The Two Biosynthetic Routes Leading to Phosphatidylcholine in Yeast Produce Different Sets of Molecular Species. Evidence for Lipid Remodeling†

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ABSTRACT: Phosphatidylcholine (PC), a major lipid class in the membranes of eukaryotes, is synthesized either via the triple methylation of phosphatidylethanolamine (PE) or via the CDP-choline route. To investigate whether the two biosynthetic routes contribute differently to the steady-state profile of PC species, i.e., PC molecules with specific acyl chain compositions, the pools of newly synthesized PC species were monitored by labeling *Saccharomyces cerevisiae* with deuterated precursors of the two routes, (*methyl*-D_3)-methionine and (D_{13})-choline, respectively. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) revealed that the two PC biosynthetic pathways yield different sets of PC species, with the CDP-choline route contributing most to the molecular diversity. Moreover, yeast was shown to be capable of remodeling PC by acyl chain exchange at the *sn*-1 position of the glycerol backbone. Remodeling was found to be required to generate the steady-state species distribution of PC. This is the first study demonstrating a functional difference between the two biosynthetic routes in yeast.

Phosphatidylcholine (PC)[1] is a major phospholipid present in membranes of eukaryotic cells. Alterations in PC metabolism have been associated with several physiological and pathological conditions, including oncogenic transformation (*1*), apoptosis (*2*), and Alzheimer's disease (*3*). In *Saccharomyces cerevisiae*, the synthesis of PC is intimately involved with the trafficking of intracellular vesicles (*4*), and mutations in the PC biosynthetic genes lead to respiratory deficiency (*5*). Like higher eukaryotes, yeast is able to synthesize PC via the triple methylation of the headgroup of phosphatidylethanolamine (PE) and via the CDP-choline pathway (*6*) (Figure 1). In the absence of exogenous choline, net production of PC proceeds via the methylation of PE mediated by the methyltransferases Cho2p and Opi3p, while the CDP-choline route serves to recycle choline from PC turnover (*7*).

Although most genes and the corresponding enzymes involved in lipid synthesis have been well studied in yeast, the functional significance for having two PC biosynthetic routes remains unclear. The long held view that mutations in the CDP-choline route, but not the PE methylation pathway, suppress the temperature-sensitive phenotype of a *sec14*^{ts} mutation (*2*) was refuted (*8*). Recently, elevated temperature was reported to accelerate the synthesis and turnover of CDP-choline derived PC but not of PC formed via methylation of PE (*9*). One explanation for the existence of two biosynthetic routes might be that they yield structurally different PC species, i.e., PC molecules with different acyl chains. The profile of acyl chains in yeast is simple compared to higher eukaryotes and is predominantly composed of the fatty acids C16:1, C18:1, C16:0, and C18:0 (*10*).

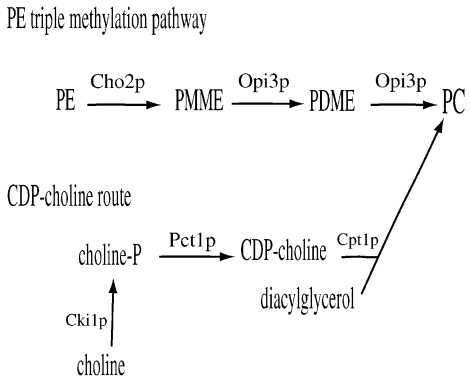

FIGURE 1: Scheme of the routes and enzymes involved in PC biosynthesis in yeast.

Recently, electrospray ionization tandem mass spectrometry (ESI-MS/MS) was shown to be an excellent tool for phospholipid species analysis because of its sensitivity and the fact that it can be used on total lipid extracts (*11*−*13*). Here we combined ESI-MS/MS with in vivo deuterium pulse labeling to analyze the newly synthesized PC species. The two biosynthetic routes were found to produce different sets of PC molecular species in yeast. Moreover, we observed

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DAG, diacylglycerol; ESI-MS/MS, electrospray ionization tandem mass spectrometry; PLA_2, phospholipase A_2.

