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An Assembly of Proteins and Lipid Domains Regulates Transport of Phosphatidylserine to Phosphatidylserine Decarboxylase 2 in *Saccharomyces cerevisiae*[*]

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Background: The machinery for interorganelle phosphatidylserine (PtdSer) transport is poorly defined at the molecular level.
Results: Molecular interaction studies identify specific protein-protein and protein-lipid interactions in PtdSer transport.
Conclusion: A protein and lipid interaction network defines key participants in PS transport to the locus of PtdSer-decarboxylase 2.
Significance: This study identifies important molecular participants involved in non-vesicular phospholipid traffic.

Saccharomyces cerevisiae **uses multiple biosynthetic pathways for the synthesis of phosphatidylethanolamine. One route involves the synthesis of phosphatidylserine (PtdSer) in the endoplasmic reticulum (ER), the transport of this lipid to endosomes, and decarboxylation by PtdSer decarboxylase 2 (Psd2p) to produce phosphatidylethanolamine. Several proteins and protein motifs are known to be required for PtdSer transport to occur, namely the Sec14p homolog PstB2p/Pdr17p; a PtdIns 4-kinase, Stt4p; and a C2 domain of Psd2p. The focus of this work is on defining the protein-protein and protein-lipid interactions of these components. PstB2p interacts with a protein encoded by the uncharacterized gene YPL272C, which we name Pbi1p (PstB2p-interacting 1). PstB2p, Psd2, and Pbi1p were shown to be lipid-binding proteins specific for phosphatidic acid. Pbi1p also interacts with the ER-localized Scs2p, a binding determinant for several peripheral ER proteins. A complex between Psd2p and PstB2p was also detected, and this interaction was facilitated by a cryptic C2 domain at the extreme N terminus of Psd2p (C2-1) as well the previously characterized C2 domain of Psd2p (C2-2). The predicted N-terminal helical region of PstB2p was necessary and sufficient for promoting the interaction with both Psd2p and Pbi1p. Taken together, these results support a model for PtdSer transport involving the docking of a PtdSer donor membrane with an acceptor via specific protein-protein and protein-lipid interactions. Specifically, our model predicts that this process involves an acceptor membrane complex containing the C2 domains of Psd2p, PstB2p, and Pbi1p that ligate to Scs2p and phosphatidic acid present in the donor membrane, forming a zone of apposition that facilitates PtdSer transfer.**

Membrane biogenesis involves two distinct but intimately connected and coordinated processes: polar lipid biosynthesis and lipid trafficking from the site of synthesis to the site of organelle membrane assembly. The major pathways of glycerophospholipid synthesis in eukaryotes have been elucidated, and the genes encoding almost all of the respective biosynthetic enzymes have been identified in the model yeast *Saccharomyces cerevisiae* (1). This is in contrast to our knowledge regarding the various means of interorganelle lipid trafficking, for which mechanistic information is incomplete. Defining the mechanisms of lipid trafficking between organelles has historically been a difficult problem, but in the past decade, the development of genetic approaches in yeast and cultured mammalian cells has led to rapid advancement in our knowledge regarding certain lipid transport events (2–6).

Specifically, our laboratory has taken advantage of the spatial organization of the enzymes of the *de novo* pathway of phosphatidylethanolamine (PtdEtn)[3] biosynthesis in yeast. In this scheme, phosphatidylserine (PtdSer) is synthesized in the endoplasmic reticulum (ER) and transported to the sites of the PtdSer decarboxylases; Psd1p is localized in mitochondria (7), and Psd2p was originally thought to be associated with membranes consistent with Golgi and/or vacuolar compartments (8, 9). Improved localization data provided by Gulshan *et al.* (10) now shows that the Psd2p enzyme functions in endosomes and regulates the PtdEtn content of the vacuolar membrane in an indirect fashion. PSD enzymes convert PtdSer to PtdEtn, and in the absence of exogenous ethanolamine (Etn) (8) or lyso-PtdEtn (11), production of PtdEtn by at least one of these enzymes constitutes an essential function in yeast. Mitochondrial PSD activity is essential for viability in mice regardless of

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[3] The abbreviations used are: PtdEtn, phosphatidylethanolamine; Etn, ethanolamine; PtdCho, phosphatidylcholine; mbSUS, mating-based split-ubiquitin two-hybrid system; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PSD, phosphatidylserine decarboxylase; NTRD, N-terminal regulatory domain; ER, endoplasmic reticulum; Cer, ceramide; FFAT, two phenylalanines in an acidic tract.

compensation by other PtdEtn biosynthetic pathways, highlighting the importance of this pathway for mitochondrial function in animal cells (12, 13).

Deletion of the gene encoding the yeast mitochondrial Psd1p enzyme results in all PtdSer biosynthetic flux being forced through the trafficking pathway that leads to the Psd2p enzyme (14). In strains lacking Psd1p, mutations affecting the enzymatic or trafficking function of Psd2p or in other components of the lipid trafficking pathways leading to it result in Etn auxotrophy. This scheme prompted genetic screens for the isolation of mutants defective in the ER to endosome PtdSer trafficking pathway, which define the class of PtdSer transport B (PstB) pathway genes. Among the proteins and protein motifs implicated in PtdSer trafficking by this genetic approach are the Sec14p homolog PstB2/Pdr17 (15), the PtdIns 4-kinase Stt4p (16), and a C2 domain present on Psd2p (17). Further studies (18) have suggested the involvement of specialized membrane regions with specific lipid compositions as being critical for competency as a PtdSer donor membrane.

The present work expands on the previous genetic and biochemical studies and provides new information regarding 1) the identification of a previously unrecognized C2 domain at the N terminus of Psd2p, 2) the identification and genetic dissection of protein-protein interactions between known components of the PstB pathway, 3) identification of novel interaction partners of these proteins, 4) investigation of the protein-lipid interactions of these proteins, and 5) the functional implications of these protein-protein and protein-lipid interactions for the transport of PtdSer. We use a combination of standard (split-transcription factor) and membrane based (split-ubiquitin) two-hybrid methods as well as co-purification assays with recombinant proteins to demonstrate a number of novel interactions between proteins involved in PtdSer trafficking to the site of Psd2p. We also make use of liposome binding assays to determine the lipid-binding specificity of these proteins and permeabilized cell-based transport assays to demonstrate the functional implications of these protein-protein interactions. The emerging picture of this lipid trafficking event is one in which a complex of proteins on the acceptor membrane interacts with a complex of proteins and lipid motifs on the donor membrane. This set of interactions leads to the close apposition of the two membranes, which facilitates lipid transfer.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise noted, all chemicals, solvents, and amino acids for media were purchased from Sigma or Fisher. Yeast extract, peptone, and yeast nitrogen base were from Difco. Silica-60 TLC plates were from EM Sciences. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Permeabilized Cell Assays—Strains RYY52 (SEY6210 Δ*psd1*) and WWY66 (SEY6210 Δ*psd1* Δ*pstb2*) were permeabilized, and transport assays were performed as described by Achleitner *et al.* (19), as modified by Wu and Voelker (18). PstB2 and Pbi1 proteins were expressed in recombinant form and purified as described below and added to transport assays as described in individual experiments.

