Doctoral thesis

Study on molecular remodeling and function of phosphatidylethanolamine in *Saccharomyces cerevisiae*

（酵母 ***Saccharomyces cerevisiae*** におけるホスファチジルエタノールアミンの
分子リモデリングと機能に関する研究）

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List of abbreviations

Gly-3-P	glycerol-3-phosphate
LPA	lysophosphatidic acid
PA	phosphatidic acid
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PS	phosphatidylserine
PI	phosphatidylinositol
GroPEtn	glycerophosphoethanolamine
GroPCho	glycerophosphocholine
CL	cardiolipin
MBOAT	membrane-bound O-acyltransferase
LPLAT	lysophospholipid acyltransferase
LPAAT	lyso-PA acyltransferase
DGAT	diacylglycerol acyltransferase
POPE	1-palmitoyl-2-oleoyl-PE
CDP-DAG	CDP-diacylglycerol
TLC	thin-layer chromatography
EUROSCARF	EUROPEAN SACCHAROMYCES CEREVISIAE ARCHIVES FOR FUNCTIONAL ANALYSIS
ESI-MS/MS	Electrospray ionization-mass spectrometry
NBD-PE	1-myristoyl-2-(6-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino) caproyl)-*sn*-glycero-3-phosphoethanolamine

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List of abbreviations

Chapter 1

Introduction

1.1 Cell membrane and phospholipid

Cell membranes are crucial to the life of the cell and have several important functions. First, they serve as a diffusion barrier between the interior of the cell and its environment, and between the lumen of organelles and the cytosol. Second, membranes harbor proteins that catalyze selective transport of molecules or act as enzymes in metabolic and regulatory pathways. Third, membranes harbor receptors, which contribute to recognition process.

Despite their different functions, biological membranes have a common general structure: comprised of a phospholipid bilayer with proteins interspersed within it. Phospholipids are the basic building blocks of cellular membranes, and their chemical structure and diversity are well suited for this important physiological role. These molecules consist of a glycerol backbone esterified with fatty acids in the *sn*-1 and *sn*-2 positions, and a phosphate group in the *sn*-3 position. The two fatty acyl chains make up the hydrophobic region of phospholipids. The hydrophobic regions of the two phospholipid layers are oriented towards each other so that the polar phosphate-head group, the hydrophilic regions of phospholipids, face out to the environment as well as into the cytoplasm of the cell's interior, where they form hydrogen bonds with surrounding water molecules.

Eukaryotic membranes contain various phospholipids, with head groups that differ in size, shape, and charge, besides with fatty acid that differ in length and saturation. The different head groups determine the phospholipid classes, and the variety of fatty acyl chains contribute to the diversity of species within a class (Fig. 1.1) (Forrester et al., 2004). Although all glycerophospholipids contain a glycerol backbone, the diversity of head groups, acyl chains, and degree of unsaturation can produce hundreds of different lipid species existing within a given cell. This enormous number of structural combination allows for a large variety of physical and chemical membrane properties, including membrane permeability, fluidity, and curvature.

1.2 Synthesis of phospholipid in *Saccharomyces cerevisiae*

Budding yeast *Saccharomyces cerevisiae* is perhaps the most relevant yeast for mankind, both for its use since ancient times in baking and brewing, and for being one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *Escherichia coli* as the model prokaryote. *S. cerevisiae* is easy to culture but as a eukaryote, it shares the complex internal cell structure of plants and animals. *S. cerevisiae* was the first eukaryote with genome completely sequenced, and its phospholipid synthesis mechanism was almost elucidated (Fig. 1.2) (Daum et al., 1998).

In *S. cerevisiae*, phospholipid synthesis is initiated by glycerol-3-phosphate (Gly-3-P) acyltransferase, Gat1p and Gat2p, through the transfer of a fatty acid from fatty acyl-CoA to the *sn*-1 position of Gly-3-P to form lysophosphatidic acid (LPA, lyso-PA). LPA can also be formed by the acylation of dihydroxyacetone phosphate (DHAP) by the same two proteins and subsequent reduction of the product.

LPA is further fatty acylated by LPA acyltransferase, Ale1p or Slc1p, to form phosphatidic acid (PA). Then PA is converted to CDP-diacylglycerol (CDP-DAG), a central metabolite in phospholipid biosynthesis, by CDP-diacylglycerol synthase, encoded by *CDS1* gene. From CDP-DAG, the major phospholipids in *S. cerevisiae*, including phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) are synthesized. The biosynthetic pathway of these phospholipids in *S. cerevisiae* is outlined below.

Synthesis of PI

PI is an essential phospholipid for *S. cerevisiae*. It was suggested that its essential function is not the structural requirement for membrane assembly but its role in cellular signaling and as a membrane sensor. PI is synthesized from CDP-DAG and inositol by PI synthase (Pis1p) (Fig. 1.2). Inositol is derived from the inositol-1-phosphate produced by inositol-1-phosphate synthase (Ino1p), which utilizes glucose-6-phosphate as a precursor (Majumder et al., 1997). *PIS1* is essential for growth in *S. cerevisiae* (Daum et al., 1998).

Synthesis of PS

PS is only a minor component of total cell phospholipid, but an important intermediate in the *de novo* synthesis of the two main yeast phospholipids, PE and PC. In *S. cerevisiae*, the PS synthase, which is encoded by the *PSS1/CHO1* gene, forms PS from CDP-DAG and serine. The *pss1/cho1* mutant is not lethal when it was grown in the presence of ethanolamine or choline, with which PE or PC can be formed via the

so-called Kennedy pathway (see below). The *pss1/cho1* null mutant has no detectable PS, suggesting that PS, by itself, is non-essential and *PSS1/CHO1* gene encodes the only PS synthase in *S. cerevisiae* (Atkinson et al., 1980) (Kiyono et al., 1987).

Synthesis of PE

In *S. cerevisiae*, the major route of PE synthesis is the *de novo* pathway through decarboxylation of PS (Fig. 1.2). PS decarboxylase was encoded by two genes, *PSD1* and *PSD2* (Trotter et al., 1993) (Trotter and Voelker, 1995). Psd1p is localized to the mitochondrial inner membrane and responsible for 90% of the PS decarboxylase activity in a certain genetic background. Psd2p is localized to Golgi/vacuole compartment. Although it only accounts for 5~10% of the cellular PS decarboxylase activity, it was suggested that it could synthesize enough PE to support the growth of *psd1* null mutant (Trotter and Voelker, 1995). An alternative route for the synthesis of PE in yeast is the Kennedy pathway (see below).

Synthesis of PC

PC, a major class of glycerophospholipids, plays critical roles in membrane structure and cellular signaling. It is synthesized through a three-step methylation of PE. These reactions are catalyzed by two independent methyltransferases, PE methyltransferase (Pem1p/Cho2p) and phospholipid methyltransferase (Pem2p/Cho3p), which are localized to the endoplasmic reticulum (Kanipes and Henry, 1997) (Kodaki and Yamashita, 1987). In a pem1/cho2 deletion strain, the activity of PE methyltransferase is greatly reduced but the ability of Pem2p/Opi2p to catalyze the first methylation of PE allows formation of some PC, albeit somewhat inefficiently (Summers et al., 1988). When *PEM2/OPI3* was deleted, phospholipid methyltransferase activity was completely lost and phosphatidylmonomethylethanolamine accumulated. The double mutant pem1/cho2 pem2/cho3 is clearly auxotrophic for choline, suggesting that PC is essential for the growth of yeast (Summers et al., 1988).

Kennedy pathway

Kennedy pathway was named after its discoverer by E. P. Kennedy. Kennedy pathway is a salvage pathway to synthesize PE and PC from free ethanolamine and choline in *S. cerevisiae*. The pathway is divided into two branches, one is using ethanolamine to synthesize PE, so-called CDP-ethanolamine pathway; the other is using choline to synthesize PC, and so-called CDP-choline pathway. The first step of the CDP-ethanolamine pathway is phosphorylation of ethanolamine by ethanolamine kinase

(Eki1p), or choline kinase (Cki1p) which has dual substrate specificity that is also involved in phosphorylation of choline in the first step of the CDP-choline pathway (Hosaka et al., 1989) (Yamashita and Hosaka, 1997). Ethanolaminephosphate is then converted to CDP-ethanolamine by the ethanolaminephosphate cytidylyltransferase (Ect1p/Muq1p) (Min-Seok et al., 1996). Finally, PE is formed in the endoplasmic reticulum by CDP-ethnolamine:1,2-diacylglycerol ethanolaminephosphotransferase (Ept1p) (McMaster and Bell, 1997). Yeast incorporates choline into PC through the Kennedy pathway similar to the utilization of free ethanolamine. The enzymes involved in the choline branch of this salvage pathway are choline kinase (Cki1p), cholinephosphate cytidylyltransferase (Pct1p/Cct1p), and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (Cpt1p) (Hosaka et al., 1989) (Yamashita and Hosaka, 1997).

Dependent on the Kennedy pathway, yeast mutant which is unable to synthesize PS or lack PS decarboxylase could grow on the ethanolamine/choline containing medium (Carman and Zeimetz, 1996). Substrate of the ethanolamine branch of the pathway can be provided by the action of phospholipase type D (Mayr et al., 1996) (Waksman et al., 1996), or by degradation of long-chain sphingoid base phosphates (Mandala et al., 1998). On the other hand, the choline branch of the pathway is not only active when exogenous choline is present but also functions continuously to recycle degradation products of PC (Cleves et al., 1991).

1.3 Turnover of phospholipid

1.3.1 Phospholipase

Although the mechanisms of phospholipids synthesis in various cells have been well elucidated, little is known about their turnover. Several phospholipases are known to have the activity to degrade phospholipids, and according to their action sites, they are divided into four classes as shown in Fig. 1.3.

Phospholipase A

Phospholipase A (PLA) catalyses the lipolytic removal of one of the two esterified fatty acid moieties of glycerol. It is represented by the two isoenzymes, phospholipase A1 and phospholipase A2, which differ on the fatty acid moiety that they remove from the phospholipid. Phospholipase A1 cleaves the *sn*-1 ester bond, removing the outer fatty acid moiety and phospholipase A2 cleaves the *sn*-2 ester bond, removing

the inner fatty acid moiety. In mammalian cells, a limited number of phospholipase A1 were characterized so far. These include PS-specific phospholipase A1 from rat platelets (Sato et al., 1997), phospholipase A1 from vespid venom (King et al., 1984) and a PA-preferential phospholipase A1 from bovine and human testis (Hiramatsu et al., 2003). The phospholipase A2 activities are found in a variety of enzymes which can be divided in several types based on their Ca^{2+} dependence for their activity; Ca^{2+}-dependent secretory phospholipases and cytosolic phospholipases, and Ca^{2+}-independent phospholipase A_2s. These enzymes also show diverse size and substrate specificity (i.e., in the fatty acid chain length and extent of saturation). In *S. cerevisiae*, the product of *YOR022C* gene is homologous to the bovine phospholipase A1 (Higgs et al., 1998). The genome-scale GFP tagging localization analysis by Huh *et al.* indicated that Yor022cp is localized to the mitochondria (Huh et al., 2003), but little has been reported about its function. In addition, Per1p possesses the glycosylphoshatidylinositol (GPI)-phospholipase A2 activity that functions in the lipid remodeling from normal PI to a C26 fatty acid-containing PI in the GPI anchor (Fujita et al., 2006).

Phospholipase B

Phospholipase B (PLB) catalyzes the hydrolytic cleavage of both acylester bonds of glycerophospholipids (both *sn*-1 and *sn*-2 sites). Products of phospholipase B activity are two fatty acids and water-soluble glycerophosphodiesters. Four genes encoding enzymes with phospholipase B/lysophospholipase activity have been characterized in *S. cerevisiae.* Three of those genes, *PLB1*, *PLB2* and *PLB3*, bear >60% homology on the DNA level. Base on the analysis of the glycerophosphodiesters produced in strains bearing deletion mutations in those genes, Plb3p was shown to be primarily responsible for the production of extracellular glycerophosphoinositol (GroPIns), with Plb1p playing a lesser role (Merkel et al., 2005). Deletion of *PLB1*, in contrast, greatly reduced the production of extracellular glycerophosphocholine (GroPCho) and glycerophosphoethanolamine (GroPEtn) (Lee et al., 1994) (Merkel et al., 1999). Deletion of *PLB2* has no effect upon the production of extracellular glycerophosphodiesters (Merkel et al., 2005). However, a role for Plb2p in the hydrolysis of exogenous phospholipids is indicated by the finding that overexpression of the gene results in resistance to lysophosphatidylcholine (Merkel et al., 1999) (Fyrst et al., 1999). Plb1p and Plb3p have been localized to the plasma membrane and periplasmic space, and Plb2p to the plasma membrane, the periplasmic space, and the culture supernatant (Lee et al., 1994) (Merkel et al., 1999) (Patton-Vogt, 2007).

