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Phospholipids in mitochondrial membranes from *Saccharomyces cerevisiae*

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Nothing in life is quite as important as you think it is while you're thinking about it.

Daniel Kahneman

Acknowledgments

I spent the last years of my life chasing all the goals that I set for myself. I travelled from place to place, met great people and faced the small challenges of everyday life. Writing my PhD thesis is giving me the opportunity to sum up and reflect on all the successes, failures and the vicissitudes of these last three and a half years. It is interesting and at times scary having to look back and retrace the different paths that led me up to this point.

Obviously in this journey back in time I cannot help but begin from my personal starting point, from my roots and centre of gravity that is my family. My parents, my grandmother and my brother Paolo are the most important thing for me; what they have given me and what they have done for me is so great that any comment is superfluous and any adjective is inappropriate. My thoughts automatically fly towards Sebastién. He is the person that during the past four years has been the backbone of my inner growth. Seb has always been an unfailing support and an inexhaustible source of advices. Each time I felt like drowning in my fears and fragility Seb lend me a hand in all circumstances and without hesitation.

My life would not be the same without my great friends; they filled my life and gave a sense to my days. A special thank to my "Best acquaintance ever" Rafi. Following, a unique place in my heart is reserved for my two fantastic girls Sophie and Nuria; the amazing “trio cosentino” Fabrizio-Ettore-Emanuale; Daniela my favourite Altoatesina, a volcano of energy and vitality; and to Kim and Colin who stole my heart and that I simply adore.

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Abstract

Phosphatidylethanolamine (PE) is one of the most abundant phospholipids found in yeast, plant and mammalian cells. PE can be synthesized by (i) decarboxylation from the precursor phosphatidylserine (PS) through the action of the PS decarboxylases type I and II, (ii) re-acylation of lyso-PE catalyzed by Ale1p and Tgl3p or (iii) the de novo biosynthesis starting from ethanolamine via CDP-ethanolamine (CDP-Etn) or Kennedy pathway. In *Saccharomyces cerevisiae*, the mitochondrial phosphatidylserine decarboxylase 1 (Psd1p) is the key enzyme as it produces the largest amount of cellular PE.

During its biogenesis Psd1p is synthesized as a larger precursor on cytosolic ribosomes and follows a unique processing pathway upon import into mitochondria. The transition to an active form of the enzyme goes through an autocatalytic cleavage which separates Psd1p into an intermembrane space localized α- and an inner membrane-bound β-subunit. The α-subunit harbors a highly conserved motif, which was proposed to be implicated in the catalysis through a specific interaction with phosphatidylserine (PS).

In the thesis I present a molecular analysis regarding the significance of this consensus motif in Psd1p, making use of mutant forms bearing either deletions or point mutations in this region of the α-subunit. The biochemical analysis performed showed that any modification in this motif differently affects the processing and stability of Psd1p. From these observations we conclude that the putative substrate recognition site of Psd1p α-subunit has a crucial role in assuring the structural integrity and the correct biogenesis of Psd1p influencing, as a consequence, also the catalytic activity of the enzyme.

Beside the work on Psd1p, my scientific interested included also some important aspects related to the interaction between phospholipids and protein complexes in mitochondrial membranes. It is well known that mitochondria contain a complex machinery that cathalyses the import of precursors proteins into the mitochondrial outer membrane. Regarding the β-barrel proteins, the outer membrane translocase (TOM complex) and the assembly machinery (SAM complex) are responsible for their proper positioning in the mitochondria.

The phospholipids PE and cardiolipin (CL) are both required for the proper function of these complexes. In this context our findings demostrate that also the bilayer-forming phosphatidylcholine (PC) is required for stability and function of the SAM complex in the biogenesis of β-barrel precursors.

Additional studies have also focused on the two protein translocases involved in the transport of precursor proteins into or across the inner mitochondrial membrane. In this case we have again shown that, unlike PE and CL, PC is able to interact directly with the complex TIM23. In fact the experiments performed revealed that the lack of PC in *S. cerevisiae pem1*Δ*pem2*Δ mutant strain, causes destabilization of the TIM23 complex without influencing the stability and activity of the respiratory supercomplexes and consequentially impacting the membrane potential.

General introduction

The work accomplished during my PhD focuses on phospholipids in mitochondria of *Saccharomyces cerevisiae.*

During the last 50 years many investigations have been performed to understand the biochemistry of mitochondria given their critical role in the generation of the metabolic energy for the eukaryotic cells. [1–3]. More recently the interest on mitochondria has shifted towards the study of mitochondrial dysfunctions, which include a broad range of disorders, as well as the implication of mitochondria in processes like apoptosis and ageing [4–8].

Mitochondria occupy up to 25% of the volume of the cytoplasm. Within these organelles many reactions, which are fundamental for the entire cell, are occurring. For example, sugars and long-chain fatty acids are metabolized, ATP is formed, steroids and lipids are synthesised and the mitochondrial DNA is replicated, transcribed and proteins are translated [1].

With respect to the whole the cell, mitochondria appear as mobile and flexible organelles that seem to be associated with microtubules [9,10]. They create an operative and dynamic reticulum whose steady-state morphology is regulated by events of fission and fusion [11,12]. These mechanisms are mainly controlled by at least three conserved GTPases called Dnm1p, Fzo1p and Mgm1p in yeast, which are also involved in the regulation of the membrane structure [12–15].

Another crucial factor, which has been the subject of many recent studies, is the influence of the phospholipid composition of mitochondrial membranes on mitochondrial function, structure and biogenesis [16–18]. The proper equilibrium in the phospholipids composition of mitochondrial membranes is pivotal not only for the mitochondrial architecture but also for the activity of protein complexes that reside in the mitochondria [19,20].

The structure of mitochondria is characterized by the presence of two membranes that delimit four distinct compartments: the outer membrane, the intermembrane space, the inner membrane and the matrix. The inner membrane comprises two morphologically distinct domains: (i) the inner boundary membrane, which is contiguous to the outer membrane, and (ii) the polymorphic tubular membrane invaginations termed cristae membrane, where specific protein complexes are restricted in a semi enclosed area of the inner membrane [21]. These two domains are connected to each other by tubular openings called cristae junctions [22,23]. A large inner membrane protein complex known as *Mitochondrial* Contact Site (MICOS) is important to stabilize, position and control the number of cristae junctions and organize the inner membrane into an efficient respiratory machine [24–26]. MICOS consists of at least six different proteins, most of which are found in yeast and animal species. In yeast, MICOS is made up of six sub-complexes exposed to the intermembrane space (Mic10p, Mic12p, Mic26p, Mic27p, and Mic60p) plus one peripheral inner membrane protein called Mic19p, which controls the number and positions of the cristae junctions that hold the folds of the inner membrane in place [25,27,28] (Fig. 1).

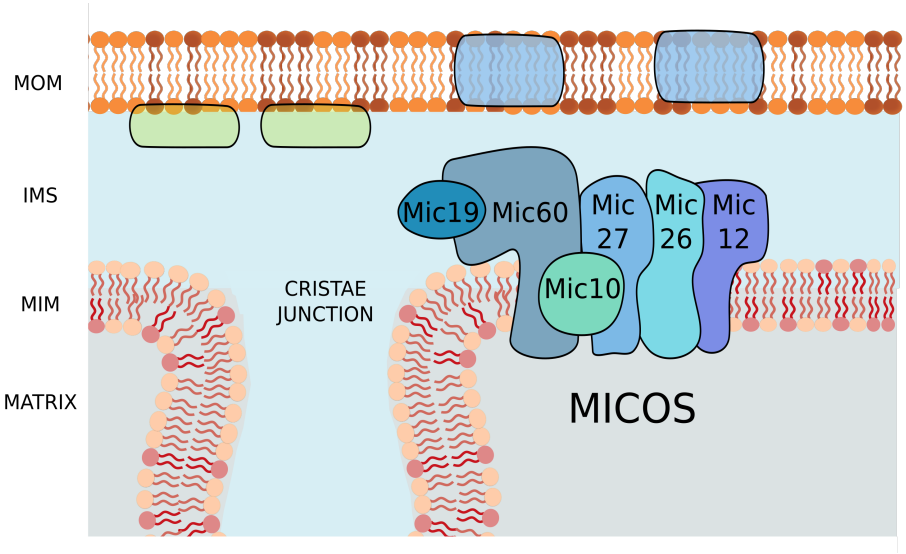

Figure 1 The MICOS complex. MICOS is a protein complex located in the inner mitochondrial membrane and responsible for preserving the architecture of the mitochondrial inner membrane and secure the cristae junctions. In *Saccharomyces cerevisiae* MICOS is composed by six subunits, five are integral inner membrane proteins (Mic10, Mic12, Mic26, Mic27, and Mic60), and one is a peripheral inner membrane protein (Mic19).

The phospholipid cardiolipin together with the respiratory chain complex of the inner mitochondrial membrane, are interacting with MICOS and play a role in the correct assembly of its two independent sub-complexes. This multipart interaction is crucial for the coordinated construction of a functional and properly assembled mitochondrial inner membrane [25,29].

The mitochondrial outer membrane consists of approximately 80% lipids and 20% proteins. The distinctive presence of pore proteins allows the free passage of ions and small, uncharged molecules, i.e. proteins, through the membrane [1,30]. The lack of impermeability in the outer mitochondrial membrane is the reason for the absence of a membrane potential across the outer membrane [31]. By contrast, the inner membrane is a tight diffusion barrier for both ions and molecules, which can cross the membrane only via specific membrane transport proteins [32]. As a result of its ion selectivity, an electrochemical membrane potential builds up across the inner mitochondrial membrane [17].

Distinctively from the outer mitochondrial membrane the inner counterpart is characterized by a protein-lipid ratio of 3 : 1. Several studies demonstrate that especially in this region phospholipids and proteins share a functional association that contributes to the stability and assembly of mitochondrial respiratory protein complexes, in the energy production and the protein import processes. For instance the activity of the inner membrane-associated proteins involved in the oxidative

phosphorylation depends on the phospholipid composition of the membrane, and changes in the phospholipid composition affect mitochondrial respiration [33,34].

The major phospholipids in the mitochondrial membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG) and cardiolipin (CL). PC and PE are the most abundant phospholipids, comprising respectively the 40% and 30% of total mitochondrial phospholipids, followed by PI and CL that are covering the 10-15% of the phospholipids component. PA and PS make up only the 5% of the total mitochondrial phospholipids, while PG is present in trace amounts [30] (Fig. 2). Mitochondrial membranes diverge drastically from the plasma membrane because of its high content in cardiolipin and the very low levels of sterols and sphingolipids that in *Saccharomyces cerevisiae* amount to 0.01 mg/mg protein and 0.02 mg/mg protein, respectively [30,35,36]. The phospholipid diversity in the mitochondrial membrane is also influenced by variation in length and degree of unsaturation of fatty acyl chain present within each class of phospholipid [37]. In the yeast *Saccharomyces cerevisiae*, palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1) are the most recurrent fatty acids found in phospholipids. Changes in acyl chain and head group are responsible for the variety and the different physical properties that characterize the different membrane phospholipids [38–41]. A general distinction is made between the cylindrical shaped phospholipids (PC, PS and PG) and conical shaped phospholipids like PE and CL, which are characterized by smaller head to tail area and the ablity to induce in the membrane the formation of inverted hexagonal phase structures (H_{II}) [42]. PE and CL are often referred to as non-bilayer forming lipids. Such non-bilayer forming phospholipids can strongly modulate the membrane flexibility and the propensity to create curvatures [43]. It is no surprise that mitochondrial membranes are characterized by the presence of CL,

as well as a particularly high concentration of the non-bilayer forming phospholipid PE compared with other organelles and the plasma membrane [30]. The synthesis of CL is a process that occurs exclusively in mitochondria [44,45]; while PE is mainly synthesized in the mitochondria by the enzyme Psd1, and to a lower extent also in different cellular compartments like the Golgi/vacuolar compartment, and the endoplasmic reticulum [45–48] (Fig. 2).

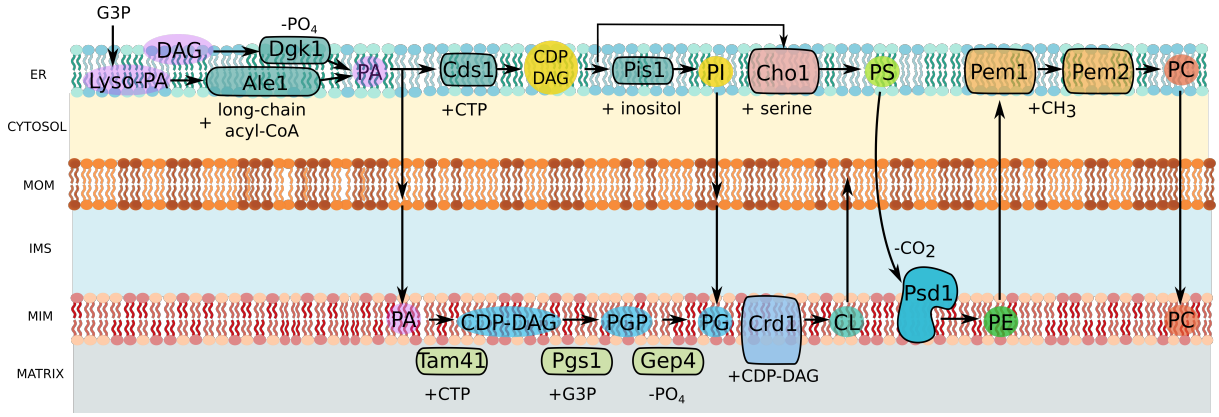

Figure 2 Biosynthesis of phospholipid in ER and mitochondria. Phospholipid biosynthetic pathway in *Saccharomyces cerevisiae*. G3P, glycerol-3-phosphate; lyso-PA, lysophosphatidic acid; Dgk1p, diacylglycerol kinase 1; Ale1p, lysophospholipid acyltransferase 1; PA, phosphatidic acid; Cds1p, CDP-diacylglicerol synthase 1; Tam41p, translocator assembly and maintenance protein 41; CDP-DAG, CDP-diacylglycerol; Pgs1p, phosphatidylglycerolphosphate synthase 1; PGP, phosphatidylglycerolphosphate; Gep4, genetic interactors of prohibitins 4; PG, phosphatidylglycerol; Pis1p, phosphatidylinositol synthase 1; PI, phosphatidylinositol; Crd1p, cardiolipin synthase; CL, cardiolipin; Cho1, phosphatidylserine synthase 1; PS, phosphatidylserine; Psd1p, phosphatidylserine decarboxylase 1; PE, phosphatidylethanolamine; Pem1p; phosphatidylethanolamine methyltransferase 1; Pem2p, phosphatidylethanolamine methyltransferase 2; PC, phosphatidylcoline.

The first chapter of this thesis is a collection of the state-of-the-art developments concerning the cell biology, physiology and enzymology of phosphatidylserine decarboxylases (PSD) type I and II. PSDs are the major class of enzymes involved in the

synthesis of PE, have an important role in the cellular lipid metabolism, and are spread among different organisms ranging from bacteria to humans [45]. A large part of Chapter 1 is concentrated on the role of mitochondrial Psd1 providing a broad illustration of its enzymology, biogenesis and assembly into the mitochondrial inner membrane, and also the contribution and importance of Psd1 for cellular phospholipid homeostasis [48].

After the exhaustive description of Psd enzymes, Chapter 2 steps into the molecular details of phosphatidylserine decarboxylases 1 from *Saccharomyces cerevisiae*. In yeasts the mitochondrial enzyme Psd1p produces the largest amount of cellular PE through decarboxylation of phosphatidylserine [49]. As the majority of mitochondrial proteins also Psd1p is first synthesized as larger precursor on cytosolic ribosomes and then imported into mitochondria directed by a specific mitochondrial targeting sequence [50]. The import of Psd1p within the outer mitochondrial membrane involves the recognition of its N-terminal mitochondrial targeting sequence by Tom70 and Tom22, which are the two main receptors of the translocases of the outer membrane (TOM complex). The subsequent passage of Psd1p through the inner mitochondrial membrane requires the mitochondrial inner membrane potential ($\Delta\Psi$), which is the only source of energy that drives the sorting of Psd1p into the inner mitochondrial subcompartment. At this stage of its biogenesis, Psd1p is embedded into the inner mitochondrial membrane via an hydrophobic sequence located in the β-subunit. The N-terminal targeting sequence protruding into the mitochondrial matrix, is removed by the Matrix Processing Peptidase MPP, and the further removal of an octapeptide stretches by Octapeptidyl Aminopeptidase Oct1p stabilize the mature form of the enzyme [50]. During the biogenesis and import of Psd1p an endoproteolitic autocatalytic process separate the precursor form of Psd1p into two separate subunits[50] (Fig. 3).

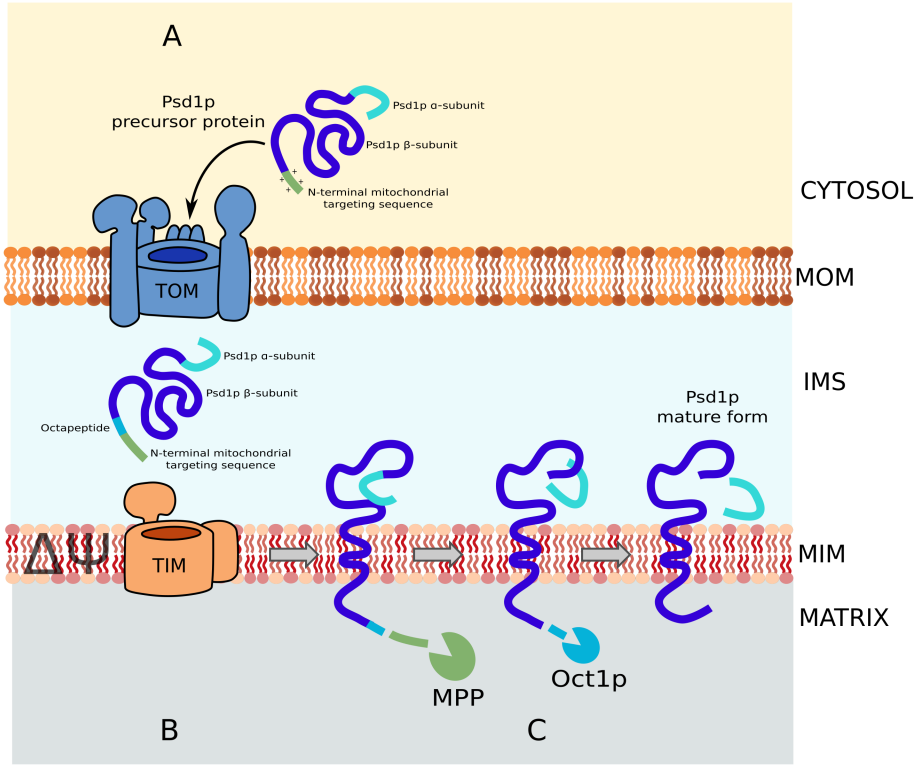

Figure 3 Psd1p biogenesis and import into mitochondria. (A) Psd1p precursor form is synthesized in the cytoplasm and then recognized by the TOM complex's receptors via its N-terminal mitochondrial targeting signal. (B) The passage of Psd1p into the inner mitochondrial membrane is performed via the translocase of the inner membrane (TIM complex) driven by the mitochondrial membrane potential ($\Delta\Psi$) (C) The positioning of Psd1p in the inner mitochondrial membrane is followed by the removal of the N-terminal signal peptide and of a octapeptide by the matrix localized peptidases MPP and the octapeptidyl aminopeptidase Oct1p.

The mature active form of Psd1p is anchored in the inner mitochondrial membrane and constituted by two distinct subunits: the inner membrane-bound β-subunit and the 4 kDa small α-subunit which is located in the intermembrane space (Fig. 3) [50]. The research described in the chapter 2 addresses an attentive analysis of the Psd1p α-subunit, which harbors a highly conserved motif. Speculations made on the role of this conserved amino acidic sequence portray this motif as the locus of the

interaction between Psd1p and its substrate, PS [51]. Despite these preliminary assumptions about the so-called *putative substrate recognition sequence*, this conserved motif has never been biochemically investigated. The data shown in this chapter highlight the importance of the conserved motif of the α-subunit for the structural integrity, the processing and biogenesis of Psd1p, as well as for the catalytic activity of this enzyme.

While the first part of this thesis is exclusively dedicated on PSDs enzymes and more specifically on Psd1p from yeast, the two following chapters (chapters 3 and 4) are exploring the direct interaction of the membrane phospholipids on the mitochondrial machinery for protein sorting and assembly.

It is well established that the majority of the proteins that reside in the mitochondria are initially synthesized as inactive precursors in the cytosol and then imported. At the level of the outer mitochondrial membrane, the translocase of the outer membrane (TOM complex) (Fig. 4), is the border line where the import process of these molecules into the mitochondria starts. The TOM complex is composed by a central channel-like subunit (Tom40), two main receptors (Tom20 and Tom70) and a fourth domain (Tom22) which stabilizes the complex and acts as a central receptor mediating the transfer of precursor proteins from Tom20 and Tom70 to the main central channel, Tom40 [52–56] (Fig. 4). After crossing the TOM complex, the majority of precursors are translocated into the intermembrane space, where they are transferred to other specialized protein sorting machineries which redirect the proteins to the right submitochondrial compartments [57,58] (Fig. 4).

The proteins characterized by a β-barrel conformation are first imported through the TOM complex and then subsequently transferred to the outer membrane sorting and assembly machinery (SAM complex) [59] . The main components of the SAM complex are the essential pore-forming domain, Sam50 and two other additional peripheral

membrane proteins, Sam35 and Sam37 [60–62]. Sam35 is crucial for initial binding of a conserved β-signal within the last β-strand of the incoming precursor; whereas Sam37 seems to mediate the release of the substrate proteins from the SAM complex [63,64] (Fig. 4).

In Chapter 3 it is explained how the biogenesis of β-barrel precursors is particularly sensitive to changes of the phospholipid composition of the outer membrane. Previous studies mainly focused on CL and PE, have shown that both phospholipids have a major impact on the import of mitochondrial β-barrel proteins. In fact, the lack of CL causes destabilization of TOM and SAM complexes, while depletion of PE affects β-barrel biogenesis at the stage of transport across the TOM complex, but it does not lead to its destabilization [65,66]. The experimental work presented in chapter 3 demonstrates that also the bilayer forming phospholipid PC plays a crucial role in assuring stability and functionality of the TOM and SAM complexes. The importance of PC in the biogenesis of β-barrel proteins is proved by the inability of a *S. cerevisiae* *pem1*Δ*pem2*Δ strain to correctly import β-barrel proteins. It is interesting to notice that the lack of PC does not interfere with the interaction between the the precursor protein and TOM complex, as it happens when PE is absent. This evidence suggests that the different membrane phospholipids have different roles and are involved in different steps of β-barrel biogenesis in mitochondria from *S. cerevisiae* [67].

After their first passage across the mitochondrial entry gates represented by the TOM and SAM complex, a substantial number of proteins are sorted into the inner membrane and the matrix [68]. The import within the inner mitochondrial membrane is operated by two translocases of the inner membrane (TIM): TIM23 is the presequence translocase, which transports precursor proteins with a cleavable presequence into the inner membrane and the matrix; and TIM22 is the carrier translocase, which inserts proteins with multiple transmembrane segments into the inner membrane [69,70] (Fig.

3). The activity of both proteins is triggered and supported by the membrane potential ($\Delta\Psi$) generated by the electron transfer through *respiratory chain* complexes. The TIM23 complex is composed by the main translocation channel Tim23, associated with Tim17. Three additional elements (Tim23, Tim50 and Tim21) stabilized by the subunits Mgr2, enable the transfer of the precursor protein from the TOM complex towards Tim23 which then releases the protein in the inner mitochondrial membrane [70]. The eventual transport of the protein into the mitochondrial matrix is accomplished thanks to the interaction between TIM23 and the presequence translocase-associated motor (PAM) [71].

The precursor protein directed to the complex TIM22 are recognized by the domain Tim54 which transfer the protein to its core structure formed by the a twin-pore Tim22. The two extra components of TIM22 (Tim18 and Sdh3) have a role in the stability and assembly of this complex [72] (Fig. 4).

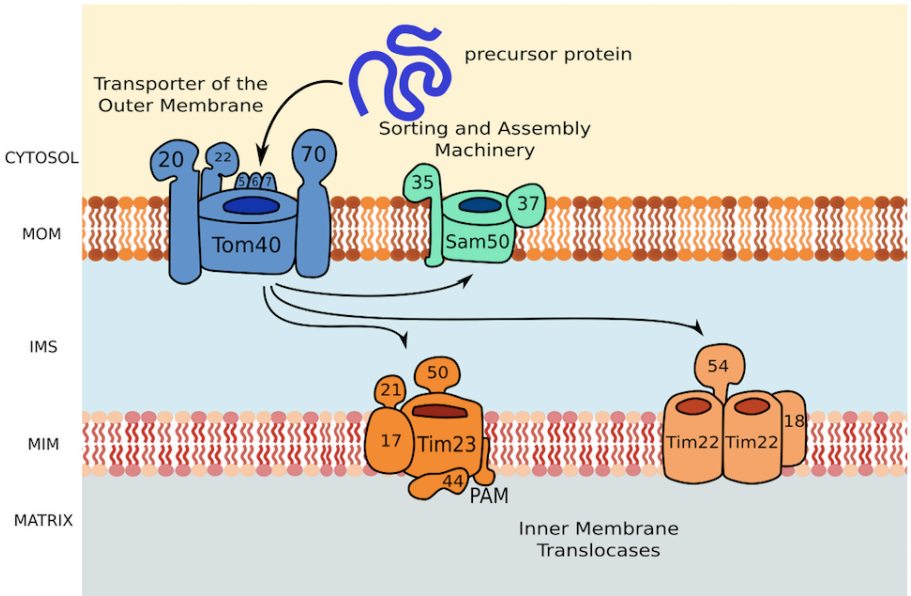

Figure 4 Import and sorting machinery of the outer and inner mitochondrial membrane. The precursors of β-barrel proteins are first imported through the TOM

complex, and then inserted into the outer membrane by the SAM complex. Proteins directed to the inner mitochondrial membrane or to the matrix are first transfered in the outer membrane through the TOM complex, then specific presequence are directing them to the TIM23 complex and motor PAM. Cleavable inner membrane proteins are laterally released from the TIM23 complex. Instead the TIM22 complex is responsible for the insertion of carrier proteins with internal targeting signals that are recognized by the receptor Tom70.

Chapter 4 examines the specific roles of PC on the activity of the described protein complexes (TIM23 and TIM22) involved in the protein translocation across the inner mitochondrial membrane (Fig. 4). PC is the most abundant phospholipid of the mitochondrial membranes. In mutant cells of *Saccharomyces cerevisiae*, which are defective in PC biosynthesis, the import of proteins precursors with a cleavable presequence as well as carrier proteins is reduced. Different from CL and PE, whose influence on the translocases TIM23 or TIM22 is a direct consequence of their ability to stabilize the respiratory chain complexes, depletion of PC does not decrease either the activity of the respiratory chain nor the membrane potential across the inner membrane [73]. This chapter describes how the decreased PC levels differentially affect the stability of TIM23 and TIM22 translocases. Among the two complexes, the TIM23 translocase is the most destabilized. Moreover the transfer of a preprotein in the TOM-TIM23 supercomplex is impaired, indicating that the lack of PC disturbs the initial binding of precursor proteins to TIM23, and in general the function of inner membrane protein translocases of mitochondria [74].

REFERENCES

[1] H. Lodish, A. Berk, S. Lawrence Zipursky, P. Matsudaira, D. Baltimore, J. Darnell, Molecular cell biology - NCBI Bookshelf, (2002).

[2] M.D. Brand, P. Couture, P.L. Else, K.W. Withers, A.J. Hulbert, Evolution of energy metabolism. Proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile, Biochem J. 275 (1991) 81–86.

[3] V.V. Emelyanov, Mitochondrial connection to the origin of the eukaryotic cell, Eur. J Biochem. 270 (2003) 1599-1618.

[4] M.T. Lin, M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, Nature 443 (2006) 787-795.

[5] X. Chen, J. Li, J. Hou, Z. Xie, F. Yang, Mammalian mitochondrial proteomics: insights into mitochondrial functions and mitochondria-related diseases, Expert Rev. Proteomics 7 (2010) 333-345.

[6] D.C. Chan, Mitochondria: dynamic organelles in disease, aging, and development, Cell 125 (2006) 1241-1251.

[7] S.A. Susin, H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D.R. Goodlett, R. Aebersold, D.P. Siderovski, J.M. Penninger, G. Kroemer, Molecular characterization of mitochondrial apoptosis-inducing factor , Nature 397 (1998) 441-446.

[8] M.F. Beal, Mitochondria take center stage in aging and neurodegeneration, Ann. Neurol. 58 (2005) 495-505.

[9] W.M. Saxton, P.J. Hollenneck, The axonal transport of mitochondria, J Cell. Sci. 125 (2012) 2095-2104.

[10] E.H. Ball, S.J. Singer, Mitochondria are associated with microtubules and not with intermediate filaments in cultured fibroblasts,Proc. Natl. Acad. Sci. USA 79 (1982) 123-126.

[11] H.M. McBride, M. Neuspiel, S. Wasiak, Mitochondria: More Than Just a Powerhouse, Curr. Biol. 16 (2006) 551-560.

[12] D.C. Chan, Mitochondrial fusion and fission in mammals, Annu. Rev. Cell. Dev. Biol. 22 (2006) 79-99.

[13] H. Chen, D.C. Chan, Emerging functions of mammalian mitochondrial fusion and fission, Hum. Mol. Genet. 14 (2005) 283-289.

[14] E.E. Griffin, D.C. Chan, Domain interactions within Fzo1 oligomers are essential for mitochondrial fusion, J. Biochem. Chem. 281 (2006) 16599-16606.

[15] E. Smirnova, L. Griparic, D.L. Shuland, A.M. van der Bliek, Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell 12 (2001) 2245-2256.
[16] L.C. Schenkel, M. Bakovic, Formation and regulation of mitochondrial membranes, Int. J. Cell Biol. (2014), Article ID 709828.
[17] W. Kühlbrandt, Structure and function of mitochondrial membrane protein complexes BMC Biol. 13 (2015) n/a.
[18] V.M. Gohil, M.L. Greenberg, Mitochondrial membrane biogenesis: phospholipids and proteins go hand in hand, J. Cell Biol. 184 (2009) 469–472.
[19] T. Tatsuta, M. Scharwey, T. Langer, Mitochondrial lipid trafficking, Trends Cell Biool. 24 (2014) 44-52.
[20] C. Osman, D.R. Voelker, T. Langer, Making heads or tails of phospholipids in mitochondria, J. Cell Biol. 192 (2011) 7–16.
[21] T.G. Frey, C.A. Mannella, The internal structure of mitochondria, Trends Biochem. Sci. 25 (2000) 319–324.
[22] C.A. Mannella, D.R. Pfeiffer, P.C. Bradshaw, I.I. Moraru, B. Slepchenko, L.M. Loew, C.E. Hsieh, K. Buttle, M. Marko, Topology of the Mitochondrial Inner Membrane: Dynamics and Bioenergetic Implications, 52 (2001) 93-100.
[23] G. Perkins, C. Renken, Electron Tomography of Neuronal Mitochondria: Three-Dimensional Structure and Organization of Cristae and Membrane Contacts, J. Struct. Biol. 119 (1997) 260-272.
[24] M. Harner, C. Körner, D. Walther, D. Mokranjac, J. Kaesmacher, U. Welsch, J. Griffith, M. Mann, F. Reggiori, W. Neupert, The mitochondrial contact site complex, a determinant of mitochondrial architecture. EMBO J. 21 (2011) 4356-4370.
[25] J.R. Friedman, A. Mourier, J. Yamada, J.M. McCaffery, J. Nunnari, MICOS coordinates with respiratory complexes and lipids to establish mitochondrial inner membrane architecture, eLife 4 (2015) e07739.
[26] M.A. Huynen, M. Mühlmeister, K. Gotthardt, S. Guerrero-Castillo, U. Brandt, Evolution and structural organization of the mitochondrial contact site (MICOS) complex and the mitochondrial intermembrane space bridging (MIB) complex, Biochim. Biophys. Acta 1863 (2016) 91-101.
[27] R.M. Zerbes, P. Höß, N. Pfanner, M. van der Laan, M. Bohnert, Distinct roles of Mic12 and Mic27 in the mitochondrial contact site and cristae organizing system, J. Mol. Biol. 428 (2016) 1485-1492.
[28] M. Bohnert, R.M. Zerbes, K.M. Davies, A.W. Mühleip, H. Rampelt, S.E. Horvath, T. Boenke, A. Kram, I. Perschil, M. Veenhuis, W. Kühlbrandt, I.J. van der Klei, N.

Pfanner, M. van der Laan, Central role of Mic10 in the mitochondrial contact site and cristae organizing system, Cell Metab. 21 (2015) 747-755.

[29] M.E Harner, A.K. Unger, T. Izawa, D.M. Walther, C. Özbalci, S. Geimer, F. Reggiori, B. Brügger, M. Mann, B. Westermann, W. Neupert, Aim24 and MICOS modulate respiratory function, tafazzin-related cardiolipin modification and mitochondrial architecture, eLife 3 (2014) e01684.

[30] E. Zinser, G. Daum, Isolation and biochemical characterization of organelles from the yeast, Saccharomyces cerevisiae, Yeast. 11 (1995) 493–536.

[31] E.J. Weeber, M. Levy, M.J. Sampson, K. Anflous, D.L. Armstrong, S.E. Brown, J.D. Sweatt, W.J. Craigen, The role of mitochondrial porins and the permeability transition pore in learning and synaptic plasticity, J. Biol. Chem. 277 (2002) 18891-18897.

[32] M. Meinecke, R. Wagner, P. Kovermann, B. Guiard, D.U. Mick, D.P. Hutu, W. Voos, K.N. Truscott, A. Chacinska, N. Pfanner, P. Rehling, Tim50 maintains the permeability barrier of the mitochondrial inner membrane, Science 312 (2006) 1523-1526.

[33] E. Zinser, C.D. Sperka-Gottlieb, E.V. Fasch, S.D. Kohlwein, F. Paltauf, G. Daum, Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae, J. Bacteriol. 173 (1991) 2026–2034.

[34] C.U. Mårtensson, K.N. Doan, T. Becker, Effects of lipids on mitochondrial functions, Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids. (n.d.). doi:10.1016/j.bbalip.2016.06.015.

[35] A.S. Joshi, J. Zhou, V.M. Gohil, S. Chen, M.L. Greenberg, Cellular functions of cardiolipin in yeast, Biochim. Biophys. Acta 1793 (2009) 212-218.

[36] J.T. Hannich, K. Umebayashi, H. Riezman, Distribution and functions of sterols and sphingolipids, Cold Spring Harb. Perspect Biol. 5 (2011) 3.

[37] W.J. van Blitterswijk, B.W. van der Meer, H. Hilkmann, Quantitative contributions of cholesterol and the individual classes of phospholipids and their degree of fatty acyl (un)saturation to membrane fluidity measured by fluorescence polarization, Biochemistry 26 (1987) 1746-1756.

[38] R. Homan, H.J. Pownall, Transbilayer diffusion of phospholipids: dependence on headgroup structure and acyl chain length, Biochim. Biophys. Acta 938 (1988) 155-166.

[39] D.G. Cameron, E.F. Gudgin, H.H. Mantsch, Dependence of acyl chain packing of phospholipids on the head group and acyl chain length, Biochemistry 20 (1981) 4496-4500.

[40] M. Koivusalo, P. Haimi, L. Heikinheimo, R. Kostiainen, P. Somerhariu, Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response, 42 (2001) 663-672.

[41] M. Kodama, T. Miyata, Effect of the head group of phospholipids on the acyl-chain packing and structure of their assemblies as revealed by microcalorimetry and electron microscopy, Colloids Surf., A 109 (1996) 283-289.

[42] P.R. Cullis, B. de Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta 559 (1979) 399-420.

[43] E. van den Brink-van der Laan, J.A. Killian, B. de Kruijff, Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile, Biochim. Biophys. Acta. 1666 (2004) 275–288.

[44] M. Schlame, D. Haldar, Cardiolipin is synthesized on the matrix side of the inner membrane in rat liver mitochondria, J. Biol. Chem. 268 (1993) 74-79.

[45] S.E. Horvath, G. Daum, Lipids of mitochondria, Prog. Lipid Res. 52 (2013) 590–614.

[46] E.A. Dennis, E.P. Kennedy, Intracellular sites of lipid synthesis and the biogenesis of mitochondria, J. Lipid Res. 13 (1972) 263–267.

[47] G. Daum, N.D. Lees, M. Bard, R. Dickson, Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*, Yeast Chichester Engl. 14 (1998) 1471–1510.

[48] F. Di Bartolomeo, A. Wagner, G. Daum, Cell biology, physiology and enzymology of phosphatidylserine decarboxylase, Biochim. Biophys. Acta (2016) In press.

[49] P.J. Trotter, D.R. Voelker, Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 6062–6070.

[50] S.E. Horvath, L. Böttinger, F.N. Vögtle, N. Wiedemann, C. Meisinger, T. Becker, G. Daum, Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1, J. Biol. Chem. 287 (2012) 36744–36755.

[51] K. Igarashi, M. Kaneda, A. Yamaji, T.C. Saido, U. Kikkawa, Y. Ono, K. Inoue, M. Umeda, 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 (1995) 29075–29078.

[52] K. Hill, K. Model, M.T. Ryan, K. Dietmeier, F. Martin, R. Wagner, N. Pfanner, Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins, Nature 395 (1998) 516-521.

[53] A.B. Harbauer, R.P. Zahedi, A. Sickmann, N. Pfanner, C. Meisinger, The protein import machinery of mitochondria-a regulatory hub in metabolism, stress, and disease, Cell Metab. 19 (2014) 357-372.

[54] D. Stojanovski, M. Bohnert, N. Pfanner, M. van der Laan, Mechanisms of Protein Sorting in Mitochondria, Cold Spring Harb. Perspect. Biol. 4 (2012) a011320.

[55] N. Bolender, A. Sickmann, R. Wagner, C. Meisinger, N. Pfanner, Multiple pathways for sorting mitochondrial precursor proteins, EMBO Rep. 9 (2008) 42-49.

[56] T. Shiota, H. Mabuchi, S. Tanaka-Yamano, K. Yamano, T. Endo, In vivo protein-interaction mapping of a mitochondrial translocator protein Tom22 at work, Proc. Natl. Acad. Sci. USA, 108 (2011) 15179-15183.

[57] A.P. van Loon, G. Schatz, Transport of proteins to the mitochondrial intermembrane space: the "sorting" domain of the cytochrome c1 presequence is a stop-transfer sequence specific for the mitochondrial inner membrane., EMBO J. 6 (1987) 2441–2448.

[58] K. Diekert, G. Kispal, B. Guiard, R. Lill, An internal targeting signal directing proteins into the mitochondrial intermembrane space, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 11752–11757.

[59] N. Wiedemann, V. Kozjak, A. Chacinska, B. Schönfisch, S. Rospert, M.T. Ryan, N. Pfanner, C. Meisinger, Machinery for protein sorting and assembly in the mitochondrial outer membrane, Nature. 424 (2003) 565–571.

[60] D. Ishikawa, H. Yamamoto, Y. Tamura, K. Moritoh, T. Endo, Two novel proteins in the mitochondrial outer membrane mediate β-barrel protein assembly, J. Cell Biol. 166 (2004) 621–627.

[61] V. Kozjak, N. Wiedemann, D. Milenkovic, C. Lohaus, H.E. Meyer, B. Guiard, C. Meisinger, N. Pfanner, An Essential Role of Sam50 in the Protein Sorting and Assembly Machinery of the Mitochondrial Outer Membrane, J. Biol. Chem. 278 (2003) 48520–48523.

[62] S. Kutik, D. Stojanovski, L. Becker, T. Becker, M. Meinecke, V. Krüger, C. Prinz, C. Meisinger, B. Guiard, R. Wagner, N. Pfanner, N. Wiedemann, Dissecting Membrane Insertion of mitochondrial β-barrel proteins, Cell. 132 (2008) 1011–1024.

[63] L.S. Wenz, L. Ellenrieder, J. Qiu, M. Bohnert, N. Zufall, M. van der Laan, N. Pfanner, N. Wiedemann, T. Becker, Sam37 is crucial for formation of the mitochondrial TOM-SAM supercomplex, thereby promoting β-barrel biogenesis, J. Cell Biol. 210 (2015) 1047–1054.

[64] N.C. Chan, T. Lithgow, The peripheral membrane subunits of the SAM complex function codependently in mitochondrial outer membrane biogenesis, Mol. Biol. Cell 19 (2008) 126-136.

[65] N. Gebert, A.S. Joshi, S. Kutik, T. Becker, M. McKenzie, X.L. Guan, V.P. Mooga, D.A. Stroud, G. Kulkarni, M.R. Wenk, P. Rehling, C. Meisinger, M.T. Ryan, N. Wiedemann, M.L. Greenberg, N. Pfanner, Mitochondrial cardiolipin involved in outer membrane protein biogenesis: implications for Barth syndrome, Curr. Biol. CB. 19 (2009) 2133–2139.

[66] T. Becker, S.E. Horvath, L. Böttinger, N. Gebert, G. Daum, N. Pfanner, Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins, J. Biol. Chem. 288 (2013) 16451–16459.

[67] M.H. Schuler, F. Di Bartolomeo, L. Böttinger, S.E. Horvath, L.S. Wenz, G. Daum, T. Becker, Phosphatidylcholine affects the role of the sorting and assembly machinery in the biogenesis of mitochondrial β-barrel proteins, J. Biol. Chem. 290 (2015) 26523–26532.

[68] J.M. Herrmann, W. Neupert, Protein Insertion into the inner membrane of mitochondria, IUMBM Life 55 (2008) 219-225.

[69] R.E. Jensen, C.D. Dunn, Protein import into and across the mitochondrial inner membrane: role of the TIM23 and TIM22 translocons, Biochim. Biophys. Acta BBA - Mol. Cell Res. 1592 (2002) 25–34.

[70] D. Mokranjac, W. Neupert, The many faces of the mitochondrial TIM23 complex, Biochim. Biophys. Acta 1797 (2010) 1045–1054.

[71] Y. Li, J. Dudek, B. Guiard, N. Pfanner, P. Rehling, W. Voos, The presequence translocase-associated protein import motor of mitochondria Pam16 function in an antagonistic manner to Pam18, J. Biol. Chem. 279 (2004) 38047–38054.

[72] P. Kovermann, K.N. Truscott, B. Guiard, P. Rehling, N.B. Sepuri, H. Müller, R.E. Jensen, R. Wagner, N. Pfanner, Tim22, the essential core of the mitochondrial protein insertion complex, forms a voltage-activated and signal-gated channel, Mol. Cell. 9 (2002) 363–373.

[73] L. Böttinger, S.E. Horvath, T. Kleinschroth, C. Hunte, G. Daum, N. Pfanner, T. Becker, Phosphatidylethanolamine and Cardiolipin Differentially Affect the Stability of Mitochondrial Respiratory Chain Supercomplexes, J. Mol. Biol. 423 (2012) 677–686. doi:10.1016/j.jmb.2012.09.001.

[74] M.H. Schuler, F.D. Bartolomeo, C.U. Martensson, G. Daum, T. Becker, Phosphatidylcholine affects inner membrane protein translocases of mitochondria, J. Biol. Chem. (2016) jbc.M116.722694.

CHAPTER 1

Cell Biology, Physiology and Enzymology of Phosphatidylserine Decarboxylase

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Cell Biology, Physiology and Enzymology of Phosphatidylserine Decarboxylase

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ABSTRACT

Phosphatidylethanolamine is one of the most abundant phospholipids whose major amounts are formed by phosphatidylserine decarboxylases (PSD). Here we provide a comprehensive description of different types of PSDs in the different kingdoms of life. In eukaryotes, type I PSDs are mitochondrial enzymes, whereas other PSDs are localized to other cellular compartments. We describe the role of mitochondrial Psd1 proteins, their function, enzymology, biogenesis, assembly into mitochondria and their contribution to phospholipid homeostasis in much detail. We also discuss briefly the cellular physiology and the enzymology of Psd2.

Key words: Phosphatidylethanolamine, phosphatidylserine decarboxylase, lipids, mitochondria

Abbreviations Ale1, acyltransferase for lyso-phosphatidylethanolamine; Cho, choline; CHO, Chinese hamster ovary cell; CL, cardiolipin; Crd1, cardiolipin synthase; Dnm1, Dynamin-related GTPase; Eki1, ethanolamine kinase; Dpl1, sphingosine phosphate lyase; ER, endoplasmic reticulum; Ept1, sn-1,2-diacylglycerol ethanolamine and choline phosphotransferase; GR, Golgi retention; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MPP, Matrix Processing Peptidase; Oct1, Octapeptidyl Aminopeptidase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Prn, propanolamine; PS, phosphatidylserine; PSD, phosphatidylserine decarboxylase; PA, phosphatidic acid; SAM, sorting and assembly machinery; TIM, translocase of the inner mitochondrial membrane; TLR4, Toll-like receptor 4; TOM, translocase of the outer mitochondrial membrane

1. INTRODUCTION

The sophisticated and highly regulated network of metabolic pathways, which lead to the synthesis of biological membranes, has gained more and more attention during the last decades as many biological processes were found to be membrane associated. Phospholipids play an important role in that respect, because they are major structural and functional components of biomembranes and play a dynamic role in many regulatory processes [1,2]. In many cell types including yeasts, parasites, plant cells and mammalian cells, such as *Saccharomyces cerevisiae* [3], *Plasmodium falciparum* [4], *Toxoplasma gondii* [5], *Arabidopsis thaliana* [6,7], mouse cells [8] and mammalian epithelial cells [9] phosphatidylcholine (PC) is the major phospholipid,

followed by phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS).

PE is specific among the other zwitterionic phospholipids because of its strong tendency to induce a curvature in the membranes due to its conical molecular structure [10–12]. Characteristically, PE forms inverted hexagonal phase structures triggering high tension within the membrane [13,14]. This effect seems to favor membrane fusion, vesicle formation and movement of proteins across membranes. Indeed, absence or presence of PE can influence these processes [15,16]. Especially in mitochondrial membranes where PE occurs at high abundance [17–19] structural properties of this phospholipid seem to play an important role for various functions. In the mitochondrial inner membrane (MIM) the amount of PE is specifically high [20]. Compared with the mitochondrial outer membrane (MOM), the curvature of MIM is more pronounced, the protein content is much higher and the ratio of PE to PC is increased [21,22].

