Post-translational processing of the phosphatidylserine decarboxylase gene product in Chinese hamster ovary cells

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We have isolated a full-length cDNA clone of the Chinese hamster ovary (CHO) *pssC* gene, which encodes mitochondrial phosphatidylserine decarboxylase. The cDNA clone is capable of increasing phosphatidylserine decarboxylase activity to 11-fold in CHO-K1 cells. The *pssC* gene product predicted from the cDNA sequence is composed of 409 amino acid residues. In an *in vitro* translation system coupled with *in vitro* transcription, the cDNA clone directs the formation of a protein with an apparent molecular mass of 46 kDa. In CHO-K1 cells, the cDNA clone leads to the production of two major peptides with apparent molecular masses of 38 and 34 kDa, as determined by Western blotting with an antibody raised against a recombinant *pssC* protein. When CHO-K1 cells transfected with the cDNA clone are labelled with [³⁵S]methionine for a short period, proteins immunoprecipitated with the antibody lack radioactive 38 and

34 kDa peptides, but contain two radioactive peptides with apparent molecular masses of 46 and 42 kDa instead. The *pssC* gene product predicted from the cDNA sequence has, near its C-terminus, a unique Leu-Gly-Ser-Thr sequence which is known as a processing site for *Escherichia coli* phosphatidylserine decarboxylase. A mutant *pssC* cDNA clone, in which Ser³⁷⁸ in the conserved sequence is replaced by Ala, leads to overproduction of 46, 42 and 38 kDa peptides, but not a 34 kDa peptide. This mutant clone is incapable of increasing phosphatidylserine decarboxylase activity, in contrast to the wild-type clone. These results indicate that the processing at the Leu-Gly-Ser-Thr sequence is essential for formation of the active enzyme. Thus, the *pssC* gene product is converted into mature phosphatidylserine decarboxylase through multiple steps of post-translational processing.

INTRODUCTION

Phosphatidylserine decarboxylase catalyses the formation of phosphatidylethanolamine in both prokaryotes and eukaryotes. In *Escherichia coli*, the phosphatidylserine decarboxylase gene, *psd*, and its product have been extensively studied [1–3]. The *psd* gene encodes a proenzyme which is cleaved post-translationally into α - and β -subunits. The α -subunit is derived from the C-terminal 69 amino acids of the proenzyme and carries a pyruvoyl prosthetic group at its N-terminus. Ser²⁵⁴ of the proenzyme is critical for both the cleavage and formation of the pyruvoyl group, which contributes to the catalytic carbonyl group of the mature enzyme.

Recently, two phosphatidylserine decarboxylase genes of *Saccharomyces cerevisiae*, *PSD1* and *PSD2*, were isolated [4–7]. The *PSD1* gene encodes mitochondrial phosphatidylserine decarboxylase, which contributes to the major phosphatidylserine decarboxylase activity *in vitro* (more than 95% of the total activity). The *PSD2* gene encodes non-mitochondrial phosphatidylserine decarboxylase.

Subcellular localization studies on mammalian cells indicated that the phosphatidylserine decarboxylase activity is localized to the inner mitochondrial membrane [8,9]. However, if, in analogy to yeast, mammalian cells have two different phosphatidylserine decarboxylases and one exhibits very low enzymic activity *in vitro*, the localization of each phosphatidylserine decarboxylase should be re-examined using specific antibodies. Because the substrate for the decarboxylase, phosphatidylserine, is synthesized extramitochondrially [10–12] from phosphatidylethanolamine and phosphatidylcholine [13,14], determination of the

subcellular localization of phosphatidylserine decarboxylase is important for understanding the metabolic regulation and inter-organelle trafficking of phosphatidylserine and phosphatidylethanolamine.

