The C2 Domain of Phosphatidylserine Decarboxylase 2 Is Not Required for Catalysis but Is Essential for *in Vivo* Function*

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Phosphatidylserine decarboxylase 2 (Psd2p) is currently being used to study lipid trafficking processes in intact and permeabilized yeast cells. The Psd2p contains a C2 homology domain and a putative Golgi retention/localization (GR) domain. C2 domains play important functions in membrane binding and docking reactions involving phospholipids and proteins. We constructed a C2 domain deletion variant (C2Δ) and a GR deletion variant (GRA) of Psd2p and examined their effects on *in vivo* function and catalysis. Immunoblotting confirmed that the predicted immature and mature forms of Psd2(C2Δ)p, Psd2(GRA)p, and wild type Psd2p were produced *in vivo* and that the proteins localized normally. Enzymology revealed that the Psd2(C2Δ)p and Psd2(GRA)p were catalytically active and could readily be expressed at levels 10-fold higher than endogenous Psd2p. Both Psd2p and Psd2(GRA)p expression complemented the growth defect of *psd1Δpsd2Δ* strains and resulted in normal aminoglycerophospholipid metabolism. In contrast, the Psd2(C2Δ)p failed to complement *psd1Δpsd2Δ* strains, and [*3H]*serine labeling revealed a severe defect in the formation of PtdEtn in both intact and permeabilized cells, indicative of disruption of lipid trafficking. These findings identify an essential, non-catalytic function of the C2 domain of Psd2p and raise the possibility that it plays a direct role in membrane docking and/or PtdSer transport to the enzyme.

The aminoglycerophospholipids, phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho) are important components of membranes in both prokaryotes and eukaryotes that collectively can comprise more than 70% of the phospholipids in many cells (1, 2). In both prokaryotes and eukaryotes that collectively can comprise more than 70% of the phospholipids in many cells (1, 2). In prokaryotes, major pathways elucidated by Kennedy and co-workers (5, 6). The yeast *Saccharomyces cerevisiae* can make the majority of its aminoglycerophospholipids from either a PtdSer, an Etn, or a Cho precursor (7). An intriguing aspect of aminoglycerophospholipid metabolism in eukaryotes is the segregation of the synthetic enzymes among different organelles (8). In yeast Psd1p is synthesized in the endoplasmic reticulum (ER) or a related membrane associated with the mitochondria (MAM) (9, 10). This nascent PtdSer must be transported to the mitochondria or the Golgi/vacuole to be converted to PtdEtn (11, 12). The mitochondrial PtdSer decarboxylase is denoted Psd1p, and the Golgi/vacuole enzyme is denoted Psd2p.

Both Psd1p and Psd2p have been extremely useful as tools for monitoring the interorganelle traffic of nascent PtdSer (13). Recent work from this laboratory has used Psd2p to characterize some of the parameters required for interorganelle transfer of PtdSer and to identify additional genes/proteins required for the process. One protein that is required for movement of nascent PtdSer to Psd2p is a phosphatidylinositol (PtdIns)-binding protein we named PstBp (phosphatidylserine transport) whose encoding gene (*PSTB2*) was previously identified in a pleiotropic drug resistance screen and is named *PDR17* (14). This same gene has also been identified as a homolog of the yeast PtdIns/PtdCho transfer protein, Sec14p, and is alternatively named *SFIH4* (15). A second protein that has been identified in genetic screens for strains exhibiting aminoglycerophospholipid transport defects is a PtdIns 4-kinase, Stt4p (16).

Our recent work has led us to consider the possibility that a protein complex may form on the donor and/or acceptor membranes involved in interorganelle lipid transfer processes. Some support for this general idea comes from experiments demonstrating that PstBp must be present on acceptor membranes for PtdSer to be transferred to Psd2p (17).