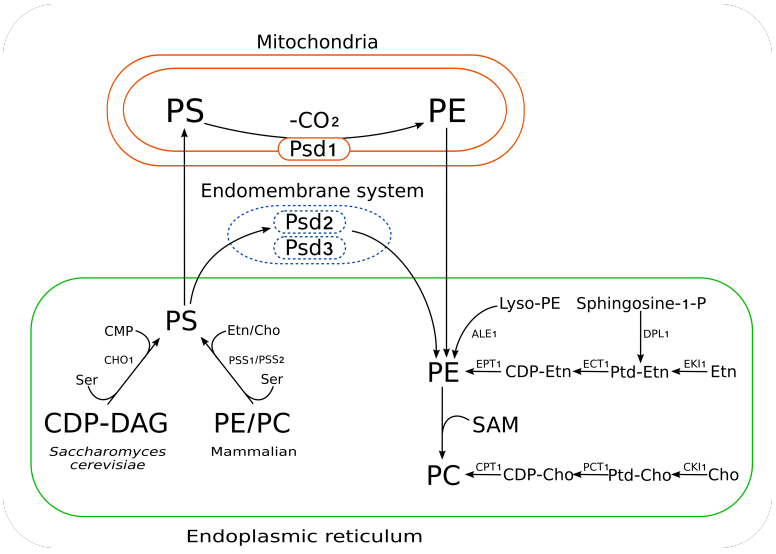


Figure 1: Overview of pathways of aminoglycerophospholipid biosynthesis In the yeast Saccharomyces cerevisiae phosphatidylserine (PS) is formed by the PS synthase from CDP-diacylglycerol (CDP-DAG) in the endoplasmic reticulum (ER). In mammalian

cells the synthesis of PS occurs via a base-exchange mechanism, where starting from PE or PC phosphatidylserine synthase 1 (Pss1) and 2 (Pss2) exchange ethanolamine or choline for serine. In either case the newly synthesized PS is transported to mitochondria where it can be decarboxylated by phosphatidylserine decarboxylase type I (Psd1) to form Phosphatidylethanolamine (PE). Minor amounts of PE are formed outside the mitochondria by phosphatidylserine decarboxylase type II (Psd2 and Psd3). PE can also be formed in the ER via the CDP-ethanolamine branch of the Kennedy pathway. Starting from ethanolamine (Etn) the enzyme ethanolamine kinase (Eki1) catalyzes phosphorylation of ethanolamine to ethanolaminephosphate (Ptd-Etn) which is further converted to CDP-ethanolamine (CDP-Etn) by ethanolamine-phosphate cytidylyltransferase (Ect1). The final synthesis of PE is catalyzed by the highly specific enzyme ethanolamine phosphotransferase 1 (Ept1). The Kennedy pathway is linked to sphingolipid catabolism through a reaction catalyzed by dihydrosphingosine-phosphate lyase (Dpl1). This enzyme cleaves phosphorylated sphingosid base to long chain aldehyde and ethanolaminephosphate (Etn-P). An alternative pathway for PE synthesis is the acylation of lyso-PE catalyzed by the enzyme lyso-PE acyltransferase (Ale1), localized in the mitochondria associated ER (MAM). PE can be further converted to phosphatidylcholine (PC) by phosphatidylethanolamine methyltransferases. Besides the methylation pathway, PC is also formed from choline (Cho) via the CDP-choline branch of the Kennedy pathway. The first step is catalyzed by choline kinase (Cki1) which phosphorylates choline to choline-phosphate (Ptd-Cho). Ptd-Cho is the substrate of choline-phosphate cytidylyltransferase (Pct1) which produces CDP-choline (CDP-Cho). The final step in the CDP-choline route leading to phosphatidylcholine (PC) is catalyzed by cholinephosphotransferase 1(Cpt1). Ser, serine; SAM S-adenosyl methionine.

In eukaryotes, PE can be synthesized via two different major pathways. First, PS can be decarboxylated by phosphatidylserine decarboxylases (PSD); and secondly diacylglycerol can be converted to PE in a reaction with CDP-ethanolamine involved [3,23]. Fig. 1 shows the main routes for the formation of PE and PC. The majority of PE is formed in the mitochondria via decarboxylation of PS, catalyzed by PSD type I. Minor amounts of PE are formed in the extramitochondrial space by other types of PSDs. PC and PE are also formed in the ER via methylation of PE and the CDP-ethanolamine/CDP-choline pathway (Kennedy pathway), respectively.

In this review article we will describe PSD enzymes and specifically focus on eukaryotic mitochondrial Psd1 proteins. We will provide recent evidence about structural and functional properties of these enzymes from different cellular sources and discuss the pivotal role of PSDs in the lipid metabolic pathways as the major producer of PE [24,25]. The importance of PE as mitochondrial and cellular phospholipid will be discussed. Furthermore, we will provide examples for the influence of this phospholipid on various cellular processes. For more information on this topic the reader is referred to other review articles [26–28].

2. PHOSPHATIDYLSERINE DECARBOXYLASES FROM BACTERIA TO HUMANS

PSDs are the major enzymes of PE synthesis in most types of cells, and they play a central role in phospholipid metabolism from bacteria to humans. Their evolutionary conservation suggests that these enzymes fulfill a central role in lipid metabolism and membrane biogenesis [29]. The similarity of bacterial and mitochondrial PSDs may be explained by the endosymbiotic theory. The import of PS, which is the substrate of PSDs, from the extramitochondrial space to mitochondria as well the supply of PE to the whole cell may also be part of this view [30].

Many cell types contain two types of PSDs, namely type I and type II. The two PSDs can be distinguished by their specific structure, sequence and subcellular localization. Analysis of Psd1 enzymes from different organisms belonging to various species and phyla shows that it is possible to characterize some identity clusters along the sequence with highly conserved and/or similar amino acids. In an overall identity of 3.93 % with 26 identical and 21 similar residues for Psd1s among the organism considered can be seen. The same sequence similarity check has been performed for Psd2 proteins. In this case the sequence of 8 different PSD type II enzymes has been

aligned and compared, showing an identity of 1.308 % for 22 identical and 29 similar positions. Unlike Psd1, the sequence alignment computed for Psd2 proteins displays an identity cluster concentrated only at the carboxy terminus of the protein sequence. For both enzyme types the site of major identity is at the autocatalytic cleavage motif (Figure 3). To explain the evolutionary relationship of Psd1 and Psd2 enzymes, a phylogenetic three was constructed using PSD Type I and II sequences of the different organisms. The reconstruction of the evolution and the ancestral interconnection of the enzymes shows that the PSD type I and II have a common ancestor but they were separated very early in the evolutionary scale.

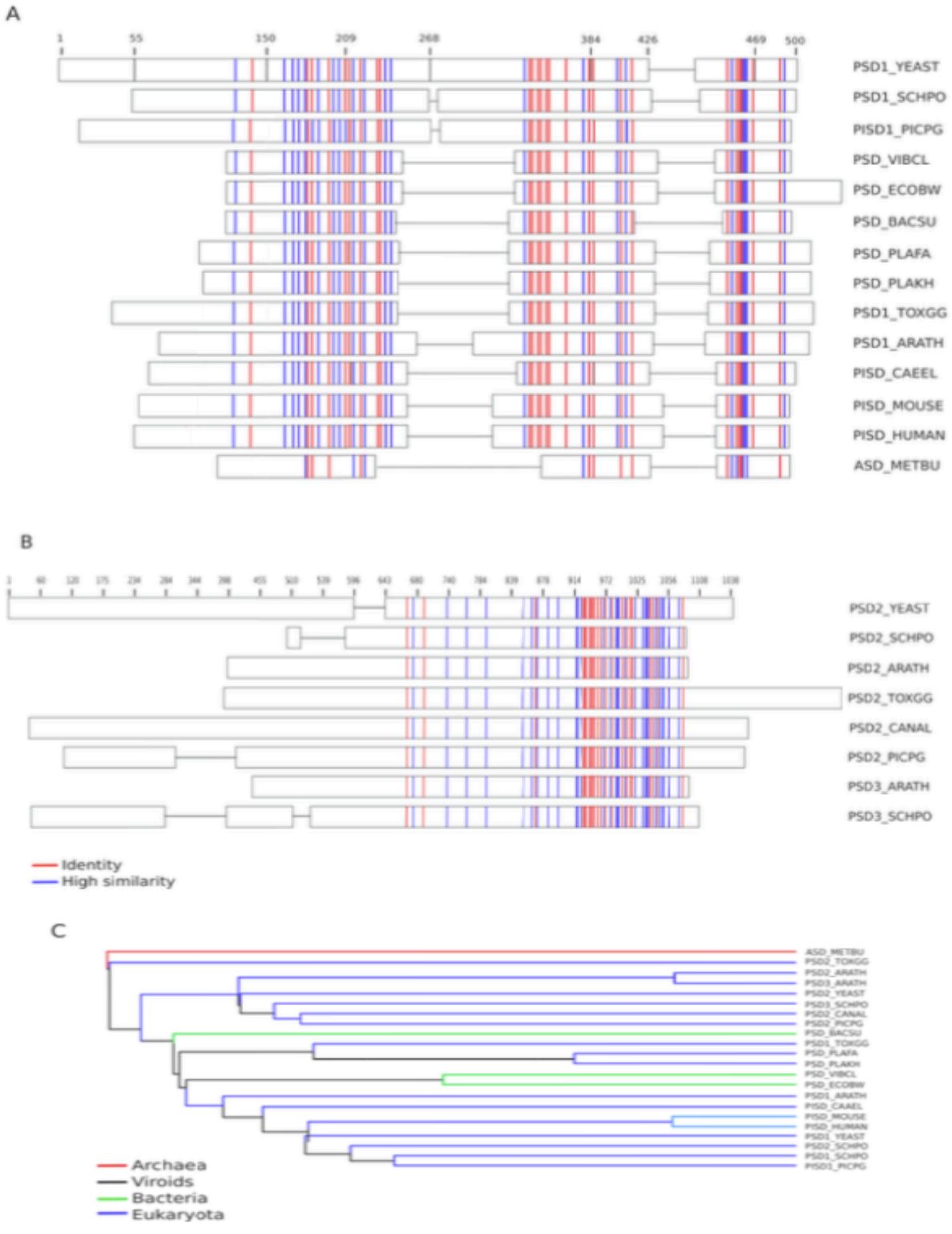

A
1
55
150
209
268
384
426
469
500
PSD1_YEAST
PSD1_SCHPO
PISD1_PICPG
PSD_VIBCL
PSD_ECOBW
PSD_BACSU
PSD_PLAFA
PSD_PLAKH
PSD1_TOXGG
PSD1_ARATH
PISD_CAEEL
PISD_MOUSE
PISD_HUMAN
ASD_METBU
B
PSD2_YEAST
PSD2_SCHPO
PSD2_ARATH
PSD2_TOXGG
PSD2_CANAL
PSD2_PICPG
PSD3_ARATH
PSD3_SCHPO
Identity
High similarity
C
Archaea
Viroids
Bacteria
Eukaryota

Figure 2 Sequence alignment and phylogenetic tree of PSDs in different organisms (A) Psd1 sequences alignment of the following selected organism is shown: *Saccharomyces cerevisiae* (YEAST), *Schizosaccharomyces pombe* (SCHPO), *Pichia pastoris* (PICPG), *Vibrio cholerae* (VIBCL), *Escherichia coli* (ECOBW), *Bacillus subtilis* (BACSU), *Plasmodium falciparum* (PLAFA), *Plasmodium knowlesi* (PLAKH), *Toxoplasma gondii* (TOXGG), *Arabidopsis thaliana* (ARATH), *Caenorhabditis elegans* (CEEL), *Mus musculus* (MOUSE), *Homo sapiens* (HUMAN) and Archaea *Methanococcoides burtonii* (METBU). Proteins analyzed have an overall identity of 3.93 % with 26 identical and 21 similar amino acids. Archaetidylserine decarboxylase (ADS) from M. burtonii shares 12 identical and 8 similar positions with Psd1 sequences of the rest of the organisms analyzed. (B) Sequence alignment of Psd2 and Psd3 proteins from the organisms *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Arabidopsis thaliana, Toxoplasma gondii, Candida albicans* and *Pichia pastoris*. Psd2 enzymes considered have an identity of 1.31 % with 22 identical positions and 29 similar positions. (C) The phylogenetic tree created by using the Clustal Omega software [34] shows the evolutionary relationships and proximity of Psd1 and the Psd2 proteins.

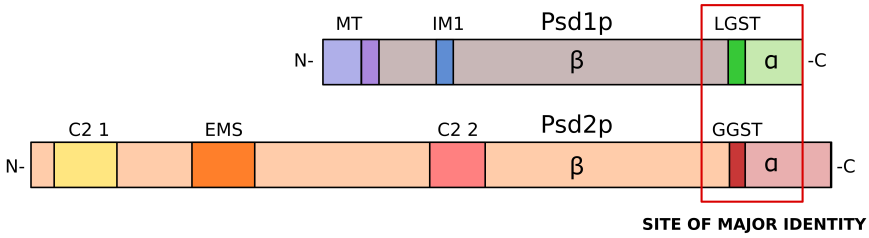
A very interesting case it the one regarding the Archaea domain. Experiments performed by Nishihara et al. [31] suggested that archaetidylethanolamine (AE), the equivalent of PE in Archaea, is generated through a pathway similar to that found in other organisms. An extensive comparative study, between archaeal and bacterial genomes, of the enzymes involved in the synthesis of polar lipids, revealed the existence of the genes or ORFs for phosphatidylserine synthase (PSS) then called Archaetidylserine synthase (ASS) and Phosphatidylserine decarboxylase (PSD) identified as Archaetidylserine decarboxylase (ASD) [32,33]. From Fig. 2 A it is possible to observe that ASD share 12 identical and 8 similar positions with the other Psd1 protein sequence analyzed and most of them are concentrated at the level of the GS(S/T) motif. In fact, it is important to notice that also the Archaetidylserine decarboxylase is subjected to autocatalytic cleavage into α- and β-subunit.

Bacteria are the only cells that are not able to synthesize PE in a PSD independent reaction. The bacterial Psd1 which is associated with the cytoplasmic

membrane [35,36] is closely related to type I PSDs from eukaryotic cells. In fact PSDs from *E. coli* and *B. subtilis* exhibit close sequence similarity to mitochondrial PSDs. The PSD enzyme from *E. coli* encodes for a polypeptide with 322 amino acids (35.9 kDa), and the PSD from *B. subtilis* for a protein with 263 amino acids (29.7 kDa) [35]. The PSD sequence from *B. subtilis* shares 27 % identity with PSDs from *E. coli* and CHO cells, and 29 % with S. *cerevisiae* Psd1 [32,37,38]. PSD from *Vibrio cholera*, a gram-negative bacterium well known for his virulence, is essential for bacterial viability. *V. cholera* PSD displays 31.7 % sequence identity with PSD from mouse and 60 % sequence identity with *E. coli* PSD [39].

Much of the research aimed at the understanding of the molecular biology of eukaryotic PSDs has been performed with yeast cells and more recently with parasites. The advantage of these unicellular organisms is their ease of genetic manipulation and biochemical handling. In *S. cerevisiae* two PSDs were identified, the mitochondrial Psd1 and the extramitochondrial Psd2 protein [25,40,41]. The more potent PSD of type I is localized to mitochondria and synthesizes the majority of PE under standard conditions. Like PSDs from other cell types, the yeast Psd1 consists of an α- and a β-subunit [42] (Figure 3). The β-subunit is the anchor fixing the protein in the MIM by one or more membrane spanning domains with a hydrophilic part that protrudes into the intermembrane space. Horvath et al. [17] showed that correct localization of Psd1 in the mitochondrial membrane of S. cerevisiae is crucial for the in vivo function of Psd1 and for achieving full enzymatic activity. As for other PSDs, the LGST motif of the yeast Psd1 is essential for correct cleavage and formation of the α- and β-subunit. Interaction of α- and β-subunit leads to the formation of the active site of the enzyme. Deletion of PSD1 results in PE depletion in mitochondria and in other cellular membranes, but growth is not affected when cells are grown in liquid and solid glucose medium at 30°C [19,43].

At 37°C the psd1Δ strain shows minimal growth without ethanolamine or choline supplementation, but supplementation with ethanolamine or choline can restore wild-type growth [14]. When non-fermentable carbon sources such as lactate are used as a carbon source, mitochondrial functions, especially respiration become more stringent and ethanolamine must be supplemented to rescue cell survival (see Figure 1).

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PSD1_YEAST KNIHL-LSQLSLNYFSNGFSCSEPHDTELFFAVIYLAPGDYHHFHSPVDWVCKVRRHFPG  363
PSD2_YEAST KEIWVKGRKFSIKKLANNYNPETFNDNNCSIGIFRLAPQDYHRFHSPCNGTIGKPVYVDG  971
           *:* :   ::*:: ::* :      * :  : :: *** ***:**** :       :  *

PSD1_YEAST DLFSVAPYFQRNFPNLFVLNERVA-LLGSWKYGFFSMTPVGATNVGSIKLNFDQEFVTNS  422
PSD2_YEAST EYYTVNPMAVRSELDVFGENIRVIIPIDSPQFGKLLYIPIGAMMVGSILLTCKENDVV--  1029
           : ::* *    *   ::*  * **   : * :: * :   *:**  **** *   :: *

PSD1_YEAST KSDKHLEPHTCYQAVYENASKILGGMPLVKGEEMGGFELGSTVVLCFEAPTEFKFDVRV-  481
PSD2_YEAST ----------------------------ESGQELGYFKFGGSTIIIIIPHNNFMFDSDLV  1061
                                       *:*:* *::* : :: :    :* **  :

PSD1_YEAST -------GDKVKMGQKLGIIGK-NDLK---------------------------------  500
PSD2_YEAST KNSSERIETLVKVGMSIGHTSNVNELKRIRIKVDDPKKIERIKRTISVSDENAKSTGNVT  1121
                     **:*  :*   : *:**

PSD1_YEAST -----------------
PSD2_YEAST WEYHTLREMMNKDFAGL  1138
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Figure 3: Sequence and position of important sites of yeast Psd1 and Psd2 Comparison of Psd1 and Psd2 from Saccharomyces cerevisiae shows 6.8 % identity with 87 identical and 139 similar amino acid positions. The highest sequence identity is found at the C terminus and specifically at the cleavage site of the two subunits, the LGST motif in Psd1 and at the GGST sequence in Psd2. Further functional domains identified in Psd1 are the mitochondrial targeting sequence (MT) and the transmembrane domain (IM1) which anchors the β-subunit of Psd1 to the MIM. In Psd2 the EMS sequence corresponds to the endomembrane system targeting domain while C2 1 and C2 2 are characteristic functional domains of Psd2 which are possibly involved in the binding of PS.

The extramitochondrial counterpart of the yeast Psd1 was named Psd2 and has been first localized to a Golgi/vacuolar compartment [20,40]. More recently, the enzymatic activity of Psd2p was rather attributed to endosomes [44,45]. Both Psd1 and Psd2 are synthesized as inactive proenzymes, but despite the fact that they catalyze the same reaction of PS decarboxylation, the primary structures exhibit low identity [40]. The yeast Psd1 protein shows more homology to the mammalian PSD than to Psd2 [46], and only the site of the self-maturation process exhibits highest homology between Psd1 and Psd2. The last 238 carboxyl terminal amino acid residues of Psd1 have 19 % identity with Psd2. Instead of the LGST motif Psd2 contains a GGST motif which is exactly the site where the reaction of autocatalytic cleavage into α- and β-subunit of the enzyme occurs [41] (see Figure 3). Besides the GGST site for the endoproteolytic cleavage, Psd2 has other relevant domains that are important for function and localization of the enzyme. A potential Golgi targeting/retention sequence (EFDIYNEDEREDSDFQSK) has been detected from amino acid residue 435 to 453 in the N-terminal part of Psd2, but has not yet been confirmed as such [41,47]. Another specific domain that characterizes and distinguishes Psd2 from Psd1 is a C2 domain from amino acid 534 to 577. Many speculations were made about the functional relevance of this domain. As the first C2 domain described in the literature [48] for the protein kinase C family was found to be responsible for Ca^{2+} binding and regulation, the same role was hypothesized for Psd2. However, this assumption has been challenged as the yeast Psd2 is not regulated by Ca^{2+} [49].

In the yeast *Pichia pastoris* two PSDs have been identified [50]. Deletion of the PSD1 gene resulted in decreased mitochondrial PSD activity to 25% of wild type and in ethanolamine auxotrophy. Presence of ethanolamine in the medium supported the CDP-ethanolamine pathway and compensated for the loss of PSD activity. As in *S.*

cerevisiae, deletion of PSD2 had only a minor effect on the total cellular PSD activity. Strains deleted of PSD2 grew like wild type and did not require ethanolamine as a supplement. These results indicated that Psd2 alone was not able to compensate for the loss of the major PE synthesizing enzyme in *P. pastoris*, the Psd1. Surprisingly a *P. pastoris* Δpsd1Δpsd2 mutant has never been described. The reason may be that this double mutation may be lethal and too stringent. The strong requirement for PE in the strictly aerobic *P. pastoris* cell may not allow such a rigorous depletion of PE.

Schizosaccharomyces pombe, a fission yeast, contains three genes with high sequence similarity to the PSDs from *S. cerevisiae* [51,52]. Although these three PSDs from *S. pombe* named Psd1, Psd2 and Psd3 fulfill overlapping functions, they are not completely redundant. Regarding the localization of these enzymes, Psd1 has been detected in mitochondria [52,53], whereas Psd2 was dually localized to mitochondria and to the nuclear envelope. Psd3 has been reported to be localized to the cytosol. In dividing cells it seems to be shifted to the cell periphery to the site of septum formation. Psd1 of *S. pombe* shows high homology to the Psd1 from *S. cerevisiae*, even though it is most similar in structure to the mammalian PSD. Psd3 from *S. pombe* is structurally comparable to the *S. cerevisiae* Psd2. The two enzymes share sequence homology in the predicted protein kinase C conserved region 2 (C2) Ca^{2+}-binding domain. The C2 domains of Psd3 from *S. pombe* and Psd2 from *S. cerevisiae* have been shown to bind Ca^{2+} as they contain all four amino acid residues that constitute the conserved metal binding pocket [54].

In the past many studies about PSDs focused on the discovery of pathways for PE synthesis. More recently, attention was shifted to the understanding of possible applications to PSD enzymes from parasites. An example for such investigations are studies with the parasitic protozoan *Trypanosoma brucei* [55]. In this study, a PSD

enzyme has been detected as a target for the development of new antimicrobial substances and for the development of strategies aimed at the containment and eradication of parasitic infections [4]. There are still controversial opinions about PSDs in *T. brucei*. In fact the majority of the studies assessed that the Kennedy pathway, also known as de novo synthesis of PE and PC, is the major cellular source of PE in T. brucei [56]. Studies performed by Signorell et al. [55] showed that in the *T. brucei* procyclic form, the RNAi-mediated knock-down of ethanolamine-phosphate cytidylyltransferase (ET), which is part of the Kennedy pathway for PE synthesis (see Figure 1), severely alters the mitochondrial morphology. On the other hand, in *T. brucei* gambiense the presence of a putative PSD was confirmed to be relevant for the survival of the parasite in the bloodstream [57]. Assessment of sequence similarities of *T. brucei* gambiense PSD with other PSDs enzymes showed that the PSD from this ancient eukaryote is more related to the eukaryotic PSD2 than to PSD1.

Plasmodium falciparum is a protozoan parasite that causes malaria in humans [58]. PSD from *Plasmodium falciparum* (PfPSD) has been tested using yeast cells as a host to perform an accurate screening for many substances with a possible anti-parasitic action. As a result, the 7-chloro-N-(4-ethoxyphenyl)-4-quinolinamine was identified as an effective inhibitor against *P. falciparum*. Subsequently a newly synthesized analog of this compound was tested against the mouse pathogen *Plasmodium yoelii*, and shown to be effective in infected mice by blocking the Psd1 activity and interfering with membrane biogenesis of the parasite [59]. In subsequent studies, the PSD from the parasite *Plasmodium knowlesi* was identified [60]. This protein can be distinguished from other eukaryotic PSDs because of its peculiarity to be expressed in a soluble and membrane associated form, both undergoing the classical auto-endoproteolytic maturation occurring in all PSDs.

Recent findings provided detailed evidence about the influence of PSDs in the infectiousness of *Toxoplasma gondii. T. gondii* is an obligate intracellular parasite and especially dangerous for immunosuppressed patients [61]. Studies of cellular lipid metabolism highlighted that the parasite is able to secrete a soluble form of PSD (TgPSD1) that normally resides in dense granules [62]. *T. gondii* elicits PS exposure on the surface of infected mammalian cells, which mimics a situation typical of apoptotic cells. This strategy is considered to be an evasion mechanism of parasites, also known as apoptotic mimicry. The fact that TgPSD1 is soluble, secreted and active on the surface of the parasite means that *T. gondii* is able to modulate the amount of PS exposed and creates a PS^+/ PS^- population. This heterogeneity has been proved to be successful for the infection and important to keep the inflammation and the parasite growth balanced. Secretion of TgPSD1 in *T. gondii* depends on temperature, ATP and Ca^{++} concentration [62]. In fact, incubating *T. gondii* at a temperature of 0°C inhibited TgPSD1 secretion by 90%. Depletion of ATP reduced the secretion of PSD by 92 % and exposure to the Ca^{++} chelator BAPTA-AM by 50 %. The transgenic parasite overexpressing TgPSD1-HA had 10-fold more PSD activity in homogenates than its parental strain. Besides TgPSD1, *T. gondii* is also able to synthesize PE in mitochondria via type I PSD (TgPSD1mt), which supplies the cell with PE necessary for regular cellular functions [5]. TgPSD1mt can functionally complement a psd1Δpsd2Δ yeast mutant and is required for optimal growth and replication of *T. gondii*, but dispensable for the parasite survival [40]. The mitochondrial targeting peptide in TgPSD1mt is not required for the catalytic activity of PSD [5]. Furthermore, beside these two PSDs, the parasite *T. gondii* is also able to generate PE in the ER from CDP-ethanolamine and diacylglycerol.

In plants, three types of PSDs were identified. Psd1, which is localized to mitochondria, exhibits substantial sequence similarity with the equivalent from other

cell types (see Figure 2). However, *Lycopersicon esculentum, Arabidopsis thaliana, Solanum tuberosum* and other plants can synthesize PE not only by mitochondrial but also by extramitochondrial PSDs [6,63]. A tomato protein named LePSD1 with a size of 50 kDa [63] was identified which was homologous to the mitochondrial PSDs from yeast and Chinese hamster and showed 37 % identity. LePSD1 had also 63 % identity with PSD from *Arabidopsis thaliana* (AtPSD1) showing divergence mostly in the N-terminal region, which is the region containing the mitochondrial targeting sequence. In contrast to other organisms, the model plant *Arabidopsis thaliana* harbors two additional isoforms of type II PSDs, Psd2 and Psd3 [6,63]. Psd2 and Psd3 from *A. thaliana* show 76 % sequence identity to the yeast Psd1. These proteins are therefore more related to this enzyme than to mitochondrial Psd1 from plants and other organisms. Fluorescent measurements showed that Psd2 and Psd3 from *A. thaliana* were localized to the endomembrane fraction or to the tonoplast and the ER. The mitochondrial Psd1 from *A. thaliana* showed highest expression in flowers, but was also located to roots and at low amounts in stems and leaves of the plant. Psd3 is also located to roots, stems, leaves and flowers, but present at very low amounts in siliques.

Surprisingly little evidence has been presented for mammalian PSDs. Only one mitochondrial form of PSD named Pisd was identified [64,65] which was attributed to the outer surface of the MIM. Deletion of Pisd is lethal for mice embryos after 8 to 10 days of development, and many of the mitochondria observed in these deceased embryos appear deformed [66]. Recent research in this field described an interesting relation between PE synthesis, PSD activity and degenerative diseases like Alzheimer, Parkinson and non-alcoholic liver disease [67]. It has been described that mammalian development strictly depends on the supply of PE via CDP-ethanolamine and PSD pathways, and that lack of mitochondrial PE dramatically alters mitochondrial

morphology [66]. In PSB-2 cells (CHO cells which require exogenous PS for cell growth with a chronically reduced synthesis of mitochondrial PE) a moderate depletion of mitochondrial PE impairs cell growth together with mitochondrial morphology and function [68]. A PSS I and PSS II defective PSB-2 mutant was compromised in growth without phospholipid supplementation [69]. Addition of exogenous PS, but not of exogenous PE suppressed the growth defect of the PSB-2 mutant.

Correlated studies performed with the yeast and the worm models led to the conclusion that PE is a component affecting Parkinson disease. In *Caenorhabditis elegans* it was shown that a drop of the PE level was followed by an alteration in the homeostasis of the Parkinson disease-associated protein α-synuclein (α-syn) [70]. In a *S. cerevisiae* psd1Δ mutant expressing α-syn leads to accumulation of α-syn and to the formation of α-syn foci. These results led to the hypothesis that reduction in PE caused formation of α-syn aggregates which accumulate in cells and trigger ER stress. Supplementation with ethanolamine was sufficient to restore ER functionality together with decreased α-syn foci formation. The same situation occurred in *C. elegans* where ethanolamine supplementation was sufficient to recover the activity of dopaminergic neurons that were under degeneration after RNAi depletion of PSD. The loss of PE homeostasis in biological membranes seems to be one of the reasons for neurodegeneration, and in this context the role of PSD in PE synthesis seems to be predominant [71].

3. PHYSIOLOGICAL ROLE OF PHOSPHATIDYLSERINE DECARBOXYLASES

The best strategy to understand the physiological role of PSDs is to analyze the effects in cells that are deleted of the respective genes. Such experiments can be effectively performed using microbial cells, because of the well-known ease of genetic manipulation.

In *E. coli* overexpression of PSD does not significantly alter the lipid composition of the cells [72]. In contrast depletion of PE due to mutation or deletion of PSD caused severe developmental defects. A conditionally lethal *E. coli* mutant with a temperature-sensitive mutation in the PSD gene appears to have a filamentous phenotype which is accompanied by PS accumulation and loss of viability [73,74]. In the absence of PE *E. coli* requires divalent cations to rescue growth [75,76]. In *E. coli*, PE can be replaced by glycolipids which restore certain functional properties of membranes [77]. For the Gram-positive bacterium *B. subtilis* PE is not essential for cell survival, and absence of PSD causes just an accumulation of PS [32]. A mutant of the Gram-negative nitrogen-fixing bacterium Sinorhizobium meliloti, which is deficient in PSD activity is unable to form PE and also accumulates PS.

A comparison of PE-deficient mutants of *S. meliloti* lacking either phosphatidylserine synthase (PSS) or PSD, respectively, showed that they grew like wild type on many complex media, but they exhibited a dosage dependent growth on phosphate [78]. They were unable to grow on minimal medium containing high phosphate concentrations, but the PSD deficient mutant could be cultivated on minimal medium containing low amounts of phosphate. Addition of choline to the minimal medium rescued growth of the PSS deficient mutant to some extent, but inhibited growth of the PSD deficient mutant.

With the yeast it has been demonstrated that depletion of PE in mitochondria leads to dysfunctions of respiration, defects in the assembly of mitochondrial protein complexes and loss of mtDNA [14,79]. However, a psd1Δ deletion mutant of *S. cerevisiae* grows like wild type on glucose media, but is unable to be cultivated on non-fermentable carbon sources such as lactate or ethanol without supplementation of ethanolamine, choline or serine [80]. The mitochondrial PE content in a psd1Δ yeast strain grown on lactate is decreased by 74 % of wild type [19], and over 90 % in a *psd1*Δ*psd2*Δ double mutant which is auxotrophic for ethanolamine or choline on both glucose and lactate [19,41,50,81].

A study from Storey et al. [14] shows that incorporation of the ethanolamine analogue propanolamine (Prn) into phosphatidylpropanolamine (PPrn) in *psd1*Δ*psd2*Δ and *psd1*Δ*psd2*Δ*dpl1*Δ yeast cells has different effects. This finding is due to the fact that a *psd1*Δ*psd2*Δ*dpl1*Δ can only grow in the presence of ethanolamine, while a *psd1*Δ*psd2*Δ which produces low levels of PE from a branch of sphingolipid breakdown can be rescued by ethanolamine or propanolamine. In fact *psd1*Δ*psd2*Δ cells supplemented with 2 mM propanolamine accumulate PPrn to an extent that can constitute 40% of the total phospholipid content. PPrn is structurally related to PE and like PE it is also capable of forming hexagonal phases. The inability of PPrn to replace ethanolamine in a *psd1*Δ*psd2*Δ*dpl1*Δ strain may be due to the low propensity of PPrn to form bilayer phases or by an essential role played by PE in cells that is independent of the physical properties of this lipid.

Most of the PE formed by Psd1 remains in the MIM of wild type mitochondria from *S. cerevisiae* [82]. To investigate the effect of PSD1 deletion on genome wide modifications in *S. cerevisiae* cDNA microarray analysis was performed [83]. Up-regulation of 54 genes was observed in a *psd1*Δ mutant, but significant down-

regulation of genes was not found [83]. Many of the up-regulated genes were related to transport, carbohydrate metabolism, generation of precursor metabolites and energy, and response to stress. Eighteen ORFs were related to catalytic enzyme activities like hydrolase, transferase, oxidoreductase, phosphatase and isomerase activities. Surprisingly, however, genes encoding for enzymes of the other three PE producing pathways such as PSD2, ALE1, EKI1, EPT1 and DPL1 were not up-regulated. In *S. cerevisiae* addition of inositol and choline represses several genes involved in PI, PS, and PC synthesis [84]. Also the activity of the PSD1 gene from yeast is repressed by 50–70 % by inositol in combination with choline [72]. It has been shown that OPI1 controls this repression process [72,84].

Deletion of PSD1 in yeast cells does not affect the mitochondrial morphology, but results in a changed mitochondrial phospholipid composition when cells are cultivated in rich medium containing lactate [85]. Exogenous ethanolamine does not significantly increase the mitochondrial PE content, indicating that PE is not efficiently transported to mitochondria [19]. Mitochondrial PE is required for efficient mitochondrial bioenergetics whereas cellular PE seems to be important for general cell growth. When a *psd1*Δ deletion strain is supplemented with ethanolamine, growth is partially rescued as extramitochondrial PE is increased and formed by the Kennedy pathway, but the oxidative phosphorylation or defects of mitochondrial ATP formation caused by the *psd1*Δ mutation could not be restored [85].

An unanticipated membrane property demonstrated with the yeast is the role of PE and cardiolipin (CL) in mitochondrial and cellular function. It was shown that both phospholipids can compensate for each other at least to some extent. Mitochondrial membranes have a unique phospholipid composition, and the two non-bilayer forming phospholipids CL and PE seem to play a crucial role in preserving the correct

mitochondrial morphology [86–90]. CL is a dimeric glycerophospholipid that is exclusively synthesized in mitochondria where it is involved in the processes of oxidative phosphorylation, regulation of apoptosis [86,91] and in mitochondrial biogenesis through regulation of protein import into this organelle [5,6,54]. CL also seems to be strictly associated with the fusion of mitochondria [92]. Formation of CL in *S. cerevisiae* is catalyzed by the mitochondrial cardiolipin synthase Crd1 [50,79,93], but cells lacking CRD1 do not bear major defects in mitochondrial functionality. This effect is mainly due to the compensation by PE synthesized by Psd1 [90]. A *crd1*Δ*psd1*Δ double mutation lacking both PE and CL is lethal [78,94]. In contrast other pathways of PE synthesis bearing deletions in the Psd2 and Dpl1 (sphingolipid breakdown) routes did not result in synthetic lethality with CRD1 [78] indicating the specific role of mitochondrially synthesized PE by Psd1 [6,14].

Mitochondrial morphology is regulated by the antagonistic mechanisms of fusion and fission. In *S. cerevisiae* the three proteins regulating mitochondrial fusion are Fzo1, Mgm1 and Ugo1 while the ones responsible for the regulation of the fission process are Dnm1, Fis1 and Mdv1. To demonstrate the role of CL and PE in the mitochondrial fusion process, a *crd1*Δ*psd1*Δ conditional mutant was created [95]. This strain expressed the CRD1 gene on a plasmid under control of the TET_{OFF} promoter. In the presence of tetracycline the strain lacks both PE and CL which led to the appearance of highly fragmented mitochondria, accompanied by the loss of mitochondrial DNA and a reduced membrane potential. To understand whether mitochondrial fragmentation was result of an increased fission or decreased fusion further experiments using the conditional mutant *crd1*Δ*psd1*Δ*dnm1*Δ were performed. In this strain the tubular mitochondrial morphology was restored because the fission-regulating gene DNM1 is deleted. In all the experiments performed, the *crd1*Δ*psd1*Δ*dnm1*Δ cells exhibited a

fusion defect due to loss of CRD1 and PSD1 indicating that the mitochondrial fragmentation observed in *crd1*Δ*psd1*Δ cells is result of defective fusion and not due to increased fission.

It was also shown that Fmp30 from *S. cerevisiae*, a MIM protein, is involved in the maintenance of mitochondrial morphology and required for the accumulation of CL in the absence of mitochondrial PE synthesis. FMP30 encodes a yeast homologue of the mammalian N-acylPE-specific phospholipase D (NAPE-PLD). It was found that a large population of *fmp30*Δ, *psd1*Δ and *fmp30*Δ*psd1*Δ cells exhibits abnormal mitochondria [86]. In a mutant strain lacking PSD1, deletion of FMP30 results in a synthetic growth defect accompanied by defects in mitochondrial morphology. Although *fmp30*Δ cells grew normally and exhibited a slightly decreased CL level, *fmp30*Δ*psd1*Δ cells exhibited a severe growth defect and a 20-fold reduction of the CL level compared to wild type [6]. Mitochondria from *fmp30*Δ cells were fragmented (22 %) and 56 % harbored aggregates of fragmented mitochondria, but still 5 % of psd1Δ cells and 21 % of fmp30Δ cells had normal tubular mitochondria. In cells of the *fmp30*Δ*psd1*Δ strain, mitochondria with normal structure were not observed, and 84 % of *fmp30*Δ*psd1*Δ cells contained aggregates of fragmented mitochondria [86]. Thus, both Psd1 and Fmp30 are involved in the maintenance of mitochondrial morphology, and the combined loss of the two genes results in a severe morphological defect of mitochondria.

Recent studies demonstrated the fundamental role of CL and PE in maintaining the proper functionality of mitochondrial protein complexes. An example is the translocase of the outer membrane (TOM complex) whose activity of β-barrel protein transport is markedly reduced in the absence of PE and CL [88]. Furthermore, CL but not PE is very important for the stability of the sorting and assembly machinery known

as SAM. Fully functional TOM and SAM complexes are necessary for the correct biogenesis and assembly of β-barrel and some α-helical proteins of the MOM. Indeed, it has been observed that *psd1*Δ mitochondria have an impaired biogenesis of β-barrel proteins, while the biogenesis of α-helical MOM proteins was not influenced. The key role of PE in the functionality of the TOM complex is stabilization at the early stage of protein import which involves docking and translocation of the precursor protein through the TOM complex. In mitochondria from *psd1*Δ strains the TOM complex binds precursor proteins with reduced efficiency. Lack of CL, on the other hand, causes destabilization of TOM and SAM complexes which determines a drastically reduced affinity of both translocases towards the precursor proteins [94]. Furthermore, strains of *S. cerevisiae* which lack genes for the synthesis of PE and CL undergo a reduction of the membrane potential. This phenomenon is associated with the difficulty for these mutants to import the β-subunit of F_1/F_0 ATP synthase into the MIM, thus correlating the presence of PE in the MIM with the establishment of the membrane potential [18].

Another major consequence related with the abundance of PE is the regulation of autophagy [85,96,97]. In yeast and mammalian cell cultures, the reduction of intracellular PE was shown to associate with augmented chronological cell ageing. After inducing the synthesis of extra PE by providing ethanolamine or by overexpression of PSD1, an increment of autophagy and a longer life expectancy was noticed.

Gulshan et al. [42] provided evidence for the bifunctional nature of yeast Psd1 in multidrug resistance in *S. cerevisiae*. In particular these authors studied Pdr3 dependent retrograde regulation of PDR5 expression. Pdr3 is a zinc cluster-containing transcription factor, and Pdr5 acts as a phospholipid floppase catalyzing the net outward movement of PE. Overproduction of PSD1 induced PDR5 transcription and drug resistance in a Pdr3-dependent manner, whereas loss of the PSD1 gene prevented activation of PDR5

expression. Surprisingly, expression of a catalytically inactive form of Psd1 still supported PDR5 transcriptional activation, suggesting that PE levels were not the signal triggering PDR5 induction. The authors argued that Psd1 has a double role and it is required both for PE biosynthesis and regulation of multidrug resistance.

Gulshan et al. [45] also showed that compartment-specific formation of PE is required for heavy metal resistance in *S. cerevisiae*. The authors demonstrated localization of Psd2 to the endosome, where the enzyme specifically controls the vacuolar membrane phospholipid composition without changing total cellular amounts of PE. Deletion of PSD2 caused sensitivity to cadmium even though Psd1 remained intact. This cadmium sensitivity was attributed to the loss of activity of a vacuolar ATP binding cassette transporter protein called Ycf1. The presence of the PI transfer protein Pdr17 was shown to form a complex with Psd2 and to be required for Psd2 function and normal cadmium tolerance. Disturbance of this regulation of intracellular phospholipid balance led to selective loss of membrane protein function in the vacuole.

Muthukumar et al. [98] observed that exposure of yeast cells to cadmium resulted in a noticeable increase of the major phospholipids, especially of PE. In a yeast *psd2*Δ deletion strains exposed to cadmium, amounts of all phospholipids including PE were decreased. Phospholipid fluctuation resulted in a strong influence of cadmium on the ER. In wild type cells, cadmium treatment increased ER stress, which stimulated autophagy. In *psd2*Δ, however, the same level of stress in the ER was not accompanied by the autophagic process. Autophagy was only restored by overexpression of PSD2 but not by supplementation with ethanolamine.

Brown et al. [94] identified Vid22 as a plasma membrane protein required for the fructose-1,6-bisphosphatase degradation pathway. Miyata et al. [99] demonstrated that a *S. cerevisiae psd1*Δ mutant strain which was also lacking Vid22 was synthetically

lethal. The catalytic activity of PSD in the cell extract of *vid22*Δ strains was about 70 % of wild type similar to that of *psd2*Δ cells. Furthermore, *vid22*Δ cells were shown to be defective in the expression of PSD2. This evidence suggested that Vid22 affects the function of PSD2 and acts as a factor required for transcriptional activation of the PSD2 gene.

In *Pichia pastoris* deletion of the PSD1 gene resulted in a decrease of mitochondrial PSD activity to 25 % of wild type and a severe growth defect on minimal media [50]. The presence of Psd2 was not sufficient to rescue this growth defect, but supplementation with ethanolamine, which is the substrate of the CDP-ethanolamine pathway, was required. As in *S. cerevisiae*, deletion of PSD2 had only a minor effect on the total cellular PSD activity.

Schizosaccharomyces pombe mutants carrying deletions in all three PSD genes (*psd1*Δ*psd2*Δ*psd3*Δ mutants) grow slowly on rich medium and are not viable on minimal medium, indicating that PSD1, PSD2 and PSD3 share overlapping essential cellular functions [54]. Supplementation of growth media with ethanolamine restored growth of *psd1*Δ*psd2*Δ*psd3*Δ cells in minimal medium, indicating that PE is essential for *S. pombe*. As *psd1*Δ*psd2*Δ*psd3*Δ cells supplemented with ethanolamine produce lower amounts of PE than wild type the CDP-ethanolamine pathway can only partially compensate for the loss of PSDs. *S. pombe psd1*Δ*psd2*Δ*psd3*Δ mutants showed abnormally shaped cells and exhibited severe septation defects including multiple, mispositioned, deformed, and misoriented septa [54] whereas *psd1*Δ*psd2*Δ*psd3*Δ mutants cells supplemented with ethanolamine showed the same membrane morphology as wild type cells.

Investigations with plant PSDs regarding their physiological relevance are rare. PSD single mutants of *Arabidopsis thaliana* have decreased PSD activity, but they do

not exhibit any growth defect or a characteristic morphological phenotype [6]. Double mutations *psd2*Δ*psd3*Δ bearing defects in the two PSDs showed similar physiological properties as single mutants and grew similar to wild type. A *psd1*Δ*psd2*Δ*psd3*Δ triple mutant was devoid of PS decarboxylase activity, showed decreased PE levels in mitochondria, but unchanged phospholipid composition in whole leaves. It was concluded that the major portion of PE in *Arabidopsis thaliana* was formed by alternative pathways, but a significant amount of PE in mitochondria was derived from PS decarboxylation by PSDs.

Voelker et al. [100] investigated mammalian cells where PE is formed by PSD in the presence and absence of ethanolamine. The substrate of PSD is synthesized extramitochondrially and must be imported into these organelles for the catalysis to occur. PSD of mice (Pisd) is expressed in a wide range of tissues, mostly in heart, liver, lung and testis [66]. The amount of Pisd is low in livers of embryos and newborn mice, but 10-fold higher in adult mice. Deficiency in Pisd activity of mice was incompatible with the embryonic development. No Pisd -/- mice were born and no Pisd deficient embryos at stages later than 10 days were found. However, mice which were heterozygous for the Pisd mutation developed with a normal lifespan, normal vitality and fertility. In contrast to the 8 days old Pisd +/+ embryos, in Pisd deficient embryos differentiation into the three germ layers was not detectable. No extra-embryonic component like placenta or amnion was visible compared to wild type embryos. Some cell division occurred in Pisd -/- embryos as mitotic spindles were present and also the decidua was formed, although it seemed to be more compact than in Pisd +/+ embryos. At day 9 of the embryonic development it came to necrosis of tissue in Pisd deficient embryos.

In CHO cells PE is mainly derived from PS decarboxylation, and no PE is imported from the ER to mitochondria [101]. When PSD expression was silenced with DsiRNA to generate Pisd knockdown (KD) cells, transcript levels were 80-94 % lower than in wild type and Pisd activity was 44-62 % lower than in the control. Cells transfected with DsiRNA showed a growth reduction by 23 % as compared to wild type. No apoptotic cells were detected in the Pisd knockdown cells. In PE deficient cells the mitochondrial membrane potential was increased; oxygen consumption was lower in Pisd KD cells than in control cells; PE deficiency in mitochondria inhibited respiration and the activity of the electron transport chain; and ATP was 80 % lower in Pisd deficient cells than in wild type after 72 h [68]. Moderate (less than 30 %) depletion of mitochondrial PE altered mammalian mitochondrial morphology and function and impaired cell growth [66,68,101,102].