We previously isolated a genomic DNA clone of the Chinese hamster ovary (CHO) *pssC* gene that can restore phosphatidylserine biosynthesis in a CHO cell mutant defective in phosphatidylserine synthase I activity, as well as a *pssC* cDNA clone, using the genomic DNA fragment as a probe [15]. The nucleotide sequence and expression of the cDNA in yeast indicate that the isolated cDNA clone is a partial one encoding a truncated but functional phosphatidylserine decarboxylase [15]. The predicted amino acid sequence of the truncated decarboxylase contains a Leu-Gly-Ser-Thr sequence, which is known as a processing site for *Escherichia coli* phosphatidylserine decarboxylase [1–3]. In this study, we have obtained the *pssC* cDNA segment which was missing, constructed a full-length *pssC* cDNA clone, and prepared a polyclonal antibody against a recombinant *pssC* protein produced in *E. coli*. With these materials, we have provided evidence that the *pssC* gene encodes a mitochondrial phosphatidylserine decarboxylase that matures through multiple steps of post-translational processing.

EXPERIMENTAL

Strains and culture conditions

Strain CHO-K1 was obtained from the American Type Culture Collection, and maintained in Ham's F-12 medium supplemented

Abbreviations used: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; NTA, nitrilo-tri-acetic acid; RACE, rapid amplification of cDNA ends.

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with 10 % (v/v) newborn-calf serum, penicillin G (100 units/ml), streptomycin sulphate (100 µg/ml), and NaHCO₃ (1.176 g/l) under a 5 % CO₂ atmosphere at 100 % humidity and 37 °C.

Amplification of the pssC cDNA end

The cDNA end corresponding to the 5'-portion of pssC mRNA was amplified by the RACE (rapid amplification of cDNA ends) procedure [16], using poly(A)⁺ RNA prepared from CHO-K1 cells as a template. First-strand cDNA was synthesized using a primer, GTACAGGGCCACCTCCCA (codons Trp¹⁰⁶-Tyr¹¹¹, antisense). The gene-specific primers used for cDNA amplification were GTGACTGGCAAGTTTGGG (codons Pro¹⁰⁰-His¹⁰⁵, antisense) and CGGAATCTCCAATCCCAG (codon Leu⁹⁴-Pro⁹⁹, antisense).

Construction of plasmids encoding the pssC and pssC-S378A mutant proteins to be expressed in mammalian cells

The pssC cDNA segment amplified by the RACE procedure was cloned into the multicloning site of the plasmid, pBluescript II SK⁺ (Stratagene). To construct a full-length open reading frame, the resultant plasmid was cleaved at the *Hind*III site of the pssC cDNA and the *Xho*I site of the vector plasmid, and ligated to a 2.0 kb *Hind*III-*Xho*I segment of pCDPSSC [15] to yield a plasmid termed pBluescript-PSSC. pBluescript-PSSC was digested with *Avr*II and *Xho*I, and the resulting 2.4 kb fragment was inserted into the multicloning site of the mammalian expression vector, pSV-Sport1 (Gibco/BRL). The resultant plasmid was termed pSVPSSC. The S378A mutation was introduced into the wild-type pssC sequence carried by pBluescript-PSSC, by site-directed mutagenesis as described in [17]. The resulting plasmid, pBluescript-PSSC-S378A, was digested with *Avr*II and *Xho*I, and then the 2.4 kb fragment of pssC cDNA was inserted into the multicloning site of the plasmid, pSV-Sport1, to yield the plasmid, pSVPSSC-S378A. The mutated cDNA sequence was verified by DNA sequencing.

Transient expression of the pssC and pssC-S378A mutant proteins in CHO-K1 cells

DNA was introduced into CHO-K1 cells using LipofectAMINE reagent (Gibco/BRL) according to the manufacturer's instructions.

In vitro transcription and translation

pssC mRNA was prepared using SP6 RNA polymerase (Promega) and the plasmid, pSVPSSC, as a template, according to the manufacturer's recommendations. *In vitro* translation was performed using a nuclease-treated rabbit reticulocyte lysate (Promega) according to the manufacturer's recommendations.