The fourth phospholipase B enzyme characterized to date is encoded by *NTE1*

(Zaccheo et al., 2004). Nte1p, which is localized to the endoplasmic reticulum, is responsible for the GroPCho production shown previously to occur as a result of choline supplementation and temperature elevation. In addition, the product of *SPO1* gene has 25% identity to Plb1p and 26% identity to fungal phospholipase B enzymes (Tevzadze et al., 1996).

Phospholipase C

Phospholipase C is a key enzyme in phosphatidylinositol 4,5-bisphosphate (PIP_2) metabolism. It is activated either by G protein (making it a part of a G protein–coupled receptor signal transduction pathway) or by transmembrane receptors with intrinsic or associated tyrosine kinase activity. It splits the phosphoester bond of PIP_2, generating two intracellular products: inositol triphosphate (IP3), a calcium-mobilizing second messenger, and diacylglycerol, an activator of protein kinase C. The phospholipase C of *S. cerevisiae* is encoded by the *PLC1* gene, which is conserved in eukaryote, and is required for glycerol synthesis and plays a key role, through hydrolysis of PIP_2 and generation of IP3, in signaling for a cytosolic transient calcium influx spike in response to glucose (Yoko-o et al., 1993) (Lin et al., 2002) (Tisi et al., 2004).

Phospholipase D

Phospholipase D catalyses the hydrolysis of the phosphodiester bond of glycerophospholipid to generate PA and a free headgroup. Phospholipase D activities have been detected in simple to complex organisms from viruses and bacteria to yeast, plants, and mammals. Although enzymes with broader selectivity are found in some of the lower organisms, the plant, yeast, and mammalian enzymes are selective for PC (McDermott et al., 2004; Simons et al., 1998). Phospholipase D of *S. cerevisiae*, which is encoded by the *SPO14/PLD1* gene, catalyzes hydrolysis of PC to yield PA and choline, and is required for sporulation and normal formation of mating projections (Honigberg et al., 1992) (Morishita and Engebrecht, 2005) (Hairfield et al., 2001). In addition, an unconventional phospholipase D activity was identified, which is Ca^{2+}-dependent, preferentially hydrolyses PE and PS, in yeast cell bearing disruption at the *SPO14/PLD1* locus (Tang et al., 2002) (Waksman et al., 1997).

1.3.2 acyltransferase

It is proposed that after *de novo* synthesis, phospholipids undergo remodeling

which contributes to the diversity of their acyl chains. The mechanism of phospholipid remodeling is unsolved. Half a century ago, Lands *et al*. proposed that a deacylation-reacylation cycle (Lands' Cycle) was involved in the remodeling of phospholipids which consisted of a deacylation at the *sn*-2 position by phospholipase A2 and subsequent reacylation by acyl-CoA-dependent lysophospholipid acyltransferase (Lands, 1960). In the 50 years since Lands' proposal, little information was available on acyltransferase involved in phospholipid remodeling utill recently, several lysophospholipid acyltransferase (LPLAT) have been identified (Fig. 1.4).

Fig. 1.4 summerizes the characterized and predicted acyltransferases in membrane-bound O-acyltransferase (MBOAT), lyso-PA acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT2) family.

In MBOAT family, it is reported that LPIAT1 is required for incorporation of polyunsaturated fatty acid into PI in *Caenorhabditis elegans* (Lee et al., 2008); LPEAT1/MBOAT1 possesses lyso-PE and lyso-PS acyltransferase activities preference for 18:1-CoA; LPCAT3/MBOAT5 exhibits lyso-PC, lyso-PE and lyso-PS acyltransferase activity preference for polyunsaturated fatty acyl-CoAs; and LPCAT4/MBOAT2 possesses lyso-PC and lyso-PE acyltransferase activity preference for 18:1-CoA in mouse (Hishikawa et al., 2008).

In *S. cerevisiae*, several proteins are members of MBOAT family, including: Ale1p/Lpt1p/Slc4p/Lca1p, which was identified to catalyze the transfer of unsaturated fatty acids into the *sn*-2 position of various lysophospholipids (Benghezal et al., 2007) (Jain et al., 2007) (Riekhof et al., 2007b) (Tamaki et al., 2007); Gup1p, which was reported to be required for the addition of a C26 fatty acid in the *sn*-2 position of GPI anchors (Bosson et al., 2006); Are1p and Are2p, two sterol acyltransferases involved in steryl ester synthesis (Sandager et al., 2002), and Gup2p, a putative glycerol transporter involved in active glycerol uptake (Holst et al., 2000).

On the other hand, in LPAAT family, Slc1p was also reported to have acyltransferase activity against the *sn*-2 position in various lysophospholipids, although playing a lesser role than Ale1p (Benghezal et al., 2007) (Jain et al., 2007). Taz1p, a putative acyltransferase that may function in cardiolipin (CL) metabolism (Vaz et al., 2003), two Gly-3-P acyltransferase, Gat1p and Gat2p (Zaremberg and McMaster, 2002), and three function unknown proteins, Ybr042cp, Ydr018c and Yor298w, are also members of LPAAT family in yeast.

1.4 Functions of PE

PE is the major phospholipid comprising ~20% of total phospholipids in eukaryotic cells. Because of having a small polar head and bulky moiety of associated fatty acid chains, PE has a strong tendency to form nonbilayer structures, so-called hexagonal-II phase structure in which polar heads orient inside the cylinder-like structure, and is the most abundant phospholipid of this type in eukaryotic cells (Rietveld et al., 1997). The potential of membranes with high PE content to undergo laminar to hexagonal phase transition has been proposed to affect membrane-membrane contact and bilayer fusion during processes of vesicle formation and vesicle-mediated protein trafficking. In addition, nonbilayer lipids may affect integration of proteins into membranes, their lateral movement within the membrane, and folding and stabilization of certain membrane protein complex.

In *E. coli*, lack of PE can be compensated by elevated levels of CL in the presence of divalent cations, thereby maintaining the potential of bilayer-to-nonbilayer phase transition of membranes (Morein et al., 1996). A PE-deficient *E. coli* mutant displays complex phenotypic changes, including filamentous growth (Mileykovskaya et al., 1998) and decreased activity of lactose permease. The latter observation was ascribed to misfolding of the permease due to the lack of PE that acts as a molecular chaperone for this transporter (Bogdanov et al., 1999). *In vitro*, nonbilayer lipids stimulate the activity of the reconstituted bacterial protein translocase (van der Does et al., 2000).

In *S. cerevisiae*, PE is essential for growth and deletion of *PSD1* and *PSD2* with simultaneous inhibition of PE supply through Kennedy pathway is lethal (Robl et al., 2001) (Birner et al., 2001). It was reported that when propanolamine, the analogue of ethanolamine, was added to the medium, the cells could synthesize phosphatidylpropanolamine (PtdPrn), which readily forms hexagonal II-phase structures as PE, via the Kennedy pathway. The existence of PtdPrn can decrease the cellular requirements of PE, but it cannot replace all the PE requirements. These data suggested that PE performs an unidentified but essential function that is independent of the ability to form hexagonal-II phase structures in membranes (Storey et al., 2001).

PE provides phosphoethanolamine to the junction between extracellular proteins and the glycosylphosphatidylinositol (GPI) anchor, which is also essential for growth in *S. cerevisiae* (Menon and Stevens, 1992). In addition, PE is directly involved in an autophagic process through Atg8p (Ichimura et al., 2000) (Kirisako et al., 2000). Furthermore, PE plays a critical role in mitochondrial function. As mentioned above, mitochondrial Psd1p provides ~90% of total cellular phosphatidylserine decarboxylase activity. In *Δpsd1* strains, which contain decreased amount of PE, the growth rate on

nonfermentable carbon sources correlates with the content of PE in mitochondria. Although morphological and biochemical analyses revealed no obvious defects of PE-depleted mitochondria, the mutant exhibited an enhanced formation of respiration-deficient cells (Birner et al., 2001). Additionally, it was suggested that PE share a common function(s) with CL, a unique phospholipid with dimeric structure which is ubiquitous in eukaryotes and predominantly found in the mitochondrial inner membrane, by the synthetic lethal interaction of mitochondrial PE and CL biosynthetic pathway (Gohil et al., 2005) (Li et al., 2007).

In mammalian cells, it was suggested that PE becomes exposed on the cell surface at the cleavage furrow during cytokinesis and this PE movement is involved in regulation of the contractile ring disassembly (Emoto et al., 1996) (Emoto and Umeda, 2000). Iwamoto, in our laboratory, suggested that in *S. cerevisiae*, PE was exposed on the cell surface at cellular polarized ends, presumptive bud site, the emerging small bud cortex, the bud neck of the late mitotic large-budded cells, and the tip of the mating projection, using biotinylated Ro09-0198, which specifically binds to PE. In addition, when yeast cells were treated with Ro09-0198, aberrant F-actin accumulation was observed at the above sites. These results suggested that movement of PE on the plasma membrane is involved in the polarized organization of the actin cytoskeleton (Iwamoto et al., 2004).

1.5 Remodeling of phospholipid

Biological membranes contain a complicated mixture of phospholipids differing from each other with respect to their head-group structure, hydrocarbon chain length, and degree of unsaturation of the acyl chains. The complexity of these phospholipid structures is believed to be important in membrane dynamics, protein regulation, signal transduction, and vesicular secretion. Remodeling of phospholipids after their *de novo* synthesis was considered to play important role in generation or maintaining the complexity of phospholipids.

To date, biological role of phospholipid remodeling has remained unclear. In general, reactive oxygen (oxygen radical) attacks unsaturated fatty acid moiety in biological membrane and is supposed to generate fatty acid peroxide, which is then degraded to short fatty acid with aldehyde group. Phospholipids with those short fatty acyl chains could cause deleterious effects on maintenance of homeostasis in cells, and it is assumed that phospholipid remodeling is involved in removal of those harmful fatty acids. Additionally, cells are supposed to alter the hydrocarbon chain length and degree

of unsaturation of the acyl chains in phospholipids in response to environmental changes. Remodeling of phospholipids can be involved in the adaptations to environment. In addition to incorporation of unsaturated acyl chains for membrane fluidity, specific incorporation of the polyunsaturated arachidonate into the *sn*-2 position may prime phospholipase A2-inducible signal cascades that involve eicosanoid synthesis (Chilton et al., 1987). Alternatively, preferential incorporation of saturated acyl chains occurs during surfactant production in lungs (Post et al., 1983). It was pointed out that in eukaryote, the remodeling of PC plays important roles in signaling process (Cui and Houweling, 2002).

In *S. cerevisiae*, PC is synthesized either via the triple methylation of PE or via the CDP-choline route. It was suggested 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 *sn*-1 position (Boumann et al., 2003). Previous results of our laboratory showed that PC with octanoic acids (diC8PC) or decanoic acids (diC10PC) in the culture medium supported growth of *cho1*/*pss1* mutant auxotrophic for choline or ethanolamine due to its deficiency in PS synthesis and *pem1pem2* double mutant auxotrophic for choline due to its defect in PE methylation (Yon et al., 1998) (Tanaka et al., 2008). Analysis of the metabolism of *methyl*-^{13}C-labeled diC8PC by electrospray ionization tandem mass spectrometry (ESI-MS/MS) suggested that acyl chains in diC8PC was remodeled in the cells, providing a new approach to analyze the remodeling of PC in yeast (Tanaka et al., 2008). Similarly, PE and PS with short acyl residues were incorporated into a deep-rough derivative of *Escherichia coli* mutant defective in synthesis of PE, and were remodeled towards a lipid species profile resembling that of the wild-type (Kol et al., 2004). In mammalian cells, Kainu *et al.* analyzed the acyl chain remodeling of PE and PS with deuterium-labeled head group, which were introduced to cells using cyclodextrin-mediated transfer, by ESI-MS/MS (Kainu et al., 2008). In *S. cerevisiae*, it was reported that lyso-PE and lyso-PC was incorporated and acylated to PE and PC (Riekhof and Voelker, 2006) (Riekhof et al., 2007a). These results gave evidences for lipid remodeling.

However, the mode and mechanism of phospholipid remodeling is not well elucidated. Ale1p and Slc1p were reported to have acyltransferase activity against the *sn*-2 position in various lysophospholipids, including lyso-PE, lyso-PI, lyso-PS and lyso-PA (Benghezal et al., 2007) (Jain et al., 2007) (Riekhof et al., 2007b) (Tamaki et al., 2007). But there is no information available on enzyme(s) involved in the remodeling of phospholipids at *sn*-1 position.

Previous results of our laboratory showed that *methyl*-^{13}C-labeled diC8PC was remodeled in yeast and the initial substitution with saturated 16:0 acyl prefers the *sn*-1 position while the substitution by unsaturated 16:1 acyl chain starts at both *sn*-1 and *sn*-2 positions (Tanaka et al., 2008). Kainu *et al.* showed that in mammalian cells, PE species containing two saturated fatty acids are remodeled very rapidly, initially at the *sn*-2 position by substitution with mainly unsaturated 18:1 fatty acyl chain and subsequently at *sn*-1 position by substitution with similar fatty acyl chain (Kainu et al., 2008). But the remodeling mode of PE in yeast is still unknown.

