

The contributions of biosynthesis and acyl chain remodelling to the molecular species profile of phosphatidylcholine in yeast

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Abstract

Phosphatidylcholine (PC) is a very abundant membrane lipid in most eukaryotes, including yeast. The molecular species profile of PC, i.e. the ensemble of PC molecules with acyl chains differing in number of carbon atoms and double bonds, is important for membrane function. Pathways of PC synthesis and turnover maintain PC homeostasis and determine the molecular species profile of PC. Studies addressing the processes involved in establishing the molecular species composition of PC in yeast using stable isotope labelling combined with detection by MS are reviewed.

Introduction

It is widely recognized that the glycerophospholipid composition of biological membranes is important for their function. Both the phospholipid class composition, defined by the nature of the polar head group, and the composition of the hydrocarbon chains attached to the glycerol backbone determine the physical properties of a biomembrane, and are subject to adaptations in response to a changing environment in many organisms. These physical properties include membrane thickness, intrinsic curvature and fluidity, which affect key functions such as the membrane barrier function, the activity of membrane-associated enzymes, and processes like membrane fusion and fission. In view of the sheer abundance of PC (phosphatidylcholine) in eukaryotic membranes – it often constitutes >50% of total membrane phospholipids [1] – the acyl chain composition of PC is paramount for proper membrane function. As the major bilayer-forming phospholipid, PC plays an important role in preserving the membranes' barrier function. In addition, PC plays an important role in signal transduction as a source of lipid signalling molecules, such as lyso-PC, phosphatidic acid and DAG (diacylglycerol) [2,3].

The yeast *Saccharomyces cerevisiae* is an excellent model organism to elucidate the mechanisms of the establishment and maintenance of the PC acyl chain composition, because of its easily accessible molecular biology, and also because its glycerophospholipid biosynthetic machinery strongly resembles that of mammalian cells. Additional advantages of yeast include its relatively simple repertoire of acyl chains, with palmitic (C_{16:0}), palmitoleic (C_{16:1}), stearic (C_{18:0}) and

oleic acid (C_{18:1}) accounting for 95% of total fatty acids [4], its lack of sphingomyelin and alkyl-linked glycerophospholipids, and the ease of incorporating labelled lipid precursors.

Yeast has two biosynthetic pathways leading to PC that are also found in mammalian cells (Scheme 1). PC is the end-product of the CDP-DAG pathway, in which PS (phosphatidylserine) and PE (phosphatidylethanolamine) are important membrane lipid intermediates [5]. In the final steps, PE is methylated to PC by the phospholipid-*N*-methyltransferases Cho2 and Opi3 localized in the endoplasmic reticulum [6]. Using *S*-adenosyl-methionine as the methyl donor, Cho2 methylates the phosphoethanolamine head-group of PE yielding phosphatidylmonomethylethanolamine (PMME), which is subsequently converted into PC via phosphatidyl dimethylethanolamine (PDME) by Opi3 [7–9]. In the complementary CDP-choline pathway, choline is phosphorylated by the kinase Cki1 to phosphocholine, which is converted into CDP-choline by the CTP-phosphocholine cytidyltransferase Pct1. Finally, the choline phosphotransferase Cpt1p transfers CDP-choline to DAG to yield PC [5]. Ept1, the corresponding enzyme from the CDP-ethanolamine pathway, also contributes to PC synthesis, the extent depending on the strain background [10]. The CDP-choline pathway only contributes to net PC synthesis if choline is present in the culture medium. In its absence, this pathway serves to recycle choline from PC turnover [11].

Whereas the biosynthesis of PC has been extensively studied, knowledge on PC metabolism downstream of synthesis is scarce. To date, the *in vivo* metabolic conversions documented at the gene level (Scheme 1) are limited to the turnover of PC by the *SPO14*-encoded phospholipase D that is essential for sporulation [12], the deacylation of PC by Plb1p [13], and the temperature-dependent and choline-induced conversion of PC into glycerophosphocholine by Nte1 that is specific for CDP-choline-derived PC [14,15]. Remodelling of PC by acyl chain exchange was inferred from

Key words: acyl chain composition, deacylation, electrospray ionization tandem MS (ESI-MS/MS), phosphatidylcholine, reacylation, yeast.

