Biochemical and Biophysical Research Communications 445 (2014) 289–293

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Acyl-chain remodeling of dioctanoyl-phosphatidylcholine in *Saccharomyces cerevisiae* mutant defective in *de novo* and salvage phosphatidylcholine synthesis

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ARTICLE INFO

Article history:
Received 23 January 2014
Available online 31 January 2014

Keywords:
Saccharomyces cerevisiae
Phosphatidylcholine
Remodeling
Acyl chain

ABSTRACT

A yeast strain, in which endogenous phosphatidylcholine (PC) synthesis is controllable, was constructed by the replacement of the promoter of *PCT1*, encoding CTP:phosphocholine cytidylyltransferase, with *GAL1* promoter in a double deletion mutant of *PEM1* and *PEM2*, encoding phosphatidylethanolamine methyltransferase and phospholipid methyltransferase, respectively. This mutant did not grow in the glucose-containing medium, but the addition of dioctanoyl-phosphatidylcholine (diC8PC) supported its growth. Analyses of the metabolism of ^{13}C-labeled diC8PC ((*methyl*-^{13}C)$_3$-diC8PC) in this strain using electrospray ionization tandem mass spectrometry revealed that it was converted to PC species containing acyl residues of 16 or 18 carbons at both *sn*-1 and *sn*-2 positions. In addition, both acyl residues of (*methyl*-^{13}C)$_3$-diC8PC were replaced with 16:1 acyl chains in the *in vitro* reaction using the yeast cell extract in the presence of palmitoleoyl-CoA. These results indicate that PC containing short acyl residues was remodeled to those with acyl chains of physiological length in yeast.

1. Introduction

Biological membranes are composed of a wide variety of phospholipids diverse in their hydrophilic head moieties and hydrophobic acyl residues. The remodeling of acyl residues in phospholipids is considered to contribute to generation and maintenance of the diversity of phospholipid species. Improper or oxidized acyl residues in phospholipid molecules are supposed to be exchanged with appropriate fatty acids by the remodeling reaction. In higher eukaryotes, polyunsaturated fatty acids are introduced into phospholipid molecules by the remodeling reaction, but not in their *de novo* synthesis steps. Despite the importance of the remodeling of phospholipid acyl residues in the structures and functions of biological membranes, the molecular mechanisms of it remain poorly understood [1–7].

In *Saccharomyces cerevisiae*, phosphatidylcholine (PC), the most abundant bilayer-forming phospholipid, is synthesized through two pathways [8,9]. In the *de novo* pathway (CDP-diacylglycerol pathway), phosphatidylserine (PS) is synthesized by PS synthase Pss1/Cho1. PS is decarboxylated to phosphatidylethanolamine (PE) by PS decarboxylase, Psd1 or Psd2. PE is then converted to PC by the sequential methylation catalyzed by two methyltransferases, Pem1/Cho2 and Pem2/Opi3. In the salvage pathway (Kennedy pathway), PC is synthesized from diacylglycerol and choline through three reactions catalyzed by choline kinase Cki1, CTP:phosphocholine cytidylyltransferase Pct1, and cholinephosphotransferase Cpt1. PC is an essential phospholipid for yeast, and mutants defective in the PC synthesis through the CDP-DAG pathway require PC supply from the Kennedy pathway for growth.

In yeast, palmitoleic acid (C16:1) and oleic acid (C18:1) are major fatty acid moieties in PC, and palmitic acid (C16:0) and stearic acid (C18:0) are minor ones [10]. It has been shown that acyl residues in PC are remodeled in yeast [11,12], but the enzymes involved in the remodeling of acyl residues in PC remain elusive. In yeast, Ale1, a lysophospholipid acyltransferase of membrane-bound *O*-acyltransferase (MBOAT) family introduces acyl residues at *sn*-2 positions of various 1-acyl-lysophospholipids, including

