

# Role of Phosphatidylethanolamine in the Biogenesis of Mitochondrial Outer Membrane Proteins\*

Received for publication, December 5, 2012, and in revised form, April 19, 2013. Published, JBC Papers in Press, April 26, 2013, DOI 10.1074/jbc.M112.442392

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**Background:** It is unknown if phosphatidylethanolamine (PE), the major non-bilayer-forming mitochondrial phospholipid, is involved in the biogenesis of outer membrane proteins.

**Results:** Depletion of PE impairs import of  $\beta$ -barrel proteins by the outer membrane translocase TOM.

**Conclusion:** PE is required for full activity but not stability of TOM.

**Significance:** PE plays a different role in the biogenesis of mitochondrial outer membrane proteins compared with cardiolipin.

The mitochondrial outer membrane contains proteinaceous machineries for the import and assembly of proteins, including TOM (translocase of the outer membrane) and SAM (sorting and assembly machinery). It has been shown that the dimeric phospholipid cardiolipin is required for the stability of TOM and SAM complexes and thus for the efficient import and assembly of  $\beta$ -barrel proteins and some  $\alpha$ -helical proteins of the outer membrane. Here, we report that mitochondria deficient in phosphatidylethanolamine (PE), the second non-bilayer-forming phospholipid, are impaired in the biogenesis of  $\beta$ -barrel proteins, but not of  $\alpha$ -helical outer membrane proteins. The stability of TOM and SAM complexes is not disturbed by the lack of PE. By dissecting the import steps of  $\beta$ -barrel proteins, we show that an early import stage involving translocation through the TOM complex is affected. In PE-depleted mitochondria, the TOM complex binds precursor proteins with reduced efficiency. We conclude that PE is required for the proper function of the TOM complex.

Mitochondrial outer membrane proteins fulfill essential functions in the eukaryotic cell, including transport of metabolites and communication with the cytosol and other organelles. Porin, also termed VDAC (for voltage-dependent anion channel), mediates the transport of metabolites (1). TOM (translocase of the outer membrane) forms the main entry gate

for mitochondrial precursor proteins that are synthesized on cytosolic ribosomes (2–8). ERMES (endoplasmic reticulum-mitochondria encounter structure) tethers mitochondria to the endoplasmic reticulum (9), and the outer membrane harbors components important for mitochondrial fusion and fission (10–12). Additionally, the outer membrane plays an important role in apoptosis (13). Thus, the biogenesis of outer membrane proteins and their correct assembly into protein machineries are essential for mitochondrial function.

The outer membrane contains two types of integral membrane proteins: proteins with  $\alpha$ -helical transmembrane segments and  $\beta$ -barrel proteins. All outer membrane proteins are synthesized on cytosolic ribosomes and are targeted to the mitochondrial surface.  $\beta$ -Barrel precursor proteins are transported via the protein-conducting channel Tom40 of the TOM complex (14–16). Subsequently, small TIM chaperones of the intermembrane space guide the  $\beta$ -barrel precursors to SAM (sorting and assembly machinery) (15–19). The SAM complex, also called the TOB (topogenesis of mitochondrial outer membrane  $\beta$ -barrel proteins) complex, binds to a conserved  $\beta$ -signal of the precursor protein and mediates the insertion into the outer membrane (15, 16, 19–23). For  $\alpha$ -helical outer membrane proteins, a common import pathway has not been found. The receptor protein Tom70 and the mitochondrial import proteins Mim1 and Mim2 play a crucial role in the biogenesis of multispinning outer membrane proteins such as Ugo1, whereas TOM core subunits and the SAM complex are not involved (24–27). Additionally, Mim1 is also involved in the import of single-spanning outer membrane proteins such as Tom20 and small Tom proteins (25–29). A specialized SAM form containing Mdm10 (mitochondrial distribution and morphology protein 10) mediates the biogenesis of Tom22, whereas other single-spanning precursor proteins insert independently of known proteinaceous factors but can be impaired by elevated levels of ergosterol (31–38).

The outer membrane of mitochondria contains two non-bilayer-forming phospholipids, cardiolipin (CL)<sup>5</sup> and phosphati-

\* This work was supported by Deutsche Forschungsgemeinschaft Sonderforschungsbereich 746, Excellence Initiative of the German Federal and State Governments Grants EXC 294 BIOSS and GSC-4 (Spemann Graduate School), the Bundesministerium für Bildung und Forschung, and Austrian Science Fund FWF Project 21429 and DK Molecular Enzymology Grant W901-B05 (to G. D.).