Preparation of recombinant pssC proteins

The His₆-tagged pssC protein, used as an antigen to produce a polyclonal antibody, was prepared as follows. The coding region of pssC cDNA was engineered by PCR so as to add a *Bam*HI site immediately upstream of the first ATG codon and a *Bgl*II site immediately downstream of the termination codon, using pBluescript-PSSC as a template. The PCR product was digested with *Bam*HI and *Bgl*II, and then cloned into the *Bam*HI site of the pQE9 vector (Qiagen), which produced a recombinant protein containing an N-terminal affinity tag consisting of six adjacent histidine residues. The resultant plasmid was introduced into *E.*

coli cells (M15 harbouring plasmid pREP4; Qiagen). The transformant was grown to a density of $A_{600} = 0.9$ in Super medium (25 g of bactotryptone, 15 g of yeast extract and 5 g of NaCl per litre) with 100 µg/ml ampicillin and 50 µg/ml kanamycin at 30 °C, and then further cultivated for 1.5 h in the presence of 1 mM isopropyl thio-β-D-galactoside. The pssC-protein-expressing cells were collected and lysed by resuspension in buffer A (6 M guanidine hydrochloride, 0.1 M NaH₂PO₄ and 0.01 M Tris, pH adjusted to 8.0 with NaOH). The lysate was clarified by centrifugation at 10000g for 30 min and the resultant supernatant was loaded on a Ni²⁺-nitrilotriacetic acid (NTA)-agarose (Qiagen) column (8-ml bed volume) equilibrated with buffer A. The resin was washed with buffer B (8 M urea, 0.1 M NaH₂PO₄ and 0.01 M Tris, pH adjusted to 8.0 with NaOH), buffer C (same composition as buffer B, but pH adjusted to 6.3 with NaOH) and buffer B containing 20 mM imidazole, in that order, and then the His₆-tagged pssC protein was eluted with a 100 ml imidazole gradient (20–200 mM) in buffer B. The pooled His₆-tagged pssC protein was dialysed against PBS and then used as an antigen to produce a polyclonal antibody.

A fusion protein consisting of an N-terminal peptide encoded by the *E. coli lacZ* gene linked to the C-terminal portion of the pssC protein was prepared, as follows, and used to affinity purify an anti-(pssC protein) antibody. The 2.3 kb *Xho*I fragment from pCDPSSC [15] was cloned into the *Sal*I site of plasmid pUC119 and the orientation of the insert was checked by mapping of the *Hind*III sites. The 2.0 kb *Hind*III fragment from the resulting plasmid was cloned into the *Hind*III site of plasmid pUR288 to generate an in-frame gene fusion of *lacZ* and *pssC*. The plasmid carrying the fusion gene was introduced into *E. coli* BMH71-18 cells, and the transformant was grown to a density of $A_{600} = 0.9$ in 2 × YT medium (16 g of bactotryptone, 15 g of yeast extract and 5 g of NaCl per litre) with 100 µg/ml ampicillin at 37 °C, and then further cultivated for 1.5 h in the presence of 0.5 mM isopropyl thio-β-D-galactoside. The cells expressing the fusion protein were collected, resuspended in medium comprising 0.2 mg/ml lysozyme, 50 mM sodium phosphate, pH 7.0, 10 mM EDTA and 1 mM PMSF, incubated for 30 min at 4 °C, and then disrupted by sonication. Sucrose monolaurate and NaCl were added to final concentrations of 0.1 % (w/v) and 0.5 M, respectively, and then the lysate was clarified by centrifugation at 10000g for 30 min. The resultant supernatant was loaded on an immunoaffinity column containing anti-β-galactosidase antibody conjugated to Sepharose 4B (provided by Dr. K. Hanada of our Institute). The resin was washed with PBS containing 0.1 % sucrose monolaurate, and the fusion protein was eluted with buffer D (50 mM K₂CO₃ and 1 mM EDTA) and dialysed against buffer E (150 mM NaCl and 10 mM Mops, pH adjusted to 7.0 with NaOH).

Preparation of an antibody to the recombinant pssC protein

A rabbit was injected with 0.5 mg of purified His₆-tagged pssC protein emulsified in Freund's complete adjuvant, followed by 0.5 mg of purified His₆-tagged pssC protein emulsified in Freund's incomplete adjuvant every 4 weeks to boost the immune response. The antibody to the pssC protein was affinity-purified [18] by adsorption to the fusion protein comprising β-galactosidase and pssC protein conjugated to Affigel 10 (Bio-Rad), according to the manufacturer's procedure, and then dialysed against PBS.