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that the available PE species are not randomly methylated to PC in vivo. Finally, it was demonstrated that yeast has the ability to remodel newly synthesized PC species by acyl chain exchange.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions. S. cerevisiae strain *met6*Δ (*met6::KanMX*) and its congenic wild-type BY4742 (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were obtained from Research Genetics. The *PCT1* gene of *met6*Δ (nucleotides −60 to 1286) was replaced with the *LEU2* marker (*14*) to generate *met6*Δ*pct1*Δ (*met6::KanMX pct1::LEU2*). Correct integration of the *LEU2* marker was verified by PCR. Strains were grown to midlog phase (OD_{600} 1.0−2.0) at 30 °C in semisynthetic lactate (SSL) medium (*15*) supplemented with 20 mg/L histidine, 60 mg/L leucine, 230 mg/L lysine, and 40 mg/L uracil.

Labeling with Deuterated Methionine and Choline. Cells grown to OD_{600} 1.0−1.5 in 0.8 L of SSL were washed with and resuspended in 0.8 L of synthetic lactate (SL) medium [0.67% yeast nitrogen base without amino acids (Difco), 2% lactate, 0.1% glucose, pH 5.5] with the above supplements. At time zero, 40 mg/L (0.26 mM) (*methyl*-D_3)-methionine and/or 0.2 mM (D_{13})-choline (Cambridge Isotope Laboratories) was added. At various time points, cells were collected and inactivated by adding KCN, NaF, and NaN_3 at 15 mM each.

Lipid Extraction. Cell pellets corresponding to ∼300 OD_{600} units were resuspended in 1 mL of water and homogenized by vortexing with glass beads for three times 1 min with intermittent cooling on ice. Total lipid extracts were prepared (*16*), and their phospholipid−phosphorus contents were determined (*17*).

Mass Spectrometry. Lipid extracts were dissolved at 50 pmol of lipid phosphorus/μL in $CHCl_3$/CH_3OH/H_2O (2:15:3 v/v/v) and subjected to ESI-MS/MS using a Quattro Ultima triple quadrupole MS instrument (Micromass). A nanoelectrospray with a flow rate of 40−60 nL/min was started by applying 1.5 keV capillary voltage in the positive ion mode. Argon was used as collision gas at a collision energy of 30 eV. The $[M + H]^+$ ions of PC species were detected in the parent ion scan mode at *m*/*z* 184, 193, or 197 for unlabeled, (*methyl*-D_3)$_3$-labeled, and (D_{13})-choline-labeled PC, respectively. The $[M + H]^+$ and $[M + Na]^+$ molecular species of PE were detected by neutral loss scanning for *m*/*z* 141. The identities of the PC and PE species were established on the basis of data from the literature (*10*, *13*) and confirmed by daughter ion scanning. Results are presented as averages from at least three independent experiments obtained from 100 to 150 repetitive scans of 10 s. Data were quantified using Mass Lynx NT software (Micromass), taking into account the three most abundant $[M + H]^+$ isotopic signals of the PC and PE species. In the case of PE, the $[M + Na]^+$ adducts were also included in the quantification. The intensity of the monoisotope signal of each monounsaturated lipid species was corrected for the coinciding second isotope peak of the diunsaturated lipid species with an *m*/*z* value of 2 units lower. Equimolar mixtures of biologically relevant PE and PC species (Avanti Polar Lipids) dissolved at 20 pmol/μL of each were used as external standards to correct for the inverse relationship between the instrument sensitivity and molecular weight (*11*, *18*, *19*). Within the range of *m*/*z* values of interest, this correction never exceeded 4.5% of the peak intensity measured. The correction was not applied to spectra of deuterated PC, since in the concentration range at which the labeled PC species are present in the samples (<0.5 pmol/μL), the instrument response is virtually independent of the acyl chain length in the relevant *m*/*z* range (*19*). Under the experimental conditions used, the relative responses of the lipid species were independent of the total lipid concentration, as was determined by 20-fold dilution of the samples (data not shown).

