Enhancement of Transport-dependent Decarboxylation of Phosphatidylserine by S100B Protein in Permeabilized Chinese Hamster Ovary Cells*

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Phosphatidylethanolamine synthesis through the phosphatidylserine (PtdSer) decarboxylation pathway requires PtdSer transport from the endoplasmic reticulum or mitochondrial-associated membrane to the mitochondrial inner membrane in mammalian cells. The transport-dependent PtdSer decarboxylation in permeabilized Chinese hamster ovary (CHO) cells was enhanced by cytosolic factors from bovine brain. A cytosolic protein factor exhibiting this enhancing activity was purified, and its amino acid sequence was partially determined. The sequence was identical to part of the amino acid sequence of an EF-hand type calcium-binding protein, S100B. A His6-tagged recombinant CHO S100B protein was able to remarkably enhance the transport-dependent PtdSer decarboxylation in permeabilized CHO cells. Under the standard assay conditions for PtdSer decarboxylase, the recombinant S100B protein did not stimulate PtdSer decarboxylase activity and exhibited no PtdSer decarboxylase activity. These results implicated the S100B protein in the transport of PtdSer to the mitochondrial inner membrane.

The intracellular transport of phospholipids from the sites of synthesis to the final locations is an essential event for the biogenesis of functional organellar membranes. Several mechanisms have been proposed for phospholipid transport, including ones involving soluble carrier proteins, transport vesicles, and contact zones between donor and acceptor membranes (1). However, the importance of the above proposed mechanisms for membrane biogenesis remains unclear, and phospholipid transport processes are largely unknown with respect to specific mechanisms, genes, and proteins involved.

In mammalian cells, phosphatidylethanolamine (PtdEtn) synthesis through the decarboxylation of nascent phosphatidylserine (PtdSer) requires both interorganelle and intramitochondrial transport of PtdSer, because PtdSer synthase is located on the endoplasmic reticulum and mitochondrial-associated membrane (MAM) (2–4), while PtdSer decarboxylase is located on the outer face of the mitochondrial inner membrane (5, 6). Using the decarboxylation of nascent PtdSer as an index of PtdSer transport, Voelker (7–11) has extensively studied the processes of PtdSer transport from the ER or MAM to the mitochondrial inner membrane in intact cells and a permeabilized cell system and has established the following: 1) The PtdSer transport to mitochondria requires ATP (7–9). 2) The PtdSer transport to mitochondria can occur in the absence of its synthesis (9). 3) The general features of protein and PtdSer export of the ER are fundamentally different insofar as the export of proteins, but not that of PtdSer, requires cytosolic factors and guanine nucleotides (9). 4) Adriamycin, which is a potent inhibitor of the import of proteins into mitochondria, also inhibits the PtdSer transport between the outer and inner mitochondrial membranes (10), and 5) When permeabilized cells are disrupted by shearing, the PtdSer transport from the ER or MAM to mitochondria is restricted to the autologous organelle (11). In addition, studies by Vance and co-workers (12) have suggested that the PtdSer synthesized in the ER traverses the MAM en route to the mitochondria.

Although studies of the PtdSer transport from the ER or MAM to the mitochondrial inner membrane have revealed the general features of this transport process as described above, our knowledge about the genes or gene products specifically involved in this transport is very limited. Mitochondrial membrane protein(s) have been shown to be required for the transport of PtdSer from the MAM to the mitochondria (13), but such protein(s) remain to be identified. In this laboratory, a mammalian cell mutant defective in the intramitochondrial transport of PtdSer has been isolated (14); however, the mutant gene responsible for the defect has not yet been cloned. To further understand the mechanisms of PtdSer transport to mitochondria, we re-examined the effect of cytosol on the transport-dependent PtdSer decarboxylation in permeabilized CHO-K1 cells and found that cytosolic factors from bovine brain enhanced the transport-dependent PtdSer decarboxylation in permeabilized CHO-K1 cells in the presence of Ca2+. Here, we describe the purification and identification of one of the factors exhibiting this enhancing activity.