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<sup>5</sup> The abbreviations used are: CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

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dylethanolamine (PE) (39–41). In yeast mitochondria, Crd1 (cardiolipin synthase 1) and Psd1 (phosphatidylserine decarboxylase 1) catalyze the formation of CL and PE, respectively (42–48). Minor amounts of mitochondrial PE can also be provided by the activity of Psd2 at the vacuole/Golgi membranes, the acyltransferases Tgl3 and Ale1, and the CDP-ethanolamine pathway (49–53). Additionally, several mitochondrial proteins were reported to regulate the levels of CL and PE (9, 54–62). Lack of CL affects stability and function of various mitochondrial membrane protein complexes, including the respiratory chain of the inner membrane (55, 56, 58, 63–71). Although the outer membrane contains only small amounts of CL, lack of CL causes destabilization of TOM and SAM complexes and thus reduced precursor binding by the translocases. The biogenesis of  $\beta$ -barrel proteins and the assembly of some  $\alpha$ -helical outer membrane proteins are impaired in CL-deficient mitochondria (41). A double deletion of *PSD1* and *CRD1* is lethal, suggesting overlapping functions of PE and CL that are essential for cell viability (72). Depletion of CL as well as of PE leads to a decreased activity of the respiratory chain, which in turn impairs preprotein import into the inner membrane and matrix due to a reduced inner membrane potential (58, 63, 73, 74). CL and PE also have overlapping functions in the fusion of mitochondria (75). However, because neither respiratory activity nor mitochondrial fusion is strictly essential for the cell viability of yeast, the effects of CL and PE on respiration and fusion cannot explain the synthetic lethality of the double deletion.

The role of PE in the biogenesis of mitochondrial outer membrane proteins has not been addressed so far, although PE is one of the major phospholipids of the outer membrane (39, 40). For this study, we analyzed the biogenesis of outer membrane proteins in PE-depleted mitochondria. Although no defect in the biogenesis of  $\alpha$ -helical proteins was observed, the import of  $\beta$ -barrel proteins was impaired at the stage of translocation through the TOM complex. The stability of TOM and SAM complexes was not affected, yet the TOM complex bound precursor proteins with reduced efficiency. We conclude that PE is required for the efficient function of the TOM machinery.

### EXPERIMENTAL PROCEDURES

**Yeast Strains, Growth Conditions, and Isolation of Mitochondria and Outer Membrane Vesicles**—The yeast strains *crd1* $\Delta$ , *psd1* $\Delta$ , and *psd1* $\Delta$  *psd2* $\Delta$  and the corresponding wild-type strain BY4741 have been described (41, 76). Cells were grown in YPLac or YPG medium (58) at 21–30 °C to an early logarithmic growth phase. Mitochondria were isolated, adjusted to a protein concentration of 10 mg/ml, aliquoted, and shock-frozen with liquid nitrogen as described (77). Outer membrane vesicles were isolated from purified mitochondria via sucrose density centrifugation as described (40).

**Protein Import into Mitochondria**—For import studies,  $^{35}\text{S}$ -labeled precursor proteins were synthesized with the TNT coupled transcription/translation kit (Promega). The import was performed as described (77). To remove non-imported precursor proteins, proteinase K was added to a final concentration of 50  $\mu\text{g}/\text{ml}$ , and the samples were incubated for 15 min on ice. The activity of the protease was blocked by addition of PMSF to a final concentration of 2 mM. For blue native electrophoresis,

mitochondria were solubilized with 1% (w/v) digitonin in digitonin buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1 mM EDTA, and 10% (v/v) glycerol) for 15 min on ice. After a clarifying spin (16,100  $\times g$ , 10 min, 4 °C), samples were loaded on a blue native gel. The blue native gel was prepared as described (77).