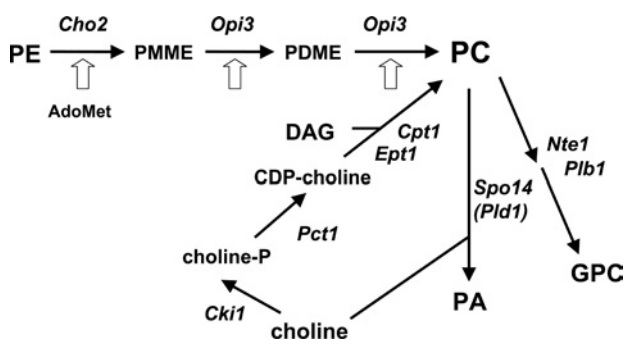
Abbreviations used: CID, collision-induced dissociation; DAG, diacylglycerol; ESI-MS/MS, electrospray ionization tandem MS; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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Scheme 1 | Metabolism of PC in yeast

The biosynthetic routes leading to PC and the conversions of PC detected *in vivo* and documented at the gene level are depicted. The enzymes catalysing the individual steps are indicated. PDME, phosphatidyl-dimethylethanolamine; PMME, phosphatidylmonomethylethanolamine.



the selective incorporation of exogenous radiolabelled acyl chains at the *sn*-2 position of PC [4].

In this paper, our current understanding of the processes involved in the establishment of the PC molecular species profile is summarized.

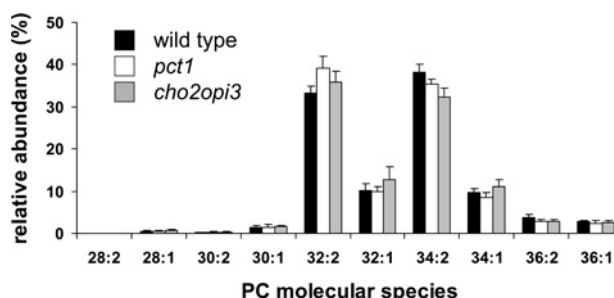
ESI-MS/MS (electrospray ionization tandem MS) analysis of PC molecular species profiles

Today, MS is the most sensitive method for a detailed structural characterization of biological molecules including phospholipids [16]. The major advantages of this technique include: (i) its sensitivity as only pmol amounts of samples are required, (ii) the use of unfractionated total lipid extracts for direct analysis of different phospholipid classes, (iii) the identification of individual molecular species, (iv) the ability to quantify data and (v) the possibility to perform metabolic labelling studies.

ESI-MS/MS has proven to be an excellent technique to perform full analysis of complex lipid mixtures [17,18]. The conventional instrument applied for MS/MS experiments is the triple quadrupole instrument, in which the first (MS1) and third (MS2) quadrupoles are identical and operate as mass analysers, while the second quadrupole serves as a collision cell. The introduction of an inert gas, e.g. argon, in the collision cell leads to the decomposition of incoming ions in a process referred to as CID (collision-induced dissociation). In most cases, phospholipids are analysed by using low-energy CID to specifically cleave off the head group moiety [17]. For quantitative analysis of PC species compositions, parent ion scanning in the positive ion mode is the method of choice. In this mode, the MS1 analyser consecutively scans a selected *m/z* range. Ions entering the collision cell will be subjected to CID, in which PC molecular species yield the characteristic phosphocholine moiety with an *m/z* of 184. The MS2 analyser is set to transfer only the preselected fragments that are typical for the phospholipid of interest. In the case of PC, recording

Figure 1 | Steady-state PC species profiles obtained by parent ion scanning for *m/z* 184 of wild-type (BY4742), *pct1* and *cho2opi3* cells cultured to mid-exponential phase in semi-synthetic lactate medium

The culture medium of the *cho2opi3* strain was supplemented with 1 mM choline. ESI-MS/MS data were obtained and quantified as described in [19,26].



proceeds if the parental ions yield a fragment with an *m/z* value of 184.

Parent ion scanning for *m/z* 184 of total lipid extracts of wild-type yeast cells (BY4742) grown to mid-exponential phase revealed a PC species profile (quantified in Figure 1) containing four prominent components: diunsaturated 32:2 (containing two C_{16:1} acyl chains) and 34:2 (containing a C_{16:1} and a C_{18:1} acyl chain), and the mono-unsaturated species 32:1 (composed of C_{16:0} and C_{16:1}) and 34:1 (composed of C_{16:0} and C_{18:1} and/or of C_{18:0} and C_{16:1}) [19]. Please note that except for 32:2, each of these components may correspond to two (32:1 and 34:2) or four (34:1) molecular species due to positional isomers, of which the relative amounts were not determined. The acyl chain composition of yeast cells strongly depends on the carbon source used to culture the cells [20]. Because of our interest in phospholipid metabolism under conditions of full mitochondrial development, lactate was used as carbon source unless indicated otherwise.

