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Phosphatidylethanolamine Biosynthesis in Mitochondria

*PHOSPHATIDYLSERINE (PS) TRAFFICKING IS INDEPENDENT OF A PS DECARBOXYLASE AND INTERMEMBRANE SPACE PROTEINS UPS1P AND UPS2P**

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Background: Phosphatidylserine is imported into mitochondria and decarboxylated during phosphatidylethanolamine biosynthesis.
Results: Phosphatidylserine is transported from the mitochondrial outer membrane to the inner membrane independently of two intermembrane space proteins, Ups1p and Ups2p, and phosphatidylserine decarboxylase, Psd1p.
Conclusion: Transport and decarboxylation of phosphatidylserine are mechanistically separable reactions.
Significance: Phosphatidylserine trafficking is important for the phosphatidylethanolamine metabolism in mitochondria.

Phosphatidylethanolamine (PE) plays important roles for the structure and function of mitochondria and other intracellular organelles. In yeast, the majority of PE is produced from phosphatidylserine (PS) by a mitochondrion-located PS decarboxylase, Psd1p. Because PS is synthesized in the endoplasmic reticulum (ER), PS is transported from the ER to mitochondria and converted to PE. After its synthesis, a portion of PE moves back to the ER. Two mitochondrial proteins located in the intermembrane space, Ups1p and Ups2p, have been shown to regulate PE metabolism by controlling the export of PE. It remains to be determined where PS is decarboxylated in mitochondria and whether decarboxylation is coupled to trafficking of PS. Here, using fluorescent PS as a substrate in an *in vitro* assay for Psd1p-dependent PE production in isolated mitochondria, we show that PS is transferred from the mitochondrial outer membrane to the inner membrane independently of Psd1p, Ups1p, and Ups2p and decarboxylated to PE by Psd1p in the inner membrane. Interestingly, Ups1p is required for the maintenance of Psd1p and therefore PE production. Restoration of Psd1p levels rescued PE production defects in *ups1*Δ mitochondria. Our data provide novel mechanistic insight into PE biogenesis in mitochondria.

Mitochondria form tubular structures consisting of two membranes, the outer membrane (OM)[4] and inner membrane (IM), and play important roles in many cellular activities. To maintain their morphology and functionality, this organelle contains a specific set of phospholipids. Most phospholipids are synthesized in the endoplasmic reticulum (ER) and mitochondria. Although the ER synthesizes the majority of phosphatidic acid, phosphatidylserine (PS), phosphatidylcholine, and phosphatidylinositol, mitochondria produce cardiolipin (CL) and phosphatidylethanolamine (PE) (1, 2). CL and PE are both cone-shaped molecules with proportionately small headgroups. Interestingly, the combined deletion of genes that produce mitochondrial CL and PE are synthetically lethal, indicating that CL and PE have some functional redundancy that is required for life (3). CL is critical for oxidative phosphorylation (4, 5), protein import (6–11), apoptosis (12–15), and organelle dynamics (16–19) in mitochondria. The role of CL in mitochondrial dynamics partially overlaps with that of PE as simultaneous, but not individual, loss of CL and PE leads to defects in mitochondrial fusion, which results in mitochondrial fragmentation (20). Demonstrating the physiological importance of CL and PE, abnormal CL metabolism causes Barth syndrome, which is manifested by cardiomyopathy (21, 22), whereas defects in PE production in mitochondria result in embryonic lethality in mice (23). The biosynthetic pathways for CL and PE are evolutionarily conserved from yeast to humans. The budding yeast *Saccharomyces cerevisiae* has been extensively used to study the mechanisms underlying the biosynthesis of CL and PE.

In yeast, a precursor of CL, phosphatidic acid is synthesized in the ER and transported to the mitochondrial IM through ER-mitochondrion contact sites although their molecular identity is controversial. In the IM, phosphatidic acid is converted to CL through several biosynthetic modifications mediated by Pgs1p, Gep4p, and Crd1p (24–26). After synthesis, the majority of CL remains in the IM. Similar to CL, the precursor of PE, PS,

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[4] The abbreviations used are: OM, outer membrane; CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; IMS, intermembrane space; IM, inner membrane; ER, endoplasmic reticulum; PAM, presequence translocase-associated motor; NBD-PS, 1-palmitoyl-2-{12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphoserine; NBD-PE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2–1,3-benzoxadiazol-4-yl).

TABLE 1
Yeast strains used in this study

Name	Genotype	Ref./source
FY833 (WT)	*MAT***a** *his3 leu2 lys2 trp1 ura3*	36
FY833 (WT ρ°)	*MAT***a** *his3 leu2 lys2 trp1 ura3 rho°*	38
*ups1*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 ups1::kanMX4*	10
*ups2*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 ups2::HIS3*	10
*ups1*Δ*ups2*Δ*ups3*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 ups1::kanMX4 ups2::HIS3 ups3::URA3*	10
*mdm35*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 mdm35::URA3*	42
*mdm31*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 mdm31::kanMX4*	34
*psd1*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 psd1::HIS3*	34
*gep5*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 gep5p::URA3*	This study
*her2*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 her2::URA3*	This study
*fzol*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 fzol::kanMX4 rho°*	38
*ugo1*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 ugo1::HIS3 rho°*	38
*mgml*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 mgml::kanMX4 rho°*	56
*fzol*Δ*dnm1*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 fzol::kanMX4 dnml::KanMX4*	56
*ugol*Δ*dnml*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 fzol::HIS3 dnml::KanMX4*	38
*mgml*Δ*dnm1*Δ	*MAT***a** *his3 leu2 lys2 trp1 ura3 mgml::kanMX4 dnml::KanMX4*	56
*ytal0*Δ	*MAT***a** *his3 leu2 lys2 ura3 yta10::kanMX4*	Open Biosystems
*ytal2*Δ	*MAT***a** *his3 leu2 lys2 ura3 yta12::kanMX4*	Open Biosystems

is produced from serine and CDP-diacylglycerol by the PS synthase Cho1p in the ER and transported to mitochondria (27, 28). PS is then decarboxylated to form PE by the mitochondrion-located PS decarboxylase Psd1p (29). After its synthesis, a fraction of PE is transferred back to the ER where PE is methylated to form phosphatidylcholine by the two phospholipid methyltransferases Cho2p and Opi3p (30). Despite its importance, it remains to be determined where PS is decarboxylated in mitochondria. PE may be generated in the inner leaflet of the OM and then transferred to the IM. Alternatively, PE may be produced in the IM after transfer of PS to the IM. Interestingly, Psd2p, another PS decarboxylase that is located in the Golgi and/or vacuoles, has been suggested to transfer PS from one membrane to another (31). However, the role of Psd1p in PS transport has not been examined.

Previous studies have identified intermembrane space (IMS) proteins Ups1p and Ups2p, which regulate the metabolism of CL and PE in mitochondria (10, 32–34). Ups1p and Ups2p are homologous to each other and evolutionarily conserved. Ups1p was originally identified as a component required for the biogenesis of Mgm1p, an IM protein that mediates mitochondrial fusion (32). Subsequent studies have shown that the defect in Mgm1p biogenesis results from partially impaired import of Mgm1p into mitochondria due to decreased amounts of CL, which stabilizes the protein import machinery located in the IM, the TIM23-PAM complex (10). Interestingly, Ups2p antagonistically functions in the metabolism of CL as the additional loss of Ups2p (*ups1*Δ*ups2*Δ) rescues decreased amounts of CL in *ups1*Δ cells. In addition to CL metabolism, Ups1p and Ups2p are also important for the metabolism of PE. It has been suggested that Ups1p promotes the export of PE from the IM to the OM after its synthesis (34). On the other hand, Ups2p negatively regulates and suppresses this process, and loss of Ups2p decreases levels of PE in mitochondria due to accelerated export (34).