Yeast Two-hybrid Analyses—PCR was used to amplify the open reading frame of *PSTB2* with flanking EcoRI and PstI restriction sites. The resulting DNA fragment was digested by EcoRI and PstI restriction enzymes and then inserted between the EcoRI and PstI sites of the bait vector, pOBD-2, to generate an in-frame fusion at the 3′-end of the GAL4-DNA binding domain coding sequence. The resulting construct ("bait") was expressed in the PJ69–4**a** yeast strain. The transformed PJ69–4**a** strain was sent to the Yeast Resource Center at the University of Washington (Seattle, WA) for screening against an array of the entire set of yeast open reading frames fused to the GAL4 transcription activation domain ("preys"). The bait strain was mated to the prey library in strain PJ49-4α, and the bait and prey proteins were co-expressed in the diploid. Strains bearing potential interacting partners were identified as histidine prototrophs due to the expression of the reporter gene, *HIS3*, under GAL1 promoter control. Prey plasmids containing the interacting clones were retrieved from histidine prototrophic diploids and sequenced to identify the open reading frame in the positive prey plasmids.

Genes to be assayed in the mating-based split-ubiquitin two-hybrid system (mbSUS) were amplified from either SEY6210 wild-type genomic DNA or plasmids bearing wild-type or mutant versions of these genes as described in the corresponding experiment, using strategies outlined in the publication describing the initial use of the mbSUS (20). These products were cloned into the appropriate mbSUS vectors by *in vivo* recombination after transformation into the strains THY.AP4 and THY.AP5. 4–5 primary transformant colonies of the appropriate bait and prey strains were pooled and grown in liquid medium. Matings were carried out using these cultures by mixing appropriate **a** and α strains in microtiter dishes and spotting the mating reactions onto SC medium lacking uracil, tryptophan, and leucine (SC −ura −trp −leu) with a 48-prong pinning tool. These plates were incubated overnight to select for the diploid strains containing both bait and prey constructs. The diploids were then replica-printed to Y-min medium (6.7 g/liter yeast nitrogen base, 20 g/liter glucose, 2% (w/v) agar) to select for fully prototrophic strains (*i.e.* exhibiting expression of marker genes (*ADE2* and *HIS3*) that would indicate a putative interacting pair of genes). These strains were also replica-printed to fresh SC −ura −trp −leu plates and incubated 2–3 days and then treated to detect LacZ reporter activity, as described previously, using X-Gal as the colorigenic reagent (20).

In some cases, X-gal was replaced with S-Gal (Sigma), which we found to be more sensitive and reproducible than the previously described protocol. In this case, the diploid strains growing on plates as described above were overlaid with an S-Gal/agarose mixture consisting of 0.3 mg/ml S-Gal, 0.5 mg/ml iron(III)ammonium citrate, 0.5% (w/v) low melting point agarose, 60 mM KPO_4, pH 7.0, 0.2% (w/v) SDS, 0.75 mg/ml KCl, and 0.25 mg/ml $MgSO_4 \cdot 7H_2O$. The solution was heated to near boiling on a hot plate to dissolve the agarose and cooled to 42 °C before pouring over the plates containing patches of yeast. LacZ activity is indicated by a black precipitate, and plates were incubated for 15 min to 2 h, depending on the signal strength, and imaged on a flat bed scanner.

Production of Recombinant Proteins and Co-purification Assays—Recombinant proteins were expressed and purified from either *Sf*-9 insect cells or *Escherichia coli*. PstB2p was expressed from vector pVL1392 in *Sf*-9 cells as a C-terminal fusion to a V5-His_6 tag. Recombinant PstB2-V5-His_6 was purified by Ni^{2+}-nitrilotriacetic acid-agarose affinity chromatography (Qiagen) according to the manufacturer's instructions. For permeabilized cell assays in which PstB2p was added, imidazole was removed by dialysis against a buffer consisting of 10 mM Tris-HCl, 1 mM EDTA, and 10% glycerol. Proteins were also produced by cloning ORFs as C-terminal His-tagged or S-tagged fusion proteins in pET series vectors and expression in *E. coli* strain Rosetta (DE3) (Novagen). PstB2p was expressed as a C-terminal S-tag (S-RNase peptide) fusion protein from vector pET-$28a^+$. Pbi1p and residues 1–620 of Psd2p were expressed as C-terminal His_6-tagged fusion proteins from vector pET$45b^+$. Cultures were grown in LB medium with 33 mg/liter chloramphenicol and either 100 mg/liter ampicillin (pET-$45b^+$) or 50 mg/liter kanamycin (pET-$28a^+$). Cultures were grown to an A_{600} of 0.5–0.7 and induced overnight with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at 22 °C. Pbi1p was purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography.

These proteins were used in the protein-protein and protein-lipid interaction assays described below, and detection was by Western blotting with appropriate antibodies, as indicated in individual experiments. The PstB2-S-tag fusion was detected with S-protein-HRP conjugate (Novagen), the His-tagged proteins were identified with α-His_6 antibody (Clontech), and V5 epitope was identified with α-V5 antibody (Invitrogen). Interactions of PstB2-S-tag protein with Pbi1-His_6 or the N-terminal 620 residues constituting the regulatory domain of Psd2 (Psd2-N-terminal regulatory domain (NTRD)-His_6) were assessed using *in vitro* interaction (pull-down) assays with S-protein-agarose beads (Invitrogen) according to the manufacturer's instructions.

Construction and Expression of Psd2p Variants in Yeast—A modification of the QuikChange approach (Stratagene) was used to introduce deletions in the *PSD2* gene, giving rise to proteins lacking the first (C2-1Δ, residues 19–86), second (C2-2Δ, residues 496–583), or both (C2-1,2ΔΔ) C2 domains. A portion (residues 95–166) of the Ser-Thr-rich acidic region that separates the C2 domains (Fig. 2*B*) was also deleted (S-TrΔ) for use as a control construct. Given that this region is not highly conserved in the Psd2 proteins of closely related yeast and other fungi, we reasoned that this deletion would have no effect on the lipid transport or catalytic functions of Psd2p. The plasmids YEp-352-Psd2 and YEp-352-Psd2-C2Δ (17) were used as templates.

Growth and PSD Assays of Mutant Psd2p Variants—Plasmids bearing wild-type and mutant Psd2 constructs in the 2μ yeast vector YEp352, as described above, were transformed into the *psd1*Δ *psd2*Δ yeast mutant PTY44 (8) using LiOAc-mediated transformation (21), and transformants were selected on SC −ura plates containing 5 mM Etn. Etn auxotrophy was determined by inoculating SC −ura liquid medium to A_{600} = 0.1, and these cultures were pregrown for 5 h. These cultures were then diluted 100-fold into SC −ura either lacking or containing 5 mM Etn and grown for 16 h. Growth of the cultures was determined by measuring A_{600}.

Transport-independent Psd2p catalytic activity was measured in crude membrane fractions prepared by bead beating, as described previously (22). This assay used NBD-Ptd-[1′-^{14}C]Ser, which spontaneously partitions into all membranes independent of facilitated transport, as described previously (7). This assay serves as a measure of total enzymatic activity without the need for facilitated transport of PtdSer transport to the Psd2p enzyme active site.