4. BIOGENESIS OF PHOSPHATIDYLSERINE DECARBOXYLASE 1

4.1. Import and sorting of Psd1 into mitochondria

Psd1 belongs to the group of proteins which are encoded by the nuclear genome, synthesized by cytosolic ribosomes and imported to the MIM where they fulfill their catalytic activities [64]. After synthesis in the cytosol, Psd1 is translocated to its proper destination by the complex mitochondrial protein translocation machinery involving the TOM and the TIM complexes (for reviews see refs [103–105]).

The process of Psd1 biogenesis was more or less entirely studied with the yeast *S. cerevisiae* [17]. The import process occurs in a series of steps as shown in Figure 4. Tom70 and Tom22 are the main receptors for recognition of the Psd1 precursor which carries a classical N-terminal mitochondrial targeting sequence. After crossing the

MOM, the Psd1 precursor is sorted into the MIM. The assembly of Psd1 into the MIM requires an energized MIM translocation system (TIM complex) using the membrane potential ($\Delta\Psi$) as the only energy source. At this stage of the import process removal of the targeting signals is performed by two matrix-localized processing peptidases, the Matrix Processing Peptidase MPP and the Octapeptidyl Aminopeptidase Oct1, which are also stabilizing the mature form of the enzyme [17,106]. The amino acid sequence removed by MPP has been predicted to be located right after leucine 48, while serine at position 56 seems to be the site recognized by Oct1, which is a mitochondrial intermediate peptidase that removes octapeptide stretches [17,107].

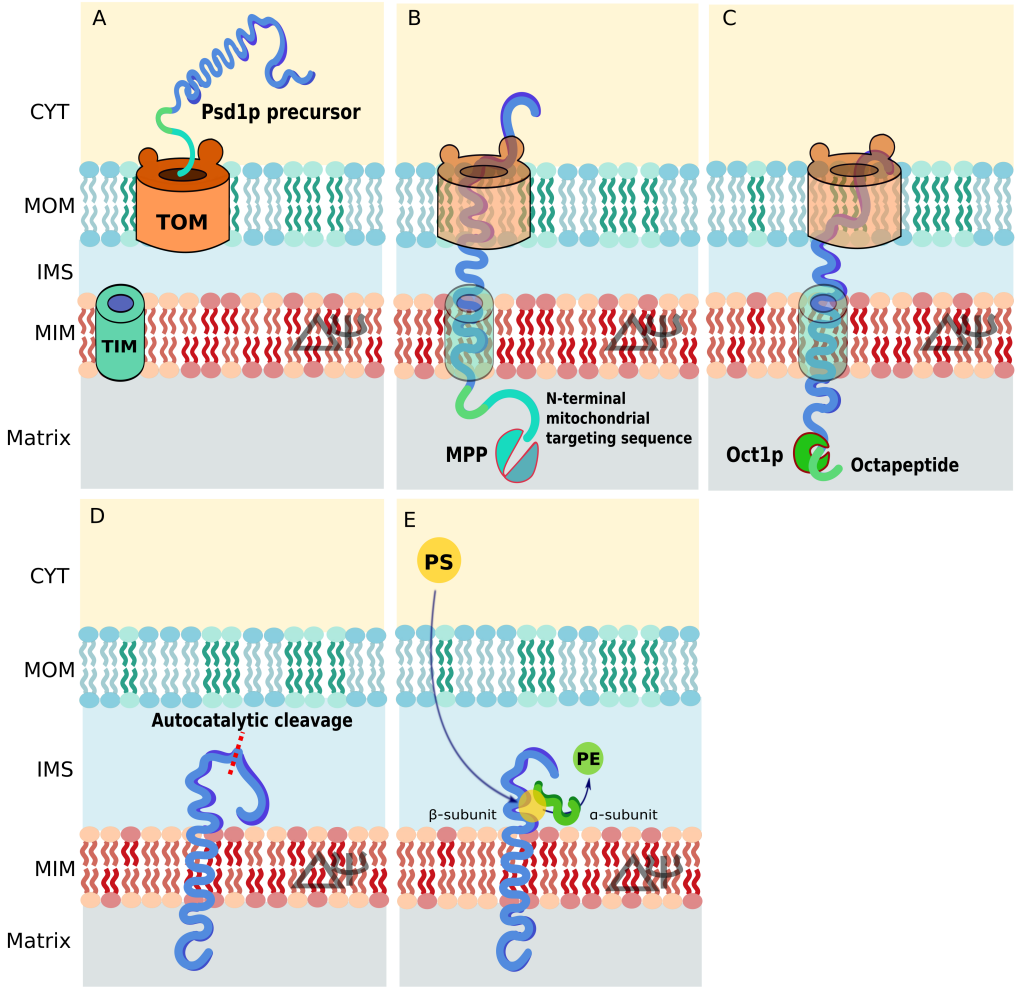

Figure 4: Import of Psd1 into mitochondria (A) The immature precursor form of Psd1 synthesized by cytoplasmic ribosomes is directed towards the MOM, where the N-terminal mitochondrial targeting signal allows docking to the TOM complex. (B) After docking of the protein, Psd1 is transferred within the organelle via the TIM complex using the membrane potential ($\Delta\Psi$) as source of energy. (C) The matrix localized peptidases MPP (Matrix Processing Peptidase) and Oct1 (Octapeptidyl aminopeptidase) remove the signal peptides and stabilize the protein structure. (D) Insertion of the protein into the MIM occurs due to a membrane spanning domain with the C-terminus protruding into the mitochondrial intermembrane space (IMS).

Nebauer et al. [97] speculated about the possible contribution of Oxa1 and Yme1 to the processing of Psd1. OXA1 encodes for a MIM protein translocase, and it was shown that deletion of this gene resulted in an impaired level of mitochondrial and cellular PE, comparable with a *psd1*Δ deletion. Experiments performed to verify the influence of the MIM protease Yme1 on Psd1 demonstrated that lack of this enzyme caused enhanced Psd1 stability. These data supported the hypothesis that Yme1 was involved in the proteolytic turnover of Psd1.

It is well established that Psd1 is embedded into the MIM. Horvath et al. [17] demonstrated with the yeast that the predicted single membrane spanning domain (IM1) from Val-81 to Ser-100 is involved in membrane anchoring. Deletion of this portion of the protein resulted in mislocalization of Psd1 to the matrix side of the MIM and reduction of enzymatic activity. A second membrane spanning domain within the β-subunit of Psd1 has been predicted between Ile-119 and Leu-140 based on bioinformatic evidence, but not confirmed by biochemical experiments.

As a key process of Psd1 maturation during import and sorting into mitochondria the enzyme undergoes internal rearrangement and autocatalytic processing leading to formation of distinct and non-covalently associated α- and β-subunits. The α-subunit is per se soluble and localized to the intermembrane space where it interacts with the

hydrophilic part of the membrane bound β-subunit [90]. As the small α-subunit harbors at least an essential part of the active site of Psd1, the recruitment to the MIM surface with orientation to the intermembrane space is essential for the ability of Psd1 to decarboxylate the polar head group of PS [108]. Indeed, experiments using a mislocalized truncated form of Psd1 showed that the enzyme was still able to perform the autocatalytic cleavage and to divide into the two subunits, but its catalytic activity was drastically reduced [17].

4.2. Processing and maturation of Psd1

An important site that is shared by all PSDs identified so far is the conserved LGST or GS(S/T) motif, which is essential for proteolytic processing to the α- and β-subunits and for the formation of the catalytic pyruvoyl group of the mature enzyme to obtain an active PSD [109,110]. With some exceptions the motif of type I PSDs is composed by a leucine, glycine, serine and threonine sequence (LGST). The sequence and position of this motif is shown in Figure 3.

The C-terminus of *E. coli* harbors a conserved LGST motif at positions Leu-252 to Thr-255, while in *B. subtilis* the processing site is composed of the sequence FGST and located at Phe-228 to Thr-231 [32,109]. Psd1 from yeast contains the specific LGST motif between the amino acid residues Leu-461 and Thr-464, similar to PSD from CHO cells where this motif is also located at the C-terminus of the enzyme [90]. Exceptions from the classical LGST motif are the Psd1 from *A. thaliana* with its MGST sequence at the C-terminus [63], and PSD from *P. falciparium* with VGSS from position Val-314 to Ser-317 [59]. *Plasmodium knowlesi* PSD contains a MGSS sequence at the C terminus. In PSDs of type II the GGST motif is more common. The best studied examples are the yeast Psd2 which contains the GGST motif at position Gly-1041 to Thr-1044 [41], and

the two type II PSDs from A. thaliana (Psd2 and Psd3) which have both a GGST motif at the C-terminus [63].

In all types of PSDs the LGST or GS(S/T) motif is used not only as a cleavage site to form α- and β-subunits, but also the active site of the enzyme with its pyruvoyl prosthetic group. The cleavage which is considered to be an autocatalytic process has been best studied with *E. coli* [109]. Figure 5 shows the process of ester bond formation between Gly-462 and Ser-463 of the LGST motif as first reaction. The next reaction is an α, β-elimination which sets the mature β-subunit free and leaves the α-subunit with a dehydroalanine residue still attached to the N-terminus. For the final formation of the pyruvoyl prosthetic group, which is attached to the N-terminus of the α-subunit, hydration is necessary and elimination of a molecule of ammonia. Mutations in the LGST motif result in an inactive and unprocessed enzyme [17]. Onguka et al. [89] showed, however, that the serine residue is the only amino acid of the LGST motif which is absolutely required for Psd1 autocatalytic cleavage and activity.

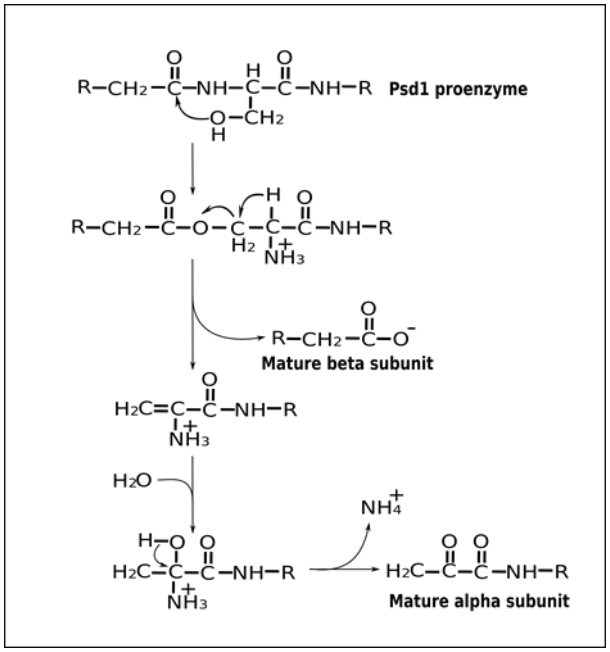

Figure 5: Maturation of Psd1 For complete maturation of Psd1 an autocatalytic endoproteolytic cleavage is necessary to form the active site of the enzyme, which consists of a pyruvoyl group. At the same time the α- and β-subunits of Psd1 are formed.

Interestingly, the reaction of autocatalytic cleavage seems to be independent of the proper localization of Psd1 to the MIM. If the Psd1 precursor is arrested at the TOM complex before reaching the MOM in the absence of the membrane potential, processing and separation between α- and the β-subunit can still be observed [17]. Deletion of the transmembrane segment leads to mislocalization of Psd1 to the matrix side of the MIM and to reduced activity, albeit processing can still occur.

It has been speculated, however, that formation of the two subunits of Psd1 may be independent of import and maturation of the enzyme, and other components might influence this process. Choi et al. [60] who studied formation of Psd1 from *Plasmodium knowlesi* highlighted the fact that in an in vitro reaction using liposomes self-processing was influenced by the surrounding lipid composition

Indeed, a positive correlation was observed between the rate of autocatalytic cleavage and the amount of dioleoyl PS (DOPS) present in the reaction. Other anionic phospholipids such as dioleoyl phosphatidic acid (DOPA) or dioleoyl phosphatidylglycerol (DOPG) showed an inhibitory effect on the self-processing and the formation of the two subunits of Psd1 [60]. Another ground-breaking study published recently by the same group explored the molecular basis of the endoproteolytic maturation of a truncated form of Psd1 from *P. knowlesi* [110]. It was shown that it is possible to inhibit the cleavage of Psd1 by adding a common inhibitor of serine proteases, PMSF, to the reaction. This result has paved the way for the identification of the amino acids which are responsible for the possible proteolytic

activity. Precise comparisons of PSD sequences from different organisms led to the identification of a highly conserved aspartic acid residue (Asp-139) and two histidines (His-195, His-198) which, together with Ser-308 of the GS(S/T) motif, form the classic catalytic triad D-H-S that is characteristic of the serine protease family [110,111]. Site direct mutagenesis experiments showed that mutations of the three amino acids led to lack of autocatalytic cleavage of the enzyme and confirmed the double activity of Psd1 as PS decarboxylase and serine protease [110].

5. ENZYMOLOGY OF PHOSPHATIDYLSERINE DECARBOXYLASES

The mechanism of PE formation from PS by PSD has been studied intensively with the gram negative model organism E.coli [37,109,112]. At the conserved GS(S/T) motif the approaching of PS leads to formation of a Schiff's base between the covalently bound pyruvoyl residue and the primary amine of the serine residue. During a successive electron rearrangement an azomethine intermediate is formed and PS is decarboxylated. At this point a protonation reaction forms PE in the Schiff's base linkage with the enzyme, and finally the addition of water across the Schiff's base regenerates the pyruvoyl prosthetic group and sets PE free from the active site of the enzyme [49,72,87].

In *E. coli* the best PSD catalysis in vitro occurs in a pH range of 6.5 to 7.0 in the presence of 10 % glycerol and 0.1 % Triton X-100 [113]. The solubilized enzyme appears to have a tendency to associate with mixed micelles made of detergent and substrate, and the optimal activity has been measured at a molar ratio of 6:1 Triton X-100 to 6 mM PS. As the catalysis depends of the carbonyl moiety of the pyruvoyl group of PSD, the presence of carbonyl reagents can inactivate the enzyme [37]. In

experiments with yeast Psd1 it was noticed that the enzyme exhibited selectivity towards different PS molecular species [23]. The ratio of unsaturated to saturated fatty acids in the substrate PS was found to be much lower than in PE.

The catalytic function of Psd1 from *Toxoplasma gondii* (TgPSD1) is comparable to other reported PSDs [62]. Secreted and soluble TgPSD exhibit a high catalytic activity with liposomal PS. PkPSD from *Plasmodium knowlesi* converts PS into PE at levels comparable to the yeast Psd1. In cell free extracts the PkPSD has nearly three times the catalytic activity of the endogenous yeast Psd1. Marked PkPSD enzyme activity (49 %) was detected in the soluble fractions of cell extracts. A PkPSD enzyme expressed in a *psd1*Δ*psd2*Δ*dpl1*Δ yeast strain was fully functional and complemented the biochemical defect of the mutant. Amino acids between positions 35 and 55 appear to be crucial for the enzymatic activity of PkPSD [60]. With PSD from *Plasmodium falciparum* it has been demonstrated that replacement of the classical LGST motif by VGSS brought an increment of the activity, demonstrating the active role of the amino acid flanking the catalytic serine. PSD from *Plasmodium falciparum* expressed in E. coli has a Km of 63 μM and a v_{max} of 680 nmol at the optimum pH of 6.8 [114].

In mammalian cells formation of PE by PS decarboxylation generates mainly species of polyunsaturated fatty acids in the sn-2 position, e. g. (18:0-20:4) PE and (18:0-20:5) PE in McArdle cells, and (18:0-20:4) PE and (18:0-22:6) PE in Chinese hamster ovary K1 cells [101]. Interestingly, the lipid precursor PS species utilized for decarboxylation, (18:0-20:4) PS and (18:0-20:5) PS, comprise only 2-3 % of total PS in whole cells [101]. Thus, mammalian PSD appears to be highly selective. Derivatives of PS like serine, phosphoserine and glycerophosphoserine are not substrates of the enzyme, but dimyristoyl PS 1 acyl, 2[N-(6[7-nitrobenz-2-oxa-1,3diazo-4-yl)]aminocaproyl

PS and 1 acyl, 2(ω-pyrene)-acyl PS are recognized and decarboxylated [72]. Partially purified rat liver PSD was affected by sulfhydryl modifying reagents and stabilized by the addition of 5 mM 2-mercaptoethanol, 1 mM ETDA and 10 % glycerol. As with other PSDs the rat liver PSD does not exhibit a substrate preference to dipalmitoyl and dimyristoyl-PS [115].

Functionality of mitochondrial Psd1 depends very much on the supply of its substrate PS from other organelles. Thus, a major prerequisite for PE formation is the conveyance of PS from its site of synthesis, the ER to the MIM. This process has been analyzed both in yeast and mammalian cells [116]. The currently best accepted mechanism of phospholipid transport between ER and mitochondria seems to be through membrane contact [117]. In yeast and mammalian cells membrane complexes form a subfraction of the ER, the mitochondria associated membrane (MAM) [118,119]. These contact sites were identified through co-sedimentation of ER and mitochondrial fractions and by direct visualization using electron microscopy [120–123]. These methods showed that 80-110 of these contact sites are present in yeast cells [124]. Gaigg et al. [125] and Vance et al. [126] demonstrated that these membrane sub-domains are enriched with enzymes of phospholipid synthesis. Additionally, it has been speculated that the existence of these membrane bridges could create a hydrophobic passage for channeling and translocation of lipid molecules [122,127].

In the yeast, molecular components localized at ER-MOM junctions were identified and named ER-mitochondrial encounter structures (ERMES) [128,129]. The ERMES complex is formed by five major proteins, namely Mmm1, Mdm34, Mdm10, Mdm12 and Gem1 [130,131]. It was hypothesized that the ERMES complex plays a role in translocation of lipids between compartments [132]. Other authors [133], however, argued that lack of ERMES does not interfere with PS transport from the ER to

mitochondria and conversion of PS to PE. Highly impaired transfer of phospholipids accompanied by their compromised steady state level was only detected when the yeast cells lacked both the ERMES complex and the ER-shaping proteins [134,135]. As the ERMES complex the ER-shaping proteins, i.e. the reticulons Rtn1p and Rtn2p and the reticulon-like protein Yop1p, are essential for keeping efficient contact at the ER-mitochondria interface and found in all eukaryotic cells [135]. Besides the reticulons, another family of proteins that play a role in maintaining ER shape are the atlastins. Sey1 is the functional ortholog of the atlastins in yeast [136]. Voss et al. [134] showed that PS transfer from the ER to mitochondria is slower in cells lacking the ERMES protein Mdm34p and either Rtn1 and Yop1 or Rtn1 and Sey1. This study also demonstrated that the set of genes encoding the ERMES proteins genetically interacts with RTN1 and YOP1, and that the ERMES complex together with the proteins required to maintain tubular ER is important for lipid exchange between ER and mitochondria.

In addition to the tethering ERMES complex, a newly identified contact site named vCLAMP (vacuole and mitochondria patch), has been described [137,138]. vCLAMP creates physical contact between mitochondria and the yeast lysosomal compartment, but is also located in vicinity to the ER-mitochondria encounter structure (ERMES) complexes creating a complex network among the different organelles. It was demonstrated that an increase in vCLAMP can rescue the growth defect of ERMES mutants. Thus, mitochondria were shown to depend on the presence of either ERMES or vCLAMP, the absence of one causing expansion of the other, whereas elimination of both is lethal.

Lahiri et al. [139] investigated the mechanism of non-vesicular mitochondrial membrane biogenesis. Using a novel genetic screening method in S. cerevisiae they demonstrated that a strain lacking different components of the conserved ER

membrane protein complex (EMC) has decreased phosphatidylserine (PS) transfer from the ER to mitochondria and consequentially also a reduction of PS and PE levels. The importance of the contact between all the EMC components with Tom5, a part of the TOM complex, in PS transfer and cell growth was also shown. This study proved the additional tethering function of the EMC complex, in fact even though the ERMES is intact. EMC mutants have a marked reduction of ER tethered to mitochondria, and cells lacking both the EMC proteins and the ERMES complex are non-viable.

In other experiments the energy requirement of lipid translocation between ER and mitochondria was investigated. A yeast strain, named pstA, which is compromised in the ER-mitochondrial route of import and export of PS and PE, respectively, was characterized by the limited amount of PE, ethanolamine auxotrophy and a lower phospholipid to protein ratio than wild type [140]. Studies in yeast cells focusing on PS delivery from the ER to the MIM showed that this activity did not require ATP [117,124], although the process appeared to be energy dependent in experiments conducted with mammalian cells [141]. Further investigations using isolated microsomes as donor membranes and mitochondria as acceptors demonstrated that in the route of lipid delivery only the final step was ATP independent in both mammalian cells and yeast cells, while initial stages of transport were functional only in the presence of ATP [92,142].

Intramitochondrial transport of lipids is a route that involves lipid transport from the MOM to MIM and backwards [143]. The import step is particularly important for the movement of the precursor phospholipids PS and PA which are required for the production of the mitochondrially synthesized phospholipids PE and CL. The lipid translocation process described here appears to be independent of the membrane potential ($\Delta\Psi$), and it is likely to occur at MOM-MIM contact sites [117,119,144–146].

The yeast proteins Ups1 and Ups2, and the human homologue known as PRELI (protein of relevant evolutionary lymphoid interest) together with the proteins Mdm35, Mdm31 and Gep5 play an important role in intra-mitochondrial lipid transfer [146–149]. When grown on fermentable carbon sources, the yeast deletion strains *ups1*Δ, *mdm31*Δ and *gep5*Δ are characterized by low expression of PSD1 accompanied by reduction of PE synthesis [146,150]. Further experiments highlighted the role of Mdm31 and Gep5 in keeping a correct mitochondrial morphology, supporting Psd1 levels and PE production [82]. PE synthesis is a time dependent process, and it has been shown that inactivation of Ups2 leads to a reduction of the PE level. In contrast, Ups1 inactivation does not interfere with this process. In pulse-chase experiments using *ups1*Δ and *ups2*Δ yeast strains Tamura et al. [147] showed that (i) loss of Ups2 supported PE export from mitochondria causing a reduction in the PE level which was balanced by an increased PC production in the ER; (ii) in *ups1*Δ strains grown under fermentable conditions the export of PE seemed to be slower than in reference strains; and (iii) in *ups1*Δ strains a reduced steady-state level of Psd1 was accompanied by a decreased membrane potential ($\Delta\Psi$), which was restored if strains lacking Ups1 were shifted to growth on a non-fermentable carbon source.

A study by Aaltonen et al. [151] describes the role of the Ups2-Mdm35 complex in PS relocation and the concomitant synthesis of PE. To investigate the influence of PS transport driven by Ups2-Mdm35 on the activity of Psd1, PE synthesis was monitored in UPS2 deficient cells in a *psd2*Δ *dpl1*Δ background. Under these conditions it was possible to observe an impaired accumulation of PE, which did not influence its conversion to PC indicating that PS decarboxylation by Psd1 can proceed despite the absence of PS transfer by Ups2. These data suggested that PS can reach Psd1 via alternative routes. In the same study it was demonstrated that Psd1 can catalyze

decarboxylation of a PS fraction in the MOM, which therefore does not need to be relocated. Psd1 activity is in this case linked to the spatial arrangement of the MOM and MIM, which is regulated by the Mitochondria Contact Site complex known as MICOS. Loss of MICOS has been shown to drastically reduce both the rate of Psd1-dependent PE synthesis and also the formation of PC from PE methylation, defining the role of MICOS in the production of PE by regulating the spatial relation between the two mitochondrial membranes. In contrast to previous studies Miyata et al. [152] showed recently that in cells grown under non fermentable conditions the Ups2-Mdm35 complex contributed to the PS transfer to mitochondria. Under these circumstances loss of activity of Ups2-Mdm35 was accompanied by a reduction in the conversion of PS to PE. Furthermore the UPS2 expression appeared to be augmented and, in support of these data, an in vitro experiment carried out with liposomes showed that the recombinant Ups2-Mdm35 fusion protein was indeed displaying phospholipid transfer activity. The new evidence suggested that the role of Ups1 and Ups2 in PE metabolism is not merely regulatory. There are now indications that Ups2 is directly involved in the transport of PS to mitochondria. Despite the data available so far, the exact mechanism driving PE transfer within mitochondria is still a matter of dispute. Especially identification of proteins which govern PE translocation still awaits elucidation.

In *S. cerevisiae* the mitochondrially localized Psd1 accounts for 70 % of PE production in the cell. Despite the apparent dominant role of Psd1, the contribution of Psd2 to cellular PE formation is noteworthy. Similarly to Psd1, also Psd2 forms PE species with a high degree of unsaturation exhibiting a preference for C34:2 and C32:2 species [153]. As the ratio of unsaturated to saturated fatty acids in PS is much lower than in PE high species selectivity of Psd2 can be anticipated. Bürgermeister et al. [153]

showed that a certain portion of PE formed by Psd2 can be imported into mitochondria, although with moderate efficiency. Interestingly, PE synthesized by Psd2 is the preferred substrate for PC synthesis, but PC derived from the different biosynthetic pathways seems to be supplied to subcellular membranes from a single PC pool.

Localization studies revealed that Psd2 is most likely a component of the endomembrane system [40]. Riekhof et al. [44] studied the supply of PS to the site of Psd2 from the ER across the endosomes. The model proposed suggests a proteins complex on the acceptor membrane consisting primarily of Psd2 and the lipid-binding protein PstB2, which are able to facilitate the lipid transfer. This interaction facilitated by a cryptic C2 domain at the extreme N-terminus (C2-1) and the C2 domain of Psd2 (C2-2). The proposed model includes PS transport requiring a docking site from the PS donor membrane to an acceptor, based on protein-protein and protein-lipid interactions. In this case the acceptor membrane complex is composed of the C2 domains of Psd2 and PstB2 that interacts with Scs2, a binding determinant for several peripheral ER proteins, and PA present in the donor membrane creating a bridge to enable PS relocation. Transfer of PS from liposomes to Psd2 fails to occur in acceptor membranes from strains lacking PstB2 or the C2 domain of Psd2. These data support a model for PS transport from planar domains highly enriched in PS or in PS plus PA [92]. We refer to a recent review paper Kannan et al. [154] for a comprehensive view on aspects related to Psd2p-specific transport pathway and PS delivery at the contact sites between the ER and Golgi/endosome membranes.

6. SUMMARY AND CONCLUSION

The aim of this review article was to summarize evidence about PSDs in different cell types, their involvement in lipid metabolism and their effect on various cellular processes. In the past, PSDs were regarded solely as producers of PE, but recently a more complex role of these enzymes evolved. Besides the unequivocally important role of PE as a membrane component governing the dynamic structure of biological membranes increasing evidence has been presented that PE is responsible for stabilization of proteins, especially in mitochondrial membranes. In that respect, PSDs of type 1 deserve our special attention. However, extramitochondrial PSDs seem to be also important for the total cellular PE metabolism although their role is not yet as well defined. Studies with mutants provide a reliable strategy to pinpoint defects which can be ascribed to individual defects in prokaryotes, eukaryotic microorganisms, parasites, plants and mammalian cells.

The best studied PSD is the mitochondrial Psd1 from different sources, especially from the yeast, *Trypanosoma*, plants and mammalian cells. Psd1 is not only the major producer of PE in most of these cell types or even the only one, e. g. in mammalian cells, but also the phylogenetically best conserved within the different kingdoms of life. Recently, much evidence about biogenesis, processing, import into mitochondria, assembly into the MIM and the topology of the Psd1 has been presented. This information helps us to understand how this enzyme works and how it may be embedded into the network of phospholipid metabolism, which is spread over different organelles. As always, however, many questions remain still unanswered. The 3D structure of Psd1 is still unsolved, and several important domains with ascribed biological functions are only defined by bioinformatic predictions. As examples, the membrane spanning sites need to be defined in more detail; the role of a putative substrate recognition sequence waits elucidation; and finally the enigmatic autocatalytic

cleavage at the LGST motif which leads to the formation of the α- and β-subunits may be worth studying in some more detail. Moreover, Psd1 may interact with other proteins from mitochondrial membranes and form complexes which need to be identified and characterized. Finally, the supply of the substrate PS to the Psd1 site is still under investigation and has not been fully understood from the mechanistic viewpoint.

Besides Psd1 other types of PSDs should not be forgotten and their role in lipid metabolism on the one hand and their possible importance as contributors to PE metabolism, regulators or stabilizing factors on the other hand should be kept in mind. Our lack of information about these enzymes is no excuse to ignore the role of these proteins in cell metabolism and physiology.

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REFERENCES

[1] P.R. Cullis, B. de Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta. 559 (1979) 399–420.

[2] R.M. Bell, L.M. Ballas, R.A. Coleman, Lipid topogenesis, J. Lipid Res. 22 (1981) 391–403.

[3] V.V. Flis, A. Fankl, C. Ramprecht, G. Zellnig, E. Leitner, A. Hermetter, G. Daum, Phosphatidylcholine supply to peroxisomes of the yeast *Saccharomyces cerevisiae*, PloS One. 10 (2015) e0135084. doi:10.1371/journal.pone.0135084.

[4] S. Déchamps, M. Maynadier, S. Wein, L. Gannoun-Zaki, E. Maréchal, H.J. Vial, Rodent and nonrodent malaria parasites differ in their phospholipid metabolic pathways, J. Lipid Res. 51 (2010) 81–96. doi:10.1194/jlr.M900166-JLR200.

[5] A. Hartmann, M. Hellmund, R. Lucius, D.R. Voelker, N. Gupta, Phosphatidylethanolamine synthesis in the parasite mitochondrion is required for efficient growth but dispensable for survival of *Toxoplasma gondii*, J. Biol. Chem. 289 (2014) 6809–6824. doi:10.1074/jbc.M113.509406.

[6] A. Nerlich, M. von Orlow, D. Rontein, A.D. Hanson, P. Dörmann, Deficiency in phosphatidylserine decarboxylase activity in the psd1 psd2 psd3 triple mutant of Arabidopsis affects phosphatidylethanolamine accumulation in mitochondria, Plant Physiol. 144 (2007) 904–914. doi:10.1104/pp.107.095414.

[7] G. Tasseva, L. Richard, A. Zachowski, Regulation of phosphatidylcholine biosynthesis under salt stress involves choline kinases in *Arabidopsis thaliana,* FEBS Lett. 566 (2004) 115–120. doi:10.1016/j.febslet.2004.04.015.

[8] Z. Li, L.B. Agellon, T.M. Allen, M. Umeda, L. Jewell, A. Mason, D.E. Vance, The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis, Cell Metab. 3 (2006) 321–331. doi:10.1016/j.cmet.2006.03.007.

[9] R.D. Hite, M.C. Seeds, R.B. Jacinto, R. Balasubramanian, M. Waite, D. Bass, Hydrolysis of surfactant-associated phosphatidylcholine by mammalian secretory phospholipases A2, Am. J. Physiol. 275 (1998) L740-747.

[10] K.A. Dill, D. Stigter, Lateral interactions among phosphatidylcholine and phosphatidylethanolamine head groups in phospholipid monolayers and bilayers, Biochemistry (Mosc.). 27 (1988) 3446–3453. doi:10.1021/bi00409a048.

[11] A.G. Rietveld, M.C. Koorengevel, B. de Kruijff, Non-bilayer lipids are required for efficient protein transport across the plasma membrane of *Escherichia coli*, EMBO J. 14 (1995) 5506–5513.

[12] E. van den Brink-van der Laan, J.A. Killian, B. de Kruijff, Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure

profile, Biochim. Biophys. Acta. 1666 (2004) 275–288. doi:10.1016/j.bbamem.2004.06.010.

[13] D.P. Siegel, R.M. Epand, The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms., Biophys. J. 73 (1997) 3089–3111.

[14] M.K. Storey, K.L. Clay, T. Kutateladze, R.C. Murphy, M. Overduin, D.R. Voelker, Phosphatidylethanolamine has an essential role in *Saccharomyces cerevisiae* that is independent of its ability to form hexagonal phase structures, J. Biol. Chem. 276 (2001) 48539–48548. doi:10.1074/jbc.M109043200.

[15] T.M. Allen, K. Hong, D. Papahadjopoulos, Membrane contact, fusion and hexagonal (HII) transitions in phosphatidylethanolamine liposomes, Biochemistry (Mosc.). 29 (1990) 2976–2985. doi:10.1021/bi00464a013.

[16] R.M. Epand, Lipid polymorphism and protein–lipid interactions, Biochim. Biophys. Acta BBA - Rev. Biomembr. 1376 (1998) 353–368. doi:10.1016/S0304-4157(98)00015-X.

[17] S.E. Horvath, L. Böttinger, F.N. Vögtle, N. Wiedemann, C. Meisinger, T. Becker, G. Daum, Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1., J. Biol. Chem. 287 (2012) 36744–55. doi:10.1074/jbc.M112.398107.

[18] L. Böttinger, S.E. Horvath, T. Kleinschroth, C. Hunte, G. Daum, N. Pfanner, T. Becker, Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes, J. Mol. Biol. 423 (2012) 677–686. doi:10.1016/j.jmb.2012.09.001.

[19] R. Birner, M. Bürgermeister, R. Schneiter, G. Daum, Roles of Phosphatidylethanolamine and of Its Several Biosynthetic Pathways in *Saccharomyces cerevisiae*, Mol. Biol. Cell. 12 (2001) 997–1007. doi:10.1091/mbc.12.4.997.

[20] E. Zinser, C.D. Sperka-Gottlieb, E.V. Fasch, S.D. Kohlwein, F. Paltauf, G. Daum, Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*, J. Bacteriol. 173 (1991) 2026–2034.

[21] D.F. Parsons, G.R. Williams, B. Chance, Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria, Ann. N. Y. Acad. Sci. 137 (1966) 643–666.

[22] T.G. Frey, C.A. Mannella, The internal structure of mitochondria, Trends Biochem. Sci. 25 (2000) 319–324.

[23] M. Bürgermeister, R. Birner-Grünberger, R. Nebauer, G. Daum, Contribution of different pathways to the supply of phosphatidylethanolamine and

phosphatidylcholine to mitochondrial membranes of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta. 1686 (2004) 161–168. doi:10.1016/j.bbalip.2004.09.007.

[24] M.A. Miller, C. Kent, Characterization of the pathways for phosphatidylethanolamine biosynthesis in Chinese hamster ovary mutant and parental cell lines, J. Biol. Chem. 261 (1986) 9753–9761.

[25] P.J. Trotter, J. Pedretti, D.R. Voelker, Phosphatidylserine decarboxylase from Saccharomyces cerevisiae. Isolation of mutants, cloning of the gene, and creation of a null allele, J. Biol. Chem. 268 (1993) 21416–21424.

[26] J.E. Vance, Thematic Review Series: Glycerolipids. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids, J. Lipid Res. 49 (2008) 1377–1387. doi:10.1194/jlr.R700020-JLR200.

[27] S.E. Horvath, G. Daum, Lipids of mitochondria., Prog. Lipid Res. (2013). doi:10.1016/j.plipres.2013.07.002.

[28] C. Osman, D.R. Voelker, T. Langer, Making heads or tails of phospholipids in mitochondria, J. Cell Biol. 192 (2011) 7–16. doi:10.1083/jcb.201006159.

[29] A. Lykidis, Comparative genomics and evolution of eukaryotic phospholipid biosynthesis, Prog. Lipid Res. 46 (2007) 171–199. doi:10.1016/j.plipres.2007.03.003.

[30] V. Kainu, M. Hermansson, S. Hänninen, K. Hokynar, P. Somerharju, Import of phosphatidylserine to and export of phosphatidylethanolamine molecular species from mitochondria, Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids. 1831 (2013) 429–437. doi:10.1016/j.bbalip.2012.11.003.

[31] M. Nishihara, H. Morii, Y. Koga, Heptads of Polar Ether Lipids of an Archaebacterium, Methanobacterium thermoautotrophicum: structure and biosynthetic relationship, (1988).

[32] K. Matsumoto, M. Okada, Y. Horikoshi, H. Matsuzaki, T. Kishi, M. Itaya, I. Shibuya, Cloning, sequencing, and disruption of the Bacillus subtilis psd gene coding for phosphatidylserine decarboxylase, J. Bacteriol. 180 (1998) 100–106.

[33] H. Daiyasu, K.-I. Kuma, T. Yokoi, H. Morii, Y. Koga, H. Toh, A study of archaeal enzymes involved in polar lipid synthesis linking amino acid sequence information, genomic contexts and lipid composition, Archaea. 1 (2005) 399–410. doi:10.1155/2005/452563.

[34] F. Sievers, A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J.D. Thompson, D.G. Higgins, Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, Mol. Syst. Biol. 7 (2011) 539. doi:10.1038/msb.2011.75.

[35] J. Kanfer, E.P. Kennedy, Metabolism and Function of Bacterial Lipids II. Biosynthesis of phospholipids in *Escherichia coli*, J. Biol. Chem. 239 (1964) 1720–1726.

[36] F. Gibellini, T.K. Smith, The Kennedy pathway—De novo synthesis of phosphatidylethanolamine and phosphatidylcholine, IUBMB Life. 62 (2010) 414–428. doi:10.1002/iub.337.

[37] Q.X. Li, W. Dowhan, Structural characterization of *Escherichia coli* phosphatidylserine decarboxylase, J. Biol. Chem. 263 (1988) 11516–11522.

[38] M. Okada, H. Matsuzaki, I. Shibuya, K. Matsumoto, Cloning, sequencing, and expression in *Escherichia coli* of the *Bacillus subtilis* gene for phosphatidylserine synthase, J. Bacteriol. 176 (1994) 7456–7461.

[39] A. Thanawastien, W.R. Montor, J. Labaer, J.J. Mekalanos, S.S. Yoon, Vibrio cholerae proteome-wide screen for immunostimulatory proteins identifies phosphatidylserine decarboxylase as a novel Toll-like receptor 4 agonist, PLoS Pathog. 5 (2009) e1000556. doi:10.1371/journal.ppat.1000556.

[40] P.J. Trotter, D.R. Voelker, Identification of a Non-mitochondrial Phosphatidylserine Decarboxylase Activity (PSD2) in the Yeast *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 6062–6070. doi:10.1074/jbc.270.11.6062.

[41] P.J. Trotter, J. Pedretti, R. Yates, D.R. Voelker, Phosphatidylserine decarboxylase 2 of Saccharomyces cerevisiáe. Cloning and mapping of the gene, heterologous expression, and creation of the null allele, J. Biol. Chem. 270 (1995) 6071–6080.

[42] K. Gulshan, J.A. Schmidt, P. Shahi, W.S. Moye-Rowley, Evidence for the Bifunctional Nature of Mitochondrial Phosphatidylserine Decarboxylase: Role in Pdr3-Dependent Retrograde Regulation of PDR5 Expression, Mol. Cell. Biol. 28 (2008) 5851–5864. doi:10.1128/MCB.00405-08.

[43] R. Birner, R. Nebauer, R. Schneiter, G. Daum, Synthetic Lethal Interaction of the Mitochondrial Phosphatidylethanolamine Biosynthetic Machinery with the Prohibitin Complex of *Saccharomyces cerevisiae*, Mol. Biol. Cell. 14 (2003) 370–383. doi:10.1091/mbc.E02-05-0263.

[44] W.R. Riekhof, W.-I. Wu, J.L. Jones, M. Nikrad, M.M. Chan, C.J.R. Loewen, D.R. Voelker, An assembly of proteins and lipid domains regulates transport of phosphatidylserine to phosphatidylserine decarboxylase 2 in *Saccharomyces cerevisiae*, J. Biol. Chem. 289 (2014) 5809–5819. doi:10.1074/jbc.M113.518217.

[45] K. Gulshan, P. Shahi, W.S. Moye-Rowley, Compartment-specific Synthesis of Phosphatidylethanolamine Is Required for Normal Heavy Metal Resistance, Mol. Biol. Cell. 21 (2010) 443–455. doi:10.1091/mbc.E09-06-0519.

[46] O. Kuge, M. Nishijima, Y. Akamatsu, A cloned gene encoding phosphatidylserine decarboxylase complements the phosphatidylserine biosynthetic defect of a Chinese hamster ovary cell mutant, J. Biol. Chem. 266 (1991) 6370–6376.

[47] C.A. Wilcox, K. Redding, R. Wright, R.S. Fuller, Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole, Mol. Biol. Cell. 3 (1992) 1353–1371.

[48] K. Igarashi, M. Kaneda, A. Yamaji, T.C. Saido, U. Kikkawa, Y. Ono, K. Inoue, M. Umeda, 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 (1995) 29075–29078.

[49] H. Kitamura, W.-I. Wu, D.R. Voelker, The C2 Domain of Phosphatidylserine Decarboxylase 2 Is Not Required for Catalysis but Is Essential for in VivoFunction, J. Biol. Chem. 277 (2002) 33720–33726. doi:10.1074/jbc.M205672200.

[50] T. Wriessnegger, A.J. Sunga, J.M. Cregg, G. Daum, Identification of phosphatidylserine decarboxylases 1 and 2 from *Pichia pastoris*, FEMS Yeast Res. 9 (2009) 911–922. doi:10.1111/j.1567-1364.2009.00544.x.

[51] S. Rajakumari, G. Daum, Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions, Mol. Biol. Cell. 21 (2010) 501–510. doi:10.1091/mbc.E09-09-0775.

[52] C.D. Sperka-Gottlieb, A. Hermetter, F. Paltauf, G. Daum, Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, *Saccharomyces cerevisiae*, Biochim. Biophys. Acta. 946 (1988) 227–234.

[53] W.R. Riekhof, J. Wu, J.L. Jones, D.R. Voelker, Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*, J. Biol. Chem. 282 (2007) 28344–28352. doi:10.1074/jbc.M705256200.

[54] J. Luo, Y. Matsuo, G. Gulis, H. Hinz, J. Patton-Vogt, S. Marcus, Phosphatidylethanolamine is required for normal cell morphology and cytokinesis in the fission yeast *Schizosaccharomyces pombe*, Eukaryot. Cell. 8 (2009) 790–799. doi:10.1128/EC.00029-09.

[55] A. Signorell, E. Gluenz, J. Rettig, A. Schneider, M.K. Shaw, K. Gull, P. Bütikofer, Perturbation of phosphatidylethanolamine synthesis affects mitochondrial morphology and cell-cycle progression in procyclic-form Trypanosoma brucei, Mol. Microbiol. 72 (2009) 1068–1079. doi:10.1111/j.1365-2958.2009.06713.x.

[56] A. Signorell, M. Rauch, J. Jelk, M.A.J. Ferguson, P. Bütikofer, Phosphatidylethanolamine in *Trypanosoma brucei* is organized in two separate pools and is synthesized exclusively by the Kennedy pathway, J. Biol. Chem. 283 (2008) 23636–23644. doi:10.1074/jbc.M803600200.

[57] L. Farine, P. Bütikofer, The ins and outs of phosphatidylethanolamine synthesis in *Trypanosoma brucei*, Biochim. Biophys. Acta. 1831 (2013) 533–542. doi:10.1016/j.bbalip.2012.09.008.

[58] R.W. Snow, C.A. Guerra, A.M. Noor, H.Y. Myint, S.I. Hay, The global distribution of clinical episodes of *Plasmodium falciparum* malaria, Nature. 434 (2005) 214–217. doi:10.1038/nature03342.

[59] J.-Y. Choi, V. Kumar, N. Pachikara, A. Garg, L. Lawres, J.Y. Toh, D.R. Voelker, C. Ben Mamoun, Characterization of *Plasmodium* phosphatidylserine decarboxylase expressed in yeast and application for inhibitor screening, Mol. Microbiol. 99 (2016) 999–1014. doi:10.1111/mmi.13280.

[60] J.-Y. Choi, Y. Augagneur, C. Ben Mamoun, D.R. Voelker, 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 (2012) 222–232. doi:10.1074/jbc.M111.313676.

[61] J.C. Boothroyd, M.E. Grigg, Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease?, Curr. Opin. Microbiol. 5 (2002) 438–442. doi:10.1016/S1369-5274(02)00349-1.

[62] N. Gupta, A. Hartmann, R. Lucius, D.R. Voelker, The obligate intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase, J. Biol. Chem. 287 (2012) 22938–22947. doi:10.1074/jbc.M112.373639.

[63] D. Rontein, W.-I. Wu, D.R. Voelker, A.D. Hanson, Mitochondrial Phosphatidylserine Decarboxylase from Higher Plants. Functional Complementation in Yeast, Localization in Plants, and Overexpression in Arabidopsis, Plant Physiol. 132 (2003) 1678–1687. doi:10.1104/pp.103.023242.

[64] A.K. Percy, J.F. Moore, M.A. Carson, C.J. Waechter, Characterization of brain phosphatidylserine decarboxylase: Localization in the mitochondrial inner membrane, Arch. Biochem. Biophys. 223 (1983) 484–494. doi:10.1016/0003-9861(83)90613-6.

[65] L.F. Borkenhagen, E.P. Kennedy, L. Fielding, Enzymatic Formation and Decarboxylation of Phosphatidylserine, J. Biol. Chem. 236 (1961) PC28-PC30.

[66] R. Steenbergen, T.S. Nanowski, A. Beigneux, A. Kulinski, S.G. Young, J.E. Vance, Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects, J. Biol. Chem. 280 (2005) 40032–40040. doi:10.1074/jbc.M506510200.

[67] E. Calzada, O. Onguka, S.M. Claypool, Phosphatidylethanolamine Metabolism in Health and Disease, Int. Rev. Cell Mol. Biol. 321 (2016) 29–88. doi:10.1016/bs.ircmb.2015.10.001.

[68] G. Tasseva, H.D. Bai, M. Davidescu, A. Haromy, E. Michelakis, J.E. Vance, Phosphatidylethanolamine deficiency in Mammalian mitochondria impairs oxidative phosphorylation and alters mitochondrial morphology, J. Biol. Chem. 288 (2013) 4158–4173. doi:10.1074/jbc.M112.434183.

[69] K. Saito, M. Nishijima, O. Kuge, Genetic evidence that phosphatidylserine synthase II catalyzes the conversion of phosphatidylethanolamine to phosphatidylserine in Chinese hamster ovary cells, J. Biol. Chem. 273 (1998) 17199–17205.

[70] S. Wang, S. Zhang, L.-C. Liou, Q. Ren, Z. Zhang, G.A. Caldwell, K.A. Caldwell, S.N. Witt, Phosphatidylethanolamine deficiency disrupts α-synuclein homeostasis in yeast and worm models of Parkinson disease, Proc. Natl. Acad. Sci. USA 111 (2014) E3976–E3985. doi:10.1073/pnas.1411694111.

[71] I. Nesic, F.X. Guix, K. Vennekens, V. Michaki, P.P. Van Veldhoven, F. Feiguin, B. De Strooper, C.G. Dotti, T. Wahle, Alterations in phosphatidylethanolamine levels affect the generation of Aβ, Aging Cell. 11 (2012) 63–72. doi:10.1111/j.1474-9726.2011.00760.x.

