FEBS Letters 395 (1996) 262–266 FEBS 17655

Immunochemical identification of the *pssA* gene product as phosphatidylserine synthase I of Chinese hamster ovary cells

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Received 21 August 1996; revised version received 10 September 1996

Abstract **We have previously shown that a Chinese hamster ovary (CHO) cell mutant defective in phosphatidylserine synthase I recovers the enzyme activity on transfection with a *pssA* cDNA clone isolated from the parental CHO-K1. The resultant transfectant, CDT-1, exhibited about 20-fold higher specific activity of the enzyme in the membrane fraction than CHO-K1 cells. Polyclonal antibodies against two peptides of the predicted *pssA* product cross-reacted with a membrane protein having an apparent molecular mass of 42 kDa, which was overproduced in CDT-1 cells. By immunoprecipitation with the antibody, phosphatidylserine synthase I activity as well as the 42-kDa protein was eliminated from solubilized membrane proteins of CDT-1 cells. Both the enzyme activity and the 42-kDa protein of CHO-K1 cells were enriched in the mitochondria-associated membrane fraction and the microsome fraction, but neither was enriched in the mitochondria fraction or the cytosol fraction. These results suggest that the *pssA* gene encodes phosphatidylserine synthase I.**

Key words: Phosphatidylserine; Phosphatidylserine synthase; *pssA*; Phospholipid base-exchange reaction; Mitochondria-associated membrane; CHO cell

1. Introduction

Phosphatidylserine (PS) is one of the major phospholipids in mammalian cell membranes, interacting with various proteins, such as protein kinase C [1], MARCKS (myristoylated alanine-rich C kinase substrate) [2], coagulation factor V [3], and synaptotagmin [4]. By using Chinese hamster ovary (CHO) cell mutants defective in PS biosynthesis, we have shown that PS is essential for CHO cell growth and is synthesized by at least two kinds of PS synthases, I and II [5,6], which catalyze an exchange reaction between free L-serine and the base moiety of preexisting phospholipids for PS formation [7,8]. In addition to the serine base-exchange reaction, PS synthase I in an extract of CHO cells catalyzes base-exchange reactions of phospholipids with ethanolamine and choline, whereas PS synthase II in the extract catalyzes the ethanolamine base-exchange reaction, but not the choline base-exchange reaction.

With respect to regulation of PS biosynthesis in mammalian cells, we found that exogenously added PS is efficiently incorporated into CHO cells and inhibits endogenous PS biosynthesis in the cells [9]. This inhibition is probably not due to a decrease in the expression level of PS synthases, because the serine base-exchange activity in an extract of cells grown with PS is not significantly different from that in an extract of cells grown without PS [9]. In addition, we isolated a CHO cell mutant in which PS synthesis is elevated about 2-fold and remarkably resistant to inhibition by exogenous PS, as compared with the parental cells [10]. These findings suggested the existence of a factor that modulates the activities of PS synthases in intact cells in response to the cellular PS level.

Cloned genes involved in PS metabolism can serve as a useful tool to understand the molecular mechanisms underlying the biosynthesis and metabolic regulation of PS. By genetic complementation with a PS-auxotrophic CHO cell mutant, PSA-3, which lacks PS synthase I activity, we isolated a *pssA* cDNA clone which transforms PSA-3 to a PS prototroph, from CHO-K1 cells [11]. The transformant, CDT-1, exhibits higher specific activity of PS synthase I than CHO-K1 cells. The *pssA* product deduced from the cDNA sequence is an integral membrane protein of 471 amino acids with several potential membrane-spanning domains. To clarify the biochemical characteristics and the function of the *pssA* product, we have here generated antibodies against peptides of the predicted *pssA* product. Using the antibodies, we obtained several lines of evidence that the *pssA* product is PS synthase I.

2. Materials and methods

2.1. Strains and culture conditions

Strain CHO-K1 was obtained from the American Type Culture Collection (ATCC CCL 61). CHO-K1, PSA-3, and CDT-1 cells were maintained as described [6,11]. For suspension culture, cells were maintained in a spinner vessel containing ES medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (ICN Biomedicals, USA), 2 mM L-glutamine, 100 units of penicillin G per ml, 100 μg of streptomycin sulfate per ml, and 2.2 mg of $NaHCO_3$ per ml at 37°C in a 5% CO_2 atmosphere at 100% humidity.