Lipid Binding Analyses—Large multilamellar liposomes of defined lipid composition were used for lipid-protein interaction assays as detailed in individual experiments. Lipids (Avanti) in chloroform were mixed in the appropriate ratio and dried under a stream of N_2 gas. A small amount of methanol was added and again taken to dryness under N_2 in order to remove traces of chloroform. The resulting lipid film was hydrated on ice for 1 h in a buffer containing 50 mM Tris-HCl, pH 7.0, and 200 mM NaCl at a total lipid concentration of 1 mg/ml. Following hydration, the preparation was vortexed vigorously, and the reconstituted liposomes were recovered by centrifugation at 15,000 × *g* for 15 min. The supernatant was discarded, and the pellet of liposomes was suspended in an equal volume of the same buffer. Recombinant proteins (as described above) were incubated with the liposomes for 30 min at 30 °C. For PstB2p binding, 50 ng of V5-His_6-tagged purified protein from *Sf*-9 cells was used. The Pbi1p binding contained 50 ng of purified His_6-tagged protein from *E. coli*. Psd2-NTRD binding was assayed with crude *E. coli* protein extract from an expressing strain, containing 10 μg of *E. coli* 100,000 × *g* supernatant protein, which gave a Western immunoblot signal equivalent to ~50 ng of Pbi1p. After incubation, the liposomes were collected by centrifugation at 15,000 × *g* for 15 min, and the supernatant was discarded. The liposomes were washed twice in 100 μl of the same buffer, and bound proteins were solubilized in SDS-PAGE sample buffer and analyzed by Western blotting with an appropriate antibody.

RESULTS

*Recombinant PstB2p Reconstitutes PtdSer Trafficking in Permeabilized pstb2*Δ *Cells*—In the reconstituted permeabilized cell system described previously (18), the competence of PtdSer acceptor membranes, but not donor membranes, was dependent on the presence of PstB2p. We asked whether recombinant epitope-tagged PstB2p produced by baculovirus-mediated expression in *Sf*-9 insect cells could support the transport of nascent PtdSer from donor to acceptor membranes in permeabilized *pstb2*Δ cells. As demonstrated in Fig. 1, *PSTB2* wild-type permeabilized cells (*gray bars*) convert ~7–8% of this newly synthesized PtdSer into PtdEtn, regardless of whether recombinant PstB2p is added to the reaction mixture. In comparison, *pstb2*Δ cells (*black bars*) convert only ~1.5% of newly synthesized PtdSer into PtdEtn in the absence of recombinant PstB2p. However, the addition of increasing amounts of recombinant purified PstB2p resulted in a dose-dependent recovery of transport activity. Recovery of transport activity was saturated at a PstB2 concentration of ~125 ng/μl and resulted in a doubling of the PtdSer transport rate of the mutant cells. This amounts to a partial recovery of activity, perhaps indicating that additional components or a specific energy-dependent assembly step are

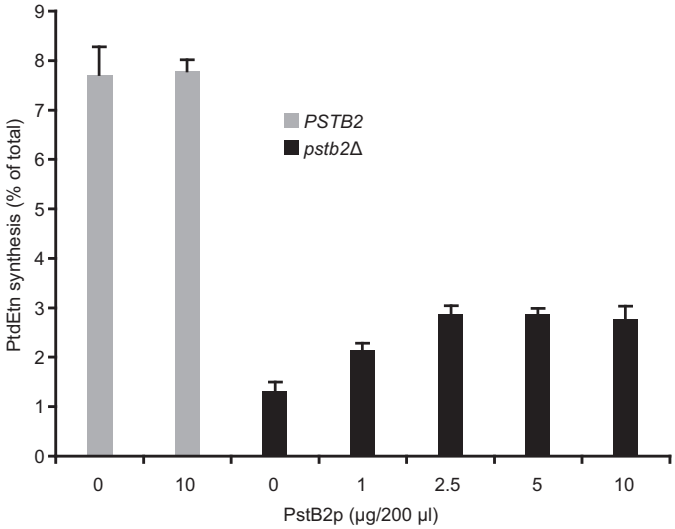

FIGURE 1. **Recombinant purified PstB2p partially rescues the PtdSer transport defect of the *psd1*Δ *pstb2*Δ mutant.** Permeabilized cells of strain RYY52 (*psd1*Δ, *gray bars*) or WWY66 (*psd1*Δ *pstb2*Δ, *black bars*) were incubated with 0–10 μg of purified recombinant PstB2p, and the synthesis and coupled transport/decarboxylation of newly formed PtdSer was measured radiochemically. *Error bars*, S.E. of three independent analyses conducted in duplicate.

necessary for the full function of the lipid transport complex in this permeabilized cell system. A newly identified PstB2-interacting protein (Pbi1p, described below) was added in purified recombinant form to the permeabilized cell transport assay to test its function as a potential regulator of transport complex assembly; however, its inclusion at up to 250 ng/μl neither enhanced nor inhibited the transport rate in this assay system.

PstB2p Physically Interacts with Psd2p—In permeabilized cell assays of ER to endosome transport of PtdSer, PstB2p is required at the acceptor membrane, and as shown above, exogenous recombinant PstB2p partially rescues PtdSer transport in permeabilized *pstb2*Δ cells in a dose-dependent and saturable manner. One hypothesis to explain these findings is that PstB2p binds a specific protein or lipid on the acceptor membrane, thus rendering it competent to facilitate the transport reaction. One potential binding partner is the Psd2p enzyme itself, and we tested the hypothesis that PstB2p interacts with Psd2p using mbSUS, which was designed specifically for testing interactions between membrane proteins (20). In this system, the bait protein, which must be cytoplasmically oriented and membrane-bound or otherwise physically excluded from the nucleus, is expressed as a C-terminal fusion to a construct consisting of the C-terminal half of ubiquitin, in frame with a synthetic Protein A-LexA-vp16 transcription factor (referred to as Cub-PLV). The prey protein is expressed as either an N- or C-terminal fusion to a mutant version of the N-terminal half of ubiquitin (referred to as NubG). If the bait and prey interact, the Cub and NubG fragments reconstitute a pseudomonomer of ubiquitin, which is then recognized by the deubiquitinating enzymes of the yeast cell. The PLV transcription factor is then released from the membrane and imported to the nucleus, where it activates the transcription of the LexA::*ADE2*, LexA::*HIS3*, and LexA::*LacZ* reporter genes. Constitutive expression of the NubG or Nub wild type (NubWT) fragments acts as negative or

