

# Processing and Topology of the Yeast Mitochondrial Phosphatidylserine Decarboxylase 1\*

Received for publication, July 4, 2012, and in revised form, September 4, 2012. Published, JBC Papers in Press, September 13, 2012, DOI 10.1074/jbc.M112.398107

Susanne E. Horvath<sup>‡§</sup>, Lena Böttlinger<sup>§¶</sup>, F.-Nora Vögtle<sup>§||</sup>, Nils Wiedemann<sup>§||</sup>, Chris Meisinger<sup>§||</sup>, Thomas Becker<sup>§||</sup>, and Günther Daum<sup>‡2</sup>

From the <sup>‡</sup>Institut für Biochemie, Technische Universität Graz, Petersgasse 12/2, A-8010 Graz, Austria, the <sup>§</sup>Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, D-79104 Freiburg, Germany, the <sup>¶</sup>Fakultät für Biologie, Universität Freiburg, D-79104 Freiburg, Germany, and the <sup>||</sup>BIOSS Centre for Biological Signalling Studies, Universität Freiburg, D-79104 Freiburg, Germany

**Background:** Although phosphatidylserine decarboxylase 1 (Psd1) is of central importance for the generation of cellular phosphatidylethanolamine (PE), its biogenesis is only poorly understood.

**Results:** Biogenesis of Psd1 involves processing by two proteases, MPP and Oct1, and an autocatalytic separation of Psd1 $\alpha$  from the membrane-anchored Psd1 $\beta$ .

**Conclusion:** Psd1 requires integration into the inner mitochondrial membrane for full enzymatic activity.

**Significance:** This study presents a new model for the biogenesis and topology of Psd1.

The inner mitochondrial membrane plays a crucial role in cellular lipid homeostasis through biosynthesis of the non-bilayer-forming lipids phosphatidylethanolamine and cardiolipin. In the yeast *Saccharomyces cerevisiae*, the majority of cellular phosphatidylethanolamine is synthesized by the mitochondrial phosphatidylserine decarboxylase 1 (Psd1). The biogenesis of Psd1 involves several processing steps. It was speculated that the Psd1 precursor is sorted into the inner membrane and is subsequently released into the intermembrane space by proteolytic removal of a hydrophobic sorting signal. However, components involved in the maturation of the Psd1 precursor have not been identified. We show that processing of Psd1 involves the action of the mitochondrial processing peptidase and Oct1 and an autocatalytic cleavage at a highly conserved LGST motif yielding the  $\alpha$ - and  $\beta$ -subunit of the enzyme. The Psd1  $\beta$ -subunit (Psd1 $\beta$ ) forms the membrane anchor, which binds the intermembrane space-localized  $\alpha$ -subunit (Psd1 $\alpha$ ). Deletion of a transmembrane segment in the  $\beta$ -subunit results in mislocalization of Psd1 and reduced enzymatic activity. Surprisingly, autocatalytic cleavage does not depend on proper localization to the inner mitochondrial membrane. In summary, membrane integration of Psd1 is crucial for its functionality and for maintenance of mitochondrial lipid homeostasis.

Mitochondrial membranes contain a high amount of non-bilayer-forming phospholipids, such as phosphatidylethanolamine (PE)<sup>3</sup> and cardiolipin (CL) (1–4). These lipids have a characteristic conical shape due to the relatively small head-group compared with the large hydrophobic acyl chains. Non-bilayer-forming phospholipids may locally induce inverted hexagonal ( $H_{II}$ ) phase structures, thereby increasing the tension in the lipid bilayer. This property seems to be important for vesicle formation, membrane fusion, transbilayer transport of lipids and polar solutes, protein interactions, topogenesis, and protein function (5, 6). PE and CL associate with different membrane-bound proteins and protein complexes, such as the respiratory chain complexes or the mitochondrial ATP/ADP carrier to maintain their stability and function (7–9). As a consequence, lack of CL affects the structural organization of the respiratory chain supercomplexes and protein translocases in both outer and inner mitochondrial membrane (2, 10–17). Simultaneous loss of both major non-bilayer forming phospholipids, PE and CL, is lethal for *Saccharomyces cerevisiae* (18). Also in mammals, biosynthesis of PE is essential (19), and alterations in content or acylation of CL lead to mitochondrial dysfunction and diseases like Barth syndrome (20, 21).

CL synthesis is restricted to mitochondria (22–27), whereas in yeast, PE biosynthesis occurs in different cellular compartments, including mitochondria, the Golgi/vacuolar compartment, and the endoplasmic reticulum with its mitochondria-associated membrane fraction (28–39). The mitochondrial phosphatidylserine decarboxylase (Psd1) synthesizes the majority of PE, which is not only supplied to mitochondrial but also to other cellular membranes (1, 40). Because the substrate of Psd1, phosphatidylserine (PS), is synthesized in the endoplasmic reticulum/mitochondria-associated membrane, transport to the site of enzymatic conversion is required. Whether

\* This work was supported by the Austrian Science Fund (project 21429 and DK Molecular Enzymology W901-B05 (to G. D.)), the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, and the Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS).

<sup>1</sup> To whom correspondence may be addressed: Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, Stefan-Meier-Str. 17, D-79104 Freiburg, Germany. Tel.: 49-761-203-5243; Fax: 49-761-203-5261; E-mail: thomas.becker@biochemie.uni-freiburg.de.

<sup>2</sup> To whom correspondence may be addressed: Institut für Biochemie, Technische Universität Graz, Petersgasse 12/2, A-8010 Graz, Austria. Tel.: 43-316-873-6462; Fax: 43-316-873-6952; E-mail: guenther.daum@tugraz.at.

<sup>3</sup> The abbreviations used are: PE, phosphatidylethanolamine; CL, cardiolipin; Psd1<sub>MET</sub>, phosphatidylserine decarboxylase 1 containing six C-terminal methionines; PS, phosphatidylserine; TOM, translocase of the outer mitochondrial membrane; MPP, mitochondrial processing peptidase.

**TABLE 1**  
Strains used in this study

| Strain              | Genotype   | Source/Reference |
|---------------------|--|------------------|
| Wild type           | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>                                 | Euroscarf        |
| <i>psd1Δ</i>        | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4</i>                   | Euroscarf        |
| WT+pYES2            | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> + pYES2                         | This study       |
| <i>psd1Δ</i> +pYES2 | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4</i> + pYES2           | This study       |
| Psd1HA              | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4</i> + pYES2-Psd1HA    | This study       |
| Psd1S463A           | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4</i> + pYES2-Psd1S463A | This study       |
| Psd1ΔIM             | BY4741 <i>MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4</i> + pYES2-Psd1ΔIM   | This study       |

this translocation process involves membrane contact sites like the endoplasmic reticulum-mitochondria tethering complex ERMES is under debate (41–43). Deletion of *PSD1* leads to ethanolamine auxotrophy during growth on non-fermentable carbon sources, reduced growth on fermentable media, and morphological alterations of mitochondria (44, 45). Moreover, Psd1 is required for the regulation of pleiotropic drug resistance by inducing *PDR5* gene expression (46). Although extramitochondrially synthesized PE can be imported into mitochondria, defects of *psd1Δ* strains cannot be completely compensated by this process, indicating that PE uptake by mitochondria has limited efficiency (44, 47).

Phosphatidylserine decarboxylases form a protein family, which is highly conserved from bacteria to humans (48). Psd1 from various cell types share a characteristic LGST motif, which is required for autocatalytic cleavage of the enzyme into  $\alpha$ - and  $\beta$ -subunits (48–50). This process has been described in some detail for Psd1 homologs of various species (9, 46, 51–54). It starts with an ester bond formation between Gly-253 and Ser-254 followed by  $\alpha,\beta$ -elimination and release of the mature  $\beta$ -subunit, leaving a dehydroalanine residue at the N terminus of the  $\alpha$ -subunit. Hydration and subsequent elimination of ammonia lead to formation of a pyruvoyl prosthetic group at the N terminus of the  $\alpha$ -subunit (9, 48, 50, 51). Mutations of the highly conserved LGST motif were shown to cause defects in Psd1 processing and formation of an inactive form of the enzyme (9, 46, 53). Surprisingly, such defects have no effect on the induction of *PDR5* expression for regulating multidrug resistance, suggesting that this process may occur independently of PS decarboxylation (46).

Although Psd1 is of central importance for the generation of cellular PE, its biogenesis is only poorly understood. Like the vast majority of mitochondrial proteins, Psd1 is synthesized on cytosolic ribosomes as a precursor containing a cleavable signal sequence (36). Upon *in vitro* import into isolated yeast mitochondria, three proteolytic products are formed from the Psd1 precursor, indicating a stepwise processing of the polypeptide (55). Psd1 contains a hydrophobic stretch, which was proposed to target the polypeptide to the inner mitochondrial membrane (49, 50, 56). It was assumed that this putative inner membrane sorting sequence is removed upon import, yielding the mature Psd1 localized to the mitochondrial inner membrane/intermembrane space (50, 55). This view was supported by enzymatic analyses assigning the enzyme activity to the intermembrane space site of the inner membrane (31, 57, 58). However, experimental evidence for the functionality of the putative inner membrane sorting sequence, the import route of the Psd1 precursor, and the nature of the three processed Psd1 forms

was missing. Moreover, the topology of the  $\alpha$ - and  $\beta$ -subunits of Psd1 remained unclear.

In a detailed biochemical study presented here, we report that in yeast the Psd1  $\beta$ -subunit (Psd1 $\beta$ ) is integrated into the mitochondrial inner membrane and serves as an anchor for the intermembrane space-localized  $\alpha$ -subunit (Psd1 $\alpha$ ). Analysis of the individual processing steps of Psd1 showed involvement of the matrix-localized peptidases mitochondrial processing peptidase (MPP) and Oct1, which remove the N-terminal signal peptides from Psd1. We demonstrate that lack of the hydrophobic stretch leads to mislocalization and partial inactivation of the enzyme. In contrast, self-cleavage of Psd1 into  $\alpha$ - and  $\beta$ -subunits can occur upon mislocalization within mitochondria but strictly depends on the LGST motif and mitochondrial membranes. Thus, correct integration of the Psd1  $\beta$ -subunit into the inner membrane is essential for proper enzymatic function.