**Experimental Procedures**

Cell Culture—Strain CHO-K1 was obtained from the American Type Culture Collection, and maintained in Ham’s F12 medium supplemented with 10% newborn calf serum, penicillin G (100 units/ml), streptomycin sulfate (100 µg/ml), and NaHCO3 (1,176 g/liter) under a 5% CO2 atmosphere of 100% humidity at 37°C.
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Preparation of Permeabilized CHO-K1 Cells—The permeabilization of CHO-K1 cells was performed by the method of Voelker (9), with slight modifications. Cells were seeded at 1.1 × 10^6 cells per 150-mm dish 20–24 h prior to permeabilization. For permeabilization, the cells were washed three times with solution A (140 mM KCl, 10 mM NaCl, 2.5 mM MgCl2, 20 mM Hepes, pH 7.5, and 0.5 μM CaCl2) for the first wash, and then solution A supplemented with 50 μg/ml of saponin (Sigma) and then incubated at 37°C for 5 min. After this treatment, the medium was aspirated off, and the cells were placed on ice and harvested in 13 ml of solution A by scraping with a rubber policeman. The harvested cells were collected by centrifugation at 400 × g for 5 min, washed twice by gentle suspension in 14 ml of solution A followed by centrifugation at 400 × g for 5 min, and then gently suspended in solution A at −1 × 10^7 permeabilized cells per ml.

PtdSer and PtdEtn Synthesis in Permeabilized CHO-K1 Cells—Unless stated otherwise, the reactions for measuring PtdSer and PtdEtn synthesis were conducted in 100 μl of solution A in the presence of permeabilized cells (5 × 10^6 cells), 0.5 mM CaCl2, 5 mM ATP, 5 mM phosphocreatine, 1 unit of creatine phosphokinase, and 0.2 μCi of [l-3H]Serine (160 μCi/μmol) (Amersham Pharmacia Biotech). The reactions were performed in glass tubes at 37°C and terminated by the addition of 1.5 ml of MeOH/CHCl3 (2:1). The lipids were extracted from the reaction mixtures by the method of Bligh and Dyer (15) and separated on a thin layer chromatography plate (Silica Gel 60, Merck) with the solvent system of chloroform/methyl acetate/1-propanol/MeOH/water (50:25:20:5:1) in water, 50:50:20:18. The radioactivity of the separated lipids was analyzed with a BAS2000 image analyzer (Fuji Film).

Purification Procedure—Fresh bovine brains were obtained from a local slaughterhouse. All procedures were carried out at 0–4°C. To measure activity, aliquots of bovine brain cytosol and purification fractions were dialyzed against solution A. The brains were cut into small pieces and then homogenized with a Polytron homogenizer in five volumes of homogenizing buffer (50 mM Heps/NaOH, pH 7.5, 0.25 mM sucrose, and 5 mM EDTA). The homogenate was centrifuged at 1,000 × g for 10 min and the supernatant was centrifuged at 18,000 × g for 15 min. The resulting supernatant was centrifuged at 100,000 × g for 1 h to obtain the soluble fraction (cytosol). The cytosol was dialyzed against solution A supplemented with 50 μg/ml of saponin (Sigma) and then incubated at 37°C for 5 min. After this treatment, the medium was aspirated off, and the cells were placed on ice and harvested in 13 ml of solution A by scraping with a rubber policeman. The harvested cells were collected by centrifugation at 400 × g for 5 min, washed twice by gentle suspension in 14 ml of solution A followed by centrifugation at 400 × g for 5 min, and then gently suspended in solution A at −2 × 10^7 permeabilized cells per ml.

Preparation of a Membrane Fraction of CHO-K1 Cells—CHO-K1 cells were washed with phosphate-buffered saline, suspended in 250 mM sucrose containing 10 mM Hepes/NaOH (pH 7.5) and 1 mM EDTA, and then homogenized with a Potter-Elvehjem Teflon homogenizer. The homogenate was centrifuged at 700 × g for 5 min, followed by centrifugation of the supernatant at 100,000 × g for 1 h. The resulting pellet was suspended in solution A and then centrifuged again at 100,000 × g for 1 h. The pellet was resuspended in solution A and subjected to biochemical characterization.