Furthermore, there is also no information available on the phospholipase involved in the remodeling of PE in *S. cerevisiae*.

This study is aimed at elucidating the mechanism of PE remodeling and the physiological role of PE in *S. cerevisiae*.

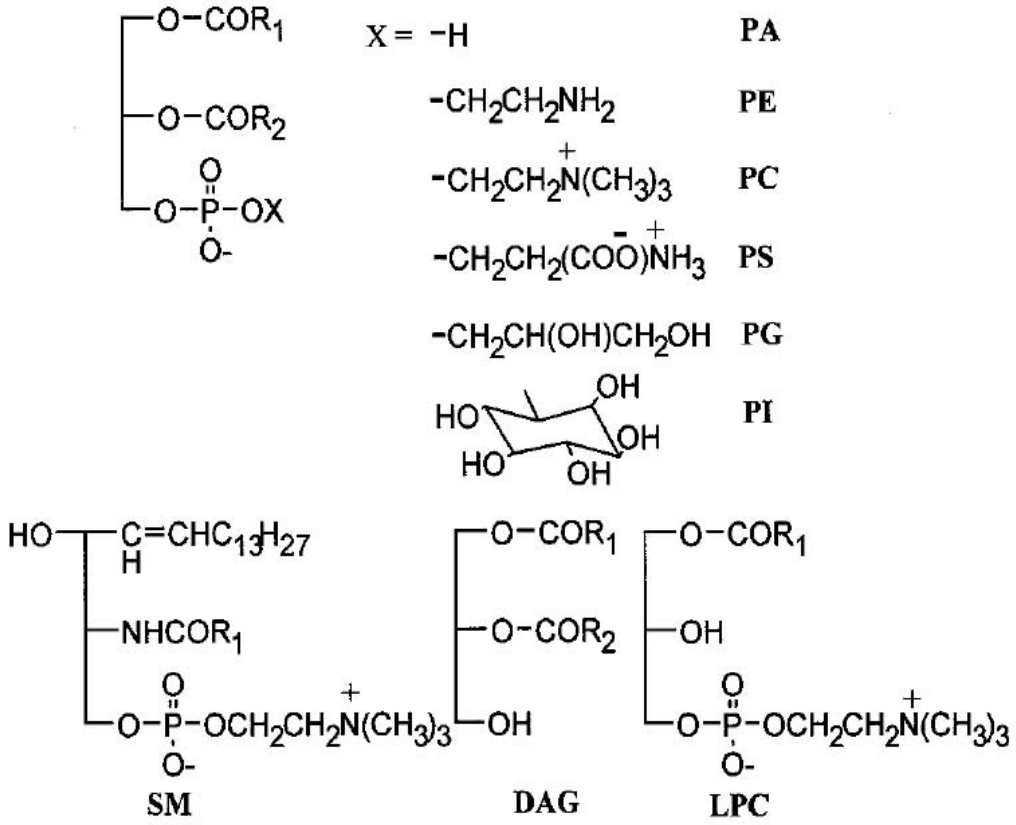


Fig. 1. Structures of glycerophospholipid classes. In the upper left segment is shown the structure of compounds commonly referred to as phosphoglycerides. The R_1 and R_2 represent long-chain carboxylic acids usually connected via an ester bond to the primary and secondary alcohol residues of glycerol. The –X moiety refers to the headgroups shown in the upper right of the figure. Joined in ester linkage to the phosphoric acid residue they yield PA, PE, PC, PS, phosphatidylglycerol (PG), and PI, respectively. In the lower figure is illustrated the structure for SM, diacylglycerol (DAG), and one configuration of LPC.

Fig. 1.1 Structure of Glycerophospholipid Classes

[Forrester et al., 2004]