## EXPERIMENTAL PROCEDURES

**Strains and Culture Conditions**—Psd1 mutant strains and plasmids used in this study are listed in Table 1. Deletion strains of mitochondrial proteases, *mas1* temperature-sensitive strain, TOM70<sub>His</sub>, TOM40<sub>HA</sub>, TOM22<sub>His</sub>, *tom22Δ*, *tom20Δ*, and *tom70Δ* strains, and their corresponding wild types have been described before (59–62). The reference wild type strain for *oct1Δ* is a re-expression of *OCT1* in the *oct1Δ* background (63). Yeast cells were grown under aerobic conditions to the logarithmic growth phase at 24 or 30 °C on YPG or YPS medium (1% yeast extract (Oxoid), 2% peptone (Oxoid), 3% glycerol (Roth), or 2% sucrose adjusted to pH 5.0 with HCl). The temperature-sensitive phenotype of a *mas1* temperature-sensitive mutant strain was induced by growth for 6 h at non-permissive temperature (61). In the case of yeast strains transformed with expression plasmids, cells were grown on minimal medium minus uracil containing 2% galactose (Roth), 0.67% yeast nitrogen base without amino acids (U. S. Biological), and 0.063% amino acid mix without uracil (Roth, Fluka).

**Plasmid and Strain Constructions**—For the generation of a yeast strain expressing a C-terminally HA-tagged Psd1, *PSD1* was amplified using gene-specific primers from genomic DNA in a standard PCR mixture containing *ExTaq* DNA polymerase (Takara). The purified PCR product was inserted via BamHI and NotI (Fermentas) into the pYES2 vector (Invitrogen), leading to pYES2-PSD1HA. For the generation of Psd1S463A and Psd1ΔIM (residues from Val-81 to Ser-100 were deleted), pYES2-PSD1HA was used as a template either for site-directed mutagenesis (QuikChange XL site-directed mutagenesis kit; Stratagene) or overlap-extension PCR. A *psd1Δ* strain was

transformed with the described constructs by lithium acetate transformation (64), generating Psd1HA, Psd1S463A, and Psd1ΔIM.

**Isolation of Mitochondria and Submitochondrial Localization of Psd1**—Mitochondria were isolated by differential centrifugation according to standard procedures (4, 65). Mitochondrial fractions were adjusted to a protein concentration of 10 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS-KOH (pH 7.2)), and aliquots were shock-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Enrichment of marker proteins and cross-contamination of subcellular fractions were assessed as described (4).

The submitochondrial localization of Psd1 was determined by accessibility of Psd1 to externally added proteinase K in intact, hypotonically swollen or lysed mitochondria. For hypotonic swelling of mitochondria, mitoplasts were generated by treating mitochondria with a 9:1 ratio of EM buffer (10 mM MOPS-KOH (pH 7.2) and 1 mM EDTA) and SEM buffer for 10 min on ice. Subsequently, samples were treated with 20–50  $\mu\text{g}/\text{ml}$  proteinase K for 15 min on ice. For lysis, mitochondria were treated with Triton X-100 at a final concentration of 0.5% (v/v) prior to the addition of proteinase K. In general, proteinase K activity was stopped by the addition of 2 mM PMSF and incubation for 10 min on ice. Subsequently, mitochondria were re-isolated by centrifugation ( $16,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ) and washed with SEM buffer. Samples were subjected to SDS-PAGE and Western blot analysis. To determine membrane association of proteins, carbonate extraction was used as described previously (65–68). In brief, isolated mitochondria were resuspended in freshly prepared 0.1 M sodium carbonate buffer at pH 10.8 or pH 11.5 and incubated on ice for 30 min. Mitochondrial membranes were re-isolated by ultracentrifugation ( $100,000 \times g$ , 40 min,  $4^{\circ}\text{C}$ ). The pellet was solubilized in SDS-PAGE loading dye, whereas proteins remaining in the supernatant were precipitated by trichloroacetic acid. Samples were subjected to SDS-PAGE and Western blot analysis.

**Import of Precursor Proteins into Isolated Mitochondria and Microsomes**—For *in vitro* transcription, a PCR-generated template containing the SP6 promoter was used. RNA was purified (MEGAclear kit; Invitrogen) and used for *in vitro* translation (TNT kit, Promega) in the presence of  $^{35}\text{S}$ -labeled methionine. Import of  $^{35}\text{S}$ -labeled precursor proteins into isolated yeast mitochondria (corresponding to 50  $\mu\text{g}$  of protein content) was performed at  $25^{\circ}\text{C}$  in the presence of 2 mM NADH, 2 mM ATP, and an ATP-regenerating system (5 mM creatine phosphate and 0.1 mg/ml creatine kinase) in import buffer (3% BSA (w/v), 250 mM sucrose, 80 mM KCl, 5 mM  $\text{MgCl}_2$ , 2 mM  $\text{KH}_2\text{PO}_4$ , 5 mM methionine, 10 mM MOPS-KOH (pH 7.2)) (65). Import reactions were stopped on ice and by dissipation of the membrane potential with 8  $\mu\text{M}$  antimycin A, 1  $\mu\text{M}$  valinomycin, and 20  $\mu\text{M}$  oligomycin. For protease treatment, samples were incubated with 50  $\mu\text{g}/\text{ml}$  proteinase K for 15 min on ice. Mitochondria were re-isolated by centrifugation ( $16,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ) and washed with SEM buffer. For pulse-chase experiments,  $^{35}\text{S}$ -labeled Psd1 was imported into mitochondria for 5 min under standard import conditions (pulse). Subsequently, mitochondria were re-isolated, washed, and incubated a second time under import conditions but without the addition of new

$^{35}\text{S}$ -labeled Psd1 precursor (chase). Samples were subjected to SDS-PAGE, and  $^{35}\text{S}$ -labeled proteins were detected by autoradiography. For blue native electrophoresis, mitochondria were solubilized with digitonin buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, 10% glycerol) containing 1% (w/v) digitonin. After a clarifying spin, mitochondrial protein complexes were separated by blue native electrophoresis as described (69, 70).

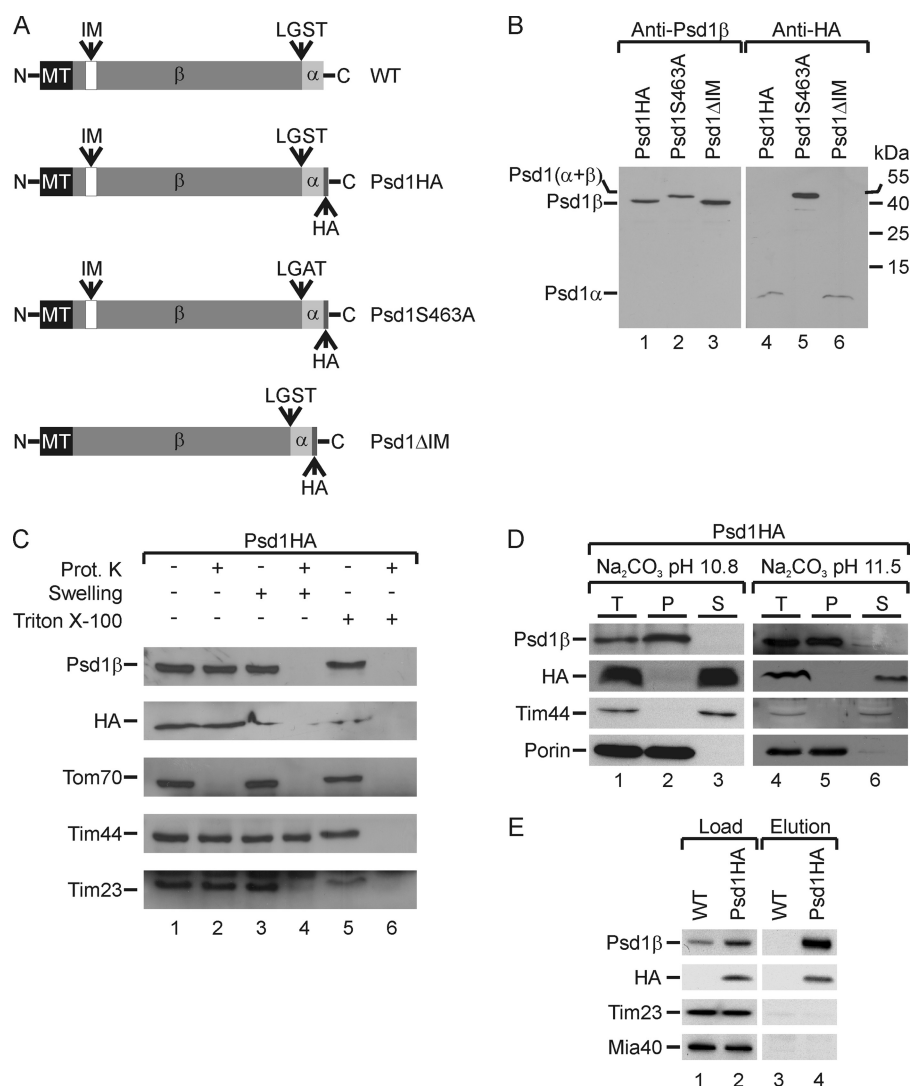
The import of  $^{35}\text{S}$ -labeled precursor proteins into dog pancreatic microsomes (Promega) was carried out following the standard import protocol for isolated mitochondria. Membranes were re-isolated by centrifugation ( $125,000 \times g$ , 30 min,  $4^{\circ}\text{C}$ ) and washed with SEM buffer. Membranes were lysed under denaturing conditions and subjected to SDS-PAGE analysis.

**Affinity Purification of Tagged Proteins**—Mitochondria were solubilized in digitonin buffer containing 1% (w/v) digitonin at a protein concentration of 1 mg/ml for 20 min on ice. After a clarifying spin, the supernatant was incubated with an anti-HA matrix (Roche Applied Science) under constant rotation for 1 h at  $4^{\circ}\text{C}$ . Following several washing steps with digitonin buffer containing 0.3% (w/v) digitonin, proteins were eluted and subjected to SDS-PAGE, Western blot analysis, and immunodetection with the indicated antisera. For affinity purification of arrested precursor,  $^{35}\text{S}$ -labeled Psd1 was imported in the absence of a membrane potential into mitochondria containing Tom22<sub>His</sub>, Tom40<sub>HA</sub>, or Tom70<sub>His</sub>. Subsequently, pull-down experiments were performed as described (59–62).

