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Biosynthetic Regulation and Intracellular Transport of Phosphatidylserine in Mammalian Cells

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In mammalian cells, phosphatidylserine (PtdSer) is synthesized through the action of the endoplasmic reticulum enzymes, PtdSer synthase 1 and 2, and the decarboxylation of PtdSer accounts for the majority of phosphatidylethanolamine (PtdEtn) synthesis. PtdSer decarboxylation for PtdEtn formation occurs in the mitochondria. In addition, the transport of PtdSer from the endoplasmic reticulum to the mitochondria is probably a rate limiting step for PtdEtn synthesis through the decarboxylation pathway. Therefore, the regulation of PtdSer synthesis and its intracellular transport appear to be essential events for the maintenance of normal cellular PtdSer and PtdEtn levels. Here we describe the current understanding of the regulation of PtdSer biosynthesis and the transport of PtdSer from the ER to the mitochondria in mammalian cells.

Key words: phosphatidylethanolamine, phosphatidylserine, phospholipid.

Abbreviations: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; MAM, mitochondria-associated membrane; PSS, phosphatidylserine synthase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

Phospholipid bilayers provide the basic structure of all biological membranes that play many essential roles in the maintenance of cell life and functions. However, in studies on the biogenesis of the phospholipid bilayer, there are two fundamental questions unresolved. One is what mechanisms regulate the composition and total content of phospholipids in biological membranes. To answer this question it seems important to clarify the regulation of the biosynthesis of each phospholipid, but the regulatory mechanisms of the biosynthesis of most phospholipids in mammalian cells are largely unknown. Another question is how phospholipids move between two biological membranes. Because phospholipid synthesis occurs in limited organelles, such as the endoplasmic reticulum (ER) and mitochondria, many biological membranes are incapable of producing their own phospholipids. Therefore, the intracellular transport of phospholipids from the sites of synthesis to their final location is an essential event in the biogenesis of functional biological 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. However, the importance of the proposed mechanisms for membrane biogenesis remains unclear, and phospholipid transport processes are largely unknown with respect to specific mechanisms, genes and proteins involved.

Phosphatidylserine (PtdSer) is one of the major membrane phospholipids in mammalian cells, comprising about 10% of the total phospholipids of various tissues and cultured cells. The structures of PtdSer and other phospholipids metabolically relevant to PtdSer are shown in Fig. 1. PtdSer is known to interact with various proteins, such as protein kinase C (*1*), myristoylated alanine-rich C kinase substrate (*2*), coagulation factor V (*3*), synaptotagmin (*4*), Raf-1 protein kinase (*5*), nitric oxide synthase (*6*), dynamin GTPase (*7*), and diacylglycerol kinase (*8*, *9*). In addition, PtdSer functions as a precursor phospholipid in the formation of lysophosphatidylserine, which is believed to act as a lipid mediator under various pathophysiological conditions (*10*). In the plasma membrane, PtdSer is localized almost exclusively in the inner leaflet of the lipid bilayer, but externalization of PtdSer to the outer leaflet is observed during cell activation, aging, and apoptosis. When externalized on the cell surface, PtdSer has been shown to act as a signal for the removal of damaged, aged, or apoptotic cells by phagocytes. Thus, PtdSer appears to play many physiological roles.

In mammalian cells, the synthesis of PtdSer occurs in the ER or mitochondria-associated membrane (MAM) (*11–15*), while the decarboxylation of PtdSer for the formation of phosphatidylethanolamine (PtdEtn) occurs in the mitochondrial inner membrane (*11*, *12*, *16*, *17*). PtdSer synthesis (*18–20*) and PtdEtn synthesis through the PtdSer decarboxylation pathway (*21*) are required for the maintenance of the normal composition of total membrane phospholipids and normal cell growth, and thus probably for the biogenesis of functional biological membranes. Using the decarboxylation of nascent PtdSer as a biochemical indicator of the transport of PtdSer, a number of studies on the transport of PtdSer from the ER or MAM to the mitochondria have been carried out. In

