antisense primer, TGGATGTCCTTGAAGCTTGC; a mutant sense primer, CCATTTACCAAACCTCATCCA; a mutant antisense primer, GGATGAGGTTTGGTAAATGGA. After digestion with *Sal*I and *Eco*RV, the resulting mutant PCR product was ligated to pSV*pssA*/*neo2* cleaved with the same restriction enzymes to replace the wild-type *pssA* sequence of the plasmid with the mutant sequence. The resutant plasmid was designated as pSV*R95K-pssA*/*neo*.

**DNA Sequencing.** DNA sequencing was performed by automated sequencing with an Applied Biosystem Prism 310 genetic analyzer and fluorescence-tagged dye terminator cycle sequencing (Perkin–Elmer). The sequences of both strands of the *pssA* and *pssB* cDNAs isolated from the mutant 29 cells were determined with walking primers. Site-directed mutant *pssA* and wild-type *pssA* sequences engineered by PCR were verified not to have extraneous mutations by DNA sequencing.

**Protein Determination.** Protein was measured according to Lowry *et al.* (16) by using BSA as a standard.

## RESULTS

**PtdSer Biosynthesis in a Hybrid Clone of the Mutant 29 and CHO-K1 Cells.** To determine whether the PtdSer-resistant PtdSer biosynthesis in the mutant 29 cells is a recessive or dominant phenotype, we constructed a hybrid clone of the mutant 29 and CHO-K1 cells carrying 6-thioguanine resistance and ouabain resistance markers. The PtdSer synthetic activity of the resultant hybrid clone, 29/K1, cultivated in the absence of PtdSer was intermediate between the activities of CHO-K1 and mutant 29 cells, as measured as the incorporation of L-[$^{14}$C]serine into PtdSer (Fig. 1*A*). When PtdSer was added to the culture medium at a concentration of 80 μM, the PtdSer formation in the CHO-K1, hybrid 29/K1, and mutant 29 cells was reduced by 98%, 46%, and 29%, respectively (Fig. 1*A*). These results indicated that the PtdSer-resistant PtdSer biosynthesis is a semidominant phenotype. Based on this semidominant phenotype, we examined whether or not a PtdSer synthase gene, *pssA* or *pssB*, of the mutant 29 has a mutation relevant to the PtdSer-resistant PtdSer biosynthesis.

**PtdSer Biosynthesis in the Mutant 29 Cells Transfected with the *pssA* and *pssB* cDNA.** Because the PtdSer-resistant PtdSer biosynthesis in mutant 29 cells was shown to be a semidominant phenotype, this resistant phenotype of the

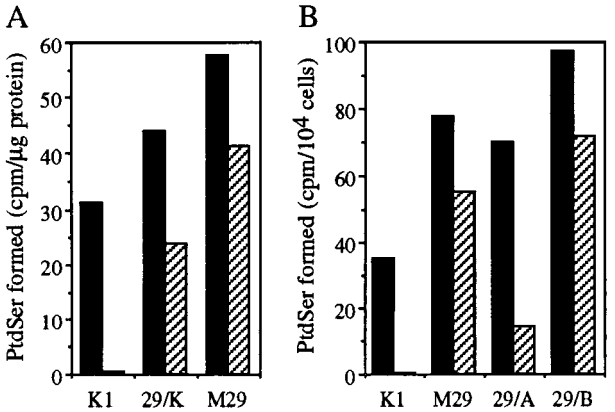


FIG. 1. PtdSer biosynthesis in a hybrid clone of mutant 29 and CHO-K1 cells (*A*) and in mutant 29 cells transfected with *pssA* and *pssB* cDNA clones (*B*). Cells were metabolically labeled with [$^{14}$C]serine for 3 hr at 37°C in the absence (filled bars) or presence (hatched bars) of 80 μM PtdSer as described. Phospholipids in the labeled cells were extracted by the method of Bligh and Dyer (12), and then the radioactivity of PtdSer was determined as described (10). Values are the averages of duplicate samples per each experiment, with variation of <10% between duplicates. K1, CHO-K1; 29/K, hybrid 29/K1; M29, mutant 29; M29/A, transformant M29/*pssA*; M29B, transformant M29/*pssB*.