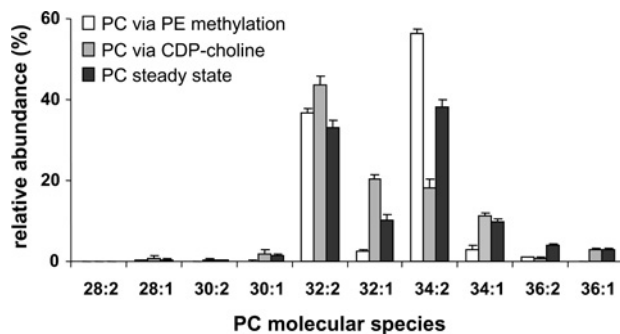
Species selectivity of the PC biosynthetic routes

To get a first insight into the possibility that the two PC biosynthetic pathways contribute differently at the level of molecular species, the steady-state PC species profile of wild-type cells was compared with those of *cho2opi3* and *pct1* mutants lacking the PE methylation and the CDP-choline route respectively (Figure 1). The three profiles are similar (in agreement with [14]), suggesting that either the contributions of the two routes are similar or that other processes compensate for differences in contribution.

Pulse labelling of cells with stable isotope-labelled precursors and subsequent analysis of phospholipid molecular species by ESI-MS/MS allows distinction between newly synthesized and pre-existing pools of most phospholipid classes, based on the difference in molecular mass conferred by the isotope labels (see e.g. [21]). To distinguish the contributions of the two PC biosynthetic routes to the PC

Figure 2 | Profiles of the newly synthesized PC species obtained via PE methylation and the CDP-choline pathway compared with the steady-state PC species distribution

Wild-type cells were pulsed with [*methyl*-²H₃]methionine and [²H₁₃]choline for 10 min. Subsequently, newly synthesized PC was detected by parent-ion scanning of total lipid extracts for *m/z* 193 and 197 respectively. The steady-state PC species profile was obtained in parent ion scans for *m/z* 184. Data were obtained and quantified as described in [19].



species profile, wild-type yeast was pulsed for 10 min with ²H-labelled substrates. The PE methylation pathway was addressed by pulse labelling with [*methyl*-²H₃]methionine yielding [*methyl*-²H₃]₃PC that is selectively visualized by ESI-MS/MS in a parent ion scan for *m/z* 193. Upon incubation with perdeuterated [²H₁₃]choline, the CDP-choline route produces [²H₁₃]PC that is detected by parent ion scanning for *m/z* 197. As shown in Figure 2, the methylation of PE and the CDP-choline route produce the PC species in different ratios. Whereas PE methylation predominantly yields diunsaturated PC, the CDP-choline route produces a more diverse profile of PC species (Figure 2). Moreover, the profiles of newly synthesized PC differ from that of steady-state PC [19].

The species profile of newly methylated PC was compared with that of its precursor PE that was determined by neutral loss scanning in the positive-ion mode for *m/z* 141. In this scanning mode, the scans by the MS1 and MS2 analysers are synchronized with a fixed *m/z* difference equivalent to the selected neutral loss fragment. Upon CID, PE ions lose the phosphoethanolamine moiety as an uncharged fragment of 141 Da. The recoding device registers only those ions that have lost the fragment of interest. Compared with the species profile of cellular PE that is dominated by 32:2 PE (~20%) and 34:2 PE (~65%), the derived newly synthesized PC is enriched in the 32:2 species (~35%, Figure 2), suggesting that the methyltransferases preferentially convert 32:2 PE. When the species composition of the PE precursor pool was varied, by using different carbon sources or a phospholipid biosynthetic mutant strain, a similar enrichment was observed [22].

To obtain further insight into the apparent preference for methylating 32:2 PE, the reaction was investigated *in vitro*, by incubating isolated microsomes for 10 min with *S*-[*methyl*-²H₃]adenosyl-methionine. Again, a similar preferential con-

version of 32:2 PE into PC was detected, indicating that it is intrinsic to the methyltransferases in microsomes, and not dependent on the intermembrane transport processes required to supply the enzymes with PE that is for the most part synthesized by mitochondrial Psd1 and by Psd2 in the Golgi/vacuole [23,24]. Consistent with this conclusion, a recent analysis of the species distributions of the aminoglycerophospholipids in yeast subcellular fractions did not reveal species selectivity in interorganelle phospholipid transport [25]. The preferential methylation of 32:2 PE is for the most part conferred by Cho2 with a minor contribution of Opi3 [22].