Assay for PtdSer Decarboxylase Activity—Exponentially growing CHO-K1 cells were washed with phosphate-buffered saline, suspended in 250 mM sucrose containing 10 mM Hepes/NaOH (pH 7.5) and 1 mM EDTA, and then homogenized with a Potter-Elvehjem Teflon homogenizer. The homogenate was centrifuged at 700 × g for 5 min, followed by centrifugation of the supernatant at 100,000 × g for 1 h. The resulting pellet was suspended in solution A and then centrifuged again at 100,000 × g for 1 h. The pellet was resuspended in solution A and subjected to biochemical characterization.

Assay for PtdSer Decarboxylase Activity—Exponentially growing CHO-K1 cells were washed with phosphate-buffered saline, suspended in 250 mM sucrose containing 10 mM Hepes/NaOH (pH 7.5), disrupted by sonication, and then used as the enzyme source. The reaction mixture, with a final volume of 100 μl, for measuring PtdSer decarboxylase activity comprised 0.05 μCi of [3H]PtdSer (Amersham Pharmacia Biosciences).
Enables the Transport-dependent Decarboxylation of PtdSer—We fractionated bovine brain cytosol on a Q- Sepharose HP ion exchange column and subjected an aliquot of each fraction to assaying for transport-dependent PtdSer decarboxylation (TPSD)-enhancing activity. The amounts of $[^{14}\text{C}]$PtdEtn and $[^{14}\text{C}]$PtdSer synthesized from $[^{14}\text{C}]$serine in permeabilized CHO-K1 cells, in the presence of an aliquot of each fraction, were determined; the increase in the ratio of $[^{14}\text{C}]$PtdEtn/$[^{14}\text{C}]$PtdEtn plus $[^{14}\text{C}]$PtdSer was used as an index of the TPSD-enhancing activity. The TPSD-enhancing activity was separated into two prominent peaks on Q-Sepharose HP column chromatography (Fig. 2A). In this study, the TPSD-enhancing factor in the peak II fraction (Fig. 2A) was further purified by successive chromatography on phenyl-Sepharose hydrophobic (Fig. 2B), Superdex 75 gel filtration (Fig. 2C), and Mono Q ion exchange (Fig. 2D) columns. The overall purification of the TPSD-enhancing activity from the cytosol (700 mg of proteins) was 300-fold, with a recovery yield of 20%, where a 1% increase in the ratio of $[^{14}\text{C}]$PtdEtn/$[^{14}\text{C}]$PtdEtn plus $[^{14}\text{C}]$PtdSer was defined as 1 unit of activity. With the third gel filtration column, the TPSD-enhancing activity was eluted as a single peak corresponding to a molecular mass of ~20 kDa. When proteins in the fractions separated by the final Mono Q chromatography were analyzed by tricine-SDS-PAGE, an ~9 kDa protein was found to comigrate with the TPSD-enhancing activity (See Figs. 2D and 3.). These results suggested that a homo dimer of the purified ~9 kDa protein exhibited the TPSD-enhancing activity.

Identification of the Purified ~9-kDa Protein as a S100B Protein.—To determine its amino acid sequence, the purified ~9 kDa protein was subjected to cyanogen bromide cleavage, and then the N-terminal amino acid sequences of the resulting two fragments were determined after purification of the fragments by reverse phase HPLC column chromatography. A search involving a protein sequence data bank indicated that the sequences determined, ITTAXHEFFEH and ETLDSDGDGEX-DFG, matched parts of the amino acid sequence of an EF-hand type calcium-binding protein, S100B. Thus, the purified ~9 kDa protein was identified as a S100B protein.