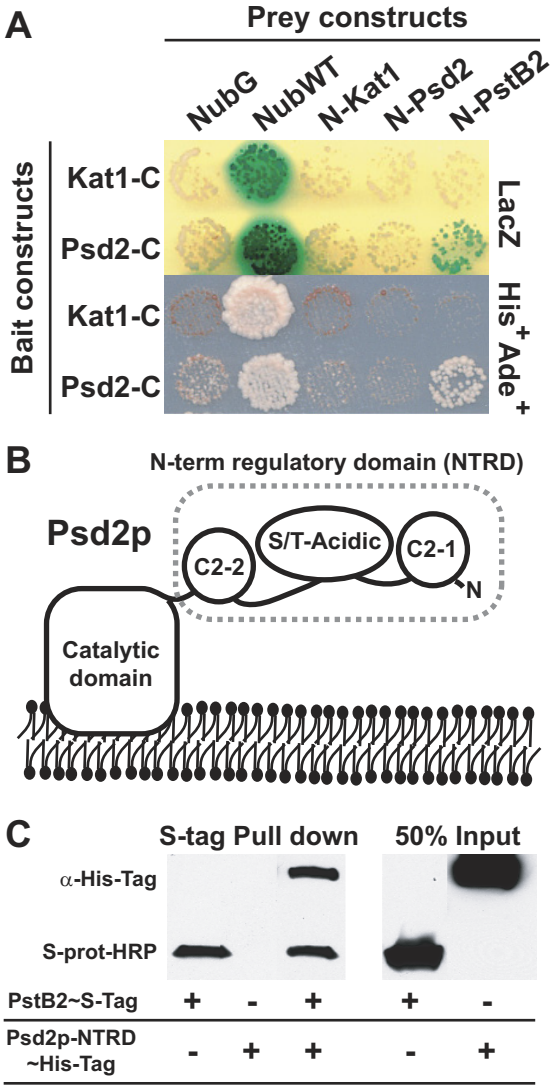

FIGURE 2. **PstB2p and Psd2p form a complex.** *A*, mbSUS was used to test for interactions between Psd2p and PstB2p *in vivo*. *NubG*, *NubWT*, and *Cub* refer to the C-terminal or N-terminal halves of ubiquitin, as described under "Results." Kat1, a K^+ channel from *Arabidopsis*, serves as an irrelevant protein negative control for both bait and prey proteins. NubG and NubWT serve as negative and positive controls for bait protein stability and expression, respectively. *B*, Psd2 is a modular protein and contains two C2 domains separated by a large acidic, hydrophilic tract. This region of the protein, referred to as the NTRD, is not present in mitochondrial or bacterial PSD enzymes and contains domains essential for the transport functions of Psd2p. *C*, protein affinity assays were conducted using the S-tagged PstB2 protein and His-tagged Psd2-NTRD protein. The interaction of Psd2-NTRD with S-protein-agarose beads was dependent on the presence of S-tagged PstB2p.

positive control, respectively, and ensures that the bait construct is both stable in the absence of an interacting NubG-fused partner and expressed at a level that would make an interaction detectable.

In the experiment shown in Fig. 2*A*, Psd2 is the bait, whereas a potassium channel subunit from the plant *Arabidopsis thaliana* (Kat1) serves as an irrelevant bait protein negative control. These were tested for interaction with NubG (negative control), Nub wild type (positive control), NubG-Kat1 (irrelevant prey protein negative control), and the experimental NubG-Psd2 and NubG-PstB2 constructs. Yeast strains bearing the appropriate plasmids were mated and replica-printed on −leu

−trp −ura dropout media to select for diploids bearing both bait and prey plasmids. These were then stained with either X-gal or S-gal, as described under "Experimental Procedures," to assay the expression of the LacZ reporter gene. Concurrently, the diploids were printed to Y-Min plates to assay the expression of the interaction-dependent HIS3 and ADE2 reporter genes. As shown in Fig. 2*A*, Psd2 fails to interact with itself, indicating that it probably functions as a monomer. However, Psd2 and PstB2 show a positive interaction, giving rise to a strong signal from all three reporter genes. This provided an initial piece of evidence that Psd2p and PstB2p form a complex on the acceptor membrane in the PtdSer transport system. Further examination and deconstruction of this interaction through mutagenesis and *in vitro* protein-protein interaction assays are detailed below.

The N-terminal Regulatory Region of Psd2p Is Sufficient for PstB2p Interaction—In order to confirm the interaction between Psd2p and PstB2p, we conducted protein co-purification assays with recombinant forms of these proteins expressed in *E. coli*. Psd2p is a membrane-associated enzyme, and because previous attempts at expression and manipulation of subdomains of this protein in soluble form have failed,[4] we took a different approach to generating a soluble fragment with which to interrogate the protein and lipid interactions of Psd2p. An updated analysis of the Psd2p sequence with the Pfam conserved domain database (23) revealed a second, highly divergent C2 domain at the extreme N terminus, which had not been identified in previous studies by our laboratory (8, 17). This N-terminal C2 domain is also present in other fungal Psd2p homologs. In addition, the presence of an acidic, hydrophilic domain that separates the C2 domains led us to express the N-terminal 620 residues as a C-terminal His_6-tagged fusion in *E. coli*. This fragment is illustrated schematically in Fig. 2*B* and is henceforth referred to as the Psd2-NTRD. Expression of both S-tagged PstB2p and the Psd2-NTRD-His_6 fusion protein in *E. coli* gave stable, soluble species of the expected size, and these proteins were routinely detected by Western blotting with S-protein-HRP conjugate or anti-His_6 primary antibody, respectively.

Fig. 2*C* shows the results of the co-purification experiment that confirms the existence of a Psd2p-PstB2p complex of significant interaction affinity. *E. coli* homogenate containing S-tagged PstB2p was incubated with S-protein-agarose beads, and the resulting PstB2p-coated beads were used to affinity-purify the Psd2-NTRD-His_6 fusion protein from 100,000 × *g* supernatants of *E. coli* homogenates. As detailed in Fig. 2*C*, Psd2-NTRD-His_6 fusion protein was precipitated from the *E. coli* extract by S-protein-agarose beads in a PstB2p-dependent manner. This result indicates that the Psd2-NTRD is sufficient for promoting the interaction with PstB2p and that the catalytic domain is dispensable for this presumably transport-related function. During the course of this work, several other reports (10, 24, 25) have appeared demonstrating the existence of an interaction between Psd2p and PstB2p. Our study thus adds additional independent evidence regarding the protein domains

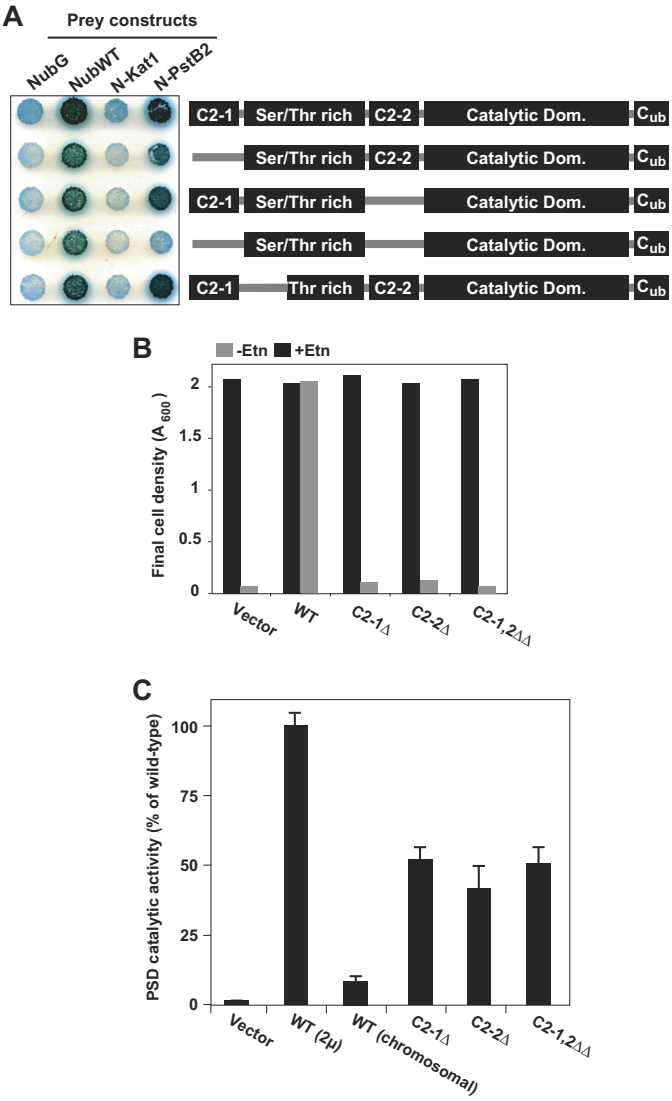

FIGURE 3. **The C2 domains of the Psd2-NTRD are required for interaction with PstB2 and *in vivo* transport functions but not for catalytic activity.** *A*, the mbSUS was used to assay the interactions between PstB2p and deletion mutants of the N-terminal regulatory domain of Psd2p (Psd2-NTRD). Schematic diagrams to the *right* of the interaction results show the sequence fragment that was deleted. *B*, growth assays were conducted as described under "Results" and show that the C2–1 domain is required for growth when the function of Psd2p is essential. *C*, transport-independent PSD assays of the Psd2p deletion mutants using NBD-PtdSer as the substrate were conducted according to previously described methods. *Error bars*, S.E. of three independent analyses conducted in duplicate.

involved in facilitating this interaction and further supports the existence and functional importance of this complex.