In this report we addressed the role of structural domains of Psd2p in the localization, catalytic activity, and *in vivo* function of the protein. The Psd2p has a domain with homology to the trans-Golgi protein Kex2p, which has been implicated in the localization of this protein (18). However, the function of this domain in Psd2p is not clear. The Psd2p also contains a C2 domain (12). The archetypical C2 domains were first identified in protein kinase C isozymes and constitute Ca<sup>2+</sup>- and phospholipid binding motifs (19). Numerous other proteins involved in signal transduction and membrane trafficking possess C2 domains (20–22). In some cases C2 domains bind lipids or membranes in Ca<sup>2+</sup>-independent reactions. C2 domains are also known to promote both protein–protein and protein–phosphoinositide interactions. The role of the C2 domain in Psd2p function has not previously been examined and has remained unclear.
The purpose of this study was to perform structure/function analyses of Psd2p using biochemistry and genetics. In this report we sought to do the following: 1) define the processing of Psd2p required for its maturation, 2) determine the role of the GR domain in processing, catalysis, targeting, and in vivo function of Psd2p, and 3) determine the role of the C2 domain in the processing, catalysis, targeting, and in vivo function of Psd2p. Our findings indicate that the GR domain is dispensable for all functions examined. In contrast, the C2 domain is not required for processing, catalysis, or targeting but is essential for in vivo function.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Simple salts, buffers, and amino acids were purchased from Sigma and Fisher. Yeast media components including yeast extract, nutrient base without amino acids, and peptone were from Difco. Zymo-lase-100T was from US Biological. The radiochemicals [3H]serine and [1-14C]serine were from Amersham Biosciences and American Radiolabeled Chemicals. The 1-acyl-2-[6-[(7-nitro-2-](3H)serine were from Amersham Biosciences and American Radiolabeled Chemicals. The 1-acyl-2-[6-[(7-nitro-2-phospholipid standards were obtained from Avanti Polar Lipids.

**Construction of Yeast Culture Conditions**—Anti-ura3 his3 lys2 leu2 psd1::TRP1 psd2::HIS3 and PTY41 (MATa trpl ura3 his3 lys2 leu2 psd1::TRP1 psd2::HIS3) and PTY41 (MATa trpl ura3 his3 lys2 leu2 psd1::TRP1 psd2::HIS3) strains were previously established in our laboratory. The synthetic complete growth medium for yeast containing 5 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was used for Western blot analysis to confirm the protein expression level. Electrophoresis was performed with Tris-glycine gels for 3 h at 30 °C with shaking. The OD_{600} of the culture media was continuously monitored for 72 h to assess the growth in liquid medium.

**Preparation of Permeabilized Yeast Cells**—Permeabilized cells were prepared in a lysis buffer (20 mM HEPES, 0.15 M potassium acetate, 2 mM magnesium acetate, 0.5 mM EDTA, 0.4 M sorbitol, pH 6.8) following the method described by Achleitner et al. (31). In brief, yeast spheroplasts were prepared by treating cells with Dithiothreitol under alkaline conditions followed by zymolase treatment. The resulting spheroplasts were regenerated for 20 min at 30 °C in the presence of 0.75% yeast extract, 1% peptone, and 0.7 M sorbitol. After washing and resuspending 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. The cells were thawed slowly on ice for 1 h prior to use in transport reactions.

**Determination of Ethanolamine Auxotrophy**—The parental PTY44 strain and its derivatives harboringYPE352-PSD2, YEp352-PSD2/GR, and YEp352-PSD2/C2A plasmids were cultured on plates supplemented with Etn (SCE-U) or without Etn (SC-U). Each of the strains (1–30 ml of culture) was streaked on one of four areas of the plate and incubated at 30 °C for 2.5 days. These strains were also inoculated into liquid SCE-U or SC-U at an initial A_{600} of 0.02 and grown at 30 °C with shaking. The OD_{600} of the culture media was continuously monitored for 72 h to assess the growth in liquid medium.