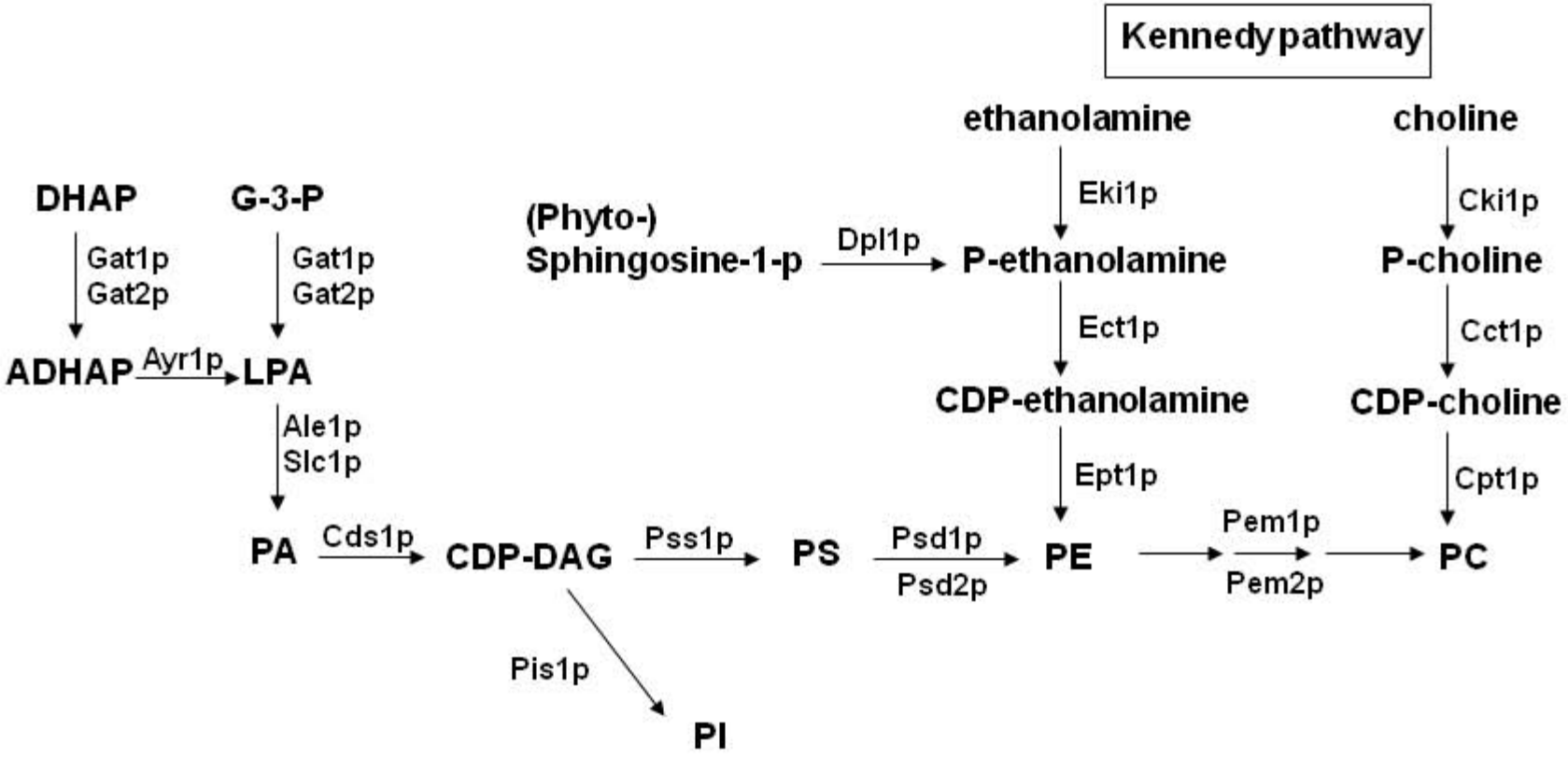


Fig. 1.2 Pathways of major phospholipid synthesis in *S.cerevisiae*

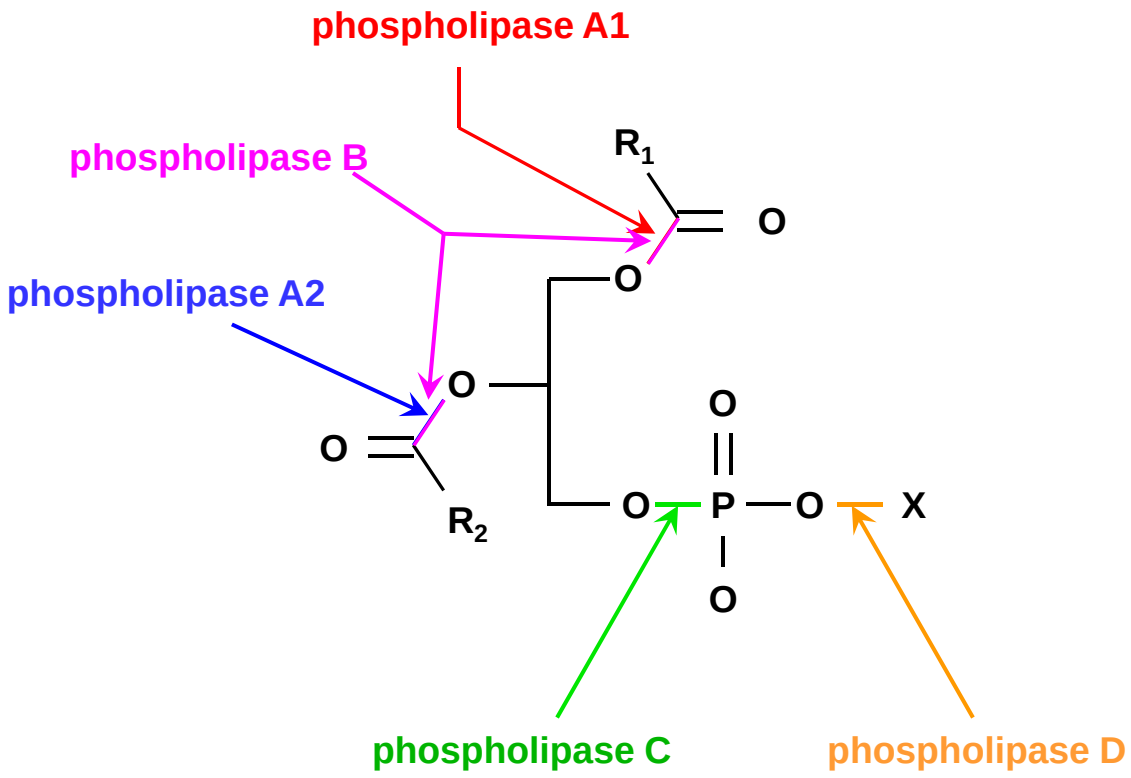


Fig. 1.3 Action sites of phospholipases

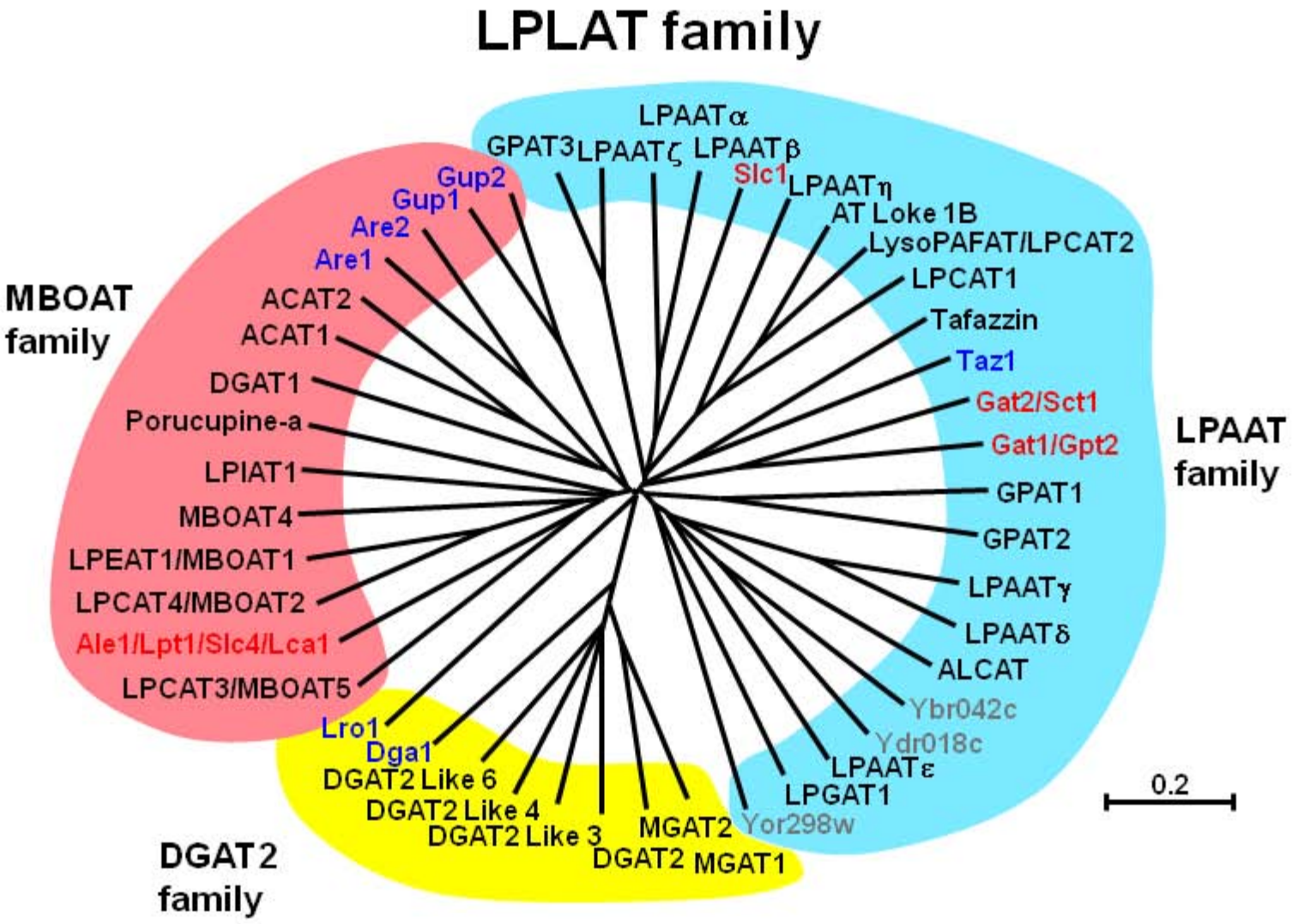


Fig. 1.4 Phylogenetic tree of LPLAT family in mouse and yeast

The phylogenetic tree of amino acid sequences of LPAAT, DGAT2 and MBOAT family members in mouse drawn by Hishikawa et al. (Hishikawa et al., 2008) was modified with yeast LPLATs using ClustalW.

Chapter 2

Uptake and remodeling of exogenous phosphatidylethanolamine in *Saccharomyces cerevisiae*

2.1 Introduction

For the research of PE remodeling, Kakihara in our laboratory constructed a yeast mutant, TKY12Ga, in which both *PSD1* and *PSD2* were deleted and the promoter of *ECT1* encoding a key enzyme in PE synthesis through the Kennedy pathway, was replaced with the galactose-inducible and glucose-repressible *GAL1* promoter in its chromosomal location (Kakihara, 2004). In a medium containing galactose as a carbon source, the expression of *ECT1* was induced and the mutant grew using PE synthesized via the Kennedy pathway in the presence of ethanolamine. In contrast, the expression of *ECT1* was repressed and its growth was arrested after 10 to 11 cell divisions in a glucose-containing medium (SD medium). This time lag before cell growth arrest was probably due to the time required to consume residual Ect1p, ethanolamine, and/or PE. However, when didecanoyl PE (diC10PE) was added to the SD medium, TKY12Ga grew despite the inability to synthesize PE (Kakihara, 2004). Since a 10-carbon-fatty acyl residue appears to be too short to maintain the structure and function of the biological membrane, diC10PE was assumed to be remodeled to those with acyl chains of normal length to support the growth of the mutant.

In this chapter, the mechanism of uptake of diC10PE into cells was analyzed. Furthermore, the remodeling of diC10PE in TKY12Ga was examined using electrospray ionization tandem mass spectrometry (ESI-MS/MS).

2.2 Materials and Methods

2.2.1 Strains

W3031A	*Mat a, his3, leu2, ura3, trp1, ade2*
TKY12Ga	*Mat a,* P_{GAL1}*ECT1::HIS3, Δpsd2::LEU2, ura3,*

	trp1, ade2, Δpsd1::KAN^r (Kakihara, 2004)
TKY12Ga*Δlem3*	*Mat a*, P_{GAL1}*ECT1::HIS3, Δpsd2::LEU2, ura3, trp1, ade2, Δpsd1::KAN*^{r}, Δlem3*::TRP1* (This study)
TKY12Ga*Δdnf1*	*Mat a*, P_{GAL1}*ECT1::HIS3, Δpsd2::LEU2, ura3, trp1, ade2, Δpsd1::KAN*^{r}, Δdnf1*::URA3* (This study)
TKY12Ga*Δdnf1Δdnf2*	*Mat a*, P_{GAL1}*ECT1::HIS3, Δpsd2::LEU2, ura3, trp1, ade2, Δpsd1::KAN*^{r}, Δdnf1*::URA3* Δdnf2*::TRP1* (This study)

2.2.2 Media

SD medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-glucose (Wako)	2%

SG medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-galactose (Wako)	2%

YPD medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-glucose (Wako)	2%

YPG medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-galactose (Wako)	2%

For adding exogenous PEs to medium, PE is dissolved in ethanol by a 30 min sonication and then added to medium with a final ethanol concentration of 1% in the medium.

For solid medium, 2% agar (Wako) was added.

When required, add the amino acid and antibiotic as the following concentration:

L-Histidine (NACALAI)	2.4 μg/ml
L-Leucine (Kanto Kagaku)	10 μg/ml
Uracil (Kanto Kagaku)	2.4 μg/ml
L-Tryptophan (Kanto Kagaku)	4 μg/ml
Adenine Hydrochloride (Kanto Kagaku)	2.4 μg/ml

When TKY12Ga or the stains derived from it were cultured, choline chloride (Kanto Kagaku) was always supplied to the glucose containing medium at the final concentration of 1 mM; 2-Aminoethanol (NACALAI) and choline chloride (Kanto Kagaku) were always supplied to the galactose containing medium both at the final concentration of 1 mM.

2.2.3 Chemicals

Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL); deuterium-labeled ethanolamine was purchased from CDN isotopes. Other chemicals used were of analytical grade or above from Wako (Tokyo, Japan), Kanto chemicals (Tokyo, Japan), Sigma chemicals (St. Louis, Mo., USA).

2.2.4 Methods

2.2.4.1 Polymerase chain reaction (PCR)

In this chapter, PCR kit of Takara Ex Taq^{TM} was used in all PCR reactions and the reactions were carried out in a PCR Thermal Cycler MP (Takara, Japan). The reaction conditions and program parameters were as indicated in the manufacturer's instruction.

2.2.4.2 Recovery of DNA fragment from agarose gel

DNA fragments were extracted from agarose gel by using Quantum Prep Freeze N Squeeze Spin Column (BIO-RAD), according to the manufacturer's instruction.

2.2.4.3 DNA sequencing

DNA sequencing was performed by the utilization of BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) and ABI PRISM 310 Genetic Analyzer, according to the manufacturer's instruction.

2.2.4.4 Construction of gene disruption cassettes

The construction of gene disruption cassettes for *LEM3, DNF1,* and *DNF2* followed the strategy of Nikawa et al. (Nikawa et al., 1998). The marker gene of deletion constructs for *LEM3* and *DNF2* genes was *TRP1* originated from pT-TRP1 and the marker gene of deletion constructs for *DNF1* was *URA3* derived from pT-URA3.

The primers that used in construction was shown as follows:

YNL323-A-f	5'-TGAGGACTATCGTAAAGAGAC-3'
YNL323-A-r	5'-CTCCTTTGTCCTTTCTACGG-3'
YNL323-B-f	5'-GGCGGTAGAAAGATTGCTGA-3'
YNL323-B-r	5'-TCCTCCAGTGTAAAGACGAC-3'
DNF1-A-f	5'-GAAGGTCATCGTAATACGCA-3'
DNF1-A-r	5'-GGGCATATTGTAATACACAGTC-3'
DNF1-B-f	5'-CTATTGCTGTAATTTCGTGT-3'
DNF1-B-r	5'-TTGATGGATATTAGCGAATG-3'
DNF2-A-f	5'-GCCAGATTTTTACTAGACGCG-3'
DNF2-A-r	5'-CTCAATATCATCCACGAAGGG-3'
DNF2-B-f	5'-CAAGCTCGTCGTCAAGTAAC-3'
DNF2-B-r	5'-AGCATCTCTTCTCTTGTGCG-3'

2.2.4.5 Transformation of *S. cerevisiae*

Transformation of *S. cerevisiae* was done according to Gietz (Gietz, R.D. and R.A.Wood., 2002) with slight modification.

① Incubate the yeast strain overnight in YPD or YPG medium at 30℃ on a shaking

incubator.

② Determine the cell density by measuring OD_{600} and dilute to 3 x 10^6 cells/ml (OD_{600}=0.2) in 10 ml YPD or YPG medium. Grow to a density of 1~2 x 10^7 cells/ml (OD_{600}=0.5~1.0).

③ Harvest the cells by centrifugation at 4000 rpm for 10 min, wash the cells in 5 ml of sterile water and resuspend in 1 ml of sterile water.

④ Boil the carrier salmon sperm DNA for 5 min and chill in an ice/water bath for at least 5 min while harvesting the cells.

⑤ Make the mixture of 240 μl of 50% PEG 4000 (w/v), 36 μl of 1 M LiOAc, 50 μl of boiled carrier salmon sperm DNA, and 34 μl of DNA plus water. Keep the mixture on ice.

⑥ Transfer the cell suspension to a 1.5 ml microcentrifuge tube, centrifuge at 10,000 rpm for 30 sec and discard the supernatant.

⑦ Add the mixture made in step ⑤ to the cells and resuspend the cells.

⑧ Incubate the cells in a 42℃ water bath for 40 min.

⑨ Collect the cells by centrifuge and plate onto proper selection medium.

2.2.4.6 Preparation of total DNA from *S. cerevisiae*

Preparation of yeast total DNA was performed by the using GenTLE Kit (TaKaRa), according to the manufacturer's instruction.

2.2.4.7 Southern blotting

Southern blotting was used to confirm the gene disruption. The total DNA of the transformants was extracted, digested with appropriate restriction enzyme and electrophoresed in agarose gel. Gel was soaked with 0.25 M HCl for 10 min and rinsed with distilled water. DNA was blotted onto a positively charged nylon membrane (Hybond N^+, Amersham Pharmacia, UK) according to standard protocols (Ausubel et al., 1990).

Labeling of the probes and detection of the signal was performed by using the AlkPhos direct nucleic acid labeling and detection systems (Amersham Pharmacia biotech, UK) according to manufacture's instruction.

2.2.4.8 Lipid extraction from *S. cerevisiae*

Lipid extraction from *S. cerevisiae* was done according to Bligh and Dyer with slight modification.

① Harvest the yeast cells by centrifugation (3600 rpm at 4℃ for 10 min).

② Wash the cells with 5ml ice-cold 0.15 M KCl for three times, and resuspend in the same solution. Transfer the yeast cells to a round bottom glass centrifuge tube with stopper.

③ Add methanol and chloroform respectively to the cell suspension, to be water:methanol:chloroform=0.5:2:1 (v/v/v).

④ Add 0.5~1.0 g 0.5 mm glass beads per tube and vortex for 30 seconds 6 times and leave at room temperature for 10 min.

⑤ Add 0.2 M KCl-0.1 M HCl (pH 2.0) and chloroform to be water:methanol:chloroform=1.8:2:2 (v/v/v).

⑥ Vortex for 30 seconds and spin at 2500 rpm for 5 min at room temperature.

⑦ Collect the lower chloroform phase and extract the upper phase two more times with chloroform.

⑧ Pool the chloroform phase and add 1.6 volume of chloroform:methanol: 0.2 M KCl-0.1 M HCl (pH 2.0) =3:48:47 (v/v/v).

⑨ Vortex for 30 seconds and spin at 2500 rpm for 5 min at room temperature.

⑩ Collect the lower chloroform phase and if necessary, extract the upper phase one more time with chloroform.

⑪ Evaporate the chloroform phase under a gentle stream of nitrogen at 40℃ in heating block.

⑫ Dissolve with chloroform:methanol=2:1 (v/v)

2.2.4.9 Thin layer chromatography

Phospholipids extracted from yeast or aqueous reaction solution was separated by thin layer chromatography (TLC). TLC plates (Merck, Kieselgel 60) were dried up 2~3 h at 180℃ in the drying oven before use. For one-dimensional TLC, the samples were spotted on a line at 1.5 cm from the bottom of the TLC plates. The TLC plates were then developed in a chamber equilibrated with 100~200 ml of chloroform/methanol/acetic acid (65/25/10, v/v/v). After air-dried, the spots on the TLC plate were visualized by staining with iodine vapor or ninhydrin spray.

For two-dimensional TLC, the sample was spotted as a dot at 2 cm from the corner. After developed in the primary developing solvent, chloroform/methanol/acetic acid (65/25/10, v/v/v), the TLC plate was placed in the draft for 1 h and air-dried. The

plate was developed with the secondly developing solvent, chloroform/methanol/formic acid (65/25/10, v/v/v). After air-dried about 15 min, the spots on the TLC plate were also visualized by stained with iodine vapor or ninhydrin spray.