In this study, we developed an *in vitro* assay for Psd1p-dependent PE production in isolated mitochondria using fluorescently labeled PS as a substrate to better understand mechanisms of PE biogenesis. We show that PS was transferred from the OM to the IM independently of Psd1p. Ups1p and Ups2p were also not involved in PS transport from the OM to the IM. Interestingly, Ups1p was required for maintaining the level of Psd1p as Ups1p loss led to decreases in Psd1p levels and therefore decreased PE production. Restoring Psd1p levels in *ups1*Δ mitochondria rescued their PE production defects. Taken together, these findings provide new insight into trafficking and biogenesis of phospholipids in mitochondria.

EXPERIMENTAL PROCEDURES

Strains, Media, and Plasmids—Yeast strains used in this study are in Table 1. Complete disruption of the *GEP5* and *HER2* genes was accomplished by PCR-mediated gene replacement (35) with a pair of primers 326/327 (5′-GCAAGCATACAAAGGCCTTCTATCAGAACAGCGATTGCGAACTTGGAGCAACAAGATTGTACTGAGAGTGCAC-3′/5′-CACGGCCACAGTGGCACGTGACATTTACTCCAGTATAACAGCCAACAACAACTGTGCGGTATTTCACACCG-3′) and 328/329 (5′-GTGCTGGAAAGATGGCCCCTCAATTGGAGTCCTAACTTTGATTATACAAAAAGATTGTACTGAGAGTGCAC-3′ /5′-GGTATAAAGTAAAAATAATAATATTTACATGATTAACGTTGTCCTCACAAATCTGTGCGGTATTTCACACCG-3′), respectively. FY833 (36) and the *URA3* (pRS306) gene were used as the parental yeast strain and disruption marker, respectively. Yeast strains lacking Yta10p and Yta12p (*yta10*Δ and *yta12*Δ) were purchased from Open Biosystems. Cells were grown in YPD (1% yeast extract, 2% polypeptone, 2% glucose), YPGE (1% yeast extract, 2% polypeptone, 3% glycerol, 3% ethanol), and SCD (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid, 2% glucose). Cells that have the *kanMX4* gene were selected on YPD containing 200 μg/ml G418 sulfate. pRS425-Psd1, a 2μ-*LEU2* plasmid expressing Psd1p, was constructed as follows. The *PSD1* gene including the own promoter and terminator was PCR-amplified from yeast genomic DNA using primers 525 (5′-AAAAGCGGCCGCGACTGGTACACCTGCAGGTGTAGGCGAG-3′) and 527 (5′-CCCGGATCCTATTTGGGTATCTAAGACTAGCTTTAAAAG-3′) and digested with NotI and BamHI. The DNA fragments were cloned into the NotI/BamHI site of pRS425.

Preparation of Liposome—1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (catalog number 850457), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (catalog number 850757),

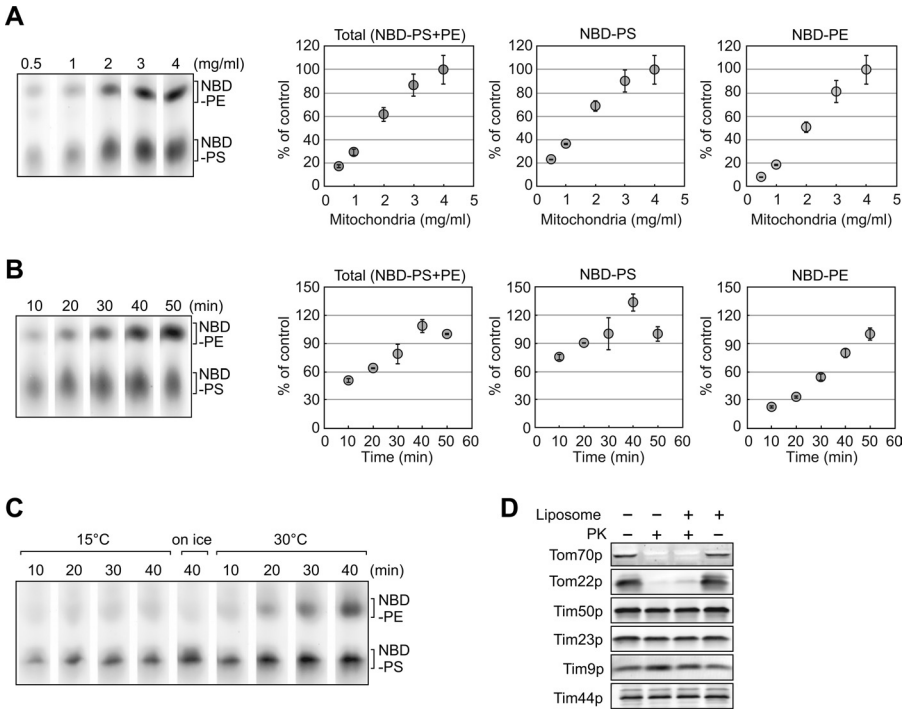

FIGURE 1. **Conversion of PS to PE in isolated mitochondria.** *A*, unilamellar liposomes containing NBD-PS were incubated with the indicated amounts of mitochondria for 30 min at 30 °C. Total phospholipids were extracted from mitochondria and separated by TLC. NBD-PS and -PE were analyzed by fluorescence imaging. Amounts of NBD-PS and -PE were quantified. 4 mg/ml mitochondria was set to 100%. Values are mean ± S.E. (*error bars*) ($n = 3$). *B*, NBD-PS liposomes were incubated with 2 mg/ml mitochondria at 30 °C for the indicated amounts of time. NBD-PS and -PE were quantified by TLC and fluorescent imaging. Values are mean ± S.E. (*error bars*) ($n = 3$). *C*, NBD-PS liposomes were incubated with 2 mg/ml mitochondria at the indicated temperatures. *D*, after incubation in the presence or absence of NBD-PS liposomes for 30 min at 30 °C, mitochondria collected by centrifugation were treated with 50 μg/ml proteinase K (*PK*) on ice for 15 min. Proteins were analyzed by immunoblotting using the indicated antibodies.

and 1-palmitoyl-2-{12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)-amino]dodecanoyl}-*sn*-glycero-3-phosphoserine (NBD-PS; catalog number 810193) were purchased from Avanti Polar Lipids. 200 μl of 3 mM phospholipid working mixture (77:20:3% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine/NBD-PS) in chloroform was dried under nitrogen gas and vortexed in 800 μl of import buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM DTT). The lipid solution was kept at room temperature for 1 h and extruded 30 times using an Avanti Mini-Extruder with 0.2-μm polycarbonate membranes to form unilamellar vesicles according to the manufacturer's instructions.