Treatment with PLA_2. To analyze the acyl chains esterified at the *sn*-1 position of PC, dried total lipid extracts corresponding to ∼200 nmol of lipid phosphorus were hydrated in 600 μL of buffer (10 mM Tris, 1.0 mM $CaCl_2$, pH 8.0), and 10 units of bee venom phospholipase A_2 (Sigma) was added. After incubation for 30 min at 37 °C, 5 mL of $CHCl_3$/CH_3OH (2:1 v/v) was added, and the mixture was dried by rotatory evaporation. ESI-MS/MS confirmed the complete hydrolysis of PC and was used to analyze the obtained lysoPC species.

Other Methods. Phospholipid compositions were determined by thin-layer chromatography (*20*).

RESULTS

PE and PC Steady-State Species Composition in Wild-Type Yeast. The distribution of the molecular species of the phospholipid classes PE and PC in total lipid extracts of wild-type yeast was analyzed by selective scanning by ESI-MS/MS (Figure 2, panels A and B, respectively). The species distributions were quantified as described in the Experimental Procedures (Figure 2C). The most prominent PE species were the 34:2 (containing a C16:1 and a C18:1 acyl chain) and 32:2 (two C16:1 acyl chains) species (Figure 2A,C, white bars). The monounsaturated species 32:1 PE (composed of C16:0 and C16:1) and 34:1 (composed of either C16:0 and C18:1 or C18:0 and C16:1) were also detected. Compared to PE, the steady-state species profile of PC was strikingly different, with a 2-fold reduced content of 34:2 and increased levels of 32:2, 32:1, and 34:1 (Figure 2B,C, black bars; cf. ref *10*). Since PE is the main precursor of PC molecules under the conditions used (*21*), this immediately raised the question of how the steady-state species composition of PC is achieved. To resolve this issue, the species profile of newly synthesized PC was analyzed.

PE Species Are Not Randomly Methylated to PC. The pool of PC species newly produced via the methylation of PE was monitored by labeling wild-type yeast with (*methyl*-D_3)-methionine. From the ESI-MS spectrum it was estimated that a 10 min pulse yielded ∼3% deuterated PC relative to the total cellular PC content (not shown). The methylation of PE produced predominantly the diunsaturated PC species 32:2 and 34:2 (Figure 3A, quantified in Figure 3C, light gray bars) in a ratio resembling that in the steady-state distribution of PC (Figure 2C, black bars) and clearly different from that of the corresponding species available in the pool of PE precursors (Figure 2C, white bars; quantified in Figure 3D). This suggests that preferential methylation of 32:2 PE could in part account for the steady-state species distribution of PC. However, the proportions of the monounsaturated PC species produced by PE methylation are much smaller than

