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The Yeast Phospholipid *N*-Methyltransferases Catalyzing the Synthesis of Phosphatidylcholine Preferentially Convert Di-C16:1 Substrates Both *in Vivo* and *in Vitro**

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Phosphatidylcholine (PC) is an important and abundant structural component of the membranes of eukaryotic cells. In the yeast *Saccharomyces cerevisiae*, the primary route for the biosynthesis of PC consists of three consecutive methylation steps of phosphatidylethanolamine (PE) catalyzed by the phospholipid *N*-methyltransferases Cho2p and Opi3p. To investigate how these biosynthetic enzymes contribute to the composition of the PC species profile, the precursor-product relationships between PE and newly synthesized PC were determined at the level of the molecular species by using electrospray ionization tandem mass spectrometry and stable isotope labeling. *In vivo* labeling of yeast cells for 10 min with [*methyl*-D_3]methionine revealed the preferential methylation of di-C16:1 PE over a range of PE species compositions. A similar preferential conversion of di-C16:1 PE to PC was found *in vitro* upon incubating isolated microsomes with *S*-adenosyl[*methyl*-D_3]methionine. Yeast *opi3* and *cho2* deletion strains were used to distinguish between the substrate selectivities of Cho2p and Opi3p, respectively. Both biosynthetic enzymes were found to participate in the species-selective methylation with Cho2p contributing the most. The combined results indicate that the selective methylation of PE species by the methyltransferases plays an important role in shaping the steady-state profile of PC molecular species in yeast.

Phosphatidylcholine (PC)[1] is a major phospholipid constituent of eukaryotic membranes. Apart from being a structural component of the membrane barrier, PC is involved in other cellular functions, including having a role as a precursor of lipid second messengers (1). In the model eukaryote *Saccharomyces cerevisiae*, PC accounts for ~40–50% of the cellular phospholipid content (2) and is required for cell viability (3). Yeast strains with defects in PC production experience respiratory deficiency, implying an important role for PC in the biogenesis and/or proper functioning of mitochondria (4). In addition, the biosynthesis of PC is involved in the regulation of vesicular transport from the Golgi via the phospholipid transfer protein Sec14p (5, 6).

The *de novo* biosynthesis of PC in yeast proceeds mainly via three sequential methylation reactions of phosphatidylethanolamine (PE) (Fig. 1) (7). When exogenously supplied choline is present, net PC production also occurs via the CDP-choline route (8). The methylation of PE is mediated by the membrane resident phospholipid *N*-methyltransferases (PNMTs), Cho2p and Opi3p, which utilize *S*-adenosyl-L-methionine as a methyl donor. Cho2p converts PE to phosphatidylmonomethylethanolamine (PMME), and Opi3p catalyzes the second and the third methylation step. Both PNMTs have been predicted to be integral membrane proteins based on hydropathy plots (9) and have been localized to the ER membrane (10).

Like the other phospholipid classes in yeast, PC is composed of molecular species that mainly possess acyl chains with a carbon chain length of C_{16} or C_{18} (11). These fatty acids are either saturated or mono-unsaturated with the saturated fatty acids predominantly esterified at the *sn*-1 position of the glycerol backbone (12). Control of the acyl chain composition is essential for creating the appropriate membrane fluidity and is expected to play a role in intracellular PC transport and in more specific functions of PC. The biosynthesis of PC plays a major role in generating the molecular species profile of PC. The contributions of the methylation of PE and the CDP-choline route at the molecular species level were recently distinguished by labeling yeast cells with deuterated methionine and choline, respectively, and subsequent analysis of the pools of newly synthesized PC species by ESI-MS/MS (13). Whereas the CDP-choline pathway produces both the di- and mono-unsaturated PC species present in the steady state profile, the methylation route yields predominantly di-unsaturated PC species. Interestingly, the species profile of the newly synthesized PC derived from PE was found to differ from that of the PE precursor pool, with the 32:2 (di-C16:1) species enriched at the expense of the other species, which is indicative for preferential methylation of the 32:2 species (13).

The aim of the present study is to investigate how the selective methylation of PE species observed *in vivo* is accomplished. This issue was addressed by ESI-MS/MS analyses of the precursor-product relationship between PC and PE molecular species *in vivo*, in intact yeast cells that differed in species composition of the PE pool, and *in vitro* in isolated microsomes. In

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PNMT, phospholipid *N*-methyltransferase; MME, monomethylethanolamine; PMME, phosphatidylmonomethylethanolamine; ER, endoplasmic reticulum; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; DME, dimethylethanolamine; PDME, phosphatidyldimethylethanolamine.