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**PDNS Transport Assays**—[3H]Serine was incorporated into the permeabilized cells by incubating the cells in a 100-μl reaction mixture containing 5 mM HEPES, 22 mM Tris-HCl, 0.6 mM MnCl<sub>2</sub>, 0.6 mM magnesium acetate, 60 mM potassium acetate, 0.1 mM KC1, 8.5 mM β-choleraomin, 0.2 mM EDTA, 0.4 mM MgCTP, 0.28 M sorbitol, 0.27 mM mannitol, 50 μM t-serine, 30 μCi/ml [3H]serine, pH 8.0, by the same method described previously (17). The final protein concentration of the permeabilized cells in the transport reaction was 0.9 mg/ml. The assay mixture was incubated at 30 °C for 100 min. The incorporation of [3H]serine was terminated by the addition of 1 ml of methanol, 0.5 mM CHCl<sub>3</sub>, and 0.3 ml of 0.2 M KC1 to generate a monophase. The extraction was completed by the further addition of 0.45 ml of 0.2 M KC1 and 0.5 ml CHCl<sub>3</sub> to create two phases that were separated by centrifugation. After aspiration of the upper phase, the lower chloroform phase was further washed with 1.9 ml of methanol/phosphate-buffered saline (1:9, v/v) twice. The resulting chloroform phase was dried under nitrogen gas, resuspended in 30 μl of chloroform/methanol (9:1, v/v), and analyzed by thin layer chromatography as described above. The ratio of PtdEtN/PtdSer + PtdEtn was used as a transport index for PtdSer.

**Fractionation of the Permeabilized Cells**—The permeabilized cells were prepared as described above. The fractionation procedure used methods previously described (14). The reaction mixture was immediately homogenized gently on ice with 15 strokes using the B pestle of a Dounce homogenizer. The cell debris was removed from the homogenate by centrifugation at 1,500 × g for 5 min. The supernatant was further centrifuged at 30,000 × g for 15 min to remove dense membranes. The supernatant was removed from the pellet, overlaid on...
a two-step gradient consisting of 1 ml of 80% sorbitol and 1 ml of 25% sorbitol, and centrifuged for 2 h at 280,000 × g in a Beckman SW41 rotor. All sorbitol densities are given as the w/v in 10 mM triethanolamine, pH 7.2. The interface between the 25 and 80% sorbitol layers was collected and adjusted to 43% sorbitol using refractometry and layered on a gradient prepared in 40, 43, 60, 70, and 80% increments. The 40% step was 3.6 ml, and the 80% step was 1 ml. All other gradient steps were 2.3 ml each. Membranes were separated by centrifuging the gradient in an SW41 rotor at 280,000 × g for 40 h. Fractions were collected by aspiration from the top of the gradient and stored at −20 °C.

RESULTS

Wild Type Psd2p and the Structural Variants Psd2(C2Δp and Psd2(ΔGR)p Are Processed to Yield Mature Enzyme—The yeast PtdSer decarboxylases contain several distinct structural domains as outlined in Fig. 1. The yeast Psd1p is organized in a similar manner to the mammalian enzyme that has been shown to be sequentially processed to yield mitochondrial outer- and inner-membrane transit intermediates and finally mature α- and β-subunits (32). The generation of α- and β-subunits in the eukaryotic enzymes is predicted to proceed via endoproteolytic cleavage of α- and β-subunits by a serinolysis mechanism that generates the pyruvyl proteinase group essential for catalysis. Predictions for eukaryotic processing are derived from definitive work performed with the E. coli enzyme that demonstrates cleavage between Glu and Ser residues residing in an LGST motif (33). The yeast Psd2p contains a GGST motif that is proposed to be the cleavage site for generating a putative Golgi localization/retention sequence and a GGST motif. The GGST motif is predicted to be the cleavage site for α- and β-subunits and generation of the active site.

As shown in Fig. 2, the bands corresponding to the mature α-subunit required for catalysis. From these data we conclude that the two structural mutants we have constructed are processed to the mature form of the protein in a process that is essentially indistinguishable from that occurring with the wild type protein. A minor immunoreactive band of slightly higher molecular size is also observed in the region of the α-subunit. The exact identity of this component is not known, but based upon the reaction sequence of pyruvyl enzymes (13), it is probably the α-subunit containing PtdSer or PtdEttn in Schiff’s base linkage to the active site.

![Figure 1](Image)

**Fig. 1.** Structures of yeast Psd1p and Psd2p. Two phosphatidyl-serine decarboxylases, Psd1p and Psd2p, are found in the yeast *S. cerevisiae*. The Psd1p contains an LGST amino acid motif that identifies the proteolysis site necessary for formation of α- and β-subunits and generation of the active site. Psd1p also has mitochondrial targeting and inner membrane (IM) sorting sequences. In contrast the Psd2p contains a C2 homology domain, a Golgi localization/retention sequence, and a GGST motif. The GGST motif is predicted to be the cleavage site for α- and β-subunits and generation of the active site.