A His$_{6}$-tagged Recombinant CHO S100B Protein Exhibits TPSD-enhancing Activity—We isolated CHO S100B cDNA clones. The nucleotide and predicted amino acid sequences of CHO S100B cDNA are shown in Fig. 4. A comparison of the predicted amino acid sequence of the CHO S100B protein with that of the S100B proteins of other species is also shown in Fig. 4. To confirm that the S100B protein exhibits the TPSD-enhancing activity, a His$_{6}$-tagged recombinant CHO S100B (His-cS100B) protein was bacterially produced and subjected to assaying for the TPSD-enhancing activity. Fig. 5 shows the tricine-SDS-PAGE patterns of soluble proteins from E. coli transformants, designated as M15/pQE9 and M15/pqE9-cS100B, which harbored an empty vector plasmid and a His-cS100B protein-expressing plasmid, respectively. A protein of 12 kDa was produced in an expression plasmid-dependent manner (Fig. 5), indicating that the 12 kDa protein was a His-cS100B protein. From a lysate of M15/pqE9-cS100B cells, the His-cS100B protein was purified on HisTrap and Superdex 75 columns successively. The tricine-SDS-PAGE pattern of the purified His-cS100B protein is shown in Fig. 5. Although the purified His-cS100B protein had no significant effect on the synthesis of $[^{14}\text{C}]$PtdSer from $[^{14}\text{C}]$serine in permeabilized CHO-K1 cells (Fig. 6), the purified His-cS100B protein remarkably enhanced the synthesis of $[^{14}\text{C}]$PtdEtn from $[^{14}\text{C}]$serine (Fig. 6). This enhancement by the His-cS100B protein occurred in a saturable manner, ~3-fold enhancement being the maximal level (Fig. 6A). Like the purified His-cS100B protein, crude

**RESULTS**

**Bovine Brain Cytosol Enhances the Transport-dependent Decarboxylation of PtdSer in Permeabilized CHO-K1 Cells**—We prepared saponin-permeabilized CHO-K1 cells according to the method of Voelker (9) and examined its ability to synthesize PtdSer and PtdEtn from serine precursor in the presence or absence of bovine brain cytosol. When the permeabilized cells were incubated with $[^{14}\text{C}]$serine at 37 °C in solution A containing 0.2 mM ATP and 0.5 mM CaCl$_2$, $[^{14}\text{C}]$PtdSer and $[^{14}\text{C}]$PtdEtn were produced in a time-dependent manner in the absence of bovine brain cytosol (Fig. 1), in agreement with Voelker’s conclusion (9) that the synthesis of $[^{14}\text{C}]$PtdEtn in permeabilized cells, which is dependent on the transport of PtdSer from the ER or MAM to the mitochondrial inner membrane, does not require cytosol. The addition of bovine brain cytosol, however, remarkably enhanced the synthesis of $[^{14}\text{C}]$PtdSer in permeabilized CHO-K1 cells, although the synthesis of $[^{14}\text{C}]$PtdSer in the presence of bovine brain cytosol was similar to that in its absence (Fig. 1). These results indicated that the bovine brain cytosol contained factor(s) that enhanced the transport-dependent decarboxylation of PtdSer in permeabilized CHO-K1 cells.

**Purification of a Bovine Brain Cytosolic Factor That Enhances the Transport-dependent Decarboxylation of PtdSer**—Permeabilized CHO-K1 cells were labeled with $[^{14}\text{C}]$serine as described under “Experimental Procedures” in the presence (closed circles) or absence (open circles) of bovine brain cytosol (180 μg of protein). The reactions were terminated at the indicated times by lipid extraction. PtdEtn and PtdSer were separated by thin layer chromatography, and the radioactivity associated with PtdSer upon a 60-min incubation in the presence of cytosol was taken as 1000. Values are the averages for duplicate assays with variation of <15% between duplicates.

**Other Methods**—Proteins were measured with a BCA protein assay kit (Fierce), with bovine serum albumin as a standard. Tricine-SDS-PAGE was performed as described (16). Silver staining of proteins in polyacrylamide gels was performed with a silver staining kit (Amer sham Pharmacia Biotech). DNA sequencing was performed by automated sequencing with an Applied Biosystem Prism 310 genetic analyzer and fluorescence-tagged dye terminator cycle sequencing.

**FIG. 1.** Bovine brain cytosol enhances the transport-dependent decarboxylation of PtdSer in permeabilized CHO-K1 cells. Permeabilized CHO-K1 cells were labeled with $[^{14}\text{C}]$serine as described under “Experimental Procedures” in the presence (closed circles) or absence (open circles) of bovine brain cytosol (180 μg of protein). The reactions were performed in glass tubes at 37 °C for 30 min and terminated by the addition of 1.5 ml of MeOH/CHCl$_3$ (2:1). The lipids were extracted from the reaction mixtures by the method of Bligh and Dyer (15) and separated on a thin layer chromatography plate (Silica Gel 60, Merck) using the solvent system described above. The radioactivity of the separated lipids was analyzed with a BAS2000 image analyzer (Fuji Film).

