Dipl.-Ing. Ariane Wagner

Formation and subcellular distribution of phospholipids in the yeast *Saccharomyces cerevisiae*

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Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Günther Daum
Institut für Biochemie
Technische Universität Graz

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Zusammenfassung

Die am häufigsten vorkommenden Phospholipide in Hefe -, Pflanzen - und Säugetierzellen sind Phosphatidylcholin (PC) und Phosphatidylethanolamin (PE). PE kann in Eukaryonten durch zwei unterschiedliche Wege synthetisiert werden: (i) Phosphatidylserin wird durch Phosphatidylserindecarboxylase Typ I (Psd1) und Typ II (Psd2) decarboxyliert oder (ii) Ethanolamin wird nach Aktivierung mit CTP über den sogenannten Kennedy Weg in PE eingebaut. Die überwiegende Menge an PE wird in den Mitochondrien durch Psd1 geformt. PSDs werden in vielen Zelltypen gefunden, einschließlich Hefen, Pflanzen, Säugetieren und Parasiten. In der Hefe *Saccharomyces cerevisiae* ist Psd1 an die innere Membran über Membrandomänen gebunden und orientiert sich in Richtung Intermembranraum. Da die Assemblierung und Funktion von Phosphatidylserindecarboxylase in Hefe nur wenig untersucht ist, haben wir in dieser Studie die Membrandomänen genauer untersucht und herausgefunden, dass Psd1 mindestens zwei Membrandomänen (IM1 und IM2) besitzt. Deletionen innerhalb der zweiten Membrandomäne (IM2) führen zu falscher Lokalisierung der Proteine mit einer Orientierung zur Matrix oder zur äußeren mitochondriellen Membran und zu Defekten in Import, Prozessierung und Aktivität von Psd1. Die Deletion von Psd1 führt zu einer Verringerung der Menge an Phosphatidylethanolamin und zu einer Hochregulierung der Glykogen Phosphorylase Gph1, welche die Umwandlung von Glykogen zu Glukose in Hefe katalysiert. Die Deletion dieses Gens hat eine Reduktion von Phosphatidylcholin (PC), Triacylglycerolen und Sterolestern zur Folge. Depletion der zwei unpolaren Lipide in einem Δ*gph1* Deletionsstamm führt zu fehlenden Lipidpartikeln und zu einer Verringerung des PC Gehalts, welche eine instabile Plasma Membran zur Folge hat. In der Hefe *Saccharomyces cerevisiae* wird Phosphatidylcholin (PC) über zwei Stoffwechselwege synthetisiert: (i) durch Methylierung von Phosphatidylethanolamin durch die Methyltransferasen Cho2p/Pem1p und Opi3p/Pem2p, und (ii) durch Inkorporation von Cholin über den CDP-Cholin-Zweig des Kennedy Stoffwechsel Weges. Bildung von PC durch die beiden Wege wird durch Deletion von Gph1 negativ beeinflusst obwohl die Expression des Gens nicht verringert ist. Zusammengefasst agiert Gph1 neben der Mobilisierung von Glykogen auch als Regulator im Lipid Metabolismus in der Hefe. Um den Beitrag der zwei Stoffwechselwege zur Bildung von PC zur Bereitstellung von PC in Peroxisomen zu untersuchen wurden Hefe Stämme mit Defekten in den zwei Stoffwechselwegen hergestellt (*cho2*Δ*opi3*Δ und c*ki1*Δ*dpl1*Δ*eki1*Δ). Diese Stämme wurden anhand ihrer Lipid Zusammensetzung und den Membraneigenschaften untersucht. Es wurde festgestellt, dass beide Wege PC für die Peroxisomen produzieren, wobei der CDP-Cholin Stoffwechselweg eine höhere Effizienz hat als der Methylierungsweg. Wir definieren den Ursprung von peroxisomalen PC und demonstrieren die Bedeutung von PC für die Bildung der peroxisomale Membran und Integrität.

Abstract

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids found in yeast, plant and mammalian cells. In eukaryotes, PE can be synthesized via two different major pathways: (i) phosphatidylserine (PS) can be decarboxylated by phosphatidylserine decarboxylases type I (Psd1) and type II (Psd2), and (ii) ethanolamine can be activated by CTP and incorporated into PE via the so-called Kennedy pathway. The majority of PE is formed in the mitochondria by Psd1. PSDs are found in many cell types, including yeasts, plants, mammalians and parasites. In the yeast *Saccharomyces cerevisiae* Psd1 is anchored to the inner mitochondrial membrane (IMM) through membrane spanning domains and oriented towards the mitochondrial intermembrane space. The incorporation of phosphatidylserine decarboxylase 1 (Psd1) in yeast into the mitochondrial membrane and its need for functionality is only poorly understood. Therefore we analyzed in this study the membrane sorting signals of Psd1 and found that the enzyme harbors at least two membrane spanning domains, named IM1 and IM2. Deletions in the second membrane sorting signal IM2 lead either to mislocalization of Psd1 to the matrix site of the mitochondria or to the outer mitochondrial membrane and to defects in import, processing and activity of Psd1. Deletion of Psd1 results in depletion of phosphatidylethanolamine and in up-regulation of the glycogen phosphorylase *GPH1*, which is catalyzing the degradation of glycogen to glucose in yeast. Deletion of this gene causes decreased levels of phosphatidylcholine (PC), triacylglycerols and steryl esters. Depletion of the two non-polar lipids in a Δ*gph1* strain lead to a lack of lipid droplets, and a decrease of the PC level results in instability of the plasma membrane. In the yeast *Saccharomyces cerevisiae*, PC can be synthesized via two different pathways: (i) methylation of phosphatidylethanolamine (PE) catalyzed by the methyl transferases Cho2p/Pem1p and Opi3p/Pem2p and (ii) incorporation of choline through the CDP-branch of the Kennedy pathway. Formation of PC via both pathways is negatively affected in a Δ*gph1* strain, although expression of the involved genes is not down regulated. Altogether, Gph1p besides its function as a glycogen mobilizing enzyme appears to play a regulatory role in yeast lipid metabolism. To determine the contribution of the two PC forming pathways to the supply of PC to peroxisomes, yeast strains bearing defects in the two pathways, namely *cho2*Δ*opi3*Δ (mutations in the methylation pathway) and c*ki1*Δ*dpl1*Δ*eki1*Δ (mutations in the CDP-choline pathway), were analyzed regarding their lipid composition and membrane properties. We found that both pathways produce PC for the supply to peroxisomes, although the CDP-choline pathway seemed to contribute with higher efficiency than the methylation pathway. We defined the origin of peroxisomal PC and demonstrated the importance of PC for peroxisome membrane formation and integrity.

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General Introduction

In eukaryotic membranes major lipid components are phospholipids, sterols, sphingolipids and glycoglycerolipids [1]. Phosphatidylcholine (PC) is the major phospholipid, followed by phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) in many cell types including yeast [1], parasite [2,3], plant [4,5] and mammalian cells [6,7]. Formation of biological membranes in all eukaryotic cells is governed by a highly regulated network of metabolic pathways. Many biological processes were found to be membrane associated. Phospholipids are the major structural and functional components of biomembranes and play an important role in many regulatory processes [8,9].

In eukaryotic cells biological membranes play an essential role in several cell functions. They serve as diffusion barrier and protect the cell from its environment [10]. Membranes consist basically of phospholipids with embedded sterols, sphingolipids and proteins. The proteins catalyze the transport of molecules and are involved in metabolic and regulatory pathways or contribute as receptors to recognition processes. Phospholipids consist of a glycerol backbone esterified with fatty acids in the *sn-1* and *sn-2* positions, and a phosphate group in the *sn-3* position. One hydroxyl group of the phosphate is linked to the polar head group, which defines the specific lipid classes. In the yeast *Saccharomyces cerevisiae* the most abundant fatty acids are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1). At the *sn-1* position preferentially a saturated fatty acid is incorporated, whereas unsaturated species are linked to the *sn-2* position. A large spectrum of different phospholipid species with different functions in the cell results from variations in the acyl chain composition and the head group. Figure 1 shows different structures of membranes, like bilayers or hexagonal phase structures, which are formed by different phospholipid species [10–12].

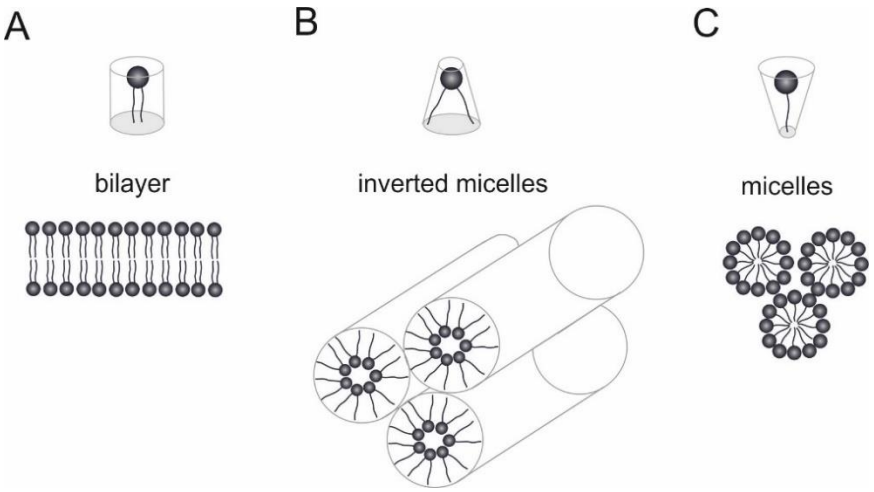

Figure 1: Membrane shapes formed by different phospholipid species. (A) Cylindrical-shaped lipid molecules, like PC, PG and PI, form bilayer structures. (B) Type II lipids (PE, PS, PA and CL) have a conical shape and induce negative curvature and lead to hexagonal phase structures. (C) Lysophospholipids (type I lipids) form positive membrane curvature, which favours micelle formation.

Phospholipids like phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylglycerol (PG) display a cylindrical shape and favour bilayer formation. Curvatures are induced by conical shaped phospholipids (type I and type II lipids) and micelle or inverted hexagonal phase structures are formed by positive (type I lipids) or negative (type II lipids) membrane curvature (Figure 1). Lysophospholipids are type I lipids and consist of only one fatty acid chain linked to a glycerol backbone resulting in a larger head to tail area. Phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA) and cardiolipin (CL) are type II lipids and form a conical shaped structure with a smaller head to tail area [8]. PE has a zwitterionic nature and a strong tendency to induce curvature in membranes due to its conical shape [12–14]. Membrane curvature favours membrane fusion, vesicle formation and movement of proteins across membranes. Absence or presence of PE or other phospholipid species can influence these processes [15,16].

The majority of phosphatidylethanolamine (PE) in *Saccharomyces cerevisiae* is formed by phosphatidylserine decarboxylase 1 (Psd1), an enzyme which is localized to mitochondrial membranes. Minor amounts are formed via Psd2, which is a component of the Golgi network. Figure 2 shows the biosynthetic pathways taking place in the yeast *S. cerevisiae*. PS is formed in the endoplasmic reticulum from cytidine-diphosphodiacylglycerol (CDP-DAG) and transported to the mitochondria, where it is decarboxylated to PE via Psd1. PE can also be formed via the CDP-ethanolamine branch of the Kennedy pathway in the endoplasmic reticulum [17]. In *S. cerevisiae* PC is formed via two main pathways, namely (i) the CDP-

choline branch of the Kennedy pathway and (ii) the methylation pathway with PE as substrate in the endoplasmic reticulum [18].

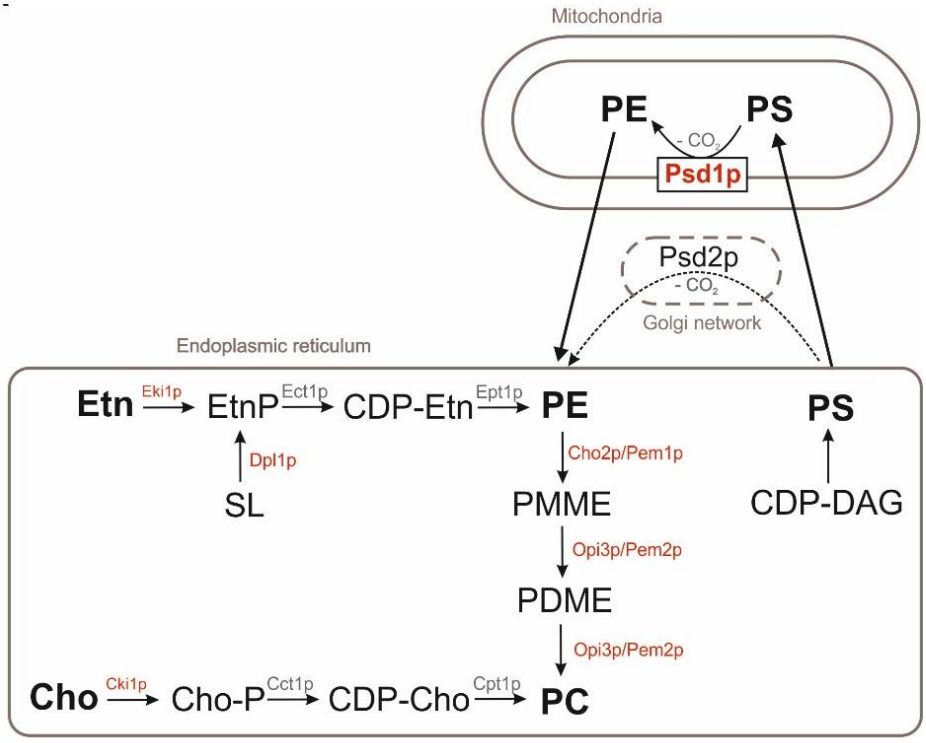

Figure 2: Pathways of phosphatidylethanolamine and phosphatidylcholine biosynthesis in the yeast *Saccharomyces cerevisiae.* Cct1, cholinephosphate cytidylyltransferase; CDP-Cho, cytidine-diphosphocholine, CDP-Etn, cytidine-diphosphoethanolamine; Cho, Choline; Cho2, phosphatidylethanolamine methyltransferase; Cho-P, phosphocholine; Cki1, choline kinase; Cpt1, cholinephosphotransferase; DAG, diacylglycerol; Dpl1, sphingosine phosphate lyase; Ect1, phosphoethanolamine cytidylyltransferase; Eki1, ethanolamine kinase; Ept1, sn-1,2-diacylglycerol ethanolamine- and cholinephosphotranferase; Etn, ethanolamine; Etn-P, phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PDME, phosphatidyldimethylethanolamine; PMME phosphatidylmonomethylethanolamine; PS, phosphatidylserine; Psd1, phosphatidylserine decarboxylase 1; Psd2, phosphatidylserine decarboxylase 2; Opi3, methylene-fatty-acyl-phospholipid synthase; SAM, S-adenosyl-L-methionine; SL, sphingolipids.

Mitochondrial membranes contain high amounts of PE [19–21] and therefore structural properties of PE seem to play an important role in various functions of this organelle. The amount of PE in inner mitochondrial membranes (IMM) is high [22] and compared to the outer mitochondrial membrane (OMM) the curvature of IMM is more pronounced, the protein content is much higher and the ratio of PC to PE is increased [23,24]. Functionality of mitochondria depends on supply of proteins and lipids from other organelles, although some of these components are synthesized by the organelle itself.

The aim of this work is the deeper understanding of the role of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in membrane structure and cellular function. *Saccharomyces*

cerevisiae was used as model organism to study function and structure of Psd1 and the role of PE and PC in lipid biosynthesis.

Chapters 1 and 2 of this work focus on the phosphatidylserine decarboxylases, especially type I PSD (Psd1p) and its formation of PE. Chapter 1 will summarize the resent knowledge about the physiology and enzymology of phosphatidylserine decarboxylases (PSDs) in different organisms. Many cell types contain two types of PSDs, the type I and type II PSDs (Psd1 and Psd2). They are different in their structure and localization within the cell. For both enzyme types a characteristic domain is the autocatalytic cleavage site (LGST motif), which is the site of processing into an active enzyme. This chapter also shows the evolutionary relationship of the two enzyme types in different organisms. The role of mitochondrial Psd1 proteins, their function, enzymology, biogenesis, assembly into mitochondria and their contribution to phospholipid homeostasis will be discussed in much detail. Also the cellular physiology and the enzymology of Psd2 is discussed briefly in this work. In eukaryotes type I PSDs are localized to mitochondria, whereas other PSDs are localized to other cellular compartments. The majority of PE in the yeast *S. cerevisiae* is formed by mitochondrial Psd1. For full enzymatic activity a Psd1 precursor protein is imported into mitochondria and processed into its functional form. Psd1 is anchored to the inner mitochondrial membrane in *S. cerevisiae*. We identified two mitochondrial membrane sorting signals. Their influence on processing, functionality and integration into mitochondria was analysed and is presented in Chapter 2. The formed PE is incorporated into mitochondria, but also supplied to other subcellular compartments [18]. Deletion of *PSD1* leads to ethanolamine auxotrophy and to a reduced PE content in mitochondria, when cells are grown on non-fermentable carbon sources [25–27]. Phosphatidylserine (PS), the substrate of Psd1, is synthesized in the endoplasmic reticulum/mitochondria-associated membrane and transported to the mitochondria. Psd1p is synthesized as a larger precursor on cytosolic ribosomes [28] and transported via different targeting signals to its final localization within mitochondria (Figure 3) [29].

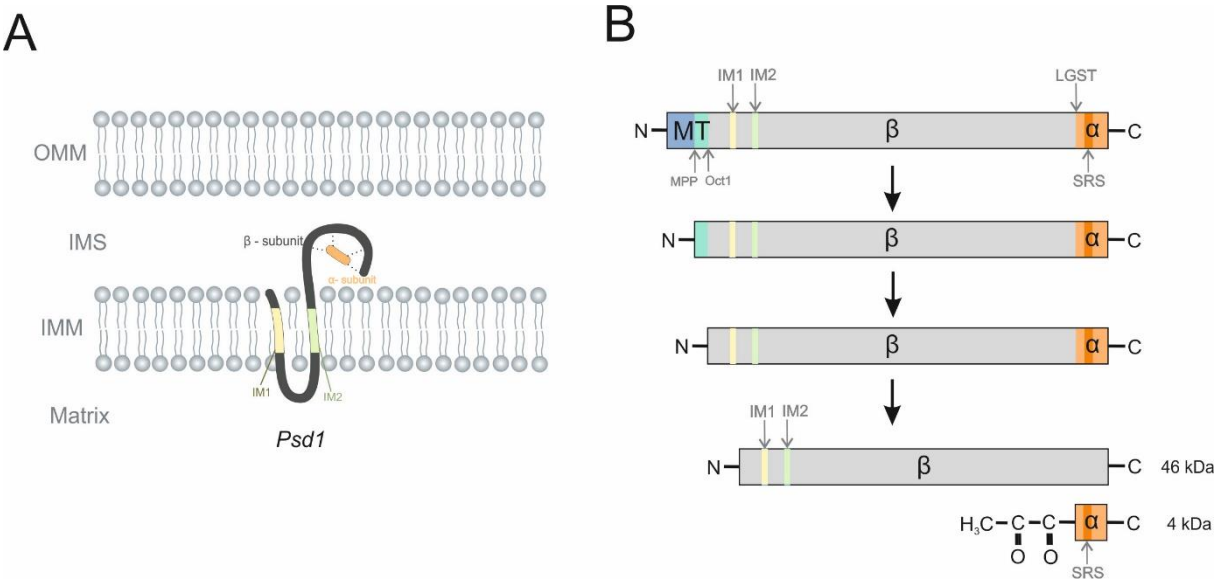

Figure 3: Localization and post-translational processing of Psd1 in the yeast *Saccharomyces cerevisiae*. (A) The β-subunit of Psd1, tethering the α-subunit to the inner mitochondrial membrane (IMM) via two membrane spanning domains (IM1 and IM2), is shown schematically. (B) Post-translational processing steps of Psd1 precurser in *S. cerevisiae*, resulting in a membrane bound β-subunit (46 kDa) and a per se soluble α-subunit (4 kDa). MT, mitochondrial targeting sequence; MPP, mitochondrial processing peptidase; Oct1, octapeptidyl aminopeptidase; IM1 and IM2, predicted inner membrane sorting signals; LGST, endoproteolytic cleavage site; SRS, predicted substrate recognition site.

During three steps of post-translational processing of the Psd1 precursor a mature α- and β-subunit with a size of 4 kDa and 46 kDa, respectively, are formed (Figure 3) [19]. During the first step, the precursor is imported into mitochondria and the N-terminal targeting sequence is cleaved off. A 2 kDa fragment, representing the intermembrane space sorting signal (MT), is removed and upon autocatalytic cleavage at the highly conserved LGST motif a mature α- and β-subunit is formed [19,30–34]. This processing step subsequently forms the N-terminal pyruvyl prosthetic group of the α-subunit and becomes the active site of the enzyme [35]. The separation into α- and β-subunit can also occur when the protein is not correctly integrated into the IMM [19]. In this study (Chapter 2), mutations in the membrane spanning domain IM2 of Psd1 were integrated and analyses regarding processing, activity, localization and import into mitochondria were performed. The membrane spanning domain IM2 is important for correct integration into the inner mitochondrial membrane, but also for processing and catalytic activity of the enzyme.

Deletion of Psd1 results in depletion of phosphatidylethanolamine and in up-regulation of the glycogen phosphorylase GPH1, which is catalysing the degradation of glycogen to glucose in

yeast (Figure 4A). Deletion of this gene causes decreased levels of phosphatidylcholine (PC), triacylglycerols and steryl esters. Depletion of the two non-polar lipids in a Δ*gph1* strain leads to lack of lipid droplets, and a decrease of the PC level results in instability of the plasma membrane. In Chapter 3 the focus is set on the influence of the deletion of *GPH1* on the lipid metabolism in the yeast *Saccharomyces cerevisiae*. Gph1p is catalysing the release of glucose-1-phosphate from glycogen, and is possibly also involved in lipid metabolism [36–39]. Deletion of *GPH1* results in increased levels of intracellular glycogen, although *GPH1* is not essential in yeast [36]. Gph1p is localized to so-called glycogen particles [36,40], whose size and amount vary during growth phases of the yeast cell [41]. Glycogen is degraded when energy is required for cell activity and growth during stationary phase. *GPH1* is induced at the end of the logarithmic growth phase in yeast and intracellular glycogen is accumulated. Gph1p is inhibited by glucose-6-phosphate, and its activity is regulated by reversible phosphorylation [36]. In a *Δgph1* deletion mutant large amounts of glycogen are accumulated during the stationary phase, which results in rapid aging and low stress tolerance [42]. In Chapter 3 we compare the influence of the high glycogen content in the Δ*gph1* mutant to the wild type strain and to a strain with inhibited glycogen synthesis, by deletion of 1,4-glucan-6-(1,4-glucano)-transferase (Δ*glc3*) (Figure 4) [43]. The strains Δ*gph1*, Δ*glc3* and wild type overexpressing *GPH1* were analysed regarding their influence on the lipid metabolism and glycogen content. We found that the metabolism of non-polar lipids is changed and that Gph1p is involved in lipid metabolism, as the Δ*gph1* yeast mutant exhibited decreased PC levels in total cell homogenates and especially in the plasma membrane.

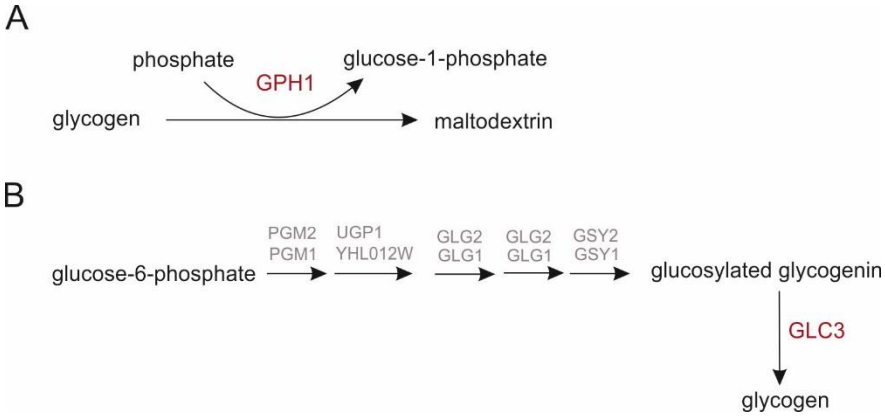

Figure 4: Glycogen catabolism (A) and glycogen biosynthetic pathway (B) in yeast with GPH1 and GLC3 involved . (A) The glycogen phosphorylase GPH1 is catalysing the degradation of glycogen to glucose in yeast. (B) The 1,4-glucan-6-(1,4-glucano)-transferase GLC3 is catalysing the last step in the glycogen biosynthetic pathway. GLG1 and 2, gycogenin glucosyltransferase; GSY1 and 2, glycogen synthase; PGM1 and PGM2, phosphoglucomutase 1 and 2; UGP1, uridinephosphoglucose pyrophosphorylase; YHL012W, UTP glucose-1-phosphate uridylyltransferase

In yeast and in plant cells, the β-oxidation of fatty acids is localized to peroxisomes, whereas in mammalian cells also mitochondria are capable of performing fatty acid degradation. Peroxisomes are the organelles were oxidative and detoxifying reactions take place [44]. Phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) are major phospholipid species of peroxisomal membranes [45–47]. In yeast cells PE can be supplied to peroxisomes from mitochondria, endoplasmic reticulum and the Golgi network [46,47]. PC is the most abundant aminoglycerophospholipid in eukaryotes [48], but little is known about its distribution within the cell. Peroxisomes belong to the group of organelles which cannot synthesize their own lipids and therefore peroxisomes rely on the supply of lipids from other organelles. In Chapter 4 we focus on the PC supply to peroxisomes in the yeast *S. cerevisiae*. To analyse the influence of the different pathways on PC supply from the endoplasmic reticulum to peroxisomes, mutants compromised in the biosynthesis of PC (*cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ) were studied in Chapter 4. Growth phenotypes, lipid composition, fatty acid composition and membrane behaviour in yeast cells were analysed. Deletion of *CHO2* and *OPI3* (*cho2*Δ*opi3*Δ) results in a defective methylation pathway. In such strains formation of PC was only possible via the Kennedy pathway. In the *cki1*Δ*dpl1*Δ*eki1*Δ strain no PC was formed via the Kennedy pathway. Although both, the methylation pathway and the CDP-choline branch of the Kennedy pathway produce PC for supply to peroxisomes, the CDP-choline pathway is slightly more efficient. Mutations in one of these pathways, each, do not cause extreme defects in peroxisomes indicating that the remaining pathway in a mutant strain can efficiently compensate for the loss of the other biosynthetic route. Both pathways are also important for the supply of PC to mitochondria and the endoplasmic reticulum. Fatty acid supply to peroxisomes, where β-oxidation occurs in *Saccharomyces cerevisiae* was not limited and energy production not affected in the two mutants. In this study also the role of PC for peroxisomal membrane properties is discussed. PE has the ability to interact with itself and neighbouring lipids via inter- and intramolecular hydrogen bonding [50] and generates a closely packed lipid bilayer with hydrogen tails [51]. DMPE and PE form bridges with each other in a lipid bilayer which leads to a change from a fluid to a gel state of the membrane [50–53]. An increased PC to PE ratio leads to a higher transition temperature of a lipid bilayer and therefore to a more rigid membrane. We can conclude that both, the Kennedy and the methylation pathway supply PC to yeast peroxisomes.

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Chapter 1

PHYSIOLOGY AND ENZYMOLOGY OF PHOSPHATIDYLSERINE DECARBOXYLASE

Francesca Di Bartolomeo[1#], **Ariane Wagner**[#], Günther Daum[1]*

[1]Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2; A-8010 Graz, Austria

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# The two authors contributed equally to this work

*Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria, phone: +43-316-873-6462,email: guenther.daum@tugraz.at

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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 of and the enzymology of Psd2.

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

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 [2,3]. In many cell types including yeasts, parasites, plant cells, and mammalian cells, such as *Saccharomyces cerevisiae* [1], *Plasmodium falciparum* [4], *Toxoplasma gondii* [5], *Arabidopsis thaliana* [6,7], mice 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 phospholipids because of its zwitterionic nature and 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 PC to PE is increased [21,22].

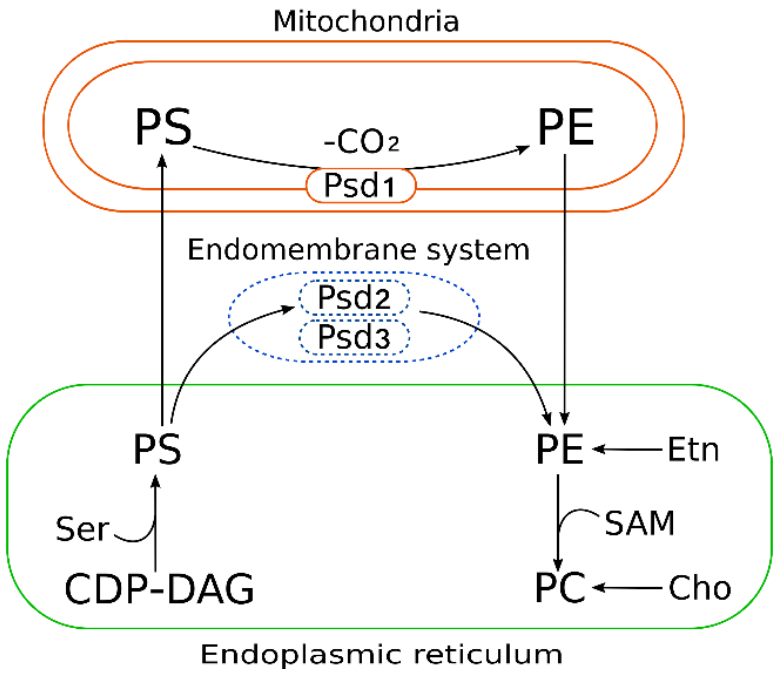

Figure 1: Schematic overview of pathways of aminoglycerophospholipid biosynthesis. Phosphatidylserine (PS) is formed by PS synthase from CDP-diacylglycerol (CDP-DAG) in the endoplasmic reticulum (ER) and then transported to mitochondria where it can be decarboxylated by phosphatidylserine decarboxylase 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 so-called Kennedy pathway from ethanolamine (Etn). PE can be further converted to phosphatidylcholine (PC) by phosphatidylethanolamine methyltransferases. Apart from the methylation pathway PC is also formed from choline. 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 involving CDP-ethanolamine [1,23]. Figure 1 shows the main routes for the formation of the PE and PC in a very schematic way. 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. PE and PC are also formed in the ER via methylation of PE and the CDP-ethanolamine or CDP-choline 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, and 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 recent review articles [26–28].

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 fulfil 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 PSDs. The two PSDs can be distinguished by their specific structure, sequence and subcellular localization. Analysis of Psd1 enzymes from different organism belonging to various species and phyla shows that it is possible to characterize some identic clusters along the sequence with highly conserved and/or similar amino acids. In Figure 2A 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 (Figure 2B). 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 end 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 (Figure 2C). The reconstruction of the enzymes evolution and the ancestral interconnection shows that the PSD type I and II have a common ancestor but they separated very early in the evolutionary scale.