**Phospholipid Analysis**—Lipids were extracted from total cell free homogenate and mitochondria by the procedure of Folch *et al.* (1957) using chloroform/methanol (2:1, v/v) (71). Remaining contaminants (e.g. proteins and salts) were removed by additional washing steps of the organic phase with 0.034%  $\text{MgCl}_2$  solution (w/v), 2 N KCl, methanol (4:1, v/v), and methanol/water/chloroform (48:47:3, v/v/v), respectively. Individual phospholipids were separated by two-dimensional thin layer chromatography on Silica gel 60 plates (Merck) by using chloroform, methanol, 25%  $\text{NH}_3$  (68:35:5, v/v/v) as the first and chloroform/acetone/methanol/acetic acid/water (53:20:10:10:5, v/v/v/v) as the second developing solvent. Phospholipids were visualized on thin-layer chromatography plates by staining with iodine vapor, scraped off, and quantified as described (72).

**Psd1 Activity Assay**—The enzymatic activity of Psd1 was determined *in vitro* by measuring the conversion of  $^3\text{H}$ -labeled PS to  $^3\text{H}$ -labeled PE in isolated mitochondria from yeast cells grown to the logarithmic growth phase as reported by Kuchler *et al.* (31). Radioactively labeled [ $^3\text{H}$ ]PS was synthesized *in vitro* by incubating isolated yeast microsomes (8 mg of protein) with 0.02/0.2 mM CDP-DAG, 5 mM hydroxylamine, 0.6 mM  $\text{MnCl}_2$ , 0.2% Triton X-100, 0.5 mM L-serine, 0.01  $\mu\text{Ci}$  of L-[ $^3\text{H}$ ]serine (specific activity 21.99 Ci/mmol), and 0.1 M Tris-HCl (pH 8.0) in a total volume of 2 ml for 2 h at  $30^{\circ}\text{C}$ . The reaction was stopped by the addition of 3 ml of chloroform/methanol (2:1, v/v), and lipids were extracted as described previously (71). The Psd1 activity assay was performed in a final volume of 1.5 ml containing 100 nmol of [ $^3\text{H}$ ]PS (specific activity, 0.64 nCi/nmol), 0.1 M Tris-HCl (pH 7.2), 10 mM EDTA, and 1 mg of protein from isolated mitochondria (31, 47). Incubations





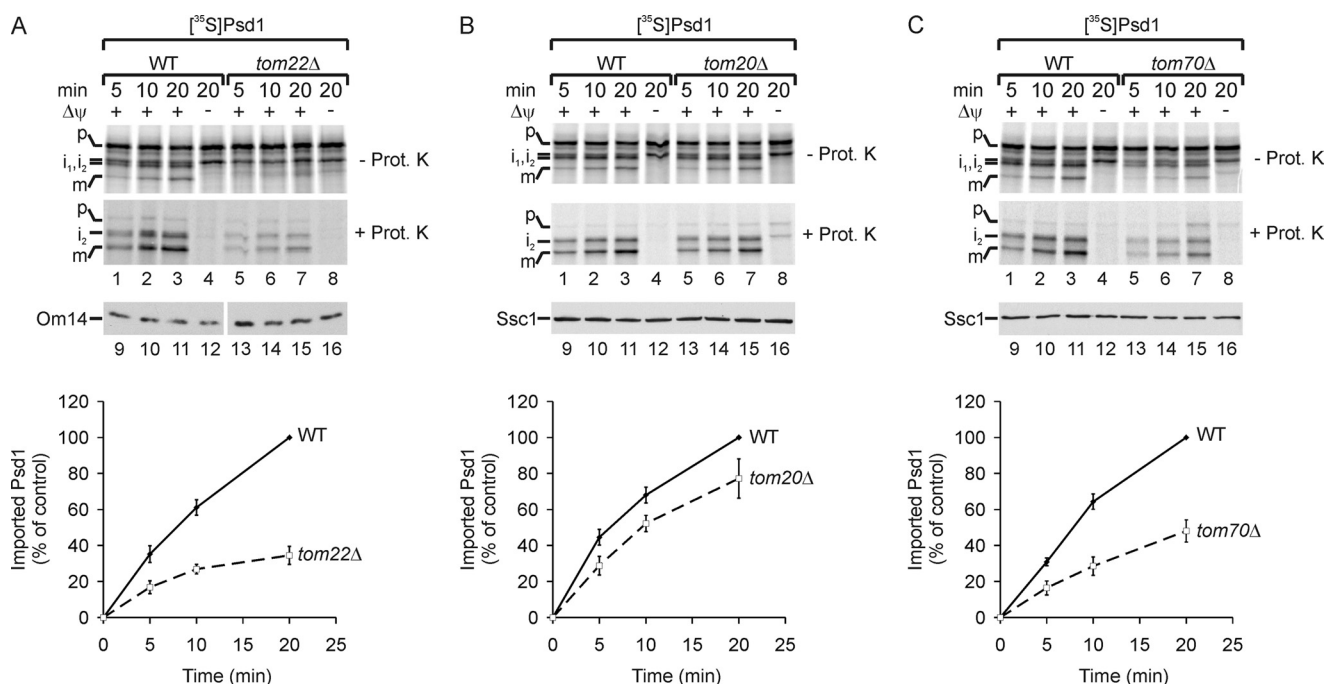
**FIGURE 1. The  $\beta$ -subunit of Psd1 tethers the Psd1  $\alpha$ -subunit to the inner membrane.** *A*, the Psd1 constructs used are shown schematically. *MT*, predicted mitochondrial targeting sequence; *IM*, putative inner membrane sorting signal. *B*, mitochondrial proteins from yeast strains expressing Psd1HA, Psd1S463A, or Psd1 $\Delta$ IM were separated by SDS-PAGE and visualized by immunodetection with the indicated antibodies. *C*, intact, swollen, or lysed (with Triton X-100) mitochondria were incubated with or without proteinase K. Subsequently, samples were subjected to SDS-PAGE, and the indicated components were visualized by immunodetection with the respective antisera. *D*, mitochondria were subjected to carbonate extraction. Total (*T*), membrane-bound (*P*), and soluble proteins (*S*) were separated by SDS-PAGE, and components were visualized by immunodetection with the indicated antisera. *E*, Psd1HA was precipitated with an anti-HA affinity matrix. Load (5%) and eluted proteins (100%) were separated by SDS-PAGE, and proteins were visualized by immunodetection with the indicated antisera.

were carried out for 7 min at 30 °C and terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v). Lipids were extracted, and individual phospholipids were separated as described above. After visualization with iodine vapor, PE was scraped off, and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker) with 5% water as scintillation mixture.

## RESULTS

**The  $\beta$ -Subunit of Psd1 Anchors the  $\alpha$ -Subunit to the Inner Mitochondrial Membrane**—A special feature of Psd1 maturation is its structural arrangement in the form of an  $\alpha$ - and a  $\beta$ -subunit (Fig. 1, *A* and *B*, lanes 1 and 4). Interestingly, the precise topology of Psd1, especially of the two non-identical subunits within mitochondrial compartments, has not yet been determined. To address this issue in detail, we used a specific

antibody recognizing the  $\beta$ -subunit of the protein (Fig. 1*B*, lane 1) and generated a yeast strain expressing Psd1 with an HA tag C-terminally fused to the  $\alpha$ -subunit (Fig. 1*A*). HA tagging did not affect maturation of Psd1 because the mature  $\beta$ -subunit of Psd1-HA behaved like wild type Psd1 (data not shown). Use of an HA-specific antibody allowed us to detect a 4-kDa  $\alpha$ -subunit of Psd1 in isolated mitochondria (Fig. 1*B*, lane 4). The appearance of the  $\alpha$ -subunit was not affected by deletion of the predicted inner membrane sorting signal of Psd1 (Psd1 $\Delta$ IM) (Fig. 1*B*, lane 6). To further test the specificity of both antibodies, we constructed a yeast strain expressing a Psd1S463A variant with a mutation at the LGST cleavage site, which cannot undergo autocatalytic processing (Fig. 1*A*) (9, 46, 53). As expected, both antibodies recognized unprocessed Psd1 (Fig. 1*B*, lanes 2 and 5) and could therefore be used to determine the submitochondrial localization of both Psd1 subunits.



**FIGURE 2. Tom70 and Tom22 are the main receptors for the import of Psd1 precursor into mitochondria.** A–C,  $^{35}\text{S}$ -labeled Psd1 precursor was imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into mitochondria from wild type, *tom22Δ* (A), *tom20Δ* (B), or *tom70Δ* (C). Subsequently, mitochondria were incubated with or without proteinase K (Prot. K).  $^{35}\text{S}$ -Labeled proteins were detected by autoradiography. The time-dependent formation of the mature form of Psd1  $\beta$  ( $m$ ) in mitochondria was quantified by using ImageJ. The longest import time point of Psd1  $\beta$  ( $m$ ) into wild type mitochondria was set to 100%. Mean values of four measurements with S.E. (error bars) are shown. Immunodetection with the indicated antisera was used as a loading control.  $p$ , precursor;  $i_1$ ,  $i_2$ , intermediates 1 and 2;  $m$ , mature Psd1  $\beta$ .

For this purpose, we treated intact, hypotonically swollen or lysed mitochondria with proteinase K (Fig. 1C). Both  $\alpha$ - and  $\beta$ -subunits were protected in intact mitochondria but accessible to the protease after rupture of the outer membrane by osmotic swelling (Fig. 1C, lanes 2 and 4). Similarly, the inner membrane protein Tim23 with a domain exposed to the intermembrane space was degraded after rupture of the outer membrane (Fig. 1C, lanes 2 and 4). The outer membrane protein Tom70 was proteolytically cleaved on intact mitochondria, whereas the matrix-localized Tim44 was digested only after lysis of mitochondria with detergent (Fig. 1C, lane 6). These results indicate that both subunits of Psd1 face the intermembrane space, which is in line with the reported detection of Psd1 activity (31, 57, 58).

Previously, it was speculated that Psd1 might be released into the intermembrane space by cleavage of a proposed inner membrane sorting signal (50, 55), although experimental evidence for this hypothesis was missing. To test a possible membrane association of Psd1, we used a well established alkaline extraction method (66, 67). This approach revealed that the Psd1  $\beta$ -subunit (Psd1 $\beta$ ) remained membrane-associated after carbonate extraction at pH 10.8 and pH 11.5 similar to the outer membrane protein porin, whereas the peripheral membrane protein Tim44 was efficiently removed (Fig. 1D). We concluded from this result that the  $\beta$ -subunit was integrated into the inner mitochondrial membrane. In contrast, the  $\alpha$ -subunit was extracted under all tested conditions and therefore localized to the intermembrane space (Fig. 1D). Furthermore, we tested attachment of both subunits to each other after autocatalytic processing in the *PSD1-HA* strain by pull-down experiments

utilizing an anti-HA affinity matrix (Fig. 1E). The Psd1  $\beta$ -subunit was efficiently co-purified with the HA-tagged  $\alpha$ -subunit (Fig. 1E, lane 4), indicating stable association of both Psd1 subunits. Thus, the  $\beta$ -subunit of Psd1 contains a membrane anchor tethering the soluble  $\alpha$ -subunit to the inner mitochondrial membrane.