mutant cells was expected to be partially suppressed by transfection with the wild-type gene corresponding to the mutant gene responsible for the resistant phenotype. Therefore, for initial examination of *pssA* and *pssB* for the mutation, we transfected the mutant 29 cells with each of plasmids pSV*pssA*/*neo* and pSV*pssB*/*neo*, which carry, respectively, *pssA* and *pssB* cDNA from CHO-K1 cells in addition to a G418 resistance determinant, and isolated G418-resistant transformants. Cell extracts of a resultant pSV*pssA*/*neo*-transformed clone (designated as M29/*pssA*) and a pSV*pssB*/*neo*-transformed clone (designated as M29/*pssB*) exhibited, respectively, 2.2- and 2.5-fold higher serine base-exchange activity than that in the cell extract of mutant 29 cells, indicating that the transfected *pssA* or *pssB* cDNA was expressed in the transformants. In the medium without exogenous PtdSer, the PtdSer biosynthetic activity of the M29/*pssA* transformant was slightly lower than that of mutant 29 cells and 2-fold that of CHO-K1 cells (Fig. 1*B*). When PtdSer was added to the culture medium, the PtdSer biosynthesis in the M29/*pssA* transformant was depressed much more efficiently than that in mutant 29, as shown in Fig. 1*B*. However, unlike CHO-K1 cells, the M29/*pssA* transformant synthesized a significant amount of PtdSer in the presence of exogenous PtdSer (Fig. 1*B*). The PtdSer biosynthetic activity of the M29/*pssB* transformant was slightly higher than that of mutant 29 in the medium with and without exogenous PtdSer (Fig. 1*B*). These results indicated that the wild-type *pssA* gene, but not the wild-type *pssB* gene, can partially suppress the PtdSer-resistant PtdSer biosynthetic phenotype of mutant 29 cells. This finding made us examine further the *pssA* gene of mutant 29 cells.

**The *pssA* cDNA from Mutant 29 Cells Induces PtdSer-Resistant PtdSer Biosynthesis in CHO-K1 Cells.** To examine the *pssA* gene of mutant 29 for the mutation, we isolated the *pssA* cDNA from the mutant cells by using reverse transcription-PCR, and transfected CHO-K1 cells with each of plasmids pSVm29-*pssA*/*neo* and pSV*pssA*/*neo2*, which carry, respectively, *pssA* cDNAs from the mutant 29 and CHO-K1 cells in addition to a G418-resistance determinant. From the resultant G418-resistant transformants, a pSVm29-*pssA*/*neo*-transformed clone (designated as K1/m29-*pssA*) and a pSV*pssA*/*neo2*-transformed clone (designated as K1/wt-*pssA*) were purified and subjected to the enzyme assay for serine base-exchange activity and a metabolic labeling experiment with [$^{14}$C]serine. Cell extracts of the transformants, K1/m29-*pssA* and K1/wt-*pssA*, exhibited, respectively, 3.8- and 6.3-fold higher serine base-exchange activity than that in the cell extract of CHO-K1, indicating that the transfected *pssA* cDNAs were expressed in the transformants. In the medium without exogenous PtdSer, the K1/m29-*pssA* transformant exhibited 5-fold higher PtdSer biosynthetic activity, relative to that observed in CHO-K1 cells (Fig. 2). In contrast to the K1/m29-*pssA* transformant, the K1/wt-*pssA* transformant did not show such elevation (Fig. 2). Furthermore, it was shown that the PtdSer biosynthesis in the K1/m29-*pssA* transformant was not depressed by exogenous PtdSer at all, although that in the K1/wt-*pssA* transformant and CHO-K1 cells was almost completely depressed by exogenous PtdSer (Fig. 2). These results indicated that the *pssA* gene of mutant 29 has a mutation that induces the PtdSer-resistant PtdSer biosynthesis.