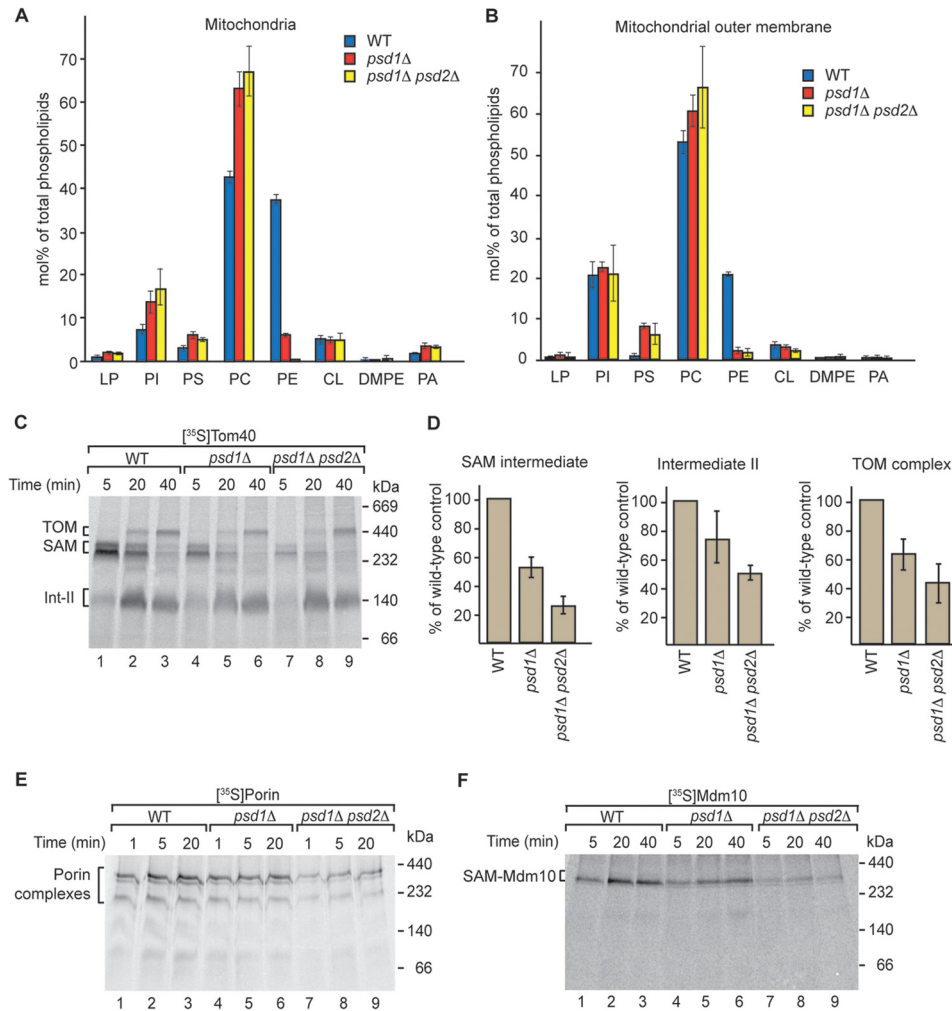
**Co-immunoprecipitation**—Mitochondria were solubilized with 1% digitonin in digitonin buffer and incubated with protein A-Sepharose (GE Healthcare) coupled to Tom5-specific antibodies or preimmune antibodies. Binding was performed for 1 h at 4 °C under constant rotation. After excessive washing, bound proteins were eluted with 0.1 M glycine (pH 2.5) and subjected to SDS-PAGE.

**Determination of Mitochondrial Phospholipid Distribution**—Lipids were extracted from isolated mitochondria with chloroform/methanol (2:1, v/v) as described (78). Subsequently, washing steps of the organic phase with 0.034% (w/v)  $\text{MgCl}_2$  solution, 2 N KCl/methanol (4:1, v/v), and methanol/water/chloroform (48:47:3, per volume) were performed. Phospholipids were separated by thin-layer chromatography as described (48). Phospholipids were detected by iodine vapor, scrapped off, and quantified (79).