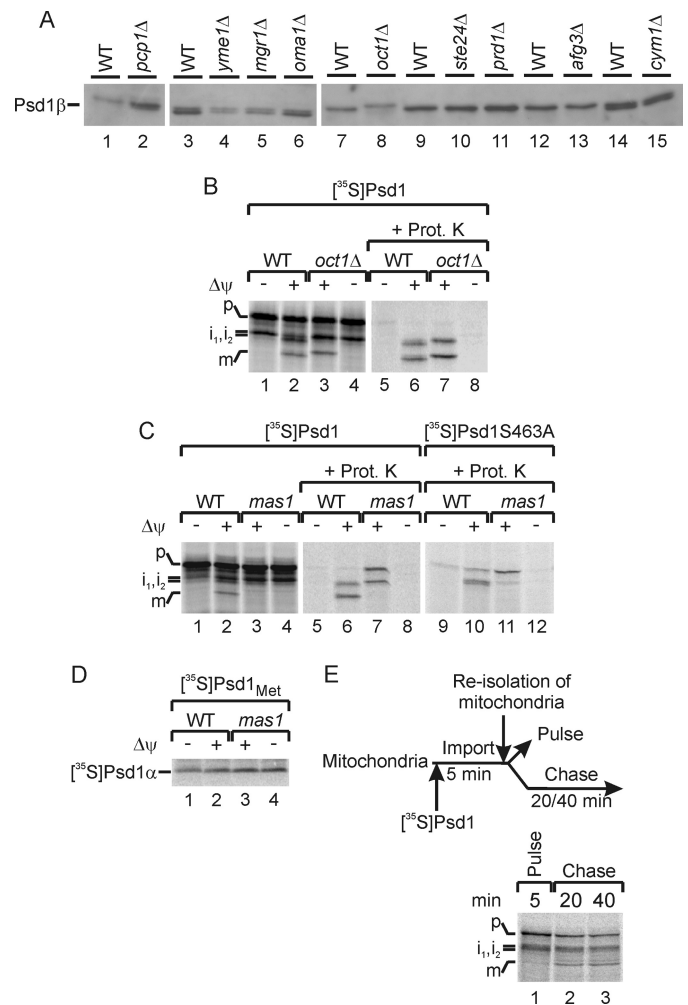
**Tom70 and Tom22 Are the Main Mitochondrial Receptors for the Psd1 Precursor**—Localization of Psd1 to the inner membrane raises the question of the import mechanism of this protein into mitochondria. In general, the majority of mitochondrial proteins are translated on cytosolic ribosomes as precursors with an N-terminal signal sequence, which is sufficient for targeting and import into mitochondria. The translocase of the outer mitochondrial membrane (TOM complex) is crucial for the translocation of precursor proteins across the outer membrane (73–77). First, we tested whether Psd1 was imported via the TOM complex at all by importing  $^{35}\text{S}$ -labeled Psd1 precursor into mitochondria isolated from a yeast strain lacking Tom22 (Fig. 2A). Deletion of Tom22 results in destabilization of the TOM complex and blocks the import of the majority of TOM-dependent substrates into mitochondria (78). Upon import into isolated mitochondria, three forms of Psd1 were detected (Fig. 2A, lanes 1–3), which were absent in the reticulocyte lysate, showing that processing does not occur spontaneously (data not shown). The three fragments represent intermediates 1 and 2 ( $i_1$  and  $i_2$ ) formed by unknown processing steps and presumably the mature Psd1  $\beta$ -subunit ( $m$ ) (Fig. 2A, lanes 1–3) (55). Intermediate 1 was not completely taken up by mitochondria because it was accessible to externally added proteinase K and predominantly formed in the absence of a mem-

brane potential (Fig. 2A, lanes 1–4). In contrast, intermediate 2 and the mature Psd1 $\beta$  were only formed in the presence of the membrane potential and showed protease resistance (Fig. 2A, lanes 1–3). Thus, we conclude that intermediate 2 and the mature Psd1 $\beta$  were fully imported into mitochondria. In the absence of Tom22, import of the precursor into isolated mitochondria was strongly reduced, indicating the general requirement of the TOM complex for biogenesis of Psd1 (Fig. 2A). The TOM complex has two peripheral receptors, Tom20 and Tom70. Tom20 was reported to recognize precursor proteins with cleavable signal information, whereas Tom70 mainly interacts with hydrophobic precursors harboring internal non-cleavable targeting information (3, 73–79). To test which peripheral receptor was involved in the import of Psd1 precursor, we performed import experiments with mitochondria from yeast strains lacking either Tom20 or Tom70. Surprisingly, import of Psd1 into mitochondria lacking Tom20 was only mildly affected (Fig. 2B) but significantly reduced in the absence of Tom70 (Fig. 2C). Thus, although Psd1 harbors a cleavable signal sequence, Tom70 and Tom22 are the main receptors for the import of Psd1 precursor into mitochondria.

**MPP and Oct1 Cleave the Signal Sequence of Psd1**—The import of  $^{35}\text{S}$ -labeled Psd1 precursor into mitochondria leads to formation of three fragments (see Fig. 2A) (53, 55). However, the molecular mechanism of proteolytic cleavage and the nature of these fragments remained unclear. Previously, it was speculated that the inner membrane sorting sequence might be removed by a protease like Yme1 (50, 55). However, the observed membrane integration of the  $\beta$ -subunit points to a different processing mode. To identify the processing proteases required for Psd1 maturation, we analyzed the protein pattern of Psd1 in mitochondria from strains lacking described or predicted mitochondrial proteases (61) using the antibody specific for Psd1  $\beta$ -subunit (Fig. 3A). Surprisingly, deletion of Yme1 did not affect the size of mature Psd1 in mitochondria at all (Fig. 3A, lane 4). Also processing of freshly imported Psd1 was not affected in the absence of Yme1 (data not shown). Similarly, the inner membrane proteases Imp1 and Imp2, both required for maturation of mitochondrial proteins destined for the inter-membrane space (74, 76, 80, 81), had no effect on Psd1 maturation (data not shown).

Whereas deletion of several other mitochondrial proteases did not affect the formation of Psd1 $\beta$ , a small size shift of Psd1 was observed in mitochondria lacking Oct1 (octapeptidyl aminopeptidase) (Fig. 3A, lane 8). It is known that the matrix-localized Oct1 typically removes an octapeptide from a precursor intermediate after cleavage by MPP to stabilize the client protein (63). To confirm processing of the Psd1 precursor by Oct1, we imported  $^{35}\text{S}$ -labeled Psd1 into mitochondria lacking this protease (Fig. 3B). We observed a size shift of intermediate 2 ( $i_2$ ) and of the mature Psd1  $\beta$ -subunit ( $m$ ), indicating that Oct1 indeed removed a peptide from the Psd1 precursor (Fig. 3B, lanes 3 and 7).

The amino acid sequence of the Psd1 precursor suggests the presence of a classical N-terminal mitochondrial signal sequence (36). Such signal sequences are typically removed by MPP of the mitochondrial matrix, which consists of the two essential subunits Mas1 and Mas2 (74, 80, 81). To address the



**FIGURE 3. MPP and Oct1 process the Psd1 precursor.** A, mitochondrial proteins from several deletion strains were analyzed on SDS-PAGE. The  $\beta$ -subunit of Psd1 was visualized by immunodetection with a Psd1-specific antibody. B,  $^{35}\text{S}$ -labeled Psd1 precursor was imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into mitochondria from wild type or *oct1* $\Delta$ . Subsequently, mitochondria were incubated with or without proteinase K (Prot. K).  $^{35}\text{S}$ -Labeled proteins were detected by autoradiography. P, precursor;  $i_1$ ,  $i_2$ , intermediates 1 and 2; m, mature Psd1 $\beta$ . C,  $^{35}\text{S}$ -labeled Psd1 and Psd1S463A precursor were imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into mitochondria from wild type or *mas1*ts mutant strain. Subsequently, mitochondria were incubated with or without proteinase K.  $^{35}\text{S}$ -Labeled proteins were detected by autoradiography. D,  $^{35}\text{S}$ -labeled Psd1<sub>Met</sub> containing six methionines at the C terminus of Psd1 was imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into mitochondria from wild type or *mas1*ts mutant strain.  $^{35}\text{S}$ -Labeled Psd1 $\alpha$  was detected by autoradiography. E, experimental scheme and pulse-chase experiment of  $^{35}\text{S}$ -labeled Psd1 into wild type mitochondria.  $^{35}\text{S}$ -Labeled Psd1 was imported into wild type mitochondria for 5 min at 25 °C under standard import conditions (lane 1; Pulse). After re-isolation and washing steps, mitochondria were further incubated under import conditions but without the addition of new  $^{35}\text{S}$ -labeled precursor (lanes 2 and 3; Chase).  $^{35}\text{S}$ -Labeled proteins were detected by autoradiography.