2.2. Assay of PS synthase I activity in cellular fractions

Cells ($1–4\times10^8$ cells) were suspended in 10 mM HEPES buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg of leupeptin per ml, and 1 μg of pepstatin A per ml, and disrupted on ice with a W-225R Ultrasonic disrupter (Heat System Ultrasonics, USA) equipped with a No. 419 microtip. Undisrupted cells were removed by centrifugation at $750\times g$ for 10 min at 4°C. The supernatant was centrifuged at $100\,000\times g$ for 1 h at 4°C in a Beckman Ti-70 rotor. The resultant supernatant was saved as a cytosolic fraction. The pellet was suspended in 10 mM HEPES buffer (pH 7.5) containing 1 mM dithiothreitol, 1 mM PMSF, 1 μg of leupeptin per ml, and 1 μg of pepstatin A per ml, and saved as a membrane fraction. To solubilize membrane proteins, the membrane fraction was suspended on ice in solubilization buffer consisting of 7 mM HEPES (pH 7.5), 0.7 mM dithiothreitol, 14% (v/v) glycerol, 10 mg of asolectin per ml, and 1% (w/v) sucrose monolaurate (Wako Pure Chemical Industries, Osaka, Japan) at a final

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Abbreviations: CHO, Chinese hamster ovary; PS, phosphatidylserine; MARCKS, myristoylated alanine-rich C kinase substrate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; MAM, mitochondria-associated membranes

PII S0014-5793(96)01049-6

protein concentration of 3 mg per ml. The mixture was then centrifuged at 45000 rpm (about 81000×*g*) for 30 min at 4°C in a Beckman TLA 100.3 rotor and the resultant supernatant was saved as a fraction of solubilized membrane proteins. About 80% of total proteins in the membrane fraction were recovered in the solubilized fraction. Protein in each fraction was measured as described [12]. PS synthase I activity in each fraction was assayed as the choline base-exchange activity as described [6] by using [*methyl*-^{14}C]choline chloride (American Radiolabeled Chemicals, USA) as a substrate.

2.3. Immunochemical analysis of the pssA product

A synthetic peptide corresponding to the amino acid residues at positions 4–18 in the *pssA* protein predicted from the cDNA sequence [11] was coupled to SuperCarrier (Pierce, USA) with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) according to the manufacturer's protocol. Another synthetic peptide corresponding to the amino acid residues at positions 447–463 in the predicted *pssA* protein was coupled to keyhole limpet hemocyanin (Sigma Chemical Co., USA) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sigma) as described [13] with slight modifications. The two peptides were provided by Sumitomo Pharmaceuticals Research Center (Osaka, Japan). Peptide [4–18] and peptide [447–463] conjugates (200–300 μg) were emulsified with alum (Pierce) and Freund's adjuvant (Difco Laboratories, USA), respectively, and injected into rabbits every 2 weeks. Antibodies against peptides [4–18] and [447–463] were affinity-purified from antisera using peptide-coupled resins as described [14] and dialyzed against phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 6.5 mM Na_2HPO_4 (pH 7.4). Resins were prepared by coupling peptides [4–18] and [447–463] to SulfoLink Coupling gel (Pierce) and CNBr-activated Sepharose 4B (Pharmacia-LKB, Sweden), respectively, according to the manufacturer's protocols. Preimmune IgG was purified from preimmune serum using protein A Sepharose CL-4B (Pharmacia-LKB) as described [14] and dialyzed against PBS.

For immunoblotting, proteins were incubated in SDS sample buffer consisting of 62.5 mM Tris-HCl (pH 6.7), 1% (w/v) SDS, 2% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue at 37°C for 30 min, separated by electrophoresis in 10% polyacrylamide slab gels containing 0.1% SDS as described [15], and then electroblotted to polyvinylidene difluoride membranes (Biorad, USA) as described [16]. The blots were incubated with the anti-peptide [447–463] antibody, and then with anti-rabbit IgG linked to horseradish peroxidase (Amersham, UK). Cross-reactive proteins were detected by enhanced chemiluminescence kit (Amersham) as recommended by the manufacturer.