**The *pssA* Gene of Mutant 29 Has a Missense Mutation Resulting in Replacement of Arg-95 by Lysine.** To determine the mutation site, the coding region of *pssA* cDNA from mutant 29 cells was subjected to nucleotide sequencing. Comparison of the nucleotide sequence determined with that of the wild-type *pssA* cDNA revealed that the *pssA* cDNA from mutant 29 cells has a transition of G → A at the second position of codon 95, which changes the codon from arginine to lysine. To verify that the R95K mutation is responsible for the PtdSer-resistant PtdSer biosynthesis, we introduced the same mutation into the wild-type *pssA* cDNA through a PCR protocol (15) and transiently transfected CHO-K1 cells with a plasmid, pSV*R95K-pssA*/*neo*, carrying the resultant R95K mutant *pssA* cDNA. As expected, the R95K mutant *pssA* cDNA but not the wild-type *pssA* cDNA induced the elevation of the PtdSer biosynthetic rate and PtdSer-resistant PtdSer biosynthesis upon transient transfection into CHO-K1 cells (Fig. 3). These results indicated that Arg-95 of PSS I plays a critical role in the PtdSer-induced depression of PtdSer biosynthesis.

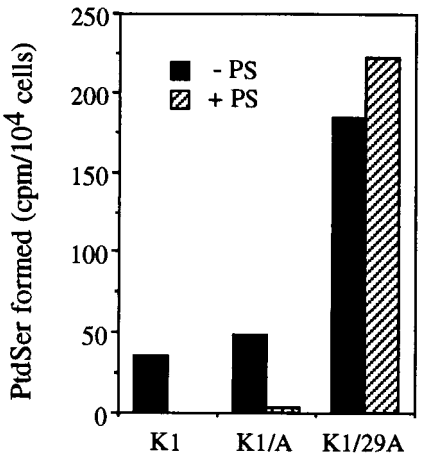


FIG. 2. The *pssA* cDNA from mutant 29 cells induces elevation of the PtdSer biosynthetic rate and PtdSer-resistant PtdSer biosynthesis in CHO-K1 cells. Cells were metabolically labeled with [$^{14}$C]serine for 3 hr at 37°C in the absence or presence of 80 μM PtdSer as described. Phospholipids in the labeled cells were extracted by the method of Bligh and Dyer (12), and then the radioactivity of PtdSer was determined as described (10). Filled and hatched bars represent the formation of [$^{14}$C]PtdSer in the absence and presence of PtdSer, respectively. Values are the averages of duplicate assays, with variation of <10% between duplicates. K1, CHO-K1; K1/A, transformant K1/wt-*pssA*; K1/29A, transformant K1/m29-*pssA*.

**R95K-Mutant PSS I Is Resistant to the Inhibition by PtdSer *in Vitro*.** We previously showed that the serine base-exchange activity in a membrane fraction prepared from CHO-K1 cells was inhibited on the addition of PtdSer to the assay mixture,

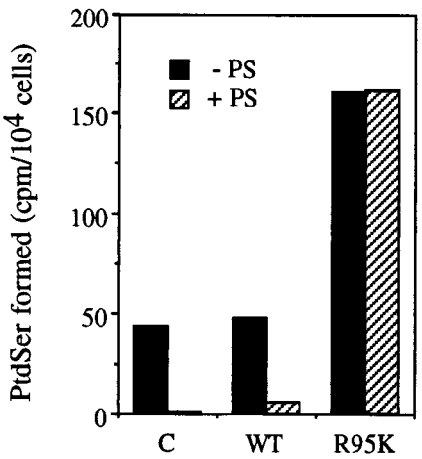


FIG. 3. The R95K mutation in *pssA* is responsible for the PtdSer-resistant PtdSer biosynthesis. CHO-K1 cells were transiently transfected with a control vector, pSVOK*neo* (C), pSV*pssA*/*neo2* (WT), or pSV*R95K-pssA*/*neo* (R95K), by using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. After transfection (1 day), the cells were harvested and then subjected to the labeling experiment with [$^{14}$C]serine, as described. Filled and hatched bars represent the formation of [$^{14}$C]PtdSer in the absence and presence of 80 μM PtdSer, respectively. Values are the averages of duplicate assays, with variation of <10% between duplicates.

but that from mutant 29 cells was resistant to the inhibition (11). To confirm that the R95K mutation in the *pssA* gene is responsible for the PtdSer-resistant serine base exchange activity observed in the cell-free system, we assessed the effect of PtdSer on the serine base-exchange activity in membrane fractions prepared from the K1/m29-*pssA* and K1/wt-*pssA* transformants, and CHO-K1 and mutant 29 cells. The serine base-exchange activities of the K1/wt-*pssA* transformant and CHO-K1 cells were inhibited by PtdSer in a dose-dependent manner, being reduced by ≈70% and 95%, respectively, on the addition of 300 μM PtdSer (Fig. 4). However, the activities of the K1/m29-*pssA* transformant and mutant 29 cells were enhanced on the addition of PtdSer (Fig. 4). These results indicated that the R95K mutation renders PSS I resistant to the inhibition by PtdSer, although it remains unknown why PtdSer exhibits the opposite effect on the wild-type and R95K-mutant PSS I.