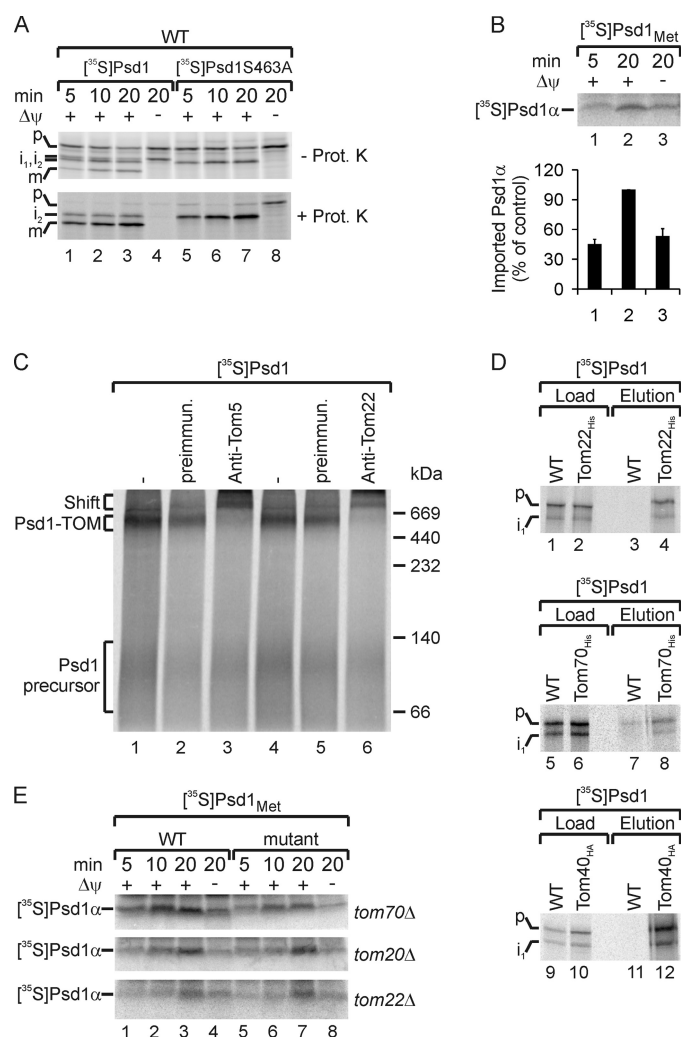
role of MPP in processing of Psd1, we imported the Psd1 precursor into mitochondria isolated from a temperature-sensitive *Mas1* mutant (61, 62). *Mas1*ts was inactivated *in vivo* by shifting the culture to non-permissive growth conditions. In isolated mitochondria of the *in vivo* heat-shocked *mas1*ts cells, removal of the signal peptide from intermediate 2 and the mature Psd1 $\beta$  was blocked (Fig. 3C, lanes 3 and 7). Thus, the N-terminal presequence of the Psd1 precursor is cleaved by the matrix-located proteases MPP and Oct1, yielding Psd1 $\beta$  ( $m$ ).



So far, we only speculated that the mature Psd1 $\beta$  is formed by self-processing, which leads to the formation of two protease-protected bands upon import of Psd1 in *mas1ts* (Fig. 3C, lane 7). To test this notion experimentally, we imported a Psd1 variant into wild type and *mas1ts* mitochondria, which contains a point mutation in the conserved LGST motif to prevent autocatalytic cleavage (9, 46, 53). This Psd1S463A construct was imported with the same efficiency as wild type, but cleavage into  $\alpha$ - and  $\beta$ -subunit was blocked (Fig. 3C, lane 10). In *mas1ts* mitochondria, only the upper band was formed, indicating that the mature Psd1 $\beta$  emerged upon self-cleavage in an MPP-independent manner (Fig. 3C, lane 11). To confirm this conclusion, we analyzed formation of the  $\alpha$ -subunit of Psd1 in *mas1ts* mitochondria directly. The C terminus of Psd1 was tagged with six methionines to allow detection of  $^{35}\text{S}$ -labeled Psd1 $\alpha$ . This Psd1<sub>Met</sub> construct was imported like wild type Psd1 (data not shown), and the formation of Psd1 $\alpha$  with a size of 4 kDa was not affected in *mas1ts* mitochondria (Fig. 3D, lanes 3 and 4). To determine at which import step autocatalysis occurred, we performed a pulse-chase experiment with  $^{35}\text{S}$ -labeled Psd1 (Fig. 3E).  $^{35}\text{S}$ -Labeled Psd1 was imported for a short time period (Fig. 3E, lane 1, Pulse). Subsequently, mitochondria were re-isolated and further incubated under import conditions but without addition of new  $^{35}\text{S}$ -labeled precursor (Fig. 3E, lanes 2 and 3, Chase). Interestingly, the mature Psd1 $\beta$  was formed more efficiently after the chase but only to a minor extent during the short pulse period (Fig. 3E). This observation indicated that self-cleavage of Psd1 occurs at late import time points and follows processing by MPP and Oct1 under normal import conditions.

**TOM Complex-arrested Psd1 Precursor Is Processed into  $\alpha$ - and  $\beta$ -Subunit**—Strikingly, Psd1 $\alpha$  is also formed in the absence of a membrane potential, which indicates that self-cleavage of Psd1 can occur independently of its import into mitochondria (Fig. 3D, lane 1). Because intermediate 1 is also formed in the absence of a membrane potential (Fig. 3B, lane 1), we wondered whether intermediate 1 represented a self-processed Psd1 precursor. To address this issue, we performed import experiments with the Psd1S463A precursor in wild type mitochondria (Fig. 4A, lanes 5–8). Interestingly, intermediate 1 was not generated during import of the Psd1S463A precursor, demonstrating that intermediate 1 is formed by self-processing of Psd1 into  $\alpha$ - and  $\beta$ -subunit. To assess the efficiency of the self-cleavage by arrested Psd1, we quantified the formation of Psd1 $\alpha$  in the presence and absence of a membrane potential (Fig. 4B). We found that albeit import into mitochondria favors self-processing, a marked amount of arrested Psd1 precursor undergoes autocatalysis as well (Fig. 4B, lane 3).

It has been shown before that upon depletion of the membrane potential, some hydrophobic precursors accumulate at the TOM complex (12, 82). To test whether or not the arrested Psd1 precursor binds to the TOM complex as well, we incubated  $^{35}\text{S}$ -labeled Psd1 precursor with isolated mitochondria in the absence of a membrane potential (Fig. 4C). After the import, mitochondria were lysed using the mild detergent digitonin, and samples were analyzed by blue native electrophoresis to detect the TOM-bound intermediate. In the absence of a membrane potential, the Psd1 precursor was stably bound to



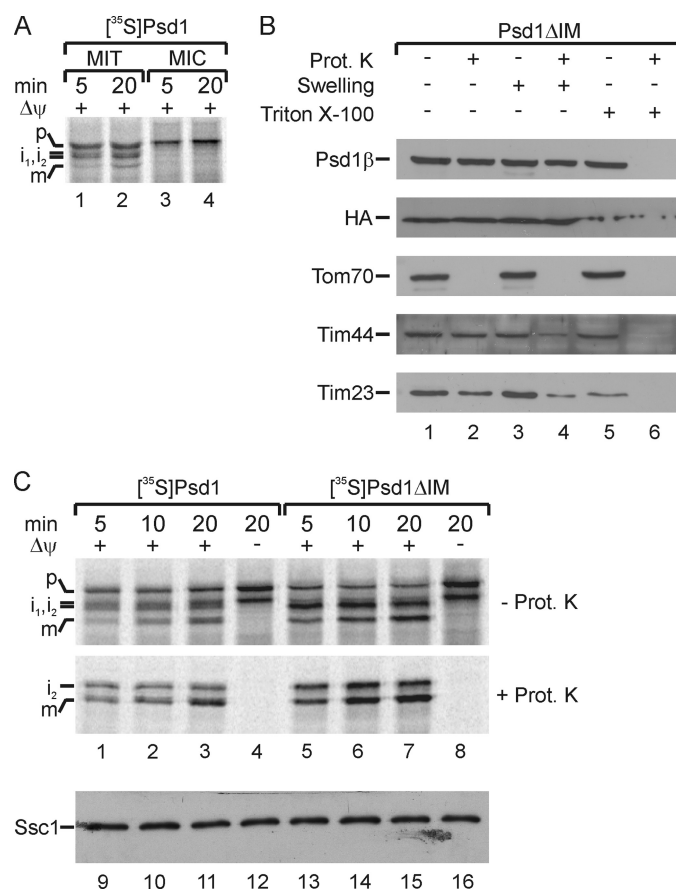
**FIGURE 4. TOM complex-arrested Psd1 precursor undergoes autocatalytic processing.** A,  $^{35}\text{S}$ -labeled Psd1 and Psd1S463A precursor were imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into wild type mitochondria. Subsequently, mitochondria were incubated with or without proteinase K (Prot. K).  $^{35}\text{S}$ -Labeled proteins were detected by autoradiography. p, precursor;  $i_1$ ,  $i_2$ , intermediates 1 and 2; m, mature Psd1 $\beta$ . B,  $^{35}\text{S}$ -labeled Psd1<sub>Met</sub> containing six methionines at the C terminus of Psd1 was imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into mitochondria from wild type.  $^{35}\text{S}$ -Labeled Psd1 $\alpha$  was detected by autoradiography. The time-dependent formation of Psd1 $\alpha$  in mitochondria was quantified by using ImageJ. The longest import time point of Psd1 $\alpha$  into wild type mitochondria in the presence of a membrane potential was set to 100%. Mean values of three measurements with S.E. (error bars) are shown. C,  $^{35}\text{S}$ -labeled Psd1 precursor was imported in the absence of a membrane potential into wild type mitochondria. Mitochondria were incubated with or without antibodies against Tom5 or Tom22 or preimmune sera as a control. Subsequently, protein complexes of lysed mitochondria were separated by blue native gel electrophoresis.  $^{35}\text{S}$ -Labeled proteins were detected by autoradiography. D,  $^{35}\text{S}$ -labeled Psd1 precursor was imported in the absence of a membrane potential into wild type, Tom22<sup>His</sup>, Tom70<sup>His</sup>, and Tom40<sup>HA</sup> mitochondria. Subsequently, mitochondria were lysed and subjected to affinity purification utilizing the His and HA tag. Load (4%) and eluted proteins (100%) were separated by SDS-PAGE and analyzed by autoradiography.  $i_1$ , intermediate 1. E,  $^{35}\text{S}$ -labeled Psd1<sub>Met</sub> precursor was imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into mitochondria from wild type, *tom70Δ*, *tom20Δ*, and *tom22Δ*.  $^{35}\text{S}$ -Labeled Psd1 $\alpha$  was detected by autoradiography.

the TOM complex, as shown by a size shift of the intermediate after adding antibodies against Tom5 or Tom22 to mitochondria (Fig. 4C, lanes 3 and 6). To analyze whether both precursor and intermediate 1 bind to the TOM complex, we arrested  $^{35}\text{S}$ -labeled Psd1 at the translocases in mitochondria containing

His-tagged Tom22, Tom70, or HA-tagged Tom40 and performed affinity purification (Fig. 4D). These experiments revealed that intermediate 1 and unprocessed precursors were co-purified with tagged TOM subunits (Fig. 4D, lanes 4, 8, and 12). To determine if Tom receptors play a role in self-cleavage of Psd1, we analyzed the formation of Psd1 $\alpha$  in mutants lacking one Tom receptor (Fig. 4E). Only the absence of Tom70 slightly affected Psd1 $\alpha$  formation (Fig. 4E, top). This observation indicated that Tom70 is involved in the initial recognition of the Psd1 precursor, whereas Tom22 is involved in later import steps. Altogether, in the absence of a membrane potential, autocatalytic cleavage of the Psd1 precursor into  $\alpha$ - and  $\beta$ -subunits occurred when the precursor was exposed on the outer membrane and bound to the TOM complex.