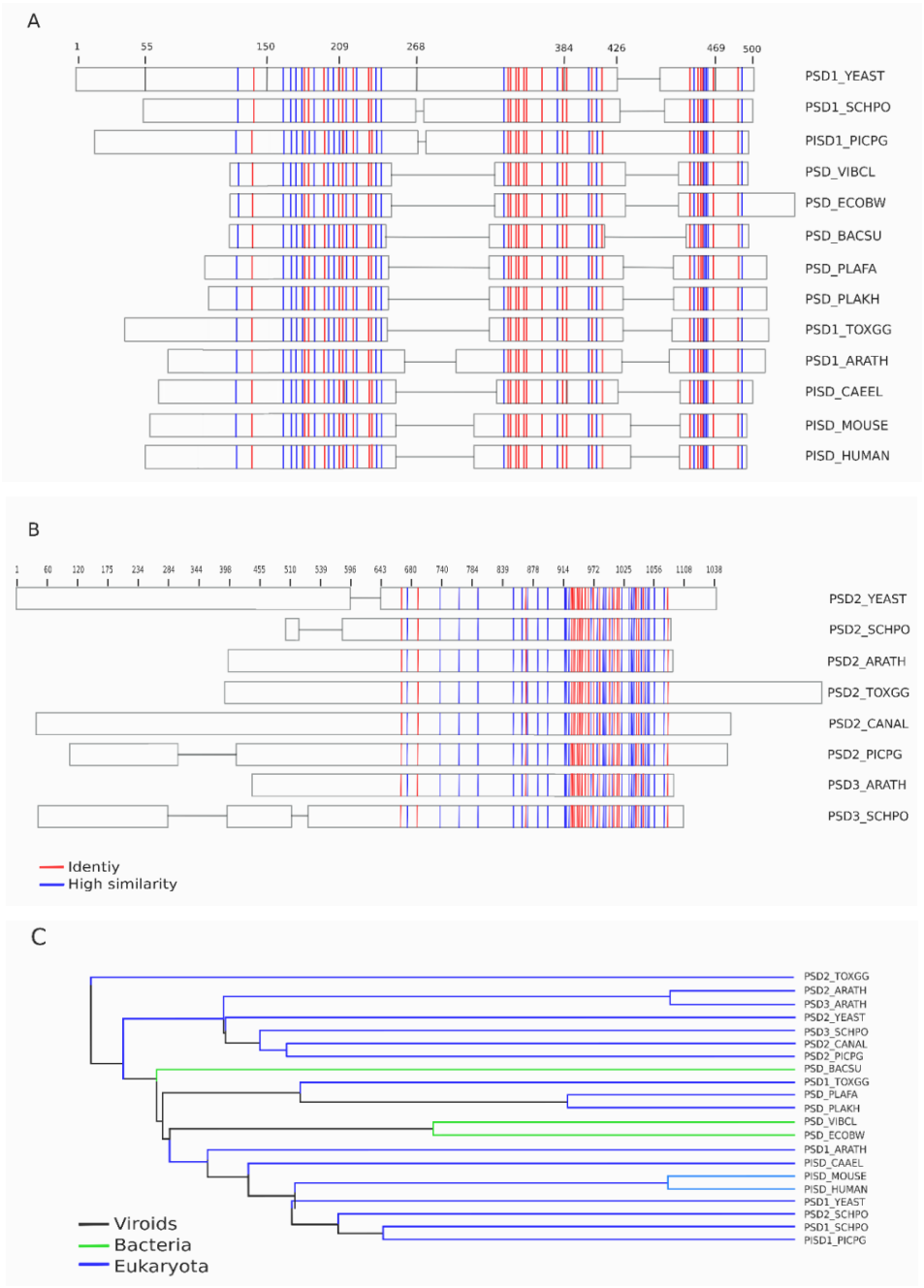

Figure 2: Sequence alignment and phylogenetic tree of PSDs in different organisms. (A) Psd1 sequences alignment of the following selected organism is shown: *Saccharomyces cerevisiae (*YEAST*)*, *Schizosaccharomyces pombe* (SCHPO), *Pichia pastoris* (PICPG), *Vibrio cholerae* (VIBCL), *Escherichia coli* (ECOBW), *Bacillus subtilis* (BACSU), *Plasmodium falciparum (*PLAFA), *Plasmodium knowlesi* (PLAKH), *Toxoplasma gondii (*TOXGG), *Arabidopsis thaliana* (ARATH), *Caenorhabditis elegans* (CEEL), *Mus musculus* (MOUSE) and *Homo sapiens* (HUMAN). Proteins analyzed have an overall identity of 3.93 % with 26 identical and 21 similar amino acids. (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 percentage of 1.31 % with 22 identical positions and 29 similar positions. (C) The phylogenetic tree created by using the Clustal Omega software [31] shows the evolutionary relationships and proximity of Psd1 and the Psd2 proteins.

Bacteria are the only cells which are not able to synthesize PE in a PSD-independent reaction. The bacterial Psd1 which is associated with the cytoplasmic membrane [32,33] 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 [34–36]. *Vibrio cholerae* is a gram-negative bacterium well known for his virulence and high diffusion. PSD from this microorganism is essential for bacterial viability. *V. cholerae* PSD displays 31.7 % sequence identity with PSD from mouse and 60 % sequence identity with *E. coli* PSD [37].

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 [25,38,39]. The more potent PSD of the 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 [40] (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 protruding into the intermembrane space. Horvath et al. [17] showed that correct localization of Psd1 in the mitochondrial membrane of *S. cerevisiae* is crucial for enzymatic activity. Like 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 as long as glucose is used as a carbon source [19,41]. Only if non-fermentable carbon sources such as lactate are used, mitochondrial functions, especially respiration, become more stringent and ethanolamine must be supplemented to rescue cell survival (see Figure 1).

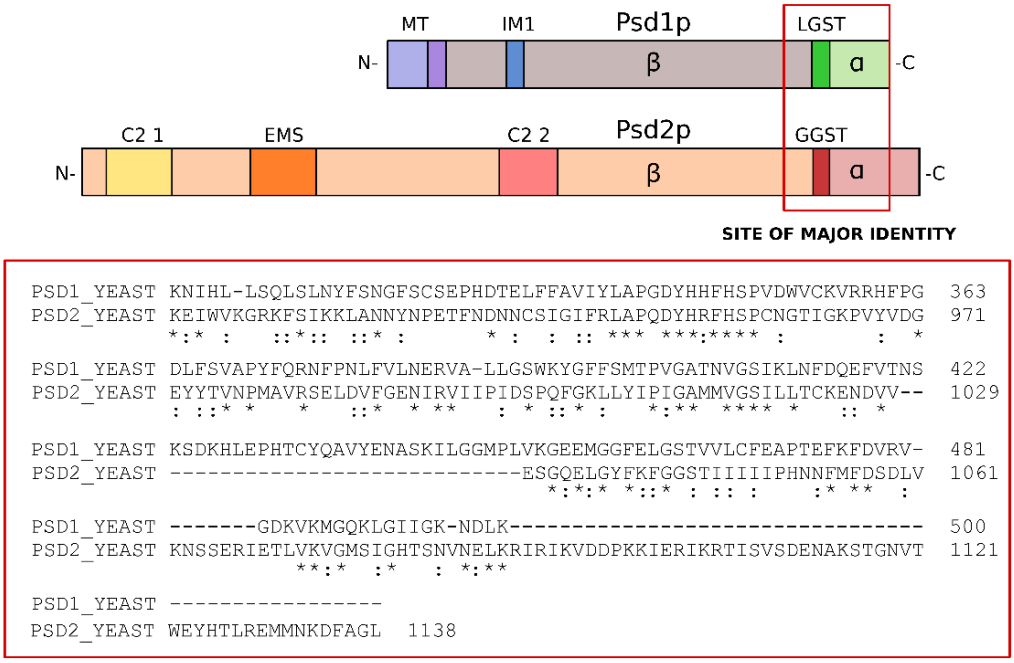

Figure 3: Sequence and position of important sites of yeast Psd1 and Psd2. Comparison of Psd1 and Psd2 from *Saccharomyces cerevisiae* shows 6.8 % identity with 87 identical and 139 similar amino acid positions. The highest sequence identity is found at the C terminus and specifically at the cleavage 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. Regarding Psd2 the EMS sequence corresponds to the endomembrane system targeting domain while C2 1 and C2 2 are the 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 [20,38]. More recently, the enzymatic activity of Psd2p was rather attributed to endosomes [42,43]. 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 [38]. The yeast Psd1 protein shows more analogy to the mammalian PSD than to Psd2 [44], 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 having the LGST motif, however, 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 [39] (see Figure 3). Besides the GGST site for the endoproteolytic cleavage, Psd2 has other relevant domains that are important for the activity and the 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 [39,45]. 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 [46] 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+} [47].

In the yeast *Pichia pastoris* two PSDs have been identified [48]. Deletion of the *PSD1* gene resulted in decreased mitochondrial PSD activity of 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* [49,50]. Although these three PSDs from *S. pombe* named Psd1, Psd2 and Psd3 *fulfil overlapping functions, they are not completely redundant. Regarding the localization of these enzymes,* Psd1 has been detected in mitochondria [50,51], whereas Psd2 was dually localized to mitochondria and to the nuclear envelope. Psd3 has been reported to be localized to the cytosol, and 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. On the other hand, Psd3 from *S. pombe* is structurally comparable to the *S. cerevisiae* Psd2. Psd3 from *S. pombe* and *S. cerevisiae* Psd2 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 [52].

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* [53]. In this study, a PSD enzyme has been detected to serve 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 is the major cellular source of PE in *T. brucei* [54]. Studies performed by Signorell et al [53] 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), is severely altering 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 [55]. 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 [56]. 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 spotted as successful inhibitor and characterized by an effective activity against *P. falciparum*. In the following, a newly synthesized analog of this compound was tested against *Plasmodium yoelii* showing its healing property towards infected mice by blocking the parasitic Psd1 activity and interfering with membrane biogenesis [57]. In subsequent studies, the PSD from the parasite *Plasmodium knowlesi* was identified [58]. 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 some 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 subjects [59]. Studies of the cellular lipid metabolism highlighted that the parasite is able to secrete a soluble form of PSD (TgPSD1) that normally resides in the dense granules [60]. *T. gondii* is able to produce PS on the cell surface, 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* is temperature, ATP and Ca^{++} dependent [61]. The enzyme secretion was inhibited by 90 % at an incubation temperature of 0°C and depletion of ATP reduced the secretion of PSD by 92 %. Exposure to the Ca^{++} chelator BAPTA-AM inhibited the secretion of PSD 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 an optimal parasite growth and replication of *T. gondii*, but dispensable for the parasite survival [38]. 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 [6,61]. A tomato protein named LePSD1 with a size of 50 kDa [61] 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 divergences 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 [6,61]. Psd2 and Psd3 from *A. thaliana* show 76 % sequence identity to the yeast Psd1 and 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 and was also located to roots and in lower amount 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 [62,63] 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 [64]. 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 [65]. 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 [64]. In PSB-2 cells (CHO cells which require exogenous PS or PE for cell growth with a further decrease in the serine base exchange activity) a moderate depletion of mitochondrial PE impairs cell growth together with mitochondrial morphology and function [66].

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) [67]. In a *S. cerevisiae psd1*Δ 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 [68].

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 [69]. 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 [70,71]. In the absence of PE *E.coli* requires divalent

cations to rescue growth [72,73]. In *E. coli*, PE can be replaced by glycolipids which restore certain functional properties of membranes [74]. For the Gram-positive bacterium *B. subtilis* PE is not essential for cell survival, and absence of PSD causes just an accumulation of PS [35]. 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 PSS or PSD, respectively, showed that they grew like wild type on many complex media, but they showed a dosage dependent growth on phosphate [75]. They were unable to grow on minimal medium containing high phosphate concentrations, but the PSD deficient mutant could grow 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 the 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 [14,76]. 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 [48]. The mitochondrial PE content in a *psd1Δ* yeast strain grown on lactate is decreased of 74 % compared to wild type [19], and over 90 % in a *psd1Δpsd2Δ* double mutant which is auxotrophic for ethanolamine or choline on both glucose and lactate [19,39,48,77]. Incorporation of the ethanolamine analogue propanolamine (Prn) into PE from *psd1*Δ*psd2*Δ and *psd1*Δ*psd2*Δ*dpl1*Δ yeast cells can replace some of the cellular requirements, but growth of *psd1*Δ*psd2*Δ*dpl1*Δ cannot be rescued [14]. This inability of Prn to replace ethanolamine in a *psd1*Δ*psd2*Δ*dpl1*Δ strain may be due to the low propensity of PE with Prn incorporated to form bilayer phases. However, PE with Prn as head group can make up to 40 % of the total membrane phospholipids, and the cells are still growing like wild type.

Most of the PE formed by Psd1 remains in the MIM of wild type mitochondria from *S. cerevisiae* [78]. To investigate the effect of *PSD1* deletion on genome wide modifications in *S. cerevisiae* cDNA microarray analysis was performed [79]. Up-regulation of 54 genes was observed in a *psd1*Δ mutant, but significant down-regulation of genes was not found [79]. 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 [80]. Also the activity of the *PSD1* gene from yeast is repressed by 50–70 % by inositol in combination with choline [69]. It has been shown that *OPI1* controls this repression process [69,80].

Deletion of *PSD1* in yeast cells does not affect the mitochondrial morphology, but results in a changed mitochondrial phospholipid composition when cells are cultured in rich medium containing lactate [81]. 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 retrieved [81].

An interesting counteraction 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 [82–86]. CL is a dimeric glycerophospholipid that is exclusively synthesized in mitochondria where it is involved in the processes of oxidative phosphorylation, regulation of apoptosis [82,87] and in mitochondrial biogenesis through regulation of protein import into this organelle [5,6,52]. CL also seems to be strictly associated with the fusion of mitochondria [88]. Formation of CL in *S. cerevisiae* is catalyzed by the mitochondrial cardiolipin synthase Crd1 [48,76,89], 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 [86]. A *crd1Δpsd1Δ* double mutation lacking both PE and CL is lethal [75,90]. 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* [75] 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 *crd1Δpsd1Δ* conditional mutant was created [91]. 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 *crd1Δpsd1Δdnm1Δ* 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 *fmp30Δ, psd1Δ* and *fmp30Δpsd1Δ* cells exhibits abnormal mitochondria [82]. 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, *fmp30Δpsd1*Δ cells exhibited a severe growth defect and an about 20-fold reduction of the CL level compared to wild type [6]. Mitochondria from *fmp30Δ* 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 *fmp30Δpsd1Δ* strain, mitochondria with normal structure were not observed, and 84 % of *fmp30Δpsd1Δ* cells contained aggregates of fragmented mitochondria [82]. 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 [84]. Furthermore, CL but not PE is also 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 *psd1Δ* 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 of the early stage of protein import which involves docking and translocation of the precursor protein through the TOM complex. In mitochondria from *psd1Δ* 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 [90]. Furthermore, strains of *S. cerevisiae* which lack the 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 F1/F0 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 [81,92,93]. 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 for yeast and mammalian cells.

Gulshan et al [40] 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 [43] 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. [94] observed that exposure of yeast cells to cadmium resulted in a noticeable increase of the major phospholipids, especially of PE. In a yeast *psd2Δ* 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 *psd2Δ*, 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. [90] identified a factor required for the transcriptional activation of the *PSD2* gene. It was demonstrated that a *S. cerevisiae psd1Δ* mutant strain which was also lacking Vid22, a plasma membrane protein required for the fructose-1,6-bisphosphatase degradation pathway, was synthetically lethal [95]. The catalytic activity of PS decarboxylase in the cell extract of *vid22Δ* strains was about 70 % of wild type cells similar to that of *psd2Δ* cells. Furthermore, *vid22Δ* cells were shown to be defective in the expression of *PSD2*. This evidence suggested that Vid22 affects the function of *PSD2*.

In *Pichia pastoris* deletion of *PSD1* gene resulted in a decrease of mitochondrial PSD activity to 25 % of wild type and a severe growth defect on minimal media [48]. The presence of Psd2 was not sufficient to rescue this growth defect, but supplementation with ethanolamine, which is the substrate for 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 (*psd1-3Δ* mutants) grow slowly on rich medium and are not vital on minimal medium, indicating that *PSD1, PSD2* and *PSD3* share overlapping essential cellular functions [52]. Supplementation of growth media with ethanolamine restored growth of *psd1-3Δ* cells in minimal medium, indicating that PE is essential for *S. pombe*. As *Psd1-3Δ* 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 psd1-3Δ* mutants showed abnormally shaped cells and exhibited severe septation defects including multiple, mispositioned, deformed, and misoriented septa [52] whereas *psd1-3Δ* 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 *psd2Δpsd3Δ* bearing defects in the two PSDs having high sequence similarity showed similar physiological properties as single mutants and grew similar to wild type. A *psd1Δpsd2Δpsd3Δ* 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. [96] investigated mammalian cells where PE is formed by PSD in the presence and absence of ethanolamine. The substrate for 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 [64]. 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 [97]. 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 % of 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 [66]. Moderate (less than 30 %) depletion of mitochondrial PE altered mammalian mitochondrial morphology and function and impaired cell growth [64,66,97,98].

BIOGENESIS OF PHOSPHATIDYLSERINE DECARBOXYLASE 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 fulfil their catalytic activities [62]. 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 [99–101]).

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 external energy source. At this stage of the import process the 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 [17,102]. 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 [17,103].

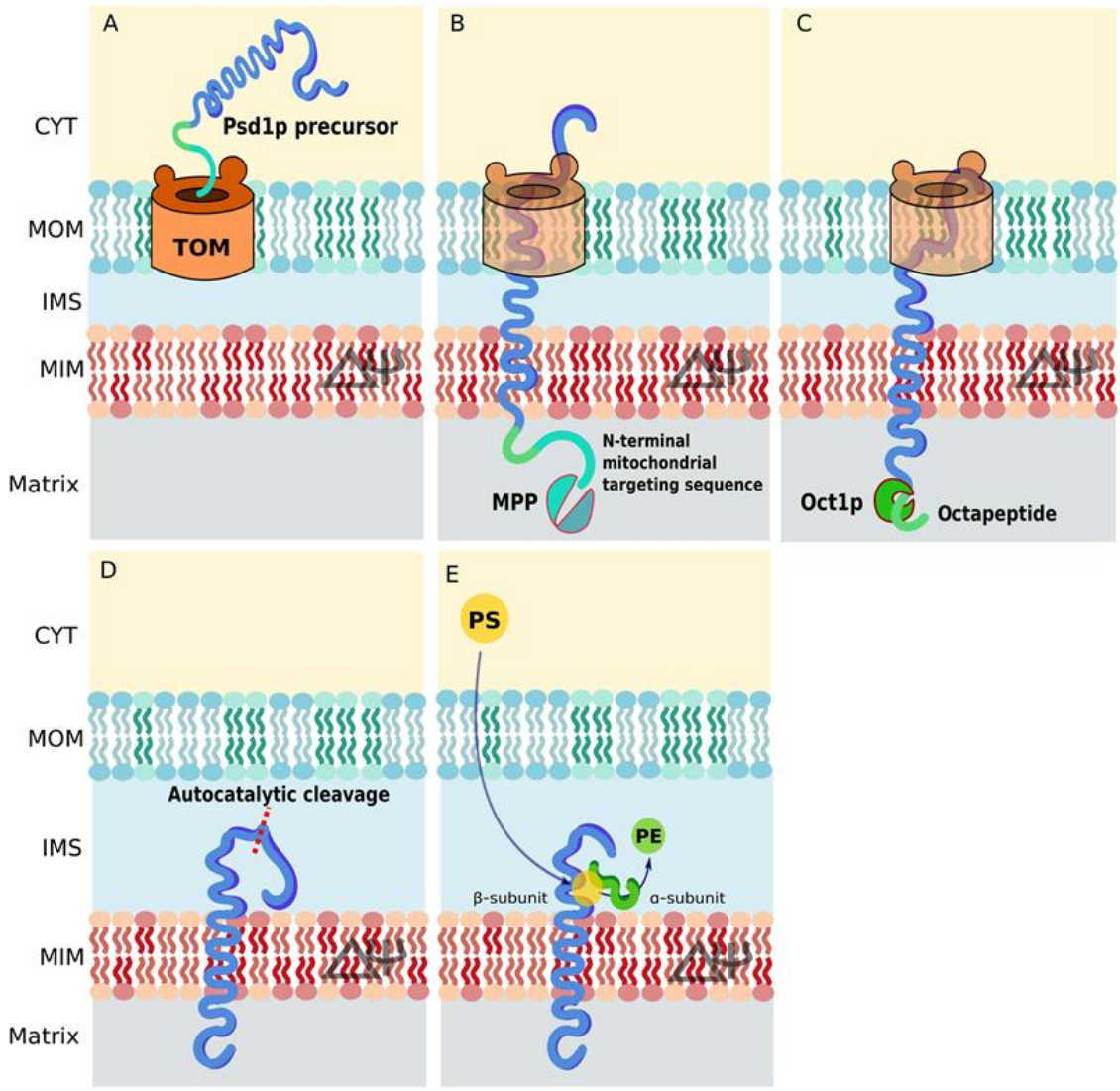

Figure 4: Import of Psd1 into mitochondria. (A) The immature precursor form of Psd1 synthesized by cytoplasmic ribosomes is directed towards the MOM, where the N-terminal mitochondrial targeting signal allows docking to the TOM complex. (B) After docking of the protein, Psd1 is transferred within the organelle. (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. [93] 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 *psd1Δ* 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 loss 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 the import and sorting into the 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 [86]. 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 crucial for the ability of Psd1 to decarboxylate the polar head group of PS [104]. 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].

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 pyruvyl group of the mature enzyme to obtain an active PSD [105,106]. 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 [35,105]. 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 [86]. Exceptions from the classical LGST motif are the Psd1 from *A. thaliana* with its MGST sequence at the C-terminus [61], and PSD from *P. falciparium* with VGSS from position Val-314 to Ser-317 [57]. *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-1.041 to Thr-1.044 [39], and the two type II PSDs from *A. thaliana* (Psd2 and Psd3) which have both a GGST motif at the C-terminus [61].

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 pyruvyl prosthetic group. The cleavage which is considered to be an autocatalytic process has been best studied with *E. coli* [105]. 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 pyruvyl prosthetic group, which is attached 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. [85] 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.

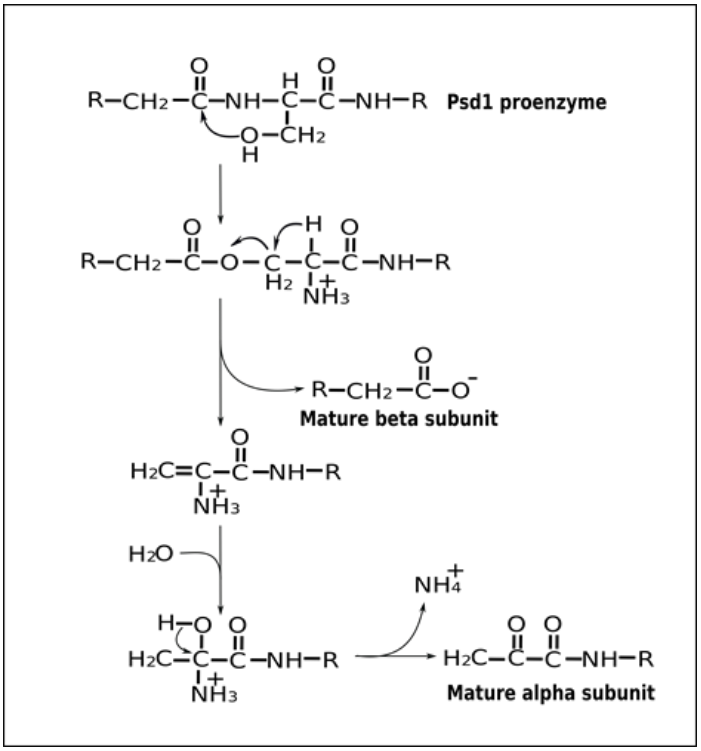

Figure 5: Maturation of Psd1. For complete maturation of Psd1 an autocatalytic endoproteolytic cleavage is necessary to form the active site of the enzyme, which consists of a pyruvyl group. At the same time the α- and β-subunits of Psd1 are formed.

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 the import and maturation of the enzyme, and other components might influence this process. Choi *et al.* [58] 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 of the self-processing and the formation of the two subunits of Psd1 [58]. 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* [106]. 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 organism 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 [106,107]. Site direct mutagenesis experiments showed that mutations in 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 [106].

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* [34,105,108]. At the conserved GS(S/T) motif the addition of PS leads to formation of a Schiff's base between the covalently bound pyruvyl 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 Schiff's base linkage with the enzyme, and ultimately the addition of water across the Schiff's base regenerates the pyruvyl prosthetic group and sets PE free from the active site of the enzyme [47,69,83].

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 [109]. 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 of Triton X-100 and 6 mM PS. As the catalysis depends of the carbonyl moiety of the pyruvyl group of PSD, the presence of carbonyl reagents can inactivate the enzyme [34].

In experiments with yeast Psd1 it was noticed that the enzyme exhibited a 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 [60]. 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 *psd1Δpsd2Δdpl1Δ* 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 activity of PkPSD [58]. 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 with the 0.057 % Triton X-100 [110].

In mammalian cells formation of PE by PS decarboxylation generates mainly species with 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 [97]. Interestingly, the 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 [97]. 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

[69]. 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 other PSDs the rat liver PSD does not exhibit a substrate preference to dipalmitoyl and dimyristoyl-PS [111].

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 [112]. The currently best accepted mechanism of phospholipid transport between ER and mitochondria seems to be through membrane contact [113]. In yeast and mammalian cells membrane complexes form a sub-fraction of the ER, the *mitochondria*-associated *membrane* (MAM) [114,115]. These contact sites were identified through co-sedimentation of ER and mitochondrial fractions and by direct visualization using electron microscopy [116–119]. These methods showed that 80-110 of these contact sites are present in yeast cells [120]. Gaigg et al. [121] and Vance et al. [122] 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 [118,123].

In the yeast, molecular components localized at ER-MOM junctions were identified and named ER-mitochondrial encounter structures (ERMES) [124,125]. The ERMES complex is formed by five major proteins, namely Mmm1, Mdm34, Mdm10, Mdm12 and Gem1 [126,127]. It was hypothesized that the ERMES complex plays a role in translocation of lipids between compartments [128]. Other authors [129], however, argued that lack of ERMES does not interfere with PS transport from the ER to mitochondria and the 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 [130,131]. Likewise 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 [131]. 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 [132]. Voss et al. [130] 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. The study also demonstrated that the set of genes encoding the ERMES proteins genetically interact with *RTN1* and *YOP1*, and that the ERMES

complex, together with the proteins required to maintain tubular ER, are important for lipid exchange between ER and mitochondria.

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 compared to wild type [133]. Studies in yeast cells focusing on PS delivery from the ER to the MIM showed that this activity did not require ATP [113,120], although the process appeared to be energy depended in experiments conducted with mammalian cells [134]. 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 [88,135].

Intramitochondrial transport of lipids is a route that involves lipid transport from the MOM to MIM and backwards [136]. 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 [113,115,137–139]. 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 [139–142]. When grown on a fermentable carbon sources, the yeast deletion strains *ups1Δ, mdm31Δ* and *gep5Δ* are characterized by low expression of *PSD1* accompanied by reduction of PE synthesis [139,143]. Further experiments highlighted the role of Mdm31 and Gep5 in keeping a correct mitochondrial morphology, supporting Psd1 levels and PE production [78]. 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 *ups1Δ* and *ups2Δ* yeast strains Tamura et al [140] 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 *ups1Δ* strains grown under fermentable conditions the export of PE seemed to be slower than in reference strains; and (iii) in *ups1Δ* strains a reduced steady-state level of Psd1 was

accompanied by a decreased membrane potential (ΔΨ), which was restored if strains lacking Ups1 were shifted to growth on a non-fermentable carbon source.

A recent study by Aaltonen et al. [144] 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 *psd2Δdpl1Δ* 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. Thus, the role of Ups1 and Ups2 in PE metabolism seems to be merely regulative, and there is no evidence that these two proteins transport PE directly within the mitochondrion. Despite the data available so far, the exact mechanism which is driving PE transfer within mitochondria is still a matter of dispute. Especially identification of proteins which govern PE translocation 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 [145]. As the ratio of unsaturated to saturated fatty acids in PS is much lower than in PE, a high species selectivity of Psd2 can be anticipated. Bürgermeister et al. [145] 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 [38]. Riekhof et al. [42] studied the supply of PS to the site of Psd2 from the ER across the endosomes. They showed that a complex between Psd2 and PstB2, the mammalian homolog

of Sec14, is involved. This interaction was 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 by 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 like phenomena 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 [88].

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. With 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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Chapter 2

IDENTIFICATION OF THE MITOCHONDRIAL MEMBRANE SORTING SIGNALS IN PHOSPHATIDYLSERINE DECARBOXYLASE 1 FROM *SACCHAROMYCES CEREVISIAE*

Ariane Wagner[a], Francesca Di Bartolomeo[a], Claudia Hrastnik[a], Kim Nguyen Doan[b], Thomas Becker[b] and Günther Daum[a*]

[1]Institute of Biochemistry, Graz University of Technology, Austria;
[2]Institute of Plant Sciences, University of Graz, NAWI Graz, Graz, Austria;
[3]Institute of Biochemistry and Molecular Biology ZBMZ, University of Freiburg, Germany

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[*]Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria; Phone: +43-316-873-6462; Fax +43-316-873-6952; E-mail: guenther.daum@tugraz.at

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ABSTRACT

Phosphatidylserine decarboxylase 1 (Psd1p) catalyzes the formation of the majority of phosphatidylethanolamine (PE) in the yeast S*accharomyces cerevisiae*. Psd1p is localized to mitochondria, oriented towards the mitochondrial intermembrane space and anchored to the inner mitochondrial membrane (IMM) through membrane spanning domains. We found that Psd1 harbors at least two such membrane spanning domains which were named IM1 and IM2. Whereas IM1 was characterized in a previous study from our lab (Horvath et al., J. Biol. Chem. 287 (2012) 36744–55) no information about IM2 has been provided so far. To discover the role of IM2 in Psd1p import, processing and assembly into the mitochondria we constructed Psd1p variants with deletions in the predicted membrane spanning domain. Deletion of the complete IM2 led to mislocalization of the protein to the matrix site and to decreased enzyme activity. Deletion of the N-terminal moiety of IM2 also led to mislocalization to the matrix site of mitochondria, but deletions at the C-terminal part of IM2 resulted in localization to the outer mitochondrial membrane and to a loss of enzyme activity. In conclusion we showed that correct integration into the inner mitochondrial membrane is essential for full functionality of the yeast Psd1p.

Abbreviations

CDP, cytidine diphosphate; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; EDTA, Ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; Etn, ethanolamine; LGST, endoproteolytic cleavage site; LP, lysophospholipids; IM1 and IM2, inner mitochondrial sorting signal 1 and 2; IMM, inner mitochondrial membrane; MMGal –ura, minimal galactose media without uracil; MMGlu –ura, minimal glucose media without uracil; MT, mitochondrial targeting sequence; OMM, outer mitochondrial membrane; MPP, mitochondrial processing peptidase; Oct1p, octapeptidyl aminopeptidase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; Psd1, phosphatidylserine decarboxylase 1; Psd2, phosphatidylserine decarboxylase 2; Psd1β, phosphatidylserine decarboxylase 1 β-subunit; *Δpsd1*, Psd1 deletion strain; SDS, sodium dodecyl sulfate; SRS, predicted substrate recognition site; TCA, trichloroacetic acid; TIM, inner mitochondrial membrane translocation system; TLC, thin layer chromatography; TOM, translocase of the outer mitochondrial membrane; YPD, complex glucose media

INTRODUCTION

In the yeast large amounts of the non-bilayer forming phospholipids phosphatidylethanolamine (PE) and cardiolipin (CL) are present in mitochondrial membranes [1–3]. The presence of these phospholipids in mitochondrial membranes has a major influence on mitochondrial functions like respiration, membrane architecture, protein transport and protein stability [4–7]. Lack of CL affects the respiratory chain super-complexes and protein translocases in both the outer (OMM) and the inner mitochondrial membrane (IMM) [8–11]. Lack of both non-bilayer forming phospholipids PE and CL is lethal for *Saccharomyces cerevisiae* [12].

