Characterization of Phosphatidylserine Transport to the Locus of Phosphatidylserine Decarboxylase 2 in Permeabilized Yeast*

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In yeast, nascent phosphatidylserine (PtdSer) can be transported to the mitochondria and Golgi/vacuole for decarboxylation to synthesize phosphatidylethanolamine (PtdEtn). In strains with a psd1Δ allele for the mitochondrial PtdSer decarboxylase, the conversion of nascent PtdSer to PtdEtn can serve as an indicator of lipid transport to the locus of PtdSer decarboxylase 2 (Psd2p) in the Golgi/vacuole. We have followed the metabolism of [3H]serine into PtdSer and PtdEtn to study lipid transport in permeabilized psd1Δ yeast. The permeabilized cells synthesize 3-H-PtdSer and, after a 20-min lag, decarboxylate it to form [3H]PtdEtn. Formation of [3H]PtdEtn is linear between 20 and 100 min of incubation and does not require ongoing PtdSer synthesis. PtdSer transport can be resolved into a two-component system using washed, permeabilized psd1Δ cells as donors and membranes isolated by ultracentrifugation as acceptors. With this system, the transport-dependent decarboxylation of nascent PtdSer is dependent upon the concentration of acceptor membranes, requires Mn2+ but not nucleotides, and is inhibited by EDTA. High speed membranes isolated from a previously identified PtdSer transport mutant, pstB2, contain normal Psd2p activity but fail to reconstitute PtdSer transport and decarboxylation. Reconstitution with permutations of wild type and pstB2Δ donors and acceptors identifies the site of the mutant defect as the acceptor side of the transport reaction.

The biochemical, molecular, and genetic details of the interorganelle transport of phospholipids are poorly understood. One approach to studying this problem utilizes the organelle-specific metabolism of the aminoglycerophospholipids, phosphatidylethanolamine (PtdEtn), phosphatidylethanolamine (PtdSer), and phosphatidylethanolamine (PtdCho) outlined in Fig. 1 (1). In yeast, PtdSer is synthesized in the endoplasmic reticulum and related membranes (2). The newly formed PtdSer is subsequently decarboxylated to form PtdEtn in the mitochondria, by PsdSer decarboxylase 1 (Psd1p) (3); and in the Golgi/vacuole, by PtdSer decarboxylase 2 (Psd2p) (4). The PtdEtn resulting from the action of the decarboxylases is exported from the mitochondria and Golgi/vacuole to the endoplasmic reticulum, where it is methylated to form PtdCho (3, 5). This sequential and subcellular location-specific metabolism of the aminoglycerophospholipids provides a rapid and convenient method for assessing lipid transport in intact cells, permeabilized cells, and isolated organelles (1). This intracellular site-specific metabolism has also provided a means to develop genetic screens for new yeast strains defective in the interorganelle transport of phospholipids (6, 7). By using yeast strains with selective genetic defects in expression of either Psd1p (psd1Δ mutants) or Psd2p (psd2Δ mutants), it is possible to study lipid trafficking events to either the Golgi/vacuole or the mitochondria, respectively, using both biochemical and genetic approaches (6–8). Currently, two mutant strains have been identified using a genetic approach, and these strains identify lesions in a phosphatidylinositol 4-kinase (Slt4p) (6), and a phosphatidylinositol transfer protein (PstB2p) (7).