Isolation of Mitochondrion-enriched Fraction—Yeast cells were cultured in YPD overnight, harvested, and washed with water. The cells were incubated in alkaline buffer (0.1 M Tris-SO_4, pH 9.3, 10 mM DTT) for 15 min at room temperature. After washing with spheroplast buffer (20 mM Tris-HCl, pH 7.5, 1.2 M sorbitol), the cells were treated with 2 units/ml Zymolyase 20T in spheroplast buffer for 45 min at room temperature. The resulting spheroplasts were washed with ice-cold spheroplast buffer and vortexed in ice-cold lysis buffer (20 mM MOPS-KOH, pH 7.2, 0.7 M sorbitol, 1 mM EDTA, 1 mM PMSF) containing ~0.5 ml of glass beads (425–600 μm in diameter; Sigma) for 1 min. After removing unbroken cells and nuclear fractions by centrifugation at 1500 × *g* for 5 min, crude mitochondrial fractions were precipitated by centrifugation at 12,000 × *g* for 10 min. The crude mitochondrial fractions were washed with SEM (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2, 1 mM EDTA), resuspended in SEM buffer, and frozen in liquid nitrogen. When yeast cells were cultivated in non-fermentable media (YPGE), mitochondria were isolated as described previously (37).

In Vitro Assay for PE Production—250 μg of crude mitochondrial fractions was suspended in 50 μl of import buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM DTT) and incubated with 50 μl of liposome at 30 °C for a certain period of time. To stop reactions, 400 μl of ice-cold SEM buffer was added to the sample. Mitochondria were then precipitated by centrifugation at 12,000 × *g* for 10 min and washed with 100 μl of SEM buffer. Phospholipids were extracted from mitochondria by vortexing in 500 μl of 2:1 chloroform/methanol for 15 min. 100 μl of water was added to the sample and vortexed for 5 min. The organic phase was separated by centrifugation at 400 × *g* for 5 min, dried in a SpeedVac, and then resuspended in 60 μl of chloroform. 20 μl of each sample was used for thin-layer chromatography (TLC) analyses. A PharosFX Plus molecular imager (Bio-Rad) was used to analyze NBD-PS and -PE.

Mitochondrial OM and IM Vesicle Separation after Incubation with NBD-PS Liposomes—4 mg of mitochondria was suspended in 2 ml of import buffer and incubated with 2 ml of NBD-PS liposome at 30 °C for 45 min. Mitochondria were then collected by centrifugation at 12,000 × *g* for 10 min at 4 °C and washed with SEM buffer. OM and IM vesicles were generated and separated as described (38). Briefly, mitochondria were first suspended in 3.7 ml of EM buffer (10 mM MOPS-KOH, pH 7.2, 1 mM EDTA) for 30 min and swollen by osmotic shock. The resulting mitoplasts were incubated for 10 min on ice after addition of sucrose to 0.45 M and then sonicated three times for

10 s using a Sonic Dismembrator (model 100, Fisher Scientific) with setting at 8. Intact mitochondria were removed by centrifugation at 18,400 rpm in an SW55Ti rotor (Beckman) for 20 min at 4 °C. OM and IM vesicles in the supernatant were pelleted by centrifugation at 47,000 rpm in an SW55Ti rotor for 40 min at 4 °C. The pellet was resuspended in EM buffer with 10 mM NaCl, placed onto a sucrose step gradient (26/31/35/40%) and then centrifuged at 39,000 rpm for 15 h with an SW55Ti rotor. After centrifugation, 320 μl-fractions were collected from the top. 20 μl of each fraction was used for protein analyses by immunoblotting. The remaining samples (300 μl) were diluted with 900 μl of EM buffer and centrifuged at 54,000 rpm in a TLA55 rotor for 30 min to precipitate OM and IM vesicles. Phospholipids were extracted from the membrane pellets and analyzed by TLC.

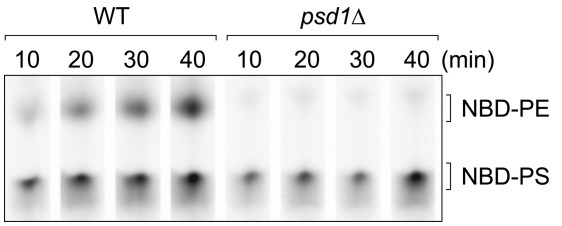

FIGURE 2. **Psd1p-dependent decarboxylation of NBD-PS.** Unilamellar liposomes containing NBD-PS were incubated with WT and *psd1*Δ mitochondria at 30 °C for the indicated amounts of time. Mitochondria were collected, and their total phospholipids were subjected to TLC and fluorescence imaging.

Immunoblotting—For immunoblotting, proteins were visualized by fluorophores conjugated with secondary antibodies (Alexa Fluor 488 or 647 goat anti-mouse or rabbit IgG (heavy + light) from Invitrogen) and analyzed using a PharosFX Plus molecular imager (Bio-Rad) and Quantity One (Bio-Rad) and Photoshop (Adobe) software.

Digitonin Extraction of Mitochondrial Proteins—The digitonin-based submitochondrial localization was performed as described previously (39). In brief, 0.25 mg of mitochondria was resuspended at 10 mg/ml in SEHK buffer (0.25 mM sucrose, 5 mM EDTA, pH 7.0, 10 mM HEPES-KOH, pH 7.4, 0.2 M KCl containing 0.2 mM PMSF) and solubilized by adding an equal volume of 2× digitonin in SEHK buffer for a final concentration of digitonin ranging from 0 to 0.5% (w/v). The samples were vortexed on low for 5 s and incubated for 1 min on ice. Solubilization was stopped by adding 8.5× volumes of cold SEHK buffer. The mixture was separated into a pellet and supernatant at 100,000 × *g* using a TLA120.1 rotor. The supernatant fractions were TCA-precipitated, and the pellet and supernatant fractions were resuspended in equal volumes of sample buffer