**FIG. 2.** Other Methods—Proteins were measured with a BCA protein assay kit (Fierce), with bovine serum albumin as a standard. Tricine-SDS-PAGE was performed as described (16). Silver staining of proteins in polyacrylamide gels was performed with a silver staining kit (Amer sham Pharmacia Biotech). DNA sequencing was performed by automated sequencing with an Applied Biosystem Prism 310 genetic analyzer and fluorescence-tagged dye terminator cycle sequencing.
soluble proteins from M15/pQE9-cS100B cells also remarkably enhanced the synthesis of [14C]PtdEtn from [14C]serine in permeabilized CHO-K1 cells (Fig. 6). In contrast, crude soluble proteins from M15/pQE9 cells, which carried a control empty vector, were incapable of significantly enhancing the synthesis of [14C]PtdEtn from [14C]serine (Fig. 6). These results indicated that the His-cS100B protein was able to enhance the transport-dependent decarboxylation of PtdSer in permeabilized CHO-K1 cells.

Effects of Ca²⁺ Concentration and ATP on the Enhancement of Transport-dependent Decarboxylation of PtdSer by the His-cS100B Protein—The assay for the TPSD-enhancing activity described above was performed in a solution containing ATP and 0.5 mM CaCl₂. This CaCl₂ concentration is 10⁻³ to 10⁻⁴ times higher than the physiological level. To determine whether or not the enhancement of the transport-dependent decarboxylation of PtdSer by the S100B protein...
requires high Ca\(^{2+}\) levels and/or ATP, permeabilized CHO-K1 cells were incubated under the several sets of conditions shown in Fig. 7, with \[^{14}C\]\textit{serine} in the presence or absence of the His-cS100B protein. In solution A, which approximates the intracellular ionic composition and Ca\(^{2+}\) level (0.1 mM), the synthesis of \[^{14}C\]\textit{PtdSer} and \[^{14}C\]\textit{PtdEtn} from \[^{14}C\]\textit{serine} in permeabilized CHO-K1 cells were negligible (Fig. 7). The addition of 2 mM ATP, 0.5 mM CaCl\(_2\), or both 2 mM ATP and 0.5 mM CaCl\(_2\), to solution A led to efficient synthesis of \[^{14}C\]\textit{PtdSer} and \[^{14}C\]\textit{PtdEtn} (Fig. 7). These results were consistent with the previous conclusion (9) that the synthesis of PtdSer and PtdEtn from \[^{14}C\]serine in permeabilized CHO-K1 cells occur at a high Ca\(^{2+}\) level. The addition of the His-cS100B protein to solution A supplemented with 2 mM ATP, 0.5 mM CaCl\(_2\), or both 2 mM ATP and 0.5 mM CaCl\(_2\), respectively, led to 2-, 3-, and 3-fold enhancement of the synthesis of \[^{14}C\]PtdEtn in permeabilized CHO-K1 cells (Fig. 7). In contrast to the \[^{14}C\]PtdEtn synthesis, the \[^{14}C\]PtdSer synthesis remained essentially unchanged upon the addition of the His-cS100B protein to these solutions. These results indicated that the enhancement of the transport-dependent decarboxylation of PtdSer by the His-cS100B protein occurred in a physiological salt solution supplemented with ATP and did not require ATP upon supplementation with a high level of Ca\(^{2+}\).