[72] D.R. Voelker, Phosphatidylserine decarboxylase, Biochim. Biophys. Acta. 1348 (1997) 236–244.

[73] E. Hawrot, E.P. Kennedy, Biogenesis of membrane lipids: mutants of *Escherichia coli* with temperature-sensitive phosphatidylserine decarboxylase, Proc. Natl. Acad. Sci. 72 (1975) 1112–1116.

[74] E. Hawrot, E.P. Kennedy, Phospholipid composition and membrane function in phosphatidylserine decarboxylase mutants of *Escherichia coli*, J. Biol. Chem. 253 (1978) 8213–8220.

[75] A. DeChavigny, P.N. Heacock, W. Dowhan, Sequence and inactivation of the pss gene of *Escherichia coli*. Phosphatidylethanolamine may not be essential for cell viability, J. Biol. Chem. 266 (1991) 5323–5332.

[76] S.K. Saha, N. Satomi, H. Matsuzaki, I. Shibuya, K. Matsumoto, A Regulatory Mechanism for the Balanced Synthesis of Membrane Phospholipid Species in *Escherichia coli*, Biosci. Biotechnol. Biochem. 60 (1996) 111–116. doi:10.1271/bbb.60.111.

[77] J. Xie, M. Bogdanov, P. Heacock, W. Dowhan, Phosphatidylethanolamine and Monoglucosyldiacylglycerol Are Interchangeable in Supporting Topogenesis and Function of the Polytopic Membrane Protein Lactose Permease, J. Biol. Chem. 281 (2006) 19172–19178. doi:10.1074/jbc.M602565200.

[78] M.A. Vences-Guzmán, O. Geiger, C. Sohlenkamp, Sinorhizobium meliloti mutants deficient in phosphatidylserine decarboxylase accumulate phosphatidylserine and

are strongly affected during symbiosis with alfalfa, J. Bacteriol. 190 (2008) 6846–6856. doi:10.1128/JB.00610-08.

[79] R. Birner, G. Daum, Biogenesis and cellular dynamics of aminoglycerophospholipids, in: B.-I.R. of Cytology (Ed.), Academic Press, 2003: pp. 273–323.

[80] S.E. Horvath, A. Wagner, E. Steyrer, G. Daum, Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast *Saccharomyces cerevisiae.*, Biochim. Biophys. Acta. 1811 (2011) 1030–7. doi:10.1016/j.bbalip.2011.08.007.

[81] P. Griac, Regulation of yeast phospholipid biosynthetic genes in phosphatidylserine decarboxylase mutants, J. Bacteriol. 179 (1997) 5843–5848.

[82] Y. Tamura, O. Onguka, K. Itoh, T. Endo, M. Iijima, S.M. Claypool, H. Sesaki, Phosphatidylethanolamine biosynthesis in mitochondria: phosphatidylserine (PS) trafficking is independent of a PS decarboxylase and intermembrane space proteins UPS1P and UPS2P, J. Biol. Chem. 287 (2012) 43961–43971. doi:10.1074/jbc.M112.390997.

[83] M. Gsell, G. Mascher, I. Schuiki, B. Ploier, C. Hrastnik, G. Daum, Transcriptional response to deletion of the phosphatidylserine decarboxylase Psd1p in the yeast *Saccharomyces cerevisiae*, PloS One. 8 (2013) e77380. doi:10.1371/journal.pone.0077380.

[84] M.L. Greenberg, L.S. Klig, V.A. Letts, B.S. Loewy, S.A. Henry, Yeast mutant defective in phosphatidylcholine synthesis, J. Bacteriol. 153 (1983) 791–799.

[85] E.Y.L. Chan, G.A. McQuibban, Phosphatidylserine decarboxylase 1 (Psd1) promotes mitochondrial fusion by regulating the biophysical properties of the mitochondrial membrane and alternative topogenesis of mitochondrial genome maintenance protein 1 (Mgm1), J. Biol. Chem. 287 (2012) 40131–40139. doi:10.1074/jbc.M112.399428.

[86] T. Kuroda, M. Tani, A. Moriguchi, S. Tokunaga, T. Higuchi, S. Kitada, O. Kuge, FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis, Mol. Microbiol. 80 (2011) 248–265. doi:10.1111/j.1365-2958.2011.07569.x.

[87] V.M. Gohil, M.N. Thompson, M.L. Greenberg, Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine and cardiolipin biosynthetic pathways in *Saccharomyces cerevisiae*, J. Biol. Chem. 280 (2005) 35410–35416. doi:10.1074/jbc.M505478200.

[88] T. Becker, S.E. Horvath, L. Böttinger, N. Gebert, G. Daum, N. Pfanner, Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins., J. Biol. Chem. 288 (2013) 16451–9. doi:10.1074/jbc.M112.442392.

[89] O. Onguka, E. Calzada, O.B. Ogunbona, S.M. Claypool, Phosphatidylserine decarboxylase 1 autocatalysis and function does not require a mitochondrial-specific factor, J. Biol. Chem. 290 (2015) 12744–12752. doi:10.1074/jbc.M115.641118.

[90] O. Kuge, K. Saito, M. Kojima, Y. Akamatsu, M. Nishijima, Post-translational processing of the phosphatidylserine decarboxylase gene product in Chinese hamster ovary cells, Biochem. J. 319 (Pt 1) (1996) 33–38.

[91] A.S. Joshi, J. Zhou, V.M. Gohil, S. Chen, M.L. Greenberg, Cellular functions of cardiolipin in yeast, Biochim. Biophys. Acta. 1793 (2009) 212–218. doi:10.1016/j.bbamcr.2008.07.024.

[92] W.-I. Wu, D.R. Voelker, Reconstitution of phosphatidylserine transport from chemically defined donor membranes to phosphatidylserine decarboxylase 2 implicates specific lipid domains in the process, J. Biol. Chem. 279 (2004) 6635–6642. doi:10.1074/jbc.M311570200.

[93] N. Gebert, A.S. Joshi, S. Kutik, T. Becker, M. McKenzie, X.L. Guan, V.P. Mooga, D.A. Stroud, G. Kulkarni, M.R. Wenk, P. Rehling, C. Meisinger, M.T. Ryan, N. Wiedemann, M.L. Greenberg, N. Pfanner, Mitochondrial cardiolipin involved in outer-membrane protein biogenesis: implications for Barth syndrome, Curr. Biol. CB. 19 (2009) 2133–2139. doi:10.1016/j.cub.2009.10.074.

[94] C.R. Brown, J.A. McCann, G.G.-C. Hung, C.P. Elco, H.-L. Chiang, Vid22p, a novel plasma membrane protein, is required for the fructose-1,6-bisphosphatase degradation pathway, J. Cell Sci. 115 (2002) 655–666.

[95] A.S. Joshi, M.N. Thompson, N. Fei, M. Hüttemann, M.L. Greenberg, Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in Saccharomyces cerevisiae, J. Biol. Chem. 287 (2012) 17589–17597. doi:10.1074/jbc.M111.330167.

[96] P. Rockenfeller, M. Koska, F. Pietrocola, N. Minois, O. Knittelfelder, V. Sica, J. Franz, D. Carmona-Gutierrez, G. Kroemer, F. Madeo, Phosphatidylethanolamine positively regulates autophagy and longevity, Cell Death Differ. 22 (2015) 499–508. doi:10.1038/cdd.2014.219.

[97] R. Nebauer, I. Schuiki, B. Kulterer, Z. Trajanoski, G. Daum, The phosphatidylethanolamine level of yeast mitochondria is affected by the mitochondrial components Oxa1p and Yme1p, FEBS J. 274 (2007) 6180–6190. doi:10.1111/j.1742-4658.2007.06138.x.

[98] K. Muthukumar, V. Nachiappan, Phosphatidylethanolamine from phosphatidylserine decarboxylase2 is essential for autophagy under cadmium stress in *Saccharomyces cerevisiae*, Cell Biochem. Biophys. 67 (2013) 1353–1363. doi:10.1007/s12013-013-9667-8.

[99] N. Miyata, T. Miyoshi, T. Yamaguchi, T. Nakazono, M. Tani, O. Kuge, VID22 is required for transcriptional activation of the PSD2 gene in the yeast *Saccharomyces cerevisiae*, Biochem. J. 472 (2015) 319–328. doi:10.1042/BJ20150884.

[100] D.R. Voelker, Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 2669–2673.

[101] O.B. Bleijerveld, J.F.H.M. Brouwers, A.B. Vaandrager, J.B. Helms, M. Houweling, The CDP-ethanolamine Pathway and Phosphatidylserine Decarboxylation Generate Different Phosphatidylethanolamine Molecular Species, J. Biol. Chem. 282 (2007) 28362–28372. doi:10.1074/jbc.M703786200.

[102] J.E. Vance, G. Tasseva, Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells, Biochim. Biophys. Acta. 1831 (2013) 543–554. doi:10.1016/j.bbalip.2012.08.016.

[103] G. Schatz, The Protein Import System of Mitochondria, J. Biol. Chem. 271 (1996) 31763–31766. doi:10.1074/jbc.271.50.31763.

[104] N. Wiedemann, A.E. Frazier, N. Pfanner, The Protein Import Machinery of Mitochondria, J. Biol. Chem. 279 (2004) 14473–14476. doi:10.1074/jbc.R400003200.

[105] J. Dudek, P. Rehling, M. van der Laan, Mitochondrial protein import: Common principles and physiological networks, Biochim. Biophys. Acta BBA - Mol. Cell Res. 1833 (2013) 274–285. doi:10.1016/j.bbamcr.2012.05.028.

[106] A. Geissler, T. Krimmer, U. Bömer, B. Guiard, J. Rassow, N. Pfanner, Membrane Potential-Driven Protein Import into Mitochondria, Mol. Biol. Cell. 11 (2000) 3977–3991.

[107] F.-N. Vögtle, C. Prinz, J. Kellermann, F. Lottspeich, N. Pfanner, C. Meisinger, Mitochondrial protein turnover: role of the precursor intermediate peptidase Oct1 in protein stabilization, Mol. Biol. Cell. 22 (2011) 2135–2143. doi:10.1091/mbc.E11-02-0169.

[108] J. Zborowski, A. Dygas, L. Wojtczak, Phosphatidylserine decarboxylase is located on the external side of the inner mitochondrial membrane, FEBS Lett. 157 (1983) 179–182. doi:10.1016/0014-5793(83)81141-7.

[109] Q.X. Li, W. Dowhan, Studies on the mechanism of formation of the pyruvate prosthetic group of phosphatidylserine decarboxylase from *Escherichia coli*, J. Biol. Chem. 265 (1990) 4111–4115.
[110] J.-Y. Choi, M.T. Duraisingh, M. Marti, C. Ben Mamoun, D.R. Voelker, From Protease to Decarboxylase: the molecular metamorphosis of Phosphatidylserine decarboxylase, J. Biol. Chem. 290 (2015) 10972–10980. doi:10.1074/jbc.M115.642413.
[111] L. Hedstrom, Serine protease mechanism and specificity, Chem. Rev. 102 (2002) 4501–4524.
[112] M. Satre, E.P. Kennedy, Identification of bound pyruvate essential for the activity of phosphatidylserine decarboxylase of *Escherichia coli*, J. Biol. Chem. 253 (1978) 479–483.
[113] T.G. Warner, E.A. Dennis, Action of the highly purified, membrane-bound enzyme phosphatidylserine decarboxylase *Escherichia coli* toward phosphatidylserine in mixed micelles and erythrocyte ghosts in the presence of surfactant, J. Biol. Chem. 250 (1975) 8004–8009.
[114] F. Baunaure, P. Eldin, A.-M. Cathiard, H. Vial, Characterization of a non-mitochondrial type I phosphatidylserine decarboxylase in *Plasmodium falciparum*, Mol. Microbiol. 51 (2004) 33–46. doi:10.1046/j.1365-2958.2003.03822.x.
[115] D.R. Voelker, E.B. Golden, [42] Phosphatidylserine decarboxylase from rat liver, in: E.A.D. and ennis E. Vance (Ed.), Methods Enzymol., Academic Press, 1992: pp. 360–365.
[116] G. Daum, J.E. Vance, Import of lipids into mitochondria, Prog. Lipid Res. 36 (1997) 103–130. doi:10.1016/S0163-7827(97)00006-4.
[117] R. Simbeni, L. Pon, E. Zinser, F. Paltauf, G. Daum, Mitochondrial membrane contact sites of yeast. Characterization of lipid components and possible involvement in intramitochondrial translocation of phospholipids., J. Biol. Chem. 266 (1991) 10047–10049.
[118] D. Brdiczka, Contact sites between mitochondrial envelope membranes. Structure and function in energy- and protein-transfer, Biochim. Biophys. Acta. 1071 (1991) 291–312.
[119] D. Ardail, J.P. Privat, M. Egret-Charlier, C. Levrat, F. Lerme, P. Louisot, Mitochondrial contact sites. Lipid composition and dynamics, J. Biol. Chem. 265 (1990) 18797–18802.
[120] P.J. Meier, M.A. Spycher, U.A. Meyer, Isolation and characterization of rough endoplasmic reticulum associated with mitochondria from normal rat liver, Biochim. Biophys. Acta BBA - Biomembr. 646 (1981) 283–297. doi:10.1016/0005-2736(81)90335-7.

[121] G.C. Shore, J.R. Tata, Two fractions of rough endoplasmic reticulum from rat liver. I. Recovery of rapidly sedimenting endoplasmic reticulum in association with mitochondria, J. Cell Biol. 72 (1977) 714–725.

[122] G. Csordás, C. Renken, P. Várnai, L. Walter, D. Weaver, K.F. Buttle, T. Balla, C.A. Mannella, G. Hajnóczky, Structural and functional features and significance of the physical linkage between ER and mitochondria, J. Cell Biol. 174 (2006) 915–921. doi:10.1083/jcb.200604016.

[123] J.E. Vance, MAM (mitochondria-associated membranes) in mammalian cells: Lipids and beyond, Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids. 1841 (2014) 595–609. doi:10.1016/j.bbalip.2013.11.014.

[124] G. Achleitner, B. Gaigg, A. Krasser, E. Kainersdorfer, S.D. Kohlwein, A. Perktold, G. Zellnig, G. Daum, Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact, Eur. J. Biochem. 264 (1999) 545–553. doi:10.1046/j.1432-1327.1999.00658.x.

[125] B. Gaigg, R. Simbeni, C. Hrastnik, F. Paltauf, G. Daum, Characterization of a microsomal subfraction associated with mitochondria of the yeast, Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria, Biochim. Biophys. Acta. 1234 (1995) 214–220.

[126] J.E. Vance, Phospholipid synthesis in a membrane fraction associated with mitochondria, J. Biol. Chem. 265 (1990) 7248–7256.

[127] S.C.J. Helle, G. Kanfer, K. Kolar, A. Lang, A.H. Michel, B. Kornmann, Organization and function of membrane contact sites, Biochim. Biophys. Acta. 1833 (2013) 2526–2541. doi:10.1016/j.bbamcr.2013.01.028.

[128] K.S. Dimmer, S. Fritz, F. Fuchs, M. Messerschmitt, N. Weinbach, W. Neupert, B. Westermann, Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*, Mol. Biol. Cell. 13 (2002) 847–853. doi:10.1091/mbc.01-12-0588.

[129] L.F. Sogo, M.P. Yaffe, Regulation of mitochondrial morphology and inheritance by Mdm10p, a protein of the mitochondrial outer membrane, J. Cell Biol. 126 (1994) 1361–1373.

[130] I.R. Boldogh, D.W. Nowakowski, H.-C. Yang, H. Chung, S. Karmon, P. Royes, L.A. Pon, A Protein Complex Containing Mdm10p, Mdm12p, and Mmm1p Links Mitochondrial Membranes and DNA to the Cytoskeleton-based Segregation Machinery, Mol. Biol. Cell. 14 (2003) 4618–4627. doi:10.1091/mbc.E03-04-0225.

[131] B. Kornmann, E. Currie, S.R. Collins, M. Schuldiner, J. Nunnari, J.S. Weissman, P. Walter, An ER-Mitochondria Tethering Complex Revealed by a Synthetic Biology Screen, Science. 325 (2009) 477–481. doi:10.1126/science.1175088.

[132] K.O. Kopec, V. Alva, A.N. Lupas, Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria, Bioinforma. Oxf. Engl. 26 (2010) 1927–1931. doi:10.1093/bioinformatics/btq326.

[133] T.T. Nguyen, A. Lewandowska, J.-Y. Choi, D.F. Markgraf, M. Junker, M. Bilgin, C.S. Ejsing, D.R. Voelker, T.A. Rapoport, J.M. Shaw, Gem1 and ERMES do not directly affect phosphatidylserine transport from ER to mitochondria or mitochondrial inheritance, Traffic Cph. Den. 13 (2012) 880–890. doi:10.1111/j.1600-0854.2012.01352.x.

[134] C. Voss, S. Lahiri, B.P. Young, C.J. Loewen, W.A. Prinz, ER-shaping proteins facilitate lipid exchange between the ER and mitochondria in *S. cerevisiae*, J. Cell Sci. 125 (2012) 4791–4799. doi:10.1242/jcs.105635.

[135] G.K. Voeltz, W.A. Prinz, Y. Shibata, J.M. Rist, T.A. Rapoport, A Class of Membrane Proteins Shaping the Tubular Endoplasmic Reticulum, Cell. 124 (2006) 573–586. doi:10.1016/j.cell.2005.11.047.

[136] G. Orso, D. Pendin, S. Liu, J. Tosetto, T.J. Moss, J.E. Faust, M. Micaroni, A. Egorova, A. Martinuzzi, J.A. McNew, A. Daga, Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin, Nature. 460 (2009) 978–983. doi:10.1038/nature08280.

[137] Y. Elbaz-Alon, E. Rosenfeld-Gur, V. Shinder, A.H. Futerman, T. Geiger, M. Schuldiner, A dynamic interface between vacuoles and mitochondria in yeast, Dev. Cell. 30 (2014) 95–102. doi:10.1016/j.devcel.2014.06.007.

[138] C. Hönscher, M. Mari, K. Auffarth, M. Bohnert, J. Griffith, W. Geerts, M. van der Laan, M. Cabrera, F. Reggiori, C. Ungermann, Cellular metabolism regulates contact sites between vacuoles and mitochondria, Dev. Cell. 30 (2014) 86–94. doi:10.1016/j.devcel.2014.06.006.

[139] S. Lahiri, J.T. Chao, S. Tavassoli, A.K.O. Wong, V. Choudhary, B.P. Young, C.J.R. Loewen, W.A. Prinz, A conserved endoplasmic reticulum membrane protein complex (EMC) facilitates phospholipid transfer from the ER to mitochondria, PLoS Biol. 12 (2014) e1001969. doi:10.1371/journal.pbio.1001969.

[140] M.M. Schumacher, J.-Y. Choi, D.R. Voelker, Phosphatidylserine Transport to the Mitochondria Is Regulated by Ubiquitination, J. Biol. Chem. 277 (2002) 51033–51042. doi:10.1074/jbc.M205301200.

[141] D.R. Voelker, Disruption of phosphatidylserine translocation to the mitochondria in baby hamster kidney cells, J. Biol. Chem. 260 (1985) 14671–14676.

[142] O. Kuge, M. Nishijima, Biosynthetic regulation and intracellular transport of phosphatidylserine in mammalian cells, J. Biochem. (Tokyo). 133 (2003) 397–403.

[143] R. Simbeni, F. Paltauf, G. Daum, Intramitochondrial transfer of phospholipids in the yeast, *Saccharomyces cerevisiae*, J. Biol. Chem. 265 (1990) 281–285.
[144] N. Pfanner, J. Rassow, U. Wienhues, C. Hergersberg, T. Söllner, K. Becker, W. Neupert, Contact sites between inner and outer membranes: structure and role in protein translocation into the mitochondria, Biochim. Biophys. Acta. 1018 (1990) 239–242.
[145] M. Lampl, A. Leber, F. Paltauf, G. Daum, Import of phosphatidylinositol and phosphatidylcholine into mitochondria of the yeast, *Saccharomyces cerevisiae*, FEBS Lett. 356 (1994) 1–4.
[146] C. Potting, C. Wilmes, T. Engmann, C. Osman, T. Langer, Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35, EMBO J. 29 (2010) 2888–2898. doi:10.1038/emboj.2010.169.
[147] Y. Tamura, T. Endo, M. Iijima, H. Sesaki, Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria, J. Cell Biol. 185 (2009) 1029–1045. doi:10.1083/jcb.200812018.
[148] J.M. Herrmann, Ups delivery to the intermembrane space of mitochondria: a novel affinity-driven protein import pathway, EMBO J. 29 (2010) 2859–2860. doi:10.1038/emboj.2010.189.
[149] Y. Tamura, H. Sesaki, T. Endo, Phospholipid transport via mitochondria, Traffic Cph. Den. 15 (2014) 933–945. doi:10.1111/tra.12188.
[150] K.S. Dimmer, S. Jakobs, F. Vogel, K. Altmann, B. Westermann, Mdm31 and Mdm32 are inner membrane proteins required for maintenance of mitochondrial shape and stability of mitochondrial DNA nucleoids in yeast, J. Cell Biol. 168 (2005) 103–115. doi:10.1083/jcb.200410030.
[151] M.J. Aaltonen, J.R. Friedman, C. Osman, B. Salin, J.-P. di Rago, J. Nunnari, T. Langer, T. Tatsuta, MICOS and phospholipid transfer by Ups2-Mdm35 organize membrane lipid synthesis in mitochondria, J. Cell Biol. 213 (2016) 525–534. doi:10.1083/jcb.201602007.
[152] N. Miyata, Y. Watanabe, Y. Tamura, T. Endo, O. Kuge, Phosphatidylserine transport by Ups2-Mdm35 in respiration-active mitochondria, J. Cell Biol. 214 (2016) 77–88. doi:10.1083/jcb.201601082.
[153] M. Bürgermeister, R. Birner-Grünberger, M. Heyn, G. Daum, Contribution of different biosynthetic pathways to species selectivity of aminoglycerophospholipids assembled into mitochondrial membranes of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta. 1686 (2004) 148–160. doi:10.1016/j.bbalip.2004.09.005.

[154] M. Kannan, W.R. Riekhof, D.R. Voelker, Transport of phosphatidylserine from the endoplasmic reticulum to the site of phosphatidylserine decarboxylase2 in yeast, Traffic Cph. Den. 16 (2015) 123–134. doi:10.1111/tra.12236.

CHAPTER 2

Involvement of a putative substrate binding site in the biogenesis and assembly of phosphatidylserine decarboxylase 1 from *Saccharomyces cerevisiae*

Manuscript submitted

Involvement of a putative substrate binding site in the biogenesis and assembly of phosphatidylserine decarboxylase 1 from *Saccharomyces cerevisiae*

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Abbreviations: CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; ER, endoplasmic reticulum; LP, lysophospholipids; MPP, mitochondrial processing peptidase; Oct1p, octapeptidyl aminopeptidase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Psd1-a, phosphatidylserine decarboxylase 1 a-subunit; Psd1-b, phosphatidylserine decarboxylase 1 b-subunit; SRS, substrate recognition site; TOM, translocase of the outer mitochondrial membrane.

ABSTRACT

In the yeast *Saccharomyces cerevisiae*, the mitochondrial phosphatidylserine decarboxylase 1 (Psd1p) produces the largest amount of cellular phosphatidylethanolamine (PE). Psd1p is synthesized as larger precursor on cytosolic ribosomes and then imported into mitochondria in a three step processing event. The α-subunit harbors a highly conserved motif, which was proposed to be involved in phosphatidylserine (PS) binding. Here, we present a molecular analysis of this consensus motif for the function of Psd1p by using Psd1p variants bearing either deletions or point mutations in this region. Our data show that mutations in this motif affect processing and stability of Psd1p, and consequently the enzyme's activity. Thus, we conclude that this consensus motif is essential for structural integrity and processing of Psd1p.

Keywords: phospholipid; mitochondria; phosphatidylserine decarboxylase 1; phosphatidylethanolamine; *Saccharomyces cerevisiae*

1. INTRODUCTION

Phospholipid composition is paramount for vital mitochondrial functions including respiration, membrane architecture, protein transport, apoptosis and mitophagy [1–3]. The non-bilayer forming phospholipid phosphatidylethanolamine (PE) is crucial for the function of several membrane-bound protein machineries. In *Saccharomyces cerevisiae* a significant reduction of cellular and mitochondrial PE causes a petite phenotype characterized by loss of respiratory capacity [4,5]. Depletion of PE also results in reduced activity of yeast and mammalian cytochrome c oxidase of the respiratory chain leading to a decrease of the inner membrane potential [6–8]. The reduced membrane potential affects in turn protein import into and across the inner membrane [6]. In addition, a reduced PE content disturbs preprotein binding but not the integrity of the translocase of the outer membrane (TOM complex) [9]. The TOM complex forms the general entry gate for most mitochondrial precursor proteins [10–13]. The impaired TOM function contributes to defective transport of β-barrel precursors into mitochondrial outer membrane with reduced PE content. Additionally, a certain level of PE in the mitochondrial inner membrane together with the prohibitin complex may be required for the attachment of mtDNA nucleoids [14]. PE also serves as a precursor for GPI-anchored proteins by providing the ethanolamine phosphate bridge which links the C-terminal amino acid of GPI-anchored proteins to glycosylphosphatidylinositol [15].

In yeast PE is synthesized by different pathways [4,16]. Phosphatidylserine decarboxylase 1 (Psd1p) of the mitochondrial inner membrane is the key player in PE synthesis [17–21]. Deletion of Pisd, the Psd1p ortholog in mice, is embryonic lethal [22]. During Psd1p biogenesis the enzyme is synthesized as precursor on cytosolic

ribosomes. An N-terminal mitochondrial targeting sequence is crucial for targeting Psd1p to mitochondria [19]. The receptor proteins Tom70p and Tom22p are important for recognition of the Psd1p precursor on the mitochondrial surface and its transfer to the TOM pore [19,23]. Subsequent insertion into the inner membrane depends on an inner mitochondrial membrane-sorting signal and the membrane potential across the inner membrane [24–27] (Fig. 1). After the import, the mitochondrial processing peptidase (MPP) cleaves the mitochondrial targeting sequence, and subsequently the mitochondrial intermediate peptidase Oct1p removes a small octapeptide to stabilize the N-terminus of the mature Psd1p [19]. In addition, Psd1p maturation comprises an autocatalytic endoproteolytic cleavage at a highly conserved LGST sequence, which occurs by a serine protease mechanism catalyzed by the catalytic triad aspartate-histidine-serine [28,29]. Cleavage of the Psd1p-precursor results in formation of two separate subunits named α and β. The β-subunit forms the membrane anchor for the intermembrane space localized α-subunit [19]. The autocatalytic cleavage is a crucial step leading to the constitution of the catalytic pyruvyl residue, which is located at the interface between the two subunits [28,30,31]. Interestingly, the autocatalytic cleavage can even occur when the protein is not correctly inserted into the inner membrane, indicating that proper insertion into the inner membrane is not prerequisite for the separation of both Psd1p subunits [16,26].

Despite the central role of Psd1p for cell survival, only little is known about the molecular mechanism of its biogenesis and activity. The aim of the investigation presented here was to shed light on the function of a reported PS-binding site within the α-subunit of Psd1p from *Saccharomyces cerevisiae*. This domain was also found in protein kinase C and binds phosphatidylserine (PS) *in vitro* [33,34]. The role of this motif for the biogenesis and activity of Psd1p in mitochondria was not yet analyzed.

Sequence analysis revealed that this motif and flanking regions are at least in part conserved in Psd1p enzymes from bacteria to eukaryotes.

Using a set of deletion and point mutants we investigated the role of this region for Psd1p biogenesis and function in mitochondria. Surprisingly, mutated Psd1p revealed a reduced stability and impaired separation of both Psd1p subunits. Consequently, the entire Psd1p enzyme including α- and β-subunit was destabilized in the mutants. Thus, in addition to the LGST motif there is a second conserved region in Psd1p α-subunit, which is crucial for maturation of Psd1p. We conclude that the conserved motif of the α-subunit is not only important for the structural integrity, but also for processing of Psd1p, which in turn has an important impact on the catalytic activity of the enzyme.

Figure 1

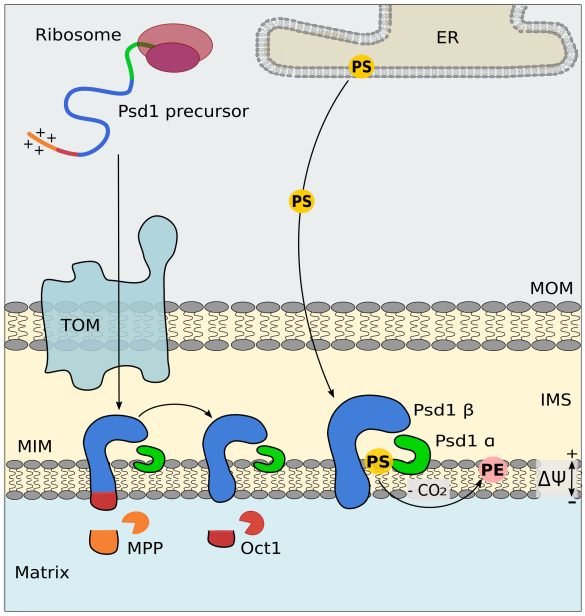

Figure 1. Biogenesis of Psd1p. The precursor form of Psd1p is synthesized on cytosolic ribosomes and imported across the outer membrane of mitochondria (MOM) by the translocase of the outer membrane (TOM complex). After a membrane-potential

(Dy) dependent insertion into the inner membrane of mitochondria (MIM) the Psd1p precursor is processed in three steps. First, the mitochondrial processing peptidase (MPP) removes the mitochondrial targeting sequence. Second, the octapeptidyl aminopeptidase Oct1p removes an octapeptide. Third, the autocatalytic cleavage separates a membrane-embedded b-subunit from the α-subunit, which harbors the active site of Psd1p. The α-subunit allows decarboxylation of PS to PE, which is distributed within mitochondria and in the whole cell.

2. EXPERIMENTAL PROCEDURES

2.1 Yeast strain construction and growth conditions

The Psd1p mutant strains used in this study are shown in Table 1. Yeast Psd1p variants were generated by overlap-extension PCR using *PSD1HA* [19] as a template for site-directed mutagenesis. The purified PCR product was inserted via the restriction sites for *Bam*HI and *Not*I into the pYES2 vector (Invitrogen, Carlsbad, California). A *Saccharomyces cerevisiae psd1*Δ strain was transformed with the constructed plasmids by lithium acetate transformation [35] resulting in yeast strains listed in Table 1. All yeast cells transformed with the plasmids were grown under aerobic conditions to the early stationary phase at 30 °C on minimal medium containing 2% (w/v) galactose (ForMedium, Hunstanton, UK), 0.67% (w/v) yeast nitrogen base without amino acids (ForMedium), and 0.063% (w/v) amino acid mix without uracil.

Tab. 1 Strains used in this study

Strain	Genotype	Source/Reference
Wild type (WT)	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
***psd1*Δ**	BY4741 *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4*	Euroscarf
WT + pYES2	BY4741 + pYES2	[19]
***psd1*Δ + pYES2**	*psd1*Δ + pYES2	[19]
Psd1HA	*psd1*Δ + pYES2-Psd1HA	[19]
Psd1ΔSRS	*psd1*Δ + pYES2-Psd1-Δ(475-487)	This study
Psd1ΔDK	*psd1*Δ + pYES2-Psd1-Δ483;Δ484	This study
Psd1AX3	*psd1*Δ + pYES2 Psd1K480A;K484A;K486A	This study
Psd1ΔSRS4	*psd1*Δ + pYES2-Psd1-Δ(480-485)	This study
Psd1ΔSRS3	*psd1*Δ + pYES2-Psd1-Δ(479-481)	This study
Psd1ΔSRS2	*psd1*Δ + pYES2-Psd1-Δ(482-484)	This study
Psd1G482P	*psd1*Δ + pYES2-Psd1G482P	This study
Psd1K486A	*psd1*Δ + pYES2-Psd1K486A	This study
Psd1G488P	*psd1*Δ + pYES2-Psd1G488P	This study
Psd1G492P	*psd1*Δ + pYES2-Psd1G492P	This study
Psd1ΔLG	*psd1*Δ + pYES2-Psd1-Δ491;Δ492	This study

2.2 Isolation of mitochondria and protein analysis

For isolation of mitochondria, cells were pre-cultured in 2% (w/v) glucose (Roth, Karlsruhe, Germany), 0.67% (w/v) yeast nitrogen base without amino acids (ForMedium), and 0.063% (w/v) amino acid mix without uracil for 6 hours at 30 °C and transferred to 2% (w/v) galactose minimal medium (see section 2.1).

Mitochondria were isolated by differential centrifugation according to standard procedures [36,37]. Mitochondrial fractions obtained 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). Aliquots were shock-frozen in liquid nitrogen and stored at -80

°C. Mitochondrial samples were concentrated by TCA precipitation, and 20 µg of protein from each sample were separated by SDS-PAGE followed by immunoblotting. Western blot analysis was performed as described [38] and immunoreactive bands were detected with chemiluminescent reagent Luminata™ Classico (Millipore, Billerica, Massachusetts). The antibodies used for detection were an anti-HA-peroxidase conjugated, high affinity (3F10) (Roche Applied Science, Penzberg, Germany) recognizing the HA-tagged α-subunit, and a monoclonal antibody directed against the Psd1 β-subunit [19].

2.3 Import of precursor proteins into isolated mitochondria

The import of precursor proteins into isolated mitochondria was performed as reported [37]. For each construct a PCR-generated template containing the SP6 promoter was used for *in vitro* transcription. The RNA produced was purified (MEGAclear kit; Invitrogen, Carlsbad, California) and used for *in vitro* translation (TNT kit; Promega, Madison, Wisconsin) in the presence of ^{35}S-labeled methionine. Import of ^{35}S-labeled precursor proteins into mitochondria isolated from wild type *Saccharomyces cerevisiae* BY4741 (containing 50 µg protein) was performed at 30 °C in the presence of 2 mM NADH and 2 mM ATP in the import buffer (3% BSA (w/v), 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH, pH 7.2). Import reactions were stopped by transfer on ice and by dissipation of the membrane potential with 8 µM antimycin A, 1 µM valinomycin, and 20 µM oligomycin (final concentrations). For protease treatment, samples were incubated with 50 µg/ml proteinase K for 15 min on ice. Proteinase K activity was stopped by addition of 2 mM PMSF and incubation for 10 min on ice. Then mitochondria were re-isolated by centrifugation (10,000 X *g*, 10

min, 4 °C) and washed with SEM buffer (see paragraph 2.2). Samples were subjected to SDS-PAGE, and ^{35}S-labeled proteins were detected by digital autoradiography (Storm imaging system, GE Healthcare, Chicago, Illinois).

2.4 Phospholipid analysis

Lipids were extracted from isolated mitochondria by Folch extraction using chloroform/methanol (2:1; v/v) [39]. The remaining contaminants, e.g., proteins and salts were removed by additional washing steps of the organic phase with 0.034% $MgCl_2$ solution (w/v), 2 N KCl/methanol (4:1; v/v), and methanol/water/chloroform (48:47:3; per vol.), respectively. Individual phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates (Merck, Darmstadt, Germany) by using chloroform/methanol/25% NH_3 (65:35:5; per vol.) as the first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as the second developing solvent. Phospholipids were visualized on two-dimensional thin-layer chromatography plates by staining with iodine vapor, scraped off and quantified as described [40].

2.5 Psd1p activity assay

The enzymatic activity of Psd1p was determined *in vitro* by measuring the conversion of ^{3}H-labeled PS to ^{3}H-labeled PE in isolated mitochondria from yeast cells grown to the logarithmic growth phase [41]. Radioactively labeled [^{3}H]PS was synthesized *in vitro* by incubating microsomes (8 mg of protein) isolated from the *Saccharomyces cerevisiae pem1*Δ*pem2*Δ strain with 0.2 mM CDP-DAG, 5 mM hydroxylamine, 0.6 mM $MnCl_2$,

0.2% Triton X-100, 0.5 mM L-serine, 0.01 mCi of L-[^{3}H]serine (specific activity 21.99 μCi/mmol; PerkinElmer, Waltham, Massachusetts) and 0.1 M Tris-HCl (pH 8.0) in a total volume of 2 ml for 2 h at 30°C. The reaction was stopped by the addition of 4 ml chloroform/methanol (2:1; v/v), and lipids were extracted as described [39]. The Psd1p activity assay was performed in a final volume of 1.5 ml containing 100 nmol of [^{3}H]PS (specific activity, 0.64 μCi/mmol), 0.1 M Tris-HCl (pH 7.2), 10 mM EDTA and 1 mg of protein from isolated mitochondria [41]. Incubations were carried out for 5 min at 30 °C and terminated by the addition of 4 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, Deventer, The Netherlands) with 5% water as scintillation mixture.

3. RESULTS

3.1 The Psd1p α-subunit contains a highly conserved motif

Multiple protein sequences alignment of the yeast Psd1p α-subunit (*S. cerevisiae*) with its homologues from bacteria (*E. coli*), plant (*A. thaliana*), mouse (*Mus musculus*) and human (*Homo sapiens)* revealed two regions of high conservation (Fig. 2). First, the LGST motif (MGST in *A. thaliana*) is present in all analyzed sequences as expected [24,28,29,32,42]. In addition, we found that a conserved motif, previously identified only in Psd1 from hamster and yeast Psd1p as putative PS binding region [33], is present in other eukaryotic Psd1 proteins too. Even though the original putative substrate-binding motif is absent in Psd1 from *E. coli*, some residues within the

sequence are nevertheless highly conserved [33] (Fig. 2A). Interestingly, not only the amino acids of this originally described substrate recognition site (SRS) of all the aligned Psd1 proteins are conserved, but also flanking residues at the C-terminal side of this domain (sequence from Gly-488 to Gly-492). To analyze the function of this region in detail, we generated several yeast variants of Psd1p. In Psd1ΔSRS the reported PS-binding site was removed, whereas in Psd1ΔSRS4, Psd1ΔSRS3, Psd1ΔSRS2, Psd1ΔDK and Psd1ΔLG distinct short portions of the conserved sequence were deleted (Fig. 2B). In a second set of Psd1p mutants conserved glycine residues were substituted by prolines (Psd1G482P, Psd1G488P, Psd1G492P). Finally, we created two mutant forms of Psd1p in which either the most conserved Lys-486, or all positively charged amino acids of this motif were substituted by alanine (Psd1AX3, Psd1K486A; Fig. 2B).

Figure 2

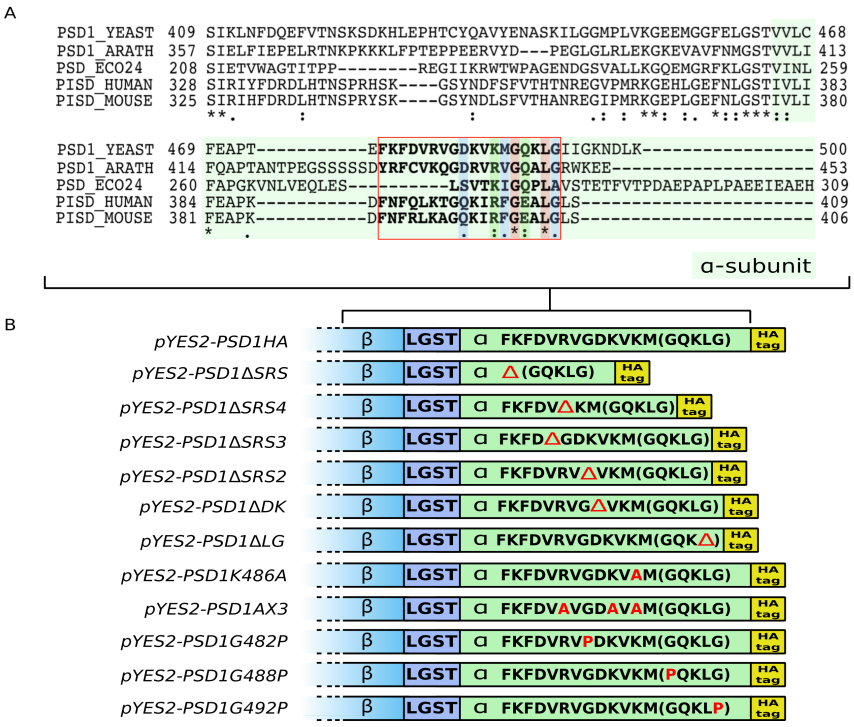

Figure 2. Conserved motives of the Psd1p α-subunit. (A) The sequence alignment of the Psd1p α-subunit with flanking regions of the β-subunit, which includes the highly conserved cleavage site LGST, is shown for the following organisms: *Saccharomyces cerevisiae* (YEAST), *Arabidopsis thaliana* (ARATH), *Escherichia coli* (ECO24), *Homo sapiens* (HUMAN) and *Mus musculus* (MOUSE). A newly identified conserved PS binding motif is highlighted by a red box. The conserved amino acids are marked: * fully conserved residue; : amino acids with strongly similar properties; . amino acids with weakly similar properties. (B) The Psd1p constructs used in this study are schematically illustrated. The different mutations introduced in the sequence are highlighted in red.

3.2 The putative PS binding site of the Psd1p α-subunit is crucial for cell growth

To analyze the role of the newly determined conserved motif of the Psd1 α-subunit, we studied the growth phenotype of the respective mutant strains (Fig. 2B) on minimal media with galactose as a carbon source. Deletion of *PSD1* results in PE depletion in mitochondria and in other cellular membranes, but growth is not affected as long as glucose is used as a carbon source [4,43]. Only if non-fermentable carbon sources such as glycerol or lactate serve as sole carbon source, mitochondrial functions, especially respiration, become more stringent and growth of the cells is impaired [4,6,44]. Even in the presence of galactose cell growth of *psd1*Δ is compromised. As shown in Figure 3A, yeast cells expressing the Psd1p variant lacking the entire putative substrate recognition sequence (Psd1ΔSRS) exhibited a growth phenotype similar to a *psd1*Δ strain on minimal medium containing galactose as carbon source. Even removal of three to six amino acids of the conserved motif compromised the function of Psd1p as reflected by impaired growth of the mutants Psd1ΔSRS4, Psd1ΔSRS3, Psd1ΔSRS2 (Fig. 3A). In contrast, deletion of two amino acids caused only a mild growth defect compared to positive control (Fig. 3A). Substitution of the three positively charged

amino acids present in the consensus motif, as well as the conserved Lys-486 hardly affected cell growth of the respective mutant strain (Psd1AX3, Psd1K486A; Fig. 3B). Similarly, mutants expressing Psd1p with glycine to proline replacements at position Gly-482 or Gly-492 showed no major growth defect (Psd1G482P, Psd1G492P; Fig. 3B). However, the point mutation G488P in Psd1p caused a dramatic growth defect of the respective mutant similar to that observed for *psd1*Δ bearing the empty vector (Psd1G488P; Fig. 3B).

Figure 3

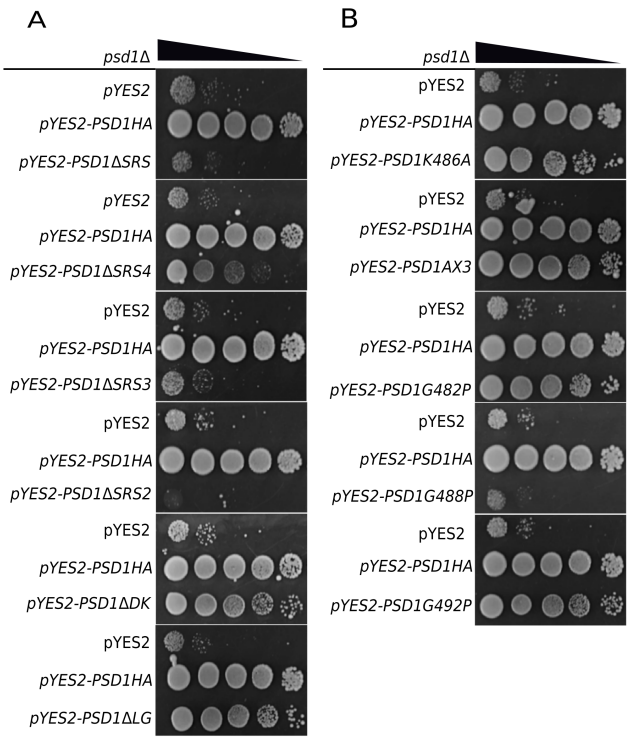

Figure 3. The putative substrate-binding motif is crucial for cell growth. (A) and (B) The indicated constructs were introduced into a *psd1*Δ strain. Cell growth at 30 °C was analyzed by serial dilution on selective media containing galactose as a carbon source.

3.3 Mutations in the putative PS binding motif of the Psd1p α-subunit affect maturation and lead to structural instability of Psd1p

It is already well known that yeast Psd1p is a mitochondrial enzyme embedded in the mitochondrial inner membrane [19]. Similar to the majority of proteins which reside in mitochondria Psd1p is synthesized as a precursor on cytosolic ribosomes. The 56 kDa large precursor undergoes three processing steps upon import into mitochondria, leading to a 4 kDa α-subunit and a 47 kDa β-subunit [19] (see Fig. 1).

As we observed that every mutation introduced into the conserved motif of the α-subunit of Psd1p compromised cell growth to some extent (see Fig. 3), we asked whether or not these mutations affected also the steady state level of the Psd1p subunits in mitochondria. To monitor both Psd1p subunits independently, we fused the α-subunit with a triple HA-tag at the C-terminus to allow immunodetection with an anti-HA antibody. For detection of the Psd1p β-subunit we used a specific antibody against this part of the polypeptide. The HA tagging did not affect maturation or function of Psd1p, as the $Psd1_{HA}$ behaved like wild type Psd1p [19]. The control strain expressing wild type Psd1p fused to an HA-tag and the mutant strains were grown to the early stationary phase. Then, mitochondria were isolated and analyzed for the presence of the Psd1p-subunits by immunoblotting with the specific antibodies.