**Increase in the Cellular PtdSer Level on Expression of the R95K Mutant *pssA* in CHO-K1 Cells.** To determine whether or not the R95K mutant product of *pssA* leads to elevation of the steady-state level of PtdSer, in addition to elevation of the biosynthetic rate, we determined the phospholipid compositions and contents of the K1/m29-*pssA* and K1/wt-*pssA* transformants, and CHO-K1 and mutant 29 cells grown without exogenous PtdSer. As shown in Table 1, the contents of PtdSer and its metabolite, PtdEtn, in the K1/m29-*pssA* transformant were both ≈2-fold those in CHO-K1 cells and similar to those in mutant 29 cells, whereas the K1/wt-*pssA* transformant did not show such significant increases in the PtdSer and PtdEtn contents.

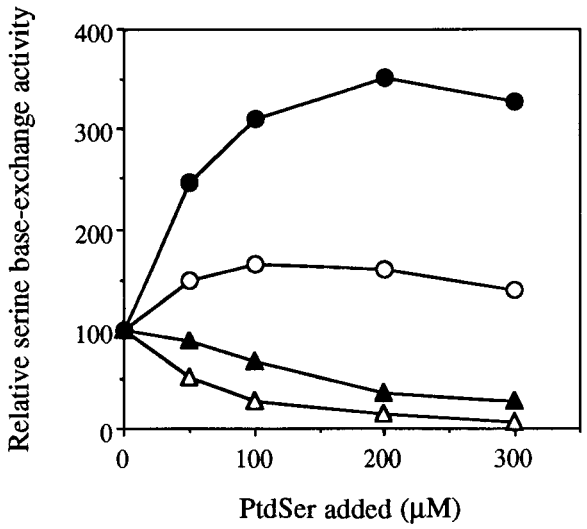


FIG. 4. Effect of PtdSer on the serine base-exchange activity *in vitro*. Cells were seeded at $1.2 \times 10^6$ cells/150-mm-diameter dish in the growth medium without exogenous PtdSer at 37°C. After 3 days, the cells were washed twice with PBS, suspended in 0.25 M sucrose containing 10 mM Hepes-NaOH (pH 7.5) and 1 mM EDTA, and then homogenized with a Potter-Elvehjem Teflon homogenizer. The homogenates were centrifuged at 700 × *g* for 5 min, followed by centrifugation of the supernatants at 100,000 × *g* for 1 hr. The resulting pellets were resuspended in 0.25 M sucrose containing 10 mM Hepes-NaOH (pH 7.5) and 1 mM EDTA, and then assayed for the serine base-exchange activity as described (1), in the presence of various amounts of PtdSer liposomes. The results are expressed as the percentages of activity relative to the specific activity of each strain, measured without exogenous PtdSer. The specific activities in the preparations from CHO-K1, mutant 29, K1/wt-*pssA*, and K1/m29-*pssA* cells, measured without exogenous PtdSer, were 6.95, 8.59, 25.78, and 18.25 nmol/hr per mg protein, respectively. Values are the averages of duplicate assays, with variation of <10% between duplicates. △, CHO-K1; ○, mutant 29; ▲, K1/wt-*pssA*; and ●, K1/m29-*pssA*.