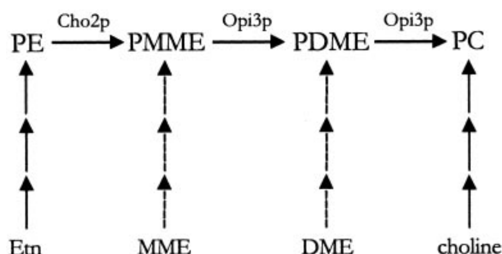

FIG. 1. **Biosynthesis of PC in yeast via the methylation of PE and the Kennedy pathways.** The enzymes and intermediates of the PE methylation route are indicated, as are the substrates of the Kennedy pathways that have been used in this study. *Etn*, ethanolamine.

addition, the contributions of the individual methyltransferases Cho2p and Opi3p in generating the PC molecular species profile have been addressed using *opi3* and *cho2* deletion strains, respectively. The results indicate that the substrate specificity of the methyltransferases, of Cho2p in particular, accounts for the species selectivity in the methylation reactions.

EXPERIMENTAL PROCEDURES

Strains—S. cerevisiae strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and the derived *cho2Δ* (*cho2::KanMX*) were obtained from Research Genetics (Carlsbad, CA). The *LEU2* marker (14) was introduced into the wild-type and the *cho2Δ* strain to delete the *OPI3* gene (nucleotides −11 to 621) to generate the *opi3Δ* (*opi3::LEU2*) and *cho2Δopi3Δ* strains (*cho2::KanMX opi3::LEU2*), respectively. The isogenic *pct1Δ* strain was obtained by replacing the *PCT1* gene with the *LEU2* marker (13). PCR was performed to verify the correct integration of the *LEU2* marker into the yeast genome.

Culture Conditions, Labeling Using (Deuterated) Precursors, and Extraction of Phospholipids—Yeast strains were cultured at 30 °C in 0.8 liters of semisynthetic lactate medium (15) supplemented with 20 mg/liter histidine, 60 mg/liter leucine, 230 mg/liter lysine, and 40 mg/liter uracil. The medium of the *cho2Δopi3Δ* strain was additionally supplemented with 1.0 mM choline. Pulse labeling experiments were performed as follows. Cells grown to midlog phase (A_{600} 0.8–1.2; Unicam Helios Epsilon spectrophotometer, MS Scientific, Berlin, Germany) were washed with and resuspended in 0.8 liter of synthetic lactate medium (0.67% yeast nitrogen base without amino acids (Difco), 2% lactate, 0.1% glucose, pH 5.5) in the presence of the supplements mentioned above. The wild-type, *pct1Δ*, and *opi3Δ* strains were labeled with 40 mg/liter [*methyl*-D_3]methionine (Cambridge Isotope Laboratories, Andover, MA) for 10 min. The wild-type strain was also cultured and pulse labeled in the above (semi)synthetic medium containing 2% glucose as a carbon source instead of lactate. The *cho2Δ* and *cho2Δopi3Δ* cells were labeled with 4.0 mM monomethylethanolamine (MME) (Fluka, Buch, Switzerland) or 4.0 mM dimethylethanolamine (DME) (Fluka) for 30 min in the presence or absence of 40 mg/liter [*methyl*-D_3]methionine as indicated. Both strains were also labeled with 2.0 mM [D_4]ethanolamine (Cambridge Isotope Laboratories) for 30 min. The labeling was ended by adding a cold mixture of KCN, NaF, and NaN_3 to final concentrations of 15 mM each to arrest cellular processes, and the cells were stored on ice. Cell pellets corresponding to ~100 A_{600} units were resuspended in 1 ml of water and homogenized by vortexing with glass beads three times for 1 min with intermittent cooling on ice. Phospholipids were extracted as described (16).

Phospholipid Methylation in Vitro—Microsomes were isolated as described (17). Briefly, midlog cells grown in semisynthetic lactate medium were harvested, and spheroplasts were prepared using zymolyase (Seikagaku, Tokyo, Japan). The spheroplasts were homogenized, and microsomes were isolated as the 32,500 × *g* pellet of the 20,200 × *g* postmitochondrial supernatant. Methylation of PE was initiated by adding microsomes at a protein concentration of 0.25 μg/μl to 50 mM Tris-HCl, pH 8.0, and 0.5 mM *S*[*methyl*-D_3]adenosyl-L-methionine (CDN Isotopes, Pointe-Claire, Canada) in a final volume of 300 μl (18). After 10 min of incubation at 30 °C, the methylation was ended by adding 1.4 ml of a mixture of chloroform, methanol, and 0.5 mM HCl (6:12:1, v/v/v), which was followed by phospholipid extraction as described above.