For immunoprecipitation, membrane fraction was prepared from ^{35}S-labelled (see below) or non-labelled cells, then membrane proteins were solubilized with solubilization buffer as described above (Section 2.2). A portion of the solubilized fraction was incubated with the anti-peptide [4–18] antibody or preimmune IgG in a total volume of 30–50 μl on ice for 3 h. The mixture was incubated with 100 μl of 20% (v/v) protein A Sepharose CL-4B suspended in PBS on ice for 1 h, then briefly centrifuged. The resultant supernatant was saved and the precipitate was washed with 500 μl of PBS three times. Proteins in the supernatant and precipitate were separated in 10% polyacrylamide gels containing 0.1% SDS as described above, and the *pssA* product was detected either by a bioimage analyzer (Fujix BAS2000) or by immunoblotting with the anti-peptide [447–463] antibody.

To label cellular proteins with [^{35}S]methionine, cells reaching 70–80% confluence in a 100 mm diameter dish were incubated with 120 μCi of [^{35}S]methionine (ICN Biomedicals) in 4.5 ml of methionine-free Dulbecco's modified Eagle medium (Gibco-Bethesda Research Laboratories, USA) supplemented with 580 μg of glutamine per ml, 110 μg of sodium pyruvate per ml, 11% (v/v) newborn calf serum (Dainippon Pharmaceuticals Co., Ltd., Osaka, Japan) dialyzed against PBS, 100 units of penicillin G per ml and 100 μg of streptomycin sulfate per ml for 8 h at 37°C.

2.4. Subcellular fractionation

Post-nuclear supernatant was prepared from CHO-K1 cells from suspension culture and separated into mitochondria, mitochondria-associated membranes (MAM), microsome, and cytosol fractions by the same procedure as described [17], except that bovine serum albumin was omitted from the buffer used for fractionation. Cytochrome *c* oxidase [18], glucose-6-phosphate phosphatase [19], and CTP:phosphocholine cytidylyltransferase [20] were assayed as described.

3. Results

3.1. Identification of the pssA protein with antibodies against pssA peptides

To identify the *pssA* protein in CHO cells, we prepared polyclonal antibodies against two *pssA* peptides corresponding to the amino acid residues at positions 4–18 and 447–463 of the *pssA* protein deduced from the *pssA* cDNA sequence [11]. By immunoblotting with the anti-peptide [447–463] antibody, a cross-reacting protein with an apparent molecular mass of 42 kDa was found in the membrane fraction of CHO-K1 cells, but not in their cytosolic fraction (Fig. 1A,B, lane 1), whereas neither of the fractions of PSA-3 cells defective in PS synthase I contained any detectable amount of the 42-kDa protein (Fig. 1A,B, lane 2). CDT-1 cells carrying the *pssA* cDNA contained a larger amount of the 42-kDa protein in their membrane fraction than did CHO-K1 cells, but no significant amount of the protein was found in their cytosolic fraction (Fig. 1A,B, lane 3). Although the 42-kDa protein was not detected by immunoblotting with the anti-peptide [4–18] antibody (data not shown), this antibody was found to precipitate a protein with an apparent molecular mass of 42 kDa from solubilized membrane proteins of CDT-1 cells that had been metabolically labelled with [^{35}S]methionine (Fig. 2A, lane 2). The 42-kDa protein precipitated with the anti-peptide [4–18] antibody cross-reacted with the anti-peptide [447–463] antibody (Fig. 2B, lane 2). The corresponding protein was not detected in the precipitates with preimmune IgG (Fig. 2A,B,

Table 1
Subcellular distribution of phosphatidylserine synthase I activity in CHO-K1 cells

Enzyme	Specific activity in				
	PNS	Mitochondria	MAM	Microsomes	Cytosol
PS synthase I	1.43	0.626	9.40	3.53	0
Cytochrome *c* oxidase	155	791	109	49.0	2.29
Glucose-6-phosphate phosphatase	14.5	12.1	33.5	26.5	6.42
CTP:phosphocholine cytidylyltransferase	122	3.28	7.32	39.4	123

Subcellular fractions were prepared from CHO-K1 cells and each fraction (50 μg of protein) was assayed for PS synthase I activity in the presence of 5 mg of asolectin per ml. The subcellular fractions were also assayed for activities of cytochrome *c* oxidase (a marker enzyme for mitochondria), glucose-6-phosphate phosphatase (a marker enzyme for the MAM and microsomes), and CTP:phosphocholine cytidylyltransferase (a marker enzyme for microsomes and cytosol). The units of the enzymes are: PS synthase I, nmol/h/mg protein; cytochrome *c* oxidase, nmol of cytochrome *c* oxidized/min/mg protein; glucose-6 phosphate phosphatase, nmol of Pi formed/min/mg protein; CTP:phosphocholine cytidylyltransferase, nmol of CDP-choline formed/h/mg protein. Specific activities are averages of duplicate assays and varied < 10% between duplicates. PNS is an abbreviation for post-nuclear supernatant.