Both C2 Domains of Psd2p Are Required for in Vivo Function but Not in Vitro Catalytic Activity—In order to further define the regions of the Psd2-NTRD that are involved in the PstB2p interaction, we coupled a deletion-mutagenesis approach to the mbSUS as described above. Fig. 3*A* gives the results of this deletion mutagenesis as well as a schematic of the deletion mutants of Psd2p used as bait. As already shown in Fig. 2*A*, wild-type Psd2p interacts with PstB2p. When the core of the newly identified C2 domain (C2-1, residues 18–86) or the previously characterized C2 domain (C2-2, residues 496–583) (17) were deleted individually, there was a slight decrease in LacZ

[4] H. Kitamura and D. R. Voelker, unpublished data.

reporter signal, but the interaction was still readily detectable. However, when both C2 domains were deleted from Psd2p, the LacZ signal indicating an interaction between Psd2p and PstB2p was barely above the negative control signal. When a portion of the hydrophilic/acidic region (residues 128–166) that separates the C2 domains was deleted, the interaction was comparable with the wild type. This region is highly divergent with regard to length and primary sequence in the Psd2 proteins of other yeast and fungi (data not shown); therefore, it may simply act as a disordered hydrophilic linker separating the highly conserved C2 domains. Taken together, these results indicate that PstB2p interacts with both of the Psd2p C2 domains and that either one is sufficient for interaction with PstB2p, as detected by the mbSUS.

Given that deletion of either one of the Psd2p C2 domains fails to disrupt the Psd2p-PstB2p interaction, as detected by the mbSUS, we asked what the consequences for *in vivo* function might be upon the deletion of these domains when Psd2p transport and catalytic functions are required for growth. For the C2-2 domain, this question has already been resolved by previous studies on the Psd2-C2-2Δ mutant (17, 26). It was found that the C2-2 domain is required for the PtdSer transport reaction in multiple experimental conditions but that it is not necessary for catalysis. Fig. 3, *B* and *C*, shows that this characteristic is shared by the newly discovered C2-1 domain. In Fig. 3*B*, the wild type and C2 deletion mutants of the *PSD2* gene were expressed in a *psd1*Δ *psd2*Δ deletion mutant, and the resulting strains were assessed for Etn prototrophy. In this case, Psd2p encoded by the plasmid must be functional in both transport and catalytic functions in order to support growth in the absence of Etn. The ability to grow in defined liquid medium with or without Etn was tested by pregrowing the cells for 5 h in media lacking Etn and then diluting these precultures 1:100 into the same media with or without Etn. These cultures were grown overnight, and then the A_{600} was recorded after 16–18 h. As shown in Fig. 3*B*, only the plasmid with the wild-type *PSD2* gene supported growth in the absence of Etn. Although the growth results make a strong case that both C2 domains are required individually, this does not provide information on whether the deficiency in *in vivo* function is due to a defect in the lipid transport or catalytic functions of the enzyme. The results presented in Fig. 3*C* demonstrate that the loss of function of the proteins encoded by the C2 deletion constructs was not due to a defect in catalytic activity, as judged by the catalytic competence of the encoded proteins to decarboxylate NBD-PtdSer, a substrate that rapidly partitions into membranes regardless of the activity of the transport complex. Although the C2 domain deletions give ~50% of the wild-type activity, this is still 4–5-fold more specific activity than that in extracts from yeast bearing a single chromosomal copy of the wild-type *PSD2* gene and thus more than enough catalytic activity to satisfy the needs of the cell for PtdEtn synthesis if coupled to a functional transport pathway.

PstB2p Physically Interacts with a Novel Protein Encoded by YPL272C (PBI1)—The PstB2p protein has been characterized genetically and biochemically as being necessary at the acceptor membrane for PtdSer transport from the ER to the site of Psd2p (18, 26) and shown to directly interact with Psd2p in this work.

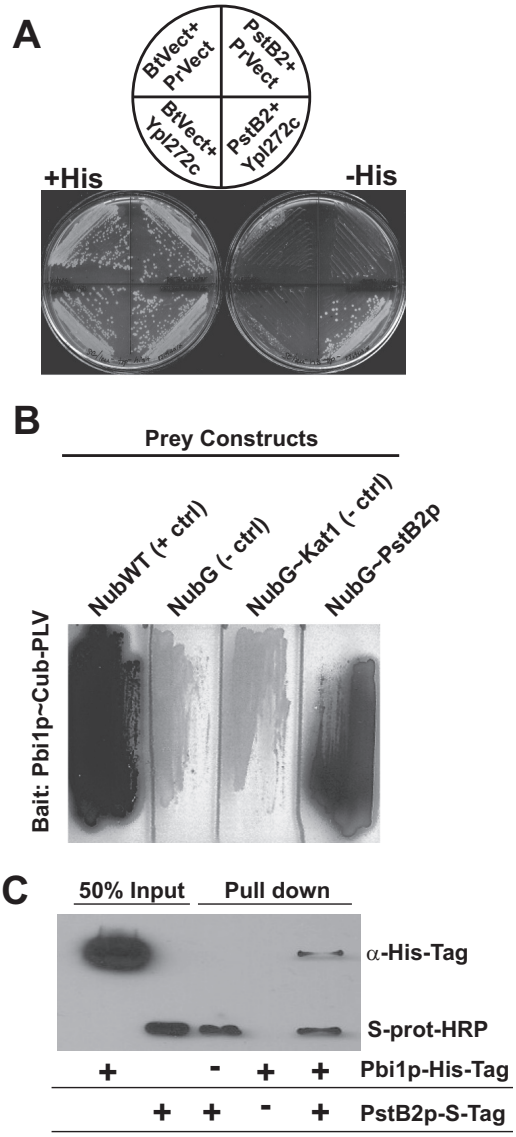

FIGURE 4. **PstB2p and Pbi1p form a complex.** *A*, the split-transcription factor-based yeast two-hybrid screen identified an interaction between PstB2p and an uncharacterized protein encoded by the YPL272C open reading frame. This open reading frame was subsequently named *PBI1*, given that the protein it encodes is a PstB2 interactor. *B*, the interaction between Pbi1p and PstB2p is also demonstrated by the mbSUS two-hybrid assay. *C*, *in vitro* interaction (pull-down) assays using S-protein-agarose beads were used to demonstrate the direct nature of the binding of PstB2p and Pbi1p, using recombinant purified proteins expressed in *E. coli*, as described under "Results."