Mass Spectrometry—ESI-MS/MS analysis of lipid extracts was performed using a Quattro Ultima triple quadrupole MS instrument (Micromass, Manchester, UK) in the positive ion mode. Lipid extracts were dissolved at 0.5 mM phospholipid phosphorus in $CHCl_3$, CH_3OH, H_2O (2:15:3, v/v/v), with 1% (v/v) formic acid added to reduce the content of $[M + Na]^+$ adducts. The samples were introduced into the MS instrument by an electrospray source with a flow rate of ~50 nL/min. The capillary and cone voltages were set at 1.5 kV and 30 V, respectively, and collision-activated dissociation was applied using argon and collision energies of 30 eV. Data are presented as averages of 50–150 repetitive scans of 10 s obtained from at least two independent experiments. Identification of phospholipid species was carried out as described (13). The $[M + H]^+$ ions of the PC species and the $[M + H]^+$ and $[M + Na]^+$ adducts of PE, PMME, and PDME were monitored as indicated in Table I. The intensity of the mono-isotope signal of each mono-unsaturated lipid species was corrected for the coinciding second isotope peak of the di-unsaturated lipid species with an *m/z* value of 2 units lower. For quantification, ESI-MS/MS data were corrected for the inverse relationship between mass and signal response of the MS instrument, which was calibrated as described previously (13, 19). This correction was not applied to spectra of the labeled phospholipids because these lipids were introduced into the instrument at low concentrations (<0.005 mM). In that concentration range the instrument response was found to be virtually independent of the acyl chain length in the relevant *m/z* range (*cf.* Refs. 13 and 20).

Other Methods—Protein concentrations were determined using the BCA method (Pierce) in the presence of 0.1% (w/v) sodium dodecyl sulfate with bovine serum albumin used as standard. Phospholipid contents were quantified according to the method of Fiske and Subbarow (21).

TABLE I
Overview of ESI-MS/MS scan modes applied for the analysis of phospholipid molecular species

Phospholipid	MS scan mode
Unlabeled PC	Parents of *m/z* 184
[D_3]PC	Parents of *m/z* 187
[D_6]PC	Parents of *m/z* 190
[D_9]PC	Parents of *m/z* 193
Unlabeled PE	Neutral loss of *m/z* 141
[D_4]PE	Neutral loss of *m/z* 145
Unlabeled PMME	Neutral loss of *m/z* 155
[D_3]PMME	Neutral loss of *m/z* 158
Unlabeled PDME	Neutral loss of *m/z* 169

RESULTS

In Vivo, 32:2 PE Is Preferentially Methylated to PC Independent of the Species Profile of the PE Substrate—To determine the substrate specificity of the PE methylation pathway *in vivo*, the molecular species composition of the PE precursor pool was varied. The profile of phospholipid acyl chains in yeast can be easily manipulated, *e.g.* by growing the cells on different carbon sources (22) or by using phospholipid biosynthetic mutants. Fig. 2*A* shows an ESI-MS/MS spectrum of total cellular PE obtained by neutral loss scanning for *m/z* 141 in wild-type BY4742 cells grown on glucose. The most abundant PE species were 32:2, 32:1, 34:2, and 34:1, which predominantly consist of combinations of the acyl chains C16:0, C16:1, C18:0, and, C18:1 (12). When wild-type cells were grown on lactate instead of glucose, the PE species profile drastically changed with strong reductions in the contents of the mono-unsaturated species 32:1 and 34:1 that were compensated for by an increase in 34:2 (Fig. 2*B*). Inactivation of the CDP-choline route by deletion of the *PCT1* gene (23) resulted in an increase of 32:2 PE at the expense of 34:2 PE compared with the wild-type (Fig. 2*B*).

To investigate how the different species profiles of PE affect the species composition of PC newly synthesized by the methylation of PE, midlog wild-type and *pct1Δ* cells were labeled for 10 min with [*methyl*-D_3]methionine. Subsequently, total lipid extracts were prepared and analyzed by ESI-MS/MS using parent ion scanning for *m/z* 193 corresponding to the phosphocholine head group with three deuterated methyl groups. The parent ion scan of [D_9]PC from wild-type cells grown on glucose (Fig. 3*A*) revealed a species composition that is enriched in the