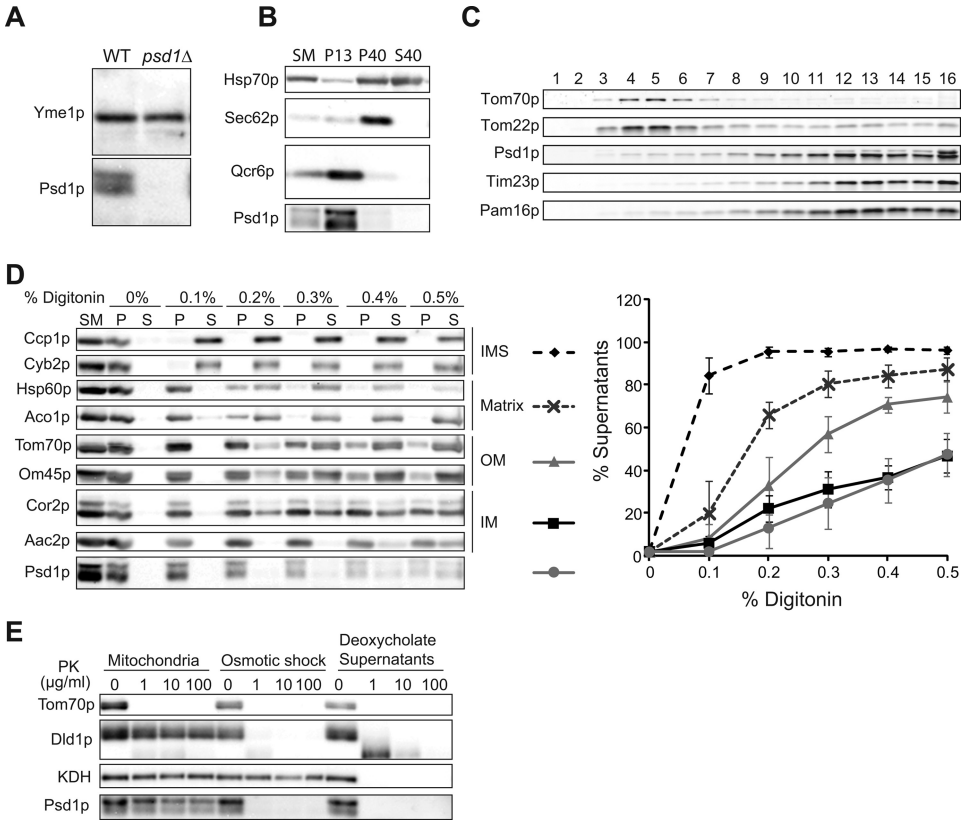

FIGURE 3. **Localization of Psd1p in the IM.** *A*, whole cell extracts of WT and *psd1*Δ cells were analyzed by immunoblotting with antibodies against Psd1p and the mitochondrial protein Yme1p. *B*, WT cells were homogenized and fractionated by differential centrifugations. Each fraction was analyzed by immunoblotting using antibodies against Hsp70p, Sec62p, Qcr6p, and Psd1p. *SM*, the whole cell extract; *P13*, the mitochondrial fraction, *P40*, the microsome fraction; *S40*, the cytosolic fraction. *C*, after osmotic swelling, mitochondria were sonicated to produce membrane vesicles derived from the OM and IM. These vesicles were separated by sucrose density gradients. Each fraction was analyzed by immunoblotting using the indicated antibodies. *D*, isolated mitochondria were incubated with the indicated amounts of digitonin on ice for 1 min. The samples were separated into the pellet (*P*) and supernatant (*S*) fractions by centrifugation. Equal volumes of pellet and TCA-precipitated supernatant fractions were analyzed by immunoblot, and the percentage of each protein in the supernatants was determined. Data from two proteins/compartment were combined (IMS, Cyb2p and Ccp1p; matrix, Hsp60p and Aco1p; OM, OM45p and Tom70p; IM, Aac2p and Cor2p). Values are mean ± S.E. (*error bars*) ($n = 3$). *E*, mitochondria were treated with proteinase K (*PK*) after osmotic shock or solubilization with deoxycholate. Mitochondrial proteins were analyzed by immunoblotting with the indicated antibodies. *KDH*, α-ketoglutarate dehydrogenase.

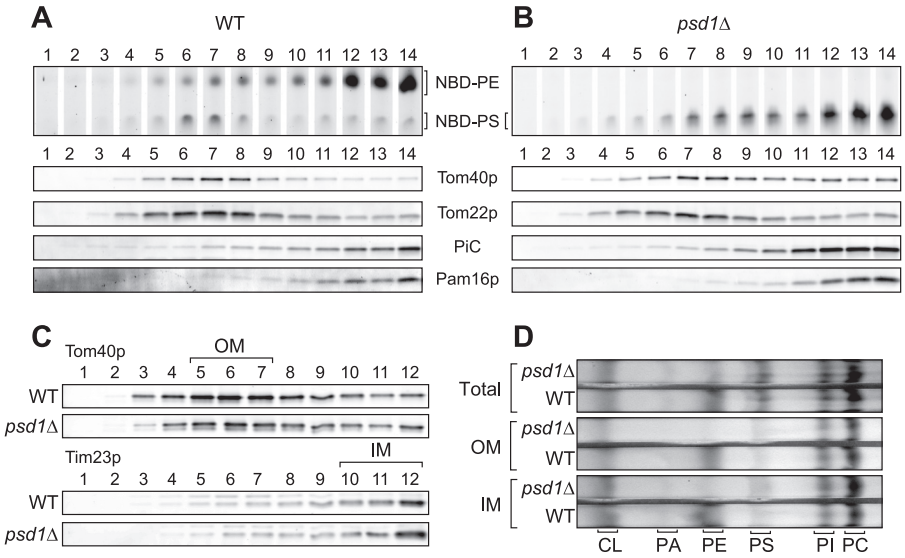

FIGURE 4. **Psd1p-independent transfer of PS from the OM to the IM.** After incubation with NBD-PS liposomes, OM and IM vesicles were prepared from WT mitochondria (*A*) and *psd1*Δ mitochondria (*B*) and separated by sucrose density gradient centrifugation. Each fraction was analyzed by TLC and fluorescence imaging for NBD-PS and -PE. Proteins were examined by immunoblotting with the indicated antibodies. *C*, OM and IM vesicles generated from WT and *psd1*Δ mitochondria by sonication were separated by sucrose density centrifugation. Fractions were collected from the gradient and analyzed by immunoblotting with antibodies against Tom40p and Tim23p. *D*, phospholipids were extracted from the OM and IM vesicle-rich fractions (*lanes 5–7* and *10–12*, respectively) and separated by TLC. After soaking in 8.5% phosphoric acid containing 470 mM $CuSO_4$, the TLC plate was baked at 180 °C for 15 min to visualize phospholipids. *PiC*, inorganic phosphate carrier; *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol.

and heated for 5 min at 95 °C. The proteins were resolved by 12% SDS-PAGE and immunoblotted as indicated, images were captured with a Fluorchem Q (Cell Biosciences, Inc.) quantitative digital imaging system, and the bands were quantified using Quantity One software (Bio-Rad).

RESULTS

In Vitro Reconstitution of Psd1p-dependent PE Biogenesis in Mitochondria—To investigate conversion of PS to PE in mitochondria, we developed an *in vitro* assay using fluorescently labeled PS (16:0–12:0 NBD-PS) and mitochondria isolated from yeast cells. First, to test whether NBD-PS can be converted to NBD-PE, we incubated unilamellar liposomes containing NBD-PS with different amounts of mitochondria for 30 min at 30 °C. Mitochondria were then washed to remove NBD-PS liposomes by centrifugation. Total phospholipids were extracted from mitochondria and analyzed by TLC. We found that NBD-PS was incorporated into mitochondria and converted to NBD-PE proportionally to the amount of mitochondria (Fig. 1*A*). Second, we incubated NBD-PS liposomes and mitochondria (2 mg/ml) for different amounts of time. Incorporation of NBD-PS into mitochondria and its conversion to NBD-PE linearly increased until 40 min (Fig. 1*B*). Finally, we found that the conversion of NBD-PS to NBD-PE depends on physiological temperatures. When we performed our *in vitro* assay at 15 °C or on ice, PE formation was greatly inhibited (Fig. 1*C*). In contrast, incorporation of NBD-PS into mitochondria was less dependent on temperatures. NBD-PS was normally incorporated into mitochondria after 40 min of incubation on ice.

To determine whether mitochondrial membranes remain intact in our *in vitro* assay, we added proteinase K to mitochondria after incubation with NBD-PS liposomes. Two OM proteins, Tom22p and Tom70p, were digested by proteinase K, whereas two IM proteins with large IMS domains, Tim50p and Tim23p, were not (Fig. 1*D*). The IMS protein Tim9 and the matrix protein Tim44p were insensitive to proteinase K treatment. These results indicate that the incubation with NBD-PS liposomes does not disrupt the integrity of mitochondrial membranes.

Yeast cells have Psd1p and Psd2p, two PS decarboxylases that are located in different membranes. Psd1p is located in mitochondria, whereas Psd2p is present in the Golgi complex and vacuoles (40). To determine whether Psd1p mediates decarboxylation of PS in our assay, we used mitochondria isolated from *psd1*Δ cells. We found that NBD-PE was no longer produced in *psd1*Δ mitochondria (Fig. 2). Therefore, Psd1p decarboxylates PS in our *in vitro* assay.