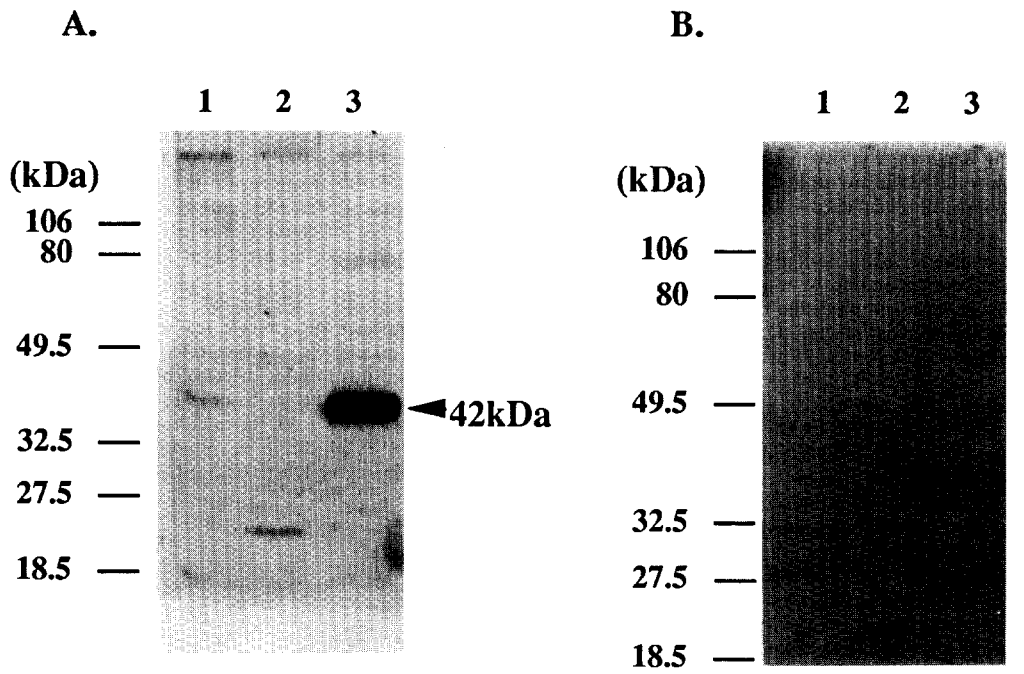

Fig. 1. Detection of the *pssA* protein by immunoblotting. Membrane (A) and cytosolic fractions (B) of CHO-K1 (lane 1), PSA-3 (lane 2) and CDT-1 (lane 3) cells (50 μg of protein per lane) were separated in a SDS-polyacrylamide gel and subjected to immunoblotting with the anti-peptide [447–463] antibody. The arrow indicates the *pssA* protein with an apparent molecular mass of 42 kDa. Molecular size markers are labelled on the left.

lane 1). These results show that the 42-kDa protein was the *pssA* gene product.

3.2. Elevation of PS synthase I activity in CDT-1 cells

As in the case of the 42-kDa *pssA* protein, PS synthase I activities in CDT-1 and CHO-K1 cells were recovered exclusively in the membrane fraction, and the specific activity of the enzyme in the membrane fraction of CDT-1 cells (77.0 ± 4.1 nmol/h/mg protein) was approximately 20-fold higher than that in the membrane fraction of CHO-K1 cells (3.89 ± 0.306 nmol/h/mg protein). The membrane and cytosolic fractions of PSA-3 cells showed no detectable activity of the enzyme. These results showed that the transfection of the *pssA* cDNA induced the simultaneous increase in PS synthase I activity and the amount of the *pssA* protein.