To complement the genetic approach and studies performed with intact cells, it is necessary to develop an in vitro assay system amenable to the addition and deletion of specific macromolecules to enable further biochemical and mechanistic dissection of the transport processes. Such a system will also allow the mixing of different donor and acceptor populations of organelles that localizes lesions topologically. Previous work by Achleitner et al. (8) described conditions for transport of nascent PtdSer from the ER to PtdEtn in the mitochondria and in permeabilized yeast cells. These preceding studies did not reveal conditions for transport of PtdSer from the ER to the locus of Psd2p in the Golgi/vacuole. The objectives of our current studies were to 1) define conditions for reconstituting PtdSer transport to PtdEtn in permeabilized cells; 2) establish conditions for defining separable donor and acceptor compartments for PtdSer synthesis and decarboxylation; 3) identify the role of nucleotides and divalent cations in the process; and 4) apply the reconstituted system to defining the site of action of PstB2p. In this report, we now describe the characteristics of PtdSer transport from the ER to the Golgi of permeabilized cells.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals, including amino acids for yeast media, were purchased from Sigma or Fisher. Other components for yeast growth media were purchased from Difco. Phospholipids were obtained from Avanti Polar Lipids. Thin layer silica gel H plates were from Analtech Corp. The radioisotopes [3-3H]serine and [1-14C]serine were purchased from Amersham Pharmacia Biotech and ICN, respectively. The lipid 1-acetyl-2,6-[7-nitro-2,1,3-benzoxadiazol-4-yl]aminocaproyl-Ptd(1-14C)serine was synthesized using Escherichia coli PtdSer synthase (9). Protein determination reagents were obtained from either Pierce or Bio-Rad. Mouse monoclonal antibody against the 100-kDa subunit of yeast vacuolar H+ -ATPase was purchased from Molecular
**FIG. 1. Biosynthesis and transport of aminoglycerophospholipids in yeast.** PtdSer is synthesized in the endoplasmic reticulum and subsequently transported to other organelles. In the mitochondria, the PtdSer is decarboxylated to form PtdEtn by PtdSer decarboxylase 1. In the Golgi/vacuole compartments, PtdEtn is formed by the action of PtdSer decarboxylase 2. PtdEtn in the mitochondria and Golgi/vacuole compartments can be subsequently exported from these organelles back to the endoplasmic reticulum for further metabolism to PtdCho by the action of PtdEtn methyltransferases. Both known and proposed mutations in the metabolic and transport process are shown in lowercase italic type: *psd1*, PtdSer decarboxylase 1; *peeA*, PtdSer transport A pathway; *peA*, PtdSer transport B pathway; *peeB*, PtdSer export A pathway; *peeB*, PtdEtn export B pathway; *pstB1*, PtdSer export A pathway; *pstB2*, PtdSer export B pathway; *pstB2*, PtdEtn methyltransferase 2. The focus of this study is upon events between PtdSer synthesis and decarboxylation by Ptd2p. For this report, *psd1*Δ mutants were used in all experiments, and the decarboxylation by the mitochondrial enzyme was completely blocked as indicated by the X through the mitochondria.

Probes, Inc. (Eugene, OR). Other reagents used for ligand blotting were from Bio-Rad and Sigma.

**Strains and Culture Conditions**—The *Psd1p*-deficient yeast strains PTY40 (MATa ura3 his3 trp1 met14 psd1Δ1::TRP1) and WWY66 (MATa lys2 trp1 ura3 his3 leu2 suc2 psd1Δ1::TRP1 pstB2::HIS3) were cultured in YPDAUE (standard YPD medium supplemented with additional adenine, uracil, and ethanolamine) (6) medium under aerobic conditions at 30 °C to an A600 of between 1 and 2.

**Preparation of Permeabilized Yeast Cells**—Permeabilized cells were prepared in 10 mM Hepes, pH 6.8, 0.15 M potassium acetate, 2 mM magnesium acetate, and 0.5 mM EDTA following the method described by Achleitner et al. (8). In brief, yeast spheroplasts were prepared by treating cells with dialysis tubing at alkaline conditions, followed by zymolyase treatment in the presence of low glucose (0.5%) YPD (10). The resulting spheroplasts were regenrated for 20 min at 30 °C in the presence of 0.75% yeast extract, 1.5% peptone, 1% glucose, and 0.7 M sorbitol before they were washed and then resuspended in lysis buffer at a concentration of 0.5 g, wet weight/mL. The cell suspensions were divided into 0.2–0.3-ml aliquots and frozen over liquid nitrogen vapor for 15 min. The frozen cells could be stored at −80 °C for at least 3 months. 