Table 1. Phospholipid compositions and contents of CHO-K1, K1/wt-*pssA*, K1/m29-*pssA*, and mutant 29 cells

| Strain | Total phospholipids, % | | | | | |
|---|---|---|---|---|---|---|
| | PS | PE | PC | SM | PI | Others |
| CHO-K1 | 5.6 | 14.2 | 58.7 | 9.7 | 7.0 | 4.7 |
| | (8.8) | (22.5) | (92.8) | (15.4) | (11.1) | (7.5) |
| K1/wt-*pssA* | 7.1 | 16.8 | 54.4 | 9.4 | 6.7 | 5.6 |
| | (10.8) | (25.3) | (81.8) | (14.1) | (10.1) | (8.4) |
| K1/m29-*pssA* | 11.1 | 23.5 | 45.0 | 11.3 | 4.0 | 5.1 |
| | (18.4) | (38.8) | (74.4) | (18.7) | (6.6) | (8.5) |
| Mutant 29 | 9.3 | 23.1 | 46.7 | 11.2 | 4.7 | 4.9 |
| | (16.4) | (40.8) | (82.4) | (19.8) | (8.3) | (8.7) |

Cells were seeded at $1.2 \times 10^6$ cells/150-mm-diameter dish in the growth medium without exogenous PtdSer at 37°C. After 3 days, the cellular phospholipids were extracted and analyzed by two-dimensional thin-layer chromatography as described (10). To quantitate the individual phospholipids, the phosphate in each spot on a chromatogram was determined chemically (17). The values in parentheses are expressed in nmol of phospholipid/mg of protein. Two independent experiments gave similar results, and one set of results is presented here. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol.

## DISCUSSION

PtdSer biosynthesis in CHO-K1 cells is depressed on the addition of PtdSer to the culture medium, but that in mutant 29 cells is highly resistant to this depression by exogenous PtdSer (11). Mutant 29 cells cultivated without exogenous PtdSer exhibited a 2- to 3-fold increase in PtdSer biosynthetic rate and an ≈2-fold higher cellular PtdSer level, relative to those in the parental strain, CHO-K1 (11). In the present study, we found a missense mutation in the *pssA* gene for PSS I of mutant 29 cells, which results in the replacement of Arg-95 by lysine (R95K mutation). The mutant *pssA* cDNA, but not the wild-type *pssA* cDNA, induces striking increases in the PtdSer biosynthetic rate and content on transfection into CHO-K1 cells. The PtdSer biosynthesis in the mutant *pssA*-transfected cells was not depressed on the addition of PtdSer to the culture medium, in contrast to that in the wild-type *pssA*-transfected cells. By using cell extracts of the mutant *pssA*- and wild-type *pssA*-transfected cells, we have shown that the R95K mutation in *pssA* renders the product, PSS I, resistant to the inhibition by PtdSer. These results indicate that the inhibition of PSS I by PtdSer is important for the regulation of PtdSer biosynthesis and essential for the maintenance of a normal PtdSer level in CHO-K1 cells. Furthermore, the results demonstrate that Arg-95 of PSS I is a crucial residue for the inhibition of PSS I by PtdSer.

In addition to the increase in the PtdSer content, the mutant *pssA*-transfected cells exhibited an ≈2-fold increase in the content of PtdEtn compared with that in CHO-K1 cells. This finding indicated that the inhibition of PSS I by PtdSer is important for the biosynthetic regulation of both PtdSer and PtdEtn, the majority of which in CHO-K1 cells is synthesized through decarboxylation of PtdSer (1, 18)

Although it is obvious that PSS I is inhibited by PtdSer, the molecular mechanism underlying the inhibition remains to be elucidated. The mechanism might comprise simple product inhibition due to the binding of PtdSer to the catalytic site of PSS I. It is also possible that PtdSer inhibits PSS I through its binding to a putative regulatory site of the synthase. Interestingly, the serine base-exchange activity in the membrane fraction of the wild-type *pssA*-transfected cells is partially resistant to the inhibition by PtdSer when compared with that in CHO-K1 cells. Fifty percent inhibition of the activity in CHO-K1 and *pssA*-transfected cells is achieved with ≈50 and ≈150 μM PtdSer, respectively. The inhibition of the activity of the *pssA*-transfected cells appears to be saturated at 70–80%,

whereas the activity of CHO-K1 cells is inhibited by >90%. These results raise another possibility that the inhibition of PSS I by PtdSer is mediated by an unknown factor saturable with overproduced PSS I.