Abbreviations: ESI-MS/MS, electrospray ionization tandem mass spectrometry; diC8PC, dioctanoyl-phosphatidylcholine; MBOAT, membrane-bound *O*-acyltransferase; (*methyl*-^{13}C)$_3$-diC8PC, *methyl*-^{13}C-labeled diC8PC; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PLC, phospholipase C; PLD, phospholipase D.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.136
0006-291X/

lysophosphatidylcholine [13–17]. Slc1, a 1-acylglycerol-3-phosphate *O*-acyltransferase, has acyltransferase activities at *sn*-2 positions of various lysophospholipids [14]. It has also been shown that Tgl3, Tgl4, and Tgl5 exhibited acyltransferase activities to certain species of lysophospholipids [18–20]. In contrast, the mechanisms to introduce acyl residues at *sn*-1 position of PC remain unknown. In addition, the phospholipase that cleaves acyl residues of phospholipids in the process of remodeling has been unclear in yeast.

This obscureness of the phospholipid remodeling is in part due to the difficulty in the evaluation of the remodeling of each phospholipid molecule *in vivo* and *in vitro*. We have established a system, in which exogenous phospholipids containing short acyl residues are incorporated into yeast cells and the remodeling of them is evaluated by observation of the growth of mutants and by analysis of the metabolism of incorporated phospholipids by mass spectrometry [12,21–23]. We have shown that *pss1*Δ mutant or *pem1*Δ*pem2*Δ mutant is able to grow in the absence of choline when diC8PC, a PC containing octanoic acids, is supplemented [12,21]. Analysis of the metabolism of ^{13}C-labeled diC8PC ((*methyl*-^{13}C)$_3$-diC8PC) in *pem1*Δ*pem2*Δ mutant by electrospray ionization tandem mass spectrometry (ESI-MS/MS) revealed that it was rapidly converted to (*methyl*-^{13}C)$_3$-PCs containing two acyl residues of 16 or 18 carbons via intermediates, in which either acyl residue at *sn*-1 or *sn*-2 position was replaced [12]. These results suggested that exogenously added diC8PC is remodeled to PC species containing physiological acyl residues and enters the cellular PC pool. However, there remained a possibility that a portion of diC8PC is degraded by phospholipase D (PLD) or phospholipase C (PLC) and generated choline or phosphocholine is used for PC synthesis through the Kennedy pathway in these mutants, and that this could interfere with accurate evaluation of acyl chain remodeling of diC8PC. In this study, we constructed a strain, in which PC synthesis through the Kennedy pathway can be repressed in the *pem1*Δ*pem2*Δ strain, and established a system to analyze the remodeling of acyl residues of diC8PC using this strain *in vivo* and *in vitro*. This system enables a quantitative and reliable evaluation of the remodeling of diC8PC in yeast.

2. Materials and methods

2.1. Strains, media and materials

S. cerevisiae strain KSY02 (*MAT*a *his3 leu2 trp1 lys2 ura3 pem1*::*HIS3 pem2*::*LEU2* P_{GAL1}-*PCT1*::*NAT*^{r}) used throughout this study was constructed from strain KEY502 (*MAT*a *his3 leu2 trp1 lys2 ura3 pem1*::*HIS3 pem2*::*LEU2*) [24]. To replace the *PCT1* promoter in KEY502, a DNA fragment containing 5′-non-coding region of *PCT1*, clonNAT resistant gene, *GAL1* promoter, *PCT1* ORF, and 3′-non-coding region of *PCT1* was constructed as follows. DNA fragment containing *GAL1* promoter was excised from pYPR3831X [25] with BamHI and HindIII, and inserted into pAG25 [26], which was digested with the same restriction enzymes, to obtain pAG25-*PGAL1*. DNA fragment containing *PCT1* ORF with restriction sites of XbaI at both ends was amplified with the primers XbaPCT-F (5′-TGCTCTAGAGCATGGCAAACCCAACAACAGG-3′) and XbaPCT-R (5′-TGCTCTAGAGCATCAGTTCGCTGATTGTTTCT-3′) using yeast total DNA as a template. The amplified fragment was digested by XbaI and cloned into pAG25-*PGAL1*, which was cut with the same enzyme, to obtain pAG25-*PGAL1-PCT1*. DNA fragment containing 78-bp 5′ flanking region of *PCT1*, clonNAT resistant gene, *GAL1* promoter, *PCT1* ORF and 80-bp 3′ flanking region was amplified with primers CCT-delta-f (5′-TATTGTATTGTTTAACTTTCAAATTTCACT CTTGCTATTCTCTCTTGGTAGTACAAAGCACAGCAGACACAAAGAAAA GCATAGGCCACTAGTGGATCTG-3′) and CCT-delta-r (5′-CGCTTGTT TATACGTGGAGGCGACAAAAAGGGTTGGTTGAAGGGAGAGAGAAAG AGACGCACAGAGATTGGAGGAGGGGCGAACGCGGCCGCCAGCTGAA-3′) using pAG25-*PGAL1-PCT1* as a template, and introduced into KEY502. Replacement of *PCT1* promoter was confirmed by PCR and Southern blot analysis (data not shown).