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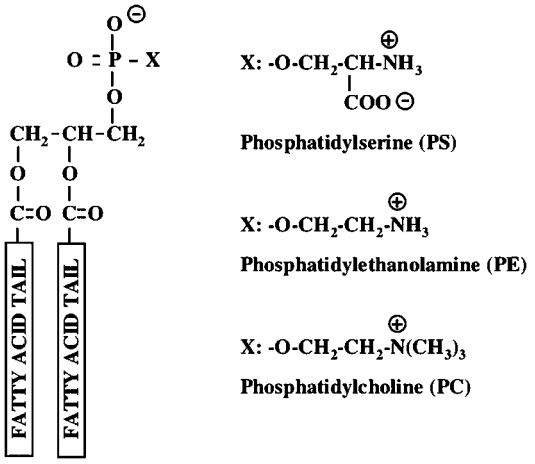

Fig. 1. **Structures of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine.**

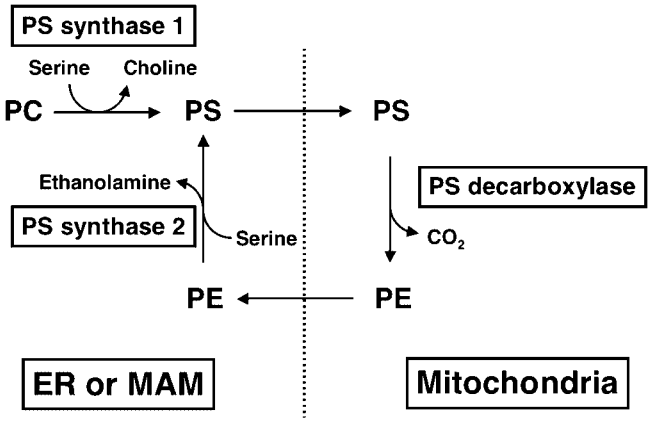

Fig. 2. **PtdSer decarboxylation pathway in mammalian cells.** PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

addition, we have found that PtdSer synthesis in mammalian cells is regulated by feedback control with PtdSer (*22*). In this review, we briefly describe the current understanding of the PtdSer decarboxylation pathway in mammalian cells, highlighting the regulation of PtdSer biosynthesis and the transport of PtdSer from the ER or MAM to the mitochondrial inner membrane.

General features of the PtdSer decarboxylation pathway in mammalian cells

The general biochemical and topological features of the PtdSer decarboxylation pathway are shown in Fig. 2. PtdSer formation in mammalian cells occurs through the exchange of L-serine with the choline moiety of phosphatidylcholine (PtdCho) or the ethanolamine moiety of PtdEtn, which are catalyzed by two different enzymes, PtdSer synthase (PSS) 1 and 2. PSS 1 is responsible for the formation of PtdSer from the PtdCho precursor, and PSS 2 is responsible for the formation of PtdSer from the PtdEtn precursor. The presence of two kinds of PtdSer synthase and their substrate specificities have been demonstrated through a series of studies involving CHO cell mutants defective in PtdSer biosynthesis (*18–20*, *23*, *24*) and cloning the cDNAs of each enzyme (*25–28*). A study on the biosynthetic site of PtdSer has identified ER and a novel ER-related fraction (*15*) as the principal intracellular site of PtdSer biosynthesis. The ER-related fraction is biochemically isolated as a fraction that is physically associated with the mitochondria and separated from the mitochondria by Percoll gradient centrifugation (*15*), and is referred to as the mitochondria-associated membrane (MAM). Immunological and biochemical studies have shown that both PSS 1 and 2 are more enriched in MAM than in bulk of ER (*13–15*).