Immunoblot analysis

Proteins were fractionated by SDS/10 %-PAGE under reducing conditions [19], and then electroblotted on to nitrocellulose

membranes in 25 mM Tris/192 mM glycine/0.02 % SDS/20 % methanol at 22 V/cm for 1 h. The pssC protein was detected using the affinity-purified anti-(pssC protein) antibody (diluted 1:1000) and a peroxidase-conjugated anti-(rabbit IgG) antibody (diluted 1:1000; Amersham). Peroxidase labelling was detected as chemiluminescence using ECL reagent (Amersham) according to the manufacturer's recommendations.

Metabolic labelling and immunoprecipitation

After transfection (50 h), cells were rinsed three times with methionine-deficient Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL) and then incubated at 37 °C for 1 h in methionine-deficient DMEM supplemented with 5 % (v/v) dialysed newborn-calf serum before the addition of the pulse medium. The pulse medium contained 1 mCi/ml of [³⁵S]-methionine (Trans-Label; ICN Biochemical Inc.) and 5 % (v/v) dialysed newborn-calf serum in methionine-deficient DMEM. Cells were incubated in the pulse medium for 5 min, and subsequently chased in Ham's F-12 medium supplemented with 10 % (v/v) newborn-calf serum and 0.3 mM methionine for the times indicated. Immediately after the chase period, the cells were lysed *in situ* with 1 % SDS and 50 mM Tris/HCl (pH 8.0). The lysates were boiled for 5 min, diluted ten times with Triton lysis buffer (1 % Triton X-100, 150 mM NaCl and 50 mM Tris/HCl, pH 8.0), and then precleared by centrifugation followed by a 1 h incubation with Protein A-Sepharose (Pharmacia LKB Biotechnology, Inc.). The Sepharose was then removed by centrifugation and the anti-(pssC protein) antibody was added to the lysate. After a 1 h incubation at 4 °C, Protein A-Sepharose was incubated with the lysate at 4 °C for a further 1 h, and subsequently collected by centrifugation. The Sepharose beads were washed five times with Triton lysis buffer. Proteins bound to the beads were fractionated by SDS/12 %-PAGE under reducing conditions [19], and examined with a bioimage analyser (Fujix BAS2000).

Preparation of subcellular fractions

Subcellular fractions were prepared from CHO-K1 cells as described [12] with slight modifications.

Other methods

Both strands of the pssC cDNA fragment amplified by PCR were sequenced by dideoxy chain termination using Sequenase (United States Biochemical Corp.). Phosphatidylserine decarboxylase [15], cytochrome *c* oxidase [20], glucose-6-phosphate phosphatase [21] and CTP:phosphocholine cytidyltransferase [22] were assayed as described. Protein was measured as described in [23], using BSA as a standard.

RESULTS

Cloning of the full-length pssC cDNA

We previously isolated a pssC cDNA clone (pCDPSSC) lacking the nucleotides corresponding to the N-terminal portion of the pssC protein [15]. In this study, we have obtained the missing cDNA segment using a PCR method known as RACE [16]. The nucleotide sequence of the longest RACE product obtained is shown in Figure 1(A). The first ATG codon of the combined nucleotide sequences of the RACE product and the pssC cDNA clone is followed by the longest open reading frame, which encodes a protein of 409 amino acid residues with a calculated molecular mass of 46745 Da. Figure 1(B) shows the amino acid

A

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-30      GAGGGCGCTGTCCACCTAGGAGGAGSGCAG
      1 M A A S V C R P Y V R S L P G V M P W R
      1 ATGCGGCGCTGTGTGTCTGCTCCGATGTTCGCTCGCTGCCTGGAGTCATGCCCTGGCG