**Effects of the His-cS100B Protein on the Transport-dependent Decarboxylation of PtdSer by Isolated Membranes and on PtdSer Decarboxylase Activity**—To examine whether or not the transport-dependent decarboxylation of PtdSer by isolated organelle membranes is enhanced by the His-cS100B protein, we prepared a membrane fraction containing ER membrane, MAM and mitochondria by centrifugation of postnuclear supernatant of CHO-K1 cells at 100,000 \(\times\) g. When the isolated membranes were incubated at 37 °C in solution A supplemented with \[^{14}C\]serine, ATP, and 0.5 mM CaCl\(_2\), \[^{14}C\]PtdSer and \[^{14}C\]PtdEtn were efficiently produced (Fig. 8), suggesting that the transport of nascent PtdSer to mitochondria had occurred. The addition of the His-cS100B protein to the incubation mixture led to 1.3-fold enhancement of \[^{14}C\]PtdEtn formation. However, this enhance-
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FIG. 7. Effects of ATP and the concentration of Ca2+ on the enhancement of transport-dependent decarboxylation of PtdSer by the His-cS100B protein in permeabilized CHO-K1 cells. Permeabilized CHO-K1 cells were prepared as described under “Experimental Procedures.” Permeabilized CHO-K1 cells (4.2 \times 10^6 cells) were labeled for 60 min at 37 °C with [14C]serine (0.2 \muCi, 160 \muCi/\mumol) in 100 \muL of solution A (bar 1), solution A containing 2 mM ATP and an ATP regenerating system (bar 2), solution A containing 0.5 mM CaCl2 (bar 3), or solution A containing 2 mM ATP, an ATP regenerating system, and 0.5 mM CaCl2 (bar 4), in the presence (hatched bars) or absence (closed bars) of 4 \muL of the His-cS100B protein. The reactions were terminated by lipid extraction. PtdEtn and PtdSer were separated by thin layer chromatography, and the radioactivity associated with PtdEtN upon incubation in solution A containing 2 mM ATP, an ATP regenerating system, and 0.5 mM CaCl2 in the absence of the His-cS100B protein was taken as 1000. Values are the averages for duplicate assays with variation of <15% between duplicates.

FIG. 8. Effect of the His-cS100B protein on the synthesis of PtdEtn and PtdSer by an isolated membrane fraction of CHO-K1 cells. A membrane fraction of CHO-K1 cells was prepared as described under “Experimental Procedures.” The isolated membranes (28 \mug of protein) were incubated in 100 \muL of solution A containing [14C]serine (0.2 \muCi, 160 \muCi/\mumol), 2 mM ATP, an ATP regenerating system, and 0.5 mM CaCl2 in the presence (closed circles) or absence (open circles) of 4 \muL of the His-cS100B protein. The reactions were terminated at the indicated times by lipid extraction. PtdEtn and PtdSer were separated by thin layer chromatography, and the radioactivity associated with PtdEtn (A) or PtdSer (B) was analyzed with an image analyzer. The results are expressed as relative radioactivity; the radioactivity associated with PtdSer upon incubation in solution A containing 2 mM ATP, an ATP regenerating system, and 0.5 mM CaCl2 in the absence of the His-cS100B protein was taken as 1000. Values are the averages for duplicate assays with variation of <15% between duplicates.

Next, we examined the effect of the His-cS100B protein on the PtdSer decarboxylase activity in a homogenate of CHO-K1 cells, which was measured under the standard assay conditions. Under such conditions, the His-cS100B protein did not stimulate PtdSer decarboxylase activity and did not exhibit PtdSer decarboxylase activity (Table I). Because the S100B protein is a Ca2+-binding protein, we also examined the effect of the His-cS100B protein on PtdSer decarboxylase activity in the presence of Ca2+ (0.5 mM). Ca2+ itself did not affect PtdSer decarboxylase activity (Table I). Even in the presence of 0.5 mM Ca2+, the His-cS100B protein did not stimulate PtdSer decarboxylase activity and did not exhibit PtdSer decarboxylase activity. These results also supported the idea that the enhancement of the transport-dependent decarboxylation of PtdSer by the His-cS100B protein in permeabilized CHO-K1 cells was not because of the stimulation of PtdSer decarboxylase.