The decarboxylation of PtdSer results in the formation of PtdEtn, and this reaction occurs through the action of mitochondrial PtdSer decarboxylase in mammalian cells. In addition to the PtdSer decarboxylation pathway, another PtdEtn biosynthetic pathway, called the CDP-ethanolamine pathway, is found in mammalian cells (*29–32*). In the CDP-ethanolamine pathway, ethanolamine is phosphorylated and converted to CDP-ethanolamine, and then the phosphoethanolamine moiety of CDP-ethanolamine is transferred to diacylglycerol for the formation of PtdEtn. Although early studies in the 1960s and 1970s on PtdEtn biosynthesis in mammalian cells focused on the CDP-ethanolamine pathway, in the 1980s several papers (*18–20*, *33*) provided evidence that PtdSer decarboxylation accounts for the majority of PtdEtn synthesis in mammalian cells. It is noteworthy that the PSS 2-catalyzed exchange of serine with the ethanolamine moiety of PtdEtn results in the production of ethanolamine as well as PtdSer, and the ethanolamine released from PtdEtn is efficiently re-incorporated into PtdEtn *via* CDP-ethanolamine (*34*).

Studies examining PtdSer decarboxylase activities in subcellular fractions have shown that the decarboxylase is localized in the mitochondria (*11*, *12*). The mitochondrial localization of the decarboxylase has been confirmed by immunochemical methods with a specific antibody (*17*). Subfractionation of the mitochondria has revealed that the decarboxylase is a mitochondrial inner membrane enzyme (*12*, *16*). Sequence analyses of the cDNA of PtdSer decarboxylase (*17*, *35*) have indicated that the decarboxylase gene product contains in its N-terminus a bipartite structure consisting of an extreme N-terminal mitochondria targeting sequence and a mitochondrial inner membrane sorting sequence, which seem to be sequentially cleaved by post-translational processing. The processing intermediates are found in CHO-K1 cells (*17*), consistent with targeting to the mitochondrial inner membrane.

Regulation of PtdSer biosynthesis

PtdSer biosynthesis in CHO-K1 cells is remarkably inhibited by the addition of PtdSer to the culture medium (*22*), indicating that feedback control is involved in the regulation of PtdSer biosynthesis. PtdSer synthase activities in homogenates of CHO-K1 cells cultivated with or without exogenous PtdSer are essentially the same (*22*), suggesting that the cellular levels of PSS 1 and 2 remain unchanged when PtdSer is added to the culture medium. The PSS 1 mRNA level is also unchanged by the addition of PtdSer to the culture medium (O. Kuge, unpublished data). (Changes in PSS 2 mRNA levels upon PtdSer addi-

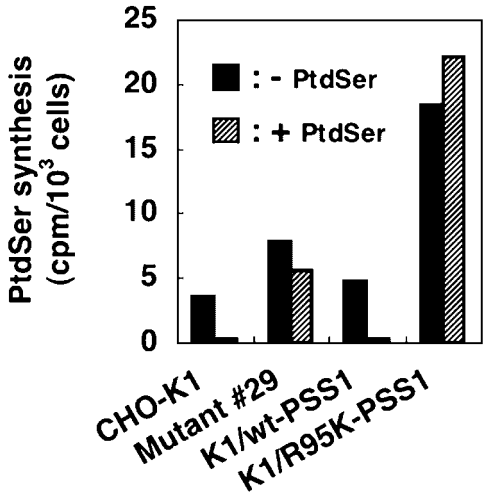

Fig. 3. **PtdSer biosynthetic rates in CHO-K1, 29 mutant, and wild-type or R95K-mutant PSS 1-overproducing CHO-K1 cells.** Cells were metabolically labeled with [^{14}C]serine for 3 h at 37°C in the absence (filled bars) or presence (hatched bars) of 80 μM PtdSer. Phospholipids in the labeled cells were extracted and separated by thin layer chromatography, and then the radioactivity of PtdSer was determined. K1/wt-PSS1, a CHO-K1 transformant that overproduces wild-type PSS 1; K1/R95K-PSS1, a CHO-K1 transformant that overproduces R95K-mutant PSS 1.