**PtdSer Transport Assays**—[3H]serine was incorporated into the permeabilized cells in a reaction mixture containing 8 mM Hepes, 22 mM Tris-HCl, pH 8, 0.6 mM MnCl2, 0.8 mM magnesium acetate, 60 mM potassium acetate, 0.1 mM KCl, 0.1 mM β-chloroalanine, 0.2 mM EDTA, 0.4 mM MgCTP, 0.28 mM sorbitol, 0.27 mM mannitol, 50 mM L-serine, 3 μCi of [3H]serine, and the permeabilized cells equivalent to 90 μg of protein in 100 μL per reaction. The purpose of β-chloroalanine is to inhibit extraneous metabolism of serine to sphingolipids. The assay mixture was incubated at 30 °C for 100 min unless stated otherwise. The radio-labeling reaction was stopped by the addition of 1 mL of chloroform, 1 mL of methanol, and 0.9 mL of 0.2 mM KCl. The organic phase was further washed with 1.9 mL of methanol/PBS (1:0.9, v/v) twice. The resulting chloroform phase was dried under nitrogen gas, resuspended in 30 μL of chloroform/methanol (9:1, v/v), and then loaded on silica gel H thin layer chromatography plates. The plates were developed using a solvent system containing chloroform, methanol, 2-propanol, 0.25% aqueous KCl, triethylamine (30:9:25:6:18, v/v/v/v/v). Individual phospholipids were identified under ultraviolet light with authentic standards, by spraying the thin layer plates with 0.1% aqueous 8-anilino-1-naphthalenesulfonic acid. The lipid spots then were scraped into vials containing scintillation fluid, and the amount of [3H]was quantified.

**Preparation of Processed Donors**—Thawed permeabilized cells were centrifuged at 1000 × g for 5 min at 4 °C. The resulting low speed supernatant (LSS) was collected for reconstituting PtdSer transport or for isolating high speed supernatants (HSS) and high speed membranes (HSM) as described below. The remaining permeabilized cell pellet was washed twice with 25-fold volume of cold lysis buffer. After the second wash, the cells were resuspended in cold lysis buffer to approximately the original volume present when they were first thawed and used as the processed donors. In the indicated experiments, washed donors were preincubated with 50 μM CDP diacylglycerol (CDP-DAG), at 30 °C for 20 min. Excess CDP-DAG was removed by 1000 × g centrifugation for 5 min at 4 °C, followed by washing the cells twice with a 20-fold volume of cold lysis buffer. Prelabeled containing [3H]PtdSer were prepared from washed permeabilized cells incubated in the PtdSer transport assay reagents, in the presence of either MgCTP or CDP-DAG at 30 °C for 45 min. Unincorporated radiolabel was removed by centrifugation and washing with cold lysis buffer as described above for CDP-DAG-treated donors.

**Preparation of LSS, HSS, and HSM**—Cellular components released from permeabilized cells were fractionated by centrifugation. LSS was collected as described above and further centrifuged at 400,000 × g for 1 h at 4 °C to obtain HSS and HSM pellet. The membrane pellet was resuspended by homogenization in cold lysis buffer using a battery-driven minihomogenizer (Kontes). 

**Analysis of Organelle Markers**—Fractions obtained from permeabilized cells, including processed donors, LSS, HSS, and HSM, were subjected to organelle marker analysis by either enzyme assays or by antigen detection. The ER marker, PtdSer synthase (Pssp), was measured by the method described by Carman and Bae-Lee (11). The vacuolar marker, H+-ATPase, was detected by enzyme-linked immunosorbent assays (12). The late Golgi marker, Kex2p protease, was determined by using Boc-Gln-Arg-Arg-7 amidomethyl coumarin as the substrate (13). Ptd2p activity was determined as described previously (14).

**RESULTS**

**PtdSer Is Synthesized and Transported to the Locus of Ptd2p in Permeabilized Yeast**—In intact cells, PtdSer is synthesized in the ER and related membranes and then transported to multiple organelles. Upon arrival at the Golgi/vacuole, the PtdSer is decarboxylated by Ptd2p to form PtdEtn. PtdEtn can also be formed from nascent PtdSer by the action of Ptd1p in the mitochondrion. In a *psd1Δ* genetic background, all decarboxylation of PtdSer occurs via the action of Ptd2p (see Fig. 1). We permeabilized *psd1Δ* cells and optimized conditions for the formation of PtdEtn by Ptd2p in the Golgi/vacuole. As shown in Fig. 2, the permeabilized strain, PTY40, efficiently synthesizes [3H]PtdSer during the first 40 min of incubation, after which the level remains relatively constant, probably reflecting the
consumption of endogenous CDP-DAG required for the reaction. Following a 20–30-min lag, the synthesis of \([^{3}H]\)PtdEtn proceeds at a linear rate between 30 and 100 min. At 100 min, \(\sim 6\%\) of the nascent \([^{3}H]\)PtdSer has been converted to PtdEtn. A preliminary description of this system (7) identified the Golgi and a novel light membrane fraction as the sites of formation/accumulation of \([^{3}H]\)PtdEtn. These experiments provide a clear demonstration that PtdSer transport to the Psd2p can be reconstituted in permeabilized cells, making the process amenable to further powerful analysis by biochemical and genetic approaches.