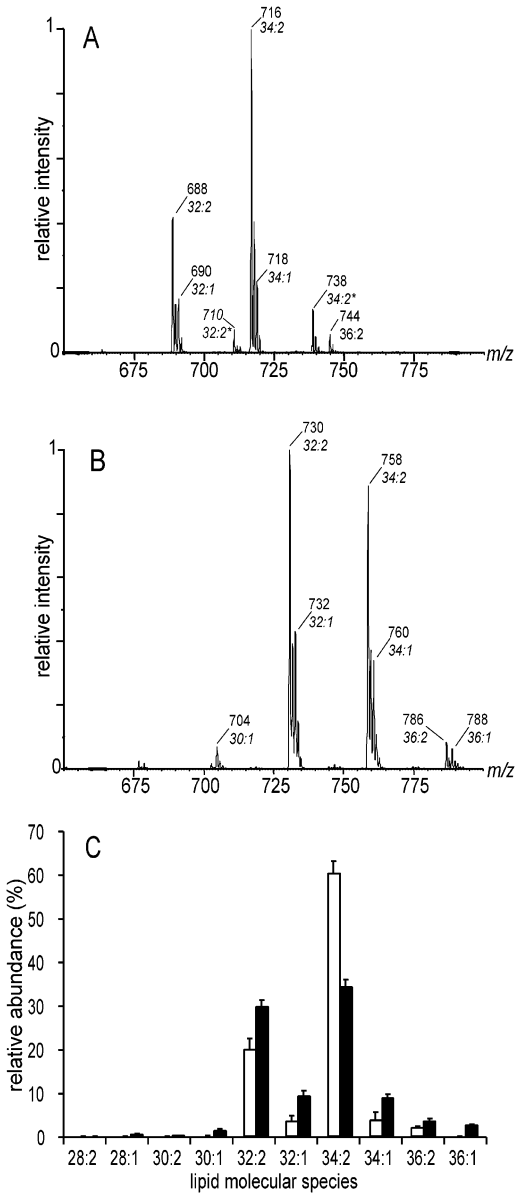

FIGURE 2: Molecular species of PE and PC in the wild-type strain BY4742. (A) ESI-MS/MS spectrum of the PE molecular species detected by scanning for neutral loss of the PE headgroup (*m*/*z* 141). The major $[M + H]^+$ ion peaks and asterisk-marked $[M + Na]^+$ adducts of PE species are denoted. (B) Parent ion scan of the PC species (*m*/*z* 184). The peaks of the most abundant PC species are identified. (C) Quantification of the relative abundance (±SD, $n \geq 3$) of the molecular species of PE (white bars) and PC (black bars).

those present in the steady-state profile (compare Figure 3C, light gray bars, to Figure 2C, black bars). Degradation of PC derived from PE and subsequent salvage of the choline headgroup by the CDP-choline route might make up for this difference.

The Two Biosynthetic Pathways Produce Different Sets of PC Species. Wild-type cells were labeled for 10 min with (D_{13})-choline to detect the PC species newly synthesized by the CDP-choline route. A parent ion scan of *m*/*z* 197 (Figure 3B) reveals a species profile which is very different from that produced by methylation of PE (Figure 3A). Compared to PE-derived and steady-state PC, the CDP-choline-derived PC is enriched in the monounsaturated species 32:1 and 34:1 (Figures 3C and 2C, black bars).

Table 1: Phospholipid Class Composition of Cell Homogenates[a]

	wt	*met6*Δ*pct1*Δ
phosphatidylcholine	40.7 ± 2.8	29.8 ± 1.2
phosphatidylethanolamine	26.2 ± 1.0	34.4 ± 1.3
phosphatidylinositol	18.4 ± 2.2	21.7 ± 1.6
phosphatidylserine	9.0 ± 1.1	9.3 ± 1.7
phosphatidic acid	1.0 ± 0.8	0.5 ± 1.0
cardiolipin	4.6 ± 0.5	4.3 ± 1.0

[a] The values (±SD, $n \geq 3$) represent the mole percent distribution of phospholipids for the wild type and the *met6*Δ*pct1*Δ strain grown to midlog phase. Other phospholipid classes were not detected at measurable levels.

At first glance, the steady-state species profile of PC could be accounted for by approximately equal contributions from the two biosynthetic routes. Thus, for example, the proportions of 34:2 (38%) and 32:1 (10%) in the steady state would reflect the weighted averages of the respective proportions of 34:2 and 32:1 produced by the methylation of PE (56% and 3%) and the CDP-choline pathway (18% and 20%) (Figures 3C and 2C, black bars). However, closer examination of the data reveals that this simple notion does not hold. Because the CDP-choline route produces most of the 32:1 and 34:1 species and does so in a ratio of approximately 2:1 (Figure 3C), the 1:1 ratio of these species in the steady state (Figure 2C) can never be attained as a weighted average of the two routes irrespective of the weighing factors. Therefore, also other processes are likely to be involved such as preferential turnover of certain PC species and/or remodeling by exchange of acyl chains. During 3 h of labeling with both deuterated PC precursors, no changes occurred in the species distribution of the preexisting unlabeled PC pool (*m*/*z* 184, data not shown), arguing against the occurrence of selective turnover of PC species in yeast.