**Psd1 Requires Integration into the Inner Mitochondrial Membrane for Full Enzymatic Activity**—The surprising finding that an arrested Psd1 precursor probably undergoes self-processing at the outer membrane tempted us to speculate that separation of  $\alpha$ - and  $\beta$ -subunit may also occur in other mitochondrial compartments or even on other membranes. To test this hypothesis, we followed the processing of  $^{35}$ S-labeled Psd1 precursor after incubation with mitochondria and microsomes under the same import conditions. We could not detect any cleavage of the Psd1 precursor when canine pancreatic microsomes were added (Fig. 5A, lanes 3 and 4). This result indicates that Psd1 maturation depends on mitochondrial membranes, which is consistent with the results obtained with *Plasmodium knowlesi* (PkPSD) (54). We also tested whether sorting of Psd1 to the inner membrane is required for self-processing and function. To address this issue, we generated a yeast strain expressing a Psd1 variant that lacks the predicted inner membrane anchor (Val-81 to Ser-100; Psd1 $\Delta$ IM) (Fig. 1A) (46). Detection of both  $\alpha$ - and  $\beta$ -subunits using the subunit-specific antibodies described above demonstrated that Psd1 $\Delta$ IM undergoes autocatalytic processing (Figs. 1B, lanes 3 and 6, and 5B). In contrast to Psd1HA, the Psd1 $\Delta$ IM  $\alpha$ - and  $\beta$ -subunit were not accessible to externally added proteases after rupturing the outer membrane by osmotic swelling (Fig. 5B, lane 4). Like the matrix-localized Tim44, Psd1 $\Delta$ IM was only degraded by proteinase K when membranes were lysed with detergent (Fig. 5B, lane 6). This result indicated that Psd1 $\Delta$ IM was fully transported to the matrix site of the inner membrane and that the predicted inner membrane anchor was crucial for correct topology. To compare the processing efficiency of both constructs, import experiments with  $^{35}$ S-labeled Psd1 $\Delta$ IM and wild type Psd1 into isolated mitochondria were performed (Fig. 5C). Deletion of the putative inner membrane anchor in Psd1 $\Delta$ IM even stimulated import and processing steps of the precursor by MPP and Oct1 (Fig. 5C, lanes 5–8).

Finally, we determined the effect of Psd1 $\Delta$ IM mislocalization on the activity of the enzyme (Fig. 6A) and on the mitochondrial phospholipid pattern (Fig. 6B). Mitochondria from a strain expressing Psd1S463A did not display detectable decarboxylase activity due to the inability to generate the active site of the enzyme. In contrast, mislocalization of Psd1 by removing the membrane anchor of the  $\beta$ -subunit (Psd1 $\Delta$ IM) led to a reduced enzymatic activity compared with wild type and HA-tagged Psd1 (Fig. 6A). The loss of Psd1 activity in Psd1S463A led to a



**FIGURE 5. Autocatalytic processing of Psd1 is restricted to mitochondria but does not depend on the proper sorting of Psd1.** A,  $^{35}$ S-labeled Psd1 precursor was imported in the presence of a membrane potential ( $\Delta\psi$ ) into wild type mitochondria (MIT) and canine pancreatic microsomes (MIC). P, precursor;  $i_1$ ,  $i_2$ , intermediates 1 and 2; m, mature Psd1 $\beta$ . B, intact, swollen, or lysed (with Triton X-100) mitochondria from a yeast strain expressing Psd1 $\Delta$ IM were incubated with or without proteinase K (Prot. K). Subsequently, samples were subjected to SDS-PAGE, and proteins were visualized by immunodetection with the indicated antisera. C,  $^{35}$ S-labeled Psd1 or Psd1 $\Delta$ IM was imported in the presence or absence of a membrane potential ( $\Delta\psi$ ) into wild type mitochondria. Subsequently, mitochondria were incubated with or without proteinase K.  $^{35}$ S-labeled proteins were detected by autoradiography. For loading controls, the mitochondrial Hsp70 (Ssc1 (stress-seventy subfamily C/mitochondrial Hsp70)) was immunodetected with specific antibodies. p, precursor;  $i_1$  and  $i_2$ , intermediates 1 and 2; m, mature Psd1.

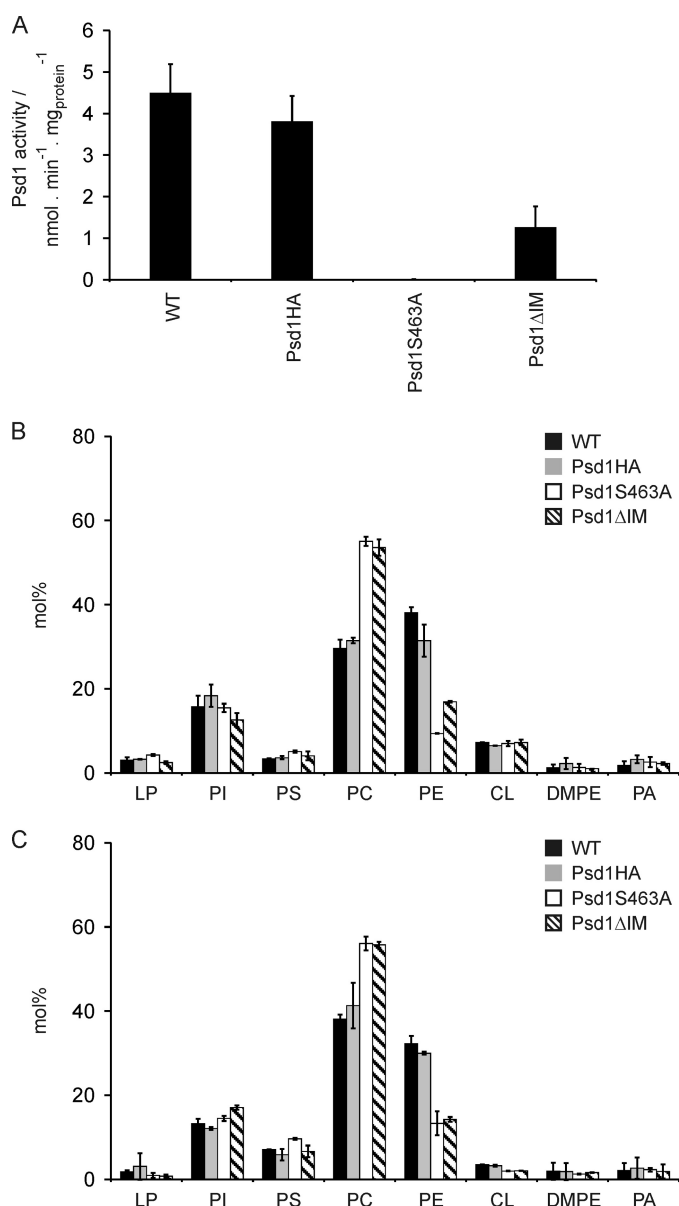
typical *psd1* $\Delta$  phospholipid pattern, which is characterized by an enrichment of phosphatidylcholine at the expense of PE in mitochondria and homogenate (Fig. 6, B and C) (18, 47). The level of PS within mitochondria was not increased because it is used as a substrate for Psd2, which is localized to the Golgi/vacuolar compartment and responsible for extramitochondrially synthesized PE (37, 38).

Interestingly, the phospholipid pattern of Psd1 $\Delta$ IM in homogenate resembled that of Psd1S463A (Fig. 6C). These findings indicated that mislocalization of Psd1 to the mitochondrial matrix leads to a reduced activity of the enzyme (Fig. 6A) and consequently to insufficient supply of mitochondrially synthesized PE to the total cellular PE pool (Fig. 6C).

## DISCUSSION

In yeast, Psd1 catalyzes formation of the majority of cellular PE by decarboxylation of PS (1, 28, 31, 37–39, 83). Deletion of *PSD1* leads to severe cell damage, including alterations of mito-





**FIGURE 6. Inner membrane localization of Psd1 is required for optimal enzymatic activity.** A, phosphatidylserine decarboxylase activity was measured using isolated mitochondria from wild type and the indicated Psd1 mutants. Strains were grown on minimal galactose medium at 30 °C. Mean values of three independent measurements and S.D. values are shown. B and C, phospholipids were extracted from mitochondria (B) and homogenate (C) isolated from wild type and the indicated Psd1 mutants. Strains were grown on minimal galactose medium at 30 °C. LP, lysophospholipids; PI, phosphatidylinositol; PC, phosphatidylcholine; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. Mean values of two independent measurements and S.D. values (error bars) are shown.

chondrial morphology and reduced growth under respiratory conditions caused by dramatically reduced PE levels in mitochondrial and other cellular membranes (44, 45). For this reason, functional Psd1 is highly important to maintain cell integrity and function.

Here we show that correct import and sorting is required for the enzymatic activity of Psd1. In previous experiments, it was shown that GFP-tagged Psd1 localizes to the inner mitochondrial membrane (47). However, the question remained open whether the protein was integrated into the membrane or

sorted into the intermembrane space, especially due to the occurrence of two non-identical subunits (46, 50, 53, 55). In the present study, we demonstrate that the  $\beta$ -subunit of Psd1 is tightly anchored into the inner membrane, most likely via a single transmembrane segment. This conclusion is supported by the prediction of at least one transmembrane region, as reported previously (53, 55). Deletion of this hydrophobic segment (amino acids Val-81 to Ser-100) (46, 54) leads to mislocalization of Psd1 to the matrix side of the inner membrane, partial loss of enzymatic activity, and, consequently, reduced PE levels (Figs. 5B and 6). In such a construct, however, cleavage of the proenzyme to its non-identical subunits was not inhibited (Fig. 1B). This result suggests that not only correct processing of Psd1 but also the membrane environment plays a crucial role for the formation of an active enzyme. Because the soluble  $\alpha$ -subunit harbors the active site of the enzyme or at least an essential part of this domain, the recruitment to the inner membrane surface with orientation to the intermembrane space appears to be crucial for the ability of Psd1 to decarboxylate the polar headgroup of PS. The soluble  $\alpha$ -subunit present in the intermembrane space interacts with the membrane-bound  $\beta$ -subunit (Fig. 1, D and E).