In yeast and in other eukaryotes PE can be synthesized via two pathways. First, phosphatidylserine (PS) can be decarboxylated by phosphatidylserine decarboxylases (PSD), and secondly diacylglycerol can be converted to PE in a reaction involving CDP-ethanolamine [2,13]. In *Saccharomyces cerevisiae* the majority of PE is synthesized by phosphatidylserine decarboxylase 1 (Psd1p), an enzyme which is located in the IMM [14–16]. Minor amounts of PE are formed in the extramitochondrial space by phosphatidylserine decarboxylase 2 (Psd2p) [17,18]. Deletion of *PSD1* leads to ethanolamine auxotrophy during growth on non-fermentable carbon sources, to reduced growth on fermentable carbon sources and to a decreased PE content in mitochondria [19–21]. The substrate of Psd1p, phosphatidylserine (PS), is synthesized in the endoplasmic reticulum/mitochondria-associated membrane and transported to the site of enzymatic conversion.

Given the fact that Psd1p is a very important enzyme of yeast phospholipid synthesis we studied this protein in some detail. Psd1p is synthesized as larger precursor on cytosolic ribosomes [22] and transported with the aid of different targeting signals to its final localization within mitochondria [23]. The precursor is translocated by the mitochondrial protein translocation machinery involving the TOM and TIM complexes (for reviews see refs [23–25]). This transport process is governed by an N-terminal mitochondrial targeting sequence [16,22]. The receptors Tom70 and Tom22 are responsible for recognition of the Psd1p precursor [22,26]. Insertion of the Psd1p precursor into the IMM depends on an energized IMM translocation system (TIM complex) which uses a membrane potential ($\Delta\psi$) as external energy source [16,27,28]. During the import process the targeting signals of the Psd1p precursor are removed by the matrix processing peptidase MPP and the octapeptidyl aminopeptidase Oct1 (Figure 1), which also stabilizes the mature form of the enzyme [16,29,30]. After import into mitochondria, Psd1p undergoes an autocatalytic endoproteolytic cleavage at its characteristic cleavage site, the LGST motif (Figure 1), to form an α- and a β-subunit [16,28,31–33]. This autocatalytic

cleavage leads to the formation of the catalytic pyruvoyl residue, which is located between the two subunits [31,34,35]. However, separation into α- and β-subunit can also occur when the protein is not correctly integrated into the IMM [16].

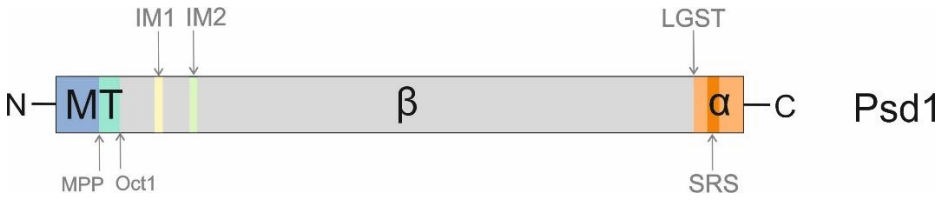

Figure 4: Psd1p is synthesized as larger precursor protein. MT, mitochondrial targeting sequence; MPP, mitochondrial processing peptidase; Oct1, octapeptidyl aminopeptidase; IM1 and IM2, predicted inner membrane sorting signal 1 and 2; LGST, endoproteolytic cleavage site; SRS, predicted substrate recognition site.

Horvath et al. [16] showed that the β-subunit of Psd1p is anchored to the IMM, tethering also the α-subunit which is per se soluble to the IMM. The authors demonstrated the importance of IM1 for this anchoring process. Using an analyzing software for transmembrane domain prediction we found that there is a second predicted membrane spanning domain in the yeast Psd1p, which we call IM2 in this study. To investigate a more general role of IM2 we generated yeast strains expressing *PSD1* with mutations in this predicted membrane domain and performed analyses addressing processing, activity, localization and import of the protein into mitochondria. We found that some mutations have an influence on growth behavior and led to growth like a *psd1Δ* deletion strain. In the mutant strains, correct processing into α- and β-subunit cannot occur. We also found that mutations in this region have an influence on the localization of Psd1p within the mitochondria, which can lead to reduced activity documented by a reduced PE level. We conclude that the membrane spanning domain IM2 is important for correct integration into the IMM, but also for processing and catalytic activity of the enzyme.

MATERIALS AND METHODS

Strains and culture conditions

Yeast strains used in this study are described in Table 1. Wild type cells were cultivated in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) to the stationary phase. Cells containing the pYES2 plasmid were cultivated in minimal medium containing glucose without uracil (MMGlu –ura) (0.67 % yeast nitrogen base without amino acids, 0.063 % amino acid mix without uracil, 2% glucose) under aerobic conditions with shaking at 30°C. Induction of protein expression was performed in minimal medium containing galactose without uracil (MMGal –ura) (0.67 % yeast nitrogen base without amino acids, 0.063 % amino acid mix without uracil, 2% galactose). Growth tests on solid media were performed on 0.67 % yeast nitrogen base without amino acids, 0.063 % amino acid mix without uracil, 2 % glucose, and 2 % agar and on 0.67 % yeast nitrogen base without amino acids, 0.063 % amino acid mix without uracil, 2 % galactose and 2 % agar. For growth phenotype analysis cell suspensions of overnight cultures, grown on YPD or MMGlu –ura, were spotted at dilutions of 1, 1/10, 1/100, 1/1000 and 1/10000 on YPD, MMGlu-ura and MMGal –ura with or without supplementation of 5 mM ethanolamine (Etn). Incubations were carried out at 30°C.

Table 1: Yeast strains used in this study

Name	Genotype	Origin
Wild type	BY4741 *MAT**a**his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf (Frankfurt, Germany)
BY4741 [pYES2]	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2*	[36]
Δpsd1	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4*	Euroscarf (Frankfurt, Germany)
Δpsd1 [pYES2]	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4 + pYES2*	This study
Psd1HA	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4 + pYES2-Psd1HA*	[16]
Psd1S463A	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 psd1Δ::KanMX4 + pYES2-Psd1S463A*	[16]
Psd1ΔIM2	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2-HIS*	This study
Psd1ΔIM2_T1	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2_T1-HIS*	This study
Psd1ΔIM2_T2	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2_T2-HIS*	This study
Psd1ΔIM2_T3	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2_T3-HIS*	This study
Psd1ΔIM2_T4	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2_T4-HIS*	This study
Psd1ΔIM2_T5	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2_T5-HIS*	This study
Psd1ΔIM2ΔGAP	BY4741 *MAT**a**his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2 - Psd1ΔIM2ΔGAP-HIS*	This study

Plasmid and strain constructions

Yeast strains expressing C-terminally HIS-tagged PSD1 variants were generated by overlap-extension PCR by amplifying *PSD1HA* [16] using gene specific primers in a standard PCR mixture containing ExTaqTM-DNA polymerase (Takara). The purified PCR product was inserted via BamHI and NotI (ThermoFisher, Waltham, Massachusetts) restriction sites into the pYES2 vector (Invitrogen, Carlsbad, California), forming pYES2-PSD1 variants Psd1ΔIM2 (residues from Ile-118 to Leu-140 were deleted), Psd1ΔIM2_T1 (residues from Ile-118 to Asn-122 were deleted), Psd1ΔIM2_T2 (residues from Asn-123 to Phe-126 were deleted), Psd1ΔIM2_T3 (residues from Phe-127 to Thr-131 were deleted), Psd1ΔIM2_T4 (residues from Leu-132 to Asn-135 were deleted), Psd1ΔIM2_T5 (residues from Ala-136 to Leu-140 were deleted) and Psd1ΔIM2ΔGAP (residues from Ser-96 to Leu-140 were deleted) (Figure 2). The plasmid was transformed into the *BY4741Δpsd1* strain by lithium acetate transformation [37]. Correct insertion of the cassette was tested by growing strains on selective media. Identity of strains was confirmed by marker-dependent growth, colony PCR and sequencing.

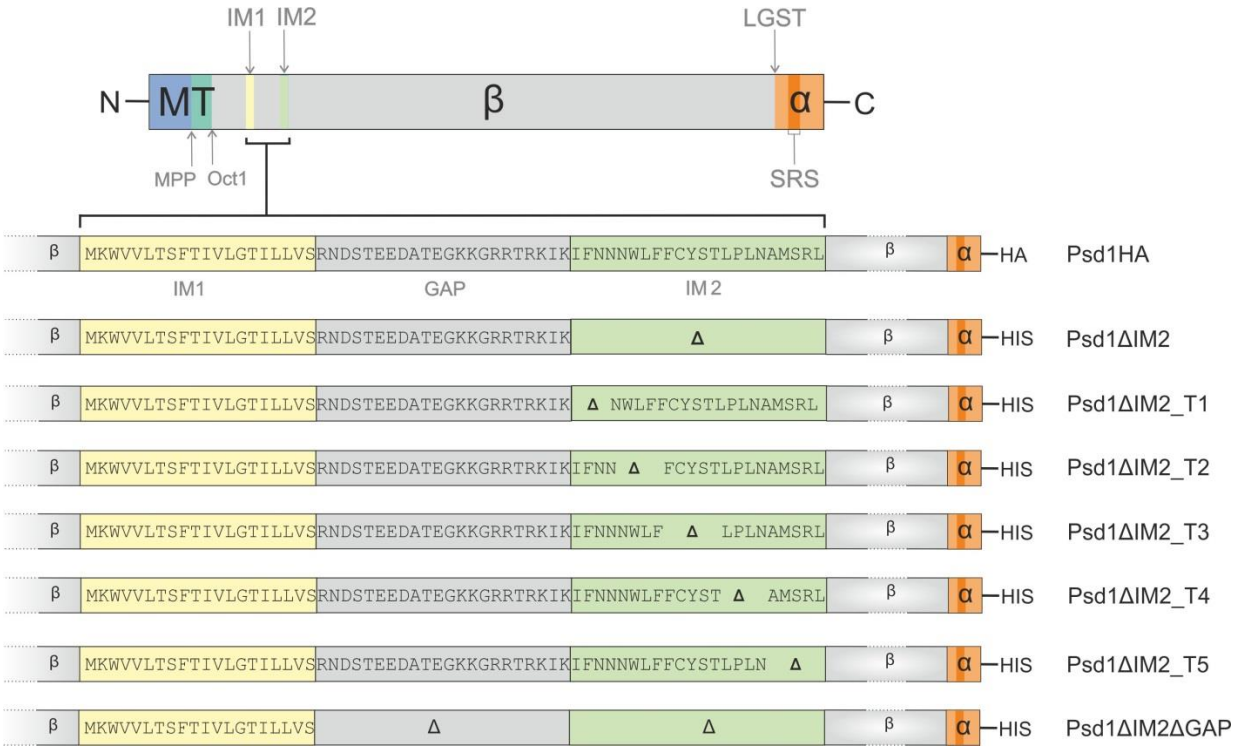

Figure *2*: The β-subunit of Psd1 tethers the Psd1 α-subunit to the inner mitochondrial membrane via two predicted membrane spanning domains. Psd1 variants with deletions in the predicted membrane spanning domains are shown schematically. MT, mitochondrial targeting sequence; MPP, mitochondrial processing peptidase; Oct1, octapeptidyl aminopeptidase; IM1 and IM2, predicted inner membrane sorting signal 1 and 2; LGST, endoproteolytic cleavage site; SRS, predicted substrate recognition site.

Isolation of mitochondria

Yeast cells were grown aerobically in MMGlu–ura for 48 h at 30°C. Cells were shifted to MMGal–ura and proteins were expressed at 30°C. To isolate mitochondria, cells were harvested and spheroplasted as described by Zinser and Daum et al. [1]. Zymolyase 20T (4 mg/g cell wet weight) was used to spheroplast the cells. Spheroplasts were homogenized in breaking buffer consisting of 0.6 M mannitol, 10 mM Tris (pH 7.4) and 1 mM phenylmethylsulfonyl fluoride (PMSF) by using a Dounce homogenizer as described previously [38]. Unbroken cells and debris were removed by centrifugation at 3000 x g for 5 min. The resulting supernatant was used to isolate mitochondria by differential centrifugation as published previously [1,39].

Protein analysis

Proteins from isolated subcellular fractions were precipitated with trichloroacetic acid (TCA) at a final concentration of 10 % for 1 h at 4°C. For protein quantification, the pellet was solubilized in 0.1 % SDS, 0.1 M NaOH and analyzed by the method of Lowry et al. [40] using bovine serum albumin as a standard. Proteins were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described at Laemmli et al. [41]. Electrophoresis was performed with 12.5 % or 15 % separation gels, and SDS-PAGE was carried out at 30 mA for 1 h. For purification studies of mitochondria Western blot analysis was performed [42] with primary rabbit antibodies directed against yeast Por1p, Cytb2p and ER-40 kDa protein. For Expression studies primary rabbit antibodies directed against Psd1 β-subunit, GAPDH and His_6 was used. Submitochondrial localization studies were performed with primary rabbit antibodies directed against yeast Tom70, Tim44 and Tim23. Immunoreactive proteins were visualized by ELISA using a peroxidase-linked secondary antibody (Sigma) following the manufacturer's instructions (SuperSignal, Pierce Chemical Company, Rockford, IL, USA).

Submitochondrial localization of proteins

For submitochondrial localization studies mitochondrial fractions were diluted in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS-KOH, pH 7.2). The submitochondrial localization of Psd1 variants was determined by accessibility to externally added proteinase K in intact, hypotonically swollen (mitoplasts) or lysed mitochondria. Mitochondria corresponding to 50 µg protein were re-isolated by centrifugation (16,000 × *g*, 10 min, 4°C). For hypo-osmotic swelling of mitochondria, mitoplasts were generated by treating mitochondria with a 9:1 ratio EM buffer (10 mM MOPS-KOH, pH 7.2, 1 mM EDTA) and SEM

buffer for 10 min on ice. Mitochondria were lysed by treatment with Triton X-100 at a final concentration of 0.5% (v/v) for 10 min on ice. Samples were treated with 50 µg/ml proteinase K for 15 min on ice. Proteinase K activity was stopped by the addition of 2 mM PMSF (phenylmethylsulfonyl fluoride) and incubation for 10 min on ice. Mitochondria were re-isolated by centrifugation (16,000 x *g*, 10 min, 4°C) and washed with SEM buffer and 2 mM PMSF. Then samples were subjected to SDS PAGE and Western blot analysis.

To determine membrane association of proteins, carbonate extraction was carried out as described previously [16,43–46]. Isolated mitochondria were suspended in 0.1 M sodium carbonate buffer (pH 11.5) and incubated on ice for 30 min. Mitochondrial membranes were re-isolated by ultracentrifugation at 100,000 x *g* and 4°C for 40 min. The pellet was solubilized in SDS-PAGE loading buffer, whereas proteins remaining in the supernatant were precipitated by trichloroacetic acid. Samples were subjected to SDS-PAGE and Western blot analysis.

Import of precursor proteins into isolated mitochondria

The import of precursor proteins into isolated mitochondria was performed according to Wenz et al. [47]. For *in vitro* transcription of each construct, a PCR-generated template containing the SP6 promoter was used. The RNA was purified (MEGAclear kit; Invitrogen) and used for *in vitro* translation (TNT kit, Promega) in the presence of ^{35}S-labeled methionine. Import of ^{35}S-labeled precursor proteins into isolated mitochondria of *S. cerevisiae* BY4741 cells (corresponding to 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 on ice and by dissipation of the membrane potential with a final concentration of 8 µM antimycin A, 1 µM valinomycin, and 20 µM oligomycin. Samples were incubated with 50 µg/ml proteinase K for 15 min on ice. Proteinase K activity was stopped by incubation with 2 mM PMSF for 10 min on ice. Mitochondria were re-isolated by centrifugation (10,000 x g, 10 min, 4°C) and washed with SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS-KOH, pH 7.2). Samples were subjected to SDS-PAGE, and ^{35}S-labeled proteins were detected by digital autoradiography (Storm imaging system, GE Healthcare, Chicago, Illinois).

Lipid analysis

Lipids from yeast cells were extracted as described by Folch et al. [48]. For phospholipid analysis 1 to 2 mg protein from total cell homogenate or 0.5 to 1 mg protein from mitochondrial fractions, respectively, were extracted using 3 ml chloroform/methanol (2:1; v/v). Individual

phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25 % NH_3 (50:25:6; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvents. Phospholipids were stained with iodine vapor, scraped off the plate and quantified by the method of Broekhuyse [49].

RESULTS

The Psd1 β-Subunit contains two predicted transmembrane domains which anchor the protein to the inner mitochondrial membrane

During processing Psd1p forms an α- and a β-subunit [16,31,50]. The α-subunit, which is per se soluble and localized to the intermembrane space is tethered to the β-subunit and thus also bound indirectly to the IMM. Horvath et al. [16] hypothesized that the β-subunit is anchored to the IMM by a single transmembrane segment named IM1. However, using analyzing methods for transmembrane domain prediction (TMHMM Server v. 2.0) we were able to detect two predicted IMM sorting signals in yeast Psd1p, which we call IM1 [16] and IM2 in this study. To study IM2 in more detail we generated yeast strains expressing *PSD1* with mutations in the predicted IMM sorting signal (Figure 2). To test all strains for correct processing and import we used a specific antibody recognizing the β-subunit of the protein and a HIS tag C-terminally fused to the α-subunit (Figure 2). Use of a HIS-specific antibody allowed us to detect the 4-kDa α-subunit of Psd1p in isolated mitochondria. To compare the Psd1p variants to a processed and unprocessed Psd1p, the Psd1HA and Psd1S463A [16] variant were used as controls. Psd1HA is a strain overexpressing Psd1p fused with a HA–tag, and the Psd1S463A variant has a mutation in the LGST cleavage site, which avoids autocatalytic processing.

Mutations in the predicted inner mitochondrial membrane sorting signal IM2 cause growth defects

To obtain insight into the involvement of the predicted membrane anchoring domain IM2 in cell function, we first tested growth behavior of the PSD1 mutant strains. Figure 3 shows that most Psd1 mutants used in this study grew like the Δ*psd1* strain on YPD. The strains Psd1ΔIM2_T1, Psd1ΔIM2_T2 and Psd1ΔIM2_T3 grew like the wild type on YPD medium. On glucose -containing minimal medium (MMGlu-ura) all mutant strains showed a slight growth defect, except Psd1ΔIM2_T2. Supplemention with ethanolamine rescued the cell growth and led to growth like wild type on MMGlu-ura. On non-fermentable carbon sources,

however, the requirement for PE in *S. cerevisiae* is more stringent than on fermentable carbon sources [19,51]. To rescue cell growth of a Δ*psd1* strain under these conditions, ethanolamine has to be supplemented as demonstrated previously [19,20,52]. Figure 3 shows that growth of the mutant strains was inhibited on MMGal –ura which contains the non-fermentable carbon source galactose. Yeast cells expressing the variant Psd1ΔIM2ΔGAP exhibited a growth phenotype similar to the Δ*psd1* strain on MMGal-ura. This result indicated that these regions are essential for Psd1p function. The other strains showed better growth than Δ*psd1* on MMGal-ura, but less than the wild type control. Figure 3 also shows that ethanolamine rescued cell growth on the non-fermentable carbon source galactose in all strains.

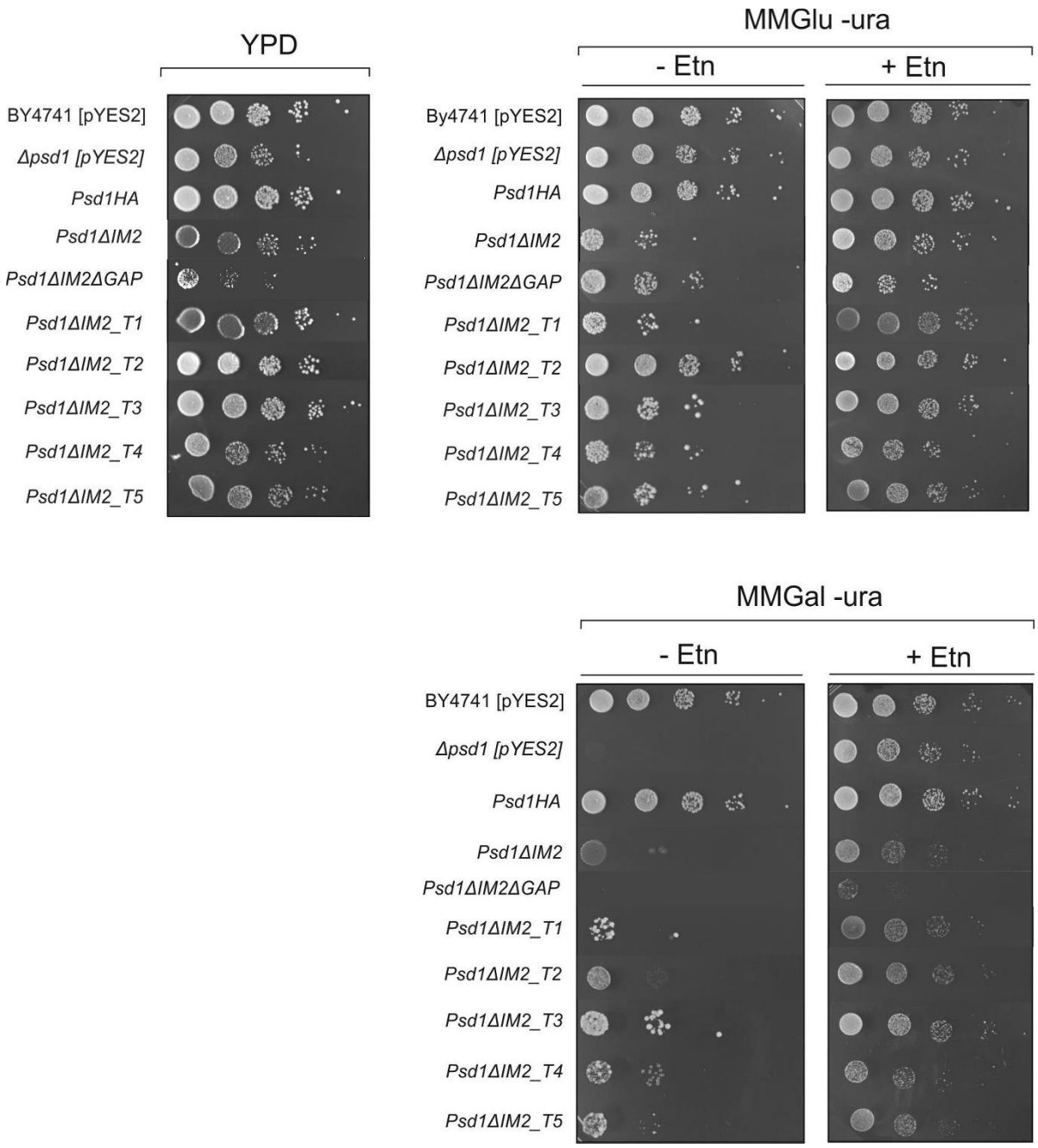

Figure 3: Phenotype analysis of BY4741, Δ*psd1*, Psd1HA and Psd1 mutants. Wild type BY4741 and mutant strains as indicated were grown on YPD, MMGlu-ura and on MMGal-ura with or without 5 mM ethanolamine. Cell suspensions of strains listed in the figure were spotted at dilutions of 1, 1/10, 1/100, 1/1000 and 1/10000 on the respective media. Incubation was carried out at 30°C. YPD, complex glucose media; MMGlu –ura, minimal glucose media without uracil; MMGal –ura, minimal galactose media without uracil; Etn, ethanolamine.

Psd1 requires the inner mitochondrial membrane sorting signal for correct integration into the inner mitochondrial membrane

To test the influence of mutations in the predicted second membrane spanning domain IM2 on localization within mitochondrial membranes, submitochondrial localization studies and carbonate extraction were performed. For this purpose we treated intact, hypotonically swollen (mitoplasts) or lysed mitochondria with proteinase K (PK) (Figure 4). Horvath et al. [16] had already shown that Psd1HA is attached to the IMM of intact mitochondria. This result was confirmed in this study as shown in Figure 4. After rupture of the outer membrane by osmotic swelling, Psd1HA was degraded by externally added proteinase K like the IMM integrated protein Tim23. The OMM protein Tom70 was digested on intact mitochondria (Figure 4, lane 1) after addition of PK and the matrix - localized protein Tim44 was degraded only after lysis of mitochondria with the detergent Triton X-100 and addition of PK (Figure 4, lane 5). This result indicates that α - and β - subunit are facing the intermembrane space. To test the membrane association of Psd1β in wild type and in the mutants we used the alkaline extraction method [43,46]. The β-subunit with an intact membrane anchor remained membrane associated after carbonate extraction at pH 11.5 like the outer membrane protein porin, whereas the soluble α-subunit, carrying the HA-tag was found in the supernatant (data not shown). We can conclude from this result that Psd1HAβ is facing the intermembrane space and anchored to the inner mitochondrial membrane, and the α-subunit is per se soluble and localized to the intermembrane space of the mitochondria.

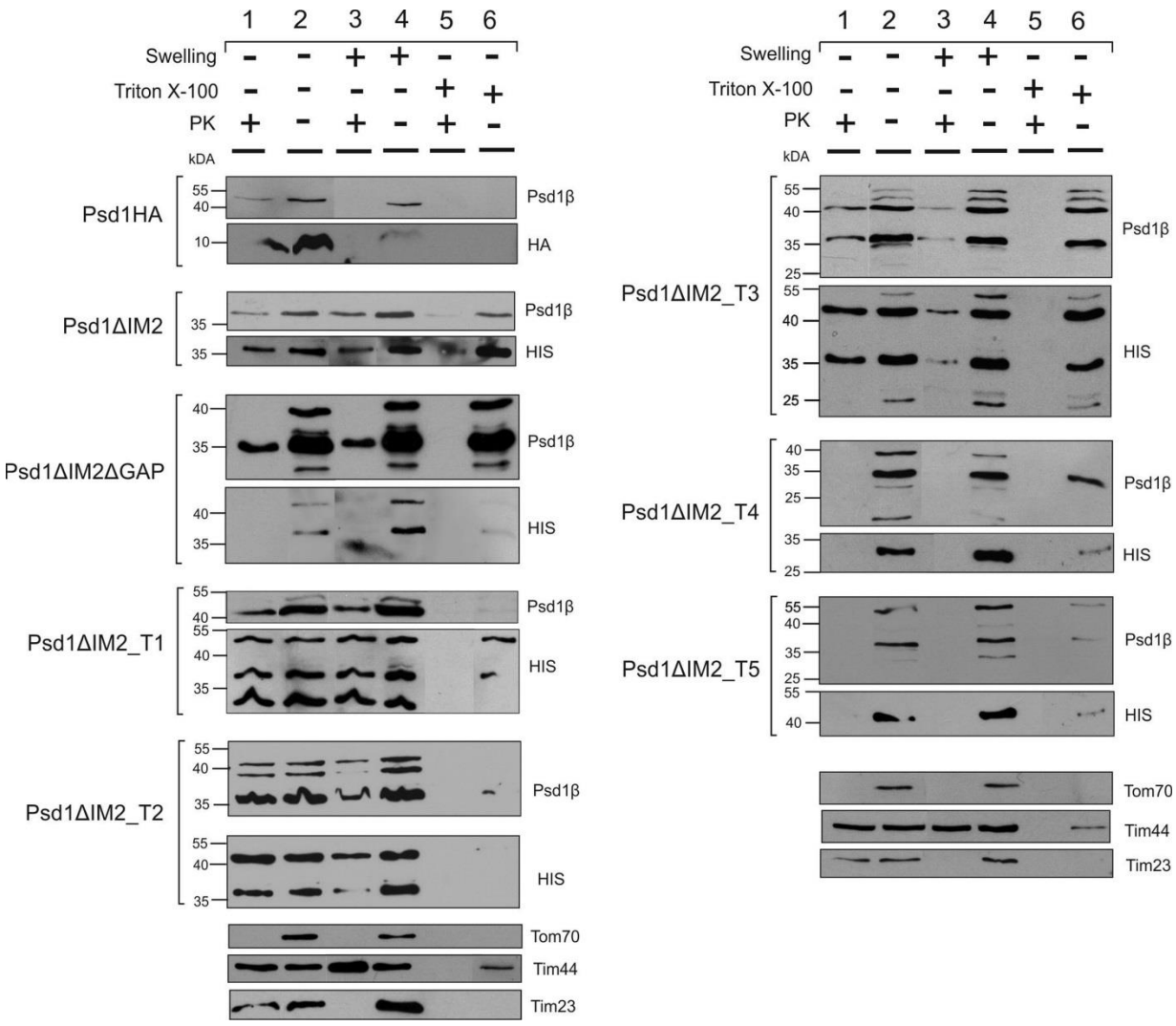

Figure 4: Psd1 requires the membrane sorting signal IM2 for integration into mitochondrial membrane and for correct processing to α – and β- subunit. Mutant strains as indicated were compared to the full length Psd1HA. Intact, swollen or Triton X-100 lysed mitochondria were incubated with or without the addition of 20 µg/ml proteinase K. Subsequently, samples were subjected to SDS-PAGE and proteins were visualized by immunodetection using the respective antibodies.

In the following all Psd1 variants bearing changes in the second membrane spanning domain IM2 were tested for their membrane topology. Psd1ΔIM2, lacking the complete IM2 domain, was found to be unprocessed and did not form separate α- and β-subunits (Figure 4, lane 2). The variant Psd1ΔIM2 was not accessible to externally added proteinases after rupture of the outer membrane by osmotic swelling (Figure 4, lane 3). The construct detected with Psd1β antibody and His antibody was degraded like Tim44, the matrix localized protein, by proteinase K only when membranes were lysed with detergent (Figure 4, lane 5). After carbonate extraction Psd1ΔIM2 was found in the pellet and therefore membrane bound (data not shown).

We can conclude from these results that Psd1ΔIM2 is unprocessed and anchored to the IMM facing the matrix site of the mitochondria. Therefore, the predicted mitochondrial sorting signal IM2 is crucial for correct localization of Psd1p within the IMM.

Psd1ΔIM2ΔGAP, lacking IM2 and the linker region to IM1, was unprocessed and partially N-terminally degraded. The protein of around 40 kDa (full length form) was degraded by proteinase K already without osmotic swelling (Figure 4, lane 1), indicating that this construct was located to the OMM. The protein of 35 kDa (partial degradation) is degraded like Tim44 only by proteinase K when membranes are lysed with the detergent (Figure 4, lane 5), indicating matrix localization. Carbonate extraction showed that the proteins were attached to the membrane as they were detected in the pellet fraction (data not shown). Thus, Psd1ΔIM2ΔGAP is mislocalized both to the OMM and to the matrix site of mitochondria.