2.2.4.10 Recovery phospholipids from TLC plate

To isolate phospholipids, phospholipid was exacted from the TLC plate after separation.

① Scrape the target spots from the TLC plate and put into the glass centrifuge tubes.
② Add 1~3 ml of 0.2 M KCl/methanol/chloroform (0.8/2/1, v/v/v).
③ Vortex for 1 min and leave at room temperature for 10 min.
④ Add 0.2 M KCl and chloroform to be 0.2 M KCl:methanol:chloroform=1.8:2:2 (v/v/v).
⑤ Vortex for 1 min and spin at 2500 rpm for 5 min at room temperature.
⑥ Collect the lower chloroform phase and if necessary, extract the upper phase one or two more times with chloroform.
⑦ Evaporate the chloroform phase under a gentle stream of nitrogen at 40℃ in heating block.
⑧ Dissolve with chloroform.

2.2.4.11 Phosphorous assay of phospholipids

The phosphorous assay was done according to the method of Bartlett (Bartlett *et al*., 1959).

① Evaporate organic solvent of the sample to dryness in 100℃ heating block. 0.5~5.0 μg phosphorus is optimal.
② Prepare for working standard: 0, 2, 5 μg phosphorus in duplicate.
③ Add 0.2 ml absolute sulfuric acid to each tube.
④ Put in 180℃drying oven for 30~60 min.
⑤ Add one drop (with transfer pipette) hydrogen peroxide directly to tubes. If color is still present after 1 min, add another drop hydrogen peroxide to every tube and check after 1 min. Do not add more than 3 drops hydrogen peroxide.
⑥ Heat tubes in 180℃ for 40 min to decompose residual hydrogen peroxide.
⑦ Take tubes from drying oven and allow to cool. Add 4.4 ml distilled water and vortex. Add 0.2 ml of 5% fresh ammonium molybdate and vortex immediately.
⑧ Add 0.2 ml of 13.7% (w/v) fresh Fiske SabbaRow and vortex.

⑨ Place tubes in 100℃ boiling water for 15 min.

⑩ Cool to room temperature. Vortex and read OD at 820 nm.

2.2.4.12 Synthesis of deuterium-labeled diC10PE

First, the ethanolamine was neutralized by hydrochloric acid. The diC10PC was dissolved in absolute diethyl ether (4 mg/ml). The lipid-ether solution was diluted with two volumes of 1.17 M ethanolamine hydrochloride in aqueous acetate buffer (100 mM, pH 5.6) containing 100 mM $CaCl_2$. Then the base-exchange reaction was initiated by the addition of cabbage phospholipase D at an enzyme/PC ratio of 3.5 units per μmol. The reaction was carried out at 25℃ on a shaking incubator. After 12 h, equal amount of phospholipase D was added, and reaction proceeded for additional 12 h. To terminate the reaction, the ether was removed by evaporation under a gentle stream of nitrogen and an aliquot of EDTA (500 mM at pH 8.0), equivalent to 20% by volume of the acetate buffer used, was added into the mixture to chelate Ca^{2+} ions. The mixture was shaken vigorously with 2.2 volume of chloroform/methanol (5:6, v/v) in a glass centrifuge tube, and the lower chloroform phase was collected. The upper phase was extracted two more times with chloroform. 1.6 volume of chloroform/methanol/water (3:48:47, v/v/v) was added to the chloroform phase, shaken vigorously, and the lower chloroform phase was collected (This step is to eliminate the residual ions that might disturb the separation of PE and PC by TLC). The chloroform phase were concentrated to 100~200 μl by evaporation under a gentle stream of nitrogen and the concentrate was spotted on a TLC plate and developed as described in 2.2.4.9. After developing, diC10PE was recovered from the TLC plate and an aliquot of it was applied to phosphorous assay for quantification as described in 2.2.4.10 and 2.2.4.11.

2.2.4.13 Mass spectrometry

Electrospray mass spectra were obtained on triple quadrupole instrument (Applied Biosystems API3000). Samples were dissolved in 1 ml acetonitrile/methanol/water (4:4:1) containing 0.1% ammonium formate and infused into the ESI source with a Harvard syringe pump at the flow rate of 10 μl /min according to the method of Houjou et al. (Houjou *et al.*, 2005). The ion spray voltage was set at 5.0 kV for positive scans and -3.8 kV for negative scans. MS/MS experiments were conducted with nitrogen as a collision gas and the collision energy of 42 Vdc. For each spectrum, 20~200 scans were summed.

2.2.4.14 Pulse-chase analysis

Cells were first seeded from SG to SD medium and precultured for 24 h to consume Ect1p and/or PE, and then was shifted to SD medium containing 20 μM non-labeled diC10PE at a starting OD_{600}=5 x 10^{-3}, and cultured till OD_{600} reached 1. Then cells were collected, washed with SD medium for 2 times, and pulse-labeled with 20 μM deuterium-labeled diC10PE for 15 min.

After pulse-labeled, cells were collected by vacuum filtration, wash with SD medium for 2 times, and shifted to SD medium containing 20 μM non-labeled diC10PE, cultured for the indicated time. Then Lipids were extracted as Bligh-Dyer method and analyzed by ESI-MS/MS.

2.2.4.15 Analysis of fatty acid and diC10PE in the whole culture and culture medium

Cells precultured as described above were seeded at a starting OD_{600}= 0.01 to SD medium containing diC10PE, and incubated for 1, 3, 6, 12, and 24 h. Lipids in the whole culture and culture medium were extracted by adding 0.1 volume of 1 M Tris-HCl (pH 8.0) and 1.1 volume of chloroform and mixing vigorously. The lower chloroform phase was collected and the upper phase was extracted two more times with chloroform. Recovery of decanoic acid and diC10PE by this extraction procedure were approximately 80% and 98%, respectively. The extracted lipids were quantified by LC-MS system LCMS-2010EV (Shimadzu, Kyoto, Japan). For separation of the lipids, RP-18 GP (ODS) column (150 x 2 mm i.d.) (Kanto Chemical, Tokyo, Japan) were used. The column was maintained at 40ºC and flow rate was 0.2 mL/min. A binary solvent gradient consisted of (A) 0.05% acetic acid in water and (B) 0.05% acetic acid in acetonitrile. The gradient program was as follows: initial A/B (40:60); linear from 0 to 20 min to A/B (5:95); held isocratic for 10 min.

2.2.4.16 *In vitro* remodeling reaction

Cells were cultured in YPG medium till OD_{600} reached 1.5, and then were collected, washed, and disrupted in buffer {50 mM Tris-HCl (pH7.4), 0.1 M KCl, 10% glycerol, 1 mM DTT, and 1% protease inhibitor} with glass beads. The protein concentration of the cell lysate was measured using Bradford reagent (Bio-Rad, CA).

The reaction mixture contained 50 mM Tris-HCl (pH7.4), 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM palmitoleoyl-CoA, 20 μM deuterium-labeled diC10PE, and cell lysate (0.5 mg protein) in a total volume of 100 μl. The reaction was carried out at 30°C and stopped at the indicated times by addition of 2 volume of chloroform, shaking vigorously. Lipids were extracted as Bligh-Dyer method and analyzed by ESI-MS/MS.

2.3 Results

2.3.1 Growth of TKY12Ga supported by exogenous PE with short acyl chains

The effect of addition of PEs with various fatty acyl chains on the growth of TKY12Ga in SD medium, in which PE synthesis of this strain is repressed, was examined (Fig. 2.1). TKY12Ga was first seeded from SG to SD medium and precultured for 24 h to consume Ect1p and/or PE, and then was seeded at a starting OD_{600}=5 x 10^{-3} to SD medium containing dihexanoyl PE (diC6PE), dioctanoyl PE (diC8PE), didecanoyl PE (diC10PE), dilauroyl PE (diC12PE) or dimyristoyl (diC14PE) at the concentration of 5 μM, 20 μM, 100 μM, or 200 μM, and cultured for 48 h. Then, OD_{600} of the yeast culture was measured. As shown in the Fig. 2.1, all PE species supported the growth of TKY12Ga on the SD medium, but the effects varied according to the length of acyl chains. Among PEs tested, the growth of TKY12Ga was supported most effectively by diC10PE, and the growth in the presence of PEs with shorter or longer acyl chains was less efficient. The addition of diC10PE with the concentration from 20 μM to 200 μM could all support the growth of TKY12Ga well and the optimum concentration was around 20 μM.

2.3.2 Phospholipid composition of TKY12Ga cultured in SD medium containing diC10PE

Kakihara showed that the amount of PE was 8% of the major phospholipids when TKY12Ga was cultured in SG medium, and the amount of PE reduced to 1%, accompanied by the growth repression when cultured in SD medium for 48 h. Here, the amount of intracellular PE of TKY12Ga when cultured in SD medium containing diC10PE was determined.

TKY12Ga was first seeded from SG to SD medium and precultured for 24 h to consume Ect1p and/or PE, and then was shifted to SD medium containing diC10PE and to SG medium at a starting OD_{600}=5 x 10^{-3}, and cultured for 48 h. After 48 h, the

phospholipid of the yeast was extracted and separated by two-dimensional TLC. The spots of phospholipids were scraped from the TLC plate and quantified by phosphorous assay as described in Materials and Methods.

In Fig. 2.2, the relative contents of major phospholipids of TKY12Ga cultured in SD medium containing diC10PE and SG medium is shown. As previously reported, the PE content of TKY12Ga grown in SG medium was approximately 8% of the major phospholipids. In contrast, the PE content of TKY12Ga cultured in SD medium containing diC10PE was approximately 2%, indicating that this amount of PE is sufficient to sustain the growth of yeast.

2.3.3 Uptake of PE with short acyl chain

It was suggested that Lem3p was required for ATP-dependent translocation of PC and PE across the plasma membrane and deletion of the *LEM3* gene resulted in a marked decrease in the uptake of fluorescence-labeled analogs of PE and PC (Kato et al., 2002). Both Dnf1p and Dnf2p have P-type ATPase activity and function in inward-directed transport of NBD-PE, NBD-PC, and NBD-PS (Paulsen et al., 1998) (Pomorski et al., 2003) (Saito et al., 2004) (Elvington et al., 2005). It was also reported that single deletion of each gene displayed reduced internalization of fluorescently-labeled PE as compared to wild type, and the double null mutant displayed greater reduction than that of either single mutant (Iwamoto et al., 2004). It is likely that these three genes would also be involved in the uptake of PE with short acyl chains. Therefore these three genes in TKY12Ga were deleted and the growth of null mutants in SD medium containing PE with short acyl chains was examined.

Gene disruption cassettes were constructed as described in 2.2.4.4, and introduced into TKY12Ga as described in 2.2.4.5. The deletion was confirmed by PCR (data not shown) and Southern analysis.

The restriction enzyme used in the Southern analysis of deletion mutant of *LEM3* gene was *Eco*T22I. Its restriction sites are shown in Fig. 2.3A. *Eco*T22I treatment resulted in a 1.5-kb detectable band for TKY12Ga, and a 2.4-kb detectable band for *lem3* null mutants (Fig. 2.3B).

In the verification Southern analysis to verify the *DNF1* gene deletion, the restriction enzyme *Eco*RV was used. Its restriction sites are shown in Fig. 2.4A. *Eco*RV treatment resulted in a 6.7-kb detectable band for TKY12Ga, and a 3.5-kb detectable band for *dnf1* null mutants (Fig. 2.4B).

To verify the deletion of *DNF2* gene, Southern analysis was performed using the

restriction enzyme *Xba*I. Its restriction sites are shown in Fig. 2.4C. *Xba*I treatment resulted in an 8.0-kb detectable band for the parent strain, and a 2.6-kb detectable band for *dnf2* null mutants (Fig. 2.4D).

The growth of these null mutants in SD medium containing diC10PE was examined (Fig. 2.5). As shown in the Fig. 2.5, deletion of *DNF2* caused slight growth impairment in the PE-containing medium, while deletion of *DNF1* did not. However, deletion of both *DNF1* and *DNF2* conferred severer growth defect, suggesting their redundant functions in the uptake of diC10PE. In contrast, deletion of *LEM3* caused most significant growth impairment. These results indicate that the growth of TKY12Ga in SD medium containing diC10PE required uptake of PE with short acyl chains or its metabolites from the medium, and the Lem3p and two P-type ATPases, Dnf1p and Dnf2p, are involved in this process.

2.3.4 Remodeling of PE

As mentioned above, the growth of TKY12Ga in SD medium can be supported by the addition of PEs with short acyl chains, especially by diC10PE, and this support was dependent on its ability to uptake PEs with short acyl chains from the media. Since it was reported that the carbon length of acyl chains of endogenous PE in yeast was mainly from 14 to 18 (Schneiter et al., 1999), it is of great interest to investigate how TKY12Ga utilizes PE with short acyl chains. Is PE with short acyl chains converted to the one with acyl chains of normal length? If converted, what is the mode of conversion, elongation of its acyl chains on PE or exchange (remodeling) of single/both short acyl chains with longer ones? To clarify the mode of PE remodeling, the metabolism of diC10PE, of which ethanolamine moiety was labeled with deuterium, after being incorporated into the cell was chased by using the electrospray ionization tandem mass spectrometry (ESI-MS/MS).