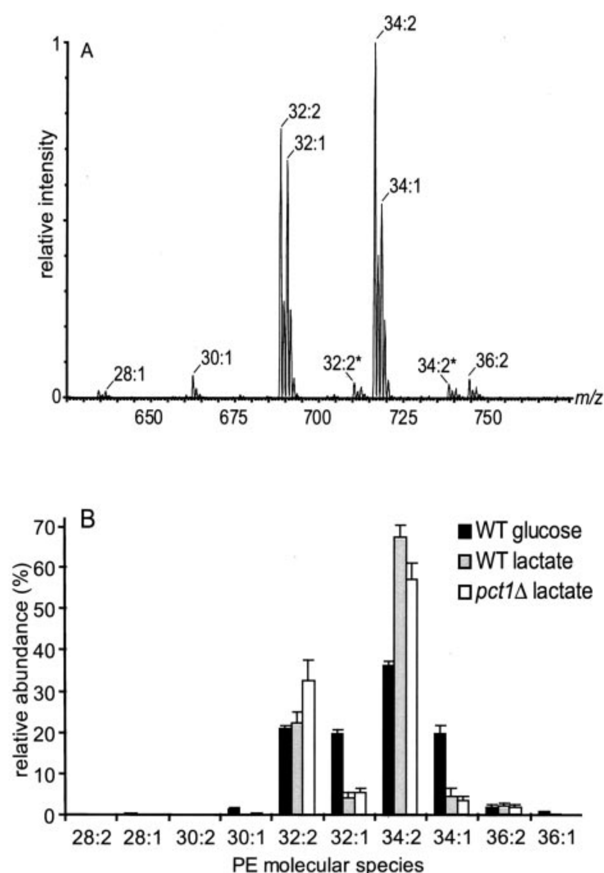

FIG. 2. **The molecular species composition of PE depends on the carbon source and varies between wild-type and *pct1*Δ cells.** *A*, ESI-MS/MS spectrum of the PE molecular species from wild-type yeast grown on semisynthetic glucose medium to the midlog phase. The PE molecular species were detected by neutral loss scanning for the PE head group at *m/z* 141. Both $[M + H]^+$ and $[M + Na]^+$ adducts were detected, and the latter are marked with an *asterisk*. The unmarked peaks represent the first isotopic signals corresponding to the preceding peaks with a mass difference of 1 Da. *B*, quantification of the relative abundance of the PE molecular species of wild-type cells grown on semisynthetic glucose (*black bars*) and semisynthetic lactate medium (*gray bars*) and of *pct1*Δ cells grown on semisynthetic lactate medium (*white bars*). *Error bars* reflect the standard deviations (n = 3).

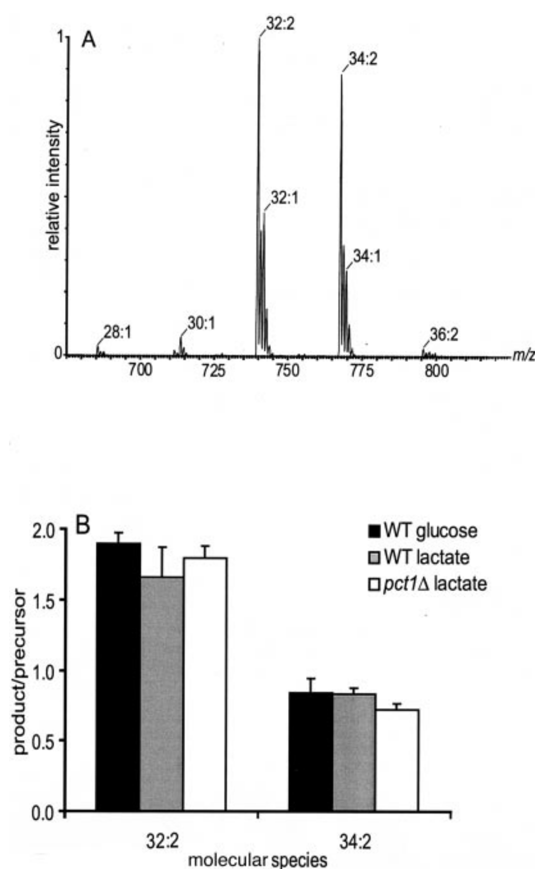

FIG. 3. **The methyltransferases preferentially convert 32:2 PE to PC to an extent that is independent of the species composition of the substrate PE.** *A*, ESI-MS/MS spectrum of the newly synthesized $[D_9]$PC molecular species in wild-type cells cultured in semisynthetic glucose medium obtained by parent ion scanning for *m/z* 193. *B*, quantification of the relative extents of conversion of the two most abundant PE species to PC for wild-type cells grown on semisynthetic glucose (*black bars*) and lactate medium (*gray bars*) and for *pct1*Δ cells grown on lactate medium (*white bars*). The product/precursor ratios were calculated by dividing the proportions of 32:2 and 34:2 PC in the $[D_9]$PC profile by the proportions of the corresponding PE species in the cell homogenate (see Fig. 2). The species compositions of PE (t = 0) and newly synthesized PC (t = 10 min) were determined as described under "Experimental Procedures." Data represent mean values from three experiments (± S.D.).