The serine base-exchange in CHO-K1 cells is catalyzed by at least two enzymes, PSS I and PSS II (7–9). Both PSS I and PSS II appear to be inhibited by PtdSer, because the serine base-exchange activity of a membrane fraction of CHO-K1 cells is almost completely inhibited on the addition of PtdSer. Therefore, if PSS II in mutant 29 cells is of the wild type, PSS II in the mutant should be also inhibited by PtdSer. Nevertheless, the serine base-exchange activity in a membrane fraction of the mutant is apparently not inhibited by PtdSer at all. To examine whether or not the wild-type PSS II is produced in mutant 29 cells, we isolated the *pssB* cDNA for PSS II from the mutant cells, by using reverse transcription–PCR and subjected the coding region of the isolated cDNA to nucleotide sequencing. We found one difference between the sequences of the *pssB* cDNAs from mutant 29 and CHO-K1 cells, namely, a transversion of C → A at the third position of codon 305. However, the transversion was of the silent substitution resulting in no change in an amino acid. Thus, mutant 29 cells can produce the wild-type PSS II. In addition, we have found that the wild-type CHO-K1 cells transfected with the mutant 29 *pssA* cDNA exhibited the serine base-exchange activity that is strikingly enhanced by PtdSer (see Fig. 4). From these observations, we speculate that the complete resistance of the serine base-exchange activity to the inhibition by PtdSer in the mutant 29 membrane is ascribed to the enhancement of the mutant PSS I activity by PtdSer, which exceeds the decrease in PSS II activity caused by PtdSer.

There is high (38%) amino acid sequence identity between PSS I and PSS II (9). PSS II has an arginine residue at position 97, which corresponds to the Arg-95 of PSS I identified as a crucial residue for the inhibition by PtdSer. In addition, the amino acid sequence, G$^{91}$PFTRPHPALWR$^{102}$, surrounding Arg-95 of PSS I is very similar to the sequence, G$^{93}$PFSRPHPAYWR$^{105}$, surrounding Arg-97 of PSS II (underlined letters represent identical amino acids between the two sequences). It is therefore likely that this amino acid motif common to PSS I and PSS II is involved in the inhibition of the synthases by PtdSer.

We thank Naoko Nobuzane for technical assistance. This work was supported in part by the Human Sciences Basic Research Project and the Integrated Study Projects on Drug Innovation Science of the Japan Health Sciences Foundation, by Grants-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture of Japan, by the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan, and by a grant from the Ichiro Kanehara Foundation.

1. Kuge, O., Nishijima, M. & Akamatsu, Y. (1986) *J. Biol. Chem.* **261,** 5790–5794.
2. Borkenhangen, J. D., Kennedy, E. P. & Fielding, L. (1961) *J. Biol. Chem.* **236,** PC28–PC30.
3. Marggraf, W. D. & Anderer F. A. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **335,** 1299–1304.
4. Kuge, O., Nishijima, M. & Akamatsu, Y. (1986) *J. Biol. Chem.* **261,** 5795–5798.
5. Kuge, O., Nishijima, M. & Akamatsu, Y. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1926–1930.
6. Voelker, D. R. & Frazier, J. L. (1986) *J. Biol. Chem.* **261,** 1002–1008.
7. Kuge, O., Nishijima, M. & Akamatsu, Y. (1991) *J. Biol. Chem.* **266,** 24184–24189.
8. Saito, K., Kuge, O., Akamatsu, Y. & Nishijima, M. (1996) *FEBS Lett.* **395,** 262–266.
9. Kuge, O., Saito, K. & Nishijima, M. (1997) *J. Biol. Chem.* **272,** 19133–19139.
10. Nishijima, M., Kuge, O. & Akamatsu, Y. (1986) *J. Biol. Chem.* **261,** 5784–5789.
11. Hasegawa, K., Kuge, O., Nishijima, M. & Akamatsu, Y. (1989) *J. Biol. Chem.* **264,** 19887–19892.
12. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917.
13. Hanada, K., Hara, T., Nishijima, M., Kuge, O., Dickson, R. C. & Nagiec, M. M. (1997) *J. Biol. Chem.* **272,** 32108–32114.
14. Lewis, W. H., Srinivasan, P. R., Stokoe, N. & Simonovitch, L. (1980) *Somat. Cell Genet.* **6,** 333–347.
15. Higuchi, R., Krummel, B. & Saiki, R. K. (1988) *Nucleic Acids Res.* **16,** 7351–7367.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
17. Gerlach, E. & Deuticke, B. (1963) *Biochem. Z.* **337,** 477–479.
18. Voelker, D. R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2669–2673.