2.3.4.1 Synthesis of Deuterium-Labeled DiC10PE

Because PE labeled with stable isotope is not commercially available, the deuterium-labeled diC10PE was synthesized by base-exchange reaction of diC10PC and deuterium-labeled ethanolamine ($HOCD_2CD_2NH_2$) which is catalyzed by phospholipase D. Phospholipase D is known with its phosphatidohydrolase activity that hydrolyze the phosphodiester bonds of phospholipids and sphingomyelin to give the corresponding phosphatidic acid *in vitro*. However, it was also reported that phospholipase D prepared

from cabbage had the base-exchange activity, but the suggested conditions were largely different to each other (Saito et al., 1974) (Xu et al., 1988). Since the deuterium-labeled ethanolamine is an expensive substrate, screening for the optimum condition to synthesize diC10PE with minimum ethanolamine was performed, for pH, concentration of Ca^{2+}, reaction temperature, reaction time, solvent to dissolve diC10PC, and amount of enzyme, diC10PC and ethanolamine.

The diC10PE was synthesized with the highest efficiency when the base-exchange reaction proceeded similarly with Xu et al., using the acetate buffer (pH 5.6) containing 100 mM $CaCl_2$ at 25°C for 24 h as described in Materials and Methods (Xu et al., 1988). Typically, 12 mg of deuterium-labeled diC10PE was obtained from 1 g of deuterium-labeled ethanolamine and 27 mg of diC10PC.

2.3.4.2 Remodeling of Deuterium-Labeled DiC10PE in TKY12Ga

The metabolism of deuterium-labeled diC10PE in TKY12Ga was analyzed using ESI-MS/MS. After cells of TKY12Ga were precultured and cultured as described in 2.2.4.14, they were pulse-labeled with 20 μM deuterium-labeled diC10PE for 15 min. Then lipids were extracted from the labeled cells and subjected to ESI-MS/MS.

To analyze the molecular species of PE, two types of scans were performed by the ESI-MS/MS. One is the neutral loss scan of *m/z* 145 in positive ion mode, specific for ethanol-1,1,2,2,-d_4-amine phosphate headgroup, giving the m/z values of all the protonated molecules that contain the polar deuterium-labeled ethanolamine phosphate headgroup (Brugger et al., 1997). With neutral loss scan for a headgroup survey, it is possible to deduce the total length of the two fatty acyl groups from the data of m/z values, but the precise information on the length of each fatty acyl group cannot be elucidated. Thus as the second type of scan, precursor ion scan in the negative ion mode was performed to detect phospholipid containing targeted carboxylate anions. Scans of precursor ion m/z 171, 199, 197, 195, 227, 225, 223, 255, 253, 251, 249, 283, 281, 279, 277, 311, 309, 307, 305, and 303 were for 10:0, 12:0, 12:1, 12:2, 14:0, 14:1, 14:2, 16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, and 20:4 fatty acid, respectively (Houjou et al., 2005).

After these scans, the molecular species profile of PE can be deduced with its m/z value and the fatty acid composition. As shown in Fig. 2.6, when TKY12Ga was incubated with deuterium-labeled diC10PE for 15 min, a portion of the deuterium-labeled diC10PE had been remodeled partially, as evidenced by the appearance of the peaks at *m/z* 610.4 and 638.5, corresponding to PEs, in which one

fatty acyl chain was replaced with a longer monounsaturated C16:1 and C18:1 acyl chains, respectively (see below). In addition, peaks corresponding to PEs, in which two fatty achy chains were replaced with C16:1 or C18:1 acyl chain, also appeared, as indicated by the peak at *m/z* 692.5, 720.5 and 748.6, respectively. These results suggest that PE with short acyl chains was remodeled to PEs containing normal acyl chains.

2.3.4.3 Structural analysis of the remodeling intermediates

Peaks at *m/z* 610.4 and 638.5 as observed in Fig. 2.6 corresponded to 26:1-PE and 28:1-PE, respectively. Both these species were also detected by precursor ion scan for *m/z* 171 in negative ion mode, which identifies phospholipid species containing acyl chain of 10:0. In addition, 26:1-PE and 28:1-PE were detected by precursor ion scans for *m/z* 253 and 281 in negative ion mode, which identify phospholipid species containing acyl chains of 16:1 and 18:1, respectively (data not shown, see below). These results strongly suggest that 26:1-PE and 28:1-PE are the remodeling intermediates, in which one fatty acyl chain was replaced with a longer monounsaturated C16:1 and C18:1 acyl chains.

Next, the structures of 10:0-16:1-PE and 10:0-18:1-PE were analyzed in detail. Product ion scan by MS/MS provides information regarding acyl chains at *sn*-1 and *sn*-2 position in PC. When an appropriate amount of collision energy is added, the acyl moiety at *sn*-2 position in PC can be more easily fragmented than that at *sn*-1 position (Ekroos et al., 2003) (Houjou et al., 2004). We analyzed the fragmentation pattern of 1-palmitoyl-2-oleoyl-PE (POPE) as a standard by product ion scan for *m/z* 716 in negative ion mode (Fig. 2.7A). The intensity of the peak at *m/z* 281.2, which corresponded to 18:1 acyl chain at *sn*-2 position of POPE, were higher than that of at *m/z* 255.2, which corresponded to 16:0 at *sn*-1 position, suggesting that this analysis is also applicable to PE. In Fig. 2.7B and C, we analyzed the fragmentation pattern of the remodeling intermediates, 10:0-16:1-PE and 10:0-18:1-PE. By product ion scan for *m/z* 608 in negative ion mode, acyl chains fragmented from 10:0-16:1-PE were detected, and the intensity of the peak at *m/z* 253.2, which corresponded to 16:1 acyl chain, was higher than that of *m/z* 171.1, which corresponded to 10:0 acyl chain (Fig. 2.7B). Similar result was obtained by the analysis of 10:0-18:1-PE, as intensity of the peak at *m/z* 281.2, which corresponded to 18:1 acyl chain, was higher than that of 10:0 acyl chain (Fig. 2.7C). These data suggest that the majority of the remodeling intermediates contained C16:1 or C18:1 acyl chain at *sn*-2 position, indicating that the PE remodeling

reaction was mainly started at *sn*-2 position.

The water-soluble metabolites of diC10PE, which were partitioned in the aqueous phase were also extracted, and their molecular profiles were analyzed. After 15 min incubation with deuterium-labeled diC10PE, the peak of m/z 374.2, corresponding to lyso-10:0-PE was observed, suggesting that lyso-10:0-PE was also an intermediate of remodeling of diC10PE (Fig. 2.8).

2.3.4.4 Pulse-chase analysis of the metabolism of deutium-labeled diC10PE in TKY12Ga

After 15 min pulse-labeling with 20 μM deuterium-labeled diC10PE, TKY12Ga was chased with 20 μM non-labeled diC10PE for 15, 30, 60 and 180 min. PE species of TKY12Ga in these incubations were analyzed by ESI-MS/MS (Fig. 2.9) and quantified (Fig. 2.10). Quantitative data was expressed as ratios of the peak intensity of each PE molecular species to that of the internal standard. As shown in Fig. 2.9 and Fig. 2.10, both of the 10:0-16:1-PE and 10:0-18:1-PE decreased immediately during chase period, suggesting that these PE species are the intermediates of remodeling reaction again. On the other hand, the 16:1-16:1-PE and 16:1-18:1-PE also decreased slightly, probably because these PE were used as substrates to synthesize PC or were degraded by some type(s) of phospholipase.

2.3.4.5 Decanoic acid was released to medium when TKY12Ga was cultured with diC10PE

Quantification of diC10PE and decanoic acid in the whole culture and the culture supernatant using LC-MS was performed when TKY12Ga was cultured in SD medium containing 20 μM diC10PE for 0, 1, 3, 6, 12 and 24 hrs. As shown in Fig. 2.11, an increasing amount of decanoic acid was detected in both the whole culture and the culture supernatant during the incubation in diC10PE containing medium, accompanied by the decrease of diC10PE. The amount of released decanoic acid in the culture supernatant was approximately twofold of the consumed diC10PE. These results suggest that most of C10 fatty acid cleaved from diC10PE were excreted into culture medium and it could be concluded that yeast remodeled diC10PE by removing C10 fatty acid and reacylating with normal length acyl chain, but not by elongating the acyl chains.

2.3.4.6 Remodeling reaction *in vitro*

Eguchi in our laboratory analyzed the remodeling of diC8PC *in vitro* using yeast lysate and palmitoleoyl-CoA (Eguchi, unpublished). The PC remodeling reaction mixture contained 25 mM Hepes-KOH (pH7.4), 1 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, 1 mM ATP, 0.4 mM palmitoleoyl-CoA, 20 μM (methyl-$^{13}C)_3$-diC8PC, and cell lysate (0.5 mg protein) in a total volume of 100 μl. Base on this system, screening for optimum condition of *in vitro* PE remodeling was performed.

Cell lysate of TKY12Ga was incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA in the presence of Ca^{2+}, Mn^{2+} and/or Mg^{2+} at various concentration for 60 min as described in Materials and Method. As shown in Fig. 2.12A, in the absence of divalent cation, a small amount of 10:0-16:1-PE was detected. Addition of Ca^{2+} caused disappearance of the substrate deuterium-labeled diC10PE, but little remodeled PE was detected. Addition of 1 or 5 mM Mn^{2+} facilitated the PE remodeling reaction, but not as efficiently as the addition of same concentration of Mg^{2+}. Addition of 1, 5, 10, or 15 mM Mg^{2+} facilitated the PE remodeling reaction, and 10 mM is most effective, suggests the enzyme(s) involved in PE remodeling is/are Mg^{2+}-dependent.

As shown in Fig. 2.12B, addition of both of Mn^{2+} and Mg^{2+} was also less efficiency than individual addition of same concentration of Mg^{2+}.Therefore, the *in vitro* PE remodeling experiment was performed in the presence of 10 mM Mg^{2+}.

In vitro PE remodeling reaction experiment was performed by incubation of cell lysate of TKY12Ga with deuterium-labeled diC10PE and palmitoleoyl-CoA for 15, 30, 60, and 180 min (Fig. 2.13). Quantitative data of this experiment was expressed as ratios of the peak intensity of each PE molecular species to that of the internal standard (Fig. 2.14). As shown in Fig. 2.14, deuterium-labeled diC10PE decreased during the incubation. A small portion of deuterium-labeled 10:0-16:1-PE was detected after 15 min incubation, and its amount was increased with the prolonged incubation. In addition, deuterium-labeled 10:0-18:1-PE, 16:1-16:1-PE and 16:1-18:1-PE were detected after 60 min incubation. These results suggest the PE remodeling reaction can be reconstituted *in vitro*. The appearance of deuterium-labeled 10:0-18:1-PE and 16:1-18:1-PE indicated that oleoyl-CoA existed in yeast cell lysate was utilized in the remodeling reaction *in vitro*.

2.4 Discussion

2.4.1 The growth of TKY12Ga in SD medium supported by diC10PE

When TKY12Ga was cultured in the SD medium in which its synthesis of PE was repressed, 20 μM diC10PE supported its growth most efficiently among PEs containing acyl chains of various carbon number. PC with octanoic acids or decanoic acids supported the growth of *cho1/pss* mutant more efficiently than that with butanoic acids, hexanoic acids, or dodecanoic acids (Yon et al., 1998). The probable reason for the inefficiency of growth support by phospholipid with longer acyl residues is that they are less soluble in water due to their longer hydrophobic acyl chains and this property interferes with their access to the plasma membrane of yeast cells. In contrast, phospholipids with shorter acyl residues, which have more hydrophilic characteristics, could be toxic to the yeast cells due to their amphipathicity as detergents.

2.4.2 Growth of TKY12Ga supported by diC10PE is dependent on Lem3p and P-type ATPases, Dnf1p and Dnf2p

Deletion of *LEM3* or double deletion of *DNF1* and *DNF2* in TKY12Ga caused severe growth defect in SD medium containing diC10PE. These suggest that Lem3p, Dnf1p, and Dnf2p, are involved in uptake of diC10PE or its metabolites. Dnf1p and Dnf2p are members of type IV P-type ATPase, which is proposed to be involved in aminophospholipid translocation in various membranes. Lem3p interacts with Dnf1p and Dnf2p, and is required for proper intracellular localization of Dnf1p, although it is not clear whether Lem3p has some additional function in regulation of Dnf proteins on the plasma membrane or not (Furuta et al., 2007; Noji et al., 2006; Saito et al., 2004). Since the growth defect of TKY12GaΔ*lem3* was severer than that of TKY12GaΔ*dnf1*Δ*dnf2* in SD medium containing diC10PE, if is likely that Lem3p has some additional functions, which is independent of Dnf1p or Dnf2p. In agreement with this, deletion of *LEM3* conferred severer effect than the double deletion of *DNF1* and *DNF2* effects both on internalization of fluorescence-labeled PE and on sensitivity to Ro09-0198, a PE-binding antibiotic (Iwamoto et al., 2004). *S. cerevisiae* has three additional type IV P-type ATPases, Neo1p, Drs2p, and Dnf3p. It was also reported that Lem3p interacts with Drs2p, which localize in Golgi membrane (Saito et al., 2004). Deletion of *LEM3* may cause dysfunction of Drs2p or other P-type ATPases.