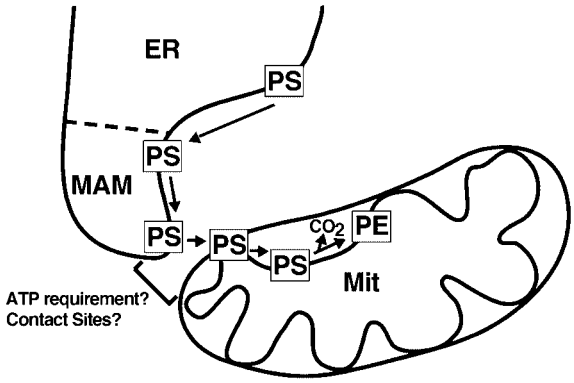

Fig. 4. **Possible PtdSer transport pathway from the ER or MAM to the mitochondrial inner membrane. Mit, mitochondria; PS, phosphatidylserine; PE, phosphatidylethanolamine.**

tion have not been examined.) In addition, it has been shown that PtdSer inhibits PtdSer synthase activity in isolated membrane fractions of CHO-K1 cells (*36*). These observations imply that the feedback control of PtdSer biosynthesis with PtdSer does not involve transcriptional or translational regulation of PtdSer synthase genes and is carried out by the inhibition of PtdSer synthase activity by PtdSer.

A CHO cell mutant, 29, which is defective in the feedback control of PtdSer biosynthesis, has been isolated from CHO-K1 cells (*36*). When PtdSer is added to the culture medium, PtdSer biosynthesis in 29 mutant cells is reduced by only 29%, although that in CHO-K1 cells is reduced by 98% (Fig. 3) (*36*, *37*). In culture medium without exogenous PtdSer, the 29 mutant cells synthesize PtdSer at a 2 to 3-fold higher rate (Fig. 3) and exhibit an approximately 2-fold higher cellular PtdSer level compared with CHO-K1 cells (*36*, *37*). In an *in vitro* assay system, the PtdSer synthase activities of CHO-K1 and 29 mutant cells are essentially the same (*36*, *37*); however, the PtdSer synthase activity of CHO-K1 cells, but not that of 29 mutant cells, is inhibited by the addition of PtdSer to the assay system reaction mixture (*36*, *37*). The 29 mutant cells have been shown to carry a point mutation in the PSS 1 gene, which results in the replacement of Arg-95 in PSS 1 with Lys (*37*). The introduction of the R95K-mutant PSS 1 cDNA into CHO-K1 cells induces about a 5-fold elevation of the PtdSer biosynthetic rate (Fig. 3) and about a 2-fold elevation of the cellular PtdSer level when the cells are cultivated without exogenous PtdSer, whereas the wild-type PSS 1 cDNA is incapable of inducing such significant elevations (*37*). Furthermore, it has been shown that the R95K mutation in the PSS 1 gene renders the product PSS 1 resistant to inhibition by exogenous PtdSer (Fig. 3) (*37*). These results indicate that the PtdSer-mediated inhibition of PSS 1 activity is critical for the feedback control of PtdSer biosynthesis observed in intact cells and that Arg-95 in PSS 1 is a critical residue for the control of PSS 1 activity.

Chinese hamster PSS 1 and 2 are similar in sequence to each other with 38% amino acid sequence identity between the two synthases (*25*, *26*). PSS 2 has an arginine residue at position 97, which corresponds to Arg-95 of PSS 1, which has been identified as a critical residue for the control of PSS 1 activity. As described later, Arg-97 of PSS 2 is also a critical residue for the control of PSS 2 activity.

The activity of PSS 2, as well as that of PSS 1, is regulated through feedback control with PtdSer, because PtdSer biosynthesis in a CHO cell mutant, PSA-3, which lacks PSS 1 but has normal PSS 2 activity, is almost completely inhibited by the addition of PtdSer to the culture medium (*38*). Interestingly, PtdSer biosynthesis in a PSS 2-overproducing stable transformant of CHO-K1 is reduced by only 35% upon the addition of PtdSer (*38*). The PtdSer synthase activity of an isolated membrane fraction of the PSS 2-overproducing CHO-K1 transformant is not inhibited by PtdSer at all, whereas the activities from the PSA-3 mutant and CHO-K1 cells are inhibited by >95% (*38*). Therefore, the overproduction of PSS 2 leads to the loss of normal control of PSS 2 activity by exogenous PtdSer.