The mutant strains Psd1ΔIM2_T1, Psd1ΔIM2_T2 and Psd1ΔIM2_T3 were unprocessed, N-terminally degraded (Figure 4, lane 2) and not accessible to externally added proteinase K after rupture of the outer membrane by osmotic swelling (Figure 4, lane 3). The proteins were degraded like Tim44, the matrix localized protein, by proteinase K only when membranes were lysed with detergent (Figure 4, lane 5). Thus, Psd1ΔIM2_T1, Psd1ΔIM2_T2 and Psd1ΔIM2_T3 are facing the matrix site of the mitochondria, but are still attached to the IMM, because they were detected in the pellet fraction after alkaline treatment (data not shown).

Psd1ΔIM2_T4 and Psd1ΔIM2_T5 were unprocessed and C-terminally degraded (Figure 4, lane 2). Both protein variants were degraded already after treatment with externally added PK (Figure 4, lane 1) and found in the pellet fraction after carbonate extraction (data not shown). These results indicate that Psd1ΔIM2_T4 and Psd1ΔIM2_T5 are located to the outer mitochondrial membrane.

In conclusion, localization studies described above indicate that the predicted IMM sorting signal IM2 has an influence on processing and localization of Psd1. In all mutants, processing into α – and β-subunit was defective and all mutants were mislocalized, either to the matrix site of the mitochondria or to the outer mitochondrial membrane.

Formation and import of proteins into isolated mitochondria is affected by mutations in the membrane spanning domain IM2

Previous studies have shown that the [^{35}S]-labeled Psd1 precursor was imported into mitochondria via the TOM complex in a multi-step process [16]. During import of the protein into isolated mitochondria in the presence or absence of a membrane potential three forms of Psd1 were detected (Figure 5A, lane 1-3). These three fragments are the intermediates 1 and 2 (i_1 and i_2), which are formed by unknown processing steps, and the mature Psd1 β-subunit (m) (Figure 5A, lane 1-3). The mature form and the intermediate 2 showed proteinase K resistance and were thus imported into mitochondria, whereas the intermediate 1 was not transported into the mitochondria because it was accessible to externally added proteinase K.

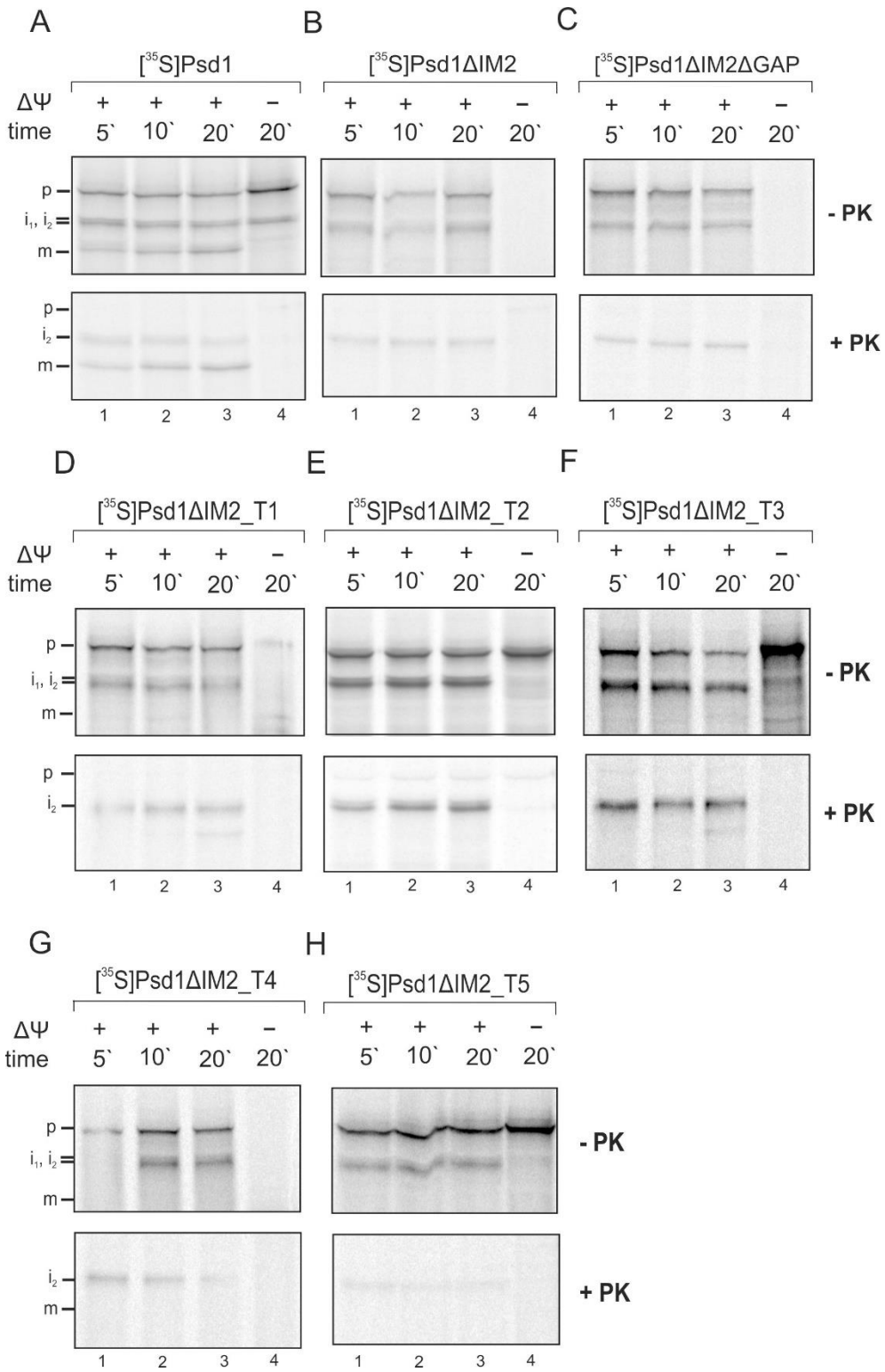

Figure 5: Mutations in the predicted membrane domain IM2 have an influence on the formation and the import of mature Psd1β into yeast mitochondria. ^{35}S-labelled Psd1HA precursor was imported in the presence or absence of a membrane potential (ΔΨ) into isolated mitochondria from wild type cells. Mitochondria were incubated with or without proteinase K (PK). The formation of the mature form (m) was time dependent and samples at time points 5, 10 and 20 minutes (min) were analyzed, ^{35}S-labelled proteins were detected by autoradiography. p, precursor; i_1,i_2, intermediates; m, mature Psd1β

In the following we tested the influence of the predicted membrane anchor IM2 on the assembly of Psd1 into the IMM. Deletion of the complete IM2 results in no formation of mature Psd1ΔIM2 α- and β-subunit and in the import of the intermediate i_2 into mitochondria (Figure 5B, lane 1-3). Also import experiments with ^{35}S-labeled Psd1ΔIM2ΔGAP showed no formation of mature protein and import of the intermediate i_2 into mitochondrial membranes (Figure 5C, lane 1-3).

Import experiments with ^{35}S-labeled variants Psd1ΔIM2_T1, Psd1ΔIM2_T2, Psd1ΔIM2_T3, Psd1ΔIM2_T4 and Psd1ΔIM2_T5 showed that processing into α- and β-subunit is defective (Figure 5D to H). Without the addition of proteinase K (PK) no mature (m) protein is formed (Figure 5D to H, lane 1-3) and only the intermediate i_2 was imported into the mitochondria.

We can conclude that deletions in the predicted membrane domain IM2 influence the ability of Psd1 to form the mature Psd1β and the import into mitochondria. In all mutant strains no mature protein was formed and only the intermediate i_2 was imported into mitochondria.

Psd1 requires integration into the inner mitochondrial membrane for full enzymatic activity

Horvath et al. [16] had already shown that mislocalization and defects in processing of Psd1p have an influence on the activity of the protein. Here we investigated whether in addition to the first membrane spanning domain IM1 also the second membrane sorting signal IM2 has an influence on the functionality of the enzyme. Phospholipid analyses were performed to compare the activities of the different variants to the wild type, the Psd1HA overexpressing strain, a *Δpsd1* deletion strain and to the unprocessed Psd1S463A variant with a mutation in the LGST motif (Figure 6). The wild type showed a PE content of 40.3 ± 1.6 % and a PC content of 44.7 ± 4.1 % in mitochondria and Psd1HA has a PE amount of 32.5 ± 1.4 % and a PC amount of 43.9 ± 2.5 % in mitochondria. The *Δpsd1* strain has a PE content in mitochondria of 6.6 ± 0.4 %, which is compensated by an increased PC content of 52.7 ± 5.3 % and the Psd1S463A variant has PE amounts of 5.5 ± 0.1 % and PC of 60.6 ± 4.9 % in mitochondria.

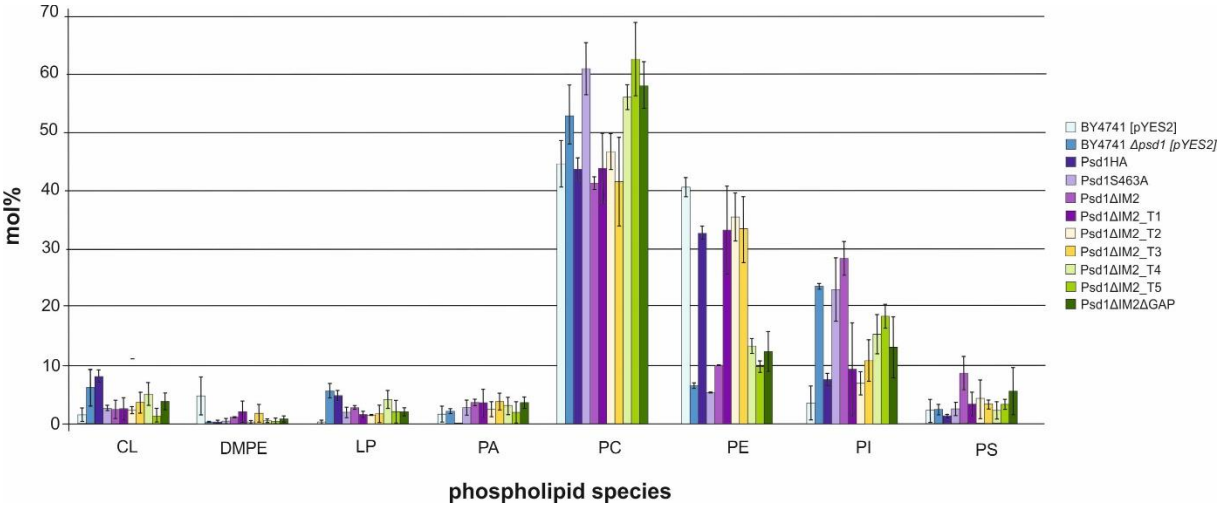

Figure 6: Localization of Psd1 to the inner mitochondrial membrane is required for optimal enzymatic activity. Phospholipids were extracted from mitochondria of wild type and the indicated Psd1 mutants. Strains were grown on MMGlu-ura and shifted to the expression medium MMGal-ura at 30°C. CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; LP, lysophospholipids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. Mean values of three independent measurements and S.D. values (error bars) are shown.

Psd1ΔIM2_T1, Psd1ΔIM2_T2 and Psd1ΔIM2_T3 are unprocessed and attached to the inner mitochondrial membrane, facing the matrix site of the mitochondria. These strains showed PE levels, and thus enzymatic activity, comparable to the wild type and the Psd1HA overexpressing strain in mitochondria (Figure 6). Defects in processing, like in the Psd1S463A strain led to a *Δpsd1* like phospholipid pattern, which is characterized by an enrichment of PC in the mitochondria and a very low amount of PE. Psd1ΔIM2_T4 and Psd1ΔIM2_T5 are unprocessed and localized to the outer mitochondrial membrane, which leads to a reduced formation of PE, which was compensated by a higher amount of PC (Figure 6). Figure 5 shows that mislocalization and defects in processing of the strains Psd1ΔIM2 and Psd1ΔIM2ΔGAP lead to reduced enzymatic activity, demonstrated by a reduced amount of formed PE in mitochondria.

We can conclude that Psd1 can only develop full enzymatic activity when the protein is transported to the IMM. Localization of an unprocessed variant to the outer mitochondrial membrane has a high influence on the activity and leads to a decreased formation of PE.

DISCUSSION

In the yeast *Saccharomyces cerevisiae* Psd1p plays an important role by forming the majority of cellular PE via decarboxylation of PS [32,33,53,54]. Deletion of *PSD1* results in reduced cell growth due to the reduced formation of PE in mitochondrial membranes and in ethanolamine auxotrophy during growth on non-fermentable carbon sources [19–21]. Thus, full functionality of Psd1 is important for cell integrity and function. In this study we show that import and correct sorting into mitochondrial membranes is crucial for full enzymatic activity of Psd1p. Previous studies had already shown that Psd1p is located to the inner mitochondrial membrane (IMM) facing the intermembrane space [16]. To reach its final shape and destination Psd1p undergoes several steps of maturation resulting in the formation of α- and β-subunit. For the ability of Psd1p to decarboxylate the polar headgroup of PS the interaction of both subunits within the intermembrane space of the mitochondria is prerequisite [55].

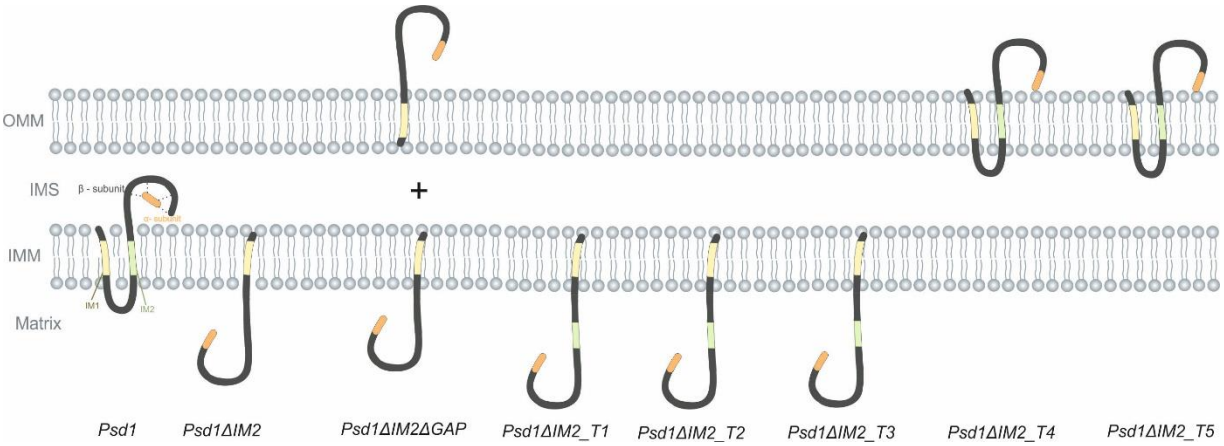

Figure 6: Scheme of wild type Psd1 and mutant strains located within the mitochondrial membrane. Mutations in the membrane anchor IM2 of Psd1 lead to different orientation of the proteins within the mitochondrial membrane. Listed mutant strains with supposed localizations within the mitochondria are shown. OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane; IM1 and IM2, inner membrane sorting signal 1 and 2.

Previously it was demonstrated that the β-subunit of Psd1p is anchored to the IMM by a single membrane spanning domain named IM1 [16]. The α-subunit is per se soluble and targeted to the IMM by attachment to the β-subunit. In this study we found that the β-subunit is anchored to the IMM by two membrane spanning domains called IM1 and IM2. Mutations in IM2 have an influence on Psd1p function, import into mitochondria and localization in the mitochondrial membranes. Figure 6 shows a scheme of the supposed localization of the Psd1 variants within the mitochondrial membrane. We found that already deletions of small parts of the second

membrane spanning domain, especially of the subdomains T4 and T5 have an influence on localization and activity of Psd1p. Only localization to the IMM leads to a fully active protein. Mislocalization of Psd1p to the OMM leads to a decreased PE formation. A reason for this observation may be that only when the protein is imported into the mitochondria an active protein is formed which can be interact with the substrate. The full enzymatic activity of the variants with deletions of the subdomains T1, T2 and T3 led us to speculate that these polypeptides are stuck in the IMM as shown in Figure 6. We assume that if the α-subunit is located to the surface of the IMM next to the intermembrane space, the substrate PS can reach the active site of the protein and can be converted to PE.

In summary we found that correct sorting of Psd1p to the IMM is required for full enzymatic function. Even small changes introduced by mutation are already sufficient to disturb the correct targeting of this enzyme.

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

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Author contributions: Conceived and performed the experiments: AW, FB, CH, KD. Wrote the manuscript: AW, TB and GD.

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Chapter 3

A YEAST MUTANT DELETED OF GPH1 BEARS DEFECTS IN LIPID METABOLISM

Martina Gsell[a#], **Ariane Fankl[a#]**, Lisa Klug[a] , Gerald Mascher[a], Claudia Schmidt[a], Claudia Hrastnik[a], Günther Zellnig[b] and Günther Daum[a,*]

[a]Institute of Biochemistry, Graz University of Technology, NaWi Graz, Petersgasse 12/2, 8010 Graz, Austria; [b]Institute of Plant Sciences, Karl Franzens University Graz, NaWi Graz, Austria

Short title: *GPH1* of the yeast

Keywords: phosphatidylethanolamine, phosphatidylserine decarboxylase, glycogen phosphorylase, triacylglycerol, lipid droplet, yeast

# Contributed equally to this study and should be regarded as co-first-authors

*Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria, Tel.: +43 316 873 6462, e-mail: guenther.daum@tugraz.at

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ABSTRACT

In a previous study we demonstrated up-regulation of the yeast *GPH1* gene under conditions of phosphatidylethanolamine (PE) depletion caused by deletion of the mitochondrial (M) phosphatidylserine decarboxylase 1 (*PSD1*) (Gsell et al., 2013, PLoS One. 8(10):e77380. doi: 10.1371/journal.pone.0077380). Gph1p has originally been identified as a glycogen phosphorylase catalyzing degradation of glycogen to glucose in the stationary growth phase of the yeast. Here we show that deletion of this gene also causes decreased levels of phosphatidylcholine (PC), triacylglycerols and steryl esters. Depletion of the two non-polar lipids in a Δ*gph1* strain leads to lack of lipid droplets, and decrease of the PC level results in instability of the plasma membrane. *In vivo* labeling experiments revealed that formation of PC via both pathways of biosynthesis, the cytidine diphosphate (CDP)-choline and the methylation route, is negatively affected by a Δ*gph1* mutation, although expression of genes involved is not down regulated. Altogether, Gph1p besides its function as a glycogen mobilizing enzyme appears to play a regulatory role in yeast lipid metabolism.

INTRODUCTION

Cellular lipids fulfill three major functions. First, they provide a depot and source of energy especially in the form of triacylglycerols (TG) and steryl esters (SE) which are stored in lipid droplets [1]. Secondly, certain classes of lipids such as glycerophospholipids, sterols, sphingolipids and glycolipids are important components of biological membrane. Finally, lipids can act as cellular messengers [2]. Depending on the environmental and nutritional conditions one or the other function of lipids becomes paramount. Importantly, pathways of lipid storage/mobilization and membrane lipid biosynthesis are interlinked thus providing a means to switch within lipid metabolism to the required branches. As examples, fatty acids (FA) from TG and/or SE can either be used for energy production through β-oxidation or as building blocks for the synthesis of membrane lipids. Diacylglycerol (DG), the other degradation product of TG hydrolysis, can be utilized as a substrate for phospholipid (PL) synthesis and consequently a key intermediate in membrane lipid formation [2-6] but also as a second messenger.

The above mentioned scenario demonstrates that lipid metabolism is a highly complex network of reactions which are subject to sophisticated regulation. During the last few years our laboratory focused on the central role of phosphatidylethanolamine (PE), which is a key component in lipid metabolism. In the yeast *Saccharomyces cerevisiae,* the model organism which we use for our studies, PE is essential and one of the major phospholipids besides phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS). The mitochondrial phosphatidylserine decarboxylase 1 (Psd1p) is the major producer of yeast PE catalyzing decarboxylation of PS to form PE [7,8]. The other three pathways of PE synthesis, namely decarboxylation of PS by Psd2p [9], the CDP-ethanolamine branch of the so-called Kennedy pathway [10], and synthesis of PE through acylation of lyso-PE catalyzed by the acyl-CoA-dependent acyltransferase Ale1p [11] are less efficient under standard growth conditions. Inactivation or deletion of the *PSD1* gene leads to a considerable decrease of PE in total cellular and especially in mitochondrial membranes and to a number of cellular defects [12,13].

To investigate effects of PE depletion caused by Δ*psd1* deletion on a genome wide basis we performed DNA microarray analysis of a Δ*psd1* mutant and compared its gene expression pattern with wild type [14]. This analysis revealed up-regulation of 54 genes in the Δ*psd1* mutant. One of the genes highlighted in this analysis was *GPH1*. Analysis of the growth phenotype and phospholipid composition suggested a possible involvement of *GPH1* in lipid metabolism. Gph1p was originally identified as glycogen phosphorylase 1 catalyzing the

release of glucose 1-phosphate from glycogen in the late stationary growth phase of the yeast to maintain the required energy for cell activity and growth during periods of nutrient starvation [15-19]. The activity of glycogen phosphorylase is regulated by cyclic AMP-mediated phosphorylation of the enzyme. *GPH1* is not essential in yeast, but Δ*gph1* mutants lacking the phosphorylase activity exhibit increased levels of intracellular glycogen [15].

In previous studies it has been shown that Gph1p is localized on so-called glycogen particles [15,20] whose size and amount vary during the growth phases of the yeast cell [14]. Glycogen, which is a storage form of carbon and energy, consists of branched glucose polymers and is synthesized by many different organisms [20]. Degradation of glycogen occurs when energy is required for cell activity and growth during stationary phase. In the yeast, expression of *GPH1* is induced at the end of the logarithmic growth phase. Almost simultaneously intracellular glycogen starts to accumulate as long as carbon sources are present. This finding suggested an important role of Gph1p in glycogen utilization as a reserve energy source during periods of nutrient starvation. Gph1p is inhibited by glucose 6-phosphate, and its activity is regulated by reversible phosphorylation [15]. The Δ*gph1* deletion mutant accumulates large amounts of glycogen during the stationary phase and shows rapid chronological aging and low stress tolerance [16].

In the present study, the influence of high glycogen content in the Δ*gph1* mutant was compared to wild type and to Δ*glc3*, a strain with inhibited glycogen synthesis [21]. Moreover, the influence of *GPH1* overexpression on phospholipid pattern and glycogen content was analyzed and compared to the other mutant strains. Analysis of Δ*gph1*, Δ*glc3* and wild type overexpressing *GPH1* [pYES_gph1] revealed changes in lipid metabolism of the mutants besides the above mentioned effects. In particular, a Δ*gph1* yeast mutant exhibited decreased PC levels in total cell homogenates (H) and especially in the plasma membrane as well as changes in the metabolism of non-polar lipids. Thus, it appears that Gph1p is involved in several branches of lipid metabolism in addition to its role as global player in carbohydrate metabolism. The multiple functions of Gph1p with emphasis on regulatory aspects in lipid metabolism are discussed in this paper.

MATERIALS AND METHODS

Strains and media

Yeast strains used in this study are listed in Table 1. Cells were grown in liquid YPD media (1% yeast extract, 2% peptone and 2% glucose) under aerobic conditions with shaking at 30°C. Growth tests on solid media were performed on 1% yeast extract, 2% peptone, and 2% agar supplemented with 2% glucose, 2.66% lactate (adjusted to pH 5.5 with KOH), 2% glycerol or 8 mM sorbitol, respectively. For SDS resistance assays on solid media, 0.05% SDS was added to the media immediately prior to pouring plates. MMLac (minimal medium with lactate) culture plates contained 0.67% yeast nitrogen base without amino acids, 0.073% amino acid mix, 2.66% lactate, adjusted to pH 5.5 with KOH, and 2% agar.

Table 1: Yeast strains used in this study

Strain	Genotype	Source / reference
BY4741	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Euroscarf (Frankfurt, Germany)
Δ*gph1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *gph1*Δ::KanMX4	Euroscarf (Frankfurt, Germany)
Δ*psd1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *psd1*Δ::KanMX4	Euroscarf (Frankfurt, Germany)
Δ*gph1*Δ*psd1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *gph1*Δ::KanMX4 *psd1*Δ::His3MX6	This study
Δ*sga1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *sga1*Δ::KanMX4	Euroscarf (Frankfurt, Germany)
Δ*gbd1*	Mata *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *gbd1*Δ::KanMX4	Euroscarf (Frankfurt, Germany)
Δ*glc3*	Mata; *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YEL011w::kanMX4	Euroscarf (Frankfurt, Germany)
BY4741 [pYES2]	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2*	This study
BY4741 [pYES2_gph1]	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 + pYES2-gph1*	This study

Yeast strains transformed with expression plasmids were grown on minimal medium minus uracil containing 2% galactose (Roth), 0.67% yeast nitrogen base without amino acids (U.S. Biological) and 0.063% amino acid mix without uracil (Roth, Fluka).
For growth phenotype analysis cell suspensions of overnight cultures grown on YPD were spotted at dilutions 1, 1/10, 1/100, 1/1000 and 1/10000 on YPD, YPLac, MMLac, YPGlycerol, YPSorbitol, and YPD with 0.05% SDS, respectively. Incubations were carried out at 30°C.

Plasmid and strain constructions

Inactivation of the *PSD1* gene in a Δ*gph1* deletion mutant was performed by transformation of the Δ*psd1*::His3MX6 disruption cassette as described by Longtine et al. [22]. The His3MX6 disruption cassette was amplified from the pFA6a-His3MX6 plasmid using the primers PSD1-koF 5'- gccagttaagaacgccttggcgcaagggaggacgctcctccggatccccgggttaattaa-3' and PSD1-koR 5'-caggtatgtggttccaagtgtttgtcgc -3'.
The disruption cassette was amplified by PCR under standard conditions using the proof reading *Ex Taq*^{TM}-DNA polymerase (Takara). The cassette was introduced into the respective strain by lithium acetate transformation [23]. Correct insertion of the cassette was tested by growing strains on selective media. Identity of strains was confirmed by marker-dependent growth, colony PCR and sequencing.
Yeast strains expressing *GPH1* were generated by amplifying *GPH1* using gene-specific primers from genomic DNA in a standard PCR mixture containing Ex TaqTM-DNA polymerase (Takara). The PCR product was purified and inserted via BamHI and XhoI (Fermentas) restriction sites into the pYES2 vector (Invitrogen), forming pYES2-GPH1.

RNA isolation and Real-Time PCR

For RNA isolation, cells were grown to the mid-logarithmic growth phase on YPD at 30°C. Total RNA was isolated using an RNeasy kit from Qiagen following the manufacturer's instructions. After DNaseI digestion, Real-Time PCR was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) as described by the manufacturer. Amplification was measured by using an ABI 7500 instrument (Applied Biosystems) in sealed MicroAmp Optical 96-Well Reaction Plates. Relative quantities of RNA were determined using the ΔΔCt method described by Livak and Schmittgen [24]. Differences in mRNA expression were calculated after normalization with *ACT1* relative to wild type control. Primers used for RT-PCR are listed in Table 2.

Table 2: Primers used for RT-PCR

Primer	Sequence (5'→ 3')
RT Act1-fwd	CCAGCCTTCTACGTTTCCATCCAAG
RT Act1-rev	GACGTGAGTAACACCATCACCGGA
RT Tgl3-fwd	GCCAACAATCCGAGCATAACGGAG
RT Tgl3-rev	TGGTGCCAAGTATGGTCTCGCCA
RT-Tgl4-fwd	TGCCCGACATGTGTATGCTTTTTAGAAT
RT-Tgl4-rev	CTTGGGCCACGTAGCTTTTGCAC
RT-Tgl5-fwd	CCGGGAGTTGACTTGGAAGAATCC
RT-Tgl5-rev	GGAGAAGGCAATGGCTGAAGAGGA
RT-Psd1fwd	GCCTCATGATACGGAACTTTTCTTTGC
RT-Psd1rev	CTGGGAAATGGCGGCGAAC
RT-Psd2fwd	GGCGCCACAAGATTATCACCGGTT
RT-Psd2rev	CGGCCATTGGATTTACAGTATAATA
RT-Cho2fwd	ATCGTGAAAAAACAAGAGTTGGATCAGGT
RT-Cho2rev	CCTTGAACGTACTTTTCAGTCGCCTTT
RT-Opi3fwd	ACCAAGCTGGGTGTGGCTCTCTTT
RT-Opi3rev	TCTCTCATCCATCAGGATGCCGAAATA

Calcofluor White sensitivity test

Sensitivity of yeast cells against Calcofluor White was tested as described by Takahashi et al. [25]. Cells were grown over night in YPD medium at 30°C with shaking. One OD_{600} unit was harvested, washed once with sterile water and diluted with 1 mL sterile water. Cell suspensions were spotted at dilutions of 1, 1/10, 1/100, 1/1000, 1/10000 onto YPD agar plates containing 20 µg/ml or 30 µg/ml Calcofluor White (CFW) and incubated at 30°C for 3 days.

Isolation of plasma membrane and mitochondria

Yeast cells were grown aerobically in YPD to the early stationary growth phase at 30°C. Then, cells were disrupted with glass beads using a Merckenschlager homogenizer under CO_2-cooling. Cell extracts were cleared of glass beads, unbroken cells and cell debris by centrifugation at 2500 x g for 5 min. The supernatant fraction represented the homogenate.

Crude plasma membrane was isolated essentially as described by Serrano [26] and further purified as reported by van den Hazel et al. [27] and Pichler et al. [28].

To isolate mitochondria, spheroplasts were prepared and homogenized in breaking buffer consisting of 0.6 M mannitol, 10 mM Tris, pH 7.4, and 1 mM phenylmethanesulfonylfluoride (PMSF) by using a Dounce homogenizer as described previously [29]. Unbroken cells and debris were removed by centrifugation at 3,000 x g for 5 min. The resulting supernatant was used to isolate mitochondria by published procedures [30].

Relative enrichment of markers and cross-contamination of subcellular fractions were assessed as described by Zinser and Daum [31]. Protein was quantified by the method of Lowry et al. [32] using bovine serum albumin (BSA) as standard. SDS-PAGE was carried out as published by Laemmli [33]. Western blot analysis of proteins from subcellular fractions was performed as described by Haid and Suissa [34]. Immunoreactive bands were visualized by ELISA using a peroxide-linked secondary antibody (Sigma-Aldrich, St. Louis, MO) following the manufacturer´s instructions.

Lipid analysis

Lipids from yeast cells were extracted as described by Folch et al. [35]. For phospholipid analysis 3 mg protein from total cell homogenate or 2 mg protein from mitochondria and plasma membrane fractions, respectively, were extracted using 3 ml chloroform/methanol (2:1; v/v). Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol/25% NH_3 (50:25:6; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvents. Phospholipids were stained with iodine vapor, scraped off the plate and quantified by the method of Broekhuyse [36].

For fatty acid analysis, cells were harvested during the logarithmic growth phase. After cell disruption using glass beads, the homogenate containing 1 mg protein was used for fatty acid analysis. Lipids were extracted as described above and fatty acids were converted to fatty acid methyl esters by methanolysis using 2.5% sulfuric acid in methanol and heating at 80°C for 90 min. Fatty acid methyl esters were extracted in a mixture of light petroleum and water (3/1; v/v) and analyzed by gas liquid chromatography (Hewlett-Packard 6890 Gas-chromatograph) using a HP-INNOWax capillary column (15 m × 0.25 mm i.d. × 0.50 μm film thickness) with helium as carrier gas. Fatty acids were identified by comparison to the fatty acid methyl ester standard

mix GLC-68B (NuCheck, Inc., Elysian, MN, USA) and hexacosanoic acid methyl ester standard (Sigma Aldrich, Vienna).