### RESULTS

**Biogenesis of  $\beta$ -Barrel Proteins Is Impaired in PE-deficient Mitochondria**—To study the role of PE in the biogenesis of mitochondrial outer membrane proteins, we isolated mitochondria from the *psd1* $\Delta$  yeast strain and the *psd1* $\Delta$  *psd2* $\Delta$  double deletion strain (47, 73, 76). As expected, the levels of PE were strongly reduced (Fig. 1A and B). The levels of phosphatidylcholine (PC), phosphatidylinositol, and phosphatidylserine were increased in the mutant mitochondria, whereas the level of CL was unchanged (Fig. 1A) (47). We also determined the phospholipid distribution in purified mitochondrial outer membrane vesicles. Similar to purified mitochondria, the levels of phosphatidylserine and PC were increased in the mutants, whereas the level of phosphatidylinositol remained unchanged (Fig. 1B). To study the biogenesis of  $\beta$ -barrel proteins, we imported the  $^{35}\text{S}$ -labeled precursor of Tom40 into isolated wild-type and mutant mitochondria. The import of this model  $\beta$ -barrel precursor allows the analysis of distinct assembly steps, which can be visualized by blue native gel electrophoresis (Fig. 1C, lanes 1–3) (14–16, 20, 21, 80–82). Upon short import times, the Tom40 precursor binds to the SAM complex. Subsequently, the precursor is released to form a second intermediate and finally assembles into the mature TOM complex of ~450 kDa (16). In the PE-depleted mutant mitochondria, all assembly steps of Tom40 were reduced (Fig. 1, C, lanes 4–9, and D). Binding of the Tom40 precursor to the SAM complex was considerably decreased, whereas formation of intermediate II and the mature TOM complex was moderately affected (Fig. 1D). We studied the assembly of two additional  $\beta$ -barrel proteins, porin and Mdm10, by monitoring formation of the mature complexes by blue native electrophoresis (16, 31). The biogenesis of both proteins was impaired by the depletion of PE (Fig. 1, E and F).

We also analyzed the biogenesis of three outer membrane proteins that contain  $\alpha$ -helical transmembrane segments:



**FIGURE 1. Biogenesis of  $\beta$ -barrel proteins is impaired in PE-depleted mitochondria.** *A*, the phospholipid distribution of isolated WT, *psd1* $\Delta$ , and *psd1* $\Delta$  *psd2* $\Delta$  mitochondria was determined. Means  $\pm$  S.E. ( $n = 4$ ) are shown for the mitochondrial preparation. *B*, the phospholipid distribution of isolated WT, *psd1* $\Delta$ , and *psd1* $\Delta$  *psd2* $\Delta$  outer membrane vesicles was determined. The means with range from two independent experiments are depicted. LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. *C*,  $^{35}$ S-labeled Tom40 was imported into isolated WT, *psd1* $\Delta$ , or *psd1* $\Delta$  *psd2* $\Delta$  mitochondria at 25  $^{\circ}$ C for the indicated time periods. The mitochondria were lysed with digitonin and analyzed by blue native electrophoresis and digital autoradiography. SAM, Tom40 precursor bound to the SAM complex; Int-II, second intermediate of the Tom40 assembly pathway. *D*, quantification of the three assembly steps of Tom40 (import was performed and analyzed as described for *C*). Means  $\pm$  S.E. ( $n = 5$ ) are shown for the formation of the SAM intermediate after 5 min of import, intermediate II after 20 min of import, and the mature TOM complex after 40 min of import. *E* and *F*, porin or Mdm10, respectively, was imported into isolated WT, *psd1* $\Delta$ , or *psd1* $\Delta$  *psd2* $\Delta$  mitochondria at 25  $^{\circ}$ C for the indicated time periods. The mitochondria were lysed with digitonin and analyzed by blue native electrophoresis and digital autoradiography.

Tom22, Tom20, and Ugo1. In contrast to the biogenesis defects of  $\beta$ -barrel precursors, the assembly of  $^{35}$ S-labeled Tom22 and Tom20 into the TOM complex was not inhibited in *psd1* $\Delta$  and *psd1* $\Delta$  *psd2* $\Delta$  mitochondria (Fig. 2, *A* and *B*). The assembly of Tom20 was mildly increased in *psd1* $\Delta$  *psd2* $\Delta$  mitochondria (Fig. 2*B*, lanes 7–9). Similarly, import of Ugo1, determined by formation of the mature dimer (25, 26), was not decreased in the mutant mitochondria and was even enhanced (Fig. 2*C*). We conclude that PE-depleted mitochondria are impaired in the import of  $\beta$ -barrel proteins but not in the biogenesis of several  $\alpha$ -helical outer membrane proteins.