DISCUSSION

PtdEtn synthesis through the PtdSer decarboxylation pathway is required for maintenance of a normal PtdEtn level in mammalian cells (14, 17–19) and normal cell growth (20), and thus probably for the biogenesis of functional organelle membranes. The PtdEtn synthesis via the decarboxylation of nascent PtdSer requires the transport of PtdSer from the ER or MAM to the mitochondrial inner membrane, because of the difference in the subcellular localization of PtdSer synthase and PtdSer decarboxylase (2–6). Although enzymes involved in the PtdSer decarboxylation pathway, PtdSer synthase and PtdSer decarboxylase, have been identified through the isolation of cDNA clones (21–23), the mammalian genes and gene products involved in the transport of PtdSer from the ER or MAM to the mitochondrial inner membrane have not been identified so far. In this study, a Ca2+-binding protein, S100B, was shown to enhance the transport-dependent decarboxylation of PtdSer in permeabilized CHO-K1 cells. This enhancement could be possibly because of the enhancement of a certain process of PtdSer transport or to the stimulation of PtdSer decarboxylase activity. However, the latter possibility was unlikely, because the His-cS100B protein did not stimulate PtdSer decarboxylase activity under the standard assay conditions, irrespective of the presence or absence of Ca2+. The enhancement of decarboxylation of PtdSer by the His-cS100B protein observed in isolated membranes was much lower than that in permeabilized cells. Thus, the S100B protein was implicated in the transport of PtdSer from ER or MAM to mitochondrial inner membrane.

The S100B protein is a member of a family of small (~10
kDa) Ca\(^{2+}\)-binding proteins of the EF-hand type known as the S100 family, which comprises 19 members (24–26). The S100B protein exists as a homodimer or a heterodimer with the S100A1 protein (27, 28), which is another S100 family member and is related most closely to the S100B protein. The S100B protein has been shown to interact with various proteins, and has been implicated in the Ca\(^{2+}\)-dependent regulation of a variety of intracellular activities such as protein phosphorylation, enzyme activities, cell proliferation, cytoskeleton assembly, neurite outgrowth, and intracellular Ca\(^{2+}\) homeostasis (24–26). Interestingly, Ca\(^{2+}\) binding to the S100B protein dimer induces a conformational change, which results in exposure of the binding surface of an individual monomer, with which target proteins of the S100B protein are believed to interact (26). Thus, the S100B protein dimer appears to expose two binding surfaces and to be capable of cross-bridging two proteins, in a Ca\(^{2+}\)-dependent manner.

Inconsistent with our finding that cytosol enhances the transport-dependent decarboxylation of PtdSer in permeabilized cells, Voelker (9) has reported that cytosol slightly inhibits it. This inconsistency may be attributed to the differences in the assay conditions. In the experiment performed by Voelker, the effect of cytosol was examined in the presence of EGTA, which chelates Ca\(^{2+}\) and arrests PtdSer synthesis. On the other hand, our assay was performed in a solution containing Ca\(^{2+}\) and in the presence of PtdSer synthesis. Thus, the enhancement of the transport-dependent decarboxylation of PtdSer by cytosol might require free Ca\(^{2+}\) and/or the presence of PtdSer synthesis.

Bovine brain cytosol appears to contain several factors that enhance the transport-dependent decarboxylation of PtdSer in permeabilized cells, because the enhancing activity has been separated into several peaks on Q-Sepharose ion exchange column chromatography. We have also tried to purify factors other than the S100B protein and found that, in addition to the S100B protein, at least two factors, which are larger than 200 kDa and smaller than 10 kDa, respectively, can enhance the transport-dependent decarboxylation of PtdSer in permeabilized cells.\(^2\) It is therefore likely that several cytosolic factors are involved in the transport or decarboxylation of PtdSer.

Studies by Voelker (9, 11) have suggested that a diffusible PtdSer transport intermediate, such as a soluble protein or a transport vesicle carrying PtdSer, is not involved in the import of PtdSer into mitochondria. Importantly, studies involving CHO-K1 cells disrupted by saponin-permeabilization followed by shearing have demonstrated that PtdSer produced in one population of disrupted cells cannot be transported to the mitochondria of a second population of disrupted cells (11). Therefore, PtdSer transport from the ER or MAM to mitochondria is physically restricted. This restriction implies that PtdSer is imported into mitochondria via a tightly associated contact site between ER or MAM and mitochondria. Then, how can the cytosolic S100B protein enhance the PtdSer transport to the inner mitochondrial membrane? One possible explanation is that the S100B protein regulates a putative PtdSer transport machinery existing in the ER membrane, MAM, or outer mitochondrial membrane. Another possible explanation is that the S100B protein stabilizes the contact site or increases the number of contact sites between the ER or MAM and mitochondria through crossbridging membrane proteins of these organelles. Identification of S100B-interacting membrane protein(s) that exist in these organelles might provide new insights into the transport of PtdSer from the ER or MAM to mitochondria.

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