3.3. Immunodepletion and inhibition of PS synthase I activity by the anti-pssA peptide antibody

The membrane fraction of CDT-1 cells was solubilized, then immunoprecipitated with the anti-peptide [4–18] antibody as described in Section 2. The resultant supernatant was assayed for the enzyme activity and also analyzed for the amount of the *pssA* protein by immunoblotting with the anti-peptide [447–463] antibody. As shown in Fig. 3A, PS synthase I activity in the supernatant was decreased by nearly 50% after immunoprecipitation with the anti-peptide [4–18] antibody, but not with preimmune IgG (Fig. 3A). The *pssA* protein in the supernatant was also decreased by nearly 50% after immunoprecipitation (Fig. 3B). These results show that PS synthase I activity in the supernatant was proportional to the residual amount of the *pssA* protein. The partial depletion of the *pssA* protein was possibly due to incomplete exposure of epitopes for the anti-peptide [4–18] antibody even after solubilization with sucrose monolaurate. In addition to the immunodepletion, the anti-peptide [4–18] antibody inhibited the activity of the solubilized PS synthase I by about 70%, whereas an excess of preimmune IgG did not at all (Fig. 4).

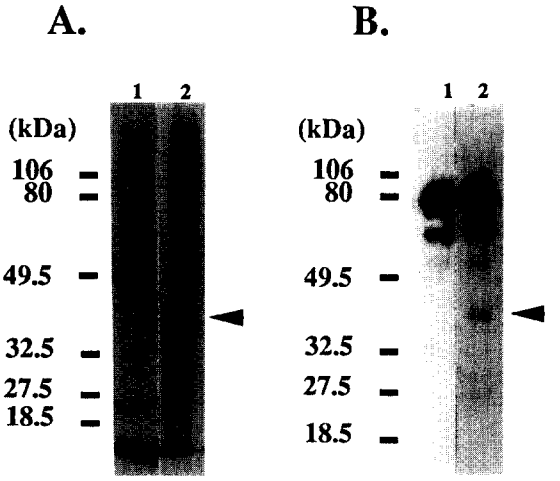

Fig. 2. Immunoprecipitation of the *pssA* protein from CDT-1 cells. (A) Membrane proteins were prepared from one 100-mm dish culture of [^{35}S]methionine-labelled CDT-1 cells and solubilized with 250 μl of solubilization buffer. A portion of the solubilized fraction (45 μl) was immunoprecipitated with 18 μg of the anti-peptide [4–18] antibody (lane 2) or preimmune IgG (lane 1). A half portion of the immunoprecipitated proteins was separated in a SDS-polyacrylamide gel. The arrow indicates a protein with an apparent molecular mass of 42 kDa. Molecular size markers are labelled on the left. (B) Membrane proteins from non-labelled CDT-1 cells were solubilized and the solubilized fraction (15 μl) were immunoprecipitated with 15 μg of the anti-peptide [4–18] antibody (lane 2) or preimmune IgG (lane 1). A half portion of the precipitated proteins was separated in a SDS-polyacrylamide gel and subjected to immunoblotting with the anti-peptide [447–463] antibody. The arrow and molecular size markers are the same as presented in (A).

3.4. Subcellular distribution of PS synthase I activity

The post-nuclear supernatant prepared from CHO-K1 cells was separated into mitochondria, MAM, microsome, and cytosol fractions. The MAM fraction, which is a microsome-like fraction isolated from a crude mitochondrial preparation by centrifugation on a Percoll density gradient [17], has been shown to exhibit high specific activities of PS synthase and several other lipid-biosynthetic enzymes [17,21]. Fractionation into the mitochondria, MAM, microsome, and cytosol fractions was verified by enrichment of marker enzymes into the corresponding fractions (Table 1). The MAM fraction exhibited much lower specific activities of cytochrome *c* oxidase and CTP:phosphocholine cytidylyltransferase than the mitochondria and microsome fractions, respectively, and higher specific activity of glucose-6-phosphate phosphatase than the microsome fraction. These characteristics of the MAM fraction from CHO-K1 cells are consistent with those of the MAM fraction from rat liver [17].