Remodeling of PC Produced by PE Methylation Is Required To Yield the Steady-State Species Distribution of PC. The proposed contribution of remodeling to the steady-state PC species profile was investigated by focusing on the methylation of PE, the main route of net PC biosynthesis. For this purpose, the CDP-choline route, which introduces most of the species diversity, was inactivated by deleting the *PCT1* gene encoding phosphocholine cytidylyltransferase (*22*). The *MET6* gene was also deleted to render the cells auxotrophic for methionine, thereby enabling maximal incorporation of deuterated methionine. Compared to the parental strain, the *met6*Δ*pct1*Δ strain had a decreased level of PC that was compensated for by a rise in PE (Table 1). Remarkably, the inactivation of the CDP-choline route hardly affected the steady-state species profile of PC, the only difference between *met6*Δ*pct1*Δ and the wild type being a slight shift toward 32:2 at the expense of 34:2 (compare Figures 4A and 2C, black bars). This indicates that a broad range of PC species is required for yeast growth and that the mutant is a valid system for investigating remodeling of PC.

Upon pulsing the *met6*Δ*pct1*Δ cells with (*methyl*-D_3)-methionine, the newly synthesized PC species were analyzed and found to be composed almost exclusively of 32:2 and 34:2 (Figure 4A). Prolonged labeling with (*methyl*-D_3)-methionine for up to 3 h showed that the abundance of 32:2 and 34:2 deuterated PC species decreased, while 32:1 and 34:1 appeared (Figure 4B). Thus, the species distribution of