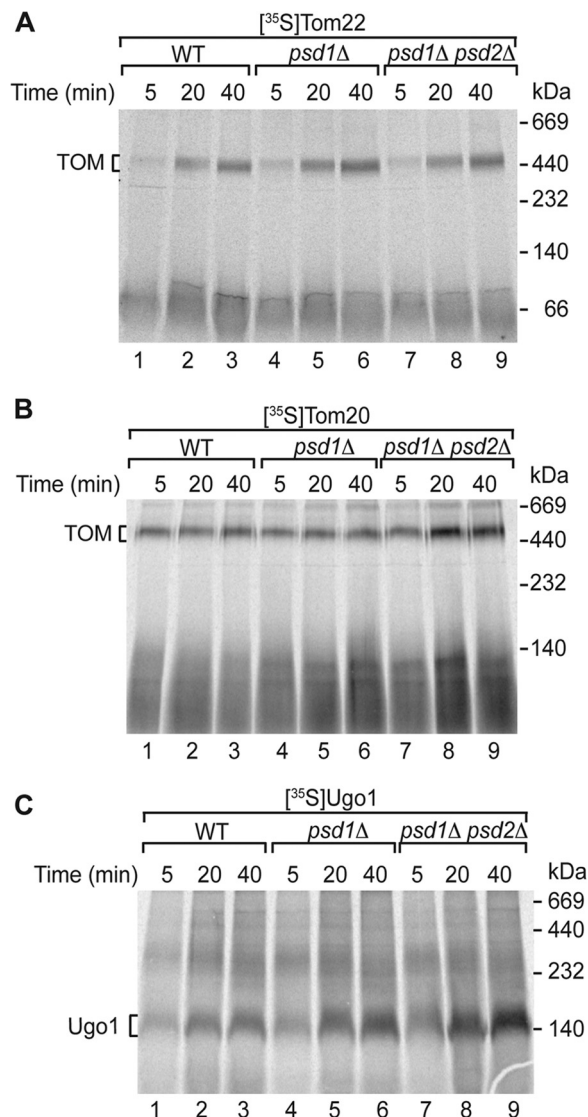
**Transport of  $\beta$ -Barrel Proteins across the TOM Complex Is Impaired in PE-depleted Mitochondria**—To address how PE depletion may impair  $\beta$ -barrel biogenesis, we first analyzed the steady-state levels of mitochondrial proteins, including TOM and SAM subunits, Mim1, and control proteins of different mitochondrial compartments. The protein levels were not

markedly altered and thus could not explain the defect in  $\beta$ -barrel assembly (Fig. 3*A*).

In *crd1* $\Delta$  mitochondria lacking CL, the assembly of Tom40 is compromised. In this case, the assembly defect is at least partially caused by a destabilized SAM complex (41). We asked if the SAM complex is affected in PE-depleted mitochondria. We lysed mitochondria with the nonionic detergent digitonin and studied outer membrane protein complexes by blue native electrophoresis (Fig. 3*B*). The two SAM complexes, SAM<sub>core</sub> and SAM-Mdm10, as well as additional outer membrane protein complexes, Mim1, Om45, and porin (25, 31, 36, 83–87), were not or only moderately affected by depletion of PE (Fig. 3*B*).

To identify the PE-dependent stage of  $\beta$ -barrel biogenesis, we dissected the biogenesis of Tom40 into distinct steps. To analyze binding of the  $\beta$ -barrel precursor to the SAM complex, we imported a mutant form of Tom40 that is blocked in release from the SAM complex due to a single amino acid exchange in

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**FIGURE 2. Biogenesis of  $\alpha$ -helical outer membrane proteins is not inhibited in PE-depleted mitochondria.**  $^{35}\text{S}$ -Labeled Tom22 (A), Tom20 (B), or Ugo1 (C) was imported into isolated WT, *psd1* $\Delta$ , or *psd1* $\Delta$  *psd2* $\Delta$  mitochondria at 25 °C for the indicated time periods. The mitochondria were lysed with digitonin and analyzed by blue native electrophoresis and digital autoradiography.

the  $\beta$ -signal (22). The mutant Tom40 precursor efficiently accumulated at the SAM complex of wild-type mitochondria (Fig. 3C, lanes 1–3). Binding of the Tom40 precursor to the SAM complex was impaired in *psd1* $\Delta$  mitochondria and strongly inhibited in *psd1* $\Delta$  *psd2* $\Delta$  mitochondria (Fig. 3C, lanes 4–9). Thus, PE is involved in an early step of the Tom40 assembly pathway, at the stage of binding to the SAM complex or at an earlier stage leading to the SAM complex.