Psd1p Is an IM Protein Exposed to the IMS—We determined the submitochondrial localization and membrane topology of Psd1p. We first confirmed that Psd1p is a mitochondrial protein by subcellular fractionation followed by immunoblotting with anti-Psd1p antibodies (Fig. 3, *A* and *B*). Psd1p was found in the mitochondrial fraction along with a mitochondrial protein, Qcr6p, but not with the ER protein Sec62p or the cytosolic protein Hsp70p (Fig. 3*B*). To examine the submitochondrial localization of Psd1p, we generated OM and IM vesicles by osmotic shock and subsequent sonication of mitochondria. We separated these vesicles using sucrose density gradient centrifugation as described (38). Immunoblotting of each fraction showed that two OM proteins (Tom70p and Tom22p) were mainly collected in fractions 4–6, whereas two IM proteins (Tim23p and Pam16p) and Psd1p penetrated into fractions 10–16 (Fig. 3*C*), indicating that Psd1p is an IM protein. We further confirmed the IM localization of Psd1p using extraction of mitochondrial proteins with a mild detergent, digitonin. Isolated mitochondria were treated with different concentrations of digitonin and separated into supernatant and pellet fractions

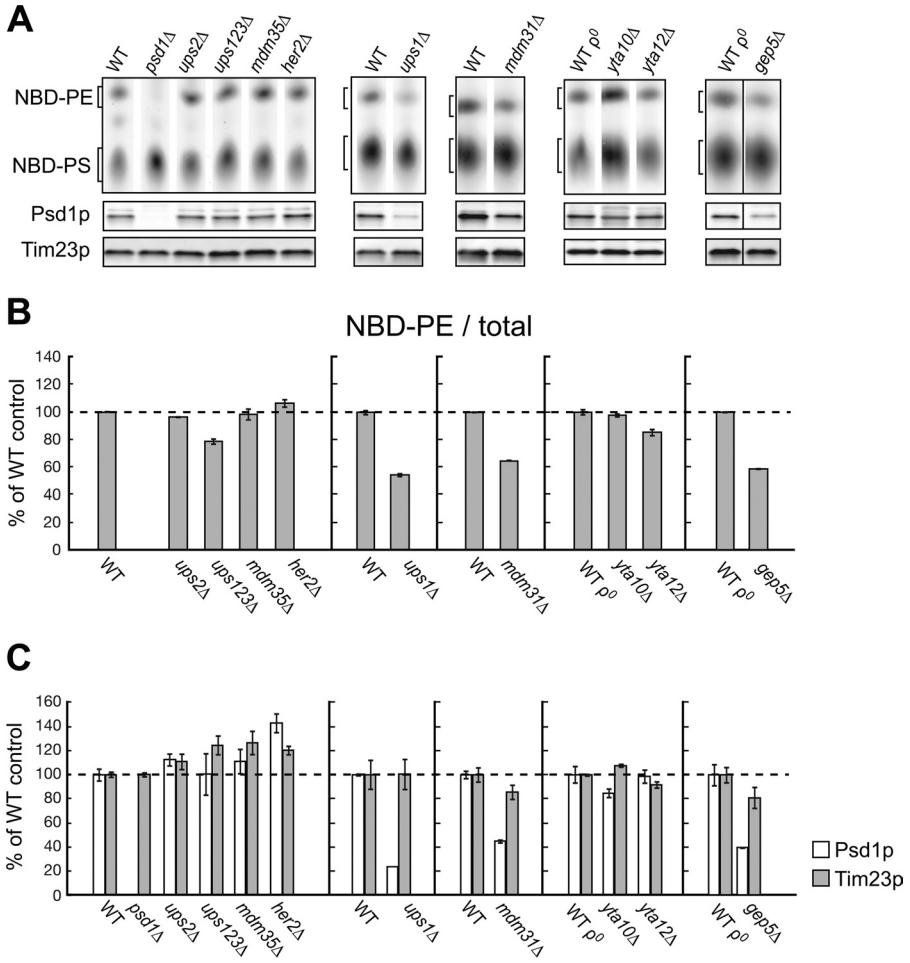

FIGURE 5. **Decreases in Psd1p abundance and PE production in mitochondria lacking Ups1p, Mdm31p, and Gep5p.** *A*, NBD-PS liposomes were incubated with mitochondria (2 mg/ml) isolated from the indicated deletion mutants for 30 min at 30 °C. The mitochondria were collected, and total phospholipids were analyzed by TLC and fluorescence imaging. Psd1p abundance was examined by immunoblotting. Tim23p was used as a loading control. Amounts of NBD-PE relative to total NBD lipids (NBD-PS and -PE) were quantified in *B*. Amounts of Psd1p and Tim23p were quantified in *C*. WT mitochondria were set to 100%. Values are mean ± S.E. (*error bars*) ($n = 3$).

by centrifugation. It has been shown that digitonin extracts mitochondrial proteins with different efficiencies depending on the submitochondrial localization of a protein (39). As shown in Fig. 3*D*, 0.1% digitonin preferentially released soluble IMS proteins (Ccp1p and Cyb2p) into the supernatant fractions. Increasing digitonin concentrations extracted soluble matrix proteins (Hsp60p and Aco1p), OM proteins (Tom70p and Om45p), and finally IM proteins (Cor2p and Aac2p). The extraction profile of Psd1p was similar to the IM proteins. Finally, to determine the membrane topology of Psd1p, we examined the sensitivity of Psd1p to proteinase K. When intact mitochondria were treated with 1 μg/ml proteinase K, the OM protein Tom70p was completely digested, whereas Psd1p was not, similar to the IMS protein Dld1p and the matrix protein α-ketoglutarate dehydrogenase (Fig. 3*E*). However, Psd1p and Dld1p, but not α-ketoglutarate dehydrogenase, were digested by proteinase K after the OM was disrupted by osmotic shock. α-Ketoglutarate dehydrogenase was digested only after mitochondrial membranes were solubilized by a detergent, deoxycholate. Taken together, these results show that Psd1p is an IM protein exposed to the IMS, consistent with its amino acid sequence, which contains a predicted matrix-targeting sequence and a potential transmembrane domain.

Psd1p Is Not Required for the Transfer of PS from the OM to the IM—It has been suggested that the non-mitochondrial PS decarboxylase Psd2p transfers PS between membranes through its C2 domain (31). We tested whether Psd1p, which lacks a C2 domain, is required for transfer of PS from the OM to the IM in our *in vitro* assay. After incubation with NBD-PS liposomes, OM and IM vesicles were produced from WT and *psd1*Δ mitochondria and separated by sucrose density gradient centrifugation. We analyzed proteins and phospholipids in each fraction using immunoblotting and TLC, respectively (Fig. 4*A*). The mitochondrial OM proteins Tom40p and Tom22p were collected in lighter fractions (Fig. 4*A*, fractions 5–8), whereas the IM proteins inorganic phosphate carrier and Pam16p penetrated into heavier fractions (Fig. 4*A*, fractions 10–14). When WT mitochondria were used, the majority of NBD-PE comigrated with the IM proteins, whereas NBD-PS cofractionated with the OM proteins. In contrast, in *psd1*Δ mitochondria, which only contain NBD-PS, NBD-PS was mainly found in the IM fractions (Fig. 4*B*). Therefore, NBD-PS was transferred from

the OM to the IM independently of Psd1p. Our data also suggest that most NBD-PE remains in the IM after conversion from NBD-PS in WT mitochondria.