Although overproduced PSS 2 is not normally controlled by exogenous PtdSer, PSS 2-overproducing cells exhibit a normal PtdSer biosynthetic rate in the culture medium without exogenous PtdSer (*38*). In contrast, another stable transformant of CHO-K1 that overproduces the R97K mutant PSS 2, exhibits an approximately 4-fold higher PtdSer biosynthetic rate compared with that in CHO-K1 cells (*38*). These results suggest that for the maintenance of a normal PtdSer biosynthetic rate, the activity of the overproduced wild-type PSS 2 is depressed by as yet unknown mechanisms other than those for the exogenous PtdSer-mediated inhibition, and also suggest that Arg-97 in PSS 2 is critical for this depression of overproduced PSS 2 activity.

When the cDNA-directed wild-type and R97K mutant PSS 2 activities are expressed at nonoverproduction levels in a PSS 1- and 2-defective mutant of CHO-K1 cells,

the expression of the mutant PSS 2 activity but not that of the wild-type PSS 2 activity induces PtdSer resistant PtdSer biosynthesis (*38*). It is, therefore, likely that Arg-97 in PSS 2 is a critical residue for the exogenous PtdSer-mediated inhibition of PSS 2.

PtdSer transport from ER or MAM to the mitochondrial inner membrane

Utilizing the decarboxylation of nascent PtdSer as an indicator of the transport of PtdSer, Voelker has shown that the transport of PtdSer from the ER or MAM to the mitochondrial inner membrane in BHK cells is inhibited by the depletion of cellular ATP (*39*), suggesting for the first time that this transport requires ATP. To address the site of the ATP requirement for PtdSer transport, Shiao *et al*. (*40*) pulse-labeled CHO-K1 cells with [^{3}H]serine and subjected the pulse-labeled cells to subcellular fractionation after chase in the presence or absence of energy poisons, which deplete cellular ATP levels by ~90%. Upon chase in the absence of energy poisons, [^{3}H]PtdSer can be found in both microsomes and MAM, and the amount of [^{3}H]PtdSer in both the microsome and MAM fractions gradually decrease over the chase period (*40*). Upon chase in the presence of energy poisons, [^{3}H]PtdSer accumulates in the MAM fraction and is blocked from reaching the mitochondria and subsequent decarboxylation, although the amount of [^{3}H]PtdSer in the microsome fraction decreases during the chase period (*40*). These results suggest that MAM plays a role as an intermediate compartment in PtdSer transport to the mitochondria and that ATP is required for the exit of PtdSer from MAM.

Isolated organelles have also been used to study PtdSer transport. Upon incubation with isolated mitochondria, [^{3}H]PtdSer generated in isolated microsomes and MAM is efficiently converted to [^{3}H]PtdEtn (*41*, *42*), indicating the efficient import of PtdSer into the mitochondria. However, in contrast to PtdSer import into the mitochondria in intact cells, PtdSer import into isolated mitochondria does not require ATP (*41*, *42*). To address this discrepancy, Voelker developed a permeabilized cell system in which to study PtdSer transport (*43*, *44*). CHO-K1 cells permeabilized by saponin treatment reproduce the synthesis, transport and decarboxylation of PtdSer with remarkable fidelity to the intact cells (*43*, *44*). Using this permeabilized cell system, Voelker has shown the following: (i) ATP is required for PtdSer transport to mitochondria in permeabilized cells (*43*, *44*). (ii) The general features of protein and PtdSer export from the ER are fundamentally different insofar as the export of proteins, but not that of PtdSer, requires cytosolic factors and guanine nucleotides (*43*, *44*). (iii) A diffusible PtdSer transport intermediate, such as a soluble protein or transport vesicle carrying PtdSer, is probably not involved in the import of PtdSer into the mitochondria (*43–45*). (iv) When CHO-K1 cells are disrupted by saponin-permeabilization followed by shearing, PtdSer produced in one population of disrupted cells cannot be transported to the mitochondria of a second population of disrupted cells (*45*), indicating that PtdSer transport from the ER or MAM to the mitochondria is physically restricted. This restriction suggests that some membrane structure tightly associated with the mitochondria is the preferred donor of PtdSer to the mitochondria.