Wild type $Psd1_{HA}$ was correctly processed leading to the formation of a β-subunit of 47 kDa and the α-subunit of 10 kDa (4 kDa mature α-subunit + HA-tag) [19] (Fig. 4A, a; 4B, a). Unexpectedly, neither the α-subunit nor the β-subunit was detected in mitochondria of the strain expressing Psd1ΔSRS lacking the entire consensus motif (Fig. 4A, b; 4B, b). This finding indicated that loss of the SRS dramatically affected the stability of the entire protein. Similarly, in Psd1ΔSRS4, Psd1ΔSRS3, and Psd1ΔSRS2

mitochondria, no mature α- and β-subunits were detected (Fig. 4A, c; d; e; 4B, c; d; e). However, in these strains, a prominent band of 35 kDa was observed instead. As the 35 kDa fragment of Psd1p was recognized by both antibodies this result revealed that parts of the two subunits were not autocatalitically cleaved. Noteworthy, whereas the antibody directed against the b-subunit revealed only a single 35 kDa band, the anti-HA antibody recognized several additional bands of lower molecular mass. This observation suggested that Psd1p was degraded from the N-terminus, because the HA-tag is located to the C-terminus, whereas the peptide-epitope recognized by the Psd1p specific antibody is located within the first 240 amino acids of the protein. Accordingly, only the HA-specific antibody detected fragments of smaller size than 35 kDa. In addition, it has to be mentioned that the anti HA-tag antibody was more sensitive than the one recognizing the β-subunit, as it also detected the unprocessed Psd1p precursor form of 56 kDa, as well as other sizes between 35 and 56 kDa.

In contrast to the Psd1p mutant forms described above, we observed that in the mutant Psd1ΔDK both Psd1p subunits were only partly destabilized but the autocatalytic cleavage was largely impaired (Fig. 4A, f; 4B, f). Western blot analysis revealed that the α- and the β-subunit of Psd1ΔLG were more effectively separated than of Psd1ΔDK, but still not fully processed, because the 56 kDa band of unprocessed Psd1p was still detected (Fig. 4A, g; 4B, g). Substitutions of the positively charged amino acids by alanine (Psd1K486A and Psd1AX3) reduced stability and processing of Psd1p moderately (Fig. 4A, h; i; 4B, h; i). Finally, individual replacements of the conserved glycine residues affected Psd1p proteins differentially. In the Psd1G482P mutant, processing and stability were mildly impaired, while no mature β-subunit was detected for Psd1G492P. Strikingly, the replacement of Gly-488 by proline caused a severe loss of protein stability (Fig. 4A, j; k; l; 4B, j; k; l). The absence of an

intact α- and β-subunit pointed to an impaired autocatalytic processing of Psd1G488P (Fig. 4A, l; 4B, l). The pattern observed for this latter mutant form of Psd1p is similar to that of Psd1p mutants lacking 3 to 6 amino acids of SRS (Fig. 4A, c; d; e; 4B, c; d; e). These data suggest that Gly-488 of the conserved flanking region is crucial for cleavage of the α- and β-subuni and for the stability of the entire protein.

Figure 4

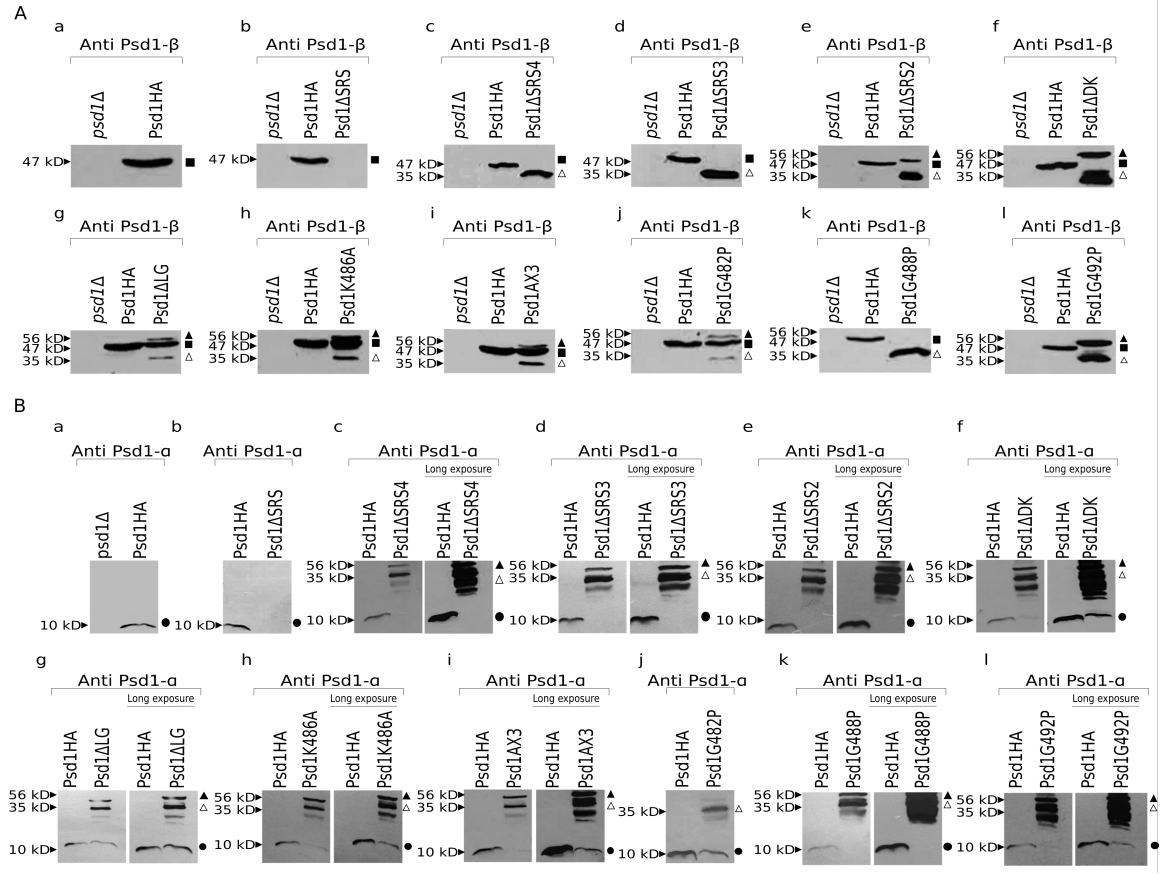

Figure 4. The putative PS binding domain in the α-subunit is crucial for stability and maturation of Psd1p. Mitochondria were isolated from the indicated yeast strains and an equal amount of 20 µg mitochondrial protein were analyzed by SDS-PAGE and immunodetection. (A) The detection of the Psd1p β-subunit was performed using an antibody specific for the Psd1 β-subunits. (B) An anti-HA-specific antibody was used to detect the C-terminally HA-tagged Psd1 α-subunit.

▲, non-cleaved form of Psd1p, in which α- and β-subunits are not separared. ■, mature Psd1p β-subunit. Δ, non-cleaved Psd1p fragment of 35 kDa ●, mature Psd1p α-subunit fused to the HA-tag.

Based on our Western blot studies we hypothesized that mutations in the conserved motif of the α-subunit affect the autocatalytic processing of Psd1p. To test this idea directly, we performed *in vitro* import of the different Psd1p mutant proteins. To this end, [^{35}S]Psd1p was incubated with isolated wild type yeast mitochondria. Several maturation steps can be monitored by SDS-PAGE and autoradiography. First, upon import the wild type Psd1p precursor is processed by MPP and Oct1p leading to the formation of two intermediates of 51 and 50 kDa [19,26,45]. Second, autocatalytic cleavage of the 4 kDa α-subunit can be detected by the formation of a mature β-subunit of 47 kDa. Finally, the import of Psd1p can be controlled by depletion of the membrane potential, which results in the accumulation of the 56 kDa Psd1p precursor. All imported intermediate and mature forms are protease resistant, whereas the precursor form is sensitive to added proteinase K.

Precursors of Psd1p deletion mutants of the putative substrate recognition site (Psd1ΔSRS, Psd1ΔSRS4, Psd1ΔSRS3 and Psd1ΔSRS2) as well as Psd1G488P were imported, albeit with slightly reduced efficiency (Fig. 5 B; C; D; E; K). In all these cases removal of the presequence occurred, but the autocatalytic processing was blocked (see Fig. 4). Remarkably, the imported proteins were rapidly degraded as indicated by the decreasing signal intensity upon longer import times. Thus, the mutations affected the stability of Psd1p but did not compromise the import into mitochondria. Interestingly, imported precursors of Psd1ΔLG, Psd1ΔDK, Psd1G482P and Psd1G492P formed intermediate stages (Fig. 5 F; G; L), but removal of the a-subunit was delayed (see Fig. 4). In contrast to the precursors of deletion mutants and Psd1G488P, the

intermediate stages of these mutant forms accumulated over time indicating that these proteins were not degraded. Finally, Psd1K486A and Psd1AX3 were imported and processed, although the levels of the a-subunit were reduced in our Western blot studies presented above (Fig. 5 H; I; J; see Fig. 4). Thus, various effects of the Psd1p mutations can be distinguished: First, removal of the entire conserved motif or three to five amino acids blocks autocatalytic processing and destabilizes the protein. Because the biogenesis of Psd1G488P was similarly affected, this conserved residue flanking the core of the conserved motif in the α-subunit is critical for maturation of Psd1p. Second, Psd1ΔLG, Psd1ΔDK and Psd1G492P are impaired in separation of the α- and β-subunit, but not generally destabilized. Third, import and processing of Psd1K486A, Psd1AX3 and Psd1G482P are largely comparable to wild type Psd1p.

Figure 5

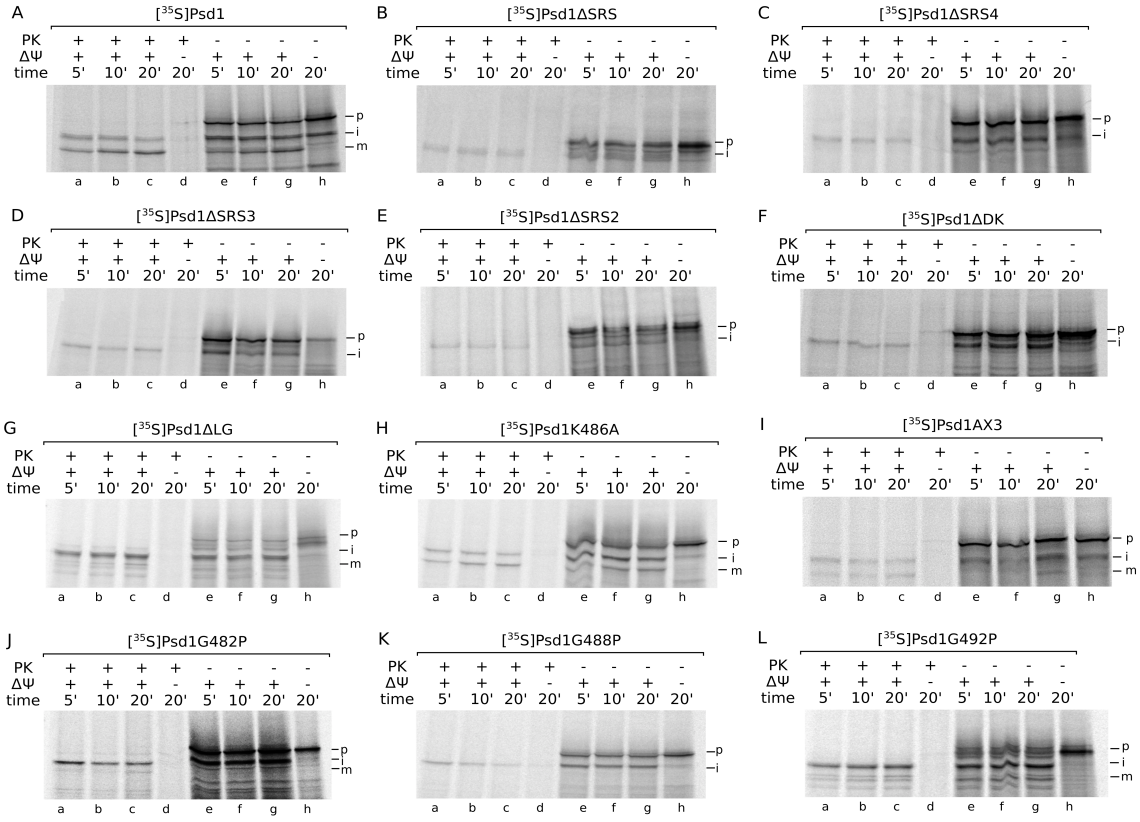

Figure 5. The conserved motif in the α-subunit is crucial for autocatalytic processing of Psd1p. ^{35}S-labeled precursors of the indicated Psd1p constructs were imported for the indicated time into wild type mitochondria in the presence or absence of a membrane potential (ΔΨ). Non-imported Psd1p precursors were removed by incubation with proteinase K (PK). The import of ^{35}S-labeled proteins were analysed by SDS-PAGE and autoradiography. p, Psd1p precursor protein; i, intermediate forms of Psd1p after processing by MPP and Oct1p; m, mature form of Psd1p after autocatalytic processing.

3.4 Mutations of the Psd1p α-subunit conserved motif affect the activity of Psd1p

We asked whether the introduced mutations affect the enzymatic activity of Psd1p. To address this question we performed two types of experiments. On the one hand, we determined the phospholipid composition of isolated mutant mitochondria, since a reduced Psd1p function is reflected by a decreased PE content [46–48]. On the other hand, we measured the activity of Psd1p *in vitro*. To this end, radiolabelled [^{3}H]PS was added to mitochondria and its conversion to radiolabelled [^{3}H]PE was measured. In mutant mitochondria with a largely destabilized Psd1p the phospholipid composition resembled that of *psd1*Δ mitochondria; the PE level was low whereas the relative amounts of PS and PC were increased (Fig. 6, A.1). Psd1p activity was not detected with mitochondria isolated from these mutants (Fig. 6 B.1). Psd1ΔDK and Psd1G492P showed reduced enzyme activity and PE content, which is in accordance with the decreased amount of the a-subunit (Fig. 6, A.2; 6, B.2). Interestingly, the mutant form Psd1ΔLG showed a reduced activity (Fig. 6, A.2; 6, B.2) although its processing and stability was only mildly affected (see Fig. 4). With mutants Psd1K486A and Psd1AX3 we tested whether positive charges of the consensus motif play an important role for binding of the negatively charged substrate PS and therefore for the activity of Psd1p [33,49]. Indeed, both mutants showed reduced Psd1p activity and a slightly decreased PE content (Fig. 6, A.3; 6, B.3). However, the stability and autoprocessing of these mutant forms of Psd1p were also clearly affected (see Fig. 4A and 4B, h; i). Therefore, the loss of activity could be rather caused by the reduced amount of Psd1p α-subunit than by a defect in substrate binding.

We conclude that we have two different types of Psd1p mutants: One set of mutations affects stability and biogenesis of Psd1p, whereas in a second set of mutants

primarily the activity of Psd1p is impaired. The loss of activity seems to be mainly related to the amount of Psd1p α-subunit detected. In fact, there is a correlation between the absence of a detectable signal corresponding to the α-subunit and the overall catalytic activity of the enzyme. Furthermore, defects in the maturation of Psd1p lead generally to lower levels of the Psd1p α-subunit with repercussions on Psd1p activity. Exceptions are the Psd1p mutants Psd1ΔLG and Psd1G482P. In these mutants the level of the α-subunits are comparable (Fig. 4B g; j), but the activity is more strongly affected in Psd1ΔLG than in Psd1G482P (Fig. 6 B.2; B.4). Therefore, it seems that some residues of the conserved motif play a dual role by affecting the stability and biogenesis of Psd1p on the one hand, and on the other hand by directly influencing its activity.

In summary, our data clearly show that the conserved motif of the α-subunit plays a crucial role for biogenesis and stability of Psd1p, which as a consequence affects the catalytic activity of the enzyme.

Figure 6

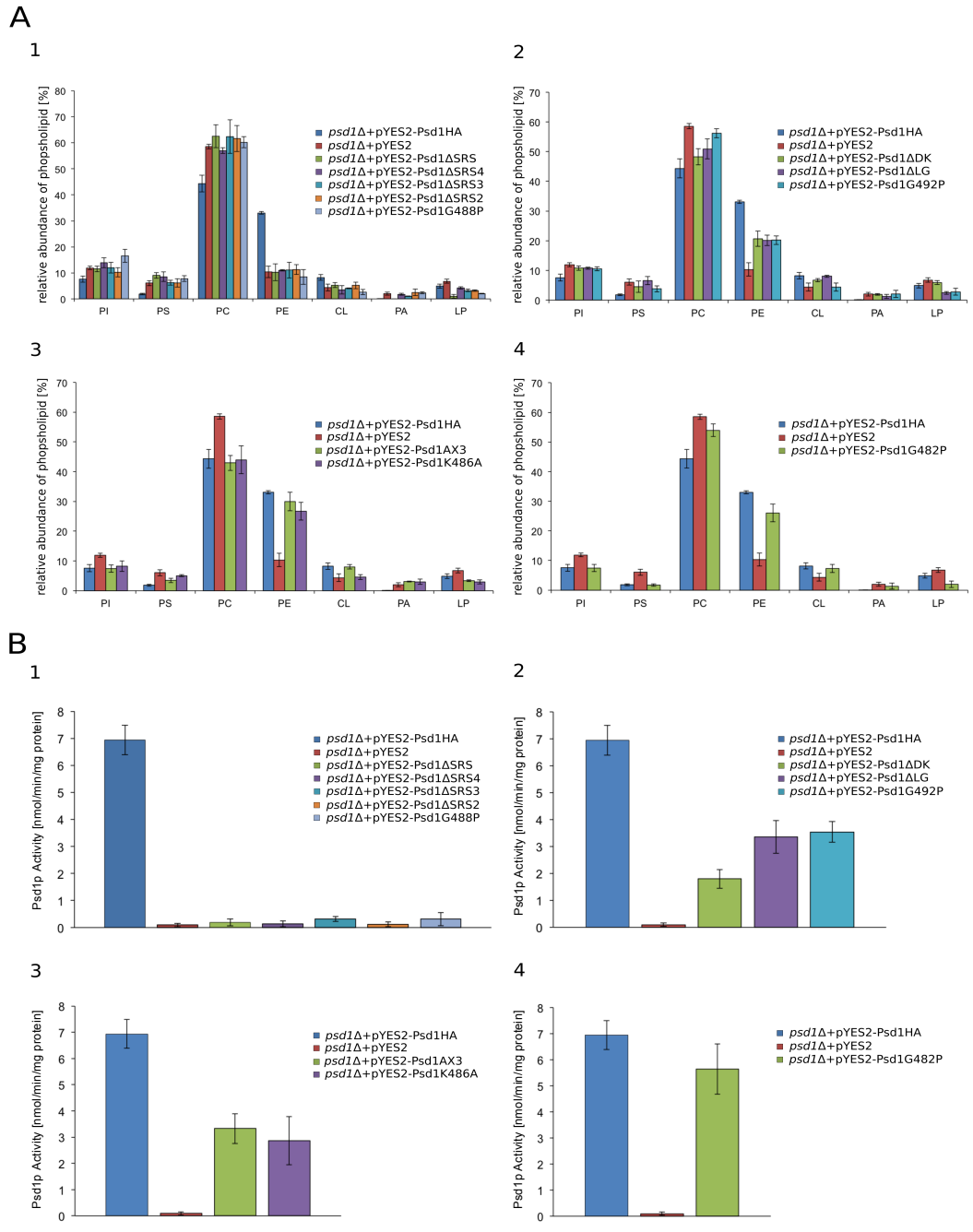

Figure 6. Studies on the enzymatic activity of Psd1p mutant forms (A.1–4) Mitochondria were isolated from the indicated yeast strains grown on selective medium

containing galactose as carbon source. Lipids were extracted and individual phospholipids were separated by thin-layer chromatography and quantified as described (see section 2.4). The data represent the mean ± SD of three independent experiments. LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. (B.1–4) The activity of phosphatidylserine decarboxylase was measured *in vitro* by using isolated mitochondria from the indicated Psd1p mutant strains as enzyme source (see section 2.5). The conversion of [^{3}H]PS to [^{3}H]PE was determined. The data represent the mean ± SD of three independent experiments.

4. DISCUSSION

In *Saccharomyces cerevisiae*, mitochondrial Psd1p plays a pivotal role by providing to the cell the majority of PE produced by decarboxylation of PS [34,50–52]. The biogenesis of Psd1p includes several steps of maturation ultimately leading to the formation of an α- and β-subunit [19]. The interaction of both subunits in the mitochondrial intermembrane space is prerequisite for the ability of Psd1p to decarboxylate the polar head group of PS yielding PE [53] (see Fig. 1).

Identification of a conserved motif within the Psd1p α-subunit was performed using a monoclonal anti-idiotypic antibody, 1d8F7, which specifically identifies PS-binding sites *in vitro* [33]. This experiment led to the speculation that this subunit harbors an important and essential part of the active domain of Psd1p. However, the effective role of the conserved motif of the Psd1p α-subunit for the activity and the functionality of the whole enzyme was never explored in detail.

The present study clearly demonstrates that the conserved motif, which was initially termed *putative PS-specific binding motif* [33], plays indeed a critical role for the enzymatic activity of Psd1p. Unexpectedly, however the mutations introduced in this

conserved motif strongly compromised the stability and processing of Psd1p which as a consequence affected the enzyme's activity. Most interestingly, the degree of Psd1p stability and its functionality strongly depended on the amino acid(s) that have been deleted or substituted.

Among the Psd1p mutant forms we tested, deletion of the reported SRS of the conserved motif within the α-subunit had the strongest impact on the functionality of Psd1p. The respective protein Psd1ΔSRS turned out to be highly unstable, as the protein amount imported *in vitro* into mitochondria declined upon longer incubation time, and the size of the imported Psd1p fragment did neither correspond to the mature Psd1p β-subunit nor to any of the intermediate forms (see Fig. 5 B). These data are in line with the results obtained from the Western blot analysis, where any Psd1p form was absent in mitochondria isolated from cells expressing *PSD1ΔSRS* (see Fig. 4A, b; Fig. 4B, b), explaining why expression of *PSD1ΔSRS* did not rescue cell growth of a *psd1Δ* mutant.

In contrast to Psd1ΔSRS, all other mutant forms investigated by this study were detected in isolated mitochondria (see Fig. 4A; 4B). However, both Psd1p-specific antibodies detected numerous bands in these mutant mitochondria. These latter finding indicated that any manipulation in the conserved motif of the α-subunit affected the stability of Psd1p. Although all Psd1p mutant forms were subject to degradation, the mutations affected the stability of Psd1p differently (see Figs 4A; 4B; 5). The strongest impact on the stability of Psd1p had the replacement of a conserved glycine at position 488 (Psd1G488P), and deletion of 3 to 6 amino acids in the core of the conserved motif of the α-subunit (Psd1ΔSRS4, Psd1ΔSRS3, Psd1ΔSRS2) (see Fig. 2). In addition, these mutations impeded the autocatalytic cleavage of the Psd1p precursor protein (see Figs 4A; 4B; 5). As separation of the a- and β-subunit is prerequisite for the

enzymatic activity of the decarboxylase, it was not surprising that these Psd1p constructs: (i) failed to rescue cell growth of a *psd1*Δ strain on media lacking a fermentable carbon source; (ii) did not contribute to the mitochondria PE level; and (iii) were catalytically dead when tested *in vitro* (see Figs 3; 6, A.1; B.1). Noteworthy, a defect in the autocatalytic processing and a strongly compromised stability of a Psd1p mutant form are not necessarily linked to each other, because a mutation in the conserved LGST motif of Psd1p, which similarly blocks separation of the α- and β-subunit, did not affect the stability of the respective Psd1p mutant form [19].

While deletion of 3 amino acids of the SRS of the α -subunit rendered Psd1p completely instable, deletion of only two amino acids in this region affected the stability of Psd1p to a lesser extent (see Figs 2; 4A; 4B; 5). The amount of the *in vitro* imported Psd1p-variant Psd1ΔDK remained constant over time. In contrast, the signal of Psd1ΔLG imported into isolated mitochondria slightly increased during the total incubation time (see Fig. 5 F; G). The higher stability of the latter construct was similarly reflected by a limited number of protein bands detected by Psd1p-specific antibodies (see Figs 4A, g; 4B, g). However, the more striking difference between these two constructs was the impact of the two-amino acids-deletion on the autocatalytic processing. Deletion of Asp-483 and Lys-484, which are localized central in the conserved motif of the α-subunit, strongly compromised self-processing of the respective Psd1p-variant *in vitro* as well as *in vivo* (see Figs 4A; 4B; 5). By contrast, Psd1ΔLG lacking Leu-491 and Gly-492 located at the extreme C-terminus of the conserved motif, formed at steady state level an amount of the α- and β-subunit which was comparable to that of wild type Psd1p (see Figs 4A, g; 4B, g). However, in the *in vitro* assay following the processing and import of Psd1ΔLG only over 20 minutes a prominent band corresponding to mature Psd1p was lacking (see Fig. 5 G). Therefore,

it seems that lack of the amino acids Leu-491 and Gly-492 mainly affects the autocatalytic processing by reducing the processing rate but not the processing itself. Deletion of the two more central amino acids Asp-483 and Lys-484 in Psd1ΔDK had a stronger impact than deletion of Leu-491 and Gly-492 (in Psd1ΔLG) at the C-terminal end of the conserved motif (see Figs 2 to 6). However, when testing the impact of individual glycine to proline replacements within the conserved motif we observed a totally different effect. Substitution of Gly-482 adjacent to the central amino acids deleted in Psd1ΔDK hardly affected stability and functionality of Psd1p (see Figs 3B; 4A, j; 4B, j; 5, J; 6, A.4; 6, B.4). By contrast, replacement of Gly-488 caused complete instability of the polypeptide; and substitution of the very last conserved glycine of the motif at position 492 also markedly disturbed the stability of Psd1p, but at a lower extent than the G488P exchange (see Figs 3B; 4A, k; l; 4B, k; l; 5, K; L; 6, A.1; 6, A.3; 6, B.1; 6, B.3). Therefore, single point mutations in the conserved motif of the α-subunit similarly affect the stability and thus functionality of Psd1p.

The highly conserved motif in the α-subunit of Psd1p was previously predicted to function in the binding of the enzyme's substrate PS [33]. As the head group of PS is negatively charged, we examined whether the proposed interaction of protein and substrate involves positively charged amino acids in the conserved motif of the α-subunit. Indeed, replacement of positively charged amino acid(s) (see Fig. 2) affected the enzyme activity of the respective Psd1p-variants (see Fig. 6 A.3; B.3). But as the respective mutation(s) reduced the amount of Psd1p α- and β-subunit to a similar extent as the activity, the specific activity of native and mutated Psd1p remained comparable (see Fig. 4A, h; i; 4B, h; i). Thus, positively charged amino acids in the conserved α-subunit of Psd1p do most likely not play a major role in binding of the substrate PS.

In summary, our data revealed that any manipulation in the conserved motif of the α-subunit compromises the stability of Psd1p. A stronger impact of Psd1p sequence manipulation on the stability of the PS decarboxylase results in a lower amount of the mature enzyme, i.e., reduced amount of α- and β-subunit. This finding suggests a competition between the machinery degrading a mutated Psd1p precursor protein and the self-processing of the enzyme. As soon as the α- and β-subunit are separated, the protein seems to be quite stable as concluded from the fact that these subunits were detected at steady state level (see Fig. 4). Interaction of the α- and β-subunit is mandatory for enzymatic catalysis [20,31,53]. As the amount of mature α-subunit (and β-subunit) clearly correlated with the enyzme activity of Psd1p, it seems that manipulations in the conserved motif of the α-subunit do not affect the interaction of the subunits. Furthermore, this latter observation questions a direct function of the conserved motif in catalysis. Therefore, although the structure of the α-subunit has not been resolved yet, our data strongly suggest that the main function of the highly conserved motif in the α-subunit is the proper positioning of amino acids which are involved in the autocatalytic processing.

Conflict of interest: The authors declare that there is no conflict of interest.

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REFERENCES:

[1] B. Mesmin, Mitochondrial lipid transport and biosynthesis: a complex balance, J. Cell Biol. 214 (2016) 9–11. doi:10.1083/jcb.201606069.

[2] E.M. Mejia, G.M. Hatch, Mitochondrial phospholipids: role in mitochondrial function, J. Bioenerg. Biomembr. 48 (2015) 99–112. doi:10.1007/s10863-015-9601-4.

[3] C.U. Mårtensson, K.N. Doan, T. Becker, Effects of lipids on mitochondrial functions, Biochim. Biophys. Acta (2016). In press. doi:10.1016/j.bbalip.2016.06.015.

[4] R. Birner, M. Bürgermeister, R. Schneiter, G. Daum, Roles of phosphatidylethanolamine and of its several biosynthetic pathways in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 997–1007. doi: 10.1091/mbc.12.4.997.

[5] P. Griac, Regulation of yeast phospholipid biosynthetic genes in phosphatidylserine decarboxylase mutants, J. Bacteriol. 179 (1997) 5843–5848.

[6] L. Böttinger, S.E. Horvath, T. Kleinschroth, C. Hunte, G. Daum, N. Pfanner, T. Becker, Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes, J. Mol. Biol. 423 (2012) 677–686. doi:10.1016/j.jmb.2012.09.001.

[7] G. Tasseva, H.D. Bai, M. Davidescu, A. Haromy, E. Michelakis, J.E. Vance, Phosphatidylethanolamine deficiency in mammalian mitochondria impairs oxidative phosphorylation and alters mitochondrial morphology, J. Biol. Chem. 288 (2013) 4158–4173. doi:10.1074/jbc.M112.434183.

[8] L. Böttinger, L. Ellenrieder, T. Becker, How lipids modulate mitochondrial protein import, J. Bioenerg. Biomembr. 48 (2015) 125–135. doi:10.1007/s10863-015-9599-7.

[9] T. Becker, S.E. Horvath, L. Böttinger, N. Gebert, G. Daum, N. Pfanner, Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins, J. Biol. Chem. 288 (2013) 16451–16459. doi:10.1074/jbc.M112.442392.

[10] J. Dudek, P. Rehling, M. van der Laan, Mitochondrial protein import: common principles and physiological networks, Biochim. Biophys. Acta 1833 (2013) 274–285. doi:10.1016/j.bbamcr.2012.05.028.

[11] W. Neupert, J.M. Herrmann, Translocation of proteins into mitochondria, Ann. Rev. Biochem. 76 (2007) 723–749. doi: 10.1146/annurev.biochem.76.052705.163409.

[12] T. Shiota, H. Mabuchi, S. Tanaka-Yamano, K. Yamano, T. Endo, *In vivo* protein-interaction mapping of a mitochondrial translocator protein Tom22 at work, Proc. Natl. Acad. Sci. 37 (2011) 15179–15183. doi: 10.1073/pnas.1105921108.

[13] V. Hewitt, F. Alcock, T. Lithgow, Minor modifications and major adaptations: the evolution of molecular machines driving mitochondrial protein import, Biochim. Biophys. Acta 1808 (2011) 947–954. doi: 10.1016/j.bbamem.2010.07.019.

[14] R. Nebauer, R. Birner-Grünberger, G. Daum, Biogenesis and cellular dynamics of glycerophospholipids in the yeast *Saccharomyces cerevisiae*, Lipid Metab. Membr. Biog. 6 (2004) 125–168. doi: 10.1007/978-3-540-40999-1_4.

[15] G. Daum, N.D. Lees, M. Bard, R. Dickson, Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*, Yeast 14 (1998) 1471–1510. doi: 10.1002/(SICI)1097-0061(199812)14:16<1471::AID-YEA353>3.0.CO;2-Y.

[16] E.A. Dennis, E.P. Kennedy, Intracellular sites of lipid synthesis and the biogenesis of mitochondria, J. Lipid Res. 13 (1972) 263–267.

[17] D.R. Voelker, Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells, Proc. Natl. Acad. Sci. USA 81 (1984) 2669–2673.

[18] M. Bürgermeister, R. Birner-Grünberger, M. Heyn, G. Daum, Contribution of different biosynthetic pathways to species selectivity of aminoglycerophospholipids assembled into mitochondrial membranes of the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1686 (2004) 148–160. doi:10.1016/j.bbalip.2004.09.005.

[19] S.E. Horvath, L. Böttinger, F.N. Vögtle, N. Wiedemann, C. Meisinger, T. Becker, G. Daum, Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1, J. Biol. Chem. 287 (2012) 36744–36755. doi:10.1074/jbc.M112.398107.

[20] F. Di Bartolomeo, A. Wagner, G. Daum, Cell biology, physiology and enzymology of phosphatidylserine decarboxylase, Biochim. Biophys. Acta (2016). In press. doi:10.1016/j.bbalip.2016.09.007.

[21] M.J. Aaltonen, J.R. Friedman, C. Osman, B. Salin, J. di Rago, J. Nunnari, T. Langer, T. Tatsuta, MICOS and phospholipid transfer by Ups2–Mdm35 organize membrane lipid synthesis in mitochondria, J. Cell Biol. 213 (2016) 525–534. doi: 10.1083/jcb.201602007.

[22] R. Steenbergen, T.S. Nanowski, A. Beigneux, A. Kulinski, S.G. Young, J.E. Vance, Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects, J. Biol. Chem. 280 (2005) 40032–40040. doi:10.1074/jbc.M506510200.

[23] D. Stojanovski, M. Bohnert, N. Pfanner, M. van der Laan, Mechanisms of protein sorting in mitochondria, Cold Spring Harb. Perspect. Biol. 4 (2012) a011320. doi:10.1101/cshperspect.a011320.

[24] O. Kuge, K. Saito, M. Kojima, Y. Akamatsu, M. Nishijima, Post-translational processing of the phosphatidylserine decarboxylase gene product in Chinese hamster ovary cells, Biochem. J. 319 (1996) 33–38.

[25] J. Zborowski, A. Dygas, L. Wojtczak, Phosphatidylserine decarboxylase is located on the external side of the inner mitochondrial membrane, FEBS Lett. 157 (1983) 179–182. doi:10.1016/0014-5793(83)81141-7.

[26] A. Geissler, T. Krimmer, U. Bömer, B. Guiard, J. Rassow, N. Pfanner, Membrane potential-driven protein import into mitochondria, Mol. Biol. Cell 11 (2000) 3977–3991. doi: 10.1091/mbc.11.11.3977.

[27] W.R. Riekhof, J. Wu, J.L. Jones, D.R. Voelker, Identification and characterization of the major lysophosphatidylethanolamine acyltransferase in *Saccharomyces cerevisiae*, J. Biol. Chem. 282 (2007) 28344–28352. doi:10.1074/jbc.M705256200.

[28] Q.X. Li, W. Dowhan, Studies on the mechanism of formation of the pyruvate prosthetic group of phosphatidylserine decarboxylase from *Escherichia coli*, J. Biol. Chem. 265 (1990) 4111–4115.

[29] J.Y. Choi, M.T. Duraisingh, M. Marti, C. Ben Mamoun, D.R. Voelker, From protease to decarboxylase: the molecular metamorphosis of phosphatidylserine decarboxylase, J. Biol. Chem. 290 (2015) 10972–10980. doi:10.1074/jbc.M115.642413.

[30] M. Satre, E.P. Kennedy, Identification of bound pyruvate essential for the activity of phosphatidylserine decarboxylase of *Escherichia coli*, J. Biol. Chem. 253 (1978) 479–483.

[31] Q.X. Li, W. Dowhan, Structural characterization of *Escherichia coli* phosphatidylserine decarboxylase, J. Biol. Chem. 263 (1988) 11516–11522.

[32] O. Onguka, E. Calzada, O.B. Ogunbona, S.M. Claypool, Phosphatidylserine decarboxylase 1 autocatalysis and function does not require a mitochondrial-specific factor, J. Biol. Chem. 290 (2015) 12744–12752. doi:10.1074/jbc.M115.641118.

[33] K. Igarashi, M. Kaneda, A. Yamaji, T.C. Saido, U. Kikkawa, Y. Ono, K. Inoue, M. Umeda, 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 (1995) 29075–29078. doi: 10.1074/jbc.270.49.29075.

[34] I. Schuiki, G. Daum, Phosphatidylserine decarboxylases, key enzymes of lipid metabolism, IUBMB Life 61 (2009) 151–162. doi:10.1002/iub.159.

[35] D. Gietz, A. St Jean, R.A. Woods, R.H. Schiestl, Improved method for high efficiency transformation of intact yeast cells, Nucleic Acids Res. 20 (1992) 1425.

[36] E. Zinser, G. Daum, Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*, Yeast 11 (1995) 493–536. doi:10.1002/yea.320110602.

[37] L.S. Wenz, L. Ellenrieder, J. Qiu, M. Bohnert, N. Zufall, M. van der Laan, N. Pfanner, N. Wiedemann, T. Becker, Sam37 is crucial for formation of the mitochondrial TOM-SAM supercomplex, thereby promoting b-barrel biogenesis, J. Cell Biol. 210 (2015) 1047–1054. doi:10.1083/jcb.201504119.

[38] A. Haid, M. Suissa, Immunochemical identification of membrane proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Methods Enzymol. 96 (1983) 192–205.

[39] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.

[40] R.M. Broekhuyse, 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 (1968) 307–315.

[41] K. Kuchler, G. Daum, F. Paltauf, Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae*, J. Bacteriol. 165 (1986) 901–910.

[42] D. Rontein, W.I. Wu, D.R. Voelker, A.D. Hanson, Mitochondrial phosphatidylserine decarboxylase from higher plants. Functional complementation in yeast, localization in plants, and overexpression in Arabidopsis, Plant Physiol. 132 (2003) 1678–1687. doi:10.1104/pp.103.023242.

[43] R. Birner, R. Nebauer, R. Schneiter, G. Daum, Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine biosynthetic machinery with the prohibitin complex of *Saccharomyces cerevisiae*, Mol. Biol. Cell 14 (2003) 370–383. doi:10.1091/mbc.E02-05-0263.

[44] C.D. Baker, W. Basu Ball, E.N. Pryce, V.M. Gohil, Specific requirements of nonbilayer phospholipids in mitochondrial respiratory chain function and formation. *Mol. Biol. Cell* 27 (2016) 2161–2171. doi: 10.1091/mbc.E15-12-0865.

[45] F.N. Vögtle, C. Prinz, J. Kellermann, F. Lottspeich, N. Pfanner, C. Meisinger, Mitochondrial protein turnover: role of the precursor intermediate peptidase Oct1

in protein stabilization, Mol. Biol. Cell 22 (2011) 2135–2143. doi:10.1091/mbc.E11-02-0169.

[46] M.A. Miller, C. Kent, Characterization of the pathways for phosphatidylethanolamine biosynthesis in Chinese hamster ovary mutant and parental cell lines, J. Biol. Chem. 261 (1986) 9753–9761.

[47] P.J. Trotter, J. Pedretti, D.R. Voelker, Phosphatidylserine decarboxylase from *Saccharomyces cerevisiae*. Isolation of mutants, cloning of the gene, and creation of a null allele, J. Biol. Chem. 268 (1993) 21416–21424.

[48] S.E. Horvath, G. Daum, Lipids of mitochondria, Prog. Lipid Res. 52 (2013) 590–614. doi:10.1016/j.plipres.2013.07.002.

[49] P.A. Leventis, S. Grinstein, The distribution and function of phosphatidylserine in cellular membranes, Annu. Rev. Biophys. 39 (2010) 407–427. doi:10.1146/annurev.biophys.093008.131234.

[50] O. Kuge, M. Nishijima, Y. Akamatsu, A cloned gene encoding phosphatidylserine decarboxylase complements the phosphatidylserine biosynthetic defect of a Chinese hamster ovary cell mutant, J. Biol. Chem. 266 (1991) 6370–6376.

[51] D.R. Voelker, Phosphatidylserine decarboxylase, Biochim. Biophys. Acta 1348 (1997) 236–244.

[52] A.G. Rietveld, M.C. Koorengevel, B. de Kruijff, Non-bilayer lipids are required for efficient protein transport across the plasma membrane of *Escherichia coli*, EMBO J. 14 (1995) 5506–5513.

[53] S.E. Horvath, A. Wagner, E. Steyrer, G. Daum, Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast *Saccharomyces cerevisiae*, Biochim. Biophys. Acta 1811 (2011) 1030–1037. doi:10.1016/j.bbalip.2011.08.007.

CHAPTER 3

Phosphatidylcholine affects the role of the sorting and assembly machinery in the biogenesis of mitochondrial β-barrel proteins

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Phosphatidylcholine affects the role of the sorting and assembly machinery in the biogenesis of mitochondrial β-barrel proteins

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Running Title: Biogenesis of mitochondria

Keywords: Mitochondria, protein import, SAM complex, TOM complex, phosphatidylethanolamine, phosphatidylcholine

Background: The role of phosphatidylcholine (PC) in the biogenesis of mitochondrial outer membrane proteins is unknown.

Results: PC is required for efficient biogenesis of b-barrel precursors.

Conclusion: Depletion of PC affects function and stability of the sorting and assembly machinery (SAM complex).

Significance: PC and phosphatidylethanolamine promote distinct steps of the import of β-barrel precursors.

ABSTRACT

Two protein translocases drive import of β-barrel precursors into the mitochondrial outer membrane: The outer membrane translocase (TOM complex) promotes transport of the precursor across the outer membrane, whereas the sorting and assembly machinery (SAM complex) mediates subsequent folding and insertion of the precursor into the target membrane. The biogenesis of β-barrel precursors is particularly sensitive towards changes of the phospholipid composition of the outer membrane. Phosphoethanolamine (PE) is required for the transport of the precursor across the TOM complex, whereas deletion of cardiolipin (CL) affects stability and function of both TOM and SAM complexes. PE and CL are both non-bilayer forming phospholipids. Whether bilayer-forming phospholipids such as phosphotidylcholine (PC), the most abundant phospholipid of the outer membrane, are important for the import of β-barrel precursors is unclear. In this study we showed that PC is required for stability and function of the SAM complex in the biogenesis of β-barrel precursors. PC further promotes the SAM-dependent assembly of the Tom22 precursor into the TOM complex, indicating a general role PC for the SAM complex. In contrast to PE-deficient mitochondria precursor accumulation at the TOM complex is not affected. Instead, We conclude that PC and PE are required for distinct steps of the biogenesis of mitochondrial β-barrel proteins.

INTRODUCTION

β-Barrel proteins are present in the outer membrane of gram-negative bacteria and cell organelles of endosymbiotic origin like mitochondria and plastids of plant cells (Schleiff and Soll, 2005; Walther et al., 2009; Webb et al., 2012; Höhr et al., 2015). In mitochondria β-barrel proteins fulfill essential functions. Tom40 forms the protein-conducting channel of the translocase of the outer membrane (TOM complex), which allows import of precursor proteins into mitochondria (Koehler et al., 2004; Baker et al., 2007; Neupert and Herrmann, 2007; Endo and Yamano, 2009; Becker et al., 2012). The voltage-dependent anion channel (VDAC; porin in yeast) transports metabolites across the outer membrane (Hiller et al., 2010; Columbini et al., 2012). The mitochondrial distribution and morphology protein Mdm10 is a core component of the ER-mitochondria encounter structure, which forms a molecular bridge between mitochondria and the endoplasmic reticulum (ER) (Osman et al., 2011; Toulmay and Prinz, 2011; Lang et al., 2015). Finally, Sam50 is the core subunit of the sorting and assembly machinery (SAM complex, also termed TOB complex for topogenesis of mitochondrial outer membrane β-barrel proteins), which is crucial for the biogenesis of several outer membrane proteins (Endo and Yamano, 2010; Dukanovic and Rapaport, 2011; Webb et al., 2012; Becker et al., 2012; Höhr et al., 2015). All β-barrel proteins are synthesized on cytosolic ribosomes as precursors and transported to the TOM receptors Tom20, Tom22 and Tom70 on the mitochondrial surface (Krimmer et al., 2001; Habib et al., 2005; Yamano et al., 2008). After passage of the outer membrane via the Tom40 channel the precursors are delivered to the SAM complex (Wiedemann et al., 2003; Paschen et al., 2003). This transfer step is promoted by two different mechanisms. First, the TOM and SAM complexes form a supercomplex to promote

efficient substrate channeling (Qiu et al., 2013). Second, small TIM chaperones of the intermembrane space bind to the precursor, probably to shield hydrophobic patches (Wiedemann et al., 2004; Hoppins and Nargang, 2004; Qiu et al., 2013). The SAM complex mediates folding and membrane integration of the β-barrel precursor (Qiu et al., 2013). The protein machinery consists of three subunits: The β-barrel forming Sam50 (Tob55, Omp85) is the central components of the SAM complex and conserved from bacteria to humans (Paschen et al., 2003; Kozjak et al., 2003; Gentle et al., 2004; Ishikawa et al., 2004). It associates with the peripheral Sam35 (Tob38, Tom38) and Sam37 (Mas37, Tom37) (Klein et al., 2012). Sam35 is crucial for initial binding of a conserved β-signal within the last β-strand of the incoming precursor (Kutik et al., 2008; Chan and Lithgow, 2008). Sam37 was reported to mediate the release of the substrate proteins from the SAM complex and is crucial for the stability of the protein translocase (Wiedemann et al., 2003; Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010; Lackey et al., 2011). Sam50 and Sam35 are essential for life, indicating the central role of β-barrel biogenesis for mitochondrial function (Kozjak et al., 2003; Paschen et al., 2003; Ishikawa et al., 2004; Waizenegger et al., 2004; Milenkovic et al., 2004). Additionally, the SAM complex promotes the assembly of the β-barrel precursor Tom40 with the small Tom proteins and Tom22 to form the mature TOM complex (Dukanovic et al., 2009; Becker et al., 2010). In particular, a subpopulation of the SAM complex that additionally contains Mdm10 is important for this process (Meisinger et al., 2004; Stojanovski et al., 2007; Thornton et al., 2010; Yamano et al., 2010; Becker et al., 2011).

The main phospholipids of mitochondrial membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and

cardiolipin (CL). Mitochondria are crucial for the biosynthesis of CL and PE, which do not form lipid bilayers when isolated and are therefore termed non-bilayer forming phospholipids (van den Brink-van der Laan, et al., 2004). The biosynthesis pathway of CL is located within mitochondria (Osman et al., 2011; Horvath and Daum, 2013). Furthermore, the PS decarboxylase (Psd1) of the mitochondrial inner membrane produces the major amount of cellular PE in yeast cells (Clancey et al., 1993; Trotter et al., 1993; Osman et al., 2011; Horvath and Daum, 2013). Studies with yeast strains defective in CL or PE synthesis revealed various functions of non-bilayer phospholipids for mitochondrial biogenesis including mitochondrial fusion and respiration (Jiang et al., 2000; Pfeiffer et al., 2003; Zhang et al., 2005; Claypool et al., 2008; Gonzalvez et al., 2008; DeVay et al., 2009; Mileykovska and Dowhan, 2009; Kuroda et al., 2011; Joshi et al., 2012; Tasseva et al., 2013; Becker et al., 2013). In addition, CL and PE affect protein transport processes driven by membrane-bound protein translocases in both mitochondrial membranes (Jiang et al., 2000; Tamura et al., 2006; Gallas et al., 2006; van der Laan et al., 2007; Kutik et al., 2008; Gebert et al., 2009; Tamura et al., 2009; Tamura et al., 2012; Böttinger et al., 2012; Becker et al., 2013; Sauerwald et al., 2015). The decreased respiratory chain activity in CL and PE depleted mitochondria leads to a reduced membrane potential, which in turn impairs protein transport into and across the inner membrane (Jiang et al., 2000; Pfeiffer et al., 2003; Kutik et al., 2008; Böttinger et al., 2013; Tasseva et al., 2013). Surprisingly, studies on the role of CL and PE in the import of mitochondrial β-barrel proteins revealed distinct functions of both phospholipids. Although present in low amounts CL affects β-barrel biogenesis by stabilizing TOM and SAM complexes (Gebert et al., 2009). In contrast, depletion of PE affects β-barrel biogenesis at the stage of transport across the TOM complex, but does not lead to destabilization of TOM and SAM complexes (Becker et al., 2013).