In order to further define the functions of PstB2p in this lipid trafficking pathway, we searched for interacting proteins through a systematic yeast two-hybrid screen, essentially as described previously (27). The *PSTB2* gene was cloned as a fusion to the Gal4 DNA binding domain in the bait vector pOBD2. The appropriate strain containing this construct was mated to a library of yeast ORFs fused to the Gal4 transcriptional activation domain in vector pOAD, and His^+ diploids indicative of a putative interacting protein pair were selected. The only ORF that gave rise to a reproducible and robust interaction was an uncharacterized gene denoted YPL272C. Because the encoded protein is a PstB2p-interacting protein, we designate this gene as *PBI1*. This putative interaction gave rise to a strong growth phenotype based on the *HIS3* reporter (Fig. 4*A*)

TABLE 1

Pairwise interaction grid among proteins in the directed mbSUS two-hybrid screen

The results of multiple experiments carried out under varying conditions of stringency were compiled and assigned a value of no interaction (−), weak interaction (+), or strong interaction (++). Interactions verified by *in vitro* co-purification assays are denoted with white symbols on a black background.

		Prey								
		Psd2	PstB2	Pbi1	Sec14	Scs2	Scs22	Osh1	Osh2	Osh3
Bait	Psd2	-	++	-	-	-	-	-	-	-
	Pbi1	-	++	-	-	+	-	-	-	-
	Stt4	-	-	-	-	-	-	-	-	-
	Osh1	-	-	-	-	++	-	-	-	-
	Osh2	-	-	-	-	++	++	-	-	-
	Osh3	-	-	-	-	++	+	-	-	-

leading to histidine prototrophy; however, the LacZ reporter gene was extremely weak (data not shown). Given this somewhat ambiguous result, this putative interaction was tested by two independent methods: the split-ubiquitin two-hybrid system and co-purification assays with purified recombinant proteins. The split-ubiquitin result is given in Fig. 4*B* and provides strong evidence corroborating the initial interaction data provided in Fig. 4*A*, with expression of the auxotrophic reporter genes (*ADE2* and *HIS3*) leading to a fully prototrophic diploid strain. Likewise, Fig. 4*C* provides additional evidence of a specific interaction between these proteins. These affinity-interaction assays with purified recombinant Pbi1p and PstB2p show that binding of Pbi1-His_6 fusion protein to S-protein-agarose beads is dependent on the presence of S-tagged PstB2p.

Directed Split-ubiquitin Two-hybrid Screening—As described above, we used a two-hybrid interaction assay designed to detect direct interactions of membrane-bound bait and prey proteins *in vivo*, mbSUS (20). We used the mbSUS to study the interactions between Psd2p and PstB2p and between PstB2p and Pbi1p. Using this system, we performed a directed screen aimed as assaying all of the possible binary interactions between proteins that had been directly or indirectly implicated in this PtdSer transport pathway. These proteins included the essential components of the pathway, Psd2p, PstB2p, and Stt4p, as well as their interacting proteins Pbi1p and Scs2p. Additionally, several oxysterol-binding protein homologs of yeast (Osh1p, Osh2p, and Osh3p) had previously been shown to bind Scs2p, and these proteins were included in the screen as well as Scs22p, a homolog of Scs2p. As shown in Table 1, this screen identified the previously described interactions between the Osh1, -2, and -3 proteins and Scs2 family members (28) and also led to the discovery of a relatively weak yet highly reproducible *in vivo* interaction between Pbi1p and Scs2p.

The Pbi1p-Scs2p interaction was novel, and co-purification assays were conducted with recombinant purified His-tagged Pbi1p and a GST-tagged soluble fragment of Scs2p expressed in *E. coli*, which lacks the C-terminal transmembrane helix. A mutant of this construct that is unable to bind "two phenylalanines in an acidic tract" (FFAT) motifs was also included as a potential negative control. We were unable to show a direct physical interaction between Pbi1p and either wild-type or mutant Scs2p (data not shown). However, given the weak reporter gene activation signal from the mbSUS analysis, the interaction between Scs2p and Pbi1p may be indirect (*i.e.* mediated by another protein). Given that the Osh1, -2, and -3 proteins had been shown to possess FFAT motifs that specify binding to Scs2p, these proteins would be prime candidates for participating in a Scs2p-Pbi1p complex. However, interactions between Pbi1p and the Osh proteins were not detected in this limited screen; therefore, the nature of the interaction between Scs2p and Pbi1p remains unclear.

Stt4p is a PtdIns 4-kinase that has been implicated in Psd2p-directed PtdSer trafficking (16) and has also been linked physically (29) and through a synthetic lethal interaction (30) to Scs2p. Interestingly, a cursory examination of the Stt4p sequence revealed that it contains an FFAT motif, which has been shown to mediate interaction with Scs2p in proteins such as the transcriptional repressor Opi1p and members of the oxysterol-binding protein homolog family of yeast (31). Stt4p was included as a bait protein in the mbSUS screen but failed to interact with any of the prey proteins tested (Table 1), including Scs2p. It might be the case that the Stt4-Cub-PLV fusion protein (bait) is not expressed at a high enough level to allow detection of weakly interacting preys. Alternatively, the large size of Stt4p and the membrane-bound nature of Scs2p may preclude the interaction of the Cub and Nub reporter proteins. Further dissection of the potential interactions of Scs2p and the Stt4p-FFAT motif and their function in PtdSer trafficking is detailed below.

The N-terminal Helical Region of PstB2 Is Necessary and Sufficient for Psd2p and Pbi1p Interaction—Members of the Sec14 family of lipid transfer proteins share high sequence similarity and a very similar structure, consisting of an N-terminal all-helical domain and a C-terminal mixed α/β domain that has been shown to bind specific lipids (32). We asked whether either or both of these domains of PstB2p could individually interact with Psd2p and Pbi1p interaction partners by expressing the N-terminal helical motif or C-terminal lipid binding domains individually as NubG-linked bait proteins. Fig. 5*A* gives a schematic view of the structure of PstB2p, using a model generated by the program SWISS-MODEL (33), in which the primary sequence of PstB2 was threaded onto the structure of Sec14p (34). Fig. 5*B* shows that the N-terminal helical region, in the absence of the lipid binding domain, is sufficient for a weak interaction with both of the PstB2-interacting partners. The lipid binding domain alone is not sufficient for these interactions, showing that the N-terminal helical region is necessary for binding to its protein partners.

Pbi1p, PstB2p, and the Psd2-NTRD Are Phosphatidic Acid-binding Proteins—A previous report (26) demonstrated specific requirements for competence of artificial PtdSer donor membranes in an *in vitro* reconstituted PtdSer transport assay, including the requirement of high concentrations of PtdSer or PtdOH. One hypothesis is that proteins on the donor or acceptor membranes act as PtdSer- or PtdOH-binding proteins, and Fig. 6 gives the results of liposome binding assays aimed at elucidating if Psd2p, PstB2p, or Pbi1p interacts with specific lipids. All three proteins robustly and reproducibly bound to PtdOH-containing liposomes, but not to PtdSer-containing liposomes (not shown). Given the polyanionic nature of PtdOH-containing liposomes, a critical test of specificity of this lipid affinity was to investigate binding of the proteins to liposomes containing other anionic lipids. For this purpose, we used phosphati-

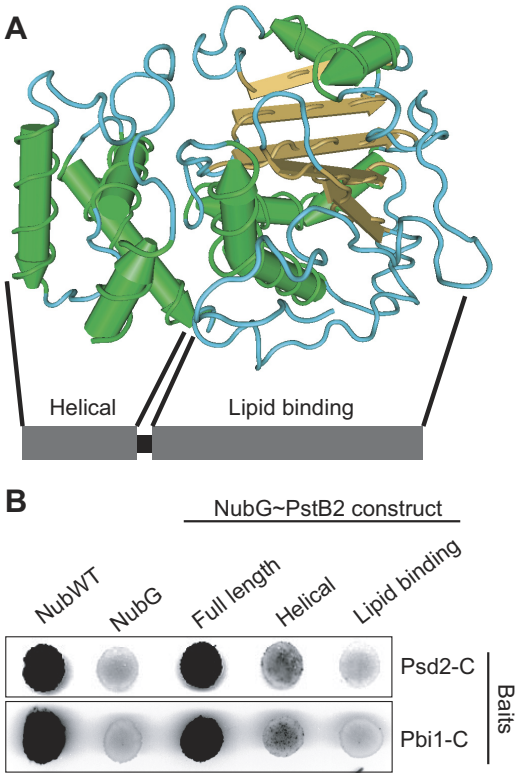

FIGURE 5. **C-terminal helical region determines PstB2 protein-protein interactions.** *A*, a homology model for PstB2 was generated with the program SWISS-MODEL by threading and optimization using the Sec14p structure as a template. *B*, N- and C-terminal mbSUS analysis with Psd2p and Pbi1p as bait proteins shows that the N-terminal helical region is both necessary and partially sufficient to support the interaction between PstB2 and its binding partners.