For quantification of non-polar lipids, lipid extracts were applied to Silica Gel 60 plates, and chromatograms were developed in an ascending manner by a two-step developing system [37]. First, chromatograms were developed using light petroleum/diethyl ether/acetic acid (70:30:2; v/v) and then light petroleum:diethyl ether (49:1; v/v) as solvents. To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2$ x 4 H_2O, 60 ml water, 60 ml methanol and 4 ml concentrated sulfuric acid, briefly dried and heated at 100°C for 20 min. Then, lipids were quantified by densitometric scanning at 400-650 nm with triolein, cholesteryl esters and ergosterol (ERG) as standards using a Shimadzu dual-wave length chromatoscanner CS-930. For DG analysis chromatograms were developed in chloroform/acetone/acetic acid (45:4:0.5; per vol.) with diolein as standard.

Analytical procedures

For the analysis of glycerol 3-phosphate (G3P), 10 OD_{600} units of yeast cells were harvested and lysed using the cellLytic Y-Yeast cell Lysis reagent (Sigma–Aldrich). Cells were incubated with 100 µl lysis buffer for 15-30 min at room temperature and centrifuged for 10 min to remove cell debris. For wild type analysis 10 – 20 µl, and for the analysis of *Δgph1* 2.5 - 5 µl of the supernatant were used for the glycerol 3-phosphate assay using the Glycerol 3-phosphate Colorimetric Assay Kit (Sigma-Aldrich).

For qualitative analysis of glycogen, cells were grown to the stationary phase and spotted onto YPD plates. Plates were grown for 48 h at 30°C prior to detection of glycogen by exposing plates to iodine vapor [38]. The intensity of the brown color was used as indicator for the cellular glycogen content.

Analysis of glycogen was performed following the instructions of the manual of the Glycogen Assay Kit (Sigma – Aldrich). For this purpose, 2 OD_{600} units of yeast cells were harvested and lysed using the cellLytic Y-Yeast cell Lysis reagent (Sigma – Aldrich). Cells were incubated with 20 µl lysis reagent for 15-30 min at room temperature and centrifuged for 10 min to remove cell debris. For the wild type strain 5 µl, and for the *Δgph1* strain 2.5 µl of homogenate were used for glycogen analysis.

Metabolic labeling of phospholipids and non-polar lipids

Labeling of aminoglycerophospholipids *in vivo* was determined by following the incorporation of L-[^{3}H]serine or [methyl-^{14}C]choline chloride, respectively, into PS, PE and PC as described previously [13,39]. An equivalent of 10 OD_{600} units from an overnight culture (~1 ml, corresponding to 1.45 x 10^{8} cells) was harvested in a Pyrex tube, washed once, suspended in 500 µl YPD and incubated for 30 min at 30°C. Cells were labeled with 10 µCi [^{3}H]serine (21.99 Ci·mmol^{-1}, Perkin-Elmer, Boston, MA) or 10 µCi [methyl-^{14}C]choline chloride (54 mCi·mmol^{-1}, Perkin Elmer Boston, MA), respectively, for 1 h at 30°C. Samples were put on ice, harvested by centrifugation and shock frozen with liquid nitrogen. Chloroform/methanol (2:1; v/v) and glass beads were added to the cell pellets and samples were vigorously shaken on an IKA® Vibrax VXR for 1 h. Then, lipids were extracted by the method of Folch et al. [35]. Individual phospholipids were separated by TLC on Silica gel 60 plates (Merck, Darmstadt, Germany) with chloroform/methanol/25% NH_3 (50:25:6, per vol.) as developing solvent. Spots on TLC plates were stained with iodine vapor, scraped off and suspended in 8 ml scintillation cocktail (Packard Bio-Science, Groningen, the Netherlands) containing 5% water. Radioactivity was determined by liquid scintillation counting using a Packard TriCarb® Liquid Scintillation Analyzer.

To estimate the incorporation of [1-^{14}C]acetic acid *in vivo* into total phospholipids and non-polar lipids an equivalent of 10 OD_{600} from an overnight culture was harvested, washed and suspended in 500 µl YPD. After an incubation of 30 min at 30°C, cells were labeled with 0.5 µCi [1-^{14}C]acetic acid (55.3 mCi mmol^{-1}, Perkin Elmer, Boston, MA) for 0, 10, 20, 30, 60 and 120 min, respectively. Lipids were extracted as described above, and individual lipids were separated by TLC with light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) and then light petroleum/diethyl ether (49:1; per vol.) as solvents. Radioactivity was determined as described above.

***In vivo* mobilization of non-polar lipids**

To measure the mobilization of non-polar lipids, cells were pre-grown for 24 h in minimal medium containing 0.67% yeast nitrogen base without amino acids, 0.073% amino acid mix and 2% glucose as the carbon source. Then, fresh minimal medium was inoculated with the pre-grown culture to an OD_{600} of 3, and cerulenin (final concentration 10 µg/ml) was added from an ethanolic stock solution. Control incubations contained the equivalent volume of ethanol only. At time points indicated, aliquots of the culture were withdrawn, and an equivalent

of 10 OD_{600} were harvested by centrifugation on a table-top centrifuge. The pellet was washed and shock frozen with liquid nitrogen. Lipids were extracted and analyzed as described above [40].

Microscopy of yeast cells

For electron microscopic inspection, cells were harvested in the early stationary growth phase by centrifugation and washed three times with double-distilled water. Subsequently, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with distilled water, and fixed in a 1% aqueous solution of $KMnO_4$ for 20 min again. Fixed cells were washed three times in distilled water and incubated in 0.5% aqueous uranylacetate overnight at 4°C. Samples were then dehydrated for 20 min in a graded series of 50%, 70%, 90% and 100% ethanol, each. Pure ethanol was then changed to propylene oxide, and specimen were gradually infiltrated with increasing concentrations (30%, 50%, 70% and 100 %) of Agar 100 epoxy resin mixed with propylene oxide for a minimum of 3 h per step. Samples were finally embedded in pure, fresh Agar 100 epoxy resin and polymerized at 60°C for 48 h. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 transmission electron microscope.

For fluorescence microscopy of lipid droplets Nile Red staining was performed as described by Greenspan et al. [41]. Yeast strains were grown to the early stationary growth phase and stained with Nile Red dissolved in ethanol. Microscopic pictures were visualized using a fluorescence microscope (Axiovert 35, Carl Zeiss, Jena, Germany) with the filter set 14 (Zeiss). Nile Red fluorescence of lipid droplets was detected at an emission wavelength of 590 nm with a 100-fold oil immersion objective.

RESULTS

Growth phenotype analysis

In a previous study from our laboratory [14] a genome wide approach revealed genetic and functional interaction of *GPH1* and *PSD1* from the yeast *Saccharomyces cerevisiae*. To obtain insight into the possible involvement of the *GPH1* gene product in lipid metabolism and especially in PE synthesis we performed a number of tests including growth behavior and lipid profiling of a Δ*gph1* mutant strain. In brief, we showed that Δ*gph1* and Δ*psd1* mutants grew like wild type on glucose-containing media. On non-fermentable carbon sources (YPLac and YPGlycerol), however, growth of Δ*psd1* was markedly reduced, whereas the Δ*gph1* mutant

grew like wild type. In the present study, growth tests were extended to the Δ*gph1*Δ*psd1* double mutant on both full media, but results largely reflected the effects of the Δ*psd1* deletion (data not shown). Growth on sorbitol containing plates revealed that Δ*gph1* as well as Δ*psd1* mutants became slightly instable [14]. Most interestingly, the Δ*gph1* deletion mutant was highly sensitive to SDS. Such an effect was not observed with the Δ*psd1* strain. This result suggested that in Δ*gph1* most likely the cell surface, the plasma membrane and/or the cell wall were compromised. In line with this view we showed that the phospholipid composition of the plasma membrane was compromised by the Δ*gph1* mutation, especially by decreasing dramatically the PC to PE ratio in this compartment [14].

To further test the sensitivity of the plasma membrane from a Δ*gph1* mutant strain we performed tests with Calcofluor White (CFW), which serves as a sensor for abnormalities of the plasma membrane [25]. Interestingly, CFW had a slight effect on the growth behavior of Δ*gph1* and Δ*psd1* (Figure 1). As a control, we also tested the effect of CFW on a Δ*glc3* strain which bears a mutation in glycogen synthesis [21]. However, growth of Δ*glc3* was also unaffected by CFW. Even higher concentrations of CFW, e.g., 30 µg/ml did not influence the growth behavior of all strains tested (data not shown).

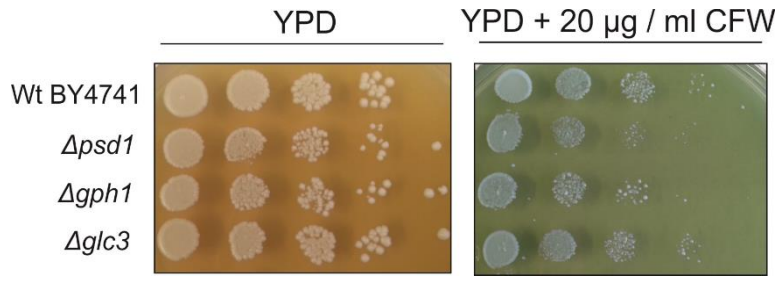

Figure 1: Calcofluor White sensitivity of Δ*gph1*, Δ*psd1* and Δ*glc3* deletion mutants. Wild type BY4741 and mutant strains as indicated were grown on YPD plates and on YPD plates containing 20 µg/ml Calcofluor White (CFW).

Phospholipid analysis of total cell homogenate and subcellular fractions

In our previous study [14] we showed that a Δ*gph1* mutation affected phospholipid profiles of total cell homogenate, mitochondria and especially the plasma membrane from cells grown on complex YPD media. The most striking effect was depletion of PC in the latter compartment. To rule out the possibility that total phospholipid production was compromised in a Δ*gph1* we quantified the total amount of phospholipids in wild type and in the mutant strain (Figure 2).

As can be seen the Δ*gph1* mutation rather led to an increased phospholipid level arguing against a negative influence of the mutation on the formation of total cellular membranes.

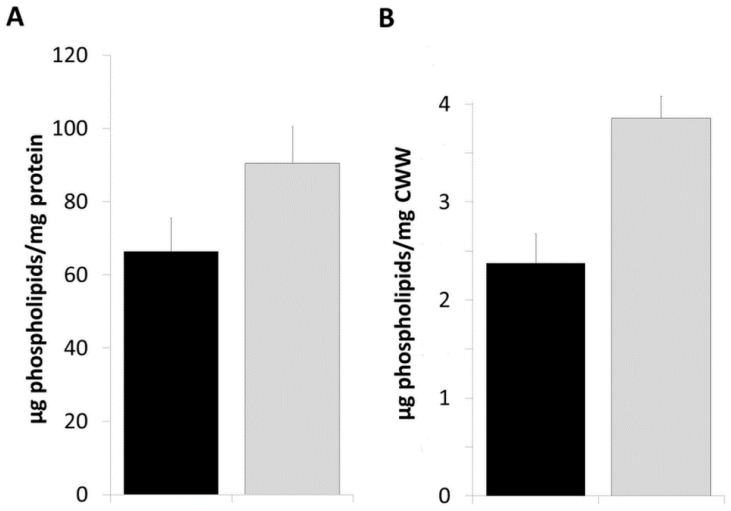

Figure 2: Total amount of phospholipids in cell free homogenate from wild type and the Δ*gph1* deletion mutant. Cells were grown on YPD to the early stationary growth phase at 30°C and disrupted with the aid of glass beads. Lipids were extracted with chloroform/methanol (2:1; v/v). Black bar, wild type BY4741; grey bar, Δ*gph1* mutant. (A) Total amounts of phospholipids were related to the amounts of protein. (B) Total amounts of phospholipids were related to cell wet weight. Inorganic phosphate was used as standard.

In the present study we extended the lipid profiling to a strain overexpressing *GPH1* (Table 3; WT [pYES_gph1]). In contrast to Δ*gph1*, overexpression of the gene led to a slight increase of PC in the total cell extract and in mitochondria. Surprisingly, the plasma membrane of WT [pYES_gph1] was rather depleted of PC. As another control, we analyzed lipids from a Δ*glc3* mutant (see Table 3). The *GLC3* gene product (1,4-glucan-6-(1,4-glucano)-transferase) is involved in glycogen metabolism catalyzing the last step in the biosynthetic pathway. As can be seen from Table 3, a Δ*glc3* deletion led to slight accumulation of PC in the total cell extract and a marked increase of PC in the plasma membrane, whereas a marked decrease of PC in the mitochondria was observed. Thus, in comparison with Δ*gph1* [14], it appears that there is no strict correlation between glycogen metabolism and PC biosynthesis. This view was confirmed by analysis of *sga1*Δ and *gdb1*Δ deletion strains. The respective gene products of *SGA1* (glucoamylase) and *GDB1* (glucotransferase) are involved in glycogen biosynthesis similar to Gph1p. However, both deletions had only minor and non-significant effects on PC formation (data not shown).

Synthesis of PC is down-regulated in the Δ*gph1* deletion mutant *in vivo*

As described above, the PC level in the Δ*gph1* deletion mutant was markedly decreased compared to wild type. As there are two pathways of PC synthesis in *Saccharomyces cerevisiae* we wished to estimate which one was affected by the Δ*gph1* deletion. PC can be synthesized (i) via the CDP-choline branch of the Kennedy pathway which utilizes choline as a substrate [10]; or (ii) through a three step methylation of PE catalyzed by Cho2p and Opi3p [42-44]. To analyze the CDP-choline pathway we labeled cells with [methyl-^{14}C]choline chloride and measured incorporation of the label into PC. As shown in Figure 3A, the synthesis of PC *in vivo* via this pathway was reduced to 60% of wild type. Interestingly, however, also the methylation pathway was affected by the Δ*gph1* deletion (Figure 3B). In this assay, cells were labeled with L-[^{3}H]serine, and sequential incorporation of the label into PS, PE (catalyzed by Psd1p or Psd2p), and PC (catalyzed by Cho2p and Opi3p) was measured. Whereas the first two steps in the biosynthetic route of aminoglycerophospholipids were reduced by 20%, the methylation of PE to PC in Δ*gph1* was approximately only 50% of wild type. In summary, formation of PC through both pathways was strongly decreased in the mutant.

Table 3: Phospholipid composition of cell-free homogenate, plasma membrane and mitochondria from cells grown on YPD or minimal medium (*). CF, cellular fraction; LPL, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; PA, phosphatidic acid. WT [pYES2], wild type bearing plasmid pYES2; WT [pYES_gph1], overexpression of *GPH1* on plasmid pYES in wild type background. Mean values of at least three measurements and standard deviations are shown.

		Phospholipids (mol%)								
CF	**Strain**	**LPL**	**PI**	**PS**	**PC**	**PE**	**CL**	**DMPE**	**PA**	**Others**
Homogenate	WT [pYES2]*	1.9 ± 1.1	7.5 ± 1.0	8.0 ± 0.8	47.5 ± 3.9	24.5 ± 5.7	3.0 ± 0.9	1.1 ± 0.9	3.6 ± 1.4	3.1 ± 0.9
	WT [pYES_gph1]*	1.4 ± 0.9	7.5 ± 1.1	6.7 ± 0.9	53.2 ± 5.9	21.8 ± 1.4	3.2 ± 0.3	1.8 ± 0.3	2.8 ± 0.8	1.5 ± 0.5
	WT	1.5 ± 0.2	9.9 ± 3.5	8.8 ±.0.5	45.1 ± 1.8	26.6 ± 2.5	3.4 ± 0.3	4.4 ± 0.7	0.7 ± 0.4	0.0 ± 0.0
	Δ*glc3*	0.1 ± 0.0	7.8 ± 3.2	5.4 ± 1.5	47.3 ± 5.1	30.4 ± 5.5	3.3 ± 1.2	4.3 ± 1.4	1.4 ± 0.8	0.0 ± 0.0
Mitochondria	WT [pYES2]*	3.5 ± 0.8	6.6 ± 1.3	3.9 ± 0.4	37.1 ± 8.6	39.6 ± 7.3	5.5 ± 1.9	0.3 ± 0.0	2.8 ± 2.2	0.8 ± 0.2
	WT [pYES_gph1]*	3.5 ± 0.7	6.1 ± 0.4	2.5 ± 0.7	41.5 ± 9.5	35.6 ± 6.2	7.3 ± 2.0	1.2 ± 1.0	1.5 ± 0.9	1.0 ± 0.5
	WT	1.9 ± 1.1	8.1 ± 1.7	4.1 ± 0.6	40.7 ± 2.4	30.4 ± 1.4	5.0 ± 3.6	6.6 ± 3.5	2.4 ± 0.4	0.7 ± 0.6
	Δ*glc3*	0.6 ± 0.1	6.5 ± 2.7	2.9 ± 0.3	27.5 ± 5.0	41.0 ± 4.3	9.1 ± 4.3	4.3 ± 0.9	8.1 ± 1.2	0.0 ± 0.0
Plasma membrane	WT [pYES2]*	2.3 ± 0.7	4.7 ± 0.3	15.9 ± 0.5	35.4 ± 5.7	33.3 ± 5.1	0.0 ± 0.0	0.6 ± 0.4	4.1 ± 0.9	3.6 ± 0.2
	WT [pYES_gph1]*	8.2 ± 1.7	9.3 ± 5.2	24.9 ± 7.8	19.5 ± 6.5	31.0 ± 9.8	3.0 ± 0.5	0.0 ± 0.0	4.3 ± 1.0	0.0 ± 0.0
	WT	2.4 ± 1.0	12.4 ± 2.1	26.2 ± 2.8	18.2 ± 1.4	32.1 ± 2.1	0.7 ± 0.1	2.2 ± 0.4	5.8 ± 0.6	0.0 ± 0.0
	Δ*glc3*	0.8 ± 0.2	7.7 ± 5.7	13.1 ± 2.3	42.3 ± 8.9	28.3 ± 6.5	0.5 ± 0.3	5.9 ± 2.9	1.4 ± 0.4	0.0 ± 0.0

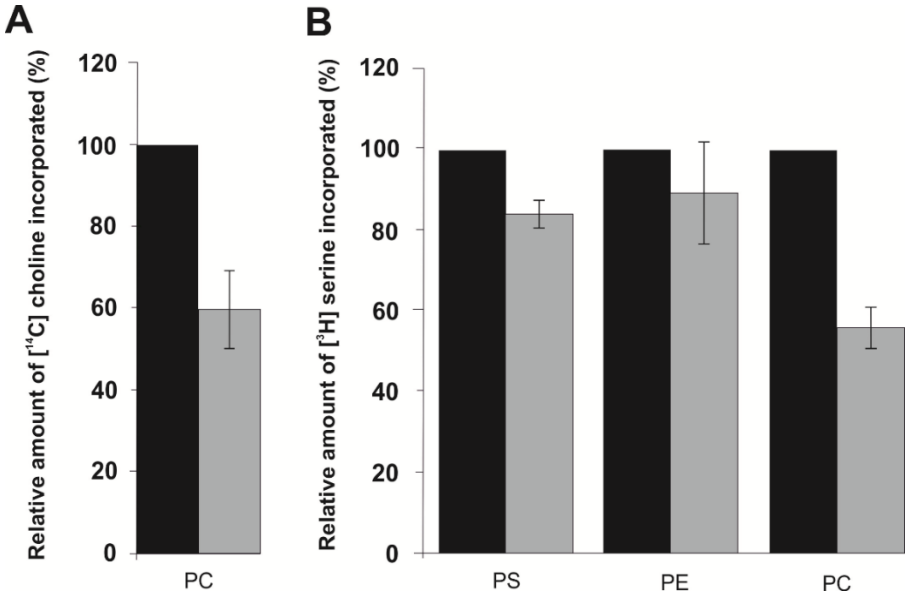

Figure 3: Synthesis of phosphatidylcholine is down regulated in the Δ*gph1* deletion mutant *in vivo.* Black bar, wild type BY4741; grey bar, Δ*gph1* deletion mutant; (A) The so-called Kennedy pathway (CDP-choline pathways) was analyzed by incorporation of [methyl-^{14}C]choline chloride into PC. (B) To analyze the methylation pathway of PC, the incorporation of L-[^{3}H]serine into PS, PE and PC was measured. Wild type BY4741 was set at 100%.

As diacylglycerol and glycerol 3-phosphate are precursors of PC their intracellular concentration was considered to have a possible influence on the cellular PC level [2, 4]. However, down regulation of PC formation in the *Δgph1* deletion strain could not be attributed to the limited availability of diacylglycerol or glycerol-3-phosphate. We found that the glycerol-3-phosphate content of the *Δgph1* mutant strain (2.412 ± 0.510 nmol/µl cell lysate) was even more the ten times higher than in wild type (0.196 ± 0.045 nmol/µl cell lysate), and also the DG content in the *Δgph1* strain (0.068 ± 0.016 µg/OD_{600} unit) was higher than in wild type (0.053 ± 0.002 µg/OD_{600} unit). These results showed that the two components were not limiting for the synthesis of PC in the *Δgph1* mutant in *vivo*.

We also tested the transcription rate of genes encoding key enzymes of aminoglycerophospholipid metabolism in wild type and Δ*gph1*. A Δ*psd1* deletion mutant was used as a negative control. As can be seen from Figure 4, genes encoding the phospholipid methyltransferase Cho2p and Opi3p as well as the PS decarboxylase Psd1p were not affected by the Δ*gph1* mutation. Interestingly, transcription of *PSD2* was strongly increased in the Δ*gph1* mutant. We may interpret this effect as compensation for the depleted activities of the aminoglycerophospholipid biosynthetic pathway (see Figure 3). Such compensation, however, was not observed with a Δ*psd1* mutant strain.

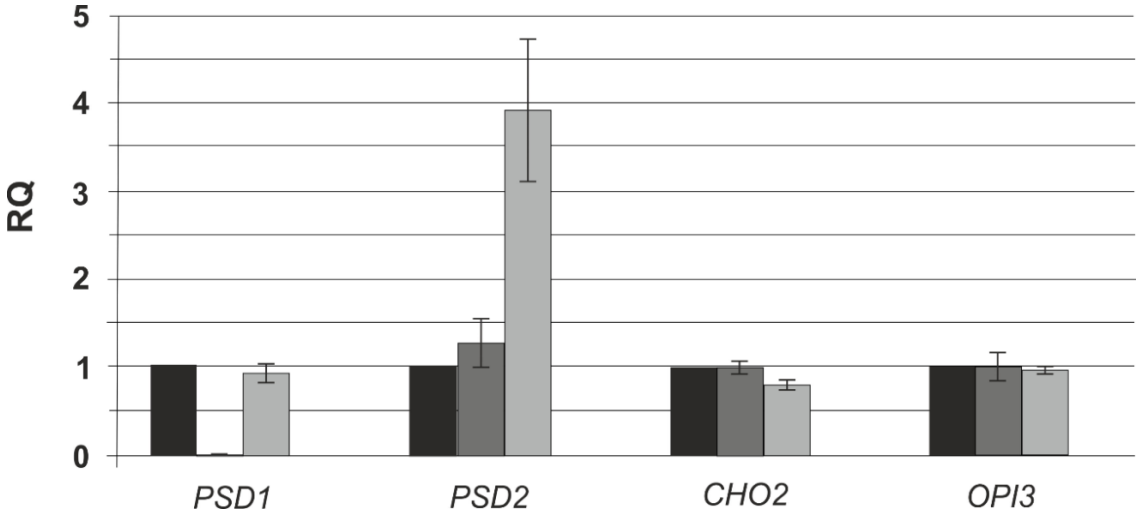

Figure 4: Expression levels of *PSD1, PSD2, CHO2* and *OPI3* in the Δ*gph1* deletion mutant. Relative gene expression of *PSD1, PSD2, CHO2* and *OPI3* in the Δ*gph1* deletion mutant was measured by RT-qPCR. A Δ*psd1* mutant was used as a negative control. Wild type was set at 1. Data are mean values from three independent experiments with the respective deviation.

Finally, we tested whether inhibition of PC synthesis (see Figure 3) was linked to the accumulation of glycogen in the *Δgph1* deletion mutant. Figure 5 shows the glycogen content of Δ*gph1,* Δ*psd1* and Δ*glc3* deletion mutants compared to the wild type *BY4741*. As expected, in the Δ*gph1* strain glycogen accumulated, whereas in the Δ*glc3* strain glycogen synthesis was inhibited. As Δ*gph1* leads to a decrease of PC [14], and Δ*glc3* to an increased PC level, one might argue that high glycogen levels correlate with low PC levels and vice versa. However, the fact that *sga1*Δ and *gdb1*Δ deletions, which also lead to the accumulation of glycogen, do not result in a significant change of PC (see above), supports the conclusion that there is no general correlation between glycogen and PC levels.

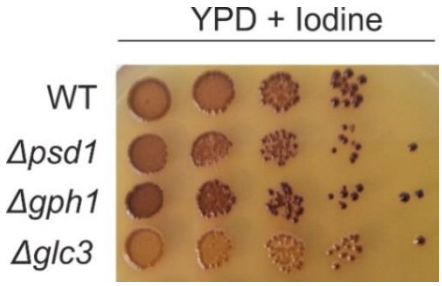

Figure 5: Glycogen content in the Δ*gph1,* Δ*psd1* and Δ*glc3* deletion mutants. Strains as indicated were grown on YPD plates. Cell suspensions were spotted at dilutions 1, 1/10, 1/100, 1/1000, 1/10000 and incubated at 30°C for 48 h. Plates were exposed to iodine vapor for 10 min.

Metabolism of non-polar lipids in the Δ*gph1* mutant

Changes in the phospholipid profile tempted us to speculate that also non-polar lipid metabolism was affected in the Δ*gph1* deletion strain. As can be seen from Figure 6 the amounts of TG and SE were indeed markedly reduced in the Δ*gph1* mutant. At the same time, the level of DG in the Δ*gph1* mutant was slightly increased (see also above) over wild type suggesting a possible involvement of Gph1p in the synthesis of TG. The amount of ergosterol was more or less the same in the mutant and in wild type.

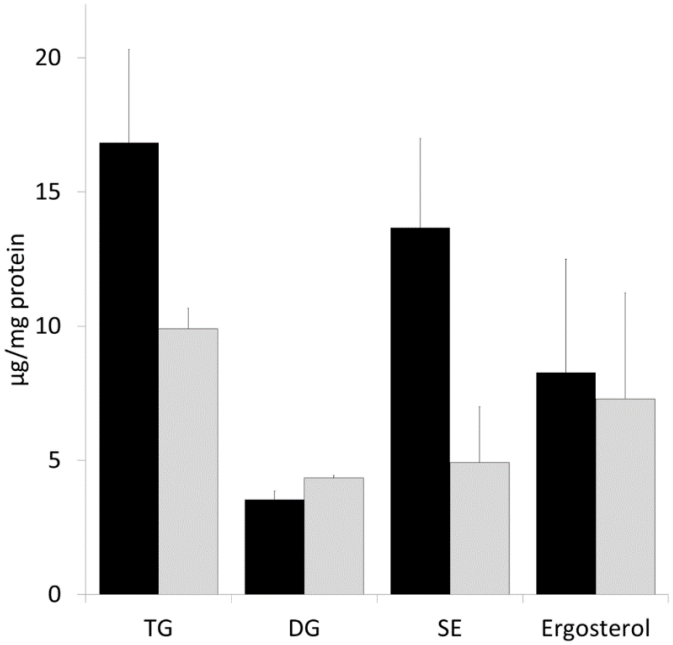

Figure 6: Amounts of triacylglycerols and steryl esters are reduced in the Δ*gph1* deletion mutant. The wild type BY4741 (black bar) and the Δ*gph1* deletion mutant (grey bar) were grown aerobically to the early stationary growth phase, and triacylglycerols (TG), diacylglycerols (DG), steryl esters (SE) and ergosterol were quantified. Results were obtained from 3 independent samples with deviations as indicated by the error bars.

As TG and SE are the main components of lipid droplets we also investigated the effect of the Δ*gph1* deletion on the formation of this compartment. Electron microscopic analysis of the Δ*gph1* mutant showed to our surprise that lipid droplets were more or less missing in the Δ*gph1* mutant strain (Figure 7A). In the wild type strain one to four distinct lipid droplets were detected per cell, whereas the Δ*gph1* deletion mutant lacked lipid droplets or contained droplets with very small size. This observation was confirmed by Nile Red staining and fluorescence microscopy (Figure 7B). The typical fluorescence signals of lipid droplets stained with Nile Red appearing as distinct spots were only detected in wild type, whereas in Δ*gph1* only diffuse fluorescence signals in the cellular background were observed.

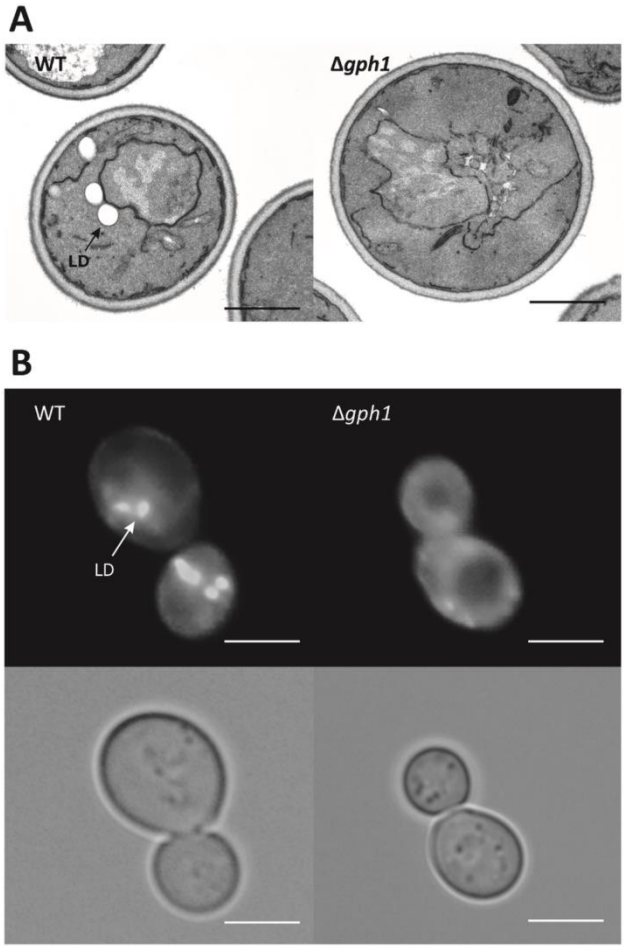

Figure 7: Number and size of lipid droplets are decreased in the Δ*gph1* deletion mutant. (A) Transmission electron microscopy images of wild type BY4741 and Δ*gph1*. (B) Nile Red staining and fluorescence microscopy of wild type BY4741 and Δ*gph1*. LD: lipid droplets. Scale bar: 2 µm.

Electron microscopic inspection of the Δ*gph1* mutant (see Figure 7A) also revealed that the total cell structure including the plasma membrane was not changed. High sensitivity of the deletion mutant to SDS [14] had been a hint for possible structural defects of the plasma membrane. However, such changes were not observed.