![Figure 2](Image)

**Fig. 2.** Psd2p, Psd2(C2Δp, and Psd2(ΔGR)p Are Catalytically Active in Vitro—To determine whether the Psd2(ΔGR)p and Psd2(C2Δp) expressed in yeast cells are catalytically active, we performed enzyme assays using cell-free extracts from the different strains. Total cell-free extracts derived from strains lacking PtdSer decarboxylases (psd1A psd2Δ) or expressing only Psd2p from the chromosome (psd1Δ psd2Δ) or expressing only high copy Psd2p, Psd2(ΔGR)p or Psd2(C2Δp) were compared. As shown in Fig. 3, the psd1Δ psd2Δ strain had no decarboxylase activity, and the strain expressing Psd2p from the chromosome had the low but measurable activity previously described (12). High level expression of Psd2p gave 15 times the enzyme activity of the control strain (PTY41) expressing only the chromosomal copy. The Psd2(ΔGR)p and Psd2(C2Δp) showed catalytic activity 12 and 10 times higher than that obtained for the chromosomal copy of the wild type gene. These data indicate that the Psd2(ΔGR)p and the Psd2(C2Δp) are catalytically active and can be expressed at very high levels within the yeast. These data clearly demonstrate that neither the GR domain nor the C2 domain are required to produce a catalytically functional enzyme.

**Psd2(ΔGR)p and Psd2(C2Δp) Colocalize with Psd2p on Sorbitol Density Gradients**—Subcellular fractionation of the permeabilized and homogenized cells was performed by density gradient centrifugation to define the localization of the Psd2(ΔGR)p, Psd2(C2Δp), and Psd2p expressed in yeast cells. The yeast strains were permeabilized and fractionated, and the localization of mature and immature Psd2p in fractionated samples was revealed by Western blot with anti-HA antibody. As shown in Fig. 4, the bands corresponding to the mature α-subunit of Psd2p, Psd2(ΔGR)p, and Psd2(C2Δp) were observed from fractions 13 to 18, with the peak of the expression at fraction 16. Within the limits of resolution of this centrifugation method, we conclude that the subcellular localization of the Psd2p variants is identical to the wild type protein. The bands corresponding to immature forms of the decarboxylases were also centered around fraction 16, demonstrating that processing of Psd2p to the mature form is not required before localization. In addition, an 80-kDa band was detected in the peak fractions for both mature and immature Psd2p and
Psd2(C2Δ)p, but not in those of Psd2(GRA)p. It is likely that the 80-kDa protein is a proteolytic product of the immature Psd2p and Psd2(C2Δ)p. This 80-kDa band could be a spurious proteolytic product, but it may also be generated from a specific cleavage within the GR domain. The identical distribution of Psd2p, Psd2(GRA)p, and Psd2(C2Δ)p strongly suggests that the deletion of either the GR domain or the C2 domain does not affect either the maturation or the localization of Psd2p. Thus, the GR and C2 domains do not participate in targeting the decarboxylase to its subcellular location.

Psd2(C2Δ)p Cannot Fulfill the in Vivo Function of Psd2p—We next examined whether the Psd2(C2Δ)p and Psd2(GRA)p could replace the wild type enzyme in vivo. In these studies strains lacking chromosomal copies of PtdSer decarboxylases (psd1Δpsd2Δ) were transformed with high copy plasmids (YEp352) either lacking an insert (Vect.) or encoding the C2 deletion variant (C2Δ) or encoding the Golgi localization/retention (GRA) variant or encoding the wild type enzyme (PSD2). The strains were initially grown to mid-log phase in the presence of 2 mM Etn. An inoculum of 1 x 10^6 cells was streaked on SC minus uracil plates with (+Etn) or without (−Etn) 2 mM Etn. Growth was scored after 3 days at 30 °C, as shown in panel A. Growth was also examined in liquid cultures for 3 days as shown in panel B.