Voelker's group reported that lyso-PE and lyso-PC are incorporated into yeast cells by the mechanism dependent on Lem3p and two P-type ATPases, Dnf1p and Dnf2p, and are acylated to PE and PC, respectively (Riekhof and Voelker, 2006;

Riekhof et al., 2007b). Therefore, it is possible that diC10PE is extracellularly converted to lyso-10:0-PE and then incorporated into cells. In agreement with this, lyso-10:0-PE was also detected in the culture supernatant of the Δ*lem3* cells that appear to have defects in the incorporation of diC10PE or its metabolites (data not shown). Alternatively, diC10PE might be incorporated into cells in intact form and remodeled in the cells.

2.4.3 PE content of TKY12Ga cultured in SD medium containing diC10PE

Kakihara showed when TKY12Ga was cultured in SD medium, the amount of PE was reduced to 1% of major phospholipids, accompanied by the growth repression. Storey *et al.* reported a *Δpsd1* strain grew normally at 30^{o}C in glucose in the absence of exogenous choline or ethanolamine, but at elevated temperature (37^{o}C), the growth of the mutant decreased largely to about 10% of wild-type strain; besides, on a nonfermentable carbon source, lactate, *Δpsd1* mutant did not grow in the absence of ethanolamine or choline, but supplementation with either nutrient restored wild-type growth in both conditions. The reduced growth and viability correlate with a PE content below 4% of total phospholipid. In addition, a *Δpsd1Δpsd2* strain, which makes low levels of PE from sphingolipid breakdown, its growth in SD medium is very limited even at 30^{o}C, but could be rescued by supplement of ethanolamine, choline, or the ethanolamine analogue propanolamine. *Δpsd1Δpsd2* cells grown in the presence of propanolamine accumulated a novel lipid, phosphatidylpropanolamine (PtdPrn). The absolute level of PE required for growth when PtdPrn was present appeared to be 1% of the total phospholipid content (Storey et al., 2001). All these data suggested that there is a critical level of PE required to support growth. As shown in 2.3.2, the amount of intracellular PE was about 2% of the major phospholipids when TKY12Ga was cultured in SD medium containing diC10PE. These results suggest that 2% PE could sustain the growth of yeast.

2.4.4 Remodeling of diC10PE in TKY12Ga

The MS/MS analysis indicated that the deuterium-labeled diC10PE was rapidly converted to PEs containing acyl chains of normal length. Since the ethanolamine branch of the Kennedy pathway is blocked in TKY12Ga grown in the medium containing glucose by repression of *ECT1* encoding the rate limiting enzyme of this pathway, it is highly unlikely that diC10PE is degraded to ethanolamine or

phosphoethanolamine by phospholipase D or phospholipase C activities, respectively, and utilized to synthesize PE through this pathway. In accordance with this, the peaks corresponding to the potential remodeling intermediates, lyso-10:0-PE, 10:0-16:1-PE, and 10:0-18:1-PE, were detected, and both of 10:0-16:1-PE and 10:0-18:1-PE were decreased during the chase period (Fig. 2.9 and 2.10). In addition, substantial amount of decanoic acids were recovered in the culture supernatant when the growth of TKY12Ga was supported by diC10PE (Fig. 2.11). These compelling evidence strongly suggest that C10:0 acyl residues in diC10PE are substituted with normal acyl residues in the cells and the decanoic acid, probably an unfavorable byproduct, is excreted into the culture medium.

The detailed analysis of the structure of the remodeling intermediates, 10:0-16:1-PE and 10:0-18:1-PE suggested that remodeling of most diC10PE starts with the exchange of *sn*-2 acyl chain followed by the exchange of *sn*-1 acyl chain. This is in good agreement with the remodeling of PE in mammalian cells observed by Kainu et al., in which PE species containing two saturated fatty acids are remodeled very rapidly, initially at the *sn*-2 position by substitution with mainly unsaturated 18:1 fatty acyl chain and subsequently at *sn*-1 position by substitution with similar fatty acyl chain (Kainu et al., 2008). In this respect, importantly, the remodeling pathway of PE containing short acyl chains differs from that of PC containing short acyl chains in yeast. The initial substitution with saturated 16:0 acyl chain in PC containing octanoic acids in yeast cells prefers the *sn*-1 position while the substitution by unsaturated 16:1 acyl chain starts at both *sn*-1 and *sn*-2 positions (Tanaka et al., 2008). The difference in the order of the *sn*-1 *versus sn*-2 remodeling and in molecular species of introduced acyl residues can be attributed to the substrate specificities of phospholipases and acyltransferases involved in the remodeling of these phospholipids and the composition of the pool of acyl donors, as proposed by Kainu et al (Kainu et al., 2008).

In the pulse-chase analysis of the metabolism of deuterium-labeled diC10PE in TKY12Ga, it was shown that the remodeling intermediates, both 10:0-16:1-PE and 10:0-18:1-PE, decreased immediately during chase period. But the amount of 16:1-16:1-PE and 16:1-18:1-PE did not increased, instead, it decreased slightly (Fig. 2.9). One possibility is that these PE were methylated by Pem1p and Pem2p to synthesize PC. Another possibility is that they were hydrolyzed by phospholipase C or D, and since Kennedy pathway is blocked in TKY12Ga, the degraded metabolite, deuterium-labeled ethanolamine or phosphoethanolamine, cannot be recycled, which lead to the decrease of deuterium-labeled PEs.

2.4.5 Remodeling reaction *in vitro*

PE remodeling reaction was reconstituted *in vitro*. In the optimum divalent cation concentration screening process, it was found that addition of Ca^{2+} caused disappearance of the substrate deuterium-labeled diC10PE, but little remodeled PE was detected. The probabe reason is that in the presence of Ca^{2+}, diC10PE was hydrolyzed by some type of phospholipase, consistent with the previous reports that yeast has a Ca^{2+}-dependent phospholipase D which preferentially utilizes PE over PC as a substrate and that Ca^{2+} increased the activity of Plb1p *in vitro* at pH 7.0 (Tang et al., 2002) (Merkel et al., 2005; Waksman et al., 1997). In addition, although no PE-specific phospholipase A and C activity has been reported in S. *cerevisiae*, various phospholipase A2 are Ca^{2+}-dependent in mammalian cells, and Plc1p, the PI-specific phospholipase C in yeast, is also Ca^{2+}-dependent (De Maria et al., 2007; Hirabayashi et al., 2004; Mueller-Roeber and Pical, 2002).

It has been shown that *in* vitro PE remodeling reaction was performed with the highest efficiency when the reaction mixture contained 10 mM Mg^{2+}. It is quite different with that in the *in vitro* PC remodeling system, which contained 1 mM Mg^{2+}, 5 mM Mn^{2+} and 5 mM Ca^{2+}, suggesting that different enzyme(s) is/are involved in the remodeling of PE and PC in yeast. Alternatively, different cation compositions are required for the same enzyme to maintain its specific structure for different substrates, since shape of PE and PC is quite different in that PE contains a much smaller hydrophilic head than PC.

In the *in vitro* analysis of PE remodeling, as shown in Fig. 2.14, diC10PE decreased immediately, but the increase of remodeled PEs was very limited. Since large amount of C10FA and lyso-10:0-PE was detected in reaction solution (data not shown), reacylation might be the limiting step of PE remodeling or this reaction system was not optimum for acytranferase activity of the enzyme(s) involved in PE remodeling. In the analysis of acyltransferase activity of Ale1p and Slc1p, 5 mM Mg^{2+} was added to reaction mixture by Benghezai's group and no addition of divalent cations by Jain's and Riekhof's groups (Benghezal et al., 2007) (Jain et al., 2007) (Riekhof et al., 2007b). In the analysis of acyltransferase activity of proteins of MBOAT family, conditions of divalent cations were similar to those of Ale1p and Slc1p, addition of 5 mM Mg^{2+} or no addition of divalent cations to reaction mixture (Soupene et al., 2008) (Lee et al., 2008) (Hishikawa et al., 2008).

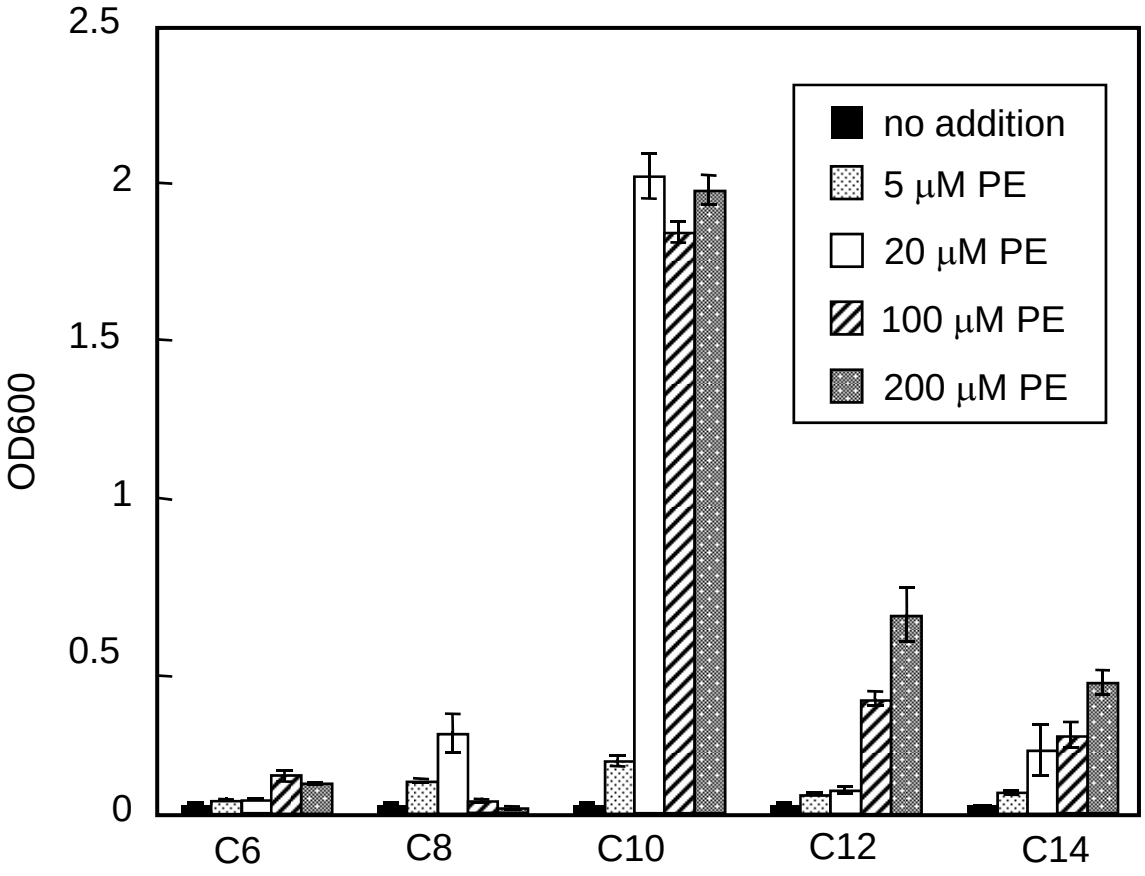


Fig. 2.1 Effect of PE with Short Acyl Chains on Growth of TKY12Ga Defective in PE Synthesis.

TKY12Ga was first seeded from SG medium to SD medium at a starting OD_{600}=5 x 10^{-3} and precultured for 24 h to consume Ect1p and/or PE. After that, TKY12Ga was shifted to SD media containing diC6PE, diC8PE, diC10PE, diC12PE, or diC14PE with concentration of 5 μM, 20 μM, 100 μM, or 200 μM, at a starting OD_{600}=5 x 10^{-3} and cultured for 48 h. Then, OD_{600} of the yeast culture was measured. Results represent the means of three parallel experiments ± SE.