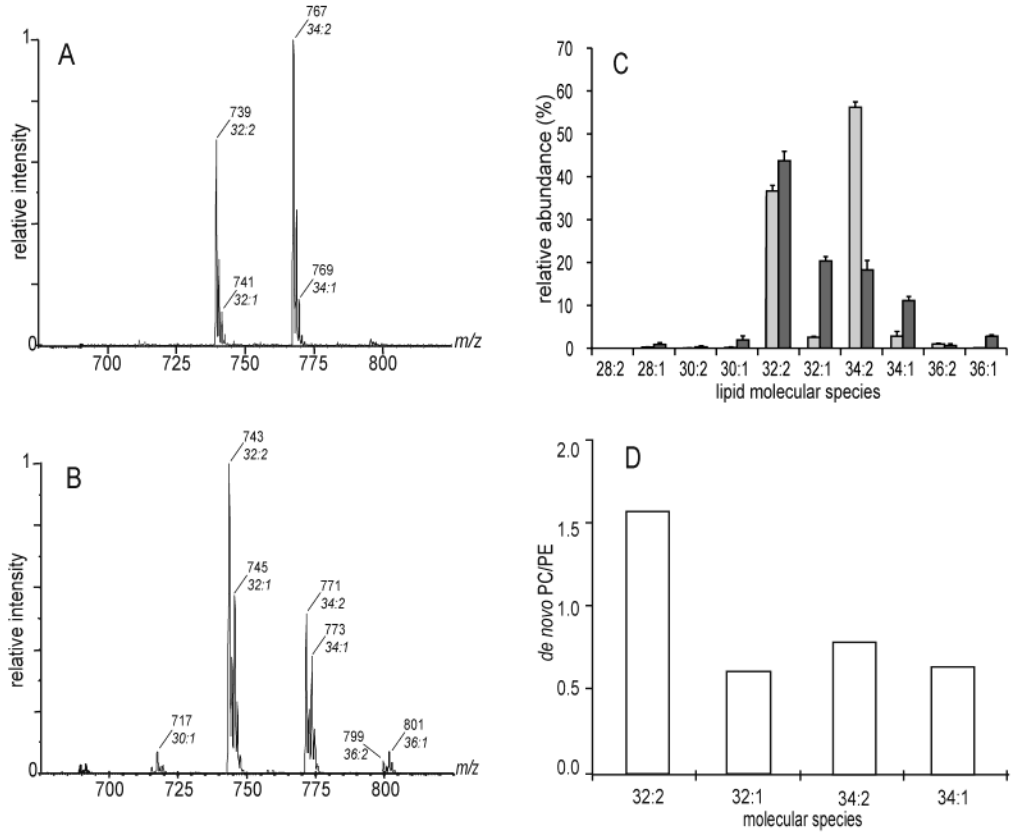

FIGURE 3: The species composition of PC synthesized by the methylation of PE differs from that produced by the CDP-choline route. ESI-MS/MS analysis of newly synthesized PC species after a 10 min pulse with (*methyl*-D_3)-methionine and/or (D_{13})-choline. (A) Molecular species of PC synthesized via PE triple methylation monitored by parent ion scanning for the (*methyl*-D_3)$_3$-choline headgroup (*m*/*z* 193). (B) Molecular species of CDP-choline-derived PC detected by parent ion scanning for the (D_{13})-choline headgroup (*m*/*z* 197). (C) Quantification of the distribution (±SD, $n = 3$) of newly synthesized PC species obtained via PE methylation (light gray bars) and the CDP-choline route (dark gray bars). (D) Species-selective methylation of PE in vivo. The relative extents of conversion to PC were determined for the four most prominent PE species by relating the relative abundance of each PE-derived PC species to the proportion of the corresponding PE species in the steady-state distribution (averaged values, $n = 3$).

newly formed PC gradually shifted toward the steady-state species composition. This cannot be attributed to preferential degradation of diunsaturated PC species, as the monounsaturated PC species are virtually absent from the pool of newly synthesized PC (Figure 4A). We therefore conclude that yeast is able to remodel newly synthesized PC species by replacing monounsaturated acyl chains by saturated acyl chains.

Remodeling of Newly Formed PC Occurs at the sn-1 Position. The presence of only 32:2 and 34:2 deuterated PC in the *met6*Δ*pct1*Δ strain upon 10 min labeling with (*methyl*-D_3)-methionine enabled the straightforward analysis of the acyl chains esterified at the *sn*-1 position of newly formed PC. For this purpose, the *sn*-2 linked acyl chains were removed by PLA_2. A parent ion scan of the (*methyl*-D_3)$_3$-choline headgroup (*m*/*z* 193) of the resulting lysoPC species revealed a peak at 503 Da corresponding to C16:1 lysoPC, whereas no signal at 531 Da corresponding to C18:1 lysoPC was present (Figure 5A). The absence of deuterated C18:1 lysoPC implies that the methylation of PE in the *met6*Δ*pct1*Δ strain yields only two PC molecular species, 32:2 (C16:1, C16:1) and 34:2 (*sn*-1 C16:1, *sn*-2 C18:1). Examination of the acyl chains present at the *sn*-1 position of steady-state PC (headgroup *m*/*z* 184) revealed, besides C16:1, also C16:0, C18:1, and C18:0 (Figure 5B), which is in agreement with previous findings (*10*). These results demonstrate the occurrence of deacylation/reacylation at the *sn*-1 position of new PC molecules in yeast.

DISCUSSION

ESI-MS/MS combined with deuterium pulse labeling revealed a functional difference between the two PC biosynthetic pathways in *S. cerevisiae* in that they produce different pools of newly synthesized PC species. Whereas the methylation of PE results predominantly in 32:2 and 34:2 PC, the CDP-choline route produces a more diverse set of species. The steady-state composition of PC species could arithmetically not be accounted for by the contributions of the two pathways alone, implicating the involvement of other mechanism(s). One possibility is species-selective degradation of PC by phospholipases D and/or B, which are known to catabolize PC in yeast (*9*, *23*). We consider this possibility unlikely because of the unvarying species profile of preexisting PC and because turnover of PC in yeast grown at 30 °C is slow compared to the growth rate (*9*).

Thus, remodeling of PC, i.e., the exchange of acyl chains, is left as the most probable mechanism to accomplish the steady-state PC species distribution. Indeed, prolonged labeling of PC in the *met6*Δ*pct1*Δ strain showed that the pool of newly formed PC comprising only 32:2 and 34:2 gradually evolved toward the steady-state profile. On the