*PtdEtn Formation Requires Cellular Components Released from Permeabilized psd1Δ Yeast*—Following permeabilization, some cellular components, including cytosol and membranes, were released from the cells. The released components were initially separated from the permeabilized cells by a low speed centrifugation. We next examined the role of the released material in PtdSer transport and decarboxylation. Permeabilized cells, washed free of released cellular contents by a 1000 \(\times\) g centrifugation, lost their ability to transport PtdSer to Psd2p (Fig. 3A). However, the transport activity was restored when the cells were reconstituted with their autologous LSS (Fig. 3B). The relative efficiency of PtdEtn formation after reconstitution with LSS approximated that of the unmanipulated cells. Both the rate and extent of PtdEtn formation by LSS-supplemented permeabilized cells were comparable with the results obtained in the experiments shown in Fig. 2.

The synthesis of PtdSer in the permeabilized cells requires a CDP-DAG precursor (8). Supplementation of the permeabilized cells with CTP in the standard reaction described in Fig. 2 relies upon the action of CDP-DAG synthase as well as endogenous CDP-DAG to drive the PtdSer synthase reaction. With prolonged incubation, it is likely that the generation of CDP-DAG by the corresponding synthase will deplete the requisite phosphatidic acid pool. To circumvent the reliance upon phosphatidic acid and endogenous CDP-DAG pools, we examined the efficacy of preincubating the washed permeabilized cells with CDP-DAG. The results of these experiments are shown in Fig. 4. In these studies, we compared the formation of PtdEtn in permeabilized cells, prepared as described in Fig. 2, to that found from washed donors supplemented with MgCTP or CDP-DAG, in either the presence or absence of LSS. Under all conditions of reconstitution, the formation of PtdEtn is strongly dependent upon the addition of LSS. Preincubation of washed donors with CDP-DAG consistently gives better synthesis of PtdEtn. We believe this is due, in part, to the more proximal nature of the reactions to PtdSer formation compared with CTP supplementation. Most significantly, we also find that preincubation of the washed donors with \([^{3}H]\)serine and CDP-DAG...
followed by centrifugation to remove precursors allows us to effectively pulse-label the PtdSer pool in this compartment, thereby permitting the independent examination of events occurring between PtdSer synthesis and PtdEtn formation.

**LSS Contains both Acceptor Membranes and Cytosol**—We next sought to characterize the components present in LSS and the manner in which they contribute to the transport-dependent conversion of nascent PtdSer to PtdEtn. Initially, we examined the LSS for the presence of Psd2p and marker enzymes for the ER (Pssp), Golgi (Kex2p), and vacuoles (H^+ ATPase). As shown in Fig. 5, Pssp was retained by the washed permeabilized cells and absent from the LSS. The essentially complete absence of Pssp from the LSS makes it an ideal acceptor compartment because it is devoid of precursor synthesis. The LSS contains ~40% of the recoverable Golgi, ~30% of the recoverable vacuolar marker, and ~51% of the recoverable Psd2p. The total recovery of all the organelle markers between the processed permeabilized cell and its autologous LSS is between 44 and 70%, implying that significant amounts of the relevant enzymes in permeabilized cells may be either inactivated or lost during the preparation of the washed donor compartment and the LSS. Although significant amounts of the Golgi, the vacuole, and Psd2p remained with the processed donors, nascent PtdSer was not efficiently transported to these loci (Fig. 4). One likely explanation of this finding is that the factors involved in the release of the acceptor membranes may also be mechanistically relevant to the lipid transfer process.

**Acceptor Membranes Alone Are Sufficient to Reconstitute PtdSer Transport**—For further characterization, the LSS was subjected to ultracentrifugation at 400,000 × g for 1 h to generate HSM and HSS. The distribution of the ER, Golgi, and vacuolar enzymes and Psd2p in the HSM relative to other fractions is shown in Fig. 5. Almost all of the Golgi-Kex2p, vacuolar H^+ ATPase, and Psd2p present in the LSS was recovered in the HSM. Essentially none of these enzymes were recovered in HSS. This further partitioning of LSS allowed us to test the roles of the acceptor membranes and the cytosol individually.