Precursors of  $\beta$ -barrel proteins are initially imported by the TOM complex to the intermembrane space side (14–18, 88, 89). This initial import step cannot be resolved by blue native electrophoresis, as no blue native-stable intermediate is formed, but can be analyzed by protection of the precursor against externally added protease (15–18, 21, 81). Therefore, we imported Tom40 into the mutant mitochondria and determined the accessibility to added proteinase K. The amount of

protease-protected Tom40 precursor was reduced in the PE-depleted mitochondria (Fig. 3D, lower panel). Moreover, the binding of the Tom40 precursor to the mitochondrial surface was moderately decreased (Fig. 3D, upper panel). We conclude that PE is required at an early stage of Tom40 import into mitochondria that takes place before binding to the SAM complex, *i.e.* for the initial translocation of the precursor across the outer membrane by the TOM complex. The analysis of Tom22 assembly supports the conclusion that the SAM complex was not generally (unspecifically) damaged by lack of PE because the biogenesis of Tom22 depends on each SAM subunit (31, 33, 36, 81, 84, 90) but is not altered in PE-depleted mitochondria (Fig. 2A).

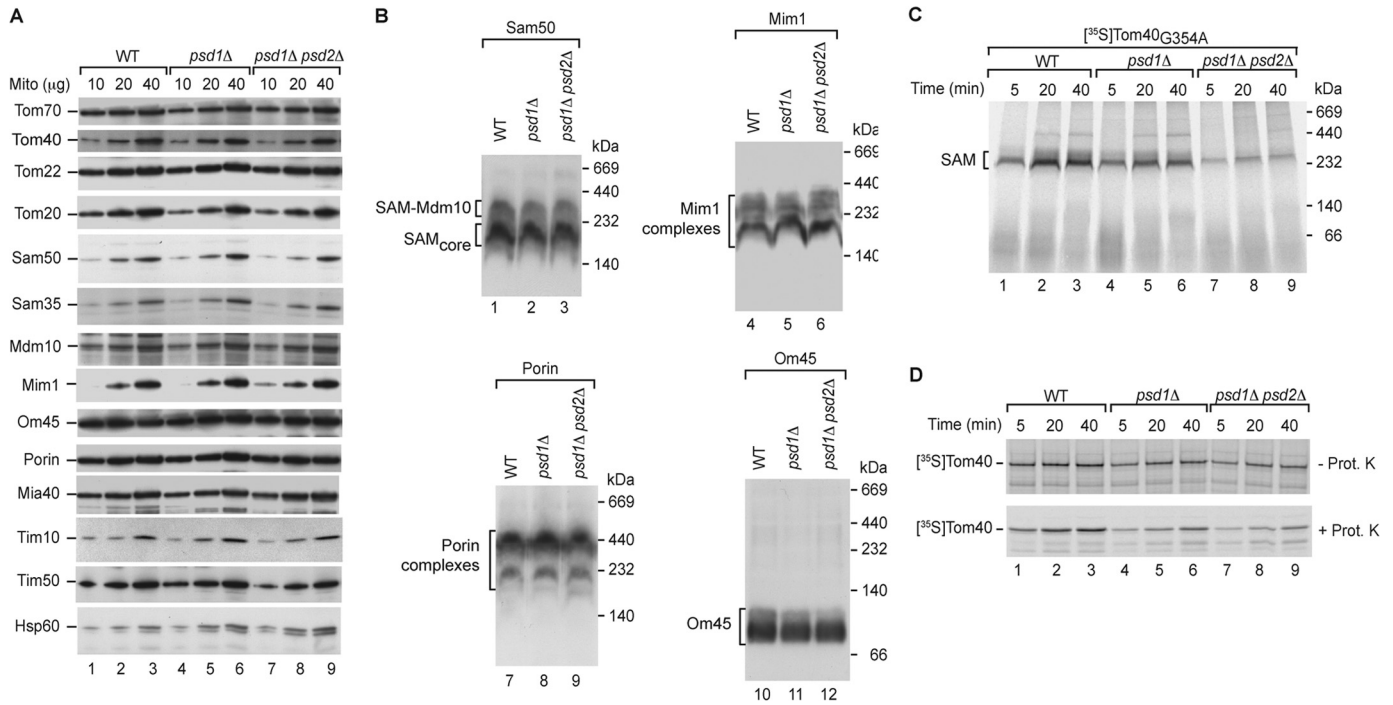
**PE Is Required for the Function but Not the Stability of the TOM Complex**—We asked if PE is required for the composition and/or function of the TOM complex. We resolved the TOM complex by blue native electrophoresis. For *crd1* $\Delta$  mitochondria, it has been shown that the interaction of Tom20 with the TOM complex is disturbed (Fig. 4A, lane 11) (41). In contrast, the TOM complex of *psd1* $\Delta$  and *psd1* $\Delta$  *psd2* $\Delta$  mitochondria was not altered on blue native electrophoresis. Tom40, Tom22, and Tom20 were present in the mature TOM complex in both mutants to a similar extent as in wild-type mitochondria (Fig. 4A, lanes 1–9). Furthermore, co-immunoprecipitation with Tom5-specific antibodies revealed that the reduced PE content did not disturb the association of the Tom20, Tom22, and Tom70 receptors with the Tom40-Tom5 core of the TOM complex (Fig. 4B) (91). Thus, the stability of the TOM translocon is not altered in *psd1* $\Delta$  and *psd1* $\Delta$  *psd2* $\Delta$  mitochondria.