To support the topological model of Psd1 presented in this study, we investigated the mitochondrial import route of this protein and identified components required for its processing. Here, we show that the two matrix-localized processing peptidases MPP and Oct1 remove the targeting signals from the Psd1 precursor (Fig. 3C). Finally, the processed Psd1 undergoes self-cleavage into  $\alpha$ - and  $\beta$ -subunits (Fig. 3E). MPP usually cleaves the mitochondrial signal sequence, and subsequently Oct1 removes a small peptide, typically an octapeptide (80, 81). The predicted MPP cleavage site is after leucine 48 of Psd1. Moreover, the Psd1 precursor contains a sequence typical for an Oct1 cleavage motif, namely arginine at position -10 and phenylalanine at position -8 of the putative Oct1 cleavage site after serine at position 56. In general, the second processing step catalyzed by Oct1 is considered to stabilize the N terminus (63). Sequential cleavage by MPP and Oct1 presumably leads to a stabilizing glycine at the N terminus of the mature Psd1 (61, 63). Proteases, which typically remove inner membrane sorting sequences, like Imp1 and Imp2 (80, 81), had no effect on Psd1 processing. This finding is in line with the observation that the hydrophobic inner membrane sorting sequence is not proteolytically removed and can therefore serve as a membrane anchor for Psd1. Thus, the yeast mitochondrial Psd1  $\beta$ -subunit is embedded into the inner membrane like its bacterial counterpart (48).

Surprisingly, we discovered that cleavage of the Psd1 precursor into  $\alpha$ - and  $\beta$ -subunits, which is a crucial step in the formation of the active center of the enzyme, can also take place when the precursor is arrested at the TOM complex of the outer mitochondrial membrane by depletion of the membrane potential (Fig. 4, B and D). Even mislocalization of the protein to the matrix side of the inner membrane did not affect its self-cleavage (Fig. 5, B and C). Thus, separation of the two subunits does not depend on proper maturation and targeting of Psd1 within mitochondria. Interestingly, self-processing of the Psd1 precursor into Psd1 $\alpha$  at the outer membrane is only mildly

affected by deletion of Tom70, whereas deletion of other Tom receptors had no effect on this processing step (Fig. 4E). Thus, Tom70 provides an initial docking site for the incoming Psd1 precursor but is not strictly required for autocatalysis of Psd1. Other components of the mitochondrial surface or specific membrane properties may be important for the autocatalytic processing of Psd1 as well. Choi *et al.* (54) reported that processing of Psd from *P. knowlesi* (PkPSD) into  $\alpha$ - and  $\beta$ -subunits was influenced by its lipid surrounding. *In vitro* experiments using liposomes demonstrated that processing of PkPSD to the mature enzyme form and Psd activity were enhanced by increasing concentrations of dioleoyl phosphatidylserine, whereas other anionic phospholipids, such as dioleoyl phosphatidic acid or dioleoyl phosphatidylglycerol, displayed an inhibitory effect (54). Future studies will be necessary to reveal whether a mitochondria-specific lipid composition contributes to induce the self-processing of Psd1 because this step does not occur when the precursor is incubated with microsomal membranes (Fig. 5A).

In summary, our studies demonstrate clearly that insertion of Psd1 into the inner membrane/intermembrane space is paramount for the formation of an enzymatically active protein. The specific membrane environment may contribute to this process. Correct topology of Psd1 at the inner membrane-intermembrane space interface appears to be important for proper access of the amphipathic substrate PS with its polar headgroup and hydrophobic tails to the active center of the enzyme.

**Acknowledgments**—We thank Drs. Nikolaus Pfanner, Martin van der Laan, Michael P. Yaffe, and Trevor Lithgow for materials and discussions. We thank Nicole Zufall for expert technical assistance.

## REFERENCES

- Bürgermeister, M., Birner-Grünberger, R., Nebauer, R., and Daum, G. (2004) Contribution of different pathways to the supply of phosphatidylethanolamine and phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1686**, 161–168
- Osman, C., Voelker, D. R., and Langer, T. (2011) Making heads or tails of phospholipids in mitochondria. *J. Cell Biol.* **192**, 7–16
- van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: Where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* **9**, 112–124
- Zinser, E., and Daum, G. (1995) Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. *Yeast* **11**, 493–536
- Dowhan, W., and Bogdanov, M. (2009) Lipid-dependent membrane protein topogenesis. *Annu. Rev. Biochem.* **78**, 515–540
- van den Brink-van der Laan, E., Killian, J. A., and de Kruijff, B. (2004) Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. *Biochim. Biophys. Acta* **1666**, 275–288
- Klingenberg, M. (2009) Cardiolipin and mitochondrial carriers. *Biochim. Biophys. Acta* **1788**, 2048–2058
- Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) Specific roles of protein-phospholipid interactions in the yeast cytochrome *bc<sub>1</sub>* complex structure. *EMBO J.* **20**, 6591–6600
- Li, Q. X., and Dowhan, W. (1990) Studies on the mechanism of formation of the pyruvate prosthetic group of phosphatidylserine decarboxylase from *Escherichia coli*. *J. Biol. Chem.* **265**, 4111–4115
- Claypool, S. M., Oktay, Y., Boontheung, P., Loo, J. A., and Koehler, C. M. (2008) Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J. Cell Biol.* **182**, 937–950
- Gallas, M. R., Dienhart, M. K., Stuart, R. A., and Long, R. M. (2006) Characterization of Mmp37p, a *Saccharomyces cerevisiae* mitochondrial matrix protein with a role in mitochondrial protein import. *Mol. Biol. Cell* **17**, 4051–4062
- Gebert, N., Joshi, A. S., Kutik, S., Becker, T., McKenzie, M., Guan, X. L., Mooga, V. P., Stroud, D. A., Kulkarni, G., Wenk, M. R., Rehling, P., Meisinger, C., Ryan, M. T., Wiedemann, N., Greenberg, M. L., and Pfanner, N. (2009) Mitochondrial cardiolipin involved in outer membrane protein biogenesis. Implications for Barth syndrome. *Curr. Biol.* **19**, 2133–2139
- Gohil, V. M., and Greenberg, M. L. (2009) Mitochondrial membrane biogenesis. Phospholipids and proteins go hand in hand. *J. Cell Biol.* **184**, 469–472
- Kutik, S., Rissler, M., Guan, X. L., Guiard, B., Shui, G., Gebert, N., Heacock, P. N., Rehling, P., Dowhan, W., Wenk, M. R., Pfanner, N., and Wiedemann, N. (2008) The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. *J. Cell Biol.* **183**, 1213–1221
- Mileykovskaya, E., and Dowhan, W. (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. *Biochim. Biophys. Acta* **1788**, 2084–2091
- Pfeiffer, K., Gohil, V., Stuart, R. A., Hunte, C., Brandt, U., Greenberg, M. L., and Schagger, H. (2003) Cardiolipin stabilizes respiratory chain supercomplexes. *J. Biol. Chem.* **278**, 52873–52880
- Tamura, Y., Harada, Y., Yamano, K., Watanabe, K., Ishikawa, D., Ohshima, C., Nishikawa, S., Yamamoto, H., and Endo, T. (2006) Identification of Tam41 maintaining integrity of the TIM23 protein translocator complex in mitochondria. *J. Cell Biol.* **174**, 631–637
- Gohil, V. M., Thompson, M. N., and Greenberg, M. L. (2005) Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine and cardiolipin biosynthetic pathways in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 35410–35416
- Steenbergen, R., Nanowski, T. S., Beigneux, A., Kulinski, A., Young, S. G., and Vance, J. E. (2005) Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects. *J. Biol. Chem.* **280**, 40032–40040
- Barth, P. G., Valianpour, F., Bowen, V. M., Lam, J., Duran, M., Vaz, F. M., and Wanders, R. J. (2004) X-linked cardioskeletal myopathy and neutropenia (Barth syndrome). An update. *Am. J. Med. Genet. A* **126A**, 349–354
- Chicco, A. J., and Sparagna, G. C. (2007) Role of cardiolipin alterations in mitochondrial dysfunction and disease. *Am. J. Physiol. Cell Physiol.* **292**, C33–C44
- Chang, S. C., Heacock, P. N., Clancey, C. J., and Dowhan, W. (1998) The PEL1 gene (renamed PGS1) encodes the phosphatidylglycerophosphate synthase of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 9829–9836
- Jiang, F., Rizavi, H. S., and Greenberg, M. L. (1997) Cardiolipin is not essential for the growth of *Saccharomyces cerevisiae* on fermentable or non-fermentable carbon sources. *Mol. Microbiol.* **26**, 481–491
- Kelly, B. L., and Greenberg, M. L. (1990) Characterization and regulation of phosphatidylglycerol phosphate phosphatase in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1046**, 144–150
- Osman, C., Haag, M., Wieland, F. T., Brügger, B., and Langer, T. (2010) A mitochondrial phosphatase required for cardiolipin biosynthesis. The PGP phosphatase Gep4. *EMBO J.* **29**, 1976–1987
- Tamai, K. T., and Greenberg, M. L. (1990) Biochemical characterization and regulation of cardiolipin synthase in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1046**, 214–222
- Tuller, G., Hrastnik, C., Achleitner, G., Schiefthaler, U., Klein, F., and Daum, G. (1998) YDL142c encodes cardiolipin synthase (Cis1p) and is non-essential for aerobic growth of *Saccharomyces cerevisiae*. *FEBS Lett.* **421**, 15–18
- Daum, G., Lees, N. D., Bard, M., and Dickson, R. (1998) Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* **14**, 1471–1510
- Kennedy, E. P., and Weiss, S. B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipides. *J. Biol. Chem.* **222**, 193–214
- Kim, K., Kim, K. H., Storey, M. K., Voelker, D. R., and Carman, G. M. (1999) Isolation and characterization of the *Saccharomyces cerevisiae* *EK11* gene encoding ethanolamine kinase. *J. Biol. Chem.* **274**,