These observations in intact and permeabilized cells suggest that contact zones between the MAM and mitochondrial outer membrane play a critical role in the import of PtdSer produced in both ER and MAM into the mitochondria. Interestingly, it has been suggested that the zones of association between the MAM and mitochondria are also sites of contact between the outer and inner mitochondrial membranes (*46*, *47*). In addition, the transport of PtdSer from the outer mitochondrial membrane to the inner mitochondrial membrane is inhibited by 1,4-dinitrophenol (*48*), which diminishes the number of contact sites between the outer and inner mitochondrial membranes. Furthermore, radiolabeled PtdSer introduced into isolated mitochondria accumulates in a contact site-enriched subfraction of mitochondria when PtdSer decarboxylase activity is inhibited (*46*). It is, therefore, likely that PtdSer moves from the outer mitochondrial membrane to the inner mitochondrial membrane *via* contact sites between the outer and inner mitochondrial membranes.

The mitochondrial contact sites between the outer and inner membranes are also well known to participate in protein import into the mitochondria. In addition, Adriamycin, which is a potent inhibitor of the import of proteins into mitochondria, also inhibits PtdSer transport between the outer and inner mitochondrial membrane in the permeabilized cell system (*49*). Thus, there are some common features to lipid and protein import.

The PtdSer transport pathway from the ER to the mitochondrial inner membrane in mammalian cells, which is suggested from these observations, is summarized in Fig. 4.

The identification of the genes and gene products specifically involved in the PtdSer transport is a largely undeveloped area of investigation. Shiao *et al.* have suggested that a mitochondrial outer membrane protein(s) is involved in the uptake of PtdSer by mitochondria (*42*), but such protein(s) remains to be identified. In this laboratory, both biochemical and genetic approaches to identifying the genes involved in PtdSer transport have been carried out. Recently, we found that the transport-dependent decarboxylation of PtdSer in permeabilized CHO-K1 cells is remarkably enhanced by cytosolic factors from bovine brain. A cytosolic protein factor exhibiting this enhancing activity has been purified, and identified as an EF-hand type calcium-binding protein, S100B (*50*). A His_6-tagged recombinant CHO S100B protein is able to enhance remarkably the transport-dependent decarboxylation of PtdSer in permeabilized CHO cells (*50*). Under the standard assay conditions for PtdSer decarboxylase, the recombinant protein does not stimulate PtdSer decarboxylation and exhibits no PtdSer decarboxylase activity (*50*). These results implicate S100B in the transport of PtdSer to the mitochondria from the ER or MAM.

As described above, contact zones between the MAM and mitochondrial outer membrane appear to play a critical role in the import of PtdSer into mitochondria. Then, how can the cytosolic S100B protein enhance PtdSer transport to the inner mitochondrial membrane? One possible explanation is that the S100B protein regulates

putative PtdSer transport machinery existing in the ER membrane, MAM, or outer mitochondrial membrane. S100B exists as a homodimer and has been shown to expose two protein binding surfaces in a Ca^{2+}-dependent manner. Therefore, another possible explanation is that the S100B protein stabilizes the contact site or increases the number of contact sites between the MAM and mitochondria by cross-linking membrane proteins of these organelles. The identification of an S100B-interacting membrane protein(s) that exists in the ER, MAM, or mitochondria might provide new insight into the transport of PtdSer from the ER or MAM to the mitochondria.