32:2 species compared with the profile of the precursor PE (Fig. 2*A*). The relative extents of conversion of the PE species to PC were quantified by dividing the proportions of the most prominent $[D_9]$PC species by those of their corresponding PE species and have been summarized for 32:2 and 34:2 in Fig. 3*B*. Irrespective of the species composition of the PE precursor pool, the 32:2 species was prefentially methylated to PC to similar extents (Fig. 3*B*), demonstrating species-selective methylation by the PNMTs *in vivo*. The product/precursor ratios of the monounsaturated species 32:1 and 34:1 did not yield statistically reliable numbers because of the relatively small amounts of these species in the PE precursor pool of the lactate-grown cells (Fig. 2*B*). The ratios in wild-type cells grown on glucose amounted to 0.8 (±0.1) and 0.4 (±0.1) (mean ± variation, n = 2) for 32:1 and 34:1, respectively.

Methylation of PE in Vitro Also Leads to an Enrichment of 32:2 PC—To test whether the preferential methylation of 32:2 PE is an intrinsic property of the enzymes in the ER, similar experiments were carried out using microsomes. The PE species profile of microsomes isolated from wild-type cells grown on lactate was almost similar to that of the corresponding cell homogenate (compare the *gray bars* in Fig. 2*B*) with mainly 32:2 (26.9 ± 2.4%), 32:1 (5.3 ± 1.1%), 34:2 (57.5 ± 2.8%), and 34:1 (7.9 ± 1.6%) (mean ± S.D., n = 9). Incubation of microsomes with 0.5 mM *S*[*methyl*-D_3]adenosyl-L-methionine for 10 min at 30 °C revealed that the relative extents of conversion of the microsomal PE species to $[D_9]$PC were similar to those found *in vivo* (Fig. 4). This was even more pronounced when the relative extents of conversion were calculated by relating the species profile of newly synthesized PC *in vivo* to the microsomal PE species rather than to the total cellular PE species (not shown). Hence, the preferential methylation of 32:2 PE observed *in vivo* is retained in isolated microsomes.

*Substrate Preference of Cho2p in opi3*Δ *Cells*—To get insight into the stage at which the non-random methylation of PE species occurs, we studied the contributions of the three methylation steps individually. For this purpose, a strategy was set up using yeast deletion strains lacking either Cho2p, Opi3p, or both (Fig. 1). To establish the product/precursor ratios for Cho2p, the *opi3*Δ strain was pulse labeled; the strain is defective in performing the second and third methylation reactions and accumulates PMME (24). Fig. 5*A* shows the neutral loss scan for the D_3-labeled head group PMME at *m/z* 158, which revealed the formation of predominantly 32:2 and 34:2 PMME. Comparison of the profile of newly synthesized $[D_3]$PMME to

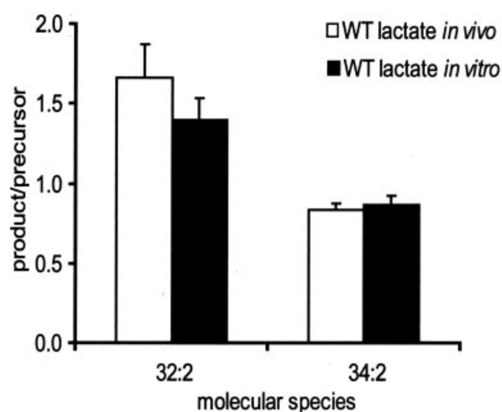

FIG. 4. **The preference of the phospholipid *N*-methyltransferases for converting 32:2 PE is similar *in vivo* and *in vitro*.** The relative degrees of methylation of 32:2 and 34:2 PE were determined *in vitro* as explained in the legend to Fig. 3. Microsomes isolated from the wild-type strain grown on lactate medium were pulse labeled with *S*[*methyl*-D_3]adenosyl-L-methionine as described under "Experimental Procedures." The product/precursor ratios *in vitro* (*black bars*) are compared with the *in vivo* conditions (*white bars*; data taken from Fig. 3*B*). *Error bars* represent the S.D. ($n \geq 3$).