Altogether, these studies revealed that non-bilayer phospholipids are important for several central processes of mitochondrial biogenesis and function.

Whereas the role of non-bilayer phospholipids for mitochondrial biogenesis was explored to some extend, the role of bilayer-forming phospholipids like PC in these processes was not investigated yet. PC is the most abundant phospholipids of mitochondrial membranes (Sperka-Gottlieb et al., 1988; Zinser, et al., 1991). Two pathways produce PC in the ER of yeast. First, enzymes of the CDP-choline pathway (Kennedy pathway) utilize free choline to generate PC (Cole et al., 2012; Daum and Horvath, 2013). Second, two PE methyltransferases (Pem1/Cho2 and Pem2/Opi3) perform a three-step methylation of PE using S-adenosylmethione as donor of methyl group. Pem1 catalyzes the first methylation step, while Pem2 is capable to promote all three steps, but the last two steps with higher efficiency (Greenberg et al., 1983; Kodaki and Yamashita, 1987; Summers et al., 1988; Kodaki and Yamashita, 1989; Gaynor and Carman, 1990; Preitschopf et al., 1993). The biosynthesis of PC is essential for the survival of the yeast cell (Kodaki and Yamashita, 1989). Mice deficient in PE methyltransferase developed non-alcoholic steatohepatitis when fed with choline-deficient diet (Li et al., 2006). In humans defects in the synthesis of PC were found in patients suffering congenital muscular dystrophy (Mitsuhashi et al., 2011). Interestingly, the patient cells contain mitochondria with abnormal morphology, pointing to a role of PC in mitochondrial biogenesis (Mitsuhashi et al., 2011). Since PC is the most abundant phospholipid it is important to build up the lipid bilayer of biological membranes. However, it is unclear whether PC might also be required for the function of specific membrane-integrated proteins.

We investigated the role of PC in the biogenesis of mitochondrial β-barrel proteins. Upon depletion of PC, the biogenesis of β-barrel proteins is severely impaired. PC is specifically required for stability and function of the SAM complex in β-barrel biogenesis as well as in the assembly of the TOM complex. In contrast to PE depleted mitochondria the precursor binding to the TOM complex was not impaired. We conclude that PC and PE are involved in different steps of b-barrel biogenesis in mitochondria.

EXPERIMENTAL PROCEDURES

Yeast strains, growth conditions and isolation of mitochondria

The yeast strains *pem1*Δ and *pem2*Δ strains and their corresponding wild-type BY4741 were obtained from Euroscarf. The *psd1*Δ strain and its corresponding wild-type were described (Böttinger et al., 2012). Cells were grown to an early stationary growth phase at 30°C in minimal medium containing 2% [v/v] glycerol and 0.1-0.2% [w/v] glucose as carbon source. Mitochondria were isolated by differential centrifugation as described (Böttinger et al., 2012), shock frozen in SEM buffer (10 mM MOPS/KOH, pH 7.2, 1 mM ethylenediaminetetraacetic acid [EDTA], 250 mM sucrose) and stored at -80°C until use.

Protein import into isolated mitochondria

For import reaction ^{35}S-labelled proteins were synthesized in the presence of ^{35}S-methionine using a cell-free coupled transcription/translation system based on reticulocyte lysate (TNT, Promega). Subsequently, ^{35}S-labelled precursors were

incubated with isolated mitochondria in import buffer (20mM Tris/HCl pH 7.4, 3% [w/v] BSA, 250 mM sucrose, 5 mM methionine, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS/KOH, pH 7.2, 2 mM KH_2PO_4) containing 4 mM ATP and 4mM NADH. Transfer on ice stopped the import reaction. Mitochondria were reisolated by centrifugation and washed with SEM buffer. The mitochondrial pellet was resuspended in lysis buffer (50 mM NaCl, 0.01mM EDTA, 10% [v/v] glycerol) containing 0.4-1% [w/v] digitonin for 15 min on ice. Unsoluble material was removed by centrifugation and the import was analyzed by blue native electrophoresis. To study membrane-insertion of the imported precursor, mitochondria were subjected to carbonate extraction as described (Thornton et al., 2010). Before the import reaction aggregated precursor proteins were removed by preincubation in import buffer without mitochondria. After removal of unsoluble proteins by centrifugation mitochondria were added to start the reaction.

Co-immunoprecipitation

For co-immunoprecipiation antisera against Tom22 and its corresponding preimmunserum were covalently coupled to protein A-Sepharose (GE Healthcare) with dimethyl pimelimidate. Isolated mitochondria were resuspended in lysis buffer containing 1% [w/v] digitonin and incubated for 15 min on ice. Unsoluble material was removed by centrifugation. The supernatant was incubated with the antibody matrix for 1h at 4°C under constant rotation. Subsequently, protein A-Sepharose beads were washed with an excess amount of lysis buffer containing 0.1% [w/v] digitonin. Bound proteins were eluted with 0.1 M glycine (pH 2.5). The eluate was immediately neutralized by addition of 1M TRIS-Base and analyzed by SDS-PAGE and Western Blotting.

Determination of the phospholipid content

The lipids of isolated mitochondria were extracted with a 2:1 (v/v) mixture of chloroform/phenol as described (Horvath et al., 2012). Subsequently, the organic phase was washed with 0.034 % [w/v] $MgCl_2$ solution, 4:1 (v/v) ratio of 2N KCl/methanol and a mixture of methanol/water/chloroform (48:47:3, per volume). The individual phospholipids were separated by thin-layer chromatography (Folch et al., 1957). For detection, phospholipids were stained with iodine vapor, scrapped off and quantified (Broekhuyse, 1968).

Miscellaneous

All antibodies used in this study have been described (Becker et al., 2013; Qiu et al., 2013). After separation proteins were transferred from the gel onto a PVDF membrane (Millipore) by semi-dry Western blotting. For immunodetection, signals of the bound antibodies were detected by chemoluminescence staining. ^{35}S-labelled proteins were detected by digital autoradiography (Storm imaging system, GE Healthcare). The experiments were analyzed by the Image J software, which is freely available on the internet.

RESULTS

Depletion of PC affects biogenesis of β-barrel precursors

To study the role of PC in the biogenesis of mitochondrial β-barrel precursors we used yeast deletion strains *pem1*Δ and *pem2*Δ, which are defective in the first and the two last methylation steps of PE, respectively. To block production of PC by the CDP-choline pathway we grew the cells on minimal medium lacking free choline. We used glycerol as the major carbon source to ensure that mitochondrial function is required for growth of the cells. The growth of *pem2*Δ is decreased under all temperatures tested, whereas *pem1*Δ grew poorly at lower temperatures (Fig. 1A). The double deletion of *pem1*Δ and *pem2*Δ is not viable without the supply of free choline, revealing the central role of PC for yeast survival (Kodaki and Yamashita, 1989). We isolated mitochondria from yeast cells grown at 30°C and analyzed the relative content of the main phospholipids. As expected the levels PC are strongly decreased in the mutants, whereas the relative levels PE and PI are increased (Fig. 1B) (in *pem2*Δ cells predominantly the monomethylated form of PE accumulates (Kodaki and Yamashita, 1987; Summers et al., 1988; Kodaki and Yamashita, 1989; Gohil et al., 2005), which could not be separated here from PE). The PC content in *pem2*Δ mitochondria is more severely reduced compared to *pem1*Δ since Pem2 can also catalyze the first methylation step, albeit with low efficiency, and therefore partially compensate for loss of Pem1 (Preitschopf et al., 1993; Kodaki and Yamashita, 1987). We used these mitochondria to study the impact of PC-depletion on the biogenesis of the model b-barrel precursors Tom40 and porin. Upon import radiolabelled Tom40 forms three assembly stages, which can be resolved by blue native electrophoresis: The Tom40

precursors binds to the SAM complex (Fig. 1C, SAM), is subsequently released in association with Tom5 (Int. II) before it assembles into the mature TOM complex of 450 kDa size (TOM) (Wiedemann et al., 2003; Becker et al., 2010). In PC-deficient mitochondria the integration of the Tom40 precursor into intermediate II and the TOM complex was largely compromised (Fig. 1C, lanes 1-9). In addition, in *pem2*Δ mitochondria already the formation of the SAM intermediate was decreased (Fig. 1C, lanes 1-9). Similarly, the assembly of imported porin precursor was impaired in PC depleted mitochondria (Fig. 1D, lanes 1-9). For comparison, we studied β-barrel biogenesis in *psd1*Δ mitochondria from cells grown on minimal medium like PC-deficient mutants. In *psd1*Δ mitochondria the PC levels are increased, whereas PE is decreased (Figure 1B) as described (Gohil et al., 2005; Becker et al., 2013). The import of Tom40 and porin is blocked in *psd1*Δ mitochondria (Fig. 1C and 1D, lanes 10-15) like it was observed in mitochondria from *psd1*Δ cells grown in complete medium (Becker et al., 2013). We conclude that the biogenesis of β-barrel precursors depends on the presence of both PC and PE.

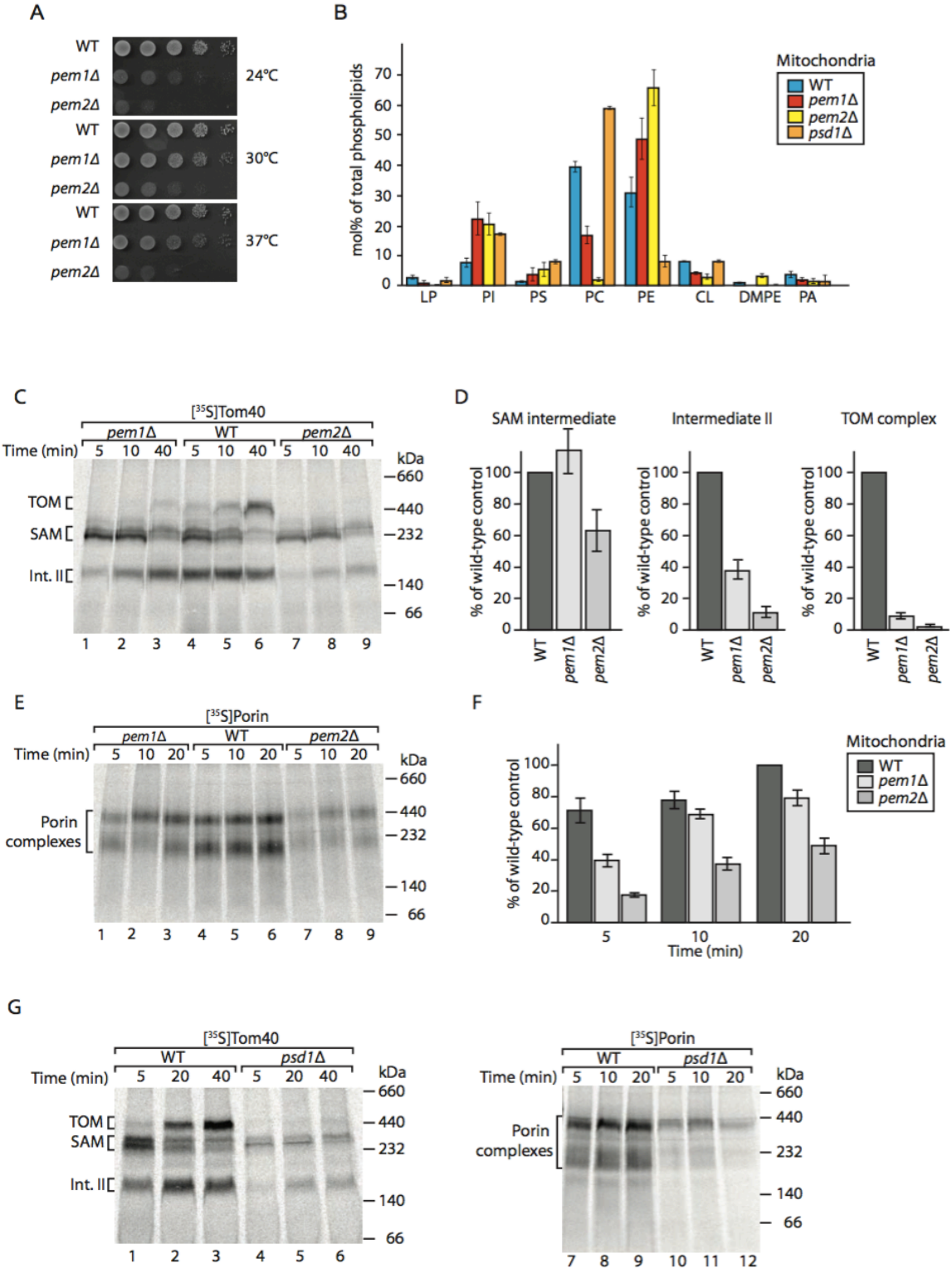
A
WT
pem1Δ
pem2Δ
24°C
30°C
37°C
B
mol% of total phospholipids
Mitochondria
WT
pem1Δ
pem2Δ
psd1Δ
LP
PI
PS
PC
PE
CL
DMPE
PA
C
[35S]Tom40
Time (min)
kDa
TOM
SAM
Int. II
D
SAM intermediate
Intermediate II
TOM complex
% of wild-type control
E
[35S]Porin
Porin complexes
F
G
[35S]Tom40
[35S]Porin

Figure 1. A balanced PC/PE ratio is crucial for the biogenesis of mitochondria β-barrel proteins. (A) Serial dilution of WT, *pem1*Δ and *pem2*Δ cells were spotted on minimal medium containing 2% glycerol and 0.2% glucose as carbon source. (B) The phospholipid profile of WT, *pem1*Δ and *pem2*Δ mitochondria were analyzed by thin-layer chromatography. Shown is the quantification of the relative amounts of the major phospholipid classes. Means +/- S.E. (?) (n=?). (C) + (D) ^{35}S-labelled Tom40 (C) or porin (D) were imported for the indicated time periods into WT, *pem1*Δ, *pem2*Δ and *psd1*Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography.

PC is required for the stability of the SAM complex

We asked, which step of the biogenesis of β-barrel precursors is affected by depletion of PC. We first analyzed the steady state levels of various mitochondrial proteins. Protein levels of TOM and SAM subunits and other mitochondrial proteins are largely comparble in the *pem1*Δ and *pem2*Δ to wild-type mitochondria (Fig. 2A). Importantly, the content of the small TIM protein Tim10 of the intermembrane space was unchanged, which promotes transfer of β-barrel precursor protein to the SAM complex (Wiedemann et al., 2004; Hoppins and Nargang, 2004; Qiu et al., 2013). Altogether, we can exclude the possibility that a decreased steady state levels of translocase subunits leads to impaired β-barrel biogenesis in the mutant mitochondria. Since the SAM complex is crucial for β-barrel biogenesis, we wondered whether the integrity of the SAM complex is affected upon PC-depletion. To tackle this issue we analyzed the SAM complex in the mutant mitochondria by blue native electrophoresis. Two main populations of the SAM can be resolved on blue native gels: The SAM_{core} complex of 200 kDa consisting of the three SAM subunits and the SAM-Mdm10 complex of 350 kDa, which additionally contains Mdm10 (Fig. 2B; Meisinger et al., 2004; Thornton et al., 2010; Yamano et al., 2010; Wideman et al., 2010; Becker et al., 2011; Klein et al.,

2012). In the PC-deficient mitochondria an increased of Sam50-Sam35 subcomplex was observed. In contrast, less Sam37 was detected at the SAM complexes in the mutant mitochondria (Fig. 2B), although protein levels of Sam37 were not reduced in the mutant mitochondria (Fig. 2A). We conclude that PC stabilizes the association of Sam37 to the Sam50/Sam35 subcomplex.

We analyzed whether the accumulation of precursors at the SAM complex requires PC. To this end, we imported a Tom40 variant ($Tom40_{G354A}$), which is not efficiently released from the SAM machinery (Kutik et al., 2008). Indeed, the binding of $Tom40_{G354A}$ to the SAM complex is decreased in *pem1*Δ and *pem2*Δ mitochondria (Fig. 3A). Precursor transfer to the SAM complex occurs via a TOM-SAM supercomplex (Qiu et al., 2013). We have established an experimental system to study the precursor binding to the TOM-SAM supercomplex. We imported a fusion construct of cytochrome b_2(220) and Tom40 (b_2(220)Tom40) into mitochondria in the absence of a membrane potential to prevent further transport of theb_2(220)Tom40 construct via the presequence pathway (Shiota et al., 2012; Qiu et al., 2013). Under these conditions b_2(220)Tom40 accumulates at the TOM-SAM supercomplex, which can be studied by blue native electrophoresis (Qiu et al., 2013). Performing this assay in PC-deficient mitochondria, we observed that the binding of b_2(220)Tom40 to the TOM-SAM supercomplex is impaired in the mutant mitochondria (Fig. 3B). We conclude that already the binding of β-barrel precursors to the TOM-SAM supercomplex requires PC.

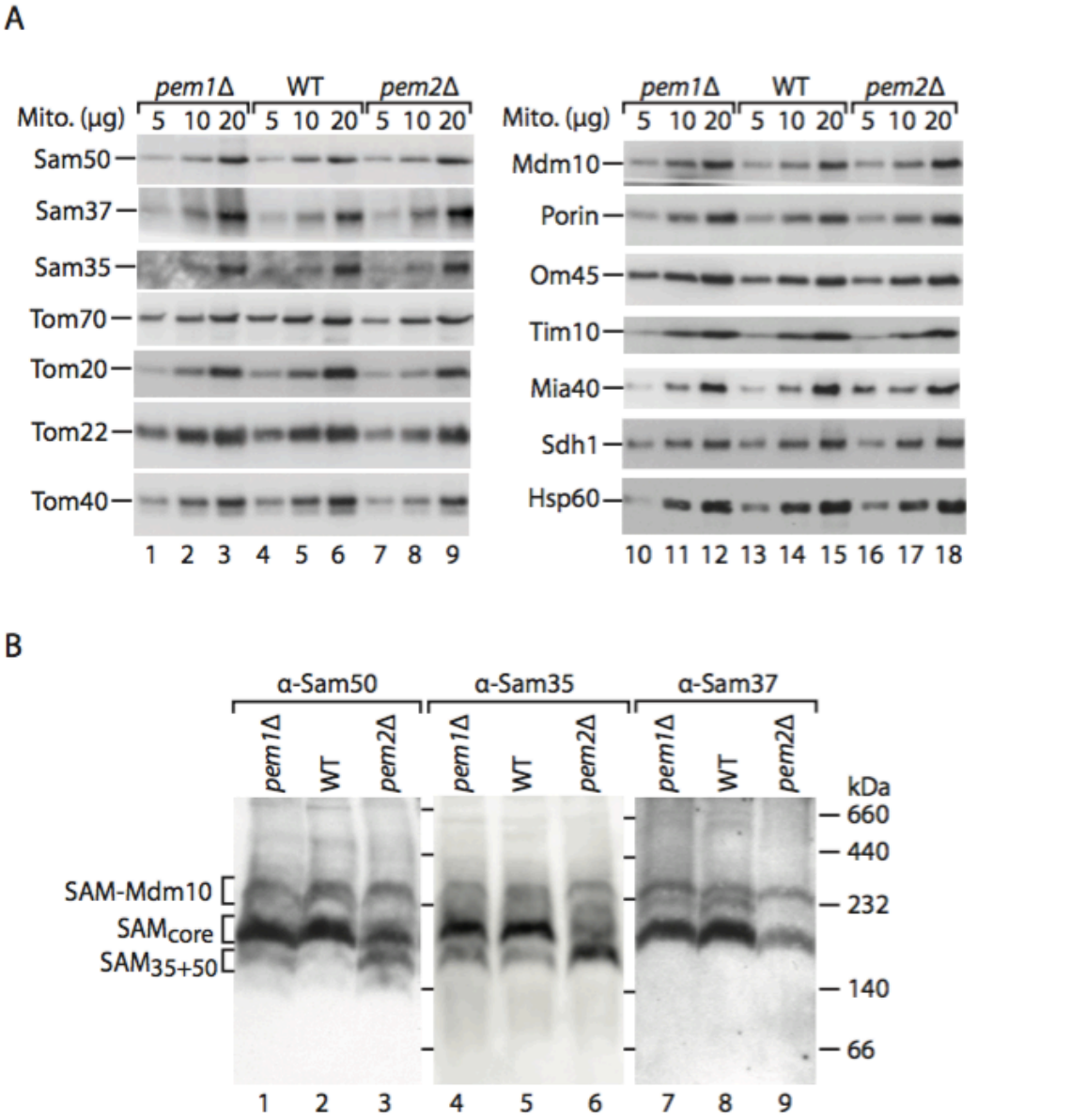

Figure 2. The SAM complex is destabilized in PC-depleted mitochondria. (A) The indicated amounts of WT, *pem1*Δ and *pem2*Δ mitochondria were subjected to SDS-PAGE and immunodetection with the indicated antisera. (B) WT, *pem1*Δ and *pem2*Δ mitochondria were subjected to blue native electrophoresis and immunodetection with the indicated antisera.

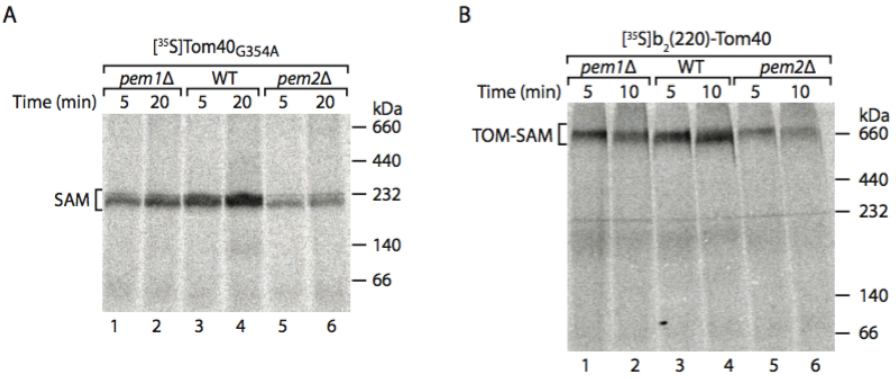

Figure 3. Transfer of precursor proteins to the SAM complex is impaired in PC-depleted mitochondria. (A) + (B) ^{35}S-labelled Tom40$_{G354A}$ (A) and b_2(220)-Tom40 (B) were imported for the indicated time periods into WT, *pem1*Δ and *pem2*Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography. Import of b_2(220)-Tom40 was performed after depletion of the membrane potential.

PC and PE differentially affect the function of the TOM complex

The import of β-barrel precursor into PE-depleted mitochondria is blocked at the TOM stage (Becker et al., 2013). We wondered whether depletion of PC affect stability and function of the TOM complex, leading to a decreased binding of β-barrel precursors to the TOM-SAM supercomplex. The levels of the TOM complex of 450 kDa on blue native gels are only mildly affected in PC-deficient mitochondria (Fig. 4A). Importantly, no subcomplex of lower molecular weight was detected, reflecting that the TOM complex remains stable in the mutant mitochondria. However, under these conditions the association of the peripheral receptors Tom20 and Tom70 is lost (Meisinger et al., 2001). To study the association of the peripheral TOM receptors, we performed co-immunprecipiation using antibodies against Tom22. Both Tom20 and Tom70 are

efficiently co-immunoprecipiated with antibodies against Tom22, but not with the corresponding preimmunserum (Fig. 4B, lanes 5-8). We conclude that the TOM complex remains largely intact in PC-depleted mitochondria. To analyze the capability of the TOM complex to bind precursor proteins, we imported Oxa1 into the mutant mitochondria in the absence of a membrane potential. Under these conditions the hydrophobic Oxa1 precursor accumulates at the TOM complex, which can be detected by blue native electrophoresis (Frazier et al., 2003). Strikingly, the binding of the Oxa1 precursor to the TOM complex is not impaired in PC-deficient mitochondria (Fig. 4C, lanes 1-9). For comparison, the Oxa1-TOM formation was largely compromised in *psd1*Δ mitochondria (Fig. 4C, lanes 10-15; Becker et al., 2013). We conclude that the stability and function of the TOM complex are not impaired in PC-deficient mitochondria. Thus, PC affects the biogenesis of mitochondria β-barrel proteins at the stage of the SAM complex and not at the TOM complex. In contrast, PE is predominantly required for the transport of β-barrel precursor proteins across the TOM complex (Becker et al., 2013). Thus, PC and PE are required for distinct steps in the import of β-barrel proteins.

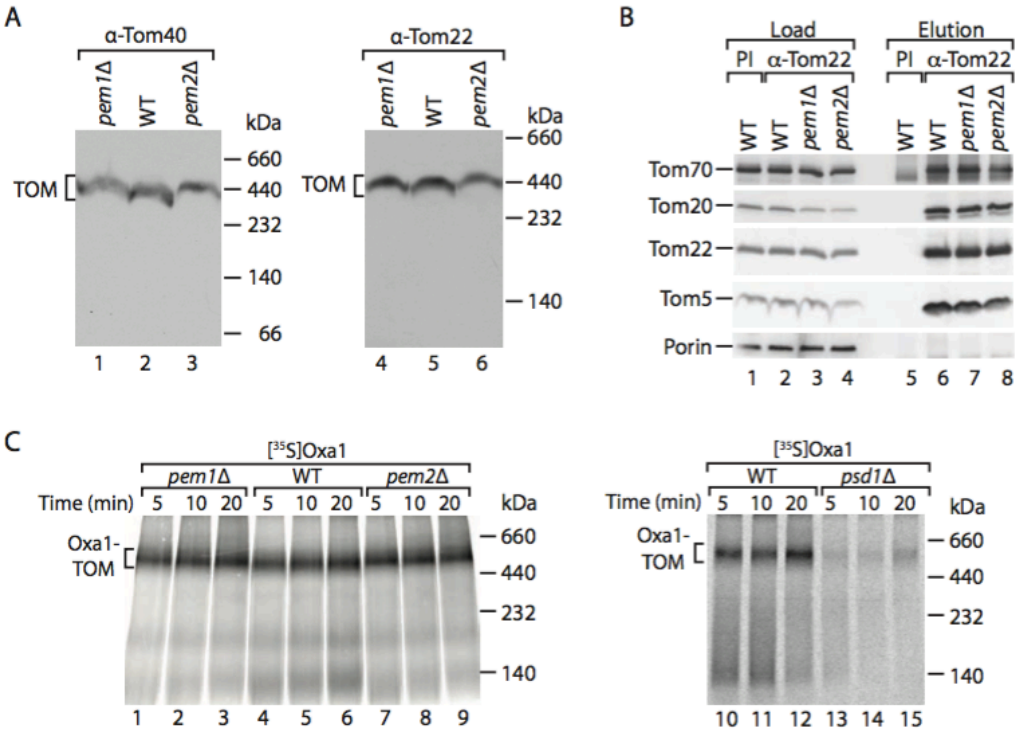

Figure 4. Precursor binding to the TOM complex is not affected in PC-depleted mitochondria. (A) WT, *pem1*Δ and *pem2*Δ mitochondria were subjected to blue native electrophoresis and immunodetection with the indicated antisera. (B) Lysed WT, *pem1*Δ and *pem2*Δ mitochondria were subjected to co-immunoprecipitation with Tom22 specific antibodies or with the corresponding preimmunserum (PI). Load and elution fractions were analyzed by SDS-PAGE and immunodetection with the indicated antisera. (C) ^{35}S-labelled Oxa1 precursor was imported for the indicated time periods into WT, *pem1*Δ and *pem2*Δ and *psd1*Δ mitochondria after depletion of the membrane potential. The import reaction was analyzed by blue native electrophoresis and autoradiography.

PC promotes the assembly of Tom22 into the TOM complex

We wondered whether PC is generally required for the function of the SAM complex. In addition to the biogenesis of β-barrel precursors the SAM complex promotes insertion

and assembly of the Tom22 precursor into the mature TOM complex (Stojanovski et al., 2007; Thornton et al., 2010; Becker et al., 2011; Lackey et al., 2011). We imported ^{35}S-labelled Tom22 precursor into PC-deficient mitochondria. Strikingly, the assembly of radiolabelled Tom22 into the mature TOM complex is largely compromised in *pem1*Δ and *pem2*Δ mitochondria (Fig. 5A, lanes 1-9). For comparison, the integration of Tom22 into the TOM complex is only mildly delayed in *psd1*Δ mitochondria (Fig. 5A, lanes 10-15) (Becker et al., 2013), revealing a specific role of PC in the function of the SAM complex. Moreover, the assembly of the Tom20 was not affected in PC-deficient mitochondria (Fig. 5B), which is imported into the outer membrane in a SAM-independent manner (Wiedemann et al., 2003). SAM subunits promote membrane integration of the Tom22 precursor (Stojanovski et al., 2007). Indeed, we found by carbonate extraction that the insertion of Tom22, but not of the Tom20 precursor into the outer membrane is compromised in *pem1*Δ and *pem2*Δ (Fig. 5C). We conclude that PC promotes both functions of the SAM complex: the biogenesis of β-barrel precursors as well as the assembly of Tom22 into the TOM complex.

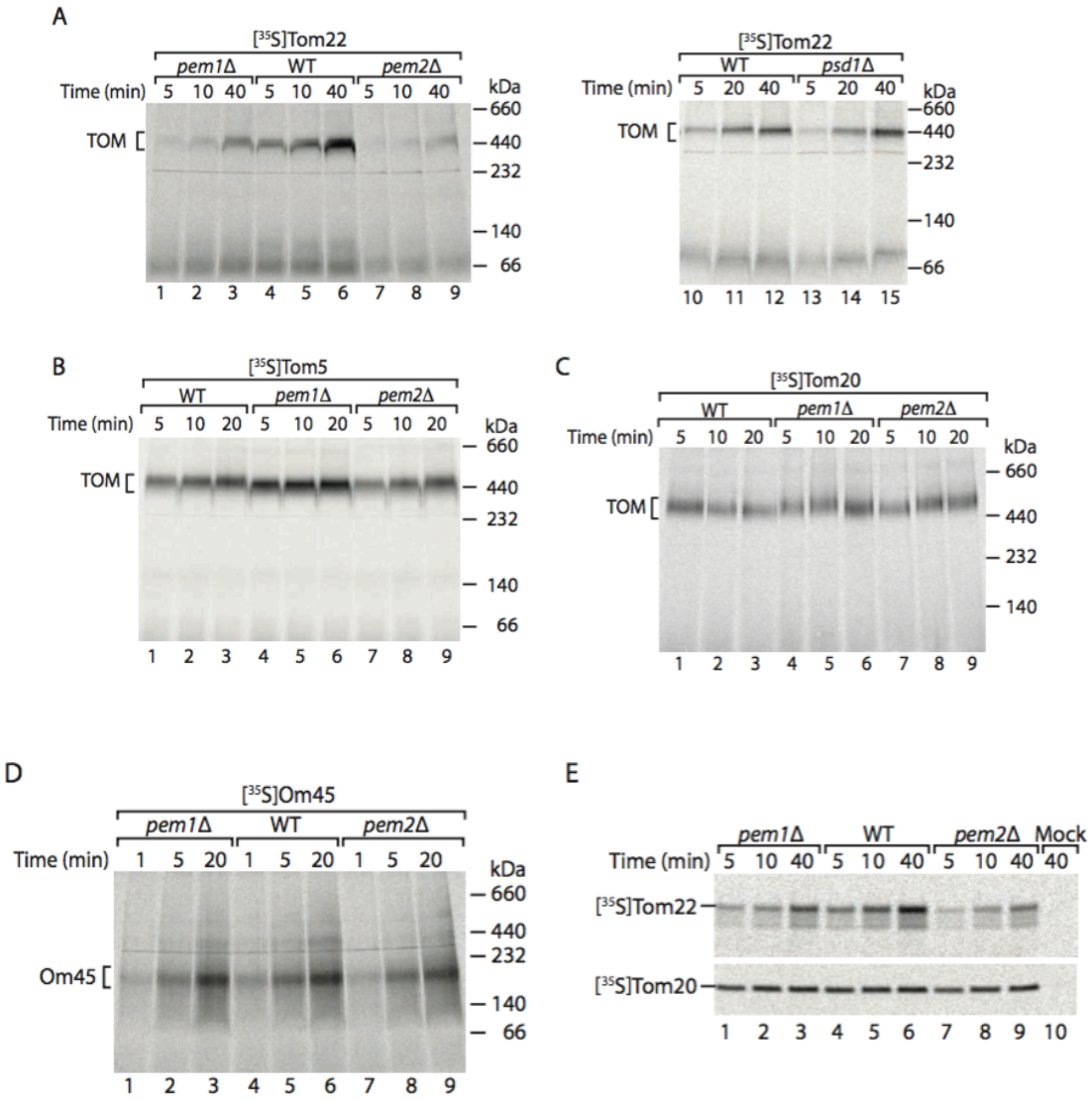

Figure 5. Biogenesis of Tom22 is impaired in PC-deficient mitochondria (A) ^{35}S-labelled Tom22 precursor was imported for the indicated time periods into WT, *pem1*Δ, *pem2*Δ and *psd1*Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography. (B) ^{35}S-labelled Tom20 precursor was imported for the indicated time periods into WT, *pem1*Δ, *pem2*Δ and *psd1*Δ mitochondria. The import reaction was analyzed by blue native electrophoresis and autoradiography. (C) ^{35}S-labelled Tom22 (upper panel) and Tom20 precursor (lower panel) were imported for the indicated time periods into WT, *pem1*Δ and *pem2*Δ mitochondria. Subsequently, mitochondria were subjected to carbonate

extraction. Proteins of the membrane pellets were analyzed by SDS-PAGE and autoradiography. In the mock control no mitochondria were added to import reaction.

DISCUSSION

PC is the most abundant phospholipid of cellular membranes. PC is core structural component to build up the phospholipid bilayer of biological membranes. Besides its structural function the impact of PC on integral membrane proteins have not been analyzed yet. Our study revealed that PC is specifically required for the biogenesis of mitochondrial β-barrel proteins. This observation could have important implications for pathologies like non-alcoholic steatohepatatis and congenital muscular dystrophy, which developed in tissues defective in PC synthesis (Li et al., 2006; Mitsuhashi et al., 2011).

Biogenesis of β-barrel proteins into the mitochondrial outer membrane is particularly sensitive towards alterations of the phospholipid composition of the target membrane. This protein import pathway involves the coordinated activity of two membrane-bound protein translocases and the integration of precursor proteins into a lipid bilayer. All these membrane-localized steps might render the biogenesis of β-barrel sensitive towards alteration of the phospholipid composition. So far, a role of the non-bilayer forming phospholipids CL and PE was reported (Gebert et al., 2009; Becker et al., 2013). Here, we identified a novel function of PC in this process. Surprisingly, depletion of these three phospholipids affect different steps in the biogenesis of β-barrel proteins. CL is required for the stabilization of TOM and SAM complexes (Gebert et al., 2009). Depletion of PE affects binding of precursor proteins to the TOM complex, but not the stability of the translocases (Becker et al., 2013). In this study, we

showed that PC promotes function of the SAM complex, whereas the precursor accumulation at the TOM complex remains largely unaffected upon depletion of PC. Interestingly, import and assembly of the Tom22 precursor are largely compromised in the PC-deficient mitochondria. Tom22 is imported via the SAM complex, indicating that PC is generally important for SAM function in mitochondria. In contrast, the non-bilayer forming phospholipids CL and PE are not required for the biogenesis of Tom22 (Gebert et al., 2009; Becker et al., 2013). Moreover, the SAM-independent insertion of Tom20 occurs independently of CL, PE and PC (Gebert et al., 2009; Becker et al., 2013; Sauerwald et al., 2015). Thus, PC is specifically required for the SAM-dependent protein import pathways.

How do phospholipids affect TOM and SAM complexes? It was assumed that non-bilayer forming property of CL and PE is important for the function of membrane-bound proteins by increasing the curvature stress (van den Brink-van der Laan, 2004). Here, we found that the bilayer-forming phospholipid PC promote the function of the SAM complex, indicating that further properties of the phospholipids are crucial. The charge of the headgroup is important feature of phospholipids. For instance, the activity of the respiratory chain depends on both CL and PE (Pfeiffer et al., 2003; Zhang et al., 2005; Böttinger et al., 2012; Tasseva et al., 2013). However, the negatively charged headgroup of CL stabilizes the supercomplex of cytochrome c reductase and oxidase (Wenz et al., 2004), whereas depletion of PE does not affect the supercomplex formation (Böttinger et al., 2012). PC and PE are the major phospholipids of the mitochondrial outer membrane and contain both a neutrally charged headgroup. Depletion of PE causes an accumulation of PC and vice versa, pointing to a close link between both phospholipids (Gohil et al., 2005; Becker et al., 2013). Nevertheless, PE and PC differentially affect outer membrane protein translocases in the biogenesis of β-

barrel proteins. Since depletion of PC does not affect the TOM complex, one can speculate that the activity of the TOM complex specifically depends on the presence of non-bilayer forming character of PE. Thus, charge of the head group and the bilayer versus non-bilayer forming character seems to be important for the specific role of phospholipids in the biogenesis of mitochondrial β-barrel proteins.

Altogether, phospholipids are required for distinct steps of the import of β-barrel proteins into the mitochondrial outer membrane. Phospholipids specifically affect the function of membrane-bound protein translocases and protein import steps. Thus, not only protein components but also phospholipids are important for mitochondrial protein biogenesis.

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REFERENCES

1. Schleiff, E., and Soll, J. (2005) Membrane protein insertion: mixing eukaryotic and prokaryotic concepts. EMBO Rep. 6, 1023-1027
2. Walther, D.M., Rapaport, D., and Tommassen, J. (2009) Biogenesis of b-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. Cell Mol. Life Sci. 66, 2789-2804
3. Webb, C.T., Heinz, E., and Lithgow, T. (2012) Evolution of the b-barrel assembly machinery. Trends. Microbiol. 20, 612-620
4. Höhr, A.I.C., Straub, S.P., Warscheid, B., Becker, T., and Wiedemann N. (2015) Assembly of b-barrel proteins in the mitochondrial outer membrane. Biochim. Biophys. Acta 1853, 74-88
5. Koehler, C. M. (2004) New developments in mitochondrial assembly. Annu. Rev. Cell Dev. Biol. 20, 309-335
6. 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
7. Neupert, W., and Herrmann, J. M. (2007) Translocation of proteins into mitochondria. Annu. Rev. Biochem. 76, 723-749
8. Endo T., and Yamano, K. (2009) Multiple pathways for mitochondrial protein traffic. Biol. Chem. 390, 723-730
9. Becker, T., Böttinger, L., and Pfanner, N. (2012) Mitochondrial protein import: from transport pathways to an integrated network. Trends Biochem. Sci. 37, 85-91
10. Hiller, S., Abramson, J., Mannella, C., Wagner, G., and Zeth, K. (2010) The 3D structure of VDAC represent a native conformation. Trends. Biochem. Sci. 35, 514-521.
11. Colombini, M. (2012) VDAC structure, selectivity, and dynamics. Biochim. Biophys. Acta 1818, 1457-1465
12. Osman, C., Voelker, D.R., and Langer, T. (2011) Making heads or tails of phospholipids in mitochondria. J. Cell. Biol. 192, 7-16
13. Toulmay, A., and Prinz, W.A. (2011) Lipid transfer and signaling at organelle contact sites: the tip of the iceberg. Curr. Opin. Cell Biol. 23, 458-463
14. Lang, A., John Peter, A.T., and Kornmann, B. (2015) ER-mitochondria contact sites in yeast: beyond the myths of ERMES. Curr. Opin. Cell Biol. 35, 7-12

15. Dukanovic, J., and Rapaport, D. (2011) Mutiple pathways in the integration of proteins into the mitochondrial outer membrane. Biochim Biophys. Acta 1808, 971-980.
16. Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E., and Pfanner, N. (2001) Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. J. Cell Biol. 152, 289-300
17. Habib, S. J., Waizenegger, T., Lech, M., Neupert, W., and Rapaport, D. (2005) Assembly of the TOB complex of mitochondria. J. Biol. Chem. 280, 6434-6440
18. Yamano, K., Yatsukawa, Y., Esaki, M., Hobbs, A.E., Jensen, R.E., and Endo T. (2008) Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. J. Biol. Chem. 283, 3799-3807.
19. Wiedemann, N., Kozjak, V., Chacinska, A., Schönfisch, B., Rospert, S., Ryan, M. T., Pfanner, N., and Meisinger, C. (2003) Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature 424, 565-571
20. Paschen, S. A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003) Evolutionary conservation of biogenesis of b-barrel membrane proteins. Nature 426, 862-866
21. Qiu, J., Wnez, L.S., Zerbes, R.M., Oeljeklaus, S., Bohnert, M., Stroud, D.A., Wirth, C., Ellenrieder, L., Thornton, N., Kutik, S., Wiese, S., Schulze-Specking, A., Zufall, N., Chacinska, A., Guiard, B., Hunte, C., Warscheid, B., van der Laan, M., Pfanner, N., Wiedemann, N., and Becker, T. (2013) Coupling of mitochondrial import and export translocases by receptor-mediated supercomplex formation. Cell 154, 596-608
22. Wiedemann, N., Truscott, K.N., Pfannschmidt, S., Guiard, B., Meisinger, C., and Pfanner, N. (2004) Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway. J. Biol. Chem. 279, 18188-18194
23. Hoppins, S. C., and Nargang, F. E. (2004) The Tim8-Tim13 complex of Neurospora crassa functions in the assembly of proteins into both mitochondrial membranes. J. Biol. Chem. 279, 12396-12405
24. Kozjak, V., Wiedemann, N., Milenkovic, D., Lohaus, C., Meyer, H.E., Guiard, B., Meisinger, C., and Pfanner, N. (2003) An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. J. Biol. Chem. 278, 48520-48523

25. Gentle I., Gabriel, K., Beech, P., Waller, R., and Lithgow, T. (2004) The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. J. Cell Biol. 164, 19-24
26. Ishikawa, D., Yamamoto, H., Tamura, Y., Moritoh, K., and Endo, T. (2004) Two novel proteins in the mitochondrial outer membrane mediate b-barrel protein assembly. J Cell Biol. 166, 621-627
27. Klein, A., Israel, L., Lackey, S.W., Nargang, F.E., Imhof, A., Baumeister, W., Neupert, W., and Thomas, D.R. (2012) Characterization of the insertase of b-barrel proteins of the outer mitochondrial membrane. J. Cell Biol. 199, 599-611
28. Chan, N. C., and Lithgow, T. (2008) The peripheral membrane subunits of the SAM complex function codependently in mitochondrial outer membrane biogenesis. Mol. Biol. Cell 19, 126-136
29. Kutik, S., Stojanovski, D., Becker, L., Becker, T., Meinecke, M., Krüger, V., Prinz, C., Meisinger, C., Guiard, B., Wagner, R. Pfanner, N., and Wiedemann, N. (2008) Dissecting membrane insertion of mitochondrial b-barrel proteins. Cell 132, 1011-1024
30. Dukanovic, J., Dimmer, K. S., Bonnefoy, N., Kumpe, K., and Rapaport, D. (2009) Genetic and functional interactions between the mitochondrial outer membrane proteins Tom6 and Sam37. Mol. Cell. Biol. 29, 5975-5988
31. Becker, T., Guiard, B., Thornton, N., Zufall, N., Stroud, D. A., Wiedemann, N., and Pfanner, N. (2010) Assembly of the mitochondrial protein import channel: role of Tom5 in two-stage interaction with the SAM complex. Mol. Biol. Cell 14, 3106-3113
32. Lackey, S. W. K., Wideman, J. G., Kennedy, E. K., Go, N. E., and Nargang, F. E. (2011) The Neurospora crassa TOB complex: analysis of the topology and function of Tob38 and Tob37. PLoS One 6, e25650
33. Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H.E., Guiard, B., Pfanner, N, and Meisinger, C. (2004) Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. J. Biol. Chem. 279, 22781-22785
34. Waizenegger, T., Habib, S.J., Lech, M., Mokranjac, D., Paschen, S.A., Hell, K., Neupert, W., and Rapaport, D. (2004) Tob38, a novel essential component in the biogenesis of the b-barrel proteins of mitochondria. EMBO Rep. 5, 704-709
35. Meisinger, C., Rissler, M., Chacinska, A., Sanjuán Szklarz, L. K., Milenkovic, D., Kozjak, V., Schönfisch, B., Lohaus, C., Meyer, H. E., Yaffe, M. P. Guiard, B., Wiedemann, N., and Pfanner, N. (2004) The mitochondrial morphology protein

Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Dev. Cell 7, 61-71
36. Stojanovski, D., Guiard, B., Kozjak-Pavlovic, V., Pfanner, N., and Meisinger C. (2007) Alternative function for the mitochondrial SAM complex in biogenesis of a-helical TOM proteins. J. Cell Biol. 179, 881-893
37. 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 a-helical outer membrane proteins. J. Mol. Biol. 396, 540-549
38. Yamano, K., Tanaka-Yamano, S., and Endo, T. (2010) Mdm10 as a dynamic constituent of the TOB/SAM complex directs coordinated assembly of Tom40. EMBO Rep. 11, 187-193
39. Wideman, J.G., Goo, N.E., Klein, A., Redmond, E., Lackey, S.W.K., Tao, T., Kalbacher, H., Rapaport, D., Neupert, W., and Nargang, F.E. (2010) Role of Mdm10, Tom7, Mdm12 and Mmm1 proteins in the assembly of mitochondrial outer membrane proteins in Neurospora crassa. Mol. Biol. Cell 21, 1725-1736
40. Becker, T., Wenz, L.S., Thornton, N., Stroud, D., Meisinger C., Wiedemann, N., and Pfanner, N. (2011) Biogenesis of mitochondria: dual role of Tom7 in modulating assembly of the preprotein translocase of the outer membrane. J. Mol. Biol. 405, 113-124
41. 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
42. Horvath, S.E., and Daum, G. (2013) Lipids of mitochondria. Prog. Lipid. Res. 52, 590-614.
43. Clancey, C. J., Chang, S. C., and Dowhan, W. (1993) Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from Saccharomyces cerevisiae by complementation of an Escherichia coli mutant. J. Biol. Chem. 268, 24580-24590
44. Trotter, P. J., Pedretti, J., and Voelker, D. R. (1993) Phosphatidylserine decarboxylase from Saccharomyces cerevisiae: isolation of mutants, cloning of the gene, and creation of a null allele. J. Biol. Chem. 268, 21416-21424
45. Horvath, S. E., Böttinger, L., Vögtle, F. N., Wiedemann, N., Meisinger, C., Becker, T., and Daum, G. (2012) Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1. J. Biol. Chem. 287, 36744-36755
46. Jiang, F., Ryan, M.T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., and Greenberg, M.L. (2000) Absence of cardiolipin in the crd1 null mutant

results in decreased mitochondrial membrane potential and reduced mitochondrial function. J. Biol. Chem. 275, 22387-22394