The specific questions that we wanted to address were whether 1) cytosol contains factors that could stimulate PtdSer transport between the donor membranes and the acceptor membranes that remained in the processed permeabilized cells, 2) isolated acceptor alone is sufficient to reconstitute PtdSer transport, and 3) both cytosol and acceptor membranes are required for PtdSer transport. When HSM alone, as the acceptor membrane, was reconstituted with processed donors, it resulted in a concentration-dependent increase of PtdSer transport activity as shown in Fig. 6. Due to the volume limitation of our assay, we were only able to test two concentrations of HSS. HSS alone weakly stimulated PtdSer transport and was significantly less effective than HSM alone. When both HSS and HSM were present to reconstitute PtdSer transport, HSS modestly enhanced PtdSer transport at low HSM concentration. However, the HSS effect was diminished when higher concentrations of HSM were present to support PtdSer transport (Fig. 6). This result demonstrates that, although there may be some cytosolic factors that can weakly stimulate PtdSer transport from the ER to the locus of Psd2p, the addition of acceptor membranes (i.e., HSM) alone, is sufficient to reconstitute the transport process in the permeabilized cell assay system.

**Transport of Nascent PtdSer to Psd2p Is Nucleotide-independent**—Achleitner et al. (8) showed that PtdSer transport to
yeast mitochondria is independent of ATP. However, PtdSer translocation in permeabilized mammalian cells has been demonstrated to be ATP-dependent (15–17). In addition, ATP as well as GTP are also involved in the trafficking and docking of vesicles effecting interorganelle protein transport (18–20). We examined whether the translocation of PtdSer from the ER to the Psd2p locus in yeast is nucleotide-dependent. The addition of Mg$^{2+}$ salts of ATP, ADP, CTP, or UTP (at 1 mM) or of GTP, GDP, or GTP$\gamma$S (at 0.2 mM) failed to significantly alter PtdSer transport reconstituted between the donor membranes and acceptors. These findings demonstrate that our reconstituted system does not require nucleotides as cofactors for PtdSer transport.

EDTA Inhibits PtdSer Transport to the Psd2p-containing Acceptor Membranes—Previously, only a trace amount of PtdSer transport to the Psd2p locus could be detected in the permeabilized psd1D cells (8). As described under “Experimental Procedures,” one alteration we made to optimize the measurement of PtdSer translocation to Psd2p was to omit EDTA from the incubations. Our success raised the question of whether PtdSer movement to the Golgi/vacuole Psd2p is sensitive to EDTA. Indeed, the addition of EDTA not only inhibited nascent PtdSer synthesis but also arrested further conversion of PtdSer into PtdEtn in unmanipulated permeabilized cells (Fig. 7). This inhibitory effect is not due to the inhibition of catalysis of Psd2p, since the enzyme does not require divalent cations for activity and is unaffected by the presence of EDTA (4). One possible explanation is that ongoing PtdSer synthesis is required for continued PtdSer transport, and the chelation of the Pssp cofactor, Mn$^{2+}$ ions, by EDTA thus arrests both activities. However, this explanation is unlikely, since reconstituted PtdSer transport, using processed donors with a prelabeled PtdSer pool (Figs. 4 and 6), bypasses continuous PtdSer synthesis. The elimination of the two possibilities described above implies that EDTA may inhibit the PtdSer transport process directly. To test this hypothesis, we measured reconstituted PtdSer transport activity with prelabeled donors either in the absence or the presence of Mn$^{2+}$ ions. The use of prelabeled donors allows us to exclude any effects on Pssp activity and restricts the conclusions to events occurring after PtdSer formation. As shown in Fig. 8, the transport activity was dependent upon Mn$^{2+}$ ions. In addition, the transport activity was abolished when EDTA was added together with Mn$^{2+}$. These results demonstrate a specific requirement for a divalent cation in the reconstituted transport reaction.

PtdSer Transport Requires Manganese Ions—Although Mn$^{2+}$ was the only divalent cation added to our reconstituted washed donor/acceptor assay system described in Fig. 8, it does not elucidate whether this is a general or specific requirement for cations as cofactors for PtdSer transport. Therefore, we
HSM from cells containing amounts of Psd2p activity comparable with As shown in Fig. 9, HSM from permeabilized PtdSer into PtdEtn in both intact and permeabilized cells (7). This demonstrates that the permeabilized cell system recapitulates processes found with intact cells.