Under the conditions used, cell growth is absolutely dependent upon the Psd2p- and -C2Δp-expressing strains grew equally well on both solid and liquid media. When Etn is included in the medium, cells can bypass the need for Psd2p function by synthesizing PtdEtn by the Kennedy pathway (5, 6, 30). High copy plasmids were chosen for this experiment to bias the results to reveal complementation of the PtdSer decarboxylase null mutant even with weakly active forms of the Psd2p variants lacking either the GR or C2 domains. In Fig. 5, A and B results are presented from experiments conducted with solid and liquid media, respectively. In the presence of Etn (permissive conditions) all strains harboring a plasmid bearing a Psd2p variant were capable of growing on either solid or liquid medium. In the absence of Etn (non-permissive conditions) the Psd2p- and Psd2(GRA)p-expressing strains grew equally well on both solid
and liquid medium, and the growth was comparable to that found under permissive conditions. In contrast to the other constructs, the Psd2(C2Δ)p variant was unable to support growth on either solid or liquid medium in the absence of Etn. These data clearly demonstrate that the C2 domain of Psd2p plays an essential role in the in vivo function of the enzyme.

Yeast Cells Harboring Psd2(C2Δ)p Fail to Metabolize PtdSer to PtdEtn—The inability of the Psd2(C2Δ)p to support cell growth, despite high level catalytic activity and normal subcellular localization, suggested a defect in the transport-dependent decarboxylation of PtdSer. We next examined the effects of the Psd2p structural variants on aminophospholipid metabolism in vivo. In these experiments, shown in Fig. 6, cells were labeled for 1–2 generations with [3H]serine in the absence of Etn. The majority of [3H]serine is directly incorporated into the head groups of aminophospholipids via the de novo synthesis of PtdSer. In psd1Δ Δpsd2Δ strains lacking the decarboxylases, PtdSer is synthesized. However, there is little PtdSer turnover, and there is a marked defect in the formation of PtdEtn. The residual PtdEtn formed in psd1Δ Δpsd2Δ strains occurs via sphingolipid labeling and turnover (26). In strains containing a chromosomal copy of the PSD2 gene, PtdEtn labeling is four S.E. for three experiments performed in duplicate.

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PtdSer generated within the permeabilized cells is not transported to the locus of Psd2(C2Δ)p.

DISCUSSION

Yeast genetics is an extremely powerful tool for dissecting complex biochemical and biological processes. We are using a combination of yeast genetics, molecular biology, and biochemistry to address fundamental mechanistic questions about interorganelle aminoglycerophospholipid traffic. We have devised and applied genetic screens that selectively use psd1Δ and psd2Δ mutants to examine aminoglycerophospholipid transport to and from specific organelles (30). In this report we focused upon the structure of the Psd2p enzyme and the potential role that specific subdomains of the protein play in the lipid transport process.

Examination of the deduced primary sequence of Psd2p reveals three potentially important sequences that could be involved in catalysis, protein traffic, and lipid recognition. In the initial work describing cloning and sequencing of the Psd2p gene (12), amino acid residues (1041–1044) comprising a GGST motif were proposed as candidates comparable to the LGST motif in E. coli (33). Cleavage of the G-S peptide linkage by serinolysis generates the large β-subunit and the smaller α-subunit containing a pyruvyl residue that is part of the active site. In this report we now provide evidence, shown in Fig. 2, that Psd2p is initially made as a high molecular weight precursor of 130 kDa that is processed to yield an 11-kDa small subunit. This result is entirely consistent with previous structural predictions about the enzyme. The immature form of Psd2p appears relatively long lived, because the protein was detected by immunoblot analysis. The unprocessed form of Psd2p is also located on gradients where the mature α-subunit is found, suggesting that maturation is not a prerequisite for targeting Psd2p to Golgi/vacuolar membranes but can occur after arrival of the precursor.

A putative GR sequence (EFDDNEDEREDSFQSK) in Psd2p with homology to a Kex2p sequence (EFDDIITDSEYDSTLDNK) implicated in its membrane targeting (18) was also identified in the original description of the PSD2 gene (12). Because subcellular fractionation indicated colocalization of a subpopulation of Psd2p molecules with the Kex2p, this GR motif was tested for function. Deletion of the GR motif in Psd2p did not alter the catalysis, processing, or subcellular localization of the enzyme. These findings clearly demonstrate that the putative GR motif of Psd2p is not required for any identifiable function.