Yeast strains were grown in YPG or SG medium, which contains 2% galactose as a carbon source, or SD medium with appropriate supplements as described previously [12].

Phospholipids were purchased from Avanti Polar Lipids Inc., and stored in −20 °C until use. diC8PC was added to the medium as described previously [12].

2.2. Measurement of growth

Cells from 2 ml overnight culture in YPG medium were collected by brief centrifugation, washed three times with 1 ml of SD medium, and suspended in 2 ml SD medium, SG medium with 1 mM choline, or SD medium with 0.1 mM diC8PC at a starting OD_{600} = 0.05. After 24 h preculture at 30 °C to reduce Pct1, choline, and/or PC, cells were collected with brief centrifugation, washed three times with 1 ml SD medium, and suspended in 5 ml of the same medium at a starting OD_{600} = 0.05. Cells were incubated at 30 °C for 48 h and growth curve was obtained with an automatically recording incubator TN1506 (Advantec).

2.3. Quantitation of phospholipids

Cells from overnight culture in YPG medium were seeded to SD medium with diC8PC or SG medium with choline, and cultivated at 30 °C for 24 h to reduce Pct1, choline, and/or PC, as described above. Cells were then seeded to the same medium and cultivated at 30 °C for 10 h to a final $OD_{600} \approx 1$. Cells were harvested and broken with glass beads in chloroform/methanol/water (2:4:1). Total lipids were extracted from organic layer by the method of Bligh and Dyer [27]. Separation and quantitation of phospholipids were carried out as described previously [12,28].

2.4. Analysis of (*methyl*-^{13}C)$_3$-diC8PC remodeling in vivo

(*Methyl*-^{13}C)$_3$-diC8PC and (*methyl*-^{13}C)$_3$-diC10PC were synthesized by methylation of diC8PE and diC10PE (Avanti), respectively, with *methyl*-^{13}C-iodine (Cambridge Isotope Laboratories, Inc.) as described previously [12].

For analysis of incorporation and remodeling of (*methyl*-^{13}C)$_3$-diC8PC, KSY02 cells from overnight culture in YPG medium were suspended in SD medium with non-labeled diC8PC at a starting OD_{600} = 0.05, and precultured for 10 h at 30 °C. Cells were collected, washed twice with SD medium, and labeled in SD medium containing 30 μM (*methyl*-^{13}C)$_3$-diC8PC for 30 min. Cells were washed three times with 0.15 M KCl, and suspended in chloroform/methanol/water (2:4:1). After adding (*methyl*-^{13}C)$_3$-diC10PC as an internal standard at a concentration of 8 ng/OD_{600}, cells were broken with glass beads. Lipids were extracted as described above, and analyzed by ESI-MS/MS. MS/MS spectra were obtained on API3000 triple quadrupole instrument (Applied Biosystem), as described previously [12].

2.5. In vitro remodeling reaction

Cell from overnight culture in YPG medium were seeded to SD medium with diC8PC and cultivated at 30 °C for 10 h to a final $OD_{600} \approx 1$. Cells were collected, washed twice with PBS, and disrupted in breaking buffer [25 mM Hepes-KOH (pH 7.4), 0.1 M KCl, 10% glycerol, 1 mM DTT, and 1% protease inhibitor cocktail (Sigma)] with glass beads. Lysates were centrifuged at 1000*g* for 10 min at 4 °C, and supernatants were used as crude cell extracts.