Because our assay utilizes the product of the Psd2p activity is reported as the -fold increase in PtdSer transport relative to prelabeled donors. Results shown are means ± S.E. of 3-6 individual experiments.

tested a broad range of divalent cations for their ability to stimulate PtdSer transport. The additional divalent cations examined included Mg2+, Cu2+, Ni2+, Zn2+, and Ca2+. With the exception of Ca2+, all divalent cations were tested at 1 mM. Because 1 mM Ca2+ caused extensive degradation of the labeled PtdSer pool, 50 µM Ca2+ was tested. The degradation of PtdSer by the addition of calcium may be due to the activation of a calcium-dependent phospholipase (21, 22). Among all of the ions examined, only Mn2+ ions supported PtdSer transport activity. These findings demonstrate that Mn2+ plays a unique role as a cofactor in reconstituted PtdSer transport.

The Acceptor Membrane Pool Requires Elements Other than Psd2p—Because our assay utilizes the product of the Psd2p reaction as our transport signal, one could argue that the apparent dose-dependent transport activity of HSM is simply due to the increasing amount of Psd2p activity added to the assay system and does not truly reflect a transport process. To investigate whether such an argument is valid, we compared HSM isolated from permeabilized psd1Δ and psd1Δ/pstB2Δ cells. We have previously reported that the PSTB2 gene encodes a protein involved in the transport-dependent metabolism of PtdSer (7). Strains harboring psd1Δ/pstB2Δ mutations have normal Psd2p activity but are defective in converting PtdSer into PtdEtn in both intact and permeabilized cells (7). As shown in Fig. 9, HSM from permeabilized psd1Δ/pstB2Δ cells containing amounts of Psd2p activity comparable with HSM from psd1Δ strains failed to reconstitute PtdSer transport. Most importantly, the addition of excess Psd2p from psd1Δ/pstB2Δ strains is completely ineffective at overcoming any of the transport defect. This result indicates that the transport activity of HSM does not solely depend on the Psd2p activity but clearly involves other factors that have been specifically implicated in lipid transport by in vivo biochemical and genetic experiments. This latter finding emphatically demonstrates that the permeabilized cell system recapitulates processes found with intact cells.

The Permeabilized Cell System Localizes the pstB2 Defect to the Acceptor Membrane Compartment—The experiments described above do not permit localization of the pstB2 defect.

However, the ability to segregate the components of the permeabilized cell system affords the opportunity to critically test whether the pstB2 defect resides in the donor or the acceptor compartment. In these experiments, donors and acceptors were prepared from cells with either wild type or null PSTB2 alleles. The wild type donors were tested for their ability to transport nascent [3H]PtdSer to either wild type or mutant acceptors. Conversely, pstB2Δ donors were also tested for their ability to transfer [3H]PtdSer to either wild type or mutant acceptors. As shown in Fig. 10, the wild type donors could transfer PtdSer to wild type acceptors but not pstB2Δ acceptors. When pstB2Δ donors were used as a source of [3H]PtdSer, the lipid was readily transferred to wild type acceptors but not mutant acceptors. These results clearly demonstrate that the pstB2Δ-harboring strains produce competent donor membranes but incompetent acceptor membranes.

**DISCUSSION**

The heterogeneity of the lipid composition of different organelle membranes has been known as a general principle of cell biology for decades (23). However, the molecular mechanisms and gene products responsible for generating this membrane diversity remain poorly defined. We have focused on the lipid aspect of membrane biogenesis by examining aminoglycosidophospholipid synthesis in Saccharomyces cerevisiae. We have conducted both biochemical and genetic approaches to this problem in an effort to identify new genes and their products and mechanisms of actions. In this report, we describe the successful reconstitution of PtdSer transport between the ER or closely related membranes and Psd2p.

The permeabilized cell system that we developed shows time-dependent synthesis of PtdSer and PtdEtn. The level of nascent [3H]PtdSer formed plateaus after 40 min of incubation presumably due to the depletion of phosphatidic acid and CDP-DAG pools. The formation of [3H]PtdEtn proceeds after a lag of 20–30 min. This lag period is likely to comprise some feature of...
PtdSer movement between elements of the ER and the Golgi apparatus. Recent studies have demonstrated that most of the nascent [3H]PtdEtn is found in the Golgi and a novel light membrane fraction (7). Although 70% of the Ptd2p activity colocalizes with the vacuole compartment (7), we do not see the appearance of [3H]PtdEtn in this organelle in the permeabilized cell system (7). These findings may mean that the permeabilization procedure destroys the machinery for PtdSer transport to the vacuole. Alternatively, the results may indicate that there is interorganelle cooperation between the Golgi and the vacuole with respect to the pool of Ptd2p available for use in transport-dependent lipid metabolism. The formation of [3H]PtdEtn provides a relatively strong biochemical signal for PtdSer transport. This signal was probably missed in earlier studies with permeabilized cells as a consequence of inadequate Mn²⁺ in the reaction. We estimate that the rate of PtdEtn formation is ~20% of that occurring in intact cells.