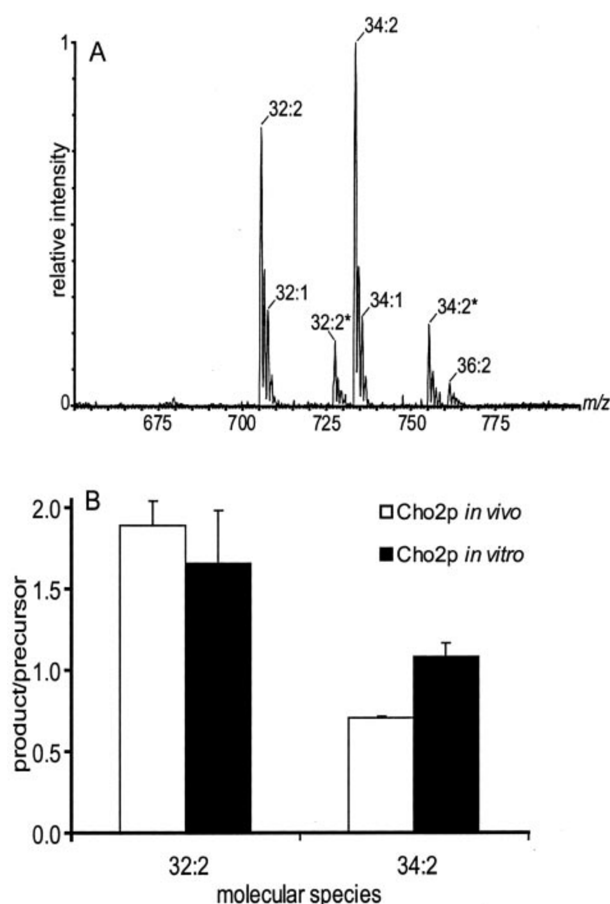

FIG. 5. **The Cho2p-mediated methylation of PE to PMME exhibits similar preferences for 32:2 PE both *in vivo* and *in vitro*.** *A*, ESI-MS/MS spectrum of newly synthesized PMME in the *opi3*Δ strain. Cells grown on lactate medium were pulse labeled with 40 mg/liter [*methyl*-D_3]methionine for 10 min. The newly synthesized PMME species were analyzed by neutral loss scanning for the [*methyl*-D_3]monomethylethanolamine moiety (*m/z* 158). Both $[M + H]^+$ and *asterisk*-marked $[M + Na]^+$ species are indicated. *B*, comparison of the relative extents of conversion of the two most prominent PE species to PMME *in vivo* (*white bars*) and *in vitro* (*black bars*). Microsomes derived from the *opi3*Δ cells grown on lactate medium were pulse labeled with *S*-[*methyl*-D_3]adenosyl-L-methionine as described under "Experimental Procedures." *Error bars* reflect the variation ($n = 2$).

that of its precursor PE in terms of product/precursor ratios showed an enrichment of 32:2 species (Fig. 5*B*, *white bars*). Similar results were obtained *in vitro* using microsomes isolated from the *opi3*Δ cells (Fig. 5*B*, *black bars*). Together, these data demonstrate that the first methylation by Cho2p already leads to an enrichment of the di-unsaturated 32:2 species.

The Species Composition of the Substrates of Opi3p in cho2Δ Cells—To examine whether Opi3p also contributes to the species-selective methylation of PE to PC, the *cho2*Δ strain was used, which allows studying the methylation of phospholipids by Opi3p exclusively. Pulsing *cho2*Δ cells with [*methyl*-D_3]methionine for up to 30 min did not yield a detectable [D_9]-PC pool (data not shown) in agreement with the limited ability of Opi3p to methylate PE (3, 18). Therefore, the *cho2*Δ cells were pulsed in the presence of MME to supply the cells with the Opi3p substrate PMME via the Kennedy pathway (Fig. 1). However, under these conditions PMME was rapidly chased into PC, preventing the analysis of the pool of PMME species available for Opi3p.

To solve this problem, the PMME species profile was determined in a *cho2*Δ*opi3*Δ double deletion strain pulsed for 30 min with MME in which PMME was not further methylated. As shown in Fig. 6, the species profile of PMME in *cho2*Δ*opi3*Δ strongly resembled that of PE newly synthesized via the CDP-ethanolamine route, which was determined after pulsing the cells with [D_4]ethanolamine. In turn, the]D_4]PE profiles were very similar between the *cho2*Δ and *cho2*Δ*opi3*Δ strains (Fig. 6). These results indicate that similar pools of diacylglycerol species are used for the synthesis via the Kennedy pathway of PMME and PE and that deletion of the second methyltransferase does not affect the species composition of this diacylglycerol pool. Based on this, the PMME species profile determined in the *cho2*Δ*opi3*Δ cells was taken as a reliable reflection of the PMME precursor pool for Opi3p in *cho2*Δ cells. Similarly, the species profile of the PDME precursor pool in the *cho2*Δ cells was established and was found to resemble that of CDP-choline derived PC (25).