The protein concentrations of the crude extracts were measured using Bradford reagent (Bio-Rad).

The reaction mixture for *in vitro* remodeling reaction contained 25 mM Hepes-KOH (pH 7.4), 1 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, 1 mM ATP, 175 μg/ml creatine kinase, 17.5 mM creatine phosphate, 0.4 mM palmitoleoyl-CoA, 20 μM (*methyl*-$^{13}C)_3$-diC8PC, 1 mM DTT, and cell extract (0.5 mg protein) in a total volume of 100 μl. The reaction was carried out at 30 °C for 30 min and stopped by the addition of 6 volumes of methanol/chloroform (2:1) and vigorous mixing. Lipids were extracted by Bligh–Dyer method [27] after adding 8 ng (*methyl*-$^{13}C)_3$-diC10PC per sample as an internal standard and analyzed by ESI-MS/MS.

3. Results

3.1. Construction and characterization of a mutant strain in which PC synthesis is controllable

We constructed a yeast strain, KSY02, in which both *PEM1* and *PEM2* were deleted and the promoter of *PCT1* encoding CTP:phosphocholine cytidylyltransferase, a key enzyme in PC synthesis through the Kennedy pathway, was replaced with *GAL1* promoter, in its chromosomal location. The KSY02 strain grew in the minimal medium containing galactose as a carbon source (SG medium) in the presence of choline (Fig. 1), but it arrested growth after several cell divisions when the cells was seeded from the SG medium with choline to the minimal medium containing glucose (SD medium) (data not shown). This time lag before the growth arrest is probably due to residual Pct1, choline, and/or PC. When the KSY02 strain was seeded in the SD medium after preculture in the same medium for 24 h, no growth was observed (Fig. 1, open squares), suggesting that PC synthesis in this mutant is repressed in the medium containing glucose. In contrast, when the KSY02 strain was cultured in the SD medium containing diC8PC after 24-h preculture in the same medium, cell growth was observed (Fig. 1, closed triangles). These results indicate that diC8PC supports the growth of the strain that is defective in PC synthesis through both the CDP-DAG pathway and the Kennedy pathway.

Next, we analyzed the phospholipid composition of the KSY02 cells that were cultured in the SG medium containing choline or the SD medium containing diC8PC (Fig. 2). The relative contents of major phospholipids, PC, PE, PS, and phosphatidylinositol (PI) in the cells that were cultured in SG medium containing choline were 45%, 34%, 11%, and 10% respectively. In contrast, when the KSY02 cells were grown in the SD medium containing diC8PC, the relative content of PC was decreased to 7%, indicating that this level of PC supports the growth of the yeast. Instead the relative

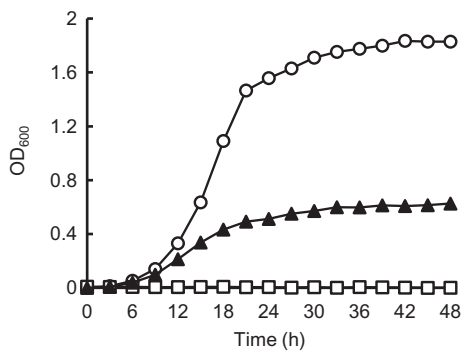

Fig. 1. Growth of KSY02 in diC8PC-containing medium. The growth of KSY02 in SG medium containing choline (open circles), SD medium (open square), or SD medium containing diC8PC (closed triangles) was analyzed as described in Section 2. Similar results were obtained in three independent experiments.

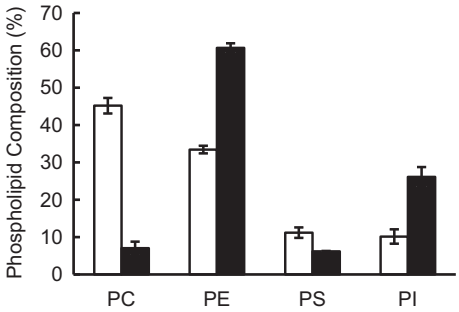

Fig. 2. Phospholipid composition of KSY02. KSY02 was cultured at 30 °C for 10 h in SG medium containing choline (white bars) or SD medium containing diC8PC (black bars) after preincubation in the same media for 24 h. Phospholipid composition was analyzed as described in Section 2. Results represent an average of three independent experiments ± SE.

contents of PE and phosphatidylinositol (PI) were increased to 61% and 26% respectively. The relative content of PS was decreased to 6%.