21 S S S C H Y E Y T A M H H F L G S F Q K
61 AGCAGCTCCTGCCACTATGAATATATACTGCCATGCACCATTTCCTGGGTCCTTCAGAA

41 L P F E E P F N T G A R K I H T A P V R S
121 CTGCCCTTTGAACCCCTTTAAACACAGGTGCCAGAAAAATCCATATCTGCTCCTGTCGCATCC

61 L F L L R P V P I L L A T G G G Y A G Y
181 TTGTTCTCTGCTGCGCCCTGTGCCATCCTGTGGCAACAGGTGGTGGGTATGCAGGGTAC

81 R Q Y E K A R T R D Q K L E K L G L E I P
241 CGGCAGTATGAGAAGTACAGGATCAGAAGCTGAGAAGCTGGGAATGGAGATTCCG

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B

1 MAASVCRPVYRSLGGVYWRSSSCHEYETAMHHFLGSGFKLPFEFFNTGARKIHITAPVRS
61 LFLRLRPVILLATPGGVAQYRQEYKRLQDKLEGLLEIPKLASHVEVALYKSPVTRLLS
121 RAWGLRGLQVELPYLWRPVYSYLITWGVNTEAAVEDLHHNRLSEFVKKLPQARPV
181 CGLHVSIVSPDGKLLTGQKNCVEQVGTWYSLESLFGRTYITDLSFPFASRSDSFR
241 NQLVITRENGELHYCVILAPGDYHCFSHTDVTYVSHRRHFPGSLMSVNPNGAKWIKELF
301 HNRVVLSGDWKGHGFSLTAVGATNGVIRYFDQDLHTNSPYKSGYNDLSVFVTHANK
361 EGIPMRKGEHLGNEFSTLIVLFEKSPDKNRFLKAGOKIRFGEAGLSL

C

Figure 1 Nucleotide and predicted amino acid sequences of the pssC cDNA segment amplified by PCR (A), predicted amino acid sequence of the pssC protein (B), and construction of the full-length pssC cDNA in a mammalian expression vector (C)

The underlined pssC cDNA segment was isolated in this study. Open, closed and striped boxes represent the pBluescript II SK⁺ vector, pCD vector and pSV-Sport1 vector, respectively. The abbreviations for restriction enzymes are: A, *AvrII*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Xb, *Xba*I; Xh, *Xho*I.

sequence of the predicted protein. The first ATG codon in the open reading frame is in an appropriate context for translational initiation [24], namely G residues are located at the -6 , -3 and $+4$ positions (with respect to the first base of the triplet). Thus, this ATG codon is probably used *in vivo* as a translational initiation codon and the combined sequence probably covers a full-length open reading frame.

The open reading frame, which appeared to be full-length, was reconstructed from restriction fragments of the RACE product and the *pssC* cDNA clone, in the mammalian expression vector pSV-Sport1, using the internal *Hind*III site (Figure 1C). The transient transfection of CHO-K1 cells with the pSVPSSC construct resulted in an 11-fold increase in the specific activity of phosphatidylserine decarboxylase, when compared with transfection with a control vector (Table 1). This confirmed that the *pssC* gene encoded phosphatidylserine decarboxylase and supported our contention that the reconstructed *pssC* cDNA clone contained a full-length open reading frame.

pssC RNA is translated to a 46 kDa protein *in vitro*

pssC mRNA was prepared *in vitro* with the plasmid pSVPSSC as a template and translated using rabbit reticulocyte lysates. As

Table 1 Phosphatidylserine decarboxylase activity in CHO-K1 cells transfected with the control vector, pSVPSSC, or pSVPSSC-S378A

CHO-K1 cells transfected with the control vector, pSV-Sport1 (Control), pSVPSSC (Wild-type), or pSVPSSC-S378A (S378A-mutant) were grown for 3 days, sonically disrupted in 0.25 M sucrose, 10 mM Hepes (pH 7.5), 1 mM EDTA, 0.5 mM (*p*-amidinophenyl)methane sulphonyl fluoride, 2 μ g/ml aprotinin and 2 μ g/ml leupeptin, and then centrifuged at 100 000 *g* for 1 h. The resulting pellets were suspended in the same medium and then assayed for phosphatidylserine decarboxylase activity as described [15]. Specific activities are averages of duplicate assays, with variation of 10% between duplicates.

	Specific activity (nmol/min per mg of protein)
Control	1.11
Wild-type	12.75
S378A-mutant	1.19

Figure 2 *In vitro* translation of *pssC* mRNA

pssC mRNA prepared *in vitro* was translated in the presence of [³⁵S]methionine (Amersham) using a rabbit reticulocyte lysate. The products were fractionated by SDS/10% PAGE under reducing conditions and then examined with a Bioimage analyser (Fujix BAS2000). Lane 1, no *pssC* mRNA added; lane 2, complete reaction.

shown in Figure 2, a protein exhibiting an apparent molecular mass of 46 kDa on SDS/PAGE was produced, depending on the *pssC* mRNA added. The molecular mass of the product agreed with that of the protein predicted from the nucleotide sequence, 46 745 Da.