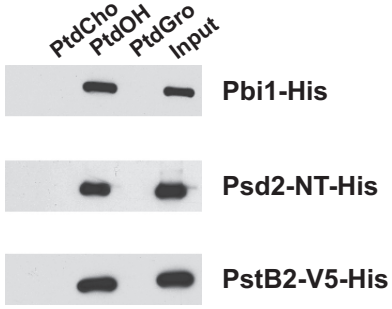

FIGURE 6. **PstB2p, Pbi1p, and the Psd2-NTRD are phosphatidic acid-binding proteins.** Liposome binding assays were carried out as described under "Results." Either pure PtdCho vesicles or vesicles containing 70 mol % of PtdCho and 30 mol % of the indicated anionic lipid were incubated with 50 ng of epitope-tagged protein. Liposomes were then collected by centrifugation and washed twice, and interacting proteins were detected by Western blotting. These proteins robustly and reproducibly bind to liposomes containing >10 mol % of PtdOH, but not other anionic lipids (*e.g.* PtdGro), and the results presented are representative of a numerous similar experiments conducted under these conditions. Input control represents ~50 ng of protein, or 100% of the total input to the binding reaction.

dylglycerol (PtdGro) at the same mole percentage used for PtdOH. The data presented in Fig. 6 represent the results of numerous lipid binding experiments with pure PtdCho liposomes (*PtdCho*); 70% PtdCho, 30% PtdOH-containing liposomes (*PtdOH*); and 70% PtdCho, 30% PtdGro-containing liposomes (*PtdGro*). The input control for each protein is also included and represents the total amount of protein (~50 ng each) added to each binding assay, thus demonstrating that the proteins bound quantitatively to the PtdOH-containing liposomes under the conditions presented. Binding was not inhibited by the presence of EDTA or enhanced by the addition of divalent cations, such as Ca^{2+}, Mg^{2+}, or Mn^{2+} (data not shown), suggesting that the PtdOH-interacting motifs of these proteins are unlikely to require metal cofactors.

The FFAT Motif of Stt4p Is Not Required for Its Function in PtdSer Transport—As described above, physical (29) and synthetic genetic (30) interactions have been detected between Scs2p and Stt4p. Analysis of the primary sequence of Stt4p (Fig. 7*A*) revealed a canonical FFAT motif, which is known to mediate binding of other proteins to Scs2p (28, 31, 35). However, we were unable to confirm the Stt4p-Scs2p interaction via the mbSUS assay (data not shown), possibly due to technical limitations regarding the inability to overexpress Stt4p. To determine if either Scs2p or the FFAT motif of Stt4p is essential for Stt4p function in trafficking of PtdSer to the site of Psd2p, we designed a series of complementation experiments shown in Fig. 7, *B–D*. Fig. 7*B* gives a schematic of the four scenarios we tested, which consisted of perturbations of Scs2 and Stt4 in a *psd1*Δ background. If the combination of mutations in a given strain results in a defect in lipid trafficking, the resultant strain should be an Etn auxotroph. These genetic constructs in the *psd1*Δ background consisted of 1) the wild-type condition, 2) *psd1*Δ *stt4*^{PstB1} (16), 3) *psd1*Δ *scs2*Δ, and 4) the FFAT mutant of Stt4p in the *psd1*Δ *stt4*^{PstB1} background. As described previously (16), the *stt4*^{PstB1} allele was identified as a mutation that results in a lipid transport defect and is complemented by introduction of a wild-type copy of the *STT4* gene on a yeast centromeric plasmid.

As shown in Fig. 7*C*, introduction of the *scs2*Δ (shown) or *scs22*Δ or *scs2*Δ *scs22*Δ alleles (not shown) into the *psd1*Δ background did not inhibit the ability of the resulting strains to grow without Etn supplementation, indicating that these yeast ER tethering proteins are not essential for PtdSer transport to the site of Psd2p. Furthermore, Fig. 7*A* shows the FFAT motif (31) of Stt4p in comparison with the *bona fide* FFAT containing and Scs2p-interacting motifs of other yeast proteins. Site-directed mutagenesis was used to change the "FF" motif to "AA," which would ablate any ability it might have to interact with Scs2p. As shown in Fig. 7*C*, when this site-directed mutant was introduced to the appropriate strain on a CEN plasmid, it was able to rescue the Etn auxotrophy of the *psd1*Δ *stt4*^{PstB1} mutant to the same degree as the corresponding wild-type *STT4* plasmid. These data suggest that, if an interaction between Scs2p and the putative Stt4p-FFAT motif exists, it is not essential for the function of Stt4p in the PtdSer trafficking pathway.

DISCUSSION

The interorganelle transport of phospholipids is an integral step in the biogenesis and homeostasis of membranes in eukaryotic cells. A developing body of evidence in the field of lipid trafficking implicates the involvement of membrane contact sites, which are thought to provide the conduit through which lipids travel between the donor and acceptor membranes (2). The presence of membrane contact sites bridging the ER and various organelles (*e.g.* the mitochondria, Golgi, chloro-