3.2. Remodeling of acyl residues in diC8PC in vivo and in vitro

We analyzed the metabolism of (*methyl*-$^{13}C)_3$-diC8PC, in which three methyl residues in choline moiety of diC8PC were labeled with ^{13}C [12], in the KSY02 strain using ESI-MS/MS. The KSY02 strain that was precultured in the SD medium containing non-labeled diC8PC was incubated in the SD medium containing 30 μM (*methyl*-$^{13}C)_3$-diC8PC for 30 min. Lipids were extracted from the cells, and subjected to ESI-MS/MS analysis. In precursor ion scan for a product ion of *m*/*z* 187 in positive ion mode to detect PC species containing three ^{13}C-labeled methyl moieties, peaks of significant intensity corresponding to remodeling products, (*methyl*-$^{13}C)_3$-32:2-PC, (*methyl*-$^{13}C)_3$-32:1-PC, (*methyl*-$^{13}C)_3$-34:2-PC, and (*methyl*-$^{13}C)_3$-34:1-PC, which most probably contain C16:0, C16:1, C18:0, or C18:1 acyl residue at *sn*-1 and *sn*-2 positions, were detected at *m*/*z* 733.5, 735.6, 761.6, and 763.6 respectively (Fig. 3A and B) [12]. In addition, peaks corresponding to potential remodeling intermediates, (*methyl*-$^{13}C)_3$-24:1-PC and (*methyl*-$^{13}C)_3$-24:0-PC, in which one C8:0 acyl residue of (*methyl*-$^{13}C)_3$-diC8PC was replaced with that of C16:1 or C16:0, were detected at *m*/*z* 623.4 and 625.5, respectively (Fig. 3A) [12]. Smaller peaks corresponding to (*methyl*-$^{13}C)_3$-26:1-PC and (*methyl*-$^{13}C)_3$-26:0-PC, in which one C8:0 acyl residue was replaced with that of C18:1 or C18:0, were also detected at *m*/*z* 651.5 and 653.5, respectively. Since PC synthesis through the Kennedy pathway is repressed in the KSY02 cells in the medium containing glucose, these results strongly suggest that C8:0 acyl residues at both *sn*-1 and *sn*-2 positions of diC8PC were replaced with physiological acyl residues and that the remodeled PC entered cellular PC pool.

We next investigated the remodeling of (*methyl*-$^{13}C)_3$-diC8PC *in vitro* (Fig. 4A and B). (*Methyl*-$^{13}C)_3$-diC8PC was incubated with the cell extract from the KSY02 strain that was cultured in the SD medium containing diC8PC, in the presence of palmitoleoyl-CoA as an acyl donor. In 30-min reaction, peaks corresponding to (*methyl*-$^{13}C)_3$-24:1-PC and (*methyl*-$^{13}C)_3$-32:2-PC, in which one or both C8:0 acyl residue(s) was/were replaced with C16:1 acyl residue(s), were detected at *m*/*z* 623.4 and 733.5 respectively. In addition, peak corresponding to (*methyl*-$^{13}C)_3$-24:0-PC, (*methyl*-$^{13}C)_3$-26:1-PC, and (*methyl*-$^{13}C)_3$-26:0-PC, which are probably derived from the replacement of one acyl residue of (*methyl*-$^{13}C)_3$-diC8PC with endogenous 16:0, 18:1, and 18:0 fatty acids in the cell extract, were detected at *m*/*z* 625.5, 651.5, and