The question remained whether an increased turnover of TG in the Δ*gph1* mutant was the reason for the decreased amount of this lipid in the mutant. To address this question, we performed mobilization assays of cellular TG *in vivo* in the presence of cerulenin, an inhibitor of fatty acid synthesis in yeast [40]. Under these conditions, fatty acids from TG get mobilized mainly to be incorporated into membrane phospholipids [45]. In both wild type and Δ*gph1* TG was properly mobilized, although the initial TG degradation rate in the mutant was slightly higher (Figures

8A and B). Thus, changed TG hydrolysis in Δ*gph1* did not contribute to the lower level of this lipid in the mutant. To correlate the glycogen content with TG degradation we also measured the concentration of glycogen over the time period of TG mobilization. As can be seen from Figure 8C, the glycogen levels in the two tested strains were more or less constant during TG degradation. However, the absolute amounts of glycogen in wild type and *Δgph1* were different as expected. In wild type, approximately 20 µg glycogen per OD_{600} unit were detected, whereas in the mutant the glycogen level was approximately 45 µg per OD_{600} unit over the inspected time period. This result suggested that TG degradation and glycogen storage occurred independently of each other, at least under cultivation conditions described here.

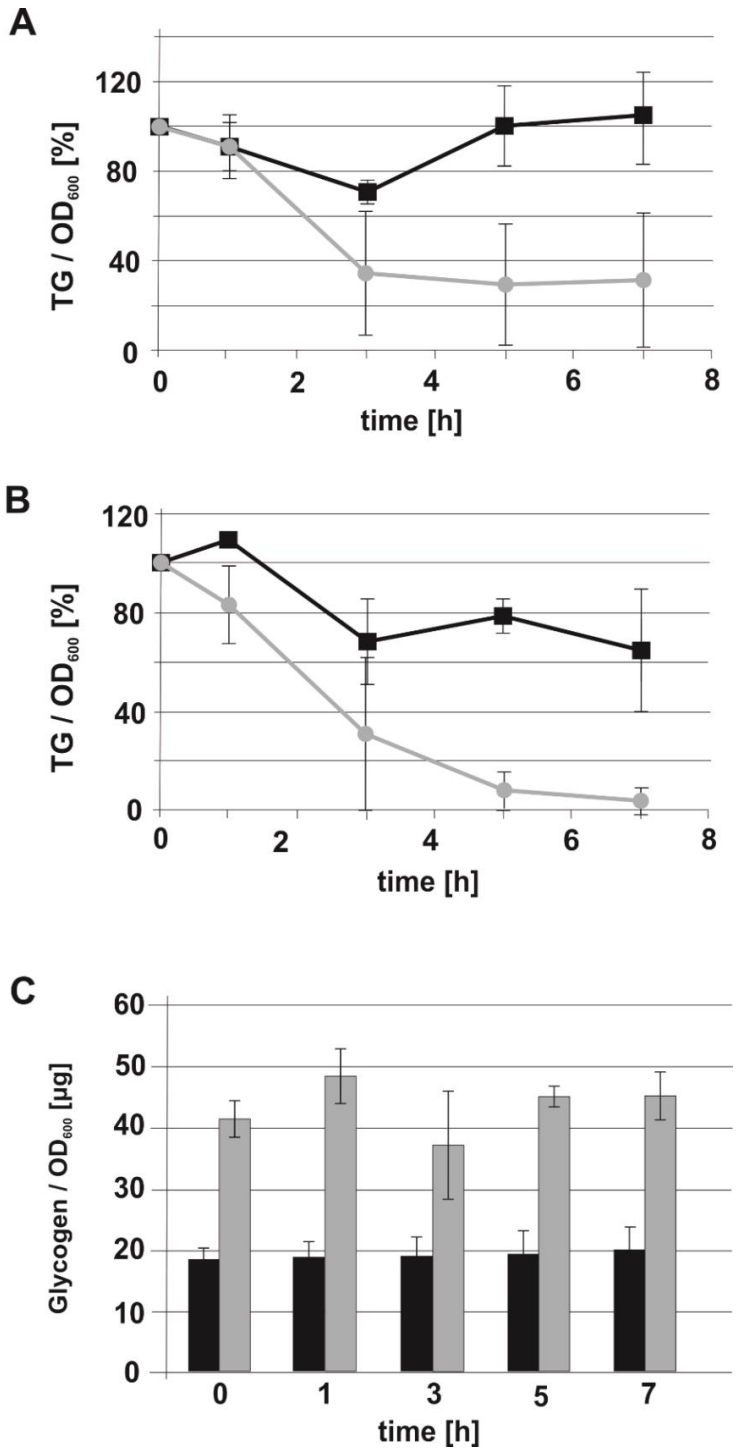

Figure 8: Triacylglycerol degradation in the *Δgph1* mutant. Wild type BY4741 (A) and *Δgph1* mutant cells (B) were grown to an OD_{600} of 3. Cerulenin was added at time point 0 to a final concentration of 10 µg/µl (grey line; -●-). The black line (-■-) shows the control without cerulenin. Over the time period of TG degradation the amount of glycogen was tested in wild type BY4741 (black bars) and *Δgph1* (grey bars). Amounts of glycogen were expressed as µg/OD_{600} unit. Results were obtained from 3 independent samples with deviations as indicated by the error bars.

Changes in the patterns of phospholipids and non-polar lipids from the Δ*gph1* mutant further led us to examine a possible role of Gph1p in fatty acid synthesis. To address this question we analyzed the fatty acid profile from wild type BY4741 and *Δgph1*. As can be seen from Figure 9 the *Δgph1* mutation did not affect the cellular fatty acids pattern. Thus, this branch of lipid metabolism was obviously not influenced by GPH1.

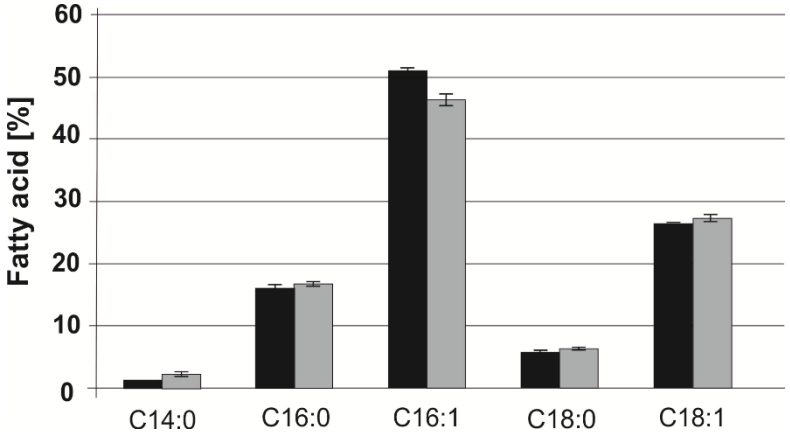

Figure 9: Fatty acid profile of the *Δgph1* mutant. The distribution of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1) was analyzed in wild type BY4741 (black bar) and in the *Δgph1* strain (grey bar). Results were obtained from 3 independent samples with deviations as indicated by the error bars.

DISCUSSION

Lipid metabolism of the yeast is a complex network of reactions with an even more complicated regulatory background. Besides genes encoding lipid metabolic enzymes a number of regulatory genes whose products are involved in synthesis and metabolic conversion of lipids have been identified (reviewed in ref. [4]). Consequently, synthesis and metabolism of the major yeast lipid classes, e.g. phospholipids, fatty acids, TG, sterols and sphingolipids are linked to each other [2]. Additionally, lipid synthesis in yeast is affected by growth conditions which influence the expression of enzymes and/or modulate their catalytic activities. As examples, expression of phospholipid biosynthetic genes in yeast is affected by carbon sources, availability of nutrient, growth phase, pH and temperature. Finally, posttranslational modifications of gene products, especially phosphorylation of key proteins involved in phospholipid synthesis, may affect metabolism of phospholipids and the balance between certain lipid precursors and final products of lipid biosynthetic pathways [5,46-51].

In previous studies from our laboratory [14] *GPH1* was identified as possible regulator gene of yeast lipid metabolism. Gph1p has originally been identified as a glycogen phosphorylase which catabolizes the branched polysaccharide glycogen used as storage carbohydrate [15-17]. In this study, we present extended evidence about the influence of *GPH1* expression on lipid metabolism in yeast. Besides the previously described effect of a Δ*gph1* deletion on PC synthesis [14] we show here that a mutant deleted of *GPH1* exhibited decreased formation of TG and SE, but increased synthesis of total phospholipids (see Figures 2, 3 and 6). Depletion of TG and SE in Δ*gph1* mutant cells led to lack of lipid droplets (see Fig. 7). Changes in the phospholipid composition, especially in the plasma membrane, were most likely the reason for the increased sensitivity of *Δgph1* against SDS. This view was confirmed by our findings that a *Δcho2Δopi3/Δpem1Δpem2* mutant which was strongly depleted of PC, also showed SDS sensitivity (supplemental Figure S1). Slight sensitivity of the *Δcho2Δopi3* mutant against Calcofluor White was also observed. Thus, PC depletion obviously leads to defects in the yeast cell periphery.

Based on the results described above we speculated about a link between carbohydrate metabolism of the yeast (glycogen storage and mobilization) and lipid metabolism through the action of Gph1p. For this reason, we first analyzed a *Δglc3* mutant strain. Glc3p is also an enzyme of glycogen metabolism, although in the biosynthetic branch. The *Δglc3* mutation caused only minor changes in the pattern of total cellular lipids (see Table 3). In mitochondria from *Δglc3*, a marked shift to lower PC and higher PE values was observed, whereas in the plasma membrane the opposite effect was seen. Furthermore, we also tested possible effects of *sga1*Δ and *gdb1*Δ deletions on lipid metabolism. Both the *SGA1* and the *GDB1* gene products are involved in glycogen metabolism similar to Gph1p. However, lipid profiles of Δ*sga1* and Δ*gdb1* deletion strains were similar to wild type indicating that the effect of Δ*gph1* was specific and unique. Altogether, we did not find a clear correlation between glycogen and PC metabolism in the yeast. Thus, Gph1p may fulfill multiple independent functions which affect carbohydrate metabolism on the one hand and lipid metabolism on the other hand.

Our investigations also addressed a possible role of Gph1p as a regulator of yeast TG lipases (see Figure 8). The decreased TG in the Δ*gph1* deletion mutant was regarded as a possible result of such an effect. However, only minor differences in TG degradation were found in wild type and in the Δ*gph1* deletion mutant. Moreover, expression levels of the three yeast genes encoding TG lipases, *TGL3*, *TGL4* and *TGL5*, were not changed in the Δ*gph1* strain (data not shown). Depletion of TG and SE are obviously the reason for the surprising observation that

the Δ*gph1* mutant lacks lipid droplets. It is even more surprising that lipid droplets are not formed although some TG and SE are still present in the mutant cells (see Figures 6 and 7). It can only be speculated that the remaining amounts of TG and SE are spread over internal membranes without leading to initiation of lipid droplet formation.

The molecular role of Gph1p in lipid metabolism is subject to speculation. Gph1p is a phosphatase and degrades glycogen leading to the formation of glucose-1-phosphate. Evidence presented here suggests, however, that Gph1p has more than one function in cell metabolism. Examples for such enzymes in lipid metabolism are the yeast TG lipases Tgl3p, Tgl4p and Tgl5p which serve simultaneously as lipases and acyltransferases [52]. It has been shown before that Gph1p is mostly active in the stationary phase of a yeast culture and not even properly expressed earlier [15]. In contrast, our study demonstrated a number of effects occurring already in earlier growth phases, e.g., sensitivity to SDS, changes in the lipid pattern, and lipid formation *in vivo*. These results largely exclude that glycogen accumulation directly affects the lipid metabolism, because glycogen accumulation does not occur in early growth phases.

Finally, we may speculate that Gph1p affects the subcellular distribution of lipid components. One piece of evidence in this line is the fact that TG, although formed at reduced quantities in Δ*gph1,* is not stored in lipid droplets at substantial amounts. The other finding supporting this view is that deletion of *GPH1* strongly and at least to some extend specifically affects assembly of PC into the plasma membrane. Altogether, Gph1p appears to cause several changes in yeast lipid metabolism whose molecular mechanisms remain to be elucidated.

APPENDIX

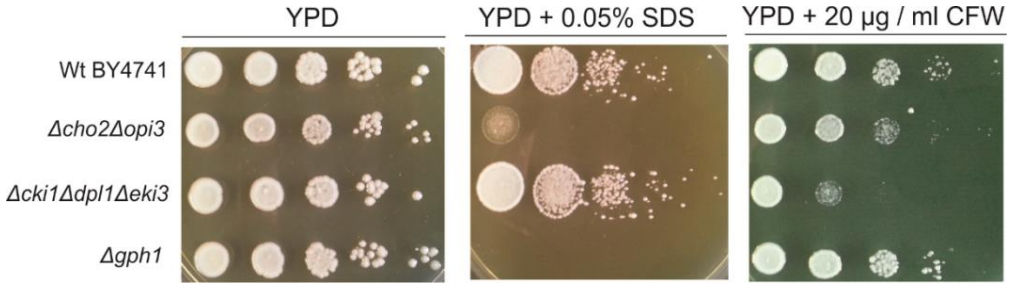

Figure S1: Sodium dodecyl sulfate and Calcofluor White sensitivity of Δ*cho2*Δ*opi3*, Δ*cki1*Δ*dpl1*Δ*eki3* and Δ*gph1* deletion mutants. Wild type BY4741 and mutant strains as indicated were grown on YPD plates and on YPD plates containing 0.05% sodium dodecyl sulfate (SDS) or 20 µg/ml Calcofluor White (CFW), respectively. The double mutant Δ*cho2*Δ*opi3* is blocked in the methylation pathway of PC synthesis, and the Δ*cki1*Δ*dpl1*Δ*eki3* triple mutant is blocked in the CDP-choline pathway of PC synthesis.

Acknowledgement

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Author contributions

Conceived and performed the experiments: MG, AF, LK, GM, CS, CH and GZ. Wrote the manuscript: MG, AF and GD. Designed the concept of the project: GD.

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Chapter 4

PHOSPHATIDYLCHOLINE SUPPLY TO PEROXISOMES OF THE YEAST *SACCHAROMYCES CEREVISIAE*

Vid V. Flis[1#], **Ariane Fankl[1#]**, Claudia Ramprecht[1], Günther Zellnig[2], Erich Leitner[3], Albin Hermetter[1], and Günther Daum[1*]

[1]Institute of Biochemistry, Graz University of Technology, NAWI Graz, Graz, Austria;
[2]Institute of Plant Sciences, University of Graz, NAWI Graz, Graz, Austria;
[3]Institute of Analytical Chemistry and Food Technology, Graz University of Technology, NAWI Graz, Graz, Austria

Short title: Phosphatidylcholine of yeast peroxisomes
Key words: phosphatidylcholine, yeast, peroxisomes, fatty acids, membrane fluidity

# Contributed equally to this study and should be considered as co-first authors
*Corresponding author: Günther Daum, Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria, Tel.: +43 316 873 6462, e-mail: guenther.daum@tugraz.at

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, phosphatidylcholine (PC), the major phospholipid (PL) of all organelle membranes, is synthesized via two different pathways. Methylation of phosphatidylethanolamine (PE) catalyzed by the methyl transferases Cho2p/Pem1p and Opi3p/Pem2p as well as incorporation of choline through the CDP (cytidine diphosphate)-choline branch of the Kennedy pathway lead to PC formation. To determine the contribution of these two pathways to the supply of PC to peroxisomes (PX), yeast mutants bearing defects in the two pathways were cultivated under peroxisome inducing conditions, i.e. in the presence of oleic acid, and subjected to biochemical and cell biological analyses. Phenotype studies revealed compromised growth of both the *cho2*Δ*opi3*Δ (mutations in the methylation pathway) and the c*ki1*Δ*dpl1*Δ*eki1*Δ (mutations in the CDP-choline pathway) mutant when grown on oleic acid. Analysis of peroxisomes from the two mutant strains showed that both pathways produce PC for the supply to peroxisomes, although the CDP-choline pathway seemed to contribute with higher efficiency than the methylation pathway. Changes in the peroxisomal lipid pattern of mutants caused by defects in the PC biosynthetic pathways resulted in changes of membrane properties as shown by anisotropy measurements with fluorescent probes. In summary, our data define the origin of peroxisomal PC and demonstrate the importance of PC for peroxisome membrane formation and integrity.

INTRODUCTION

In the yeast *S. cerevisiae* peroxisomes are important organelles for growth on fatty acids and alkaline media. In yeast and in plant cells, the β-oxidation of fatty acids is localized to peroxisomes, whereas in mammalian cells also mitochondria (M) are capable of performing fatty acid degradation. Peroxisomes are also important due to the fact that they harbor oxidative and detoxifying reactions involving oxygen and hydrogen peroxide as substrates [1].

It is believed that peroxisomes originate from the endoplasmic reticulum [2 - 4]. How this happens is still a matter of debate. Once the peroxisomes have come to maturation, they can divide autonomously [5]. Little is known about the phospholipid composition and especially the supply of phospholipids to peroxisomes. Similar to other biomembranes, peroxisomal membranes contain four major classes of phospholipids, namely phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) [6–8]. In mitochondria, cardiolipin (CL) is also a major phospholipid component. In almost all yeast membranes, PC and PE are present at 60-70% of total phospholipids [9].

Studies from our lab had shown, that PE in yeast cells grown on oleate media can be supplied to peroxisomes from three different sites of synthesis, namely the mitochondria, the endoplasmic reticulum and the Golgi apparatus [7, 10]. First, PE can be provided by the mitochondrial PS decarboxylase Psd1p; second, the vacuolar Psd2p produces PE for peroxisomes; and finally, the CDP-ethanolamine branch of the so-called Kennedy pathway synthesizes a portion of PE destined for peroxisomes. Ethanolamine required for this pathway can be used from external sources. Alternatively, phosphoethanolamine can be introduced into this pathway through degradation of sphingolipids [11]. PC supply to peroxisomes of the yeast *S. cerevisiae* has not yet been studied in detail.

In *S. cerevisiae* two main pathways of PC production exist: (i) the methylation pathway and (ii) the CDP-choline branch of the Kennedy pathway (Fig. 1) [10]. In the methylation pathway, PC is produced from PE which is synthesized either from PS or through the CDP-ethanolamine pathway. Aminoglycerophospholipid synthesis starts with the formation of PS in the endoplasmic reticulum by phosphatidylserine synthase Pss1p/Cho1p [12]. PS can then be decarboxylated by two identical reactions catalyzed by the mitochondrial phosphatidylserine decarboxylase 1 (Psd1p), or by Psd2p located to the vacuole. PE is then methylated through three steps by the methyltransferases Cho2p/Pem1p and Opi3p/Pem2p using S-adenosyl-L-methionine (SAM) as co-substrate [23]. The second pathway of PC production in the yeast is the CDP-choline branch of the Kennedy pathway. In the CDP-choline pathway, externally

added or endogenous choline is stepwise incorporated through phosphorylation by choline kinase and activation with CTP by phosphocholine cytidyltransferase. In the last step, phosphocholine is transferred from CDP-choline to diacylglycerol (DAG) by choline phosphotransferase and PC is formed [155].

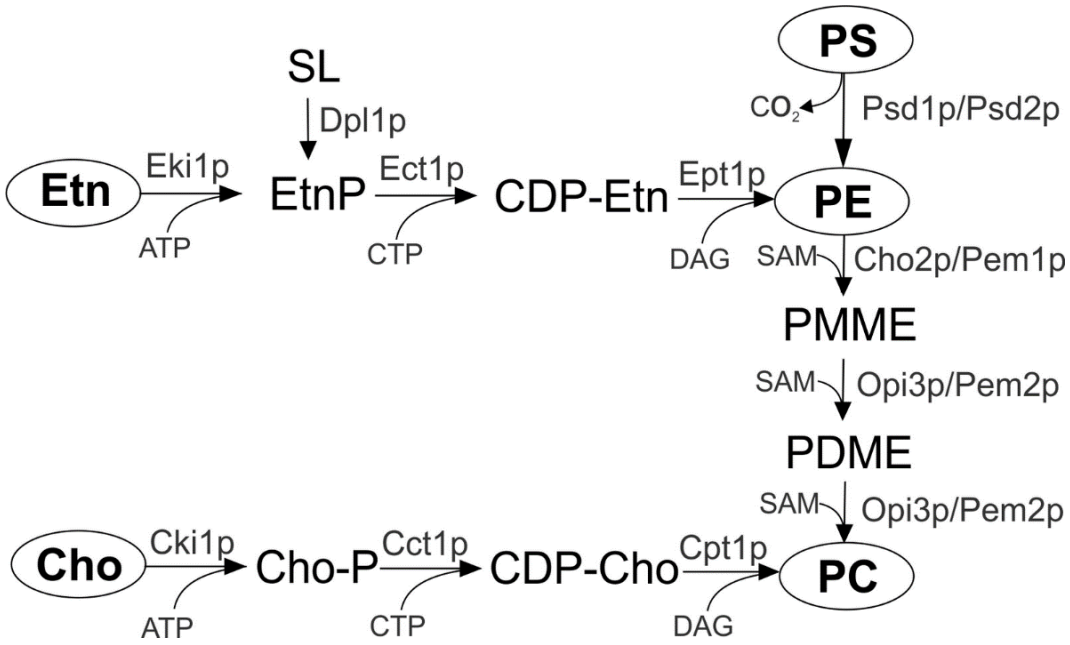

Figure 1: Pathways of phosphatidylcholine biosynthesis in the yeast *S. cerevisiae*. Cct1, cholinephosphate cytidylyltransferase; CDP-Cho, cytidine-diphosphocholine, CDP-Etn, cytidine-diphosphoethanolamine; Cho, Choline; Cho2, phosphatidylethanolamine methyltransferase; Cho-P, phosphocholine; Cki1, choline kinase; Cpt1, cholinephosphotransferase; DAG, diacylglycerol; Dpl1, sphingosine phosphate lyase; Ect1, phosphoethanolamine cytidylyltransferase; Eki1, ethanolamine kinase; Ept1, sn-1,2-diacylglycerol ethanolamine- and cholinephosphotranferase; Etn, ethanolamine; Etn-P, phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PDME, phosphatidyldimethylethanolamine; PMME phosphatidylmonomethylethanolamine; PS, phosphatidylserine; Psd1, phosphatidylserine decarboxylase 1; Psd2, phosphatidylserine decarboxylase 2; Opi3, methylene-fatty-acyl-phospholipid synthase; SAM, S-adenosyl-L-methionine; SL, sphingolipids.

While PC is the most abundant eukaryotic aminoglycerophospholipid [156] and important for the structure of membranes because of its cylindrical shape [157], little is known about the process of distribution of this lipid within the cell. The specific aim of the present study was to investigate routes of PC supply from its different sites of synthesis to peroxisomes of the yeast *S. cerevisiae* grown on oleate media, i.e. under peroxisome inducing conditions. To address this question we initiated studies with increasing specificity and analyzed growth phenotype, lipid composition and membrane properties of mutants compromised in the biosynthesis of PC.

These studies allowed us to identify the distribution of PC supply to peroxisomes and other organelles of the two mutants compromised in the specific biosynthetic pathway. The second focus of this study was on the role of PC as a component of peroxisomal membranes from the yeast *S. cerevisiae*. We show that the presence of PC in peroxisomal membranes is important for membrane properties. Especially the PC to PE ratio was identified as an important parameter for the biophysical status of the membrane.

MATERIALS AND METHODS

Strains and culture conditions

Yeast strains used in this study are described in Table 1. Cells were cultivated in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) to the stationary phase. For induction of peroxisomes, cultures were inoculated to an OD_{600} of 0.1 in YPO media containing 0.3 % yeast extract, 0.5 % peptone, 0.5 % potassium dihydrogen phosphate, pH 6, 0.1 % oleic acid (herbal oleic acid pure; Merck, Darmstadt, Germany), 0.2 % Tween 80 and 0.1 % glucose. Cells were grown to the late logarithmic phase. It has to be noted that YPD and YPO media contain low amounts of ethanolamine and choline. It also has to be noted that oleic acid preparations used routinely as carbon source contained impurities of margaric acid, myristic acid, stearic acid, palmitic acid, palmitoleic acid, linoleic acid and linolenic acid. Growth of the different strains on liquid media was followed by measuring the OD_{600}.

Table 2: Yeast strains used in this study

Name	Genotype	Origin
BY4742 (wild type)	MAT*α his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0*	Euroscarf (Frankfurt, Germany)
cki1Δdpl1Δeki1Δ	MATα *his3Δ200, leu2Δ1 trp1Δ63, ura3-52, cki1Δ::HIS3, dpl1Δ::LEU2, eki1Δ::TRP1*	[16]
cho2Δopi3Δ	*MATα his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; cho2::kanMX4*; *opi3::kanMX4*	Kindly provided by K. Athenstaedt
cho2Δ	MAT*α his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0 cho2Δ::KanMX4*	Euroscarf (Frankfurt, Germany)
opi3Δ	MAT*α his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0 opi3Δ::KanMX4*	Euroscarf (Frankfurt, Germany)

Isolation of peroxisomes, mitochondria and microsomes

For cell fractionation and isolation of peroxisomes, mitochondria and microsomes (endoplasmic reticulum) late exponential cultures of *S. cerevisiae* grown on YPO media were used. Cells were harvested and spheroplasted as described by Daum et al. [158]. 2 mg Zymolyase 20T were used per 1 g wet cell weight. Cells were homogenized on ice with a Dounce homogenizer in breaking buffer (5 mM MES; 1 mM KCl; 0.6 M sorbitol and 0.5 mM EDTA, pH 6.0-KOH) with 1 mM phenylmethanesulfonylfluoride (PMSF) added as protease inhibitor. Cell debris and nuclei were removed by centrifugation at 5000 rpm for 5 min. The resulting pellet was collected and subjected to two further rounds of resuspension in breaking buffer, homogenizing and centrifugation. The combined supernatants were centrifuged at 13,000 rpm in an SS34 rotor (Sorvall) for 30 min.

The pellets containing peroxisomes and mitochondria were collected and gently resuspended in a small Dounce homogenizer in breaking buffer plus 1 mM PMSF and centrifuged at low speed (3000 rpm) for 5 min to remove residual cellular debris. The supernatant containing the microsomal fraction was centrifuged at 15,000 rpm for 10 min. The pellet was resuspended in breaking buffer and loaded for further purification on a Nycodenz gradient (17 % - 24 % - 35 %; w/v) in 5 mM MES-KOH, pH 6.0, 1 mM KCl, and 0.24 M sucrose. Centrifugation was carried out in a swing out rotor (Sorvall AH-629) at 26,000 rpm for 90 min. A colorless-white band containing peroxisomes was collected with a syringe at the bottom, diluted in 4 volumes of breaking buffer and sedimented for 15 min at 15,000 rpm in an SS34 rotor. The procedure was repeated for mitochondria which formed a separate band at the top of the tube in the density gradient. The supernatant of the previous step containing the microsomal fraction was also centrifuged with an SS34 rotor for 45 min at 18,000 rpm to obtain a fraction containing the endoplasmic reticulum. The pellet containing the endoplasmic reticulum was collected. All organelles were stored at -70^{o}C for further analysis.

Protein analysis

Proteins from isolated subcellular fractions were precipitated with trichloroacetic acid (TCA) at a final concentration of 10 % for 1 h at 4^{o}C. For protein quantification, the pellet was solubilized in 0.1 % SDS, 0.1 M NaOH and analyzed by the method of Lowry et al. [18] using bovine serum albumin as a standard. Proteins were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described [19]. Electrophoresis was performed with 12.5 % separation gels, and SDS-PAGE was carried out at 24 mA for 1.5 h. For studies of

protein localization, Western blot analysis was performed [20] with primary rabbit antibodies directed against yeast Fox1p, Por1p, Cytb2p and ER-40kDa protein. Immunoreactive proteins were visualized by ELISA using a peroxidase-linked secondary antibody (Sigma) following the manufacturer's instructions (SuperSignal, Pierce Chemical Company, Rockford, IL, USA).

Lipid analysis

Total phospholipids were extracted from homogenate (H), endoplasmic reticulum, mitochondria and peroxisomes by the method of Folch et al. [21]. Total phospholipids were separated from non-polar lipids by one-dimensional thin layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany) using light petroleum/diethyl ether/acetic acid (35:15:1, per vol.) as solvent. Lipid bands were stained with iodine vapor, scrapped off the plate and quantified by the method of Broekhuyse [164]. For total phospholipid analysis 0.8 to 1 mg protein was used.

Individual phospholipids were separated by two-dimensional TLC on silica gel plates (Merck, Darmstadt, Germany) using chloroform/methanol/25 % NH_3 (65:35:5, per vol.) as first solvent, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second solvent. Lipid bands were visualized with iodine vapor, scrapped off the plate and quantified by the method of Broekhuyse [164]. For the quantification of individual phospholipids samples containing 1 mg protein were used.

Fatty acids were analyzed by gas liquid chromatography (GLC). Lipid extracts prepared as described above were incubated with 2.5 % sulfuric acid in methanol at 85°C for 90 min [165]. After incubation and cooling of the samples water was added, and fatty acids converted to methyl esters were extracted with light petroleum. Fatty acid methyl esters were separated using a Hewlett-Packard 6890-Gas-Chromatograph equipped with a HP-INNO Wax capillary column (15 m × 0.25 mm i.d. × 0.50 μm film thicknesses) and helium as carrier gas (20 min at 200°C, 10 min to 280°C, 15 min at 300°C). Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

Free fatty acids were extracted from homogenate, endoplasmic reticulum, mitochondria and peroxisomes by the method of Folch et al. [21]. Oleic acid was separated from other free fatty acids by one-dimensional thin layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany) using light petroleum/diethyl ether/acetic acid (35:15:1, per vol.) as first solvent and light petroleum/diethyl ether (49:1) as second solvent. Free fatty acid bands were visualized by dipping TLC plates into a charring solution (0.63 g $MnCl_2$ x 4 H_2O, 60 ml H_2O,

60 ml MeOH and 4 ml concentrated H_2SO_4) and incubating for 30 min at 100°C. Bands were quantified by scanning at 400 nm using a CAMAG TLC Scanner 3.

Sterols from whole cells or subcellular fractions were identified and quantified by gas liquid chromatography/mass spectrometry (GLC–MS) [24, 25]. In brief, a mixture of 0.6 ml methanol (Merck), 0.4 ml 0.5 % (w/v) pyrogallol (Fluka) dissolved in methanol and 0.4 ml 60 % (w/v) aqueous KOH solution was placed into 15 ml Pyrex tubes. As an internal standard 5 µl of cholesterol stock solution (2 mg/ml) were added. For analyzing sterols, organelles containing 0.5 to 1.0 mg protein were used. The respective amount of organelles was added to the reaction mixture, and tubes were heated in a sand bath for 2 h at 90ºC. Then, lipids were extracted three times with 1 ml n-heptane, each. The upper phase was transferred into a new tube and the lower phase was re-extracted. The combined upper phases were dried under a stream of nitrogen, and lipids were dissolved in 10 µl pyridine. After adding 10 µl N´O´-bis(trimethylsilyl)-trifluoracetamide (Sigma) samples were diluted with 50 µl ethylacetate and analyzed by GLC-MS. GLC–MS was performed on an HP 5890 Gas-Chromatograph equipped with a mass selective detector HP 5972, using an HP5-MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). Aliquots of 1 µl were injected in the splitless mode at 270°C injection temperature with helium as carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100°C, 10°C/min to 250°C, and 3°C/min to 310°C. Mass spectra were acquired in the scan mode (scan range 200–550 amu) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

For non-polar lipid analysis, lipids from yeast cells were extracted as described above. Lipids were applied to Silica Gel 60 plates, and chromatograms were developed in an ascending manner by a two-step developing system. First, chromatograms were developed using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) to two thirds of the plate. After drying, plates were further developed to the top using light petroleum/diethyl ether (49:1; v/v) as the second solvent system. Chromatograms for DAG analysis were developed using chloroform/acetone/ acetic acid (45:4:0.5; per vol.). To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2 \times 4H_2O$, 60 ml water, 60 ml methanol and 4 ml concentrated sulfuric acid, briefly dried and heated at 100°C for 20 min. Then, lipids were quantified by densitometric scanning at 400–650 nm with diolein and triolein as standards using a Shimadzu dual-wave length chromatoscanner CS-930.