To directly analyze the activity of the TOM complex in the interaction with preproteins, we used the presequence-carrying Oxa1 precursor that is targeted to the inner membrane. In the absence of an inner membrane potential, Oxa1 is efficiently arrested at the TOM complex of the outer membrane and forms a blue native-stable intermediate (41, 92, 93). The interaction of  $^{35}\text{S}$ -labeled Oxa1 with the TOM complex was strongly decreased in PE-depleted mitochondria (Fig. 5). In the *psd1* $\Delta$  *psd2* $\Delta$  mitochondria, the formation of the Oxa1-TOM intermediate was virtually blocked (Fig. 5), demonstrating that PE is required for the proper function of the TOM complex.

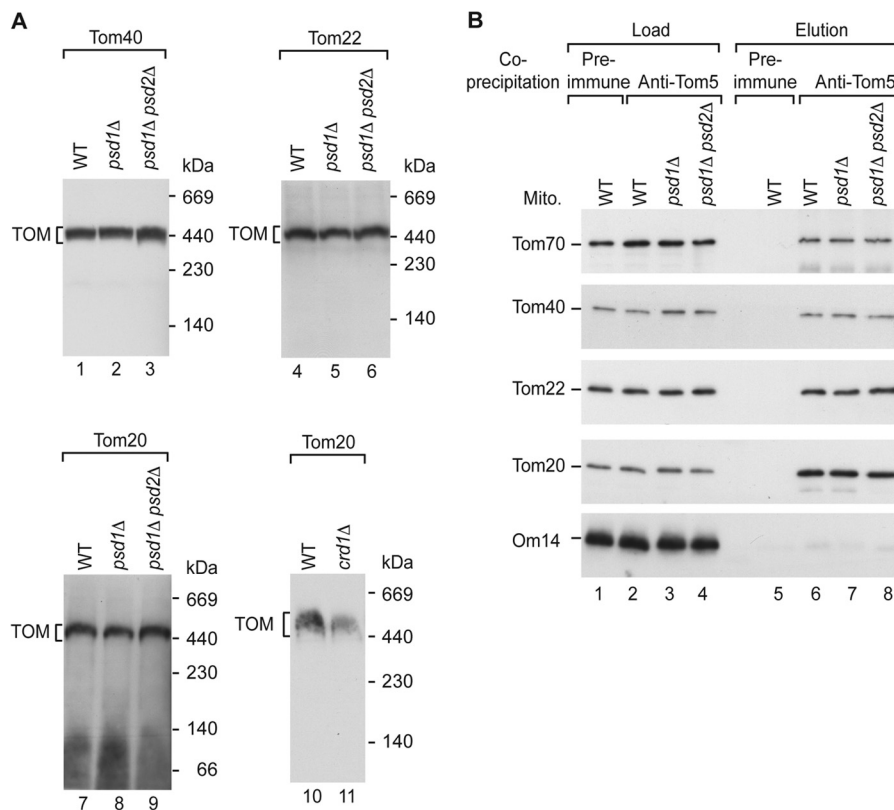
## DISCUSSION

We have demonstrated that PE is required for the full activity of the main protein entry gate of mitochondria. The capability of the TOM complex to interact with precursor proteins is strongly compromised in PE-depleted mitochondria. PE is thus required for the initial translocation of  $\beta$ -barrel precursor proteins across the outer membrane that occurs through the TOM complex. The biogenesis of outer membrane proteins with  $\alpha$ -helical membrane anchors, which are not transported through the TOM channel (25–30, 33), is not inhibited by depletion of PE, demonstrating that the protein import activity of the outer membrane is not generally impaired.