Two major products of 38 and 34 kDa are produced from the *pssC* gene *in vivo*

A recombinant *pssC* protein that has an extension containing six histidine residues at its N-terminus was expressed in *E. coli* cells, purified by chromatography on a Ni²⁺-NTA-agarose column, and used as an antigen to produce a polyclonal anti-(*pssC* protein) antibody. The anti-(*pssC* protein) antibody purified by affinity chromatography recognized two peptides with apparent molecular masses of about 34 and 38 kDa on an immunoblot of a crude membrane fraction from CHO-K1 cells (Figure 3A). The transient transfection of CHO-K1 cells with the plasmid pSVPSSC resulted in striking increases in the amounts of both the 34 and 38 kDa peptides, as shown in Figure 3(B) (lane 2). In addition, an antigenic peptide with an apparent molecular mass of approximately 46 kDa was found in the transient transfectant (Figure 3B, lane 2). These results indicated that two major products of 38 and 34 kDa were produced from the *pssC* gene.

Figure 3 Immunoblotting using the anti-(*pssC* protein) antibody

(A) CHO-K1 cells were homogenized in medium comprising 0.25 M sucrose, 10 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM PMSF, 2 μ g/ml aprotinin and 2 μ g/ml leupeptin by 30 strokes in a Potter–Elvehjem motor-driven homogenizer, and then centrifuged at 600 *g* for 5 min. The resulting supernatant was centrifuged at 100 000 *g* for 1 h and the proteins in the pellet were analysed by immunoblotting, as described under the Experimental section. (B) The same preparations as those used for the assaying of phosphatidylserine decarboxylase activity shown in Table 1 were immunoblotted, as described under the Experimental section. Lane 1, CHO-K1 cells transfected with the control vector; lane 2, CHO-K1 cells transfected with pSVPSSC; lane 3, CHO-K1 cells transfected with pSVPSSC-S378A.

Processing at the conserved Leu-Gly-Ser-Thr sequence

The *pssC* gene product deduced from the cDNA sequence had, near its C-terminus, a unique Leu-Gly-Ser-Thr sequence which is known as a processing site for *E. coli* phosphatidylserine decarboxylase [1–3]. To determine whether or not the processing at the Leu-Gly-Ser-Thr sequence is involved in the maturation of the *pssC* gene product, we introduced a mutation of Ser³⁷⁸ to alanine (S378A) into the wild-type *pssC* cDNA clone, and then transfected the mutant cDNA clone into CHO-K1 cells. The resultant transient transfectants overproduced three peptides with apparent molecular masses of 46, 42 and 38 kDa, but not a 34 kDa peptide, as examined with the anti-(*pssC* protein) antibody (Figure 3B). The phosphatidylserine decarboxylase activity of the transfectant with the mutant clone was similar to that of the untransfected cells (Table 1), while the activity of the transfectant with the wild-type clone was 11-fold higher than that of the non-transfected cells, as described above. These results indicated that the processing at the Leu-Gly-Ser-Thr sequence was essential for formation of the active enzyme.

The *pssC* gene products of 38 and 34 kDa are produced through post-translational processing

To examine the precursor–product relationship of the *pssC* gene products, CHO-K1 cells transfected with plasmid pSVPSSC were pulse-labelled with [³⁵S]methionine for 5 min and subsequently chased in the presence of an excess of unlabelled methionine. After the chase the radioactive proteins were immunoprecipitated with the anti-(*pssC* protein) antibody and resolved by SDS/PAGE, the results being shown in Figure 4. In the pulse-labelling period, the transfectants produced two radioactive peptides with apparent molecular masses of 46 and 42 kDa, which were not detected among proteins from CHO-K1 cells transfected with the control vector plasmid. During the 60 min chase period, the amounts of the radioactive 46 and 42 kDa peptides decreased, and two radioactive peptides with apparent molecular masses of 38 and 34 kDa appeared. These results

Figure 4 Post-translational processing of the wild-type and S378A-mutant forms of the *pssC* gene product

CHO-K1 cells transfected with either the control vector (Control), pSVPSSC (Wild-type), or pSVPSSC-S378A (S378A-mutant) were pulse-labelled with [35 S]methionine for 5 min and then subsequently chased in the presence of an excess of unlabelled methionine for the times indicated, as described in the Experimental section. The radioactive proteins were precipitated with the anti-(*pssC* protein) antibody, fractionated by SDS/12%-PAGE, and then examined with a bioimage analyser, as described in the Experimental section.

showed that the *pssC* gene products of 38 and 34 kDa were produced through post-translational processing.