Fluorescence anisotropy measurements

Isolated organelles (100 µg) were suspended in breaking buffer, pH 6 (5 mM MES, 1 mM KCl 0.5 mM EDTA, 0.6 M sorbitol). After addition of an organic solution of diphenylhexatriene (DPH) at a molar ratio of 1:50 (probe to phospholipid), mixtures were incubated for 5 min at 30°C. Samples were kept in the dark until fluorescence measurements were carried out using a Shimadzu RF 540 spectrofluorimeter equipped with polarizers in the excitation and emission light path. Excitation and emission wavelengths for DPH were 350 nm and 452 nm, respectively (slit width 10 nm). Fluorescence intensities were corrected for background fluorescence and light scattering from the unlabeled sample. The fluorescence anisotropy was calculated according to the equation $r = (I_{\parallel}-I_{\perp}) / (I_{\parallel}+2*I_{\perp})$. The values of $I_{\parallel}$ and $I_{\perp}$ are measured emission intensities parallel and perpendicular to the vertical polarization plane of the excitation light [26].

Electron microscopy

For electron microscopic examination, cell precultures were grown under aerobic conditions at 30°C on YPD medium containing 2 % glucose as carbon source. Cells were diluted to an OD_{600} of 0.1 in fresh YPO medium and grown to the late exponential phase. Then, cells were harvested by centrifugation and washed twice with 0.5 % BSA (fatty acid free) and 3 times with H_2O. Washed cells were fixed for 5 min in a 1 % aqueous solution of $KMnO_4$ at room temperature, washed with double distilled water and fixed again in a 1 % aqueous solution of $KMnO_4$ for 20 min. Fixed cells were washed three times in distilled water and incubated in 0.5 % aqueous uranyl acetate for the first three hours with shaking at room temperature and afterwards overnight at 4°C. Samples were then dehydrated for 20 min in a graded series of 50 %, 70 %, 90 % and 100 % ethanol, each. Pure ethanol was then changed to propylene oxide, and specimen were gradually infiltrated with increasing concentrations of Agar 100 epoxy resin (30 %, 50 %, 70 % and 100 %) mixed with propylene oxide for a minimum of 3 h per step. Samples were embedded in pure, fresh Agar 100 epoxy resin and polymerized at 60°C for 48 h. Ultra-thin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 transmission electron microscope.

RESULTS

Growth characteristics of yeast mutants compromised in one of the two phosphatidylcholine biosynthetic pathways

To analyze the influence of mutations in PC synthesis on growth of yeast cells on different carbon sources drop tests on YPD and YPO agar plates (Fig. 2A) were performed and cells were cultivated in liquid media (Fig. 2B). These tests showed that strains bearing defects in the CDP-choline pathway of PC synthesis grew normally on YPD at 30°C. The *cho2*Δ*opi3*Δ mutant showed only slight growth defects on YPD at 30°C. At a temperature of 37°C on YPD, however, *cho2*Δ, *cho2*Δ*opi3*Δ and the *cki1*Δ*dpl1*Δ*eki1*Δ mutants exhibited a growth defect. As shown in Fig. 2A growth of the *cho2*Δ*opi3*Δ mutant was more affected than growth of *cki1*Δ*dpl1*Δ*eki1*Δ. On minimal media containing oleate as carbon source and supplemented with 5 mM choline and 5 mM ethanolamine the *cho2*Δ*opi3*Δ mutant grew markedly worse than wild type (see Fig. 2A). Under these conditions only a slight growth defect of *cho2*Δ was observed, and growth of the *cki1*Δ*dpl1*Δ*eki1*Δ mutant was practically not affected. Surprisingly, on YPO rich media the growth defect of *cho2*Δ, *cho2*Δ*opi3*Δ and the *cki1*Δ*dpl1*Δ*eki1*Δ mutants was more pronounced than on minimal oleate containing media. This effect may be due to the limiting amount of ethanolamine and choline present in YPO. At 37°C, mutant strains did not grow at all on minimal oleate media supplemented with choline and ethanolamine, although the wild type strain showed normal growth (data not shown).

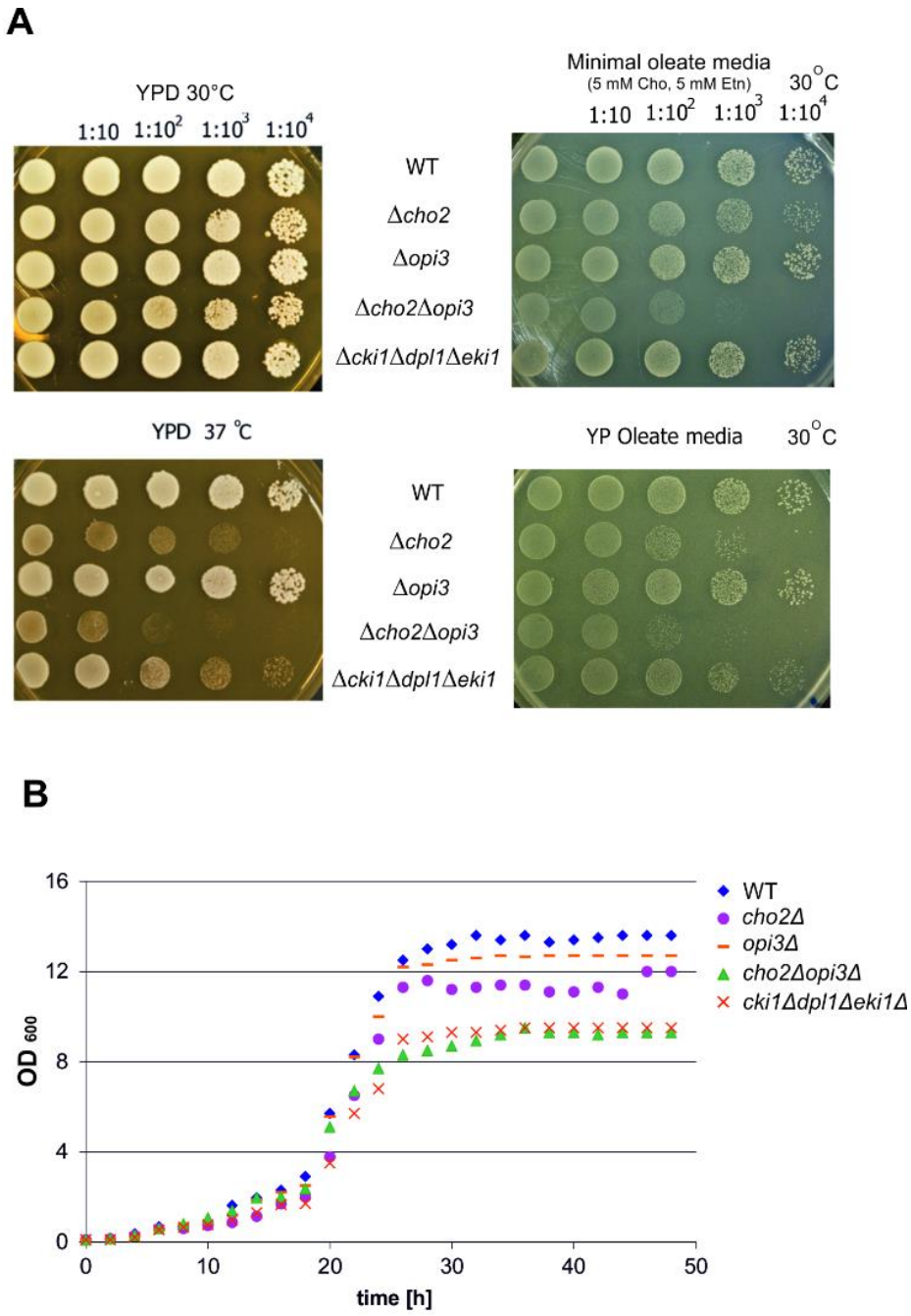

Figure 2: Growth phenotype of wild type, *cho2*Δ, *opi3*Δ, *cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ yeast strains. (A): Drop test on YPD plates (30°C and 37°C); on minimal oleate media containing choline and ethanolamine; and on YPO plates are shown. (B) Growth of liquid cultures on YPO.

The mutant strains *cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ showed similar growth in liquid media (Fig. 2B), but clear growth defects compared to the wild type strain. The *cho2*Δ mutant was only slightly affected, and the *opi3*Δ mutant behaved almost like wild type.

Taken together, these data suggest that under the chosen growth conditions defects in PE methylation result in more severe growth defects than mutations in the CDP-choline pathway

of PC formation on solid media. Noteworthy, these defects become more evident under conditions of elevated temperature and on oleate as carbon source than on YPD under standard conditions. In liquid media the two mutant strains had similar growth defects.

Electron microscopy of yeast cells bearing defects in phosphatidylcholine biosynthesis

To investigate possible morphological changes caused by mutations in the different PC biosynthetic pathways wild type, *cho2*Δ*opi3*Δ and the *cki1*Δ*dpl1*Δ*eki1*Δ mutant strains grown on oleate media were subjected to electron microscopic inspection (Fig. 3). Growth of yeast cells on fatty acids as carbon source not only induces peroxisome proliferation, but also causes enhanced formation of lipid droplets. These droplets mainly consist of non-polar lipids and are a storage compartment for triacylglycerols and steryl esters [169]. Data from our group [151] and from other laboratories had indicated that peroxisomes have a tendency to associate with other subcellular compartments, especially the endoplasmic reticulum [170]8, 29], mitochondria [171]30 - 32] and lipid droplets [173]. Especially the contact between peroxisomes and lipid droplets was considered to be relevant for the supply of fatty acids as a substrate for β-oxidation to peroxisomes, although biochemical evidence for such a route is missing. The association of peroxisomes with mitochondria and the endoplasmic reticulum [29] may be more important for the biogenesis of peroxisomes including supply of lipids from the respective sites of synthesis. Electron micrographs (see Fig. 3), however, did not show fundamental changes of the cellular structure caused by mutations in the PC biosynthetic pathways. One obvious difference between the strains analyzed was the decreased number of lipid droplets in the *cki1*Δ*dpl1*Δ*eki1*Δ mutant. These data are in line with previous results from our laboratory [34] showing that a *cki1*Δ*dpl1*Δ*eki1*Δ strain grown on YPD medium with glucose as a carbon source had a markedly lower level of triacylglycerols than wild type. The morphology and the size of mitochondria were not much affected by the mutations. Despite the fact that the *cki1*Δ*dpl1*Δ*eki1*Δ mutant contains considerably less phospholipid than wild type (see below) only minor difference in size and area of mitochondria were found (Table 2).

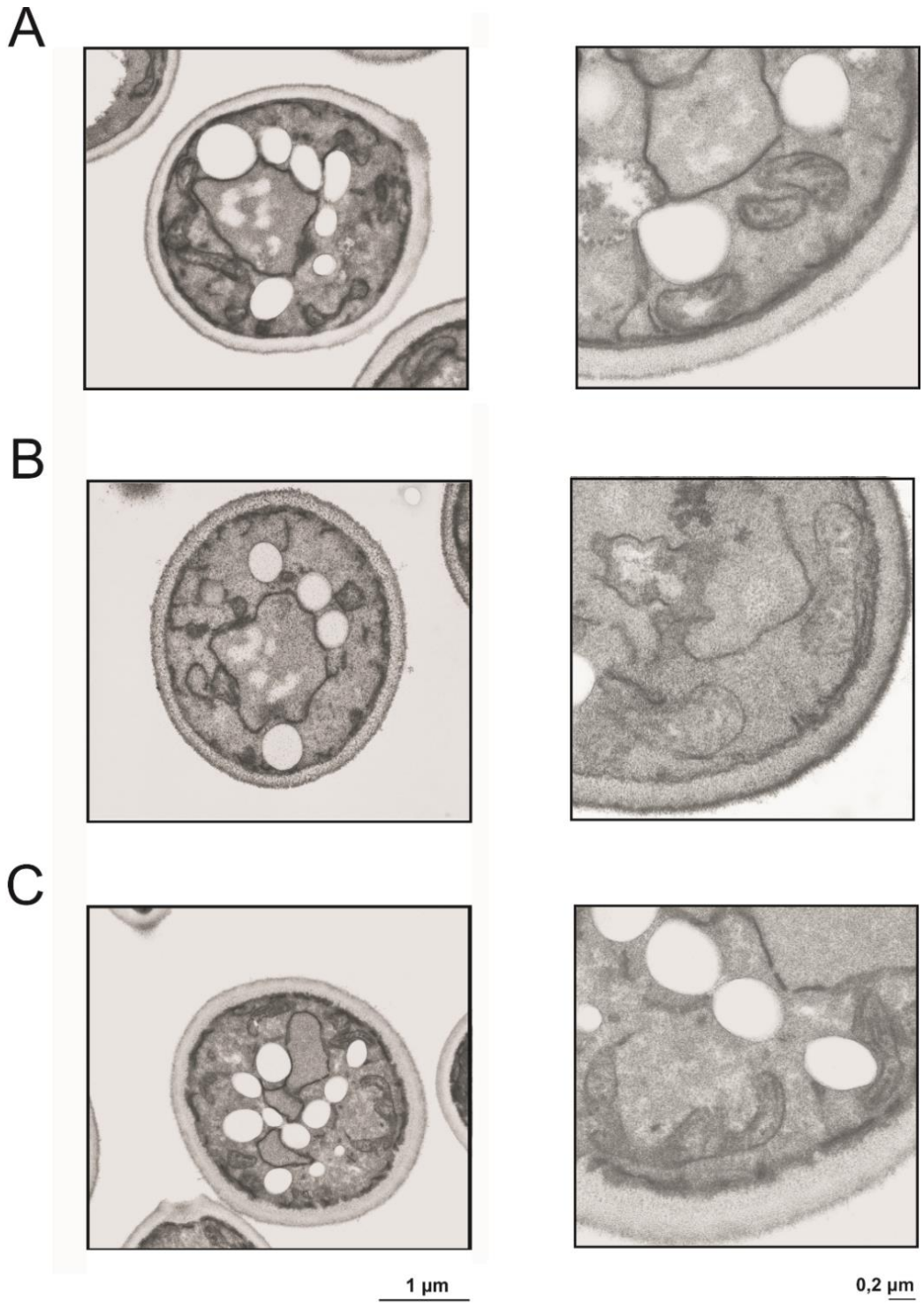

Figure 3: Transmission electron microscopy of wild type and mutant strains. Ultrathin sections of chemically fixed *S. cerevisiae* yeast cells are shown. For experimental details see Materials and Methods section. Mitochondria are marked with arrows. Wild type (A), *cki1*Δ*dpl1*Δ*eki1*Δ (B), and *cho2*Δ*opi3*Δ (C) were grown on YPO to induce formation of peroxisomes.

Table 2: Structural parameters of mitochondria from different mutant strains. Electron micrographs of the tested strains were obtained as described in the Materials and Methods section. For the analysis of mitochondrial size, circumference and area 70 to 100 mitochondria were tested.

	Size (µm)	Circumference (µm)	Area (µm²)
Wild type	0.50	1.34	0,084
cki1Δdpl1Δeki1Δ	0.50	1.41	0,105
cho2Δopi3Δ	0.56	1.51	0,087

Isolation and characterization of peroxisomes

Prerequisite for the analysis of effects on the formation of peroxisomes caused by mutations in the two PC biosynthetic pathways was the isolation of these organelles as described previously by Zinser and Daum [35]. The quality of peroxisome preparations was tested by Western blot analysis to determine the enrichment of peroxisomes and cross contamination with other organelles (Fig. 4). Antibodies used for Western blot analysis were directed against a 40 kDa protein (endoplasmic reticulum); Fox1p (multifunctional β-oxidation protein from peroxisomal membranes) and Por1p (outer mitochondrial membrane). As can be seen from Fig. 4, Fox1p was highly enriched in peroxisomal fractions from wild type cells. The degree of cross-contamination of peroxisomes with other subcellular fractions was low. Impurities caused by co-isolation of mitochondria were small as can be seen from the patterns of Por1p. Western blot analysis was also routinely performed with subcellular fractions from the mutant strains bearing defects in PC synthesis. Cross contaminations were quantified in Fig. 5. Contaminations with mitochondria were found in all fractions most likely due to close contact of mitochondria with other organelles. Endoplasmic reticulum and mitochondria were not contaminated by peroxisomes, and no contamination with endoplasmic reticulum (ER) was found with the other isolated organelles.

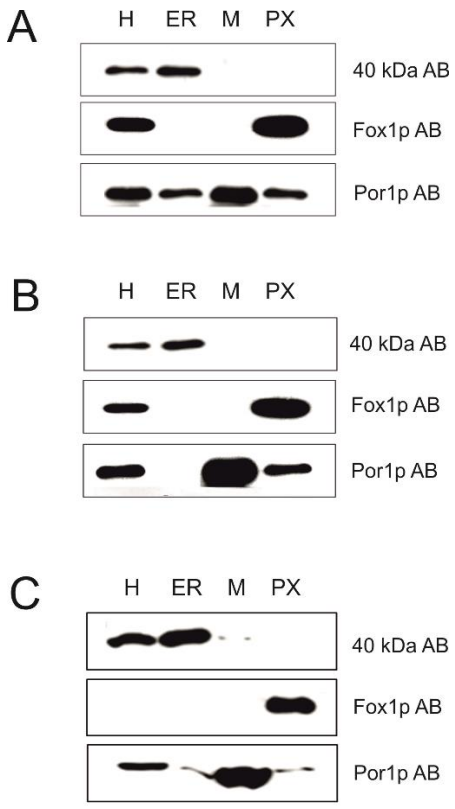

Figure 4. Western blot analysis of subcellular fractions from *S. cerevisiae*. Wild type (A) and mutant strains *cki1*Δ*dpl1*Δ*eki1*Δ (B) and *cho2*Δ*opi3*Δ (C) were grown on oleic acid as described in the Materials and Methods section. H (homogenate), ER (endoplasmic reticulum), M (mitochondria) and PX (peroxisomes) were isolated according to standard procedures [35]. For electrophoresis 10 µg protein were loaded onto each lane of the gel.

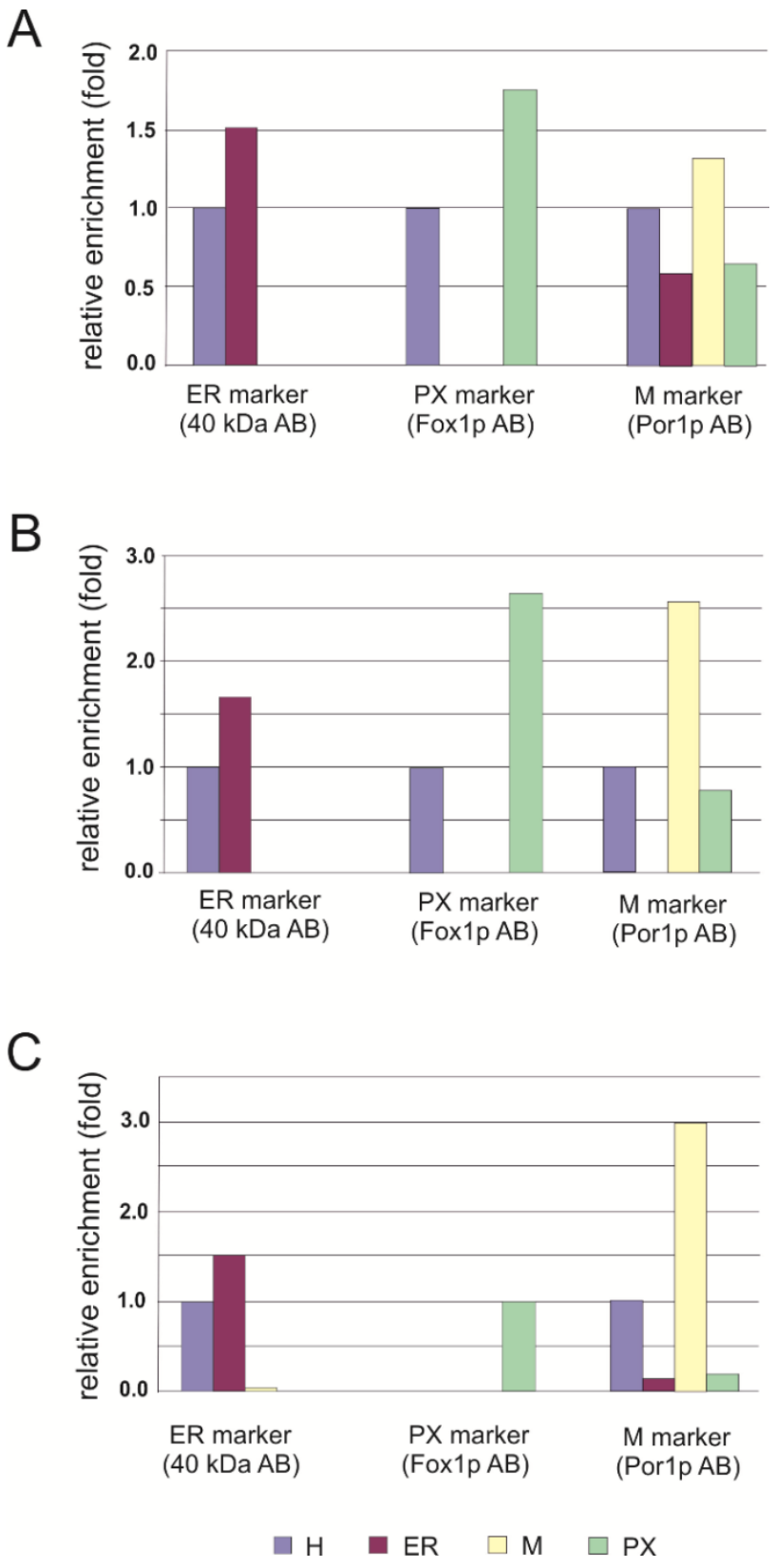

Figure 5: Quantification of Western blot analysis of subcellular fractions from *S. cerevisiae*. ER (endoplasmic reticulum), M (mitochondria) and PX (peroxisomes) were compared to the H (homogenate) of the wild type strain (A), the *cki1*Δ*dpl1*Δ*eki1*Δ (B) and the *cho2*Δ*opi3*Δ (C) mutant strains.

Phospholipid analysis of peroxisomes from cells with phosphatidylcholine biosynthetic defects

To characterize the contributions of the two PC biosynthetic pathways of the yeast to the supply of PC to peroxisomes phospholipid patterns from *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ mutants were compared to wild type. To obtain a broader view of the expected effects, we analyzed phospholipids from peroxisomes, endoplasmic reticulum and mitochondria.

A key experiment to understand PC supply to peroxisomes was the analysis of individual phospholipids from wild type (WT), the *cki1*Δ*dpl1*Δ*eki1*Δ mutant and the *cho2*Δ*opi3*Δ mutant (Fig. 6). In peroxisomes from the *cho2*Δ*opi3*Δ strain the amount of PC was even slightly increased over wild type, whereas the PC level in peroxisomes from *cki1*Δ*dpl1*Δ*eki1*Δ was slightly decreased. Thus, PC production for peroxisomal membranes through the CDP-choline pathway in the strain lacking enzymes of PE methylation appears to be more efficient than through the methylation pathway. It has to be noted, however, that according to these results both pathways of PC synthesis in the yeast supply PC to peroxisomes, although with slightly different efficiency. Interestingly, however, the PE and PS levels in peroxisomes of the *cho2*Δ*opi3*Δ mutant were markedly higher than in wild type and in the *cki1*Δ*dpl1*Δ*eki1*Δ strain. Some accumulation of PE and PS may have occurred due to the lack of further conversion to PC. In the *cki1*Δ*dpl1*Δ*eki1*Δ strain levels of all phospholipids were found to be lower than in wild type. Results described above led to an interesting effect, namely a decrease in the PC to PE ratio in the *cho2*Δ*opi3*Δ mutant. Taking into account that this ratio may affect membrane properties, the double mutation appears to cause a marked effect. In the *cki1*Δ*dpl1*Δ*eki1*Δ strain the PC to PE ratio was similar to wild type.

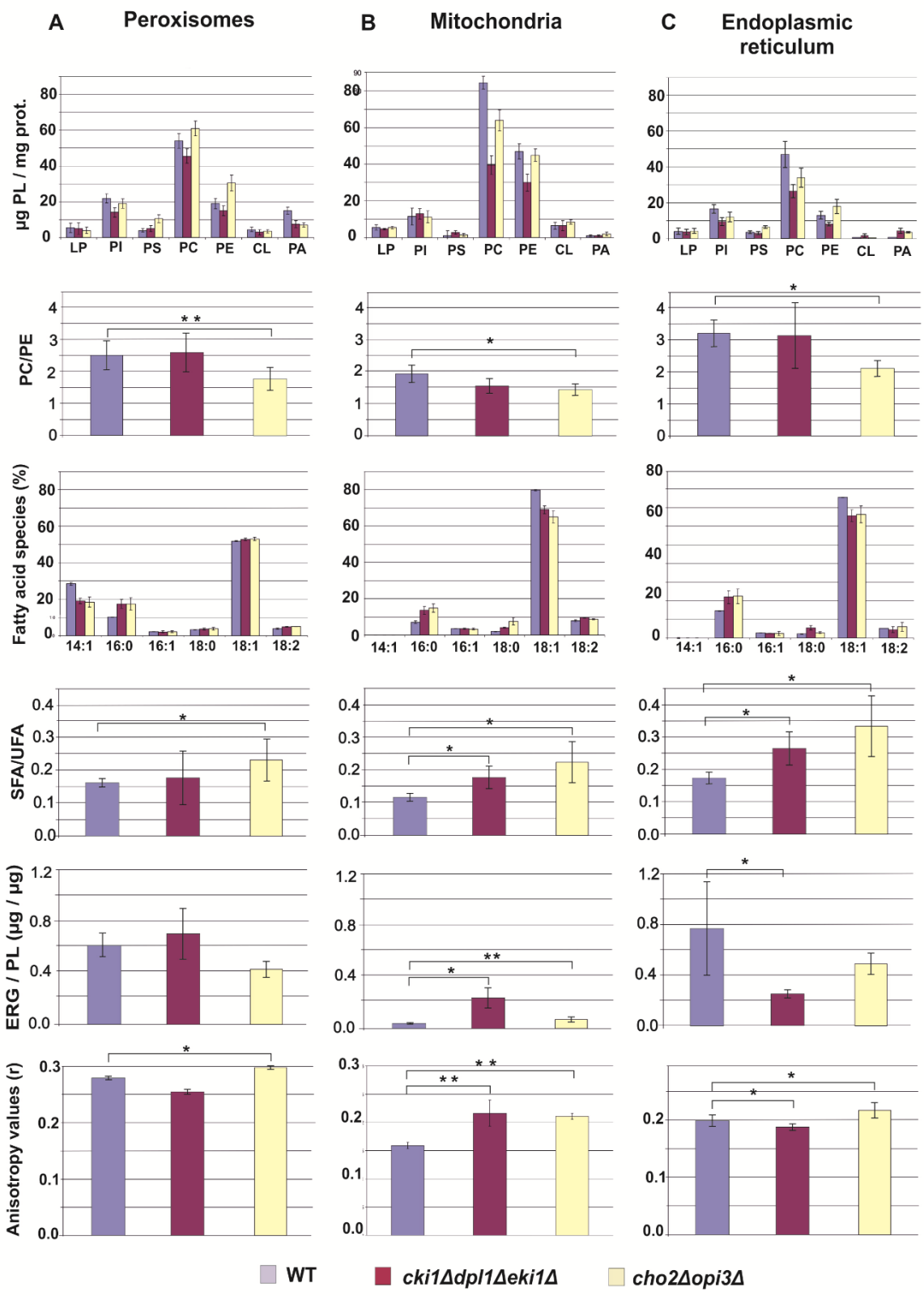

Figure 6: Analysis of organelles from wild type, *cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ yeast strains. Yeast strains used were grown on YPO media. Data obtained with isolated peroxisomes (lane A), mitochondria (lane B) and endoplasmic reticulum (lane C) are shown. Line 1: Phospholipid pattern expressed as µg of individual phospholipids per mg protein. LP, lysophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PA, phosphatidic acid. Line 2: PC to PE

ratio in different organelles and strains. Line 3: Fatty acid composition of different organelles and strains. Line 4: Ratio of saturated (SFA) to unsaturated (UFA) fatty acids in different organelles and strains. Line 5: Ergosterol (ERG) to phospholipid (PL) ratio in different organelles and strains. Line 6: Anisotropy values obtained with different organelles and strains. For experimental details see Materials and Methods section. For all experiments two independent biological samples were used which were analyzed 2 to 3 time, each.

Some of the trends described with phospholipids from peroxisomes were also observed with mitochondrial and endoplasmic reticulum membranes, in some cases even more pronounced. Both in mitochondria and in the endoplasmic reticulum *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ mutations caused a decrease of PC levels. Effects with the *cki1*Δ*dpl1*Δ*eki1*Δ triple deletion were more pronounced than with the *cho2*Δ*opi3*Δ double deletion. In mitochondria from both strains, the PC to PE ratio was lower than in wild type, whereas in the endoplasmic reticulum this ratio was more affected by the *cho2*Δ*opi3*Δ double deletion. However, in principle also mitochondria and the endoplasmic reticulum can obtain PC from both sites of synthesis.

Cardiolipin (CL) was also found in peroxisomes, although this lipid has been considered for a long time specific for mitochondria. The CL content measured in peroxisomes occurred mainly from contamination with mitochondrial membranes (71 % in wild type; 67 % in the *Δcki1Δdpl1Δeki1* mutant. In the *Δcho2Δopi3* mutant only 16 % of peroxisomal CL could be attributed to contamination with mitochondria. The remaining amounts of CL have therefore to be considered as "true" peroxisomal.

Mitochondria isolated from the *Δcki1Δdpl1Δeki1* mutant (see Fig. 6) contained considerably less phospholipids (PC and PE) than the wild type. As isolated mitochondria were not contaminated with other organelles (see Figs. 4 and 5), the purity of samples did not influence the result. Surprisingly, the changes in the phospholipid of strains did not affect the structure of mitochondria (see Fig. 3 and Table 2).

Fatty acid analysis

As phospholipid patterns of the organelle membranes from the three analyzed strains (wild type, PE methylation and CDP-choline mutant) were versatile we also tested changes in the fatty acid patterns of the respective organelles (see Fig. 6). Not surprisingly, oleate (C18:1) was the predominant fatty acid in all samples due to the fact that this fatty acid present in the medium is not only used as a carbon source but also as a component for the synthesis of membrane phospholipids. Interestingly, the level of C18:1 in peroxisomes was markedly lower than in the

endoplasmic reticulum and mitochondria indicating that some selectivity in the supply of lipids to the different organelle membranes had occurred.