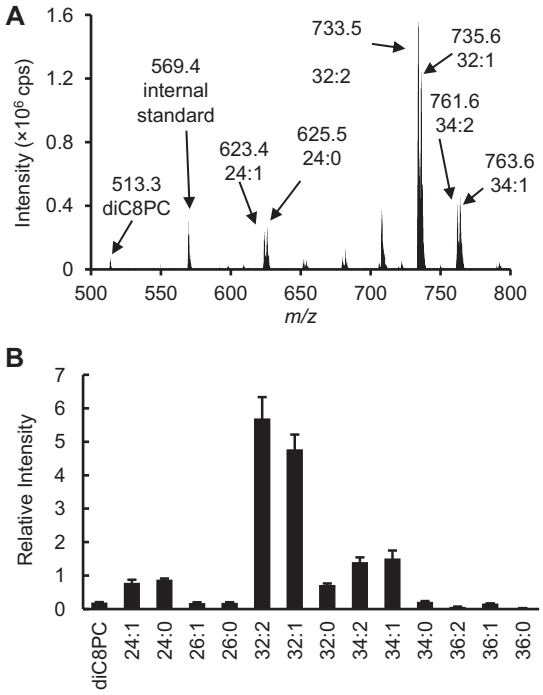

Fig. 3. Remodeling of (*methyl*-$^{13}C)_3$-diC8PC in KSY02. (A) KSY02 was labeled with (*methyl*-$^{13}C)_3$-diC8PC for 30 min after the preincubation in the SD medium containing diC8PC as described in Section 2. Phospholipids were extracted and subjected to ESI-MS/MS analysis with precursor ion scan for *m/z* 187 in positive ion mode. The theoretical *m/z* values are indicated, and experimental errors were within 0.5 mass unit. (B) (*methyl*-$^{13}C)_3$-PC species were quantified and amount of those species were expressed as ratios to internal standard, (*methyl*-$^{13}C)_3$-diC10PC. Results represent an average of three independent experiments ± SE.

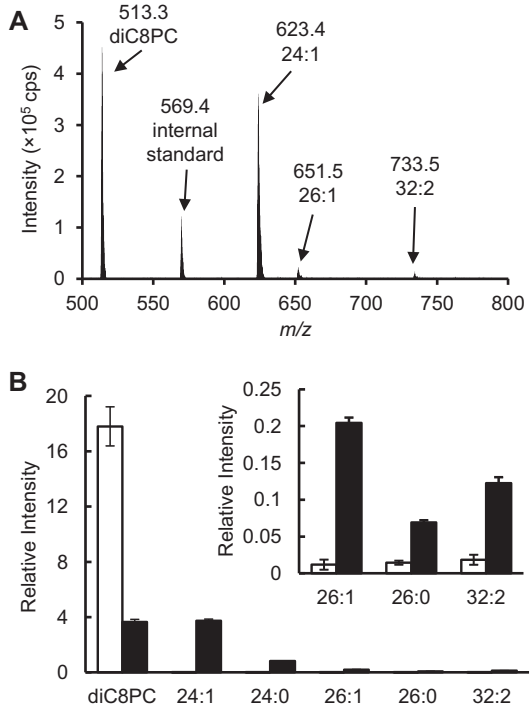

Fig. 4. Remodeling of (*methyl*-$^{13}C)_3$-diC8PC *in vitro*. (A) (*Methyl*-$^{13}C)_3$-diC8PC was incubated with the KSY02 cell extract and palmitoleoyl-CoA for 30 min as described in Section 2. Phospholipids were extracted and subjected to ESI-MS/MS analysis. The theoretical *m/z* values are indicated, and experimental errors were within 0.7 mass unit. Similar results were obtained in three independent experiments. (B) (*Methyl*-$^{13}C)_3$-PC species detected in the *in vitro* reaction using the KSY02 cell extract (black bars) or buffer (white bars) were quantified and amount of those species were expressed as ratios to internal standard, (*methyl*-$^{13}C)_3$-diC10PC. Results represent an average of three independent experiments ± SE.

653.5, respectively. The results indicate that (*methyl*-$^{13}C)_3$-diC8PC is remodeled to PCs containing longer acyl residues in the *in vitro* reaction. These *in vivo* and *in vitro* results provide convincing evidence that diC8PC is remodeled to PC species containing physiological acyl residues in yeast.