- 14857–14866
31. Kuchler, K., Daum, G., and Paltauf, F. (1986) Subcellular and mitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae*. *J. Bacteriol.* **165**, 901–910
32. Rajakumari, S., and Daum, G. (2010) Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. *Mol. Biol. Cell* **21**, 501–510
33. Riekhof, W. R., and Voelker, D. R. (2006) Uptake and utilization of lysophosphatidylethanolamine by *Saccharomyces cerevisiae*. *J. Biol. Chem.* **281**, 36588–36596
34. Riekhof, W. R., Wu, J., Jones, J. L., and Voelker, D. R. (2007) Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **282**, 28344–28352
35. Saba, J. D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y. A. (1997) The *BST1* gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. *J. Biol. Chem.* **272**, 26087–26090
36. Trotter, P. J., Pedretti, J., and Voelker, D. R. (1993) Phosphatidylserine decarboxylase from *Saccharomyces cerevisiae*. Isolation of mutants, cloning of the gene, heterologous expression, and creation of the null allele. *J. Biol. Chem.* **268**, 21416–21424
37. Trotter, P. J., Pedretti, J., Yates, R., and Voelker, D. R. (1995) Phosphatidylserine decarboxylase 2 of *Saccharomyces cerevisiae*. Cloning and mapping of the gene, heterologous expression, and creation of the null allele. *J. Biol. Chem.* **270**, 6071–6080
38. Trotter, P. J., and Voelker, D. R. (1995) Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 6062–6070
39. Zinser, E., Sperka-Gottlieb, C. D., Fasch, E. V., Kohlwein, S. D., Paltauf, F., and Daum, G. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. *J. Bacteriol.* **173**, 2026–2034
40. Voelker, D. R. (1984) Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2669–2673
41. Kornmann, B., Currie, E., Collins, S. R., Schuldiner, M., Nunnari, J., Weissman, J. S., and Walter, P. (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* **325**, 477–481
42. Nguyen, T. T., Lewandowska, A., Choi, J. Y., Markgraf, D. F., Junker, M., Bilgin, M., Ejsing, C. S., Voelker, D. R., Rapoport, T. A., and Shaw, J. M. (2012) Gem1 and ERMES do not directly affect phosphatidylserine transport from ER to mitochondria or mitochondrial inheritance. *Traffic* **13**, 880–890
43. Vance, J. E. (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* **265**, 7248–7256
44. Birner, R., Bürgermeister, M., Schneiter, R., and Daum, G. (2001) Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **12**, 997–1007
45. Kuroda, T., Tani, M., Moriguchi, A., Tokunaga, S., Higuchi, T., Kitada, S., and Kuge, O. (2011) FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. *Mol. Microbiol.* **80**, 248–265
46. Gulshan, K., Schmidt, J. A., Shahi, P., and Moye-Rowley, W. S. (2008) Evidence for the bifunctional nature of mitochondrial phosphatidylserine decarboxylase. Role in Pdr3-dependent retrograde regulation of PDR5 expression. *Mol. Cell. Biol.* **28**, 5851–5864
47. Birner, R., Nebauer, R., Schneiter, R., and Daum, G. (2003) Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine biosynthetic machinery with the prohibitin complex of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14**, 370–383
48. Dowhan, W. (1997) Phosphatidylserine decarboxylases. Pyruvoyl-dependent enzymes from bacteria to mammals. *Methods Enzymol.* **280**, 81–88
49. Schuiki, I., and Daum, G. (2009) Phosphatidylserine decarboxylases, key enzymes of lipid metabolism. *IUBMB Life* **61**, 151–162
50. Voelker, D. R. (1997) Phosphatidylserine decarboxylase. *Biochim. Biophys. Acta* **1348**, 236–244
51. Dowhan, W., and Li, Q. X. (1992) Phosphatidylserine decarboxylase from *Escherichia coli*. *Methods Enzymol.* **209**, 348–359
52. Igarashi, K., Kaneda, M., Yamaji, A., Saido, T. C., Kikkawa, U., Ono, Y., Inoue, K., and Umeda, M. (1995) A novel phosphatidylserine-binding peptide motif defined by an anti-idiotypic monoclonal antibody. Localization of phosphatidylserine-specific binding sites on protein kinase C and phosphatidylserine decarboxylase. *J. Biol. Chem.* **270**, 29075–29078
53. Kuge, O., Saito, K., Kojima, M., Akamatsu, Y., and Nishijima, M. (1996) Post-translational processing of the phosphatidylserine decarboxylase gene product in Chinese hamster ovary cells. *Biochem. J.* **319**, 33–38
54. Choi, J. Y., Augagneur, Y., Ben Mamoun, C., and Voelker, D. R. (2012) Identification of gene encoding *Plasmodium knowlesi* phosphatidylserine decarboxylase by genetic complementation in yeast and characterization of *in vitro* maturation of encoded enzyme. *J. Biol. Chem.* **287**, 222–232
55. Nebauer, R., Schuiki, I., Kulterer, B., Trajanoski, Z., and Daum, G. (2007) The phosphatidylethanolamine level of yeast mitochondria is affected by the mitochondrial components Oxa1p and Yme1p. *FEBS J.* **274**, 6180–6190
56. Rontein, D., Wu, W. I., Voelker, D. R., and Hanson, A. D. (2003) Mitochondrial phosphatidylserine decarboxylase from higher plants. Functional complementation in yeast, localization in plants, and overexpression in *Arabidopsis*. *Plant Physiol.* **132**, 1678–1687
57. Simbeni, R., Tangemann, K., Schmidt, M., Ceolotto, C., Paltauf, F., and Daum, G. (1993) Import of phosphatidylserine into isolated yeast mitochondria. *Biochim. Biophys. Acta* **1145**, 1–7
58. Zborowski, J., Dygas, A., and Wojtczak, L. (1983) Phosphatidylserine decarboxylase is located on the external side of the inner mitochondrial membrane. *FEBS Lett.* **157**, 179–182
59. Becker, T., Wenz, L. S., Krüger, V., Lehmann, W., Müller, J. M., Goroncy, L., Zufall, N., Lithgow, T., Guiard, B., Chacinska, A., Wagner, R., Meisinger, C., and Pfanner, N. (2011) The mitochondrial import protein Mim1 promotes biogenesis of multispanning outer membrane proteins. *J. Cell Biol.* **194**, 387–395
60. Meisinger, C., Ryan, M. T., Hill, K., Model, K., Lim, J. H., Sickmann, A., Müller, H., Meyer, H. E., Wagner, R., and Pfanner, N. (2001) Protein import channel of the outer mitochondrial membrane. A highly stable Tom40-Tom22 core structure differentially interacts with preproteins, small tom proteins, and import receptors. *Mol. Cell. Biol.* **21**, 2337–2348
61. Vögtle, F. N., Wortelkamp, S., Zahedi, R. P., Becker, D., Leidhold, C., Gevaert, K., Kellermann, J., Voos, W., Sickmann, A., Pfanner, N., and Meisinger, C. (2009) Global analysis of the mitochondrial N-proteome identifies a processing peptidase critical for protein stability. *Cell* **139**, 428–439
62. Witte, C., Jensen, R. E., Yaffe, M. P., and Schatz, G. (1988) *MAS1*, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. *EMBO J.* **7**, 1439–1447
63. Vögtle, F. N., Prinz, C., Kellermann, J., Lottspeich, F., Pfanner, N., and Meisinger, C. (2011) Mitochondrial protein turnover. Role of the precursor intermediate peptidase Oct1 in protein stabilization. *Mol. Biol. Cell* **22**, 2135–2143
64. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**, 1425
65. Stojanovski, D., Pfanner, N., and Wiedemann, N. (2007) Import of proteins into mitochondria. *Methods Cell Biol.* **80**, 783–806
66. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) Isolation of intracellular membranes by means of sodium carbonate treatment. Application to endoplasmic reticulum. *J. Cell Biol.* **93**, 97–102
67. Thornton, N., Stroud, D. A., Milenkovic, D., Guiard, B., Pfanner, N., and Becker, T. (2010) Two modular forms of the mitochondrial sorting and assembly machinery are involved in biogenesis of  $\alpha$ -helical outer membrane proteins. *J. Mol. Biol.* **396**, 540–549
68. Truscott, K. N., Voos, W., Frazier, A. E., Lind, M., Li, Y., Geissler, A., Dudek, J., Müller, H., Sickmann, A., Meyer, H. E., Meisinger, C., Guiard, B., Rehling, P., and Pfanner, N. (2003) A J-protein is an essential subunit of the presequence translocase-associated protein import motor of mitochondria. *J. Cell Biol.* **163**, 707–713
69. Dekker, P. J., Ryan, M. T., Brix, J., Müller, H., Hönlinger, A., and Pfanner, N. (1998) Preprotein translocase of the outer mitochondrial membrane. Molecular dissection and assembly of the general import pore complex.



- Mol. Cell. Biol.* **18**, 6515–6524
70. Schägger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **199**, 223–231
  71. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497–509
  72. Broekhuysse, R. M. (1968) Phospholipids in tissues of the eye. I. Isolation, characterization, and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids. *Biochim. Biophys. Acta* **152**, 307–315
  73. Baker, M. J., Frazier, A. E., Gulbis, J. M., and Ryan, M. T. (2007) Mitochondrial protein-import machinery. Correlating structure with function. *Trends Cell Biol.* **17**, 456–464
  74. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009) Importing mitochondrial proteins. Machineries and mechanisms. *Cell* **138**, 628–644
  75. Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006) Evolution of the molecular machines for protein import into mitochondria. *Science* **313**, 314–318
  76. Neupert, W., and Herrmann, J. M. (2007) Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* **76**, 723–749
  77. Schleiff, E., and Becker, T. (2011) Common ground for protein translocation. Access control for mitochondria and chloroplasts. *Nat. Rev. Mol. Cell Biol.* **12**, 48–59
  78. van Wilpe, S., Ryan, M. T., Hill, K., Maarse, A. C., Meisinger, C., Brix, J., Dekker, P. J., Moczko, M., Wagner, R., Meijer, M., Guiard, B., Hönliger, A., and Pfanner, N. (1999) Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase. *Nature* **401**, 485–489
  79. Yamamoto, H., Fukui, K., Takahashi, H., Kitamura, S., Shiota, T., Terao, K., Uchida, M., Esaki, M., Nishikawa, S., Yoshihisa, T., Yamano, K., and Endo, T. (2009) Roles of Tom70 in import of presequence-containing mitochondrial proteins. *J. Biol. Chem.* **284**, 31635–31646
  80. Gakh, O., Cavadini, P., and Isaya, G. (2002) Mitochondrial processing peptidases. *Biochim. Biophys. Acta* **1592**, 63–77
  81. Mossmann, D., Meisinger, C., and Vögtle, F. N. (2012) Processing of mitochondrial presequences. *Biochim. Biophys. Acta* **1819**, 1098–1106
  82. Frazier, A. E., Chacinska, A., Truscott, K. N., Guiard, B., Pfanner, N., and Rehling, P. (2003) Mitochondria use different mechanisms for transport of multispinning membrane proteins through the intermembrane space. *Mol. Cell. Biol.* **23**, 7818–7828
  83. Schuiki, I., Schnabl, M., Czabany, T., Hrastnik, C., and Daum, G. (2010) Phosphatidylethanolamine synthesized by four different pathways is supplied to the plasma membrane of the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1801**, 480–486