We also analyzed the steady state amounts of PS in the OM and IM in WT and *psd1*Δ mitochondria. As expected, we found decreased PE levels in *psd1*Δ mitochondria compared with WT mitochondria. In *psd1*Δ mitochondria, we did not observe a dramatic accumulation of PS in the IM unlike NBD-PS in the *in vitro* assay (Fig. 4*C*). In addition, steady state amounts of PE were also comparable in the OM and IM of WT mitochondria (Fig. 4*C*), consistent with a previous observation (41). These results suggest that PS and PE levels had equilibrated between the OM and IM *in vivo*. It is also possible that NBD-labeled phospholipids may preferentially accumulate in the IM.

Ups1p, Mdm31p, and Gep5p Are Required for the Maintenance of Psd1p Levels and the Production of PE—Our *in vitro* assay separated two steps involved in PE biogenesis in mitochondria. The first step is Psd1p-independent transfer of PS from the OM to the IM. The second step is Psd1p-dependent decarboxylation of PS to generate PE. Previous studies have shown that several mitochondrial proteins are important for the maintenance of PE abundance in mitochondria, including Ups1p, Ups2p, Mdm35p, Mdm31p, Yta10p, Yta12p, Her2p, and Gep5p (10, 33). Ups1p and Ups2p form protein complexes with Mdm35p and regulate export of PE from the IM to the OM (42, 43). Mdm31p is an IM protein required for mitochondrial morphology (44, 45). A recent study has shown that Mdm31p has functions similar to Ups1p in CL metabolism (34). Yta10p and Yta12p are subunits of m-AAA ATPases Associated with diverse cellular Activities protease in the IM (46). Her2p is involved in ER membrane remodeling (47). Gep5p is required for normal respiratory growth (48). However, roles of these proteins in PE metabolism are unknown. To determine whether mitochondria lacking these proteins are defective in production of PE, we examined conversion of PS to PE *in vitro* using mitochondria isolated from their deletion mutants. Because *yta10*Δ, *yta12*Δ, and *gep5*Δ cells are unable to grow in non-fermentable media (46, 48), we used mitochondria isolated from ρ^0 WT cells without mtDNA as a control, although ρ^+ and ρ^0 WT cells showed indistinguishable PE production in our assay (Fig. 5*A*). We found that conversion of PS to PE was significantly decreased in mitochondria lacking Ups1p, Mdm31p, or Gep5p (Fig. 5*B*). Consistent with their PE production defects, Psd1p levels were also decreased in *ups1*Δ, *mdm31*Δ, or *gep5*Δ mitochondria (Fig. 5*C*).

*Overexpression of Psd1p Rescues Defective PE Production in ups1*Δ *and gep5*Δ *Mitochondria in Vitro*—To test whether increases in Psd1p levels can rescue defects in PE production in *ups1*Δ or *gep5*Δ mitochondria, Psd1p was overexpressed from a multicopy plasmid in *ups1*Δ and *gep5*Δ cells. Immunoblotting showed that Psd1p levels were increased approximately 3–5-fold in control, *ups1*Δ, and *gep5*Δ mitochondria upon its overexpression compared with endogenous levels of Psd1p in WT cells (Fig. 6, *A* and *C*). In control mitochondria, Psd1p overexpression did not affect conversion of PS to PE *in vitro*, showing that Psd1p levels are not rate-limiting in these mitochondria (Fig. 6*B*). In contrast, *ups1*Δ and *gep5*Δ mitochondria restored normal PE production upon Psd1p overexpression. These

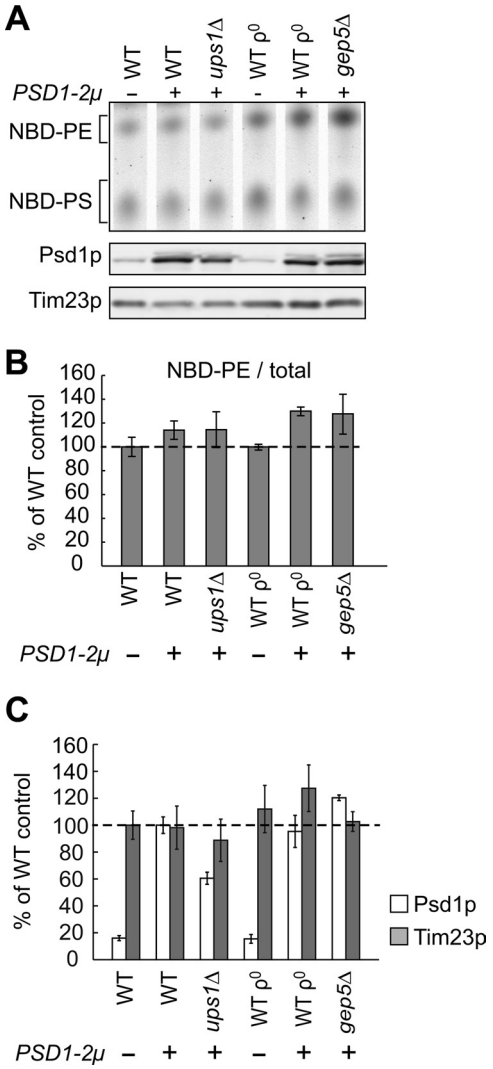

FIGURE 6. **Restoration of PE production by overexpression of Psd1p in *ups1*Δ and *gep5*Δ mitochondria.** *A*, NBD-PS liposomes were incubated with a 2 mg/ml concentration of the indicated mitochondria for 30 min at 30 °C. Total phospholipids in mitochondria were analyzed by TLC and fluorescence imaging. Psd1p and Tim23p were examined by immunoblotting. Amounts of NBD-PE relative to total NBD lipids (NBD-PS and -PE) were quantified in *B*. Levels of Psd1p and Tim23p were determined in *C*. WT mitochondria were set to 100%. Values are mean ± S.E. (*error bars*) ($n = 3$).

results suggest that defects in PE production in *ups1*Δ and *gep5*Δ mitochondria likely result from decreased levels of Psd1p.

*Non-fermentable Growth Conditions Restore Normal Psd1p Levels and PS to PE Conversion in ups1*Δ *and mdm31*Δ *Mitochondria*—It has been reported that loss of Ups1p causes decreased import of mitochondrial proteins (10). This protein import defect is restored when *ups1*Δ cells are grown in non-fermentable media likely due to increases in the membrane potential across the IM, which is important for protein import into mitochondria (10). Therefore, we reasoned that import of Psd1p may be partially compromised in *ups1*Δ mitochondria and that non-fermentable culture conditions may restore Psd1p abundance. To test this idea, we isolated mitochondria from WT and *ups1*Δ cells grown in the non-fermentable medium containing glycerol and ethanol as carbon sources (YPGE). Immunoblotting showed similar levels of Psd1p in WT