The contributions of Cpt1 and Ept1 to CDP-choline derived PC were examined by pulsing *ept1* and *cpt1* cells with [²H₁₃]choline respectively. The species profile of newly synthesized PC in the *ept1* strain was virtually identical with that in wild-type cells, and differed significantly from that obtained in the *cpt1* strain in which 34:2 PC was the major species [26]. From this result, it was concluded that in the BY4742 strain background, Ept1 does not contribute substantially to PC biosynthesis (cf. [10,11]).

Acyl chain remodelling of PC

Are the contributions of PE methylation and the CDP-choline pathway sufficient to account for the steady-state PC species distribution in wild-type cells? Inspection of Figure 2 reveals that the steady-state PC species profile cannot be fitted to any weighted average of the two profiles of newly synthesized PC, implicating other processes in addition to the biosynthetic routes in establishing the steady-state distribution. The PC acyl chain composition could be modified by species-selective degradation, and/or by acyl chain remodelling, i.e. exchange of acyl chains. In the *pct1* and *cho2opi3* mutants, lacking either of the pathways (Figure 1), the need for processing of newly synthesized PC to accomplish the steady-state profile is even more evident [19,26]. The post-synthetic processing of the newly synthesized PC profiles towards the steady state was visualized in these strains in prolonged labelling and pulse-chase experiments with [*methyl*-²H₃]methionine [19] and [²H₁₃]choline (H.A. Boumann, unpublished work) respectively, and proceeds with an apparent half-time in the order of 1 h.

In the *pct1* strain, a species profile containing relatively small proportions of newly synthesized 32:1 and 34:1 PC evolves to a steady-state profile containing at least twice the initial amounts of these species [19]. Species-selective degradation alone is unlikely to account for this process, as it would involve degradation of some 50% of newly synthesized PC. Moreover, during prolonged labelling with deuterated PC precursors, no change in species composition of the pre-existing PC pool was observed in wild-type cells, arguing against the occurrence of extensive species-selective turnover. This leaves remodelling by acyl chain exchange as the more plausible mechanism. ESI-MS/MS analysis of lyso-PC, after treatment of the *pct1* lipid extracts with phospholipase A₂, revealed the substitution of C_{16:1} acyl chains

esterified at the *sn*-1 position of newly synthesized PC by other acyl chains, unequivocally demonstrating the occurrence of acyl chain remodelling [19].

Conclusion

The PC species profile in wild-type yeast is to a large extent determined by the species selectivity of the methyltransferases converting PE into PC. The CDP-choline pathway using exogenous choline or choline from PC turnover adds to the profile by producing the major PC species in a different characteristic ratio. However, the relative contributions of the two pathways to the total PC pool are unknown and depend on the culture conditions. Stable isotope labelling combined with detection by ESI-MS/MS has revealed that, irrespective of their relative contributions, the two biosynthetic pathways are insufficient to account for the steady-state PC species profile. Acyl-chain remodelling and possibly species-selective degradation are also involved, and can be conveniently studied in mutants lacking one of the biosynthetic pathways. Remodelling of newly synthesized PC by acyl chain exchange was demonstrated in the *pct1* mutant.

Remodelling of phospholipids is generally thought to proceed via deacylation to a lyso-phospholipid mediated by a phospholipase A or B, followed by an acyl-CoA-dependent reacylation by an acyltransferase [27]. Alternatively, acyl chain shuffling via transacylation may occur in which another phospholipid serves as acyl chain donor and a lyso-phospholipid as the acceptor (see e.g. [28]). Both acyl-CoA-dependent and acyl-CoA-independent acylation of lyso-PC have been reported in yeast [29,30]. Our current research is directed at identifying the genes involved in shaping the PC species profile in order to elucidate the biological functions of PC remodelling.

We gratefully acknowledge Professor Dr Albert Heck (Utrecht University) for sharing mass spectrometric facilities and expertise and Professor Dr Ben de Kruijff (Utrecht University) and the yeast lipid group in the Department of Biochemistry of Membranes for fruitful discussions. This work was supported by the Netherlands Division of Chemical Sciences with financial aid from the Netherlands Organization for Scientific Research.

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Received 21 June 2005