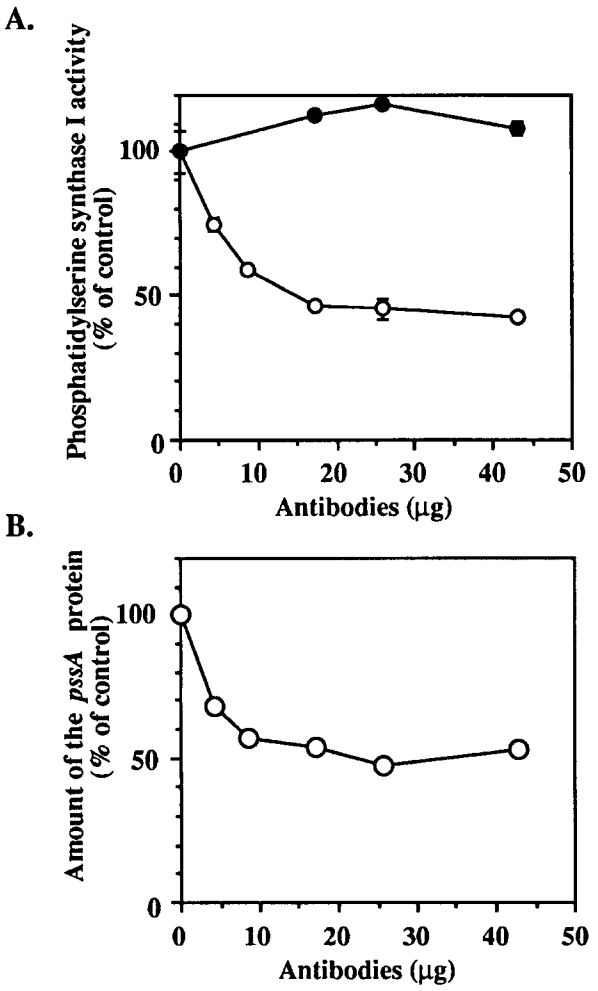

Fig. 3. Immunodepletion of PS synthase I activity (A) and the *pssA* protein (B) from solubilized membrane proteins. Membrane proteins from CDT-1 cells were solubilized and the solibilized fraction was immunoprecipitated with increasing amounts of the anti-peptide [4–18] antibody (open circles) or preimmune IgG (closed circles). A portion of the resultant supernatant (40 μl) was assayed for PS synthase I activity in the presence of 5 mg of asolectin per ml (A). Another portion of the supernatant (15 μl) was analyzed by immunoblotting with the anti-peptide [447–463] antibody (B). A control experiment was performed in the same manner except that the antibody was omitted. As for (B), the amount of the *pssA* protein detected in the immunoblot was estimated densitometrically using NIH Image (ver. 1.57).

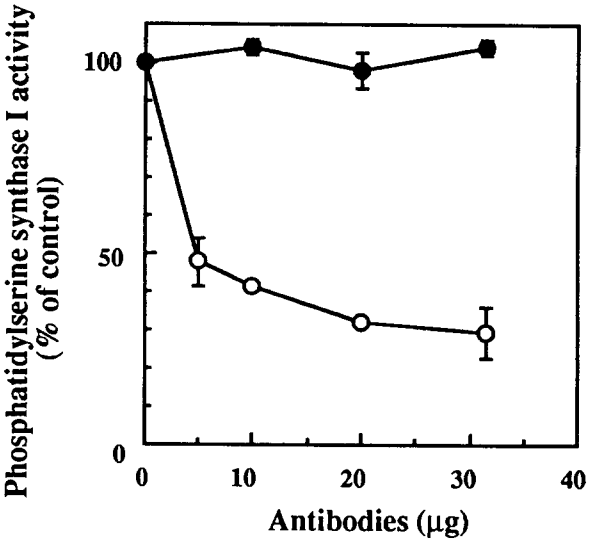

Fig. 4. Effect of the anti-peptide [4–18] antibody on PS synthase I activity. Membrane proteins from CDT-1 cells were solubilized and the solibilized fraction (7.5 μl) was incubated with increasing amounts of the anti-peptide [4–18] antibody (open circles) or preimmune IgG (closed circles) in a total volume of 15 μl on ice for 3 h. The mixture was then assayed for PS synthase I activity in the presence of 5 mg of asolectin per ml. A control experiment was performed in the same manner except that incubation with the antibody was omitted.

Using the subcellular fractions, we determined the distribution of PS synthase I activity (Table 1). The PS synthase activity was enriched in the MAM and microsome fractions, but not in the mitochondria or cytosol fractions. The specific activities of the PS synthase in the MAM and microsome fractions were respectively 6.6- and 2.5-fold higher than that in the post-nuclear supernatant. The recoveries of the PS synthase activity in the MAM and microsome fractions from the post-nuclear supernatant were 24% and 50%, respectively.