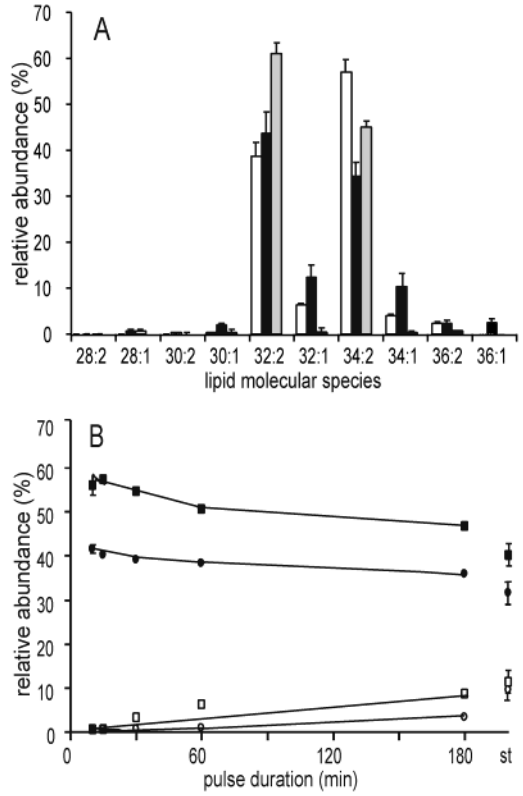

FIGURE 4: PC species are remodeled to obtain monounsaturated PC species. (A) ESI-MS/MS analysis of the molecular species of PE (white bars), PC (black bars), and PC, newly synthesized by PE methylation (light gray bars) in the *met6*Δ*pct1*Δ strain (±SD, $n \geq 3$). (B) Time course of incorporation of the *methyl*-D_3 group into newly synthesized PC species in the *met6*Δ*pct1*Δ strain. The distribution of the four most abundant molecular species of PC, 32:2 (■), 34:2 (●), 32:1 (□), and 34:1 (○), was determined at the indicated time points and compared to the steady-state distribution (st).

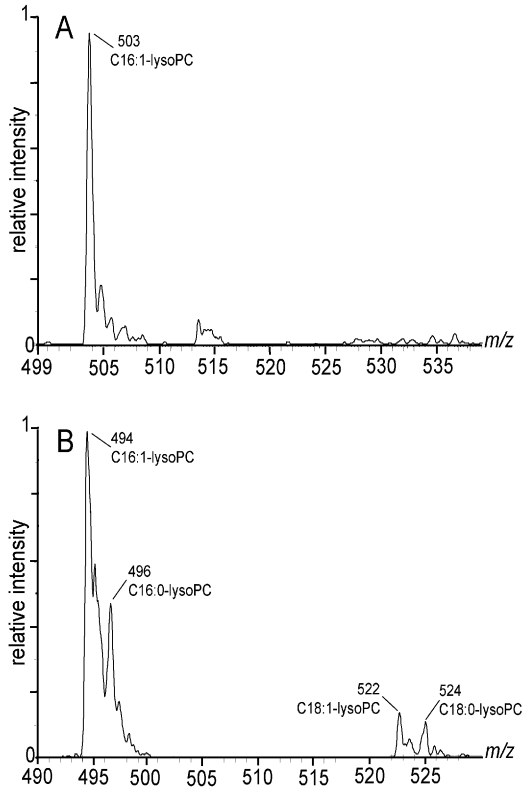

FIGURE 5: Newly synthesized PC in the *met6*Δ*pct1*Δ strain contains exclusively C16:1 esterified at the *sn*-1 position, in contrast to steady-state PC. Parent ion scans are shown of lysoPC species originating from (A) newly synthesized PC (*m*/*z* 193) and (B) steady-state PC (*m*/*z* 184). *Met6*Δ*pct1*Δ cells were labeled for 10 min with (*methyl*-D_3)-methionine, and the total lipid extract was subjected to PLA_2 treatment as detailed in the Experimental Procedures. Daughter ion scanning showed that the signal around *m*/*z* 513 in panel A does not derive from (lyso)PC. To facilitate comparison, the *x*-axis in panel A was shifted by 9 mass units relative to that in panel B.