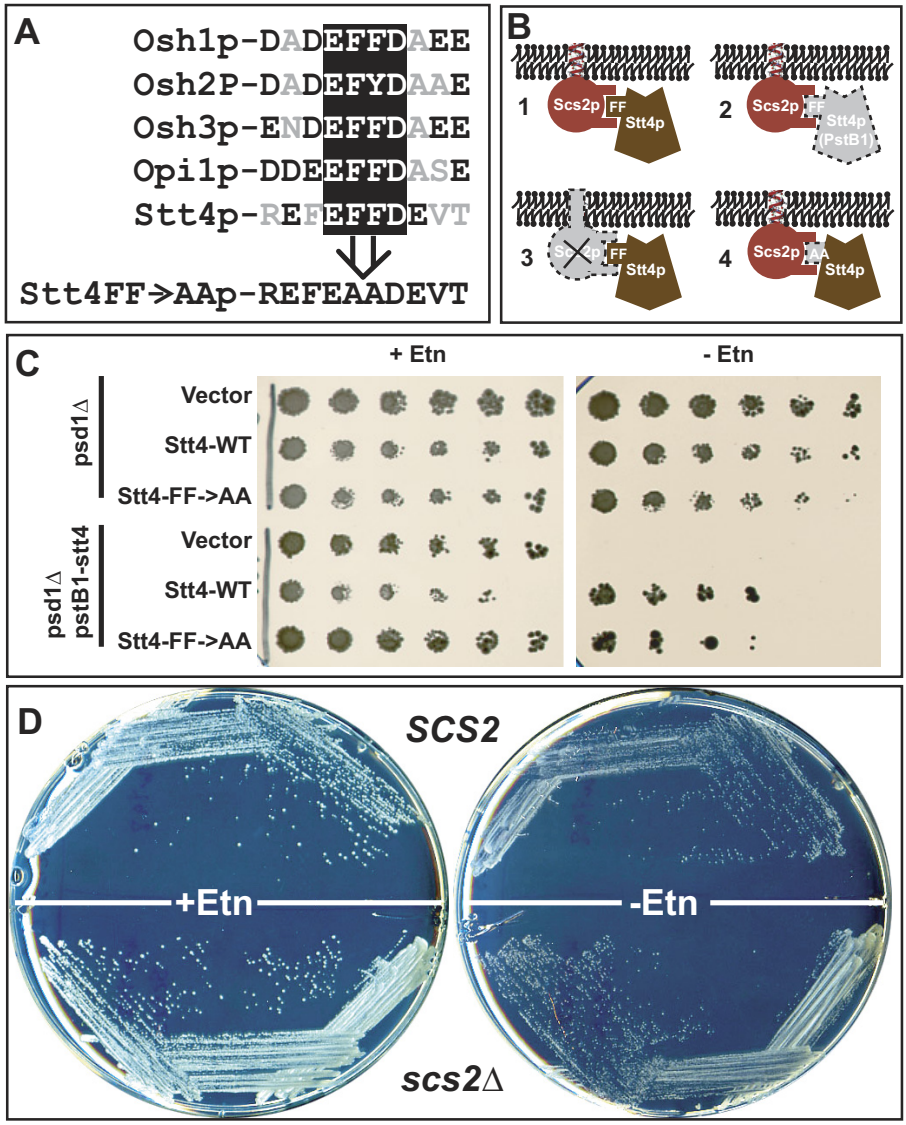

FIGURE 7. **A putative FFAT motif is not necessary for Stt4p function in the PstB trafficking pathway.** *A*, Stt4p contains a FFAT motif similar to those of *bona fide* Scs2p-interacting proteins. The central phenylalanine residues were mutated to alanine to ablate any potential interaction with Scs2p. *B*, schema showing the experimental constructs tested: wild-type Scs2p and Stt4p (*1*); wild-type Scs2p and Stt4p^{PstB1} mutant, defective in PtdSer transport (*2*); *scs2*Δ mutant and wild-type Stt4p (*3*); and wild-type Scs2 and Stt4pFFAT mutant (*4*). *C* and *D*, Etn auxotrophy assays to determine the functionality of the PstB transport event in the experimental constructs outlined in *B*. All strains contain a *psd1*Δ allele, allowing Etn prototrophy or auxotrophy to be used as a determinant of transport-dependent PtdSer transport to the site of Psd2p. *C*, results of dilution-spotting assays (5-fold serial dilutions) on Etn-containing (+*Etn*) or Etn-lacking (−*Etn*) medium to demonstrate the ability of an FFAT mutant of Stt4p to functionally rescue the Etn auxotrophy of the *psd1*Δ *stt4*^{PstB1} strain. *D* demonstrates the Etn prototrophy of the *psd1*Δ *scs2*Δ strain, providing further evidence that a functional interaction between Stt4p and Scs2p is not required for PtdSer transport in this *in vivo* assay system.

plast, plasma membrane, etc.) is well documented, but the elucidation of the molecular determinants of their formation has remained elusive.

A prominent example of recent progress in interorganelle lipid trafficking involves studies on ceramide (Cer) transport between the ER and Golgi (reviewed in Ref. 4), which have revealed some of the protein machinery involved in this process. Through genetic and biochemical approaches, the Cer transport protein (Cert) was identified as an essential component for the trafficking of Cer from its site of synthesis in the ER to the site of its conversion to sphingomyelin in the Golgi (36). Cert consists of multiple domains specifying lipid and protein binding motifs. The START lipid binding domain of this protein was shown to be necessary for extraction of Cer from a donor membrane and deposition in an acceptor membrane. It is thought that specificity with regard to the donor and acceptor membranes is achieved by the FFAT motif and pleckstrin homology domain contained in the N-terminal half of the molecule. These motifs specify binding to VAP-A protein in the ER (donor) and to PtdIns 4-phosphate in the Golgi (acceptor) (37, 38), allowing vectorial transport of Cer between membranes down its concentration gradient.

Given the data presented in this work, a similar scenario is emerging for the transport of PtdSer to the site of Psd2p in endosomes; however, even with data provided by our current study, the exact mechanism of PtdSer transport remains obscure. Previous work from our laboratory has identified a number of proteins and lipid motifs necessary *in vivo* and *in*

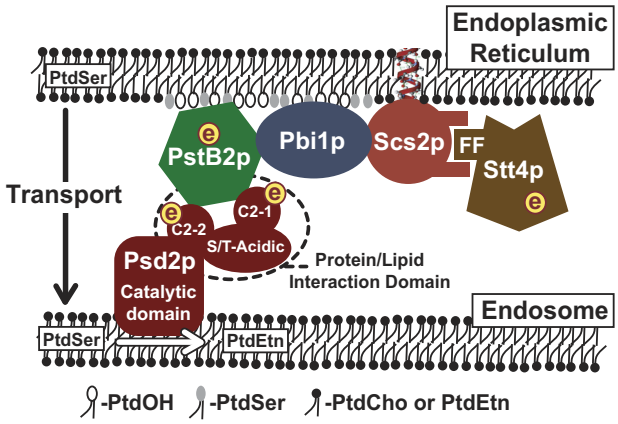

FIGURE 8. **A model of the PstB transport complex.** We propose that the PstB lipid transfer event is facilitated by a complex of proteins on the acceptor membrane consisting minimally of Psd2p and PstB2p. We further hypothesize that this acceptor-side protein complex binds to specific lipids on the donor membrane and that the lipid composition of a discrete patch of ER membrane renders it competent as a donor of PtdSer. Genetically essential components of the transfer complex are defined as those for which removal causes a measurable defect in PtdSer transport and are designated with an *e*.

vitro for the non-vesicular transport of PtdSer from the ER to the site of Psd2p. The minimum requirements for efficient transfer of PtdSer from donor to acceptor membranes *in vitro* include 1) donor liposomes of large diameter and high mole percentage of PtdSer and 2) acceptor microsomes bearing intact Psd2p and PstB2p proteins. Coupling this information with the results of the current studies, we propose an updated model for the PstB trafficking event, as depicted in Fig. 8.

In this scheme, we account for all known determinants that have been implicated in the PstB trafficking pathway, directing PtdSer from its site of synthesis by Cho1p/Pss1p in the ER to the site of Psd2p in endosomal membranes. Genetically essential components of the system (*i.e.* those denoted by an *e*) are those for which disruption in a *psd1*Δ background results in an Etn auxotrophic strain. Other components, such as Pbi1p and Scs2p are also included with the caveat that they are not essential for PtdSer trafficking *per se* but may play a role in the regulation of transport flux, act as chaperones or assembly factors for the wild-type transport complex, or act as binding partners of these proteins in unrelated cell biological contexts. This study serves to expand our understanding of the PstB lipid transport event but does not yet mechanistically describe the physical transport of PtdSer molecules. Studies informed by these newly described protein and lipid interactions using a fully reconstituted *in vitro* lipid transport system should provide additional mechanistic details to aid in our understanding of the functioning of the PtdSer lipid transfer complex.

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