PE is the most abundant non-bilayer-forming phospholipid of the outer membrane (Fig. 1B) (39, 40). Several membrane proteins require non-bilayer-forming lipids for optimal activity (94, 95). Therefore, the shift to a higher level of bilayer-forming



**FIGURE 3. Impaired transport of  $\beta$ -barrel precursors across the outer membrane of PE-depleted mitochondria.** *A*, proteins of isolated WT, *psd1Δ*, and *psd1Δ psd2Δ* mitochondria were lysed with digitonin and separated by blue native electrophoresis. Protein complexes were detected by immunodecoration with the indicated antisera. *B*, WT, *psd1Δ*, and *psd1Δ psd2Δ* mitochondria were lysed with digitonin and separated by blue native electrophoresis. Protein complexes were detected by immunodecoration with the indicated antisera. *C*, <sup>35</sup>S-labeled Tom40(G354A) was imported into WT, *psd1Δ*, and *psd1Δ psd2Δ* mitochondria. The imported proteins were analyzed by blue native electrophoresis and autoradiography. *D*, <sup>35</sup>S-labeled Tom40 was imported into WT, *psd1Δ*, and *psd1Δ psd2Δ* mitochondria, followed by proteinase K (Prot. K) treatment as indicated. Proteins were separated by SDS-PAGE.



**FIGURE 4. Depletion of PE does not affect the stability of the TOM complex.** *A*, WT, *psd1Δ*, *psd1Δ psd2Δ*, and *crd1Δ* mitochondria were lysed with digitonin and separated by blue native electrophoresis. Protein complexes were detected by immunodecoration with the indicated antisera. *B*, WT, *psd1Δ*, and *psd1Δ psd2Δ* mitochondria (Mito.) were lysed with digitonin and subjected to co-immunoprecipitation with the indicated antisera. Proteins were eluted, separated by SDS-PAGE, and detected by immunodecoration with the indicated antisera (4% load and 100% elution).

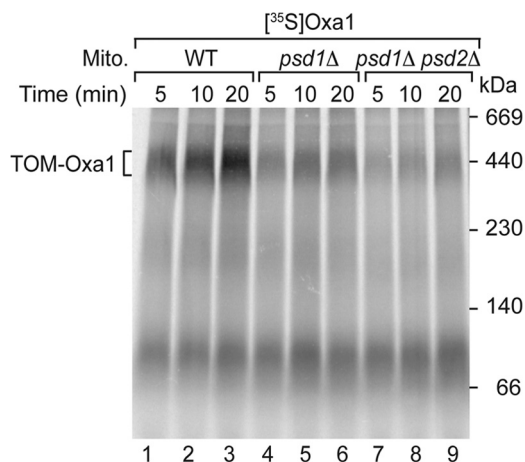


FIGURE 5. PE is required for precursor binding to the TOM complex. <sup>35</sup>S-Labeled Oxa1 was imported into WT, *psd1*Δ, and *psd1*Δ *psd2*Δ mitochondria (Mito.) in the absence of a membrane potential. The samples were analyzed by blue native electrophoresis and autoradiography.

lipids such as phosphatidylserine and PC in the outer membrane might affect the activity of the TOM complex in PE-depleted mitochondria. It has been shown that PE can assist in the refolding of integral membrane proteins (96). Although the overall composition and stability of the TOM complex do not depend on the presence of PE, it is conceivable that PE may play a more specific role in the proper conformation of some TOM subunits and thus may be required for the full function of the TOM complex.

Surprisingly, the biogenesis of some  $\alpha$ -helical outer membrane proteins such as Ugo1 and Tom20 is enhanced in PE-deficient mitochondria. These precursor proteins use the Mim1 complex on their biogenesis pathway and not the TOM channel (25–30). It will be interesting to address in future studies if PE plays an inhibitory role in the Mim1 pathway or if the increased levels of the phospholipids phosphatidylserine and PC in PE-deficient outer membranes support the activity of components of the Mim1 pathway.

PE is not required for the overall stability of the TOM and SAM complexes of the mitochondrial outer membrane, whereas lack of CL results in destabilization of both TOM and SAM (41). Thus, both non-bilayer-forming phospholipids CL and PE are required for the biogenesis of mitochondrial outer membrane proteins, but they play different roles. Whereas CL affects the stability and thus also the function of several translocase complexes (41), PE plays a selective role in the activity of the TOM complex. Because Tom40 is essential for cell viability, its biogenesis is rate-limiting for the growth of yeast (4, 5, 7, 8, 97, 98). The involvement of CL and PE in the biogenesis pathway of Tom40 provides a possible explanation for the synthetic lethality of a double deletion of *PSD1* and *CRD1* (72).

*Acknowledgments*—We thank Dr. Martin van der Laan for discussion and Nicole Zufall for expert technical assistance.

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