Opi3p Substrate Specificity in cho2Δ Cells—To investigate the contribution of the second and third methylation steps to the species-selective methylation of PE, *cho2*Δ cells were pulsed for 30 min with [*methyl*-D_3]methionine in the presence of either 4.0 mM MME or 4.0 mM DME. Newly synthesized PC species derived from PMME and PDME were detected by parent ion scanning for *m/z* 190 and 187, respectively. For both MME-derived [D_6]PC and DME-derived [D_3]PC, the relative extent of conversion to PC was slightly larger for the 32:2 precursor than for the 34:2 precursor (Fig. 7). The data demonstrate that the species-selective methylation of PE *in vivo* is accomplished by both PNMTs, although to a lesser extent by Opi3p than by Cho2p.

DISCUSSION

In the absence of exogenous choline, the net synthesis of the abundant membrane phospholipid PC in yeast proceeds via the methylation of PE (7). Because under most conditions the rates of PC turnover and acyl chain remodeling are low relative to the generation time of yeast (13, 26, 27), the methylation route is considered a major determinant of the steady state PC molecular species profile and consequently of membrane fluidity in wild-type yeast. Pulse labeling yeast with phospholipid precursors containing stable isotopes (and subsequent analysis of the newly synthesized phospholipids by ESI-MS/MS) allows the substrate use of phospholipid biosynthetic enzymes to be examined *in vivo* in intact cells. Using this approach, we showed previously that the PE molecular species are not randomly methylated to PC in live yeast by pulse labeling with

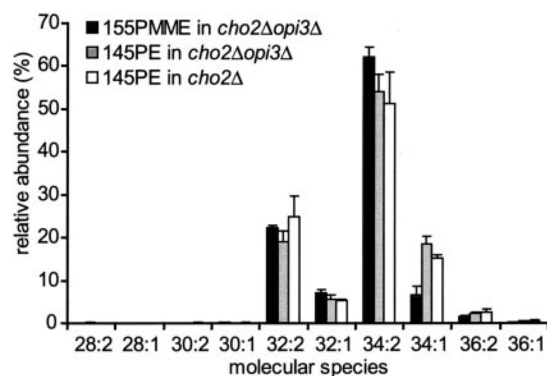

FIG. 6. **Determination of the species composition of the PMME substrate for Opi3p.** As a measure for the PMME species composition in *cho2*Δ, the PMME species profile of the strain *cho2*Δ*opi3*Δ was determined by pulsing the cells with 4.0 mM MME for 30 min (*black bars*) and subsequent ESI-MS/MS analysis by neutral loss scanning (*m/z* 155). The utilization of similar diacylglycerol species in the CDP-ethanolamine route in *cho2*Δ*opi3*Δ (*gray bars*) and *cho2*Δ (*white bars*) cells was verified by pulse labeling both strains with 2.0 mM [D_4]ethanolamine for 30 min. [D_4]PE species were detected by neutral loss scanning for *m/z* 145. *Error bars* indicate the variation ($n = 2$).

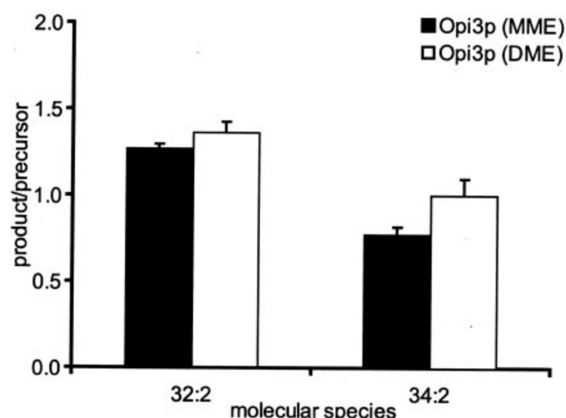

FIG. 7. **Opi3p slightly prefers 32:2 substrates in both the second and the third methylation step of PE.** The relative extents of conversion of the two most prominent PMME (*black bars*) and PDME species (*white bars*) to PC were determined by pulse labeling *cho2*Δ for 30 min with 40 mg/liter [*methyl*-D_3]methionine in the presence of either 4.0 mM MME or 4.0 mM DME. Newly formed PC species derived from PMME and PDME were detected by parent ion scanning at *m/z* 190 and 187, respectively. ($n = 2$, ± variation).

[*methyl*-D_3]methionine (13). Instead there is a preference to convert the 32:2 species of PE into PC. The extent of conversion of 32:2 PE to PC was found to exceed by 50% the extent expected based on random methylation of the available PE species (13). The present study addresses the mechanism underlying the selectivity in the methylation of different PE species. It is shown that the extent of the preferential conversion of 32:2 PE to PC is independent of the species composition of the PE precursor pool and is similar in intact cells and in isolated microsomes.