Low speed centrifugation and washing of permeabilized cells resolves a sedimentable donor compartment capable of PtdSer synthesis but only weakly competent for decarboxylation. The LSS fraction restores the transport dependent metabolism of PtdSer to Ptd2p to the washed donors. In general, reconstitution of washed donors with LSS restores PtdEtn formation to levels between 50 and 100% of those found for unprocessed permeabilized cells. The ability to physically isolate the donor compartment permitted optimization of the conditions for synthesis of the PtdSer precursor. Preincubation of the donors with [3H]serine and CDP-DAG provided a convenient and efficient method for radiolabeling the PtdSer pool in the donors. Most importantly, this approach allowed for both temporal and physical segregation of PtdSer synthesis from its transport dependent decarboxylation. As a consequence of this refinement of the permeabilized cell system, we are more accurately able to assess requirements for events occurring after PtdSer synthesis. Subfractionation of the LSS into HSS and HSM components provides additional important details about the minimal requirements for reconstitution of PtdSer transport. These experiments provide clear evidence for the existence of two pools of Ptd2p. One pool of Ptd2p is retained within the permeabilized cell and functions relatively inefficiently in the decarboxylation of nascent PtdSer. In contrast, a pool of Ptd2p released from the cells is more effective at the decarboxylation of PtdSer. We currently understand few of the details that characterize these two compartments containing Ptd2p. However, we propose that elements of the system that are permissive for the release of Ptd2p may be important components affecting PtdSer transfer between organelle domains. The current studies clearly demonstrate that the HSM derived from LSS is fully competent to import PtdSer and decarboxylate it. Varying HSM shows that the rate of PtdEtn formation is dependent upon the amount of acceptor added.

The reconstituted transport system composed of either permeabilized cells or washed donor/acceptor membranes displays marked sensitivity to chelation of Mn²⁺ with EDTA. Upon the addition of EDTA, the formation of PtdEtn is immediately arrested. The requirement for Mn²⁺ appears specific, since other common divalent cations fail to substitute for its function. The Mn²⁺ requirement appears to be an uncommon aspect of lipid transport that may distinguish the process from interorganelle protein transport.

Comparison of reconstituted transport using HSM derived from psd1Δ strains and psd1Δ/pstB2 strains provides compelling evidence that the PtdSer transport to the locus of Ptd2p is specific. In vivo studies have provided clear evidence that pstB2 mutants are profoundly defective in decarboxylation of nascent PtdSer despite the presence of wild type levels of Ptd2p. This same defect is reproduced with permeabilized psd1Δ/pstB2 cells. The reconstituted donor/acceptor system reveals that even in the presence of excess Ptd2p in HSM, there is no significant decarboxylation of nascent PtdSer by cells harboring a pstB2 mutation. This latter result effectively rules out the occurrence of nonspecific fusion and artificial decarboxylation in the permeabilized cell system.

The reconstituted transport system also allows for assignment of the subcellular site of action of PstB2p. Previous studies have demonstrated that PstB2p is an amphitropic protein present in both the cytosol and multiple membranes (7). The experiments described in this report demonstrate that donor membranes prepared from pstB2Δ mutant cells are fully competent to transfer nascent PtdSer to wild type acceptor membranes for the formation of PtdEtn. In contrast, wild type donor membranes cannot effectively transfer nascent PtdSer to Ptd2p present in the pstB2Δ acceptor membranes. Thus, the pstB2Δ defect clearly resides in the acceptor membranes. These findings are consistent with PstB2p regulating the final steps of substrate access to the PtdSer decarboxylase.

In summary, we have defined a powerful new biochemical system for examining PtdSer transport events between the ER and the Golgi apparatus. This system should prove amenable to further dissection and reconstitution. We anticipate that this system will also facilitate the identification of the sites of function of new gene products in the lipid transport process and provide a biochemical assay system for elucidating their mechanism of action.

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