In all three strains tested, the C14:1 fatty acid was only found in peroxisomes but not in mitochondria and in the endoplasmic reticulum. The origin of peroxisomal C14:1 remains unclear. As the oleic acid used as carbon source contained some C14:1, an external origin is very likely. The fact remains, however, that C14:1 accumulated in peroxisomes whereas other organelles were practically devoid of this fatty acid. These findings support some selectivity in the supply of certain lipid species to peroxisomes. C16:0 was present in all three strains and organelles at a level of ~ 10-20%, whereas C16:1 and C18:0 fatty acids were detected only at low concentrations. C18:2 was present in all samples due to impurities of oleic acid samples used as carbon source. The used oleic acid contains max. 5% of C14:1 (myristic acid), 16% of C16:0 (palmitic acid), 8 % of C16:1 (palmitoleic acid), 6 % of C18:0 (stearic acid), 65 – 88 % of C18:1 (oleic acid) and 18 % of C18:2 (linoleic acid).

Although variations in individual fatty acids from the different samples appear to be minor they had a major impact on an important parameter of membrane properties, which is the ratio of saturated to unsaturated fatty acids (see Fig. 6). In all organelle samples from *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ this ratio was markedly increased over the wild type. The highest ratio of saturated to unsaturated fatty acids was found in organelles from the *cho2*Δ*opi3*Δ strain.

Sterol analysis of organelles from cells with phosphatidylcholine biosynthetic defects

In addition to the phospholipid and fatty acid composition, sterols may have an influence on the properties of membranes. For this reason, we compared sterol levels in peroxisomes, mitochondria and endoplasmic reticulum from wild type, *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ (see Fig. 6). In all fractions tested the major sterol was ergosterol. In peroxisomes of wild type, 57 µg ergosterol/mg protein was detected. In the *cki1*Δ*dpl1*Δ*eki1*Δ mutant the sterol amount was slightly increased (61 µg ergosterol/mg protein), whereas in the *cho2*Δ*opi3*Δ mutant it was slightly decreased (48 µg ergosterol/mg protein). The more crucial value for membrane properties is the sterol to phospholipid ratio. As can be seen from Fig. 6 this value was increased in peroxisomes from *cki1*Δ*dpl1*Δ*eki1*Δ over wild type, whereas it was decreased in *cho2*Δ*opi3*Δ. Compared to peroxisomes, the ergosterol to phospholipid ratio in mitochondria was low. Also in this case the *cki1*Δ*dpl1*Δ*eki1*Δ triple deletion led to a marked increase of this value. In the endoplasmic reticulum the ergosterol to phospholipid ratio was lower in *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ than in wild type.

Organelle membrane fluidity affected by defects in phosphatidylcholine biosynthetic pathways

Evaluation of anisotropy values obtained with biological membranes is a serious task because many membrane parameters such as the phospholipid pattern, the phospholipid to protein ratio, the PC to PE ratio, the fatty acid pattern, the ratio of saturated to unsaturated fatty acids and the ergosterol to phospholipid ratio may contribute to the overall fluidity/rigidity of a membrane. High ratios of saturated to unsaturated fatty acids, high amounts of ergosterol, low phospholipid to protein ratios and low PC to PE ratios in membranes were considered as parameters that may lead to high anisotropy values indicating high rigidity of membranes. The overall fluidity/rigidity of a membrane is result of a combination of all parameters mentioned.

PC is the classical bilayer forming phospholipid, whereas the non-bilayer forming phospholipid PE may induce curvature of the membrane and disturb the order of the bilayer. Therefore, organelles from strains with compromised PC biosynthesis were good candidates for changed membrane properties. To test organelle membrane rigidity/fluidity, anisotropy measurements using the fluorophore diphenylhexatriene (DPH) were performed. High anisotropy values are indicative of membrane rigidity, whereas low anisotropy is typical for more fluid membranes.

Anisotropy measurements of peroxisomal membranes, endoplasmic reticulum and mitochondria from wild type, *cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ mutants are shown in Fig. 6. As can be seen, the fluidity of peroxisomal and endoplasmic reticulum membranes of the two mutant strains showed slight differences compared to the membranes of the wild type. In both organelles, the *cki1*Δ*dpl1*Δ*eki1*Δ triple deletion caused a slight decrease of the anisotropy, whereas a slight increase of anisotropy values was observed with *cho2*Δ*opi3*Δ. In general, anisotropy values were lower in the endoplasmic reticulum fraction indicating higher fluidity of these membranes. In mitochondrial membranes, defects in both PC biosynthetic pathways led to higher anisotropy compared to wild type. In this case, differences were more pronounced than in peroxisomes and endoplasmic reticulum indicating that changes in the mitochondrial membrane composition led to a marked increase of membrane rigidity.

The result of the anisotropy measurements of the peroxisomes was surprising, because the ratio of saturated to unsaturated fatty acids was higher in both mutants than in wild type, and the sterol content varied. However, anisotropy (fluidity/rigidity) seemed to correlate with the observed PC to PE ratio in peroxisomal membranes under the mentioned conditions, because low PC to PE ratios led to high anisotropy values, whereas high PC to PE ratios led to low

anisotropy values (see Fig. 6). The other lipid components (fatty acids and ergosterol) seemed to have less influence on the fluidity/ rigidity of the membrane in this case.

Although we have to be very cautious with the interpretation of these data, two aspects have to be mentioned. First, the amount of sterols in the membranes which were examined was rather low and may not contribute much to the overall rigidity of the membranes. Secondly, the ratio of saturated to unsaturated fatty acids is very low, because more than 85 % of fatty acids are unsaturated due to the massive incorporation of oleate into lipids. Thus, small changes in the pattern result in relatively high changes of the ratio. Finally, we are left with a possible effect of changed amounts of PC and PE in peroxisomal membranes, which might result in the observed changes of fluidity/rigidity.

The same arguments as for peroxisomes hold for mitochondria and the endoplasmic reticulum. Also in these samples the effect of fatty acids and sterols may not be crucial for the fluidity of the membranes. Again, the PC to PE ratio can be regarded as a parameter which may influence this membrane property.

DISCUSSION

Peroxisomes belong to the group of organelles which cannot synthesize all of their own lipids. In mammalian cells, peroxisomes contribute to phospholipid biosynthesis by the formation of ether lipids and to cholesterol biosynthesis (for recent reviews see refs [36] and [37]), but yeast peroxisomes are completely devoid of lipid biosynthetic activities. As a consequence of these facts, peroxisomes rely to a large extend or completely on the supply of lipids from other organelles.

The supply of PE to peroxisomes of the yeast *S. cerevisiae* from the three sites of PE synthesis in the mitochondria, the Goli/vacuole and the endoplasmic reticulum was studied previously in our lab [7]. These investigations revealed that all three pathways of PE synthesis can produce PE for peroxisomes, although with different efficiency. The supply of PC to peroxisomes and the correlation between PC and PE synthesis in the yeast *S. cerevisiae* are presented in this study. This process has not been studied before neither with the yeast nor with other cellular systems. As one important result presented here we show that both the methylation pathway and the CDP-choline branch of the Kennedy pathway produce PC destined to peroxisomes, but also to mitochondria and the endoplasmic reticulum. Interestingly, the two pathways exhibit different efficiency in PC formation for peroxisomes, at least under growth conditions chosen for this study. It appears that the CDP-choline pathway is slightly more efficient in the supply

of PC to peroxisomes than the methylation pathway. This result also suggests that different pools of PC may exist in the cell.

In both mutants, the *cki1*Δ*dpl1*Δ*eki1*Δ and the *cho2*Δ*opi3*Δ deletion strains, the PC level of peroxisomes gets close to wild type (see Fig. 6). This is not the case for the other organelles tested, because in mitochondria as well as in the endoplasmic reticulum the wild type level of PC was much higher than in mutants, especially in the Δ*cki1*Δ*dpl1*Δ*eki1* strain. It appears that in peroxisomes import and assembly of PC formed by both pathways are more balanced than in the other organelles. Thus, the phospholipid translocation network governing the traffic of phospholipids between synthesizing and accepting organelles may be regulated by individual requirements of the organelles. Not much is known about the transport mechanism of phospholipids to peroxisomes, but it was shown that non-vesicular transport of lipids to peroxisomes may be relevant [38]. Evidence has also been presented that vesicle transport from mitochondria to peroxisomes may exist [39 – 42]. Membrane contact between different organelles has also been discussed as a prerequisite for lipid translocation (for reviews see refs. [29] and [43-46]), although functionality of contact sites between peroxisomes and mitochondria or endoplasmic reticulum [45] is still a matter of dispute. Such mechanisms, however, may explain the finding that small amounts of CL were found in peroxisomes of all three strains tested in this study. Taking into account the contamination of peroxisomes with mitochondria (see Figs. 4 and 5) approximately 30 % of CL found in peroxisomes of wild type and *Δeki1Δcki1Δdpl1*; and even 80 % of CL found in peroxisomes from the *Δcho2Δopi3* mutant can be regarded as "true" peroxisomal components. This finding is in line with previous observations obtained with isolated yeast subcellular fractions. [35].

The impaired growth on oleate media of the two mutants investigated in this study, especially the *cho2*Δ*opi3*Δ strain, may be attributed to changes in the phospholipid composition of organelles. Peroxisomes are of special interest with this respect as they are the organelles which harbor the enzymes of β-oxidation and are required for the utilization of fatty acids as substrates. Thus, the amount of fatty acids present in the strains tested is of considerable importance. As can be seen from Fig. 7, however, oleic acid which is the predominant fatty acid in cells grown under the given conditions and the substrate for peroxisomal β-oxidation was not depleted in the two mutant strains compared to wild type. Thus, fatty acid supply to peroxisomes where β-oxidation occurs exclusively in *Saccharomyces cerevisiae* was not limited and energy production not affected.

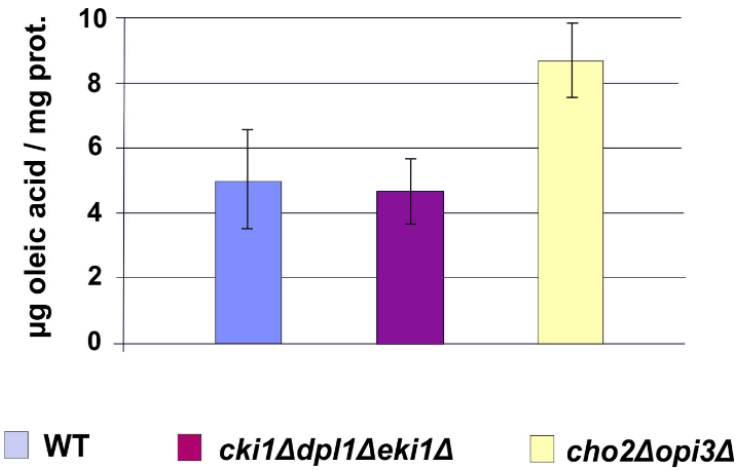

Figure 7: Fatty acids in the homogenate from wild type, *cho2*Δ*opi3*Δ and *cki1*Δ*dpl1*Δ*eki1*Δ yeast strains. Yeast strains used were grown on YPO media, and homogenate samples were prepared after cell disruption. The oleic acid content was quantified as described in the Materials and Methods section.

Although changes in the ratio of saturated to unsaturated fatty acids and the ergosterol to phospholipid ratio have also to be taken into account, the PC to PE ratio seems to have an influence on the anisotropy of peroxisomal membranes. PC and PE differ only in their head groups. However, molecular simulations demonstrated the unique ability of PE to interact strongly with itself and neighboring lipids via inter- and intramolecular hydrogen bonding [46]. This biophysical ability generates a closely packed lipid bilayer with hydrogen tails aligned, decreasing the space occupied by each lipid molecule [47]. The DMPE and PE molecules which are present in a lipid bilayer, form bridges with each other [46 – 50]. These bridges lead to a higher value of the "main" transition temperature (T_m) from a fluid to a gel state of the membrane [48]. Furthermore, in model studies insertion of DMPE or PE molecules into a lipid bilayer increased the T_m of the mixed PC/PE bilayer systems substantially [49]. Other studies indicated that hydrogen bonds within a cluster remain stable for longer time and that especially PE lipids may diffuse or reorient as groups or clusters rather than individual lipids [50]. As a result, an increased PE to PC ratio leads to a higher transition temperature of a lipid bilayer, i.e. membranes become more rigid. Such effects may apply to organelle membranes from yeast mutants described here.

In summary, our study demonstrates routes of PC transport to peroxisomes in the yeast *S. cerevisiae*. Both pathways of PC production, PE methylation and the CDP-choline pathway are able to produce PC for peroxisomes and other organelles. Interestingly, mutations in one of these pathways, each, do not cause extreme defects in peroxisomes indicating that the remaining pathway in a mutant strain can efficiently compensate for the loss of the other biosynthetic

route. Despite the flexibility of the yeast, certain consequences of PC depletion such as growth defect or disturbed membrane fluidity became evident pointing out the importance of PC for peroxisomal and other cellular membranes.

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General Discussion and Summary

Saccharomyces cerevisiae was the first eukaryote with a completely sequenced genome [1]. Therefore it became an important model organism in life science. Lipid metabolism in eukaryotic cells is often studied in *S. cerevisiae*. The main synthesis routes, storage and degradation of lipid compounds have already been studied in the yeast. Lipids are important compounds for cellular functions and for the formation of cellular membranes. In yeast, lipid metabolism is a network of reactions with genes involved which encode lipid metabolic enzymes and regulatory genes required for synthesis and metabolic conversion of lipids (reviewed in ref. [2]). Synthesis and metabolism of the lipid classes in yeast, like phospholipids, fatty acids, triacylglycerols, sterols and sphingolipids, are linked to each other [3]. Defects in phospholipid metabolism caused by defects in enzymes or metabolic routes for forming phospholipids have an influence on cell growth, various cellular functions and membrane behaviour.

The aim of this thesis was to clarify some open questions regarding the lipid metabolism in *Saccharomyces cerevisiae*. Some aspects were selected and three parts of this work focused on the influence of the enzymes phosphatidylserine decarboxylase 1 (Psd1p), responsible for the formation of the majority of phosphatidylethanolamine (PE), and Gph1p, a glycogen phosphorylase. The influence of deletion and mutation of these enzymes on lipid metabolism and other cellular functions, like growth behaviour or membrane composition were determined. Another part of this work describes the identification of routes for the supply of lipids to different cellular compartments, like the PC supply to peroxisomes and other organelles. Figure 1 summarizes the routes of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) formation in yeast and the involved enzymes discussed in this thesis. In the following each chapter will be summarized and discussed.

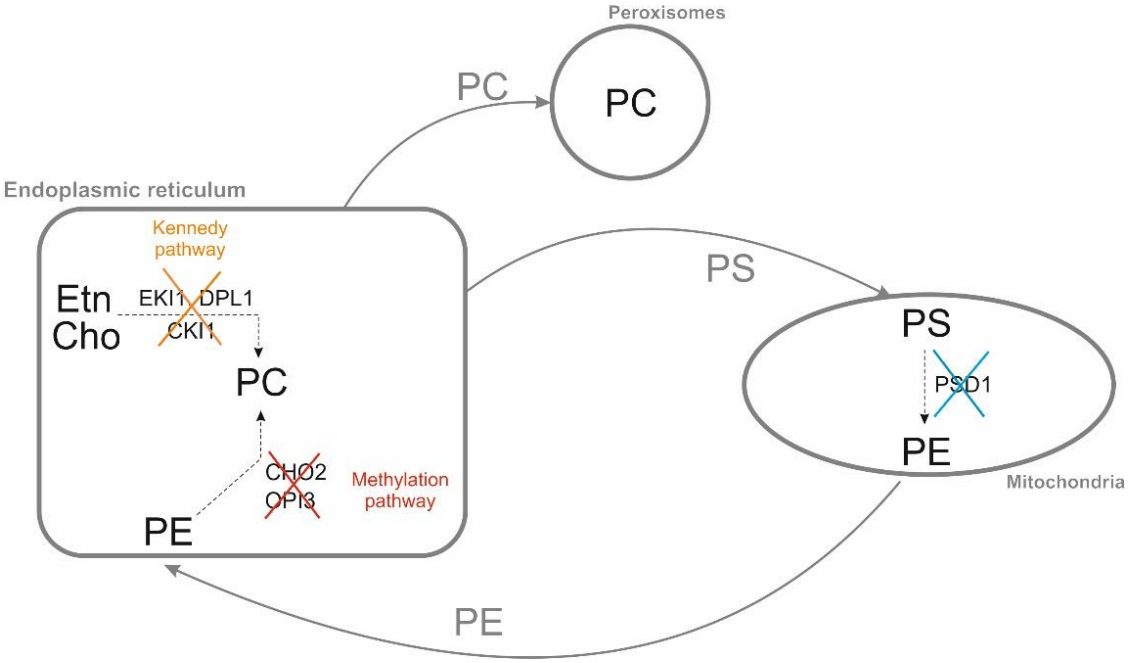

Figure 1: Routes of phospholipid formation and involved enzymes discussed in this thesis. The majority of phosphatidylethanolamine (PE) is formed in mitochondria via phosphatidylserine decarboxylase 1 (Psd1p) by decarboxylation of phosphatidylserine (PS). PE is transported to the endoplasmic reticulum and phosphatidylcholine (PC) is formed via the methylation pathway with Cho2 (phosphatidylethanolamine methyltransferase) and Opi3 (methylene-fatty-acyl-phospholipid synthase) and via the Kennedy pathway with Eki1 (ethanolamine kinase), Dpl1 (sphingosine phosphate lyase) and Cki1 (choline kinase). The influence of knockouts of these pathways in strains *Δcho2Δopi3* and *Δeki1Δdpl1Δcki1* is discussed in this thesis. PC is supplied from the endoplasmic reticulum to the peroxisomes.

THE ROLE OF INCORPORATION OF PHOSPHATIDYLSERINE DECARBOXYLASE 1 INTO THE MITOCHONDRIAL MEMBRANE FOR FUNCTION AND LIPID FORMATION

Defects in the formation of phosphatidylethanolamine (PE) are due to defects in the activity of phosphatidylserine decarboxylase 1 (Psd1), which is the enzyme responsible for the formation of the majority of PE within yeast cells [4]. Psd1 is synthesized on cytosolic ribosomes and translocated into mitochondria, where protein maturation occurs. The precursor protein of Psd1 contains a mitochondrial targeting sequence, an internal sorting sequence and membrane sorting signals, which are located at the β-subunit. The substrate recognition site is located at the α-subunit of the protein. During processing, the α- and β-subunit are separated at the LGST motif, leading to the active form of the enzyme, which contains a pyruvoyl group at the N-terminus of the α-subunit [5-8]. Psd1 is located to the inner mitochondrial membrane facing the

intermembrane space (Figure 2) [9]. In this study we determined two predicted mitochondrial membrane sorting signals (IM1 and IM2) and their effects on Psd1p processing, transport, localization within the mitochondria and activity of the enzyme. Deletion of IM2 leads to an unprocessed enzyme, to growth defects, reduced activity and mislocalization of the protein to the matrix site of the mitochondria. We also wanted to pinpoint which area of IM2 has the highest influence on Psd1 sorting and activity. Therefore we deleted four to five amino acids in the area of the second membrane spanning domain IM2 and called the strains Psd1ΔIM2_T1, Psd1ΔIM2_T2, Psd1ΔIM2_T3, Psd1ΔIM2_T4 and Psd1ΔIM2_T5. In the strain Psd1ΔIM2ΔGAP the whole IM2 was deleted and the area between IM1 and IM2 (GAP). These deletions have an influence on processing, localization, import and activity of Psd1.

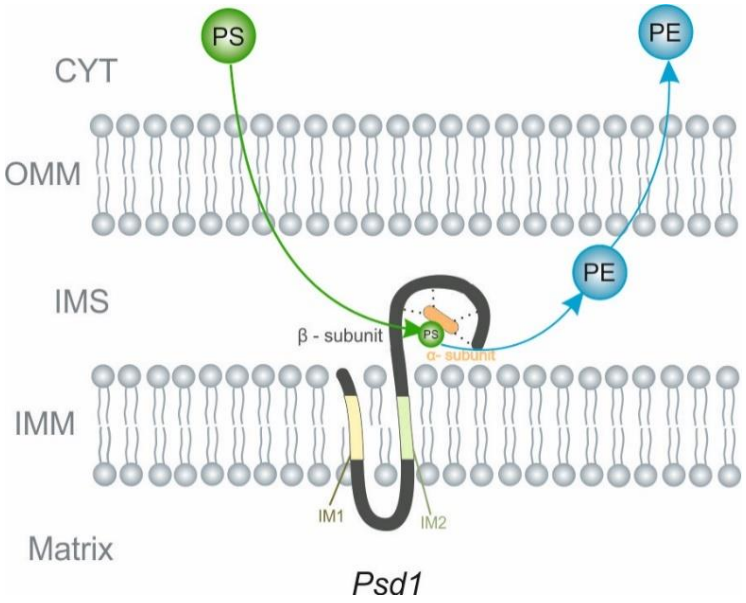

Figure 2: Localization of phosphatidylserine decarboxylase 1 in the mitochondrial membrane. Psd1p is anchored to the inner mitochondrial membrane (IMM) facing the intermembrane space (IMS) by at least two membrane spanning domains (IM1 and IM2). The α-subunit is involved in binding phosphatidylserine (PS) to its substrate recognition site (SRS) and conversion to phosphatidylethanolamine (PE).

Taken together the results of this study, we can say that deletion of the whole IM2 lead to an unprocessed protein and to mislocalization to the matrix site of the mitochondria but still to a protein anchored to the inner mitochondrial membrane (IMM). This mutant showed a decreased PE content compared to the wild type. Already deletions of small parts of the second membrane spanning domain results in changes in activity and localization of the protein. Deletion of the first three parts (Psd1ΔIM2_T1, Psd1ΔIM2_T2 and Psd1ΔIM2_T3) lead to an unprocessed protein, which was located to the IMM facing the matrix. These variants showed full enzymatic

activity and a PE amount compareable to the wild type. Psd1ΔIM2_T4 and Psd1ΔIM2_T5 are unprocessed and localized to the outer mitochondrial membrane (OMM). In case of the Psd1ΔIM2ΔGAP mutant an unprocessed protein is formed which is localized to the IMM and the OMM. The strains Psd1ΔIM2_T4, Psd1ΔIM2_T5 and Psd1ΔIM2ΔGAP showed reduced PE amounts. These results indicates that only when Psd1 is anchored to the inner mitochondrial membrane it can develop full enzymatic activity like wild type Psd1. Localization of the unprocessed protein to the outer mitochondrial membrane leads to loss of activity and reduced PE formation. Our data show that Psd1 harbors at least two inner membrane sorting signals which are crucial for correct sorting into the inner mitochondrial membrane and for correct processing into α- and β-subunit. Correct localization is important to obtain a full enzyme activity of Psd1. Even small changes introduced by mutation are already sufficient to disturb the correct targeting of this enzyme.

THE GLYCOGEN PHOSPHORYLASE GPH1P IS INVOLVED IN LIPID METABOLISM AND CELLULAR FUNCTIONS

Synthesis of lipids, their different functions and the functions of cellular proteins are linked together in the yeast *Saccharomyces cerevisiae*. The glycogen phosphorylase Gph1p acts also as possible regulator of yeast lipid metabolism [10-13]. The polysaccharide glycogen is used as storage carbohydrate within yeast cells [11-13]. Deletion of *GPH1* has an influence on PC synthesis, exhibited lowered triacylglycerol (TG) and steryl ester (SE) formation and increases formation of total phospholipids in *S. cerevisiae* [10]. In this study we analysed the influence of *GPH1* expression on lipid metabolism in yeast *S. cerevisiae*. In a Δ*gph1* mutant, depletion of TG and SE leads to a lack of lipid droplets and a changed phospholipid composition. We show that PC depletion may also lead to defects in the yeast cell periphery. Thus, Gph1p may fulfil multiple independent functions which affect the lipid and carbohydrate metabolism. Only minor differences in TG degradation were found in wild type and in the Δ*gph1* deletion mutant, because expression levels of the three yeast genes encoding TG lipases, *TGL3, TGL4* and *TGL,* were not changed in the Δ*gph1* strain. We found that Gph1p may affect the distribution of lipid components within the cell as deletion of GPH1 affects the assembly of PC to plasma membrane. Taken together Gph1p seems to cause several changes in yeast lipid metabolism.

SUPPLY OF PHOSPHATIDYLCHOLINE TO PEROXISOMES

Synthesis routes in the different cellular compartments in *S. cerevisiae* form a complex network. In some organelles lipid composition is depended on the supply from other cellular compartments. As an example, peroxisomes which are important due to the fact that they harbour oxidative and detoxifying reactions, involving oxygen and hydrogen peroxide as substrates, cannot synthesize phospholipids by their own [14]. The three sites of phosphatidylethanolamine (PE) synthesis in *Saccharomyces cerevisiae* are the mitochondria, the Golgi/vacuole and the endoplasmic reticulum as studied previously in our lab [15]. Here we show the influence of different routes of phosphatidylcholine (PC) formation on the supply of PC to peroxisomes and other organelles. There are two main routes for PC formation in the endoplasmic reticulum, namely the CDP-choline branch of the Kennedy pathway and the methylation pathway. To determine the influence of the pathways on PC supply to peroxisomes, the Kennedy pathway or the methylation pathway, respectively, were knocked out (*cki1*Δ*dpl1*Δ*eki1*Δ and *cho2*Δ*opi3*Δ*)*. We found that both pathways produce PC for the supply to peroxisomes and also to mitochondria and the endoplasmic reticulum in yeast. The CDP-choline branch of the Kennedy pathway is slightly more efficient in the supply of PC to peroxisomes than the methylation pathway. In both the *cki1*Δ*dpl1*Δ*eki1*Δ and the *cho2*Δ*opi3*Δ deletion strains the PC amount in peroxisomes is nearly as high as in the wild type. In mitochondria and endoplasmic reticulum the amount of PC in the wild type was much higher than in the two deletion strains. Thus, in peroxisomes the supply of PC from both pathways seemed to be more balanced than in the other organelles. One pathway seems to compensate for the PC decrease, caused by deletion of the other pathway. Peroxisomes harbour the enzyme of β-oxidation and are required for the utilization of fatty acids as substrates. In the two mutant strains supply of fatty acids to peroxisomes for β-oxidation was not limited and energy production not affected. We also showed that changes in the phospholipid pattern can influence membrane properties like fluidity or rigidity of membranes. The PC to PE ratio seems to have an influence on the anisotropy of peroxisomal membranes. Despite the flexibility of the yeast, certain consequences of PC depletion such as growth defect or disturbed membrane fluidity became evident, pointing out the importance of PC for peroxisomes and other cellular membranes.

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Curriculum vitae

Personal Data

Name	**Ariane Wagner (nee Fankl)**
Date of birth	06 April 1986
Nationality	Austria
E-mail	ariane.wagner@gmx.at

Education

University of Technology Graz

Since 09/2013	Studies: PhD thesis, „Phosphatidylserine Decarboxylase 1 of the yeast"
	Working group of Prof. Dr. Günther Daum, Institute of Biochemistry
2009 - 2013	Studies: Master in Biotechnology, "Expression of membrane associated proteins and structure determination"
	Diploma examination: 3. April 2013
2005 – 2009	Studies: Technical chemistry
	Bachelor examination: December 2009

University of Technology Tampere (FIN)

01/2010 – 06/2010	Studies: Master in biotechnology

HTBL Kapfenberg (Higher technical school)

2000-2005	Electrical engineering / feedback control
	School leaving examination: 21. Juni 2005

Employment

Since 09/2013	**PhD thesis of natural science**
	Institute of Biochemistry, University of Technology, 8010 Graz
08/2011 – 08/2012	**Master thesis**
	Institute of inorganic chemistry, University of Technology, 8010 Graz
05/2014 – 05/2015	**Supervisor in an undergraduate laboratory course**
	„Methods in immunology", Institute of Biochemistry, University of Technology, 8010 Graz
05/2012	**Supervisor in an undergraduate laboratory course**
	„Introduction to biotechnology", University of Technology, 8010 Graz
2007-2009	**Research assistant**
	Institute of inorganic chemistry, University of Technology, 8010 Graz
2002-2013	**short-time jobs / internship**
	Eisgenuss G u G GmbH, 8010 Graz
	Wiener Neustädter Samenhaus Natlacen, 8670 Krieglach
	Fankl Thomas KG Restauration, 8665 Langenwang
	Rettig Austria GmbH, 8662 Mitterdorf
	Bundesrechenzentrum GmbH, Wien

Academic teaching experience

02/2016	**Organization committee for the 15th DocDay** of the NAWI Graz Doctoral School of molecular Biosciences and Biotechnology
05/2014 – 05/2015	**Supervisor in an undergraduate laboratory course** „Methods in immunology“, Institute of Biochemistry, University of Technology, 8010 Graz
05/2012	**Supervisor in an undergraduate laboratory course** „Introduction to biotechnology“, University of Technology, 8010 Graz

Scientific contributions

Publications

Phosphatidylcholine supply to peroxisomes of the yeast Saccharomyces cerevisiae. (2015), Flis VV, Fankl A, Ramprecht C, Zellnig G, Leitner E, Hermetter A, Daum G, PLoS ONE 10(8):e0135084. Doi:10.1371/journal.pone.0135084
A yeast mutant deleted of GPH1 bears defects in lipid metabolism. (2015), Gsell M, Fankl A, Klug L, Mascher G, Schmidt C, Hrastnik C, Zellnig G, Daum G, PLoS ONE
Cell Biology, Physiology and Enzymology of Phosphatidylserine Decarboxylase. (2016), Bartolomeo FD, Wagner A, Daum G

Poster presentations

Poster at 13th **DocDay** (2014)
Poster at the FEBS-workshop **`Lipids as molecular switches`**, Spetses (Greece) (2014)
Poster at the **`Euro Fed Lipid`**, Montpellier (France) (2014)
Poster at the **`12th Yeast Lipid Conference`**, Ghent (Belgium) (2015)
Poster at the **`Organelle Crosstalk in Membrane Dynamicy and Cell Signalling`**, Edinburgh (United Kingdom) (2015)
Poster at the **`PYFF6-6th Conference on Physiology of Yeasts and Filamentous Fungi`**, Lisbon (Portugal) (2016)

Grants

FEMS grant for the “12th Yeast Lipid Conference” Ghent (Belgium) (2015)
Young Scientist Meeting Grant, sponsored by FEMS for “PYFF6-6th Conference on Physiology of Yeasts and Filamentous Fungi, Lisbon (Portugal)

Knowledge

Languages	German (mother tongue) English (advanced knowledge)
Software	MS Office, data bases (NCBI, Brenda, PDB,...), SeqBuilder, SeqMan, GeneDesigner, PyMol, Instrument #3 offline (MS Software), Serial cloner, Chromas Lite, desktop publishing software (CorelDraw), Zotero (Reference manager)
Laboratory techniques	Advanced cloning strategies, PCR, genome manipulation of *S.cerevisiae* (tagging); isolation of genomic DNA,

plasmid DNA and RNA from *S. cerevisiae*, *E.coli* and *P.pastoris*; experience in protein purification techniques (affinity and, size exclusion chromatography) from *S.cerevisiae* and *E.coli*, SDS-PAGE, Western blot, Lipid analysis, Fatty acid analysis, TLC analyses, organelle separation methods, sterol analyses, mikrobiological methods, GC-MS, Fluorescent microskopie, radioactivity assays,…

References

Prof. Günther Daum
University of Technology Graz,
Institute of Biochemistry, Petersgasse 12/2, 8010 Graz,
Phone: +43 316 873 6462
Email: guenther.daum@tugraz.at

Prof. Harald Pichler
ACIB GmbH
Petersgasse 14, 8010 Graz,
Phone: +43 316 873 4089
Email: harald.pichler@acib.at