What is the origin of the preferential methylation of 32:2 PE? The finding that the species selectivity in methylation is retained in isolated microsomes supplemented with *S*-adenosyl-L-methionine implies that it is intrinsic to the methyltransferases in the ER and does not depend on the entire sequence of cellular processes required for the synthesis of PC, including the trafficking of PE from its site(s) of synthesis to the ER resident enzymes. The species preference in methylation appears to be very robust, as it is maintained under a range of conditions. The species profile of PE was varied by deleting the *PCT1* gene and by growing yeast on the fermentable and non-fermentable carbon sources glucose and lactate, respectively (22). The nature of the carbon source is expected to also affect the supply of PE to the ER, as the volume of the mitochondrial network (which harbors the enzyme phosphatidylserine decarboxylase 1 (Psd1p), the main source of PE production in the cell (28, 29)) is strongly reduced in yeast cells grown on glucose (30). The extents of conversion of the available PE species to PC were found to be similar under the conditions tested, demonstrating that the preferential methylation is not affected by varying the PE species composition or by modulating the supply of PE to the ER. It should be noted that the present results do not rule out the possibility that newly synthesized PE originating from the mitochondrial Psd1p is channeled to the methyltransferases in the ER to be preferentially methylated over the existing pool of PE. However, we have no indications that such a lipid flux would contribute to the species selectivity in the methylation of PE.

The preferential methylation of 32:2 PE in ER membranes can be explained by the intrinsic substrate preferences of the methyltransferases. Alternatively, the species-selective methylation in the ER could reflect differences in accessibility of the PE molecular species to the PNMTs originating from lateral heterogeneity of the PE species in the ER membrane. In the latter scenario, the pool of PE species in the putative microdomains surrounding the PNMTs would be in equilibrium with the bulk of PE in the ER. To distinguish between these possibilities, the substrate specificities of the purified methyltransferases should be analyzed in assays using mixtures of defined PE species. However, so far attempts to purify the methyltransferases have failed because of the loss of enzymatic activity of both enzymes in the presence of detergents (31).

To distinguish between the contributions of the two methyltransferases to the species selective methylation, *cho2* and *opi3* deletion strains were pulsed with [*methyl*-D_3]methionine in the absence and presence of MME/DME, respectively. These experiments revealed that the methylation catalyzed by Cho2p contributes most of the species selectivity. The limited substrate preference of Opi3p is consistent with PMME and PDME being short-lived intermediates that do not accumulate in the presence of both PNMTs (2, 32).

In contrast to yeast, mammals possess only a single gene encoding phospholipid methyltransferase activity (reviewed in Ref. 32). To date, it remains unclear whether the two isoforms of the enzyme in rat liver, PEMT1 and PEMT2, which localize to the ER and the mitochondria associate membranes, respectively, result from post-translational modification or differential gene splicing and/or mRNA editing (34). *In vitro* experiments using either crude microsomes or purified PEMT2 revealed that the enzyme from rat liver does not possess specificity toward particular molecular species of PE, PMME, or PDME, whereas experiments using intact hepatocytes revealed a slight preference for polyunsaturated 16:0-22:6 PE (35). In mammals the methylation of PE is secondary to the CDP-choline pathway and contributes significantly to PC production only in the liver (36), explaining the lack of a need for species selective methylation. Mammals have evolved other mechanisms for maintaining PC species homeostasis, including acyl chain remodeling (see *e.g.* Refs. 37 and 38). Interestingly, *in silico* analysis showed that Opi3p (23 kDa) with its limited species selectivity is very similar to PEMT2 (23 kDa) with 44% identical residues and 68% of sequence similarity (34, 39). As the *CHO2* gene is likely to derive from an ancestor of the *OPI3* gene by gene duplication (9), we speculate that the species selective methylation in yeast evolved after this event.

The preferential methylation of 32:2 PE by the PNMTs, Cho2p in particular, provides the yeast cell with a tool to

maintain the appropriate balance between the species distributions of PC and PE. The synthesis of the shorter and more hydrophilic di-C16:1 PC species might be favored as they are more readily transported from the site of synthesis to other cellular membranes (*cf.* Ref. 40). Other processes contributing to the steady state PC species distribution comprise synthesis of PC via the CDP-choline pathway and remodeling of PC by acyl chain exchange. Also in the absence of choline the CDP-choline route contributes to PC biosynthesis by recycling choline from PC turnover (41). This recycling of choline may serve to adjust the PC species composition because PC synthesized by the CDP-choline route is enriched in the monounsaturated species 32:1 and 34:1 compared with PE-derived PC (13). The occurrence of PC remodeling by acyl chain exchange (as recently demonstrated in a yeast mutant lacking an active CDP-choline route (13)) may further modify the species profile. Efforts are now directed toward identifying the genes involved in PC remodeling and turnover to determine the relative importance of the two biosynthetic pathways and remodeling in establishing the steady state PC species distribution in wild-type yeast.

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