47. Pfeiffer, K., Gohil, V., Stuart, R. A., Hunte, C., Brandt, U., Greenberg, M. L. and Schägger, H. (2003). Cardiolipin stabilizes respiratory chain supercomplexes. J. Biol. Chem. 278, 52873-52880
48. 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
49. 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
50. 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
51. van der Laan, M., Meinecke, M., Dudek, J., Hutu, D. P., Lind, M., Perschil, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007) Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. Nat. Cell Biol. 9, 1152-1159
52. 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
53. Gonzalvez, F., Schug, Z. T., Houtkopper, R. H., McKenzie, E. D., Brooks, D. G., Wanders, R. J., Petit, P. X., Vaz, F. M., and Gottlieb, E. (2008) Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. J. Cell Biol. 183, 681-696
54. Gebert, N., Joshi, A.S., Kutik, S., Becker, T., McKenzie, M., Guan, X.L., Mooga, V.P., Stroud, D.S., Kulharni, 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 the Barth Syndrome. Curr. Biol. 19, 2133-2139
55. Tamura, Y., Endo, T., Iijima, M., and Sesaki, H. (2009) Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. J. Cell Biol. 185, 1029-1045
56. 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

57. DeVay, R.M., Dominguez-Ramirez, L., Lackner, L. L., Hoppins, S., Stahlberg, H., and Nunnari, J. (2009) Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. J. Cell Biol. 186, 793-803
58. Osman, C., Haag, M., Potting, C., Rodenfels, J., Dip, P. V., Wieland, F. T., Brügger, B., Westermann, B., and Langer, T. (2009) The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. J. Cell Biol. 184, 583-596
59. 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
60. Tamura, Y., Onguka, O., Aiken Hobbs, A. E., Jensen, R. E., Iijima, M., Claypool, S. M., and Sesaki, H. (2012) Role of two conserved intermembrane space proteins, Ups1p and Ups2p, in intra-mitochondrial phospholipid trafficking. J. Biol. Chem. 287, 15205-15218
61. Zhang, M., Mileykovskaya, E., and Dowhan, W. (2002) Gluing the respiratory chain together: Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. J. Biol. Chem. 280, 29403-29408
62. Mileykovskaya, E., and Dowhan, W. (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim. Biophys. Acta 1788, 2084-2091
63. 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
64. Böttinger, L., Horvath, S. E., Kleinschroth, T., Hunte, C., Daum, G., Pfanner, N., and Becker, T. (2012) Phosphatidylethanolamine and cardiolipin differently affect the stability of mitochondrial respiratory chain supercomplexes. J. Mol. Biol. 423, 677-686
65. Joshi, A. S., Thompson, M. N., Fei, N., Hüttemann, M., and Greenberg, M. L. (2012) Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in Saccharomyces cerevisiae. J. Biol. Chem. 287, 17589-17597
66. Tasseva, G., Bai, H.D., Davidescu, M., Haromy, A., Michelakis, E., and Vance, J.E. (2013) Phosphatidylethanolamine deficiency in mammalian mitochondria

impaires oxidative phosphorylation and alters mitochondrial morphology. J. Biol. Chem. 288, 4158-4173
67. Becker, T., Horvath, S.E., Böttinger, L., Gebert, N., Daum, G., and Pfanner, N. (2013) Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins. J. Biol. Chem. 288, 16541-16459
68. Sauerwald, J., Jores, T., Eisenberg-Bord, M., Chuartzman, S.G., Schuldiner, M. and Rapaport, D. (2015) Genome-wide screens in yeast highlight a role for cardiolipin in biogenesis of mitochondrial outer membrane multispan proteins. Mol. Cell Biol. doi: 10.1128/MCB.00107-15
69. Sperka-Gottlieb, C. D. M., Hermetter, A., Paltauf, F., and Daum, G. (1988) Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, Saccharomyces cerevisiae. Biochim. Biophys. Acta 946, 227-234
70. Zinser, E., Sperka-Gottlieb, C. D. M., Fasch, E. V., Kohlwein, S. D., Paltauf, F., and Daum, G. (1991) Phospholipid synthesis and lipid composition of subcellular membrane in the unicellular eukaryote Saccharomyces cerevisiae. J. Bacteriol. 173, 2026-2034
71. Cole, L.K., Vance, J.E., and Vance, D.E. (2012) Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochim Biophys Acta 1821, 754-761
72. Greenberg, M.L., Klig, L.S., Letts, V.A., Loewy, B.S., and Henry, S.A. (1983) Yeast mutant defective in phosphotidylcholine synthesis. J. Bacteriol. 153, 791-799
73. Kodaki, T., and Yamashita, S. (1987) Yeast phosphatidylethanolamine methylation pathways: cloning and characterization of two distict methyltransferase genes. J. Biol. Chem. 262, 15428-15435
74. Summers, E.F., Letts, V.A., McGraw, P., and Henry, S.A. (1988) Saccharomyces cerevisiae cho2 mutants are deficient in phospholipid methylation and cross-pathway regulation of insolitol synthesis. Genetics 120, 909-922
75. Kodaki, T., and Yamashita, S. (1989) Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. Eur. J. Biochem. 185, 243-251
76. Gaynor, P.M., and Carman, G.M. (1990) Phosphatidylethanolamine methyltransferase and phospholipid methyltransferase activities from Saccharomyces cerevisiae, enzymological and kinetic properties. Biochim Biophys Acta 1045, 156-163
77. Preitschopf, W., Lückl, H., Summers, E., Henry, S.A., Paltauf, F., and Kohlwein, S.D. (1993) Molecular cloning of the yeast OPI3 gene as a high copy number suppressor of the cho2 mutant. Curr. Genet. 23, 95-101

78. Li, Z., Agelion, L.B., Allen, T.M., Umeda, M., Jewell, L., Mason, A., and Vance, D.E. (2006) The ration of phosphatidylcholine and phosphatidylethanolamine influences membrane integrity and steatohepatitis. Cell. Metabol. 3, 321-331
79. Mitsuhashi, S., Ohkuma, A., Talim, B., Karahashi, M., Koumura, T., Aoyama, C., Kurihara, M., Quinlivan, R., Sewry, C., Mitsuhashi, H., Goto, K., Koksal, B., Kale, G., Ikeda, K., Taguchi, R., Noguchi, S., Hayashi, Y.K., Nonaka, I., Sher, R.B., Sugimoto, H., Nakagawa, Y., Cox, G.A., Topaloglu, H., and Nishino, I. (2011) A congenital muscular dystrophy with mitochondrial structural abnormalities caused by defective de novo phosphatidylcholine biosynthesis. Am. J. Hum. Genet. 88, 845-851
80. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509
81. Broekhuyse, 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
82. Shiota, T., Maruyama, M., Miura, M., Tamura, Y., Yamano, K., Esaki, M., and Endo, T. (2012) The Tom40 assembly process probed using attachment of different intromitochondrial sorting signals. Mol. Biol. Cell 23, 3936-3947
83. 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
84. Frazier, A. E., Chacinska, A., Truscott, K. N., Guiard, B., Pfanner, N., and Rehling, P. (2003) Mitochondria use different mechanisms for transport of multispanning membrane proteins through the intermembrane space. Mol. Cell. Biol. 23, 7818-7828
85. Wenz, T., Hielscher, R., Helwig, R., Schägger, H., Richers, S., and Hunte, C. (2009) Role of phospholipids in respiratory cytochrome bc_1 complex catalysis and supercomplex formation. Biochim Biophys Acta 1787, 609-616

FOOTNOTES

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[4] The abbreviations used are: VDAC, voltage-dependent anion channel; ER, endoplasmic reticulum; TOM, translocase of outer mitochondrial membrane; SAM, sorting and assembly machinery; Mdm10, mitochondrial distribution and morphology protein 10; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol, PS; phosphatidylserine; Pem, phosphatidylethanolamine methylase; Psd, phosphatidylserine decarboxylase.

CHAPTER 4

Phosphatidylcholine Affects Inner Membrane Protein Translocases of Mitochondria

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Phosphatidylcholine affects inner membrane protein translocases of mitochondria*

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[3]The abbreviations used are: TIM23, presequence translocase of the inner memrane; TIM22, carrier translocase of the inner membrane; TOM, translocase of the outer membrane; SAM, sorting and assembly machinery; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol, PS; phosphatidylserine; Pem, phosphatidylethanolamine methyltransferase; Psd, phosphatidylserine decarboxylase.

ABSTRACT

Two protein translocases transport precursor proteins into or across the inner mitochondrial membrane. The presequence translocase (TIM23 complex) sorts precursor proteins with a cleavable presequence either into the matrix or into the inner membrane. The carrier translocase (TIM22 complex) inserts multi-spanning proteins into the inner membrane. Both protein import pathways depend on the presence of a membrane potential, which is generated by the activity of the respiratory chain. The non-bilayer forming phospholipids cardiolipin and phosphatidylethanolamine are required for the activity of the respiratory chain and therefore to maintain the membrane potential for protein import. Depletion of cardiolipin further affects the stability of the TIM23 complex. The role of bilayer-forming phospholipids like phosphatidylcholine (PC) in protein transport into the inner membrane and the matrix is unknown. Here, we report that protein transport across and into the inner membrane is impaired in PC-deficient mitochondria. Surprisingly, depletion of PC does not affect stability and activity of the respiratory supercomplexes and the membrane potential is maintained. Instead, the dynamic TIM23 complex is destabilized when the PC levels are reduced, whereas the TIM22 complex remains intact. Our analysis revealed that initial precursor binding to the TIM23 complex is impaired in PC-deficient mitochondria. We conclude that reduced PC levels differentially affect the TIM22 and TIM23 complexes in mitochondrial protein transport.

INTRODUCTION

Mitochondria fulfill essential functions for the survival of the cell like energy conversion to produce ATP, synthesis of amino acids, lipids and heme as well as the generation of iron-sulphur clusters. They contain about 1000 proteins in yeast and 1500 proteins in humans (1, 2). More than 99% of the mitochondrial proteins are synthesized as precursors on cytosolic ribosomes. Mitochondria contain a sophisticated system of protein translocases to import precursor proteins (3-9). The translocase of the outer membrane (TOM complex) forms the general entry gate for most of the precursor proteins. After passage of the TOM channel distinct protein translocases sort the preproteins into the different subcompartments: the outer and inner membrane as well as the two aqueous compartments the matrix and intermembrane space.

The majority of mitochondrial proteins are sorted into the inner membrane and the matrix. Two inner membrane-bound protein complexes mediate protein import: The presequence translocase (also termed TIM23 complex) transports precursor proteins with a cleavable presequence into the inner membrane and the matrix, whereas the carrier translocase (also termed TIM22 complex) inserts proteins with multiple transmembrane segments into the inner membrane (3-9). The membrane potential across the inner membrane provides the driving force for both protein import pathways and is generated by the activity of the respiratory chain. Presequence-containing preproteins are directly transferred from the TOM complex to the TIM23 complex (10, 11). The TIM23 complex consists of five subunits: Tim23 forms the translocation channel, which is in close association with Tim17 (12, 13). The intermembrane space exposed domains of Tim23, Tim50 and Tim21 facilitate preprotein transfer from the TOM complex to the Tim23 channel (14-17). The fifth TIM23 subunit Mgr2 (mitochondrial genome required) controls sorting of preproteins into the inner membrane and stabilizes the association of Tim21 with the translocase

(18, 19). The TIM23 complex laterally releases precursor proteins into the inner membrane (14, 20). For transport into the mitochondrial matrix the TIM23 complex dynamically associates with the presequence translocase-associated motor (PAM). The ATP-consuming activity of the mitochondrial Hsp70 within the PAM module completes preprotein transport into the matrix (3-9). Finally, the presequence is removed by the mitochondrial processing peptidase. Precursors of carrier proteins lack such a cleavable presequence. Small TIM chaperones guide these hydrophobic preproteins from the TOM complex to the carrier translocase. The TIM22 complex consists of four membrane-bound subunits. Tim54 mediates the docking of the preprotein-loaded small TIM chaperones to the carrier translocase (21-23). Tim22 forms a twin-pore to insert preproteins into the inner membrane (24-26). Finally, Tim18 and Sdh3 are required to warrant assembly and stability of the TIM22 complex (27-29).

Mitochondrial membranes contain five major phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin (CL). The majority of these phospholipids are synthesized in the endoplasmic reticulum. CL and PE are synthesized in the inner mitochondrial membrane (30-34). The CL biosynthesis pathway consists of multiple steps (30-34), whereas PE is produced by decarboxylation of PS by the phosphatidylserine decarboxylase 1 (Psd1) (35-37). In yeast Psd1 is the major source of cellular PE under standard growth conditions (38). However, further sources for cellular PE exist. Psd2 of the vacuolar/Golgi membrane, the activity of the acyltransferases Tgl3 and Ale2 as well as the CDP-ethanomamine (Kennedy pathway) produce additional amounts of PE (38-42). Isolated CL and PE do not form membrane bilayer structures and are regarded as non-bilayer forming lipids (30, 43). Both phospholipids are crucial for mitochondrial function and morphology (44-46). Double deletion of Psd1 with the cardiolipin synthase

Crd1 is lethal confirming the closely related functions of CL and PE (47). Studies of the last years revealed specific roles of CL and PE in protein transport into mitochondrial subcompartments (30, 31, 48). In mutants defective in CL or PE synthesis the biogenesis of outer membrane b-barrel proteins is affected (49, 50). Whereas CL is also required for the import of proteins with multiple α-helical membrane spans into the outer membrane, this import pathway remains largely unaffected in PE deficient mitochondria (50, 51). Furthermore, both phospholipids promote protein transport into the inner membrane and matrix (52-57). First, binding of preproteins to the TOM complex is disturbed in PE and CL deficient mitochondria (49, 50). Second, the activity of the respiratory chain complexes, in particular of the cytochrome c oxidase (complex IV) is decreased in mitochondria with reduced PE or CL content (57-59). Consequently, the membrane potential is decreased and fails to drive protein translocation via TIM23 or TIM22 translocases (52, 55, 57). CL and PE exhibit distinct roles in the stability of protein complexes. Whereas deletion of CL affects the stability of respiratory chain supercomplexes as well as of the TOM and TIM23 translocases, these protein complexes remain largely intact in PE deficient mitochondria (49, 50, 53-58, 60-62). CL associates with respiratory chain complexes (63, 64) and stabilizes the interaction of the cytochrome c reductase (complex III) and complex IV via its negatively charged headgroup (65).

The role of bilayer-forming phospholipids in mitochondrial function is poorly understood. Phosphatidylcholine (PC) is the most abundant phospholipid of the mitochondrial membranes (66, 67). Two pathways in the endoplasmic reticulum produce cellular PC. First, within the Kennedy pathway free choline is activated via phosphorylation and subsequent binding to CDP. CDP-choline is then linked to diacylglycerol to form PC (31, 68). Second, PE can be methylated in three steps to

produce PC. Pem1/Cho2 promotes the first methylation step, whereas Pem2/Opi3 is capable of performing all three methylation steps, but the last two with higher efficiency (69-74). PC is essential for the survival of the cell (72). Recent studies revealed that cells with decreased PC levels show a reduced growth under non-fermentative conditions and that the biogenesis of outer membrane β-barrel proteins is impaired in mitochondria isolated from these cells (75). The role of PC for protein transport into the inner mitochondrial subcompartments is unknown.

We studied protein transport into the inner membrane and matrix in mitochondria isolated from mutants defective in PC biosynthesis. We found that the import of both precursors with a cleavable presequence and carrier proteins is reduced in the mutant mitochondria. Strikingly, depletion of PC does not decrease the activity of the respiratory chain nor the membrane potential across the inner membrane. Decreased PC levels differentially affect the stability of TIM23 and TIM22 translocases. Whereas TIM23 translocase is destabilized, the TIM22 complex remains intact. The arrest of a preprotein in the TOM-TIM23 supercomplex is impaired, indicating that PC affects initial binding of precursor proteins to the TIM23 translocase. Altogether, depletion of PC specifically affects the function of inner membrane protein translocases of mitochondria.

EXPERIMENTAL PROCEDURE

Yeast strains, growth conditions and isolation of mitochondria

The yeast strains pem1Δ, pem2Δ, psd1Δ and their corresponding wild-type BY4741 have been described (75). The cells were grown at 30°C on minimal medium containing a non-fermentable carbon source (0.67% [w/v] yeast nitrogen base without amino acids (Difco), 0,077% [w/v] SC amino acid mixture (MP Biomedicals), 2% [v/v] glycerol or lactate and 0.1-0.2% [w/v] glucose). Cells were harvested at mid-logarithmic growth phase and mitochondria were isolated by differential centrifugation as described (76). In brief, the cell wall was disrupted by incubation with 0.1 M Tris/HCl pH 9.4, 10 mM DTT and subsequent treatment with zymolase (Nacalai Tesque) in 1.2 M sorbitol, 20 mM KP_i pH 7.2. The generated spheroplasts were resuspended in homogenizing buffer (0.6 M sorbitol, 10 mM Tris/HCl pH 7.4, 1 mM EDTA, 1 mM PMSF and 0.2% [w/v] BSA) and disintegrated by using a Dounce Homogenizer. Subsequently, cell debris was removed by centrifugation and mitochondria were isolated. Mitochondrial protein concentration was adjusted to 10 mg/ml in washing buffer (10 mM MOPS/KOH pH 7.2, 1 mM EDTA, 250 mM sucrose). Subsequently, mitochondria were shock frozen in liquid nitrogen and stored at -80°C until use.

Protein import into isolated mitochondria and blue native electrophoresis

Radiolabeled precursor proteins were synthesized in a cell-free translation system based on reticulocyte lysate (TNT, Promega) in the presence of [^{35}S]methionine. For the import reaction isolated mitochondria were incubated with the radiolabeled precursor

protein in import buffer (3% [w/v] BSA, 250 mM sucrose, 5 mM methionine, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS/KOH pH 7.2, 10 mM KH_2PO_4) containing 2 mM ATP, 2 mM NADH, 5 mM creatine phosphate and 0.1 mg/ml creatine kinase. The import reaction was stopped by transfer on ice and by addition of 8 mM antimycin, 1 mM valinomycin and 20 mM oligomycin to dissipate the membrane potential. In control reactions the membrane potential was dissipated before the import reaction was started. Non-imported proteins were removed by treatment with proteinase K (50 mg/ml) for 15 min on ice. The protease was inactivated by an excess amount of PMSF. Mitochondria were reisolated and washed with washing buffer. The mitochondrial pellet was solubilized in Laemmli buffer and proteins were separated by SDS-PAGE. The import of carrier proteins was studied by blue native electrophoresis. To this end, mitochondria were reisolated after the import reaction, washed with washing buffer, resuspended in lysis buffer (20 mM Tris/HCl pH 7.4; 50 mM NaCl, 0.1 mM EDTA, 10% [v/v] glycerol) containing 1% [w/v] digitonin and incubated for 15 min on ice. The sample was subjected to centrifugation to remove insoluble material. Protein complexes were separated by blue native electrophoresis (77).

Arrest of precursor proteins

To arrest precursors of cytochrome b_2-DHFR, AAC-DHFR and Om45-DHFR in the TOM complex, import reactions were performed as described above. The stable folding of the DHFR-domain was induced by the presence of 5 mM methotrexate (10, 11). Oxa1 was accumulated in the TOM complex upon dissipation of the membrane potential (57, 75).

In-gel activity assays

The activity of the F_1F_O-ATP-synthase was determined by in-gel activity assays (78, 79). Mitochondrial protein complexes were separated by blue native electrophoresis. The blue native gel was washed in ATP buffer (5 mM $MgCl_2$, 50 mM glycine pH 8.4, 20 mM ATP) for 20 minutes at room temperature. Subsequently, the gel was incubated with 10% [w/v] $CaCl_2$ solution until the CaP_i precipitate appeared. The activity of the cytochrome c oxidase was determined by in-gel activity stain (80). Protein complexes were separated by blue native electrophoresis. The gel was washed with 50 mM KP_i pH 7.2. Subsequently, the gel was incubated with 1 mg/ml reduced horse cytochrome c in 50 mM KP_i pH 7.2 in the presence of 1 mg/ml diaminobenzidine to stain active cytochrome c oxidase.

Measurement of the membrane potential

To determine the membrane potential across the inner mitochondrial membrane, isolated mitochondria were incubated with the fluorescent dye 3,3-dipropylthiadicarbocyanine iodide ($DISC_3$) in potential buffer (0.6 M sorbitol, 0.1% [w/v] BSA, 10 mM $MgCl_2$, 0.5 mM EDTA, 20 mM KP_i pH 7.2) supplemented with 5 mM succinate and 5 mM malate (55, 57). $DISC_3$ is taken up by mitochondria in a membrane-potential-dependent manner, which results in quenching of the fluorescence signal at 670 nm (52). To determine the specificity of the measured signals, valinomycin was added to 1 mM final concentration, which dissipates the membrane potential leading to a release of $DISC_3$ from mitochondria. All measurements were performed with an

Aminco Bowman II Luminescence Spectrometer (Thermo Electron) using a Hellma-101.OS cuvette and the AB2 software (Thermo Electron).

Phospholipid analysis

Extraction of phospholipids from isolated mitochondria was performed with a 2:1 (v/v) mixture of chloroform/methanol as described (37, 75, 81). The lipids in the organic phase were washed with 0.034 % [w/v] $MgCl_2$, a 4:1 (v/v) mixture of 2 N KCl/methanol and subsequently with a mixture of methanol/water/chloroform (48:47:3, per vol.). Two-dimensional thin-layer chromatography was used to separate the individual phospholipid classes (82). Finally, phospholipids were stained with iodine vapor, scrapped off and quantified (83).

Miscellaneous

For the detection of mitochondrial proteins we used a large set of polyclonal antisera. To exclude cross-reactions all immunosignals were validated with mitochondria isolated from the corresponding mutant strains (57,84). Proteins were transferred from SDS-PAGE and blue native gels onto a PVDF membrane (EMD Milipore) via semi-dry Western blotting. Immunosignals were detected by enhanced chemiluminescence (85) and visualized on x-ray films (Medix XBU) or via LAS3000 image reader (FujiFilm). Radiolabeled proteins were visualized by autoradiography (Storm imaging system, GE Healthcare and FLA-9000, FujiFilm). We used the Image J version 1,46r (National Institutes of Health) software to analyze autoradiograms. Images were processed with Photoshop CS5 (Adobe) and arranged in figures using Illustrator CS5 (Adobe). Where

indicated by separating white lines non-relevant bands were digitally removed. Quantifications were performed with the Image Quant version 5.2 (GE Healthcare) software.

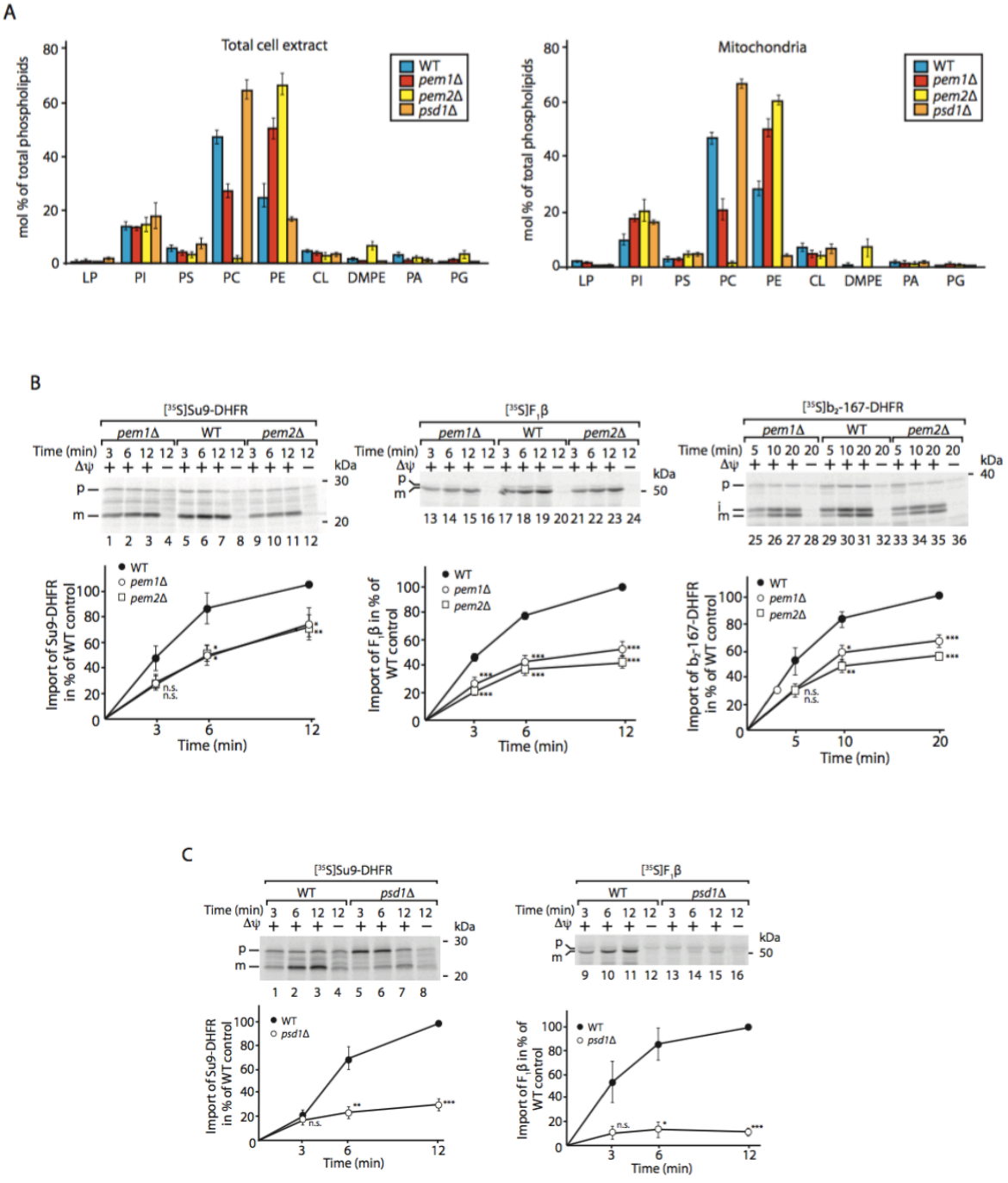
RESULTS

Depletion of PC impairs protein transport into the inner membrane and the matrix

To study the role of PC in protein transport into and across the inner mitochondrial membrane, we chose *pem1*Δ and *pem2*Δ strains, which are defective in the methylation pathway of PE to produce PC (69-74). Yeast cells were grown in minimal medium to block the synthesis of PC from free choline via the Kennedy pathway. We used a non-fermentable carbon source to promote mitochondrial function. We determined the phospholipid profiles in total cell extract and in isolated mitochondria. The relative amounts of PC were strongly reduced in the cell extract and mitochondria of both mutants, but more severely in *pem2*Δ mitochondria (Fig. 1A). As reported the content of PE was drastically increased (47, 69-72, 75) (Fig. 1A). In *pem2*Δ mitochondria the monomethylated form of PE accumulated (47, 69-72), but was not separated here from PE. For comparison, we determined the phospholipid profile of *psd1*Δ mutant, which was grown under the same conditions like *pem1*Δ and *pem2*Δ cells. Particularly, mitochondrial PE is strongly reduced, whereas the relative levels of total cellular PE are only mildly decreased in the *psd1*Δ mutant (Fig. 1A). The PC content is strongly increased in cell extract and mitochondria compared to wild-type cells (Fig. 1A). Under these growth conditions PC in *psd1*Δ cells is synthesized by methylation of PE, which is predominantly produced by Psd2 (38). Thus, it is crucial for the yeast cells to maintain

certain amounts of PC/PE although the ratio can vary drastically. In the PC and PE-deficient mutants the mitochondrial PI levels are increased, whereas other the content of other phospholipids remains largely comparable to wild-type (Fig. 1A).

We isolated mitochondria to analyze import of presequence-containing proteins. We chose three model precursor proteins for our studies: Precursor of the model preprotein Su9-DHFR and of the F_1b-subunit of the F_1F_O-ATP-synthase are transported into the matrix, whereas the precursor of cytochrome b_2-DHFR is sorted into the inner membrane. Radiolabeled precursor proteins were synthesized and incubated with isolated mitochondria. Non-imported precursor proteins were removed by addition of proteinase K. Successful translocation into mitochondria can be monitored by the detection of the mature band, which is formed after proteolytic removal of the presequence by the mitochondrial processing peptidase. The precursor of cytochrome b_2-DHFR is processed in a second step by Imp1 (86), which results in the presence of an intermediate band. The import of all three precursor proteins was moderately reduced in *pem1*Δ and *pem2*Δ mitochondria (Fig. 1B). For comparison imports of Su9-DHFR and F1b-subunit of were strongly impaired in *psd1*Δ (Fig. 1C). Carrier proteins lack such a cleavable presequence. To determine the import of the ADP-ATP carrier (AAC) and of the dicarboxylate carrier (DiC), we monitored their assembly into the inner membrane. Therefore, mitochondria were solubilized with the mild detergent digitonin and protein complexes were separated by blue native electrophoresis. The import of both carrier proteins was impaired in *pem1*Δ and *pem2*Δ mitochondria (Fig. 2A). Again, the import of the AAC precursor was more strongly affected in *psd1*Δ mitochondria (Fig. 2B). Altogether, we conclude that reduced PC levels affect protein import via the presequence and carrier pathway. The TIM23- and TIM22-dependent protein import is more severely impaired in PE-deficient mitochondria.


A
Total cell extract
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mol % of total phospholipids
WT
pem1Δ
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psd1Δ
LP PI PS PC PE CL DMPE PA PG
B
[35S]Su9-DHFR
[35S]F1β
[35S]b2-167-DHFR
Time (min)
Δψ
kDa
p
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Import of Su9-DHFR in % of WT control
Import of F1β in % of WT control
Import of b2-167-DHFR in % of WT control
Time (min)
n.s.
C
[35S]Su9-DHFR
[35S]F1β
WT
psd1Δ

Figure 1. Import of presequence-containing precursor proteins into the mitochondrial inner membrane and matrix is affected in PC-deficient mitochondria. (A) The relative amounts of phospholipids from total cell extract (left panel) and isolated mitochondria (right panel) from *pem1*Δ, *pem2*Δ and *psd1*Δ cells were determined. Depicted are mean values of three independent experiments with their corresponding S.E.M.. LP, lyso-phospholipids; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol. (B) ^{35}S-labeled precursors of Su9-DHFR, F_1b and cytochrome b_2-167-DHFR were imported for the indicated time periods into wild-type (WT), *pem1*Δ and *pem2*Δ mitochondria. Non-imported precursor proteins were proteolytically removed by proteinase K. Upper panel, the import reaction was analyzed by SDS-PAGE and autoradiography. p, precursor form. i, import intermediate; m, mature protein. Lower panel, quantifications of the import reactions of the upper panel are shown. Depicted are the mean values and their corresponding S.E.M. of 7 (Su9-DHFR), 6 (F_1b) and 4 (b_2-167-DHFR) independent import experiments. Statistical significant differences based on an unpaired t-test of the individual import time points in mutant mitochondria related to wild-type control are depicted (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. not significant). (C) ^{35}S-labeled precursors of Su9-DHFR and F_1b were imported for the indicated time periods into wild-type (WT) and *psd1*Δ mitochondria. Non-imported precursor proteins were proteolytically removed by proteinase K. Upper panel, the import reaction was analyzed by SDS-PAGE and autoradiography. p, precursor form. i, import intermediate; m, mature protein. Lower panel, quantifications of the import reactions of the upper panel are shown. Depicted are the mean values and their corresponding S.E.M. of 4 (Su9-DHFR) and 3 (F_1b) independent import experiments. Statistical significant differences based on an unpaired t-test of the individual import time points in mutant mitochondria related to wild-type control are depicted (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. not significant).

PC is not required for stability and activity of the respiratory chain

Two scenarios are conceivable to explain the defective protein import into and across the inner mitochondrial membrane in the mutant mitochondria. First, the membrane potential could be decreased, which would cause a delayed protein transport. Second, the functions of the TIM23 and TIM22 translocases could be disturbed. To experimentally address the first possibility, we determined the membrane potential of

isolated mitochondria. In this assay, a fluorescent dye is taken up by isolated mitochondria in a membrane potential-dependent manner, which results in a quenching of the fluorescence signal (52). Fluorescence signals close to wild-type mitochondria indicate that the membrane potential is intact. Reduced membrane potential results in less quenching of the fluorescence compared to the wild-type control. Addition of the ionophore valinomycin dissipates the membrane potential, which causes a release of the dye from mitochondria and restoration of the fluorescent signal (Fig. 3). Unexpectedly, following this experimental strategy we observed that the membrane potential remained largely unaffected in *pem1*Δ and *pem2*Δ compared to wild-type mitochondria (Fig. 2). Thus, protein import into or across the inner membrane of PC-deficient mitochondria is not impaired by dissipated membrane potential. In contrast, the membrane potential was compromised in mitochondria isolated from *psd1*Δ (Fig. 3) cells like reported previously (57). The diminished membrane potential in *psd1*Δ mitochondria leads to strongly impaired protein import via the TIM23 and TIM22 complexes (Figs. 1C and 2B). We conclude that the membrane potential was differentially affected by depletion of PC or PE.

The non-bilayer forming phospholipids PE and CL are required for full activity of the respiratory chain, which generates the membrane potential across the inner membrane (52, 55, 57-59). Thus, we wondered whether the respiratory chain complexes were present and functional in PC-deficient mitochondria. The steady state levels of various subunits of the respiratory chain were largely unchanged in mutant compared to wild-type mitochondria (Fig. 4A). We only observed a mild reduction of a few components of the cytochrome c oxidase (complex IV) (Fig. 4A). Cytochrome c reductase (complex III) and complex IV of the respiratory chain form supercomplexes, which can be analyzed by blue native electrophoresis. In yeast, one or two copies of

complex IV associate with a dimer of complex III (87, 88). We found that these supercomplexes were present in *pem1*Δ and *pem2*Δ like in wild-type mitochondria (Fig. 4B, lanes 1-15). Furthermore, the reduced PC content did not affect the stability of the succinate dehydrogenase (complex II) and of the F_1F_O-ATP-synthase (Fig. 4B, lanes 16-21). We conclude that the respiratory chain complexes were not destabilized in *pem1*Δ and *pem2*Δ mitochondria. In *psd1*Δ mitochondria respiratory chain supercomplexes are stable, but the activity of particular complex IV is decreased (57). To directly visualize the activity of respiratory chain supercomplexes, we performed in-gel activity stain of complex IV (80). Strikingly, the amount of active complex IV was not reduced in *pem1*Δ and *pem2*Δ mitochondria (Fig. 4C). Similarly, the activity of the monomeric and dimeric F_1F_O-ATP-synthase (complex V) was not affected in the mutant mitochondria (Fig. 4D). We conclude that depletion of PC did not affect the stability and function of the respiratory chain supercomplexes. Consequently, the respiratory chain is capable to establish a membrane potential in PC-deficient mitochondria, which is comparable to wild-type mitochondria.

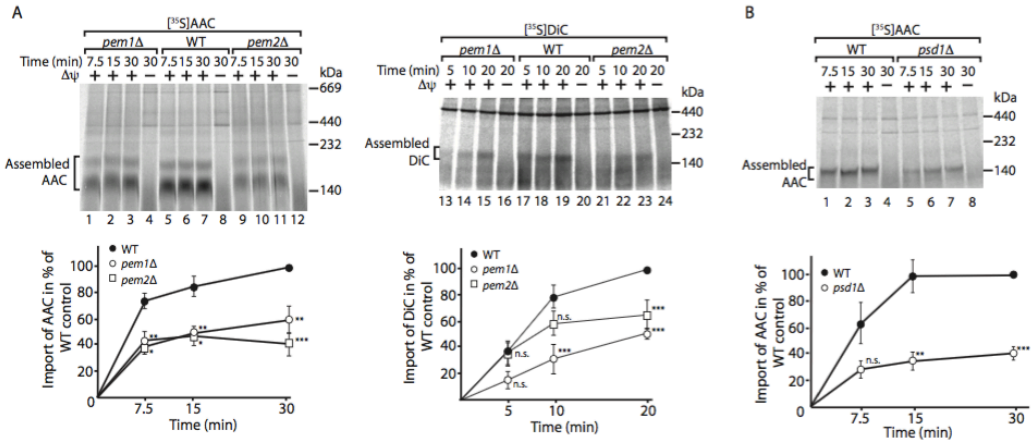


Figure 2. Import of carrier proteins into the mitochondrial inner membrane and matrix is affected in PC-deficient mitochondria. (A) ^{35}S-labeled precursors of AAC and DiC were imported for the indicated time periods into wild-type (WT), *pem1*Δ and *pem2*Δ mitochondria. Upper panel, the import reaction was analyzed by blue native electrophoresis and autoradiography. Lower panel, quantifications of the import reactions of the upper panel are shown. Depicted are the mean values and their corresponding S.E.M. of 5 independent import experiments. Statistical significant differences based on an unpaired t-test of the individual import time points in mutant mitochondria related to wild-type control are depicted (*p < 0.05; **p < 0.01; ***p < 0.001; n.s. not significant). (B) ^{35}S-labeled precursor of AAC was imported for the indicated time periods into wild-type (WT) and *psd1*Δ mitochondria. Upper panel, the import reaction was analyzed by blue native electrophoresis and autoradiography. Lower panel, quantifications of the import reactions of the upper panel are shown. Depicted are the mean values and their corresponding S.E.M. value of 5 independent import experiments. Statistical significant differences based on an unpaired t-test of the individual import time points in mutant mitochondria related to wild-type control are depicted (**p < 0.01; ***p < 0.001; n.s. not significant).

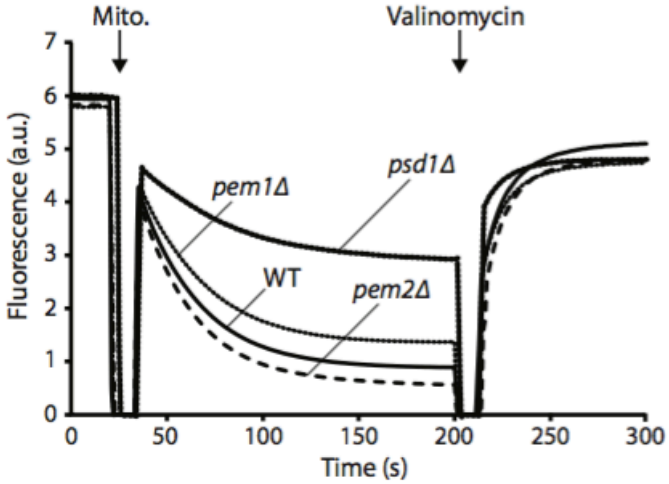


Figure 3. The membrane potential across the inner mitochondrial membrane is not affected in PC-deficient mitochondria. The membrane potential of wild-type (WT), *pem1*Δ, *pem2*Δ and *psd1*Δ mitochondria was determined by the membrane potential-dependent uptake of the fluorescent dye $DISC_3$ into mitochondria as described in

experimental procedures. When indicated valinomycin was added to release $DISC_3$ from mitochondria. Mean values of three independent measurements are depicted.

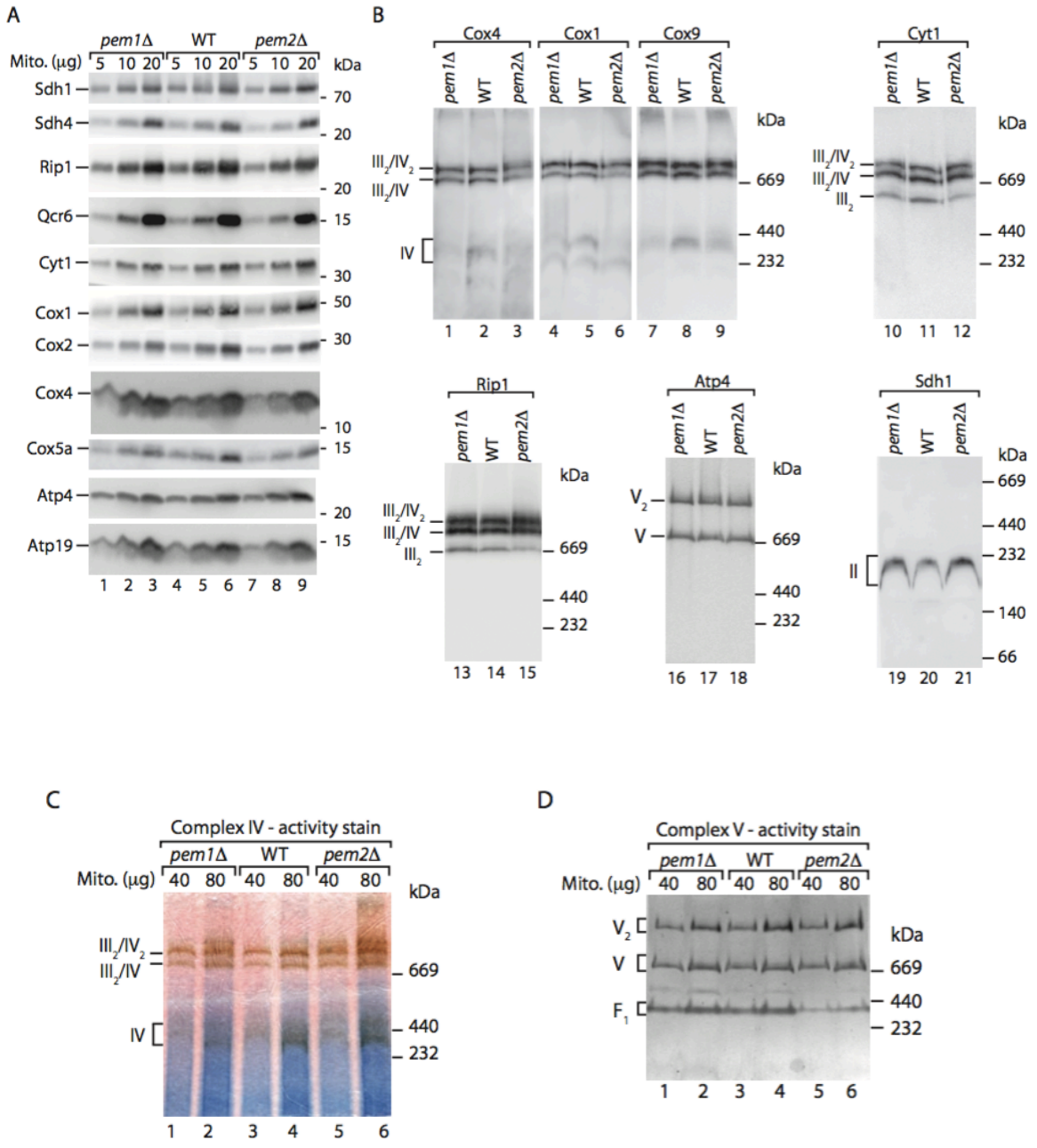


Figure 4. Depletion of PC does not perturb stability and activity of the respiratory chain supercomplexes (A) The indicated amounts of mitochondrial proteins from wild-

type (WT), *pem1*Δ and *pem2*Δ cells were separated by SDS-PAGE and detected by immunodetection with the indicated antisera. Sdh, succinate dehydrogenase, Qcr6, subunit 6 of the ubiquinol-cytochrome c oxidoreductase (complex III); Rip1, Rieske iron-sulfur protein 1 (complex III), Cox, subunit of the cytochrome c oxidase (complex IV), Atp, subunit of the F_1F_O-ATP-synthase (complex V) (B) WT, *pem1*Δ and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. Protein complexes were analyzed by immunodetection with the indicated antisera. (C) WT, *pem1*Δ and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. The activity of complex IV (cytochrome c oxidase) was detected by in-gel activity stain. (D) WT, *pem1*Δ and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. The activity of complex V (F_1F_O-ATP-synthase) was detected by in-gel activity stain.

Depletion of PC differentially affects the stability of inner membrane protein translocases

We excluded the possibility that a decreased membrane potential caused the defective protein import across and into the inner membrane of PC-depleted mitochondria. Therefore, we asked whether the stability of the protein translocases was affected in PC-deficient mutant mitochondria. The steady state levels of various subunits of the TIM23 and TIM22 translocases as well as of control proteins were unchanged in *pem1*Δ and *pem2*Δ in comparison to wild-type mitochondria (Fig. 5A). Notably, the steady state levels of Tim10 were largely unaffected in these mutant mitochondria as well (Fig. 5A) (75). Small TIM chaperones like Tim10 are essential for transport of carrier precursors through the intermembrane space to the TIM22 translocase (22, 23). Next, we analyzed the stability of the protein translocases by blue native electrophoresis and Western blotting. The TIM23 translocase is a highly dynamic protein complex, which forms two complexes on a blue native gel: The TIM23 core complex consists of Tim23,

Tim17 and Tim50, whereas the TIM23 sorting form additionally contains Tim21 (14, 53). Strikingly, in *pem1*Δ and *pem2*Δ mitochondria both TIM23 forms were reduced when detected with antibodies raised against Tim17 or Tim23 (Fig. 5B). Thus, depletion of PC affects the integrity of the TIM23 translocase. In contrast, the formation of the TIM22 complex remained largely unaltered in *pem1*Δ and *pem2*Δ mitochondria as shown by detection with antibodies specific for Tim22 and Tim54 (Fig. 5C). As control the MIA machinery, the TOM complex and the Hsp60 ring complexes were normally formed in the mutant mitochondria (Fig. 5D). We conclude that depletion of PC differentially affects the stability of the inner membrane protein translocases. Whereas the dynamic TIM23 complex is destabilized, the carrier translocase is formed normally.