basis of Figure 4B, we estimate that at least 30% of the newly synthesized PC is remodeled within one generation time to reach the steady-state species profile. A phospholipase A_1- or B-dependent deacylation−reacylation mechanism is postulated on the basis of the substitution by other acyl chains of part of the C16:1 acyl chains initially esterified at the *sn*-1 position of PC in the *met6*Δ*pct1*Δ strain (Figure 5). In addition, a phospholipase A_2-dependent remodeling mechanism is likely to play a role, as saturated acyl chains also occur at the *sn*-2 position of PC (*10*). Other than the phospholipases B, Plb1p, Plb2p, and Plb3p (*23*, *24*), and Lro1p, which in vitro catalyzes the esterification of DAG using the *sn*-2 acyl group of PC as the acyl donor (*25*), no enzymes or genes potentially involved in deacylation of phospholipids in yeast have been identified so far. Little is known about reacylating enzymes in yeast: Plb1p was reported to have some acyltransferase activity in vitro (*24*), and a Plb1p-independent lysoPC acyltransferase activity was observed (*26*).

Dowd et al. (*9*) recently raised the possibility that the two pathways yield different PC molecular species. As these authors examined the molecular species composition of steady state rather than newly synthesized PC, they only found minor differences in species composition between mutants in which either one of the two biosynthetic routes was inactivated. These findings can now be explained by the occurrence of remodeling of PC.

In higher eukaryotes, the occurrence of lipid remodeling is well established (see, e.g., ref *27*) and has been correlated with the release of arachidonic acid, the precursor of eicosanoid hormones (*28*). The discovery of the occurrence of remodeling in the unicellular eukaryote yeast with its limited repertoire of acyl chains points to a fundamental physiological role for lipid remodeling.

The results in this work seem to contradict the recent study of DeLong and co-workers (*12*). They labeled rat hepatocytes for 24 h with PC precursors and reported that the methylation of PE rather than the CDP-choline route generates a more diverse set of newly formed PC species, while assuming that remodeling was negligible. However, remodeling of ethanolamine-derived PC has been reported in rat hepatocytes (*27*, *29*). Furthermore, by the choice of label, DeLong et al. monitored only PC derived from PE originating from the Kennedy pathway, which is considered to be an auxiliary route for PE biosynthesis (*6*). In contrast, we examined all of the newly formed PC species originating from PE derived both from PS decarboxylation and from the Kennedy pathway. Next to that, it is possible that differences in PC species synthesis differ between organisms.

In yeast, the PE species are not randomly used by the methyltransferases; instead, the 32:2 PE species is preferentially methylated (Figure 3D). When the new PC species derived from PE are related to the pool of PE species present

in the endoplasmic reticulum, to account for the intracellular location of the methyltransferases (*30*), a similar preference is observed (H. A. Boumann, unpublished data). Whether this is due to the intrinsic substrate specificity of the methyltransferases or to differences in substrate−enzyme accessibility is the subject of further study. The methylation of monounsaturated PE was almost completely abolished in the *met6*Δ*pct1*Δ double mutant (Figure 4A). This is due to deletion of both the *PCT*1 and the *MET6* genes, since the *pct1*Δ and *met6*Δ single mutants have a distribution of newly synthesized PE-derived PC species resembling that of the wild type (not shown). At this point we can only speculate about the reason for this. As the production of PC in this strain is decreased (Table 1), the biosynthesis of diunsaturated, more hydrophilic PC species may be preferred to facilitate transport of PC from the ER to other subcellular destinations (cf. ref *31*). A similar adaptation to the impaired PC biosynthesis may be reflected by the enrichment of the 32:2 species in the steady-state profiles of PC and, even more pronounced, of PE in the *met6*Δ*pct1*Δ mutant relative to the wild type (Figures 4A and 2C).

The present study shows that the two PC biosynthetic pathways contribute differently to the PC species profile in *S. cerevisiae*. Together with PC remodeling, this provides yeast with the flexibility to adjust the acyl chain composition of this abundant membrane lipid in response to changing environmental conditions. For example, the reported increased deacylation of CDP-choline-derived PC but not of PE-derived PC in yeast grown at 37 °C (*9*) could be responsible for the enrichment of unsaturated acyl chains observed in response to temperature stress (*32*). The combination of stable isotope pulse labeling and ESI-MS/MS has great potential for "visualizing" the flux of phospholipid species through the biosynthetic routes in yeast.

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