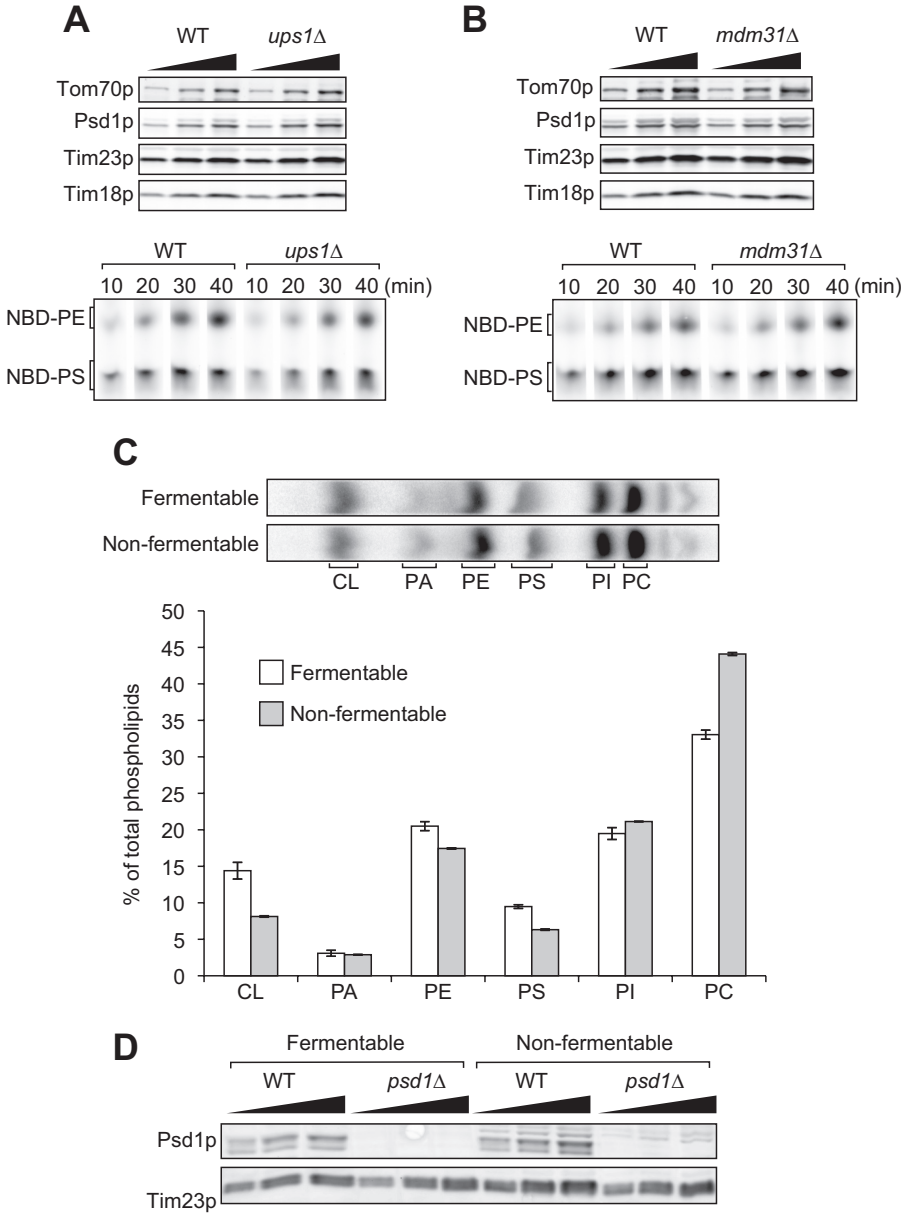

FIGURE 7. **Restoration of Psd1p levels and PE production in *ups1*Δ and *mdm31*Δ mitochondria by non-fermentable growth condition.** Mitochondria were isolated from WT, *ups1*Δ cells (*A*), and *mdm31*Δ cells (*B*), which were grown in a non-fermentable culture medium and analyzed by immunoblotting using the indicated antibodies. The indicated mitochondria (2 mg/ml) were collected and analyzed by TLC and fluorescence imaging after incubation with NBD-PS liposomes for the indicated periods of time at 30 °C. *C*, total phospholipids were extracted from WT yeast cells cultured in YPD (fermentable) and YPGE (non-fermentable) containing $^{32}P_i$ and analyzed by TLC and radioimaging. Amounts of each phospholipid relative to total phospholipids were determined. Values are mean ± S.E. (*error bars*) ($n = 3$). *D*, mitochondria isolated from WT yeast cells cultured in YPD and YPGE were analyzed by immunoblotting with antibodies against Psd1p and Tim23p. *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol.

and *ups1*Δ mitochondria (Fig. 7*A*). In the *ups1*Δ mitochondria, conversion of PS to PE was also restored (Fig. 7*A*). Similarly, the non-fermentable growth condition also increased Psd1p levels and PE production in *mdm31*Δ mitochondria (Fig. 7*B*). We examined the amounts of Psd1p and PE in mitochondria isolated from WT cells grown in YPD and YPGE and found similar Psd1p and PE levels regardless of the carbon source (Fig. 7, *C* and *D*).

Mitochondria That Are Defective in Fusion Show Decreases in Psd1p Amounts and PE Production—Cells lacking Ups proteins and Mdm31p display abnormal mitochondrial morphology (10, 32, 34, 45). Mitochondrial fusion regulates mitochondrial shape, and mitochondrial fusion defects lead to fragmentation of mitochondria due to unopposed mitochondrial division. Because *ups1*Δ cells contain fragmented mitochondria (10, 32), we sought to determine whether proteins that mediate mitochondrial fusion are required for PE production in our *in vitro* assay. The mitochondrial fusion machinery consists of two OM proteins, Fzo1p and Ugo1p, and an IM protein, Mgm1p (49). As *fzo1*Δ, *ugo1*Δ, and *mgm1*Δ mitochondria lack mtDNA, ρ^0 WT mitochondria were used as a control. We found that *fzo1*Δ, *ugo1*Δ, and *mgm1*Δ mitochondria showed decreased conversion of PS to PE with decreased Psd1p levels similar to *ups1*Δ, *mdm31*Δ, and *gep5*Δ mitochondria. As fragmentation of mito-

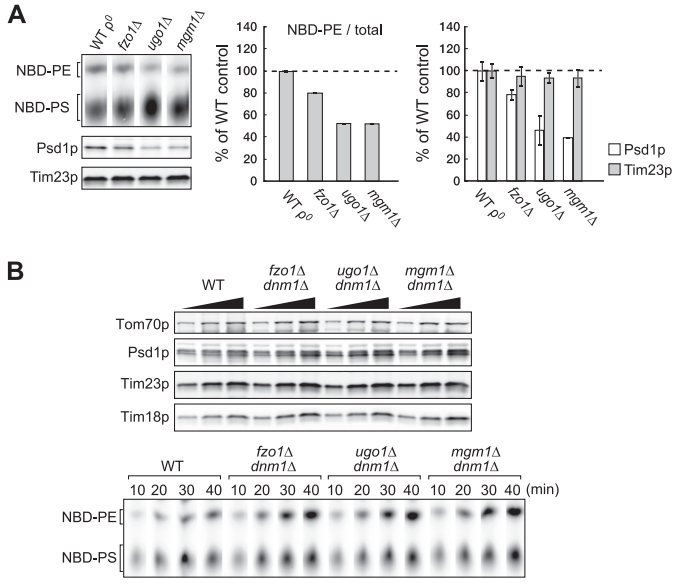

FIGURE 8. **Psd1p levels and PE conversion in fusion-defective mitochondria.** *A*, NBD-PS liposomes were incubated with a 2 mg/ml concentration of the indicated mitochondria for 30 min at 30 °C. Mitochondria were collected, and total phospholipids were analyzed by TLC and fluorescence imaging (*left panel*). Amounts of Psd1p and Tim23p were determined by immunoblotting (*right graph*). Relative amounts of NBD-PE were determined (*middle graph*). WT mitochondria were set to 100%. Values are mean ± S.E. (*error bars*) ($n = 3$). *B*, mitochondria were isolated from WT, *fzo1*Δ*dnm1*Δ, *ugo1*Δ*dnm1*Δ, and *mgm1*Δ*dnm1*Δ cells that were cultured in YPGE and analyzed by immunoblotting using the indicated antibodies. NBD-PS liposomes were incubated with the mitochondria at 30 °C. Total mitochondrial phospholipids were analyzed by TLC and visualized by fluorescence imaging.