A CHO cell mutant (designated R-41) defective in PtdEtn formation through the PtdSer decarboxylation pathway has been isolated as a variant resistant to a PtdEtn-directed antibiotic peptide (*51*). The mutant exhibits normal PtdSer synthase and decarboxylase activities (*51*), implying a defect in some step of PtdSer transport. In fact, the mutant has been shown to be defective in intramitochondrial transport of PtdSer in experiments involving a PtdSer analog, 1-palmitoyl-2-{*N*-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl}-PtdSer (C6-NBD-PtdSer), which is readily inserted into accessible membranes but does not undergo spontaneous transmembrane movement. Upon incubation with isolated mitochondria from CHO-K1 cells (the parental cells of R-41 mutant cells), C6-NBD-PtdSer is efficiently decarboxylated to form C6-NBD-PtdEtn (*51*), indicating that the PtdSer analog is partitioned into the outer leaflet of the outer mitochondrial membrane, and then transported to the inner mitochondrial membrane. The rate of decarboxylation of C6-NBD-PtdSer by the mutant mitochondria is reduced to ~40% of that by CHO-K1 mitochondria, although the disrupted mitochondria of the mutant and CHO-K1 cells exhibit similar C6-NBD-PtdSer decarboxylation activities (*51*). Furthermore, when the amount of C6-NBD-PtdSer and C6-NBD-PtdCho that are transported from the surface of mitochondria to the inside of mitochondria is examined by extracting the analogs selectively from the surface, the mutant mitochondria have been shown to be defective in the transport of C6-NBD-PtdSer, but not in the transport of C6-NBD-PtdCho (*51*). These results indicate that the mutant is defective in PtdSer transport from the outer leaflet of the outer mitochondrial membrane to the inner mitochondrial membrane, and suggest the existence of a specific mechanism for the intramitochondrial transport of PtdSer. Identification of the mutated gene responsible for the PtdSer transport defect of R-41 cells would greatly enhance understanding of the intramitochondrial transport mechanism of PtdSer.

Perspectives

The precise mechanisms for the PtdSer-mediated inhibition of PtdSer synthase activity are currently unknown. Whether the inhibition occurs through direct interaction of PtdSer with PtdSer synthase or is mediated by an unknown factor is still unresolved. To address this issue, the purification of functional PtdSer synthases seems to be important, but a PtdSer synthase from mammalian cells has not yet been purified. A search for proteins that interact with PtdSer synthase might also provide new insight into the mechanisms of the regulation of PtdSer biosynthesis. The physiological role of the PtdSer-mediated inhibition of PtdSer synthase activity in whole animals also remains to be elucidated. The construction of a transgenic mouse that produces PtdSer-resistant R95K-PSS 1 or R97K-PSS 2 would be useful in clarifying this point.

Many gene products appear to be involved in PtdSer transport from the ER or MAM to the mitochondrial inner membrane, because of the complexity of the transport process. The identification and characterization of such gene products seems to be essential for understanding the transport mechanisms. However, very little is known about the genes and gene products that participate in PtdSer transport. The isolation of PtdSer-transport mutant cells, such as R-41 cells, would be one effective approach to identifying the genes involved in PtdSer transport. R-41 mutant cells require exogenous PtdEtn or ethanolamine for growth, due to the defect in the PtdEtn formation through the PtdSer decarboxylation pathway (*21*). Therefore, the gene, in which the R-41 mutant has a mutation responsible for the PtdSer transport defect, might be isolated as a gene that confers PtdEtn prototrophy on R-41 mutant cells. Given that the basic mechanisms of PtdSer transport to the mitochondrial inner membrane are common to yeast and mammalian cell systems, transport-defective yeast mutants would also be important tools for understanding the transport mechanisms in mammalian cell systems, as well as in yeast cell systems. Recently, a mutant strain of the yeast *Saccharomyces cerevisiae* that is defective in the transport of PtdSer from the ER/MAM to the mitochondria has been isolated (*52*). The transport defect is complemented by the *MET30* gene, which encodes a subunit of a ubiquitin ligase complex (*52*), suggesting that PtdSer transport is regulated by ubiquitination. We believe that in the near future genetic and biochemical studies, together with the recent advances in genome sequence research, will reveal many molecules and molecular machineries critical for PtdSer transport from the ER or MAM to the mitochondrial inner membrane.

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