To compare it with the distribution of PS synthase I activity, the distribution of the *pssA* protein in subcellular fractions was determined by immunoblotting with the anti-peptide [447–463] antibody (Fig. 5). The *pssA* protein was enriched in the MAM and microsome fractions, but not in the mitochondria or cytosol fractions. The relative contents of the *pssA* protein in the MAM and microsome fractions were respectively 5.8- and 2.1-fold higher than that in the post-nuclear supernatant. The recoveries of the *pssA* protein in the MAM and microsome fractions from the post-nuclear supernatant were 21% and 42%, respectively. These results showed that the level of PS synthase I activity in each subcellular fraction was comparable with the amount of the *pssA* protein.

4. Discussion

We prepared polyclonal antibodies against two different peptides of the predicted *pssA* product and identified the *pssA* product as a 42-kDa membrane protein by using the antibodies. The apparent molecular mass (42 kDa) of the *pssA* protein in a SDS gel is significantly smaller than the molecular mass predicted from the cDNA sequence (55.3

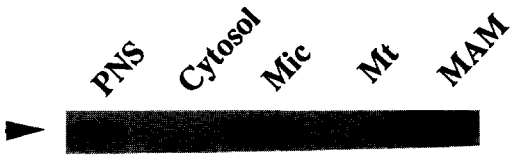

Fig. 5. Subcellular distribution of the *pssA* protein in CHO-K1 cells. Subcellular fractions (30 μg of protein) from CHO-K1 cells were electrophoresed on a SDS-polyacrylamide gel and subjected to immunoblotting with the anti-peptide [447–463] antibody. The abbreviations, PNS, Mit, and Mic are post-nuclear supernatant, mitochondria, and microsome fractions, respectively. The arrow indicates the *pssA* protein, which was quantified as described in the legend to Fig. 3B.

kDa). It is unlikely that the 42-kDa protein is produced by proteolytic processing of its precursor, because the 42-kDa protein cross-reacted with the antibodies against both peptides, which were located respectively near the N- and C-termini. Rather, the discrepancy may be due to the high content of hydrophobic amino acid residues of the *pssA* protein, because certain membrane proteins with high contents of hydrophobic amino acid residues are known to behave atypically in SDS gels as a result of their increased binding to SDS [22,23].

We conclude that the *pssA* gene encodes PS synthase I from the following results: (1) PS synthase I activity and the 42-kDa *pssA* protein were greatly elevated by introduction of the *pssA* cDNA into PSA-3; (2) both PS synthase I activity and the *pssA* protein were present exclusively in the membrane fraction; (3) the specific activity of the PS synthase in the membrane fraction of CHO-K1, PSA-3, and CDT-1 cells correlated well with the amount of the *pssA* protein in the same fraction of the respective strains; (4) PS synthase I activity in solubilized membrane proteins was inhibited by an anti-*pssA* peptide antibody; (5) the solubilized PS synthase I activity was eliminated by the antibody in proportion to the amount of the eliminated *pssA* protein; (6) the antibody did not significantly precipitate proteins other than the *pssA* protein from the solubilized membrane proteins; (7) the subcellular distribution of the PS synthase activity in CHO-K1 cells was very similar to that of the *pssA* protein. Although it remains uncertain at present whether PS synthase I is composed of a single peptide or multiple subunits including the *pssA* protein, result (6) rules out the possibility that the *pssA* protein associates with other proteins through disulfide bonds or in a detergent-resistant manner.

PS synthase I activity and the amount of the *pssA* protein were greatly elevated in the membrane fraction of CDT-1 cells. However, both the biosynthetic rate and content of PS in CDT-1 cells are very similar to those in CHO-K1 cells [11]. These results clearly show that PS biosynthesis catalyzed by PS synthase I is regulated by factors other than the expression level of the enzyme, such as concentrations of the substrates, product and Ca^{2+}. Further study of the *pssA* protein with site-directed mutagenesis may shed light on the regulation of PS metabolism as well as the catalytic mechanism of PS synthase I.

Acknowledgements: We wish to thank Dr. Yoshimasa Sakakibara (National Institute of Health, Tokyo, Japan) for his helpful comments during the preparation of the manuscript. This work was supported in part by the Human Sciences Basic Research Project and the Integrated Study Projects in Drug Innovation Science of the Japan Health Sciences Foundation, by Grants-in-Aid for General Scientific Research, Cooperative Research and Encouragement of Young Scientists from the Ministry of Education, Science and Culture of Japan, by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan, by Research Grants for Aging and Health and for Pharm Dream 21 from the Ministry of Health and Welfare of Japan, and also by research grants from the Fugaku Trust for Medical Research, the ONO Medical Research Foundation and the TERMO Science Foundation.

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