4. Discussion

In this study, we analyzed the remodeling of PC containing short acyl residues in the KSY02 strain, in which PC synthesis was repressed. (*Methyl*-$^{13}C)_3$-diC8PC was converted to (*methyl*-$^{13}C)_3$-PC species containing longer acyl residues in the KSY02 cells. Since PC synthesis through the Kennedy pathway in the KSY02 strain is repressed in the medium containing glucose, these PC species should be derived from the acyl chain remodeling of (*methyl*-$^{13}C)_3$-diC8PC, but not from the PC synthesis through the Kennedy pathway using choline or phosphocholine generated by the degradation of (*methyl*-$^{13}C)_3$-diC8PC by PLD or PLC, respectively. Thus, the system, in which the remodeling of (*methyl*-$^{13}C)_3$-diC8PC in the KSY02 strain or its derivative strains is analyzed using ESI-MS/MS, will enable a quantitative and reliable evaluation of the remodeling of acyl residues in diC8PC. In addition, diC8PC supported the growth of the KSY02 strain in the glucose-containing medium. The carbon numbers of acyl residues in the endogenous PC in *S. cerevisiae* is predominantly from C16 to C18 [10], and C8 fatty acyl residues in diC8PC appear to be too short to maintain the structures and functions of cellular membranes. Therefore, the remodeling of diC8PC could be monitored by observation of the growth of this strain in the medium containing glucose with diC8PC.

The growth of the KSY02 strain in the SD medium containing diC8PC was poorer than that in the SG medium containing choline. PC containing short acyl residues could be toxic to yeast cells due to its amphiphilic property, while the hydrophilic property is requisite for the access of diC8PC to the plasma membrane through the cell wall [21,23]. Indeed, the growth of the wild-type cells in the SD medium was slightly impaired by the addition of diC8PC [12]. Another reason for the poor growth of the KSY02 strain in the SD medium containing diC8PC could be the inability to utilize choline or phosphocholine generated by the degradation of diC8PC due to the transcriptional repression of *PCT1*. The *pem1*Δ*pem2*Δ strain, which retains the ability to synthesize PC through the Kennedy pathway, grew to higher OD in the SD medium containing diC8PC than the KSY02 strain [12]. The relatively higher PC content of 25% in the *pem1*Δ*pem2*Δ cells cultured in the SD medium containing diC8PC [12] compared to that of 7% in the KSY02 cells cultured in the same medium could be explained by the contribution of PC synthesis through the Kennedy pathway using diC8PC metabolites.

The relative content of PE and PI were increased to 61% and 26%, respectively, in the KSY02 cells cultivated in the SD medium with diC8PC. The increase in these phospholipids might compensate for the reduction in PC. Boumann et al. reported that decrease in the PC induced the increases in PE and PI, accompanied by the shortening and increased saturation of the acyl residues in PE [29]. It would be of great interest to analyze the acyl chain profiles of phospholipids in the KSY02 strain cultured in the SD medium with diC8PC.

Two reactions, removal of acyl residues and reacylation, at *sn*-1 and *sn*-2 positions are involved in the remodeling of phospholipid acyl residues. Our previous results of the growth analysis of the mutant derived from the *pem1*Δ*pem2*Δ strain suggested that Ale1 [13–17] is involved in the utilization of diC8PC [12]. However,

other acyltransferase(s) appear(s) to be involved in the reacylation at *sn*-2 position of diC8PC, since the deletion mutant of *ALE1* in a strain defective in PC synthesis could still utilize diC8PC (our unpublished results). In addition, the acyltransferase that introduces acyl residues at *sn*-1 position and the phospholipase that cleaves acyl residues in the remodeling of diC8PC remain unclear. Recently, it was reported that Plb1, one of phospholipases B of *S. cerevisiae*, is involved in the remodeling of acyl residues of PC in yeast overexpressing *SCT1*, encoding a glycerol-3-phosphate acyltransferase [30]. The system to evaluate the remodeling of PC using diC8PC will contribute to the identification of enzymes involved in the each step of PC remodeling. Dissection of a sequence of remodeling processes of diC8PC into individual reaction steps and the analysis of each reaction using specific substrates will aid our understanding of the mechanisms of the remodeling of phospholipid acyl residues.

Acknowledgments

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was performed using the facilities of the Biotechnology Research Center of the University of Tokyo.

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