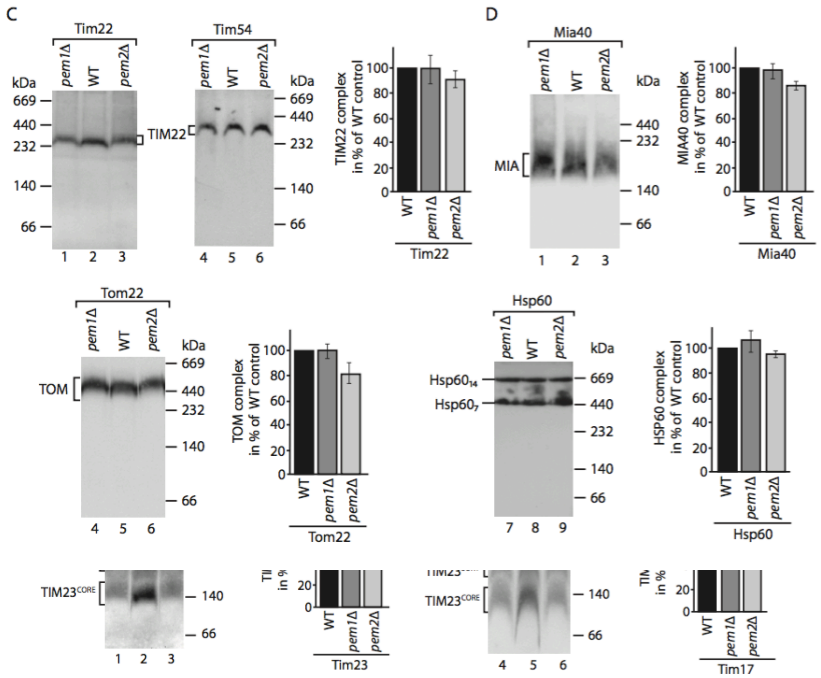


Figure 5. Depletion of PC differentially affects the stability of inner membrane protein translocases. (A) The indicated amounts of mitochondrial proteins from wild-type (WT), *pem1*Δ and *pem2*Δ cells were separated by SDS-PAGE and detected by immunodetection with the indicated antisera. Mgr2, mitochondrial genome required;

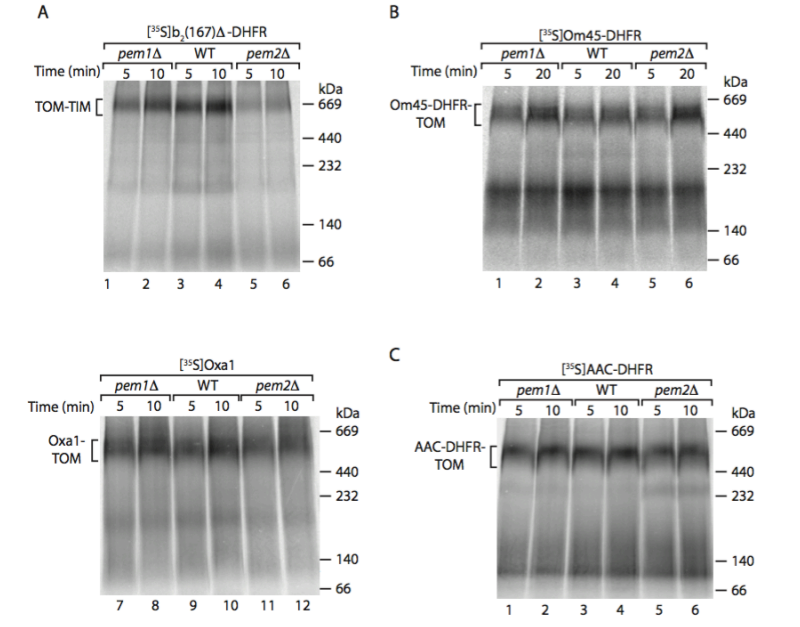
AAC, ADP-ATP carrier; Mge1, mitochondrial GrpE; Ccp1, cytochrome c peroxidase 1; Aco1, aconitase, Ilv2, isoleucine plus valine requiring (B - D) WT, *pem1*Δ and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. Protein complexes were analyzed by immunodetection with the indicated antisera. TIM23, presequence translocase; TIM22, carrier translocase; MIA, mitochondrial intermembrane space import and assembly; TOM, translocase of the outer membrane; $Hsp60_7$, Hsp60 heptamer; $Hsp60_{14}$, Hsp60 tretadecamer. Quantifications of the protein complexes were shown. Depicted are the mean values with their corresponding S.E.M. of 4 ($Hsp60_{14}$), 7 (Tim17 (TIM23core), Tim22, Mia40), 8 (Tim23 (Tim23core) and 9 (Tom22) independent experiments. Statistical significant differences based on an unpaired t-test for the TIM23 complex in mutant mitochondria related to wild-type control are depicted (**$p < 0.01$; ***$p < 0.001$).

Depletion of PC affects precursor transfer to the TIM23 complex

Precursor proteins are first transported across the outer membrane via the TOM complex and then directly transferred to the TIM23 translocase. We wondered whether the initial binding of the precursor to the TIM23 complex is affected in PC-deficient mitochondria. To this end, we arrested a variant of the precursor of cytochrome b_2 (b_2(167)D-DHFR) that lacks the inner membrane-sorting signal at an early import stage. In the presence of a membrane potential and methotrexate the radiolabeled cytochrome b_2 portion passes the TOM complex and engages the TIM23 complex. Addition of methotrexate induces a stable folding of the DHFR-moiety, which blocks its passage through the TOM channel. Consequently, the cytochrome b_2(167)D-DHFR construct gets arrested in the TOM-TIM23 supercomplex, which can be analyzed by blue native electrophoresis (10, 11). Strikingly, the accumulation of this precursor in the TOM-TIM23 supercomplex was decreased in both mutant mitochondria, but particularly in *pem*2Δ mitochondria (Fig. 6A). One possibility is that the reduced PC levels affect the function of the TOM complex. However, the accumulation of TIM23-

dependent precursors like Om45-DHFR (89, 90) and Oxa1 at the TOM complex was not decreased (Fig. 6B) (75). Furthermore, the TOM complex remains intact in PC-deficient mitochondria (Fig. 5D) (75). We conclude that depletion of PC affects initial recognition of the precursor by the TIM23 complex. To investigate whether the import of mitochondrial carrier proteins was affected at the TOM-stage, we imported AAC fused to DHFR (AAC-DHFR) into isolated mutant mitochondria. Upon import the majority of the precursor binds to the TOM complex, but is not further transported into the inner membrane (91, 92). Imported AAC-DHFR precursor efficiently accumulated at the TOM complex in *pem1*Δ and *pem2*Δ mitochondria (Fig. 6C), indicating that the binding of carrier protein to the TOM complex was not compromised. We conclude that reduced PC content does not affect initial binding of the carrier precursor to the TOM complex, but impairs the import of the precursor into the inner membrane by the TIM22 complex. Altogether, transport of precursor proteins into the inner membrane and matrix is affected at the stage of the TIM translocases in PC-deficient mitochondria.

Figure 6. Arrest of a precursor protein in the TOM-TIM23 supercomplex is



defective in PC-deficient mitochondria. (A) ^{35}S-labeled precursor of cytochrome b_2(167)D-DHFR was imported for the indicated time periods into wild-type (WT), *pem1*Δ and *pem2*Δ mitochondria in the presence of methotrexate. The import reaction was analyzed by blue native electrophoresis and autoradiography. (B) ^{35}S-labeled precursors of Om45-DHFR and Oxa1 were imported for the indicated time periods into wild-type (WT), *pem1*Δ and *pem2*Δ mitochondria. The import of Om45-DHFR was performed in the presence of methotrexate. The arrest of the Oxa1 precursor at the TOM complex was studied after dissipation of the membrane potential. The samples were analyzed by blue native electrophoresis and autoradiography. (C) ^{35}S-labeled precursor of AAC-DHFR was imported for the indicated time periods into wild-type (WT), *pem1*Δ and *pem2*Δ mitochondria in the presence of methotrexate. The import reaction was analyzed by blue native electrophoresis and autoradiography.

DISCUSSION

We report that depletion of PC affects protein import into the mitochondrial inner membrane and matrix. Surprisingly, the stability and activity of respiratory chain supercomplexes are not reduced in mitochondria isolated from *pem1*Δ and *pem2*Δ cells. Consequently, the membrane potential generated by the respiratory chain is sufficient to drive protein transport in the mutant mitochondria. However, we could demonstrate that the TIM23 complex is destabilized and that preprotein arrest in a TOM-TIM23 supercomplex is impaired in PC-deficient mutant mitochondria. Contrary, accumulation of TIM23- and TIM22-dependent precursor proteins at the TOM complex is not altered. We conclude that PC affects protein transport into and across the inner membrane at the stage of the TIM translocases.

Our studies on mitochondrial protein biogenesis revealed unexpected specific effects of reduced PC levels on protein machineries. Depletion of PC selectively affects the stability of highly dynamic protein translocases like the TIM23 complex of the inner membrane and the sorting and assembly machinery (SAM complex) of the outer

membrane (75). The SAM complex mediates folding and insertion of β-barrel proteins in the mitochondrial outer membrane (3-5, 7, 8). It interacts with different partner proteins to promote protein biogenesis. First, the SAM complex interacts with the TOM complex to facilitate transfer of β-barrel precursors (76, 93). Second, it associates with the mitochondrial division and morphology protein Mdm10 to promote the assembly of Tom22 into the TOM complex (94-96). Depletion of PC affects both the biogenesis of β-barrel proteins and the assembly of Tom22 (75). The initial binding of the β-barrel precursor to the TOM-SAM supercomplex is decreased in PC-depleted mitochondria (75). The TIM23 complex mediates protein transport into the matrix and lateral release into the inner membrane. The TIM23 subunits dynamically interact with the PAM module for transport into the matrix (3-9). Furthermore, the TIM23 complex interacts with the TOM complex and precursor proteins can be arrested in a TOM-TIM23 supercomplex (10, 11, 14). In PC-deficient mitochondria the stability of the TIM23 complex is decreased and both import pathways into the matrix and inner membrane are affected. Furthermore, binding of a precursor protein to the TOM-TIM23 supercomplex is impaired upon depletion of PC. Since precursor accumulation at the TOM complex remains unaffected the initial binding of preproteins by the TIM23 complex is impaired in mutants defective in PC biosynthesis. In contrast to the TIM23 complex the TOM translocase and the respiratory chain supercomplexes are not destabilized in PC deficient mitochondria (75). Moreover, the integration of the SAM-independent model precursors Tom20 and Om45 are not impaired in mitochondria with reduced PC levels (75). We conclude that depletion of PC selectively affects distinct protein transport pathways, but does not generally compromise protein import into mitochondria. This is an unexpected finding since PC is the most abundant phospholipid in mitochondrial membranes.

Previous studies have revealed roles of the non-bilayer forming phospholipids PE and CL for protein import into and across both mitochondrial membranes (49-57). Depletion of PE and CL affects precursor accumulation at the TOM complex and the activity of the respiratory chain (49, 57-57), which both remain unaffected upon depletion of PC. CL stabilizes respiratory chain supercomplexes and the TOM complex (49, 60-61). In contrast, respiratory chain supercomplexes and the TOM complex are formed in mitochondria with reduced content of either PE or PC (57, 75). The dynamic TIM23 and SAM complexes are particularly sensitive towards alterations of the phospholipid composition of the mitochondrial membranes. The TIM23 translocase is not destabilized when PE levels are reduced (55), whereas depletion of PC or CL affects the integrity of the TIM23 forms on blue native gels. Whether impaired activity of the TIM23 complex contributes to defective protein import into and across the inner membrane in PE-deficient mitochondria remains unclear. In these mitochondria the membrane potential is strongly reduced, which blocks protein transport via inner membrane protein translocases (57). Import of b-barrel proteins into the mitochondrial outer membrane requires the presence of CL, PE and PC (49, 50, 75). However, the SAM complex is destabilized in mutant mitochondria impaired in PC or CL but not in PE biosynthesis. Furthermore, the SAM-dependent assembly of Tom22 requires normal PC levels (75), but is unaffected in CL or PE deficient mitochondria. All these examples illustrate that phospholipids exhibits surprisingly specific roles for distinct protein translocases and respiratory chain complexes in mitochondria.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

TB conceived and coordinated the study and wrote the manuscript. MHS, FDB, CMA and TB designed, performed and analyzed the experiments together with GD. MHS and TB prepared the figures. All authors reviewed results and approved the final version of the manuscript

REFERENCES

1. Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R.P., Meyer, H. E., Schönfisch, B., Perschill, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N., and Meisinger, C. (2003) The proteome of Saccharomyces cerevisae mitochondria. Proc. Natl. Acad. Sci. U. S. A. 100, 13207-13212
2. Pagliarini, D. J., Calvo, S. E., Chang, B., Sheth, S. A., Vafai, S. B., Ong, S.-E., Walford, G. A., Sugjana, C., Boneh, A., Chen, W. K., Hill, D. E., Vidal, M., Evans, J. G., Thorburn, D. R., Carr, S. A., and Mootha, V. K. (2008) A mitochondrial protein compendium elucidates complex I disease biology. Cell 134, 112-123
3. 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
4. Neupert, W., and Herrmann, J. M. (2007) Translocation of proteins into mitochondria. Annu. Rev. Biochem. 76, 723-749
5. Endo, T., Yamano, K., and Yamano, K. (2011) Structural insights into the mitochondrial protein import system. Biochim. Biophys. Acta 1808, 955-970
6. Marom, M., Azem, A., and Mokranjac, D. (2011) Understanding the molecular mechanism of protein translocation across the mitochondrial inner membrane: still a long way to go. (2011) Biochim. Biophys. Acta 1808, 990-1001
7. Dimmer, K. S., and Rapaport, D. (2012) Unsolved mysteries in the biogenesis of mitochondrial membrane proteins. Biochim Biophys Acta 1818, 1085-1090
8. Becker, T., Böttinger, L., and Pfanner, N. (2012) Mitochondrial protein import: from transport pathways to an integrated network. Trends Biochem. Sci. 37, 85-91
9. Schulz, C., Schendzielorz, A., and Rehling, P. (2015) Unlocking the presequence import pathway. Trends Cell Biol. 25, 265-275
10. Dekker, P. J. T., Martin, F., Maarse, A. C., Bömer, U., Müller, H., Guiard, B., Meijer, M., Rassow, J., and Pfanner, N. (1997) The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70-Tim44. EMBO J. 16, 5408-5419
11. Chacinska, A., Rehling, P., Guiard, B., Frazier, A. E., Schulze-Specking, A., Pfanner, N., Voos, W., and Meisinger, C. (2003) Mitochondrial translocation contact sites: separation of dynamic and stabilizing elements in formation of a TOM-TIM-preprotein supercomplex. EMBO J. 22, 5370–5381
12. Truscott, K. N., Kovermann, P., Geissler, A., Merlin, A., Meijer, M., Driessen, A. J., Rassow, J., Pfanner, N., and Wagner, R. (2001) A presequence- and voltage-

sensitive channel of the mitochondrial preprotein translocase formed by Tim23. Nat. Struct. Biol. 8, 1074-1082
13. Martinez-Caballero, S., Grigoriev, S. M., Herrmann, J. M., Campo, M. L., and Kinnally, K. W. (2007) Tim17p regulates the twin pore structure and voltage gating of the mitochondrial protein import complex TIM23. J. Biol. Chem. 282, 3584-3593
14. Chacinska, A., Lind, M., Frazier, A., Meyer, H. E., Truscott, K. N., Guiard, B., Pfanner, N., and Rehling, P. (2005) Mitochondrial presequence translocase: switching between TOM tethering and motor recruitment involves Tim21 and Tim17. Cell 120, 817-829
15. Mokranjac, D., Popov-Čeleketić, J., Hell, K., and Neupert, W. (2005) Role of Tim21 in mitochondrial translocation contact sites. J. Biol. Chem. 280, 23437–23440
16. Tamura, Y., Harada, Y., Shiota, T., Yamano, K., Watanabe, K., Yokota, M., Yamamoto, K., Sesaki, H., and Endo, T. (2009) Tim23-Tim50 pair coordinates functions of translocators and motor proteins in mitochondrial protein import. J. Cell Biol. 184, 129-141
17. Waegemann, K., Popov-Čeleketić, D., Neupert, W., Azem, A., and Mokranjac, D. (2015) Cooperation of TOM and TIM23 complexes during translocation of proteins into mitochondria. J. Mol. Biol. 427, 1075-1084
18. Gebert, M., Schrempp, S. G., Mehnert, C. S., Heisswolf, A. K., Oeljeklaus, S., Ieva, R., Bohnert, M., von der Malsburg, K., Wiese, S., Kleinschroth, T., Hunte, C., Meyer, H. E., Haferkamp, I., Guiard, B., Warscheid, B., Pfanner, N., and van der Laan, M. (2012) Mgr2 promotes coupling of the mitochondrial presequence translocase to partner complexes. J. Cell Biol. 197, 595–604
19. Ieva, R., Schrempp, S. G., Opaliński, Ł., Wollweber, F., Höß, P., Heißwolf, A. K., Gebert, M., Zhang, Y., Guiard, B., Rospert, S., Becker, T., Chacinska, A., Pfanner, N., and van der Laan, M. (2014) Mgr2 functions as lateral gatekeeper for preprotein sorting in the mitochondrial inner membrane. Mol. Cell 56, 641-652
20. van der Laan, M., Meinecke, M., Dudek, J., Hutu, D. P., Lind, M., Perschil, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007) Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. Nat. Cell Biol. 9, 1152-1159
21. Kerscher, O., Holder, J., Srinivasan, M., Leung, R. S., and Jensen, R. E. (1997) The Tim54p-Tim22p complex mediates insertion of proteins into the mitochondrial inner membrane. J. Cell Biol. 139, 1663-1675.
22. Sirrenberg, C., Endres, M., Fölsch, H., Stuart, R. A., Neupert, W., and Brunner, M. (1998) Carrier protein import into mitochondria mediated by the

intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5. Nature 381, 912-915
23. Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmid, K., Schweyen, R.J., and Schatz, G. (1998) Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. Science 279, 369-373
24. Kovermann, P., Truscott, K. N., Guiard, B., Rehling, P., Sepuri, N. B., Müller, H., Jensen R. E., Wagner, R., and Pfanner, N. (2002) Tim22, the essential core of the mitochondrial protein insertion complex, forms a voltage-activated and signal-gated channel. Mol. Cell 9, 363-373
25. Rehling, P., Model, K., Brandner, K., Kovermann, P., Sickmann, A., Meyer, H. E., Kühlbrandt, W., Ragner, R., Truscott, K. N., and Pfanner, N. (2003) Protein insertion into the mitochondrial inner membrane by a twin-pore translocase. Science 299, 1747-1751
26. Peixoto, P. M. V., Grana, F., Roy, T. J., Dunn, C. D., Flores, M., Jensen, R. E., and Campo, M. L. (2007) Awaking TIM22, a dynamic ligand-gated channel for protein insertion in the mitochondrial inner membrane. J. Biol. Chem. 282, 18694-18701
27. Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000) Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. Mol. Biol. Cell 11, 103-116
28. Koehler, C. M., Murphy, M. P., Bally, N. A., Leuenberger, D., Oppliger, W., Dolfini, L., Junne, T., Schatz, G., and Or, E. (2000) Tim18p, a new subunit of the TIM22 complex that mediates insertion of imported proteins into the yeast mitochondrial inner membrane. Mol. Cell. Biol. 20, 1187-1193
29. Gebert, N., Gebert, M., Oeljeklaus, S., von der Malsburg, K., Stroud, D. A., Kulawiak, B., Wirth, C., Zahedi, R. P., Dolezal, P., Wiese, S., Simon, O., Schulze-Specking, A., Truscott, K. N., Sickmann, A., Rehling, P., Guiard, B., Hunte, C., Warscheid, B., van der Laan, M., Pfanner, N., and Wiedemann, N. (2011) Dual function of Sdh3 in the respiratory chain and TIM22 protein translocase of the mitochondrial inner membrane. Mol. Cell 44, 811-818
30. Osman, C., Voelker, D. R., and Langer, T. (2011) Making heads or tails of phospholipids in mitochondria. J. Cell. Biol. 192, 7-16
31. Horvath, S. E., and Daum, G. (2013) Lipids of mitochondria. Prog. Lipid Res. 52, 590-614.
32. Claypool, S. M., and Koehler, C. M. (2012) The complexity of cardiolipin in health and disease. Trends Biochem Sci. 37, 32-41
33. Tamura, Y., Sesaki, H., and Endo, T. (2014) Phospholipid transport via mitochondria. Traffic 15, 933-945.

34. Ye, C., Shen, Z., and Greenberg, M. L. (2015) Cardiolipin remodeling: a regulatory hub for modulating cardiolipin metabolism and function. J. Bioenerg. Biomembr. Doi 10.1007/s10863-014-9591-7
35. Clancey, C. J., Chang, S. C., and Dowhan, W. (1993) Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from Saccharomyces cerevisiae by complementation of an Escherichia coli mutant. J. Biol. Chem. 268, 24580-24590
36. Trotter, P. J., Pedretti, J., and Voelker, D. R. (1993) Phosphatidylserine decarboxylase from Saccharomyces cerevisiae: isolation of mutants, cloning of the gene, and creation of a null allele. J. Biol. Chem. 268, 21416-21424
37. Horvath, S. E., Böttinger, L., Vögtle, F. N., Wiedemann, N., Meisinger, C., Becker, T., and Daum, G. (2012) Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1. J. Biol. Chem. 287, 36744-36755
38. 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
39. 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
40. Riekhof, W. R., and Voelker, D. R. (2006) Uptake and utilization of lyso-phosphatidylethanolamine by Saccharomyces cerevisiae. J. Biol. Chem. 281, 36588-36596
41. 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
42. Rajakumari, S., and Daum, G. (2010) Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Mol. Biol. Cell 21, 501-510
43. 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
44. DeVay, R. M., Dominguez-Ramirez, L., Lackner, L. L., Hoppins, S., Stahlberg, H., and Nunnari, J. (2009) Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. J. Cell Biol. 186, 793-803
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. Joshi, A. S., Thompson, M. N., Fei, N., Hüttemann, M., and Greenberg, M. L. (2012) Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in Saccharomyces cerevisiae. J. Biol. Chem. 287, 17589-17597
47. 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
48. Böttinger, L., Ellenrieder, L., and Becker, T. (2015) How lipids modulate mitochondrial protein import. J. Bioenerg. Biomembr. Doi 10.1007/s10863-015-9599-7
49. 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
50. Becker, T., Horvath, S. E., Böttinger, L., Gebert, N., Daum, G., and Pfanner, N. (2013) Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins. J. Biol. Chem. 288, 16451-16459
51. Sauerwald, J., Jores, T., Eisenberg-Bord, M., Chuartzman, S. G., Schuldiner, M., and Rapaport, D. (2015) Genome-wide screens in yeast highlight a role for cardiolipin in biogenesis of mitochondrial outer membrane multispan proteins. Mol. Cell Biol. 35, 3200-3211
52. Jiang, F., Ryan, M. T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., and Greenberg, M. L. (2000) Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. J. Biol. Chem. 275, 22387-22394
53. 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
54. 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
55. 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

56. Tamura, Y., Endo, T., Iijima, M., and Sesaki, H. (2009) Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. J. Cell Biol. 185, 1029-1045
57. Böttinger, L., Horvath, S. E., Kleinschroth, T., Hunte, C., Daum, G., Pfanner, N., and Becker, T. (2012) Phosphatidylethanolamine and cardiolipin differently affect the stability of mitochondrial respiratory chain supercomplexes. J. Mol. Biol. 423, 677-686
58. Pfeiffer, K., Gohil, V., Stuart, R. A., Hunte, C., Brandt, U., Greenberg, M. L., and Schägger, H. (2003). Cardiolipin stabilizes respiratory chain supercomplexes. J. Biol. Chem. 278, 52873-52880
59. Tasseva, G., Bai, H. D., Davidescu, M., Haromy, A., Michelakis, E., and Vance, J. E. (2013) Phosphatidylethanolamine deficiency in mammalian mitochondria impairs oxidative phosphorylation and alters mitochondrial morphology. J. Biol. Chem. 288, 4158-4173
60. Zhang, M., Mileykovskaya, E., and Dowhan, W. (2002) Gluing the respiratory chain together: cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J. Biol. Chem. 277, 43553-43556.
61. Zhang, M., Mileykovskaya, E., and Dowhan, W. (2005) Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. J. Biol. Chem. 280, 29403-29408
62. 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
63. Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) Specific roles of protein-phospholipid interactions in the yeast cytochrome bc_1 complex structure. EMBO J. 20, 6591-6600
64. Shinzawa-Itho, K., Aoyama, H., Muramoto, K., Terada, H., Kurauchi, T., Tadehara, Y. Yamasaki, A., Sugimura, T., Kurono, S., Tsujimoto, K., Mizushima, T., Yamashita, E., Tsukihara, T., and Yoshikawa, S. (2007) Structures and physiological roles of 13 integral lipids of bovine heart cytochrome c oxidase. EMBO J. 26, 1713-1725
65. Wenz, T., Hielscher, R., Hellwig, P., Schägger, H., Richers, S., and Hunte, C. (2009) Role of phospholipids in respiratory cytochrome bc_1 complex catalysis and supercomplex formation. Biochim Biophys Acta 1787, 609-616
66. Sperka-Gottlieb, C. D. M., Hermetter, A., Paltauf, F., and Daum, G. (1988) Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, Saccharomyces cerevisiae. Biochim. Biophys. Acta 946, 227-234

67. Zinser, E., Sperka-Gottlieb, C. D. M., 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
68. Cole, L. K., Vance, J. E., and Vance, D. E. (2012) Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochim Biophys Acta 1821, 754-761
69. Greenberg, M. L., Klig, L. S., Letts, V. A., Loewy, B. S., and Henry, S. A. (1983) Yeast mutant defective in phosphatidylcholine synthesis. J. Bacteriol. 153, 791-799
70. Kodaki, T., and Yamashita, S. (1987) Yeast phosphatidylethanolamine methylation pathways: Cloning and characterization of two distinct methyltransferase genes. J. Biol. Chem. 262, 15428-15435
71. Summers, E. F., Letts, V. A., McGraw, P., and Henry, S. A. (1988) Saccharomyces cerevisiae cho2 mutants are deficient in phospholipid methylation and cross-pathway regulation of inositol synthesis. Genetics 120, 909-922
72. Kodaki, T., and Yamashita, S. (1989) Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. Eur. J. Biochem. 185, 243-251
73. Gaynor, P. M., and Carman, G. M. (1990) Phosphatidylethanolamine methyltransferase and phospholipid methyltransferase activities from Saccharomyces cerevisiae. Enzymological and kinetic properties. Biochim Biophys Acta 1045, 156-163
74. Preitschopf, W., Lückl, H., Summers, E., Henry, S. A., Paltauf, F., and Kohlwein, S. D. (1993) Molecular cloning of the yeast OPI3 gene as a high copy number suppressor of the cho2 mutation. Curr. Genet. 23, 95-101
75. Schuler, M.-H., Di Bartolomeo, F., Böttinger, L., Horvath, S. E., Wenz, L.-S., Daum, G., and Becker, T. (2015) Phosphatidylcholine affects the role of the sorting and assembly machinery in the biogenesis of mitochondrial b-barrel proteins. J. Biol. Chem. 290, 26523-26532
76. Wenz, L.-S., Ellenrieder, L., Qiu, J., Bohnert, M., Zufall, N., van der Laan, M., Pfanner, N., Wiedemann, N., and Becker, T. (2015) Sam37 is crucial for formation of the mitochondrial TOM-SAM supercomplex, thereby promoting b-barrel biogenesis. J. Cell. Biol. 210, 1047-1054
77. 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

78. Bornhövd, C., Vogel, F., Neupert, W., and Reichert, A. S. (2006) Mitochondrial membrane potential is dependent on the oligomeric state of the F_1F_o-ATP-synthase supracomplexes. J. Biol. Chem. 281, 13990-13998
79. Wagner, K., Perschil, I., Fichter, C. D., and van der Laan, M. (2010) Stepwise assembly of dimeric F_1F_o-ATP synthase in mitochondria involves the small F_o-subunits k and i. Mol. Biol. Cell, 21, 1494-1504
80. Wittig, I., Karas, M., and Schägger, H. (2007) High-resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol. Cell Proteomics 6, 1215-1225
81. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509
82. Schneiter, R., and Daum, G. (2006) Analysis of yeast lipids. Methods Mol. Biol. 313, 75-84
83. Broekhuyse, 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
84. Böttinger, L., Guiard, B., Oeljeklaus, S., Kulawiak, B., Zufall, N., Wiedemann, N., Warscheid, B., van der Laan, M., and Becker, T. (2013) A complex of Cox4 and mitochondrial Hsp70 plays an important role in the assembly of the cytochrome c oxidase. Mol. Biol. Cell 24, 2609-2619
85. Haan, C., and Behrmann, I. (2007) A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. J. Immunol. Methods 318, 11-19
86. Nunnari, J., Fox, T. D., and Walter, P. (1993) A mitochondrial protease with two catalytic subunits of nonoverlapping speficities. Science 262, 1997-2004
87. Schägger, H., and Pfeiffer, K. (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J. 19, 1777-1783
88. Cruciat, C.-M., Brunner, S., Baumann, F., Neupert, W., and Stuart, R. A. (2000) The cytochrome bc_1 and cytochrome c oxidase complexes associate to form a single supracomplex in yeast mitochondria. J. Biol. Chem. 275, 18093-18098.
89. Wenz, L.-S., Opaliński, L., Schuler, M.-H., Ellenrieder, L., Ieva, R., Böttinger, L., Qiu, J., van der Laan, M., Wiedemann, N., Guiard, B., Pfanner, N., and Becker, T. (2014) The presequence pathway is involved in protein sorting to the mitochondrial outer membrane. EMBO Rep. 15, 678–685
90. Song, J., Tamura, Y., Tohru, Y., and Endo, T. (2014) A novel import route for an N-anchor mitochondrial outer membrane protein aided by the TIM23 complex.

EMBO Rep. 15, 670-677.
91. Wiedemann, N., Pfanner, N., and Ryan, M. T. (2001) The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. EMBO J. 20, 951-960
92. 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 differently interacts with preproteins, small Toms, and import receptors. Mol. Cell. Biol. 21, 2337-2348
93. Qiu, J., Wenz, L.-S., Zerbes, R. M., Oeljeklaus, S., Bohnert, M., Stroud, D. A., Wirth, C., Ellenrieder, L., Thornton, N., Kutik, S., Wiese, S., Schulze-Specking, A., Zufall, N., Chacinska, A., Guiard, B., Hunte, C., Warscheid, B., van der Laan, M., Pfanner, N., Wiedemann, N., and Becker, T. (2013) Coupling of mitochondrial import and export translocases by receptor-mediated supercomplex formation. Cell 154, 596-608
94. Meisinger, C., Rissler, M., Chacinska, A., Sanjuán Szklarz, L. K., Milenkovic, D., Kozjak, V., Schönfisch, B., Lohaus, C., Meyer, H. E., Yaffe, M. P., Guiard, B., Wiedemann, N., and Pfanner, N. (2004) The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Dev. Cell 7, 61-71
95. 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 a-helical outer membrane proteins. J. Mol. Biol. 396, 540-549
96. Yamano, K., Tanaka-Yamano, S., and Endo, T. (2010) Mdm10 as a dynamic constituent of the TOB/SAM complex directs coordinated assembly of Tom40. EMBO Rep. 11, 187-193

General summary and conclusion

This thesis presents an overview on the metabolism of phospholipids and their interaction with the protein complexes located in the mitchondrial membranes of *Saccharomyces cerevisiae*. The mitochondrial membranes are differentiated for both their architecture but also for the protein and lipid composition and the ratio between these two main membrane constituents [1–4].

The foremost reactions that associate with the phosphalipids metabolism are concentrated at the level of the endoplasmic reticulum (ER) [5]. Despite the ER compartmentalization of the phospholipids metabolic pathway, mitochondria play a very central and decisive role as they are the site where cardiolipin (CL) and the majority of phosphatidylethanolamine (PE) are synthesized. In this context the mechanisms for exchange and transfer of phospholipids between ER and mitochondria is of fundamental importance and is the subject of many current studies [2,6–8].

In this thesis the following points have been taken into consideration (i) analysis of the mitochondrial enzyme Psd1p and its product, PE, through a collection of the most recent data (ii) understanding the molecular mechanisms and the role of the previously identified *putative substrate recognition seuqence* located in the α-subunit of Psd1p (iii) investigation on the interactions between the bilayer forming phospholipid phosphatidylcholine and the import/sorting machinery in the mitchondria.

The first chapter of this thesis provides with an accurate description regarding a class of enzymes, the phosphatidylserine decarboxylases (PSDs), and their relevant contribution in the phospholipid metabolism of different organisms. The high diffusion of PSDs in many kingdoms of life it's a further indication of their central role in phophoslipid metabolism [9]. The PSDs enyzmes are pyruvoyl-dependent decarboxylases whose main feature is the presence of a pyruvoyl prostetic group generated from an internal serine residue, which also represents the core of the catalytic site of the enzyme [10,11]. During the self-processing and generation of the active site, PSDs precursor proteins

are divided into two dissimilar subunits which constitute the mature form of the enzyme [12].

The second chapter describes a very detailed analysis of the biochemical and molecular mechanisms that characterize the phosphatidylserine decarboxylases 1 (Psd1p) from yeast. Psd1p is known and studied for many years, but only recently aspects concerning the topology, the import mechanism in the mitochondria and the biogenesis of Psd1p have been discovered [13].

The main aim of the research described in the second chapter is investigating the function of a consensus motif localized in the α-subunits of Psd1p, which is supposedly involved in the recognition and binding of Psd1p's substrate, phosphatidylserine (PS) [14]. Different mutations inserted along the conserved motif brought a variety of outcomes in the biochemical analysis that allowed pinpointing specific residues along the sequence that are essential for the processing, biogenesis and stability of Psd1p. The results obtained show that this *putative substrate recognition site* retains a number of highly conserved amino acids that are essential for the catalytic activity of the enzyme. Despite the many pieces of information collected on the functional domain of the Psd1p α-subunit, there are still points that await to be clarified. In fact it is not sure wheter this conserved motif indeed plays an active role in identifying and binding Psd1p's substrate. Another hypothesis is whether this conserved sequence is involved in the interaction and association of Psd1p with other mitochondrial proteins that might ensure the acitvity and stability of the enzyme. In fact, considering the key role of Psd1p, and the strategic location at the mitochondria level, it remains to be clarified whether there exist one or more direct interaction(s) with other membrane components that could be, for instance, involved in the transport of PE in the rest of the cell.

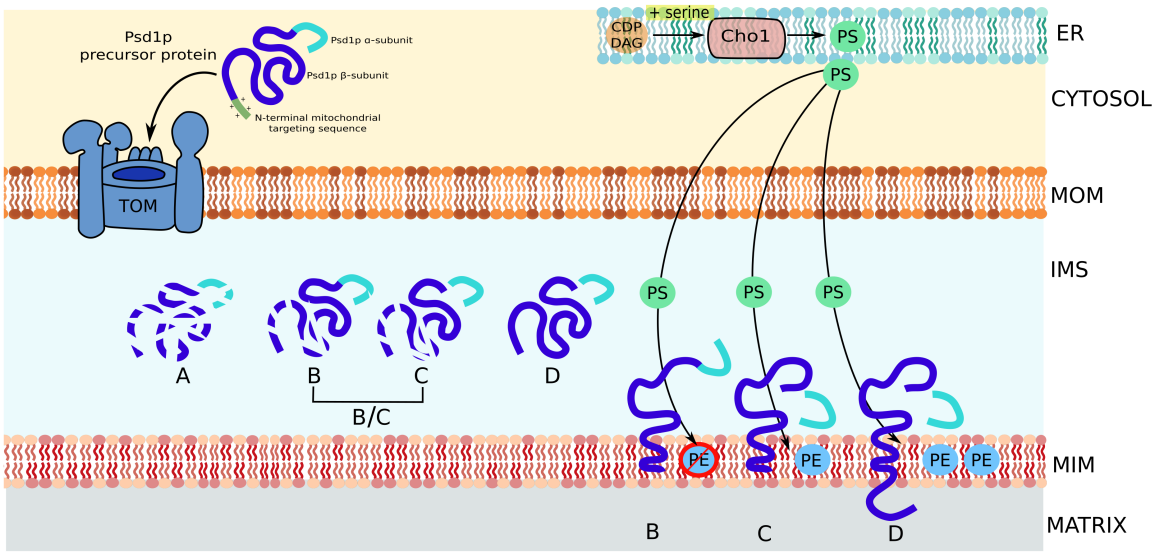

Figure 1 Various mutations in the putative substrate recognition sequence of Psd1p α-subunits differently affect processing and stability of Psd1p Manipulation in the conserved motif of the α-subunit compromises the stability of Psd1p which might results in : A, complete loss of protein stability and degradation of both a and b-subunit; B, instability of the enzyme with degradation starting from the N-terminus of the protein and lack of self processing; C, structural instability of the enzyme with slower rate of self processing; D, enzyme structurally stable and capable of self process. Since the interaction of the α- and β-subunit and the self processing are mandatory for enzymatic catalysis, the unprocessed (B) and the structurally unstable form of Psd1p (C) have an absent or a reduced catalytic activity.

The second part of this thesis is the result of a collaboration with the research group of Dr. T. Becker and Pr. N. Pfanner (Freiburg, Germany).

Compared with the previous two chapters this section is characterized by a change in perspective. In fact the study of a single key enyzme, described in the second chapter, is contextualized by exploring the intricate interactions that exist between the lipid and protein components of mitochondrial membranes.

Chapter 3 and 4 are charactrized by a common denominator that is the functional analysis of the molecular machinery located in the mitochondrial membranes, in correlation with the membrane phospholipids, which regulate the activity of these protein complexes.

It is well established that CL and PE modulate the activity of mitochondrial protein complexes involved in the import and sorting of protein within the mitochondrial membranes [15–17]. In the two studies presented as part of this thesis, there are new evidences showing that also PC plays an active role in influencing the import of β-barrels, as well as the activity of the translocases of the inner mitochondrial membrane. Furthermore, these data confirm the hypothesis that any different phospholipids can impact the protein complexes in a very specific manner, which differs from one another.

In conclusion, in order to have a complete comprehension of the phospholipid-protein functional correlation at the membrane level, it is important to untangle the molecular details related to this interaction. For instance it is still not clear yet which are the factors that govern the heterogeneous group of effects triggered by the different phspholipids. One speculation would be related to the chemical properties of the phospholipids or to the presence of specific lipid microdomains, like the microdomains of CL and PE, already identified in bacterial membranes which can self-assemble and seems to be relevant in cellular processes like cell division and sporulation [18–20].

REFERENCES

[1] E. Zinser, G. Daum, Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*, Yeast. 11 (1995) 493–536. doi:10.1002/yea.320110602.

[2] E. Zinser, C.D. Sperka-Gottlieb, E.V. Fasch, S.D. Kohlwein, F. Paltauf, G. Daum, Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*, J. Bacteriol. 173 (1991) 2026–2034.

[3] P.R. Cullis, B. de Kruijff, Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta. 559 (1979) 399–420.

[4] E.M. Mejia, G.M. Hatch, Mitochondrial phospholipids: role in mitochondrial function, J. Bioenerg. Biomembr. 48 (2015) 99–112. doi:10.1007/s10863-015-9601-4.

[5] G. Achleitner, D. Zweytick, P.J. Trotter, D.R. Voelker, G. Daum, Synthesis and intracellular transport of aminoglycerophospholipids in permeabilized cells of the yeast, *Saccharomyces cerevisiae*, J. Biol. Chem. 270 (1995) 29836–29842.

[6] M. Schlame, D. Haldar, Cardiolipin is synthesized on the matrix side of the inner membrane in rat liver mitochondria, J. Biol. Chem. 268 (1993) 74-79.

[7] V. Kainu, M. Hermansson, S. Hänninen, K. Hokynar, P. Somerharju, Import of phosphatidylserine to and export of phosphatidylethanolamine molecular species from mitochondria, Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids. 1831 (2013) 429–437.

[8] G. Achleitner, B. Gaigg, A. Krasser, E. Kainersdorfer, S.D. Kohlwein, A. Perktold, G. Zellnig, G. Daum, Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact, Eur. J. Biochem. 264 (1999) 545–553. doi:10.1046/j.1432-1327.1999.00658.x.

[9] F. Di Bartolomeo, A. Wagner, G. Daum, Cell biology, physiology and enzymology of phosphatidylserine decarboxylase, Biochim. Biophys. Acta (2016). In press.

[10] W. Dowhan, Phosphatidylserine decarboxylases: pyruvoyl-dependent enzymes from bacteria to mammals, Methods Enzymol. 280 (1997) 81–88.

[11] P.D. van Poelje, E.E. Snell, Pyruvoyl-dependent enzymes, Annu. Rev. Biochem. 59 (1990) 29–59.

[12] L.J. Rizzolo, Kinetics and protein subunit interactions of *Escherichia coli* phosphatidylserine decarboxylase in detergent solution, Biochemistry (Mosc.). 20 (1981) 868–873.

[13] S.E. Horvath, L. Böttinger, F.N. Vögtle, N. Wiedemann, C. Meisinger, T. Becker, G. Daum, Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1, J. Biol. Chem. 287 (2012) 36744–36755.

[14] K. Igarashi, M. Kaneda, A. Yamaji, T.C. Saido, U. Kikkawa, Y. Ono, K. Inoue, M. Umeda, 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 (1995) 29075–29078.

[15] L. Böttinger, L. Ellenrieder, T. Becker, How lipids modulate mitochondrial protein import, J. Bioenerg. Biomembr. 48 (2015) 125–135. doi:10.1007/s10863-015-9599-7.

[16] T. Becker, S.E. Horvath, L. Böttinger, N. Gebert, G. Daum, N. Pfanner, Role of phosphatidylethanolamine in the biogenesis of mitochondrial outer membrane proteins, J. Biol. Chem. 288 (2013) 16451–16459.

[17] L. Böttinger, S.E. Horvath, T. Kleinschroth, C. Hunte, G. Daum, N. Pfanner, T. Becker, Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes, J. Mol. Biol. 423 (2012) 677–686. doi:10.1016/j.jmb.2012.09.001.

[18] L.D. Renner, D.B. Weibel, Cardiolipin microdomains localize to negatively curved regions of *Escherichia coli* membranes, Proc. Natl. Acad. Sci. USA 108 (2011) 6264–6269.

[19] A. Nishibori, J. Kusaka, H. Hara, M. Umeda, K. Matsumoto, Phosphatidylethanolamine domains and localization of phospholipid synthases in *Bacillus subtilis* membranes, J. Bacteriol. 187 (2005) 2163–2174.

[20] K. Matsumoto, J. Kusaka, A. Nishibori, H. Hara, Lipid domains in bacterial membranes, Mol. Microbiol. 61 (2006) 1110–1117.

Curriculum Vitae

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Education

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09/2010 - 04/2013	University of Study of Molise, Italy. MSc in Molecular and Cellular Biology. Title of MSc thesis: "Functional Food: The role of Fructans".
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Professional skills and competence

- Expertise in techniques of Molecular Biology and Biochemistry: Cloning procedures, Isolation and separation of nucleic acids and protein, PCRs, Construction and screening of a gene library, Protein purification, Enzyme assays, Agarose gel Electrophoresis, SDS-PAGE and immunoassays.
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- Experience with HPLC chromatographic techniques.
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Di Bartolomeo F, Wagner A, Daum G (2016) Cell Biology, Physiology and Enzymology of Phosphatidyserine decarboxylase BBA- Molecular and Cell Biology of Lipids 2016, doi:10.1016/j.bbalip.2016.09.007

Schuler M.H, **Di Bartolomeo F**, Daum G, Becker T (2016) Phosphatidylcholine Affects Inner Membrane Protein Translocases of Mitochondria. J Biol Chem. 2016 Sep 2;291(36):18718-29. doi: 10.1074/jbc.M116.722694. Epub 2016 Jul 11.

Schuler M.H, **Di Bartolomeo F**, Böttinger L, Horvath S. E, Wenz L. S, Daum G, Becker T (2015) Phosphatidylcholine affects the role of the sorting and assembly machinery in the biogenesis of mitochondrial β-barrel proteins. J Biol Chem. 2015 Oct 30;290(44):26523-32. doi: 10.1074/jbc.M115.687921. Epub 2015 Sep 18.

Di Bartolomeo F, Van den Ende W (2015) Fructose and Fructans: Opposite Effects on Health? Plant Foods Hum Nutr. 2015 Sep;70(3):227-37. doi: 10.1007/s11130-015-0485-6. Review.

Di Bartolomeo F, Startek J & Van den Ende W (2013) Prebiotics to fight diseases: reality or fiction? Phytother Res. 2013 Oct;27(10):1457-73. doi: 10.1002/ptr.4901. Epub 2012 Dec 27. Review.

Conferences attended

09/2016	57th International Conferene on the Bioscience of Lipids (ICBL). Chamonix, Mont-Blanc, France, September 4-8, 2016. Poster presentation

06/2015	FEBS/EMBO Advanced Lecture Course "Biomembranes: Molecular Architecture, Dynamics and Function" Cargese, Corsica, France, June 15-25, 2015. Poster presentation
05/2015	12th Yeast Lipid Conference. Ghent, Belgium, May 20-22, 2015. Poster presentation
09/2014	12th EuroFed Lipids Congres, "Oils, Fats and Lipids: from Lipidomics to Industrial Innovation". 14-17 September 2014. Poster presentation
05/2013	"Fascination of Plants Day" workshop organized by the Department of Biosciences and Territory (DiBT), University of Molise. Poster presentation

Distinctions

- Recognized with the Best Poster Awards at the 57th International Conference on the Bioscience of Lipids 4th – 8th September 2016 – Chamonix, France.
- Awarded with the EMBO Short term fellowship for research funding (ASTF No: 181-2015) Project title: Functional and topological characterization of Phosphatidylserine decarboxylase 1 (Psd1p) from the yeast
- Graduated Summa Cum Laude for both BSc and MSc

References

- Pr. Günther Daum, PhD supervisor - *guenther.daum*@tugraz.at
- Pr. Nikolaus Pfanner, director of the hosting department during my research stay in the Institute of Biochemistry and Molecular Biology, University of Freiburg *nikolaus.Pfanner*@biochemie.uni-freiburg.de
- Dr. Thomas Becker, supervisor and project leader during my research stay in Institute of Biochemistry and Molecular Biology, University of Freiburg thomas.becker@biochemie.uni-freiburg.de
- Dr. Karin Athenstaedt, Group leader with long lasting cooperation with the all the members of Pr, Daum's group - karin.athenstaedt@tugraz.at