chondria in fusion-deficient mutants is rescued by additional loss of Dnm1p, a protein required for mitochondrial division (50), we tested whether PE production defects are also rescued by the loss of Dnm1p. We found that *fzo1*Δ*dnm1*Δ, *ugo1*Δ*dnm1*Δ, and *mgm1*Δ*dnm1*Δ mitochondria showed normal Psd1p levels and PE synthesis (Fig. 8). These data suggest that fragmentation of mitochondria, but not a fusion defect itself, leads to decreases in Psd1p levels and therefore compromised PE production in the absence of Fzo1p, Ugo1p, or Mgm1p.

DISCUSSION

Here, we studied trafficking and decarboxylation of PS using an *in vitro* assay for Psd1p-dependent PE production in mitochondria. We show that conversion of PS to PE occurs at physiological temperatures in a time-dependent manner. The PE formation is efficient, and its time course is consistent with a pulse-chase study with [^{14}C]serine in which conversion of PS to PE was examined in cells (34). Previously, mitochondrial PE production has been studied using permeabilized cells and purified mitochondria (51–55). Based on these studies, at least three models for PE biogenesis have been proposed. In the first model, PS is decarboxylated in the inner leaflet of the OM by Psd1p, and then PE is transferred to the IM. This model suggests that decarboxylation of PS precedes its transport from the OM to the IM. In the second model, trafficking and decarboxylation of PS are coupled and mediated by Psd1p. This model is based on the function of the Golgi/vacuole-located PS decarboxylase Psd2p, which plays roles in both trafficking and decarboxylation of PS. The C2 domain in Psd2p has been suggested to participate in the trafficking function; however, Psd1p does not have this domain. In the last model, PS is first transferred to the IM and then decarboxylated. Our *in vitro* assay supports the third model and shows that PS trafficking and decarboxylation are two separable reactions. First, PS is transferred from the OM to the IM independently of Psd1p. Decarboxylation of PS is not required for the transfer of PS from the OM to the IM. Next, PS is decarboxylated by Psd1p in the IM. In addition, our data also suggest that, once transferred to the IM, PS is immediately decarboxylated because only small amounts of PS were found in the IM.

Our findings also show that Ups1p is important for the abundance of Psd1p and therefore for PE production in mitochondria. We have shown previously that Ups1p facilitates efficient import of proteins that carry matrix-targeting sequences (10). *ups1*Δ cells that were grown in fermentable carbon sources exhibited a decreased membrane potential across the IM, which is required for mitochondrial protein import. In addition, in *ups1*Δ mitochondria, the IM protein translocase TIM23-PAM complex is partially disassembled due to lack of CL, which is required for proper assembly of many IM protein complexes. The import defect is rescued when *ups1*Δ cells are grown in non-fermentable carbon sources, which increase the membrane potential across the IM and facilitate efficient protein import even with partially disassembled TIM23-PAM (10). Because Psd1p contains a matrix-targeting sequence, decreased Psd1p levels could result from a partial defect in its import into *ups1*Δ mitochondria. Consistent with this notion, Psd1p levels were restored by non-fermentable growth conditions in *ups1*Δ mitochondria. Furthermore, it has been shown that protein import defects can be overcome by supplying more substrate proteins (42). Likewise, we found that overexpression of Psd1p increases Psd1p levels in *ups1*Δ mitochondria, suggesting that Ups1p helps normal import of Psd1p into mitochondria. Because the transfer of PS from the OM to the IM precedes PE production by IM-located Psd1p, Ups1p is likely dispensable for the transfer of PS.

We have shown previously that Ups1p promotes the export of PE from the IM to the OM in pulse-chase studies with [^{14}C]serine in cells (34). We suggest that, in PE metabolism, Ups1p is required for both the maintenance of Psd1p levels and the export of PE from the IM to the OM when cells are grown under fermentable conditions. In *ups1*Δ cells, both the production and export of PE are partially decreased, therefore maintaining a normal level of PE in mitochondria at the steady state. In contrast, when *ups1*Δ cells are grown under non-fermentable conditions, Psd1p levels are restored, but the export is still suppressed, therefore increasing levels of PE at the steady state (10, 34).

We have shown that Ups2p is not involved in PS transport and decarboxylation in cells in pulse-chase studies (34). In the same study, Ups2p has been shown to have a function antagonistic to Ups1p and to negatively control the export of PE from the IM to the OM. Consistent with these *in vivo* observations, our current study shows that *ups2*Δ mitochondria normally produce PE *in vitro*. It has been shown that steady state levels of PE are decreased in *ups2*Δ mitochondria (10, 34, 42). The func-

tion of Ups2p in PS export suggests that decreases in PE levels in *ups2*Δ mitochondria likely result from accelerated export of PE from the IM to the OM and then the ER where PE is converted to phosphatidylcholine. Similar to *ups2*Δ mitochondria, *her2*Δ, *yta10*Δ, and *yta12*Δ mitochondria, which exhibit decreased steady state PE levels (33), could synthesize PE normally in our *in vitro* assay. These findings suggest that Her2p, Yta10p, and Yta12p, like Ups2p, may play a role in PE export.

We also examined roles of mitochondrial proteins that have been shown to be important for the maintenance of PE levels. Similar to Ups1p, Mdm31p and Gep5p are required for the maintenance of Psd1p and thereby production of PE. CL levels have been shown to be lower in *mdm31*Δ and *gep5*Δ mitochondria (33, 34). Restoration of Psd1p levels rescued the defects in PE production in mitochondria lacking these proteins, and therefore, these proteins are not directly involved in transport or decarboxylation of PS. Rather, these mutant mitochondria may be partially defective in the import of Psd1p into mitochondria. It is also possible that the stability of Psd1p is affected in *mdm31*Δ and *gep5*Δ mitochondria. In addition, we found that proteins that mediate mitochondrial fusion (Fzo1p, Ugo1p, and Mgm1p) are important for Psd1p abundance. Additional loss of mitochondrial division, which restores tubular mitochondrial morphology (50, 56), rescued Psd1p defects in these fusion mutants; therefore, mitochondrial shape, rather than fusion activity, is critical for the maintenance of Psd1p.

Previous studies have suggested that trafficking of PS takes place at contact sites between the OM and IM. Ugo1p, which directly binds to both OM-located Fzo1p and IM-located Mgm1p, has been suggested to form contact sites to couple OM fusion to IM fusion (57). Furthermore, Ugo1p is associated with the mitochondrial contact site complex containing Fcj1p (58). However, because Ugo1p was not required for PS transport, PS likely moves from the OM to the IM independently of contact sites mediated by Ugo1p. In addition, we also found that Fcj1p is dispensable for PE production *in vitro*.[5] Therefore, it would be interesting to decipher the molecular basis of PS transport in future studies.

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