The Psd2p also contains a C2 domain most closely related to the C2 domains of synaptotagmin III and PKCα (12). Although C2 domains are most often associated with Ca2+-mediated binding between proteins, or proteins and lipids (20, 21), there is no requirement for Ca2+ in Psd2p catalysis. We designed experiments to test the function of the C2 domain in Psd2p by deletion analysis. A gene encoding Psd2(C2Δ)p was expressed in psd1Δ psd2Δ cells, and the function of the mutant protein was examined. When the Psd2(C2Δ)p is expressed on high copy plasmids, a catalytically active protein results. By expressing Psd2(C2Δ)p on a high copy plasmid the resultant yeast strains produce ~10 times the amount of enzyme activity that is sufficient for the replication of cells under non-permissive growth conditions. These findings clearly demonstrate that the C2 domain is not essential for catalysis. Close inspection of the immunoblot data in Fig. 3 suggests that the processing of Psd2p to the mature form may be modestly retarded compared with the wild type protein, but the C2 domain is clearly not essential for the proteolytic cleavage of the enzyme to its mature form. Subcellular fractionation of cells expressing Psd2(C2Δ)p reveals that distribution of the mutant enzyme parallels that of its wild type counterpart. From this data we conclude that the C2 domain also does not participate in directing Psd2p to its correct subcellular location.

The in vivo function of Psd2(C2Δ)p was tested in a genetic background (psd1Δpsd2Δ) where cell growth requires either Psd2p activity or Etn supplementation (30). In the absence of Etn, the Psd2(C2Δ)p failed to support cell growth even though the catalytic activity present was 10-fold higher than what is available for cell growth, and the subcellular location of the enzyme was normal. This striking effect on growth indicates that the C2 domain plays a crucial role in regulating a non-catalytic aspect of Psd2p function. Most significantly, experimentation reveals that permeabilized cells produce a pool of Psd2p that cannot be decarboxylated by Psd2p(23). However, in this same reconstituted transport system, introduction of NBD-Ptd[1-3H]serine results in significant decarboxylation of the substrate by Psd2p(23). Thus, the defect appears to be the access of the substrate to the active site. We are currently testing the idea that the C2 domain may function in docking the membranes containing Psd2p with membranes that are the proximal donors of Psd2p. The C2 domain or other elements of Psd2p may participate in the transfer of Psd2p to the membrane in which the decarboxylase resides. This proposed docking and transfer process appears to involve another recently described gene product, PstB2p, that is required on the acceptor membrane for the transport-dependent decarboxylation of Psd2p (17). We believe that the Psd2p decarboxylates PtdSer within its own bilayer (catalysis in “cis”) rather than that present in an apposed bilayer (catalysis in “trans”), because the enzyme can use Psd2p added in detergent solutions or NBD-PtdSer that readily partitions into membranes, but not liposomal Psd2p. In addition, structural predictions place the active site at the N terminus of the α-subunit in a hydrophobic patch located in the cytoplasmic leaflet of the lipid bilayer (psot.nibb.ac.jp/formz.html). The simplest model based upon structural considerations favors catalysis in cis, but rigorous testing of this model is clearly needed. Ligands recognized by C2 domains include PtdSer, PtdIns, and polyphosphoinositides (27–29). All of the aforementioned ligands have been implicated by other criteria as important elements of aminophospholipid traffic along the phosphatidylycerine B pathway (30). Psd2p is the substrate for Psd2p and as described above appears to require interaction with the C2 domain prior to catalysis. PtdIns is known to bind PstB2p, which must be present on the acceptor membrane for Psd2p to be decarboxylated. PtdIns-4-P has been implicated in the lipid transport to Psd2p because mutant strains with defects in the PtdIns-4-kinase (such as Stt4p) are defective in converting nascent PtdSer to PtdEtn. Collectively these data suggest that multiple protein-lipid interactions and probably protein-protein interactions occur between the donor and acceptor membranes to facilitate the interorganelle movement of Psd2p.

In summary, our data demonstrate that the C2 domain of Psd2p is dispensable for catalysis but essential for enzyme function in intact and permeabilized cells. These findings suggest that the C2 domain participates in interorganelle Psd2p transport, most likely in cooperation with other membrane-associated proteins.

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