For CHO-K1 cells transfected with the S378A-mutant cDNA clone, the same pulse-chase experiment was performed. The results are shown in Figure 4. In the pulse-labelling period, like the wild-type clone, the mutant clone led to the production of two radioactive peptides with apparent molecular masses of 46 and 42 kDa. During the 60 min chase period, a radioactive peptide with an apparent molecular mass of 38 kDa, but not 34 kDa, appeared. Unlike the radioactive wild-type peptide of 42 kDa, the radioactive mutant peptide of 42 kDa did not disappear during the chase period. These results suggested that the S378A mutation blocked the formation of the 34 kDa *pssC* gene product and affected the processing of the 42 kDa *pssC* gene product.

Subcellular localization of the *pssC* gene product and phosphatidylserine decarboxylase

Cytosolic, microsomal and mitochondrial fractions, and a mitochondria-associated membrane fraction, which reportedly contains several phospholipid biosynthetic enzymes [12], were prepared from CHO-K1 cells. These subcellular fractions were

Figure 5 Subcellular localization of the *pssC* gene products

Subcellular fractions were prepared from CHO-K1 cells, as described in the Experimental section, and 50 μ g of protein from each fraction was analysed by immunoblotting with the anti-(*pssC* protein) antibody, as described in the Experimental section. Abbreviation: MAM, mitochondria-associated membrane.

analysed by immunoblotting with the anti-(*pssC* protein) antibody, and assayed for phosphatidylserine decarboxylase along with the marker enzymes for mitochondria (cytochrome *c* oxidase), endoplasmic reticulum (glucose-6-phosphate phosphatase), and endoplasmic reticulum and cytosol (CTP:phosphocholine cytidyltransferase). The relative specific activities of the marker enzymes in each subcellular fraction prepared from CHO-K1 cells (Table 2) were essentially the same as those in rat liver [12]. The highest specific activity of phosphatidylserine decarboxylase was found in the mitochondrial fraction (Table 2). Immunoblotting revealed that the 34 and 38 kDa *pssC* gene products were enriched in the mitochondrial fraction (Figure 5). These results indicated that the *pssC* gene encoded mitochondrial phosphatidylserine decarboxylase.

DISCUSSION

We have isolated a full-length cDNA clone of the CHO *pssC* gene, which encodes phosphatidylserine decarboxylase. The cDNA clone directs the *in vitro* formation of a protein with an apparent molecular mass of 46 kDa. The molecular mass of the product is consistent with that predicted from the nucleotide sequence, i.e. 46745 Da. In CHO-K1 cells, the cDNA clone leads to the production of two major peptides with apparent molecular masses of 38 and 34 kDa. However, in the nascent *pssC* gene products, the 38 and 34 kDa peptides are missing, two peptides with apparent molecular masses of 46 and 42 kDa being found instead. These results suggest that the *pssC* gene product is synthesized as a 46 kDa protein and subsequently undergoes three post-translational events.

The *E. coli* phosphatidylserine decarboxylase gene encodes a

Table 2 Phosphatidylserine decarboxylase and marker enzyme activities in subcellular fractions

Subcellular fractions were prepared from CHO-K1 cells and assayed for enzyme activities as described in the Experimental section. The units for the enzyme activities are: phosphatidylserine decarboxylase, nmol/min per mg; cytochrome *c* oxidase, nmol/min per mg of protein; glucose-6-phosphate phosphatase, μ mol/h per mg of protein; CTP:phosphocholine cytidyltransferase, nmol/min per mg of protein. Specific activities are averages of duplicate assays, with variation of < 10% between duplicates. The values in parentheses are the total enzyme activities in individual fractions, expressed as percentages of the total activity in the original post-nuclear supernatant. The total enzyme activity for each fraction was calculated as the specific activity multiplied by the total amount of protein recovered in that fraction. Abbreviations: nd, not detected; MAM, mitochondria-associated membrane.

	Specific activity				
	Post-nuclear supernatant	Mitochondria	MAM	Microsomes	Cytosol
Phosphatidylserine decarboxylase	0.32 (100)	2.92 (37)	0.16 (< 1)	0.08 (5)	0.06 (12)
Cytochrome <i>c</i> oxidase	98.5 (100)	511 (21)	83 (< 1)	13 (3)	nd (< 1)
Glucose-6-phosphate phosphatase	0.96 (100)	0.60 (3)	2.26 (15)	0.58 (13)	0.43 (28)
CTP:phosphocholine cytidyltransferase	0.62 (100)	0.13 (< 1)	0.86 (23)	1.35 (38)	0.10 (13)

proenzyme which is cleaved into the α - and β -subunits of the mature enzyme [1–3]. The cleavage is autocatalytic, and occurs at a peptide bond between Gly and Ser in an Leu-Gly-Ser-Thr sequence [1–3] that is found in the *pssC* gene product. It has also been shown that the cleavage results in modification of the serine residue to a catalytic pyruvoyl group located at the N-terminus of the α -subunit [1–3]. A mutant *pssC* cDNA clone in which Ser³⁷⁸ in the conserved Leu-Gly-Ser-Thr sequence is replaced by Ala is incapable of increasing phosphatidylserine decarboxylase activity, in contrast to the wild-type *pssC* cDNA clone. This result suggests that the *pssC* gene product is activated through cleavage of the peptide bond between Gly³⁷⁷ and Ser³⁷⁸. The result also suggests that the mature *pssC* gene product consists of α - and β -subunits. The α -subunit predicted from the *pssC* cDNA sequence is derived from 32 C-terminal amino acids of the *pssC* gene product. The two major *pssC* gene products of 38 and 34 kDa, which are detected by the anti-(*pssC* protein) antibody, are candidates for the β -subunit because of their apparent molecular masses. The 34 kDa product may be the mature β -subunit, since the S378A mutant clone leads to the production of 46, 42 and 38 kDa peptides, but not a 34 kDa peptide.

The 38 kDa peptides produced by the wild-type and S378A-mutant *pssC* cDNA clones exhibit similar but slightly different mobilities on SDS/PAGE. The difference in mobility may be due to the single amino acid substitution. Alternatively, the difference may be ascribed to the processing at different sites of the wild-type and mutant forms of the *pssC* gene product, because the *pssC* gene product appears to have three processing sites (see below).

It has been shown that mitochondrial phosphatidylserine decarboxylase of mammalian cells is an inner membrane protein which is exposed to the intermembrane space [25]. Therefore, the mitochondrial phosphatidylserine decarboxylase, or its precursor protein, appears to carry a signal which directs the decarboxylase to the mitochondrial intermembrane space. The known signal sequences containing the information for the targeting of proteins to the intermembrane space have been shown to be located at the N-terminus of the precursor proteins and to have a bipartite structure [26–28]. The N-terminal parts of the signal sequences exhibit the features of a typical mitochondrial targeting sequence, and their C-terminal parts contain a hydrophobic stretch of approx. 20 amino acids that is preceded by basic residues [26–28]. We have shown that phosphatidylserine decarboxylase produced from the *pssC* gene is a mitochondrial enzyme. The N-terminal sequence of the *pssC* gene product is rich in positively charged and hydroxylated residues, and is poor in acidic amino acids. These are common features of mitochondrial targeting sequences. A hydrophobic stretch of 15 amino acids that is preceded by basic residues is found at amino acid residues 65–79 in the *pssC* gene product. Thus, the primary structure of the N-terminus of the *pssC* gene product is consistent with the bipartite structure of the known signal sequences for the targeting of proteins to the intermembrane space. It has also been shown that the signal sequences with the bipartite structure are cleaved in two steps by

different processing peptidases [26–28]. As described above, the 46 kDa *pssC* gene product appears to undergo three post-translational events, including cleavage of the peptide bond between Gly³⁷⁷ and Ser³⁷⁸. It is therefore likely that two-step proteolytic processing at the putative N-terminal signal is involved in the maturation of the *pssC* gene product.

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