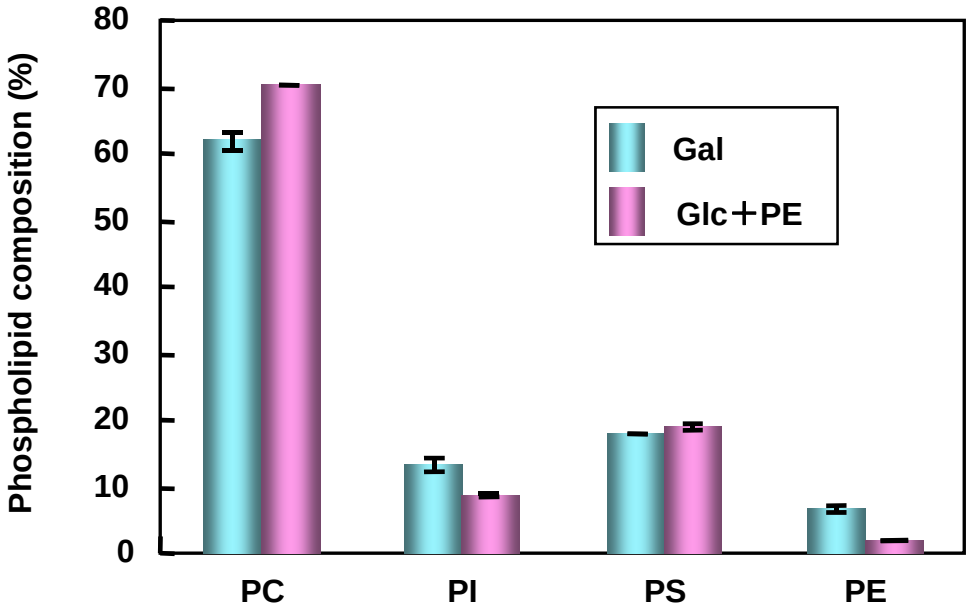
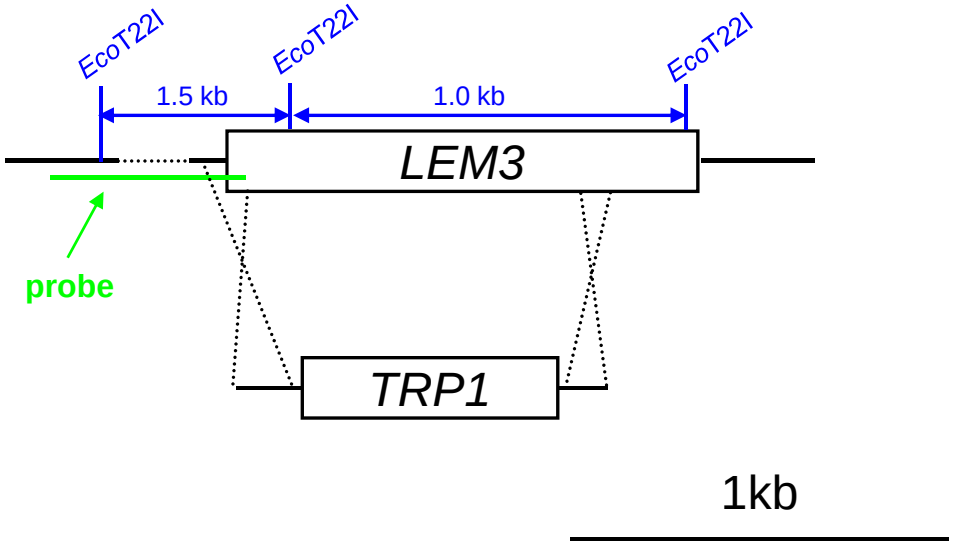


Fig. 2.2 Composition of major phospholipid of TKY12Ga.

TKY12Ga was first seeded from SG medium to SD medium at a starting OD_{600}=5 x 10^{-3} and precultured for 24 h to consume Ect1p and/or PE. After that, TKY12Ga was shifted to SD medium containing 20 μM diC10PE and SG medium at a starting OD_{600}=5 x 10^{-3} respectively, cultured for 48 h. After 48 h, the yeast phospholipids were extracted and separated by 2D-TLC. The spots of phospholipids were scraped from TLC plates and quantified by phosphorous assay. Results represent an average of three independent experiments ± SE.

(A)



(B)

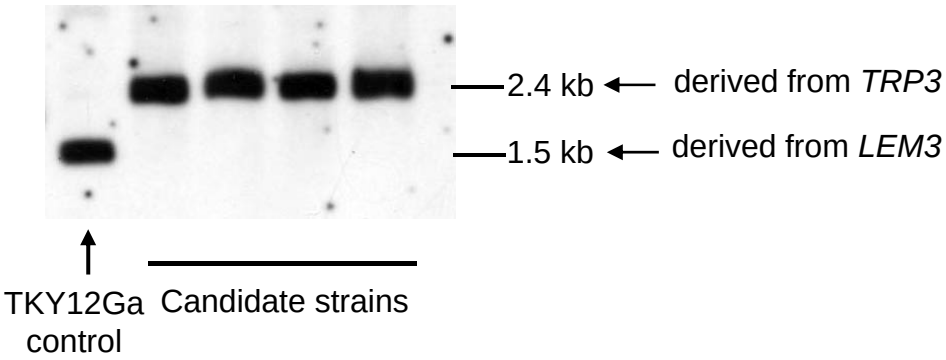


Fig. 2.3 (A) strategy of the *LEM3* gene deletion
(B) deletion verification by Southern blotting analysis

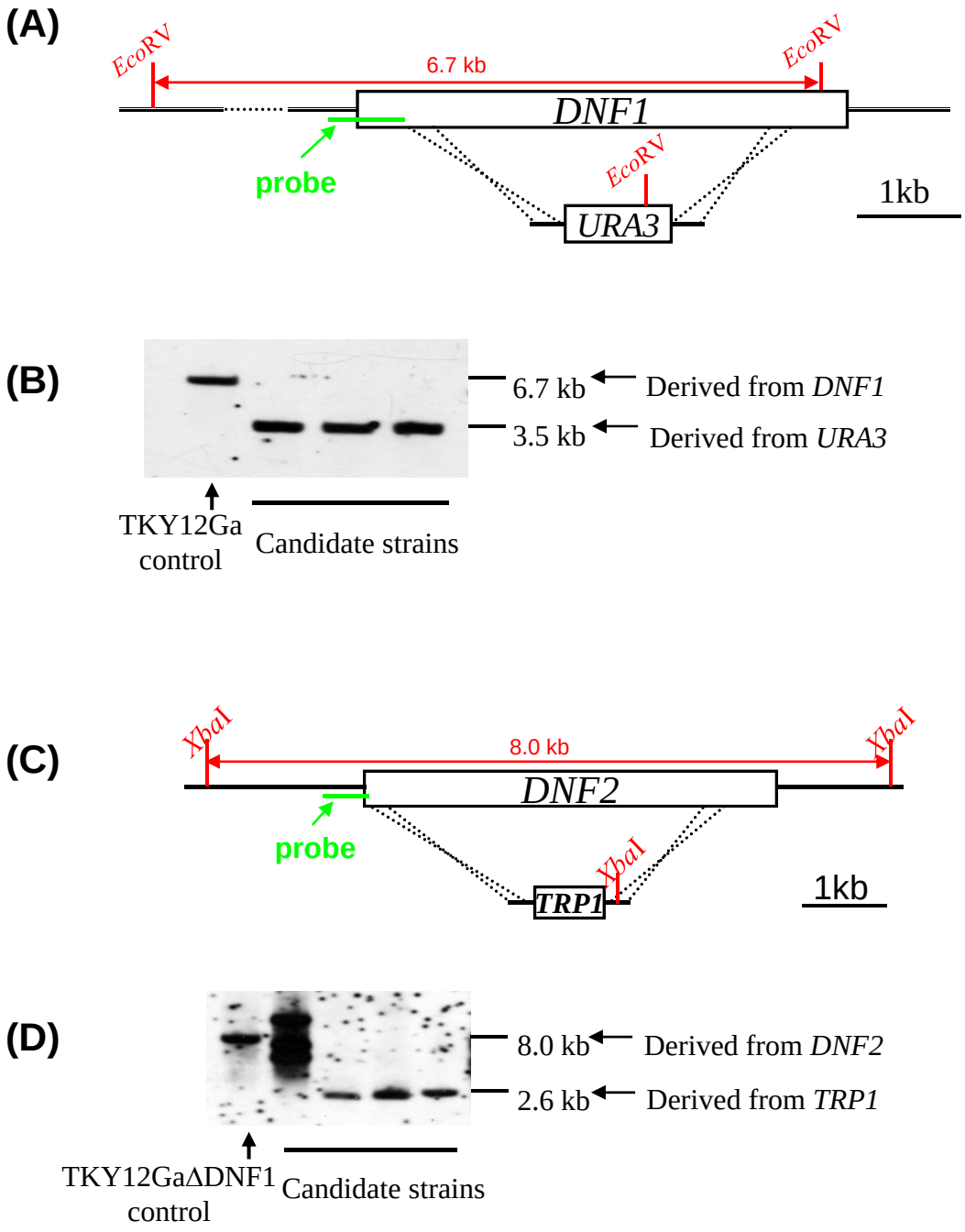


Fig. 2.4 (A) strategy of the *DNF1* gene deletion
(B) *Δdnf1* verification by Southern blotting analysis
(C) strategy of the *DNF2* gene deletion
(D) *Δdnf2* verification by Southern blotting analysis

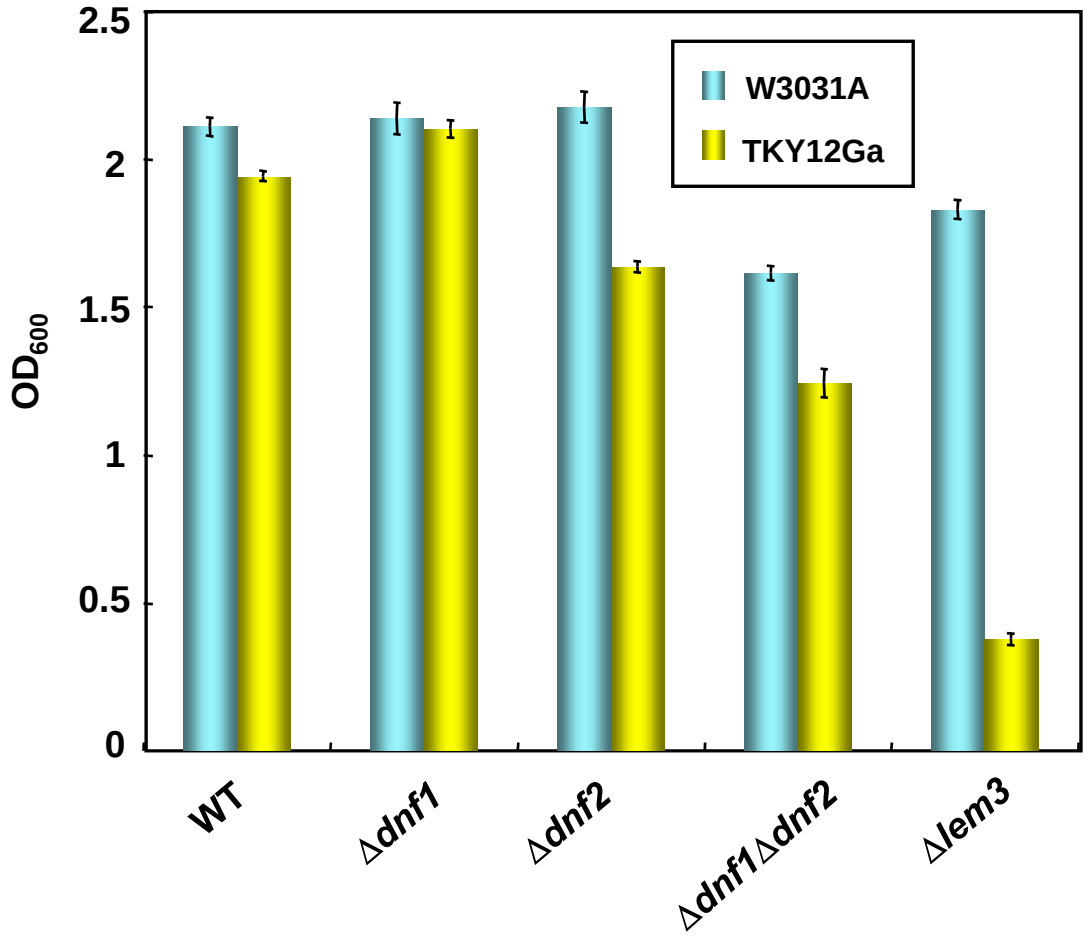


Fig. 2.5 Growth of TKY12Ga Supported by diC10PE is Dependent on Lem3p and P-type ATPases, Dnf1p and Dnf2p. The mutants were first seed from SG medium to SD medium and precultured for 24 h to consume Ect1p and/or PE, and then were seeded at a starting OD_{600}=5 x 10^{-3} to SD medium containing diC10PE and cultured for 48 h. After 48 h, OD_{600} of the cultures was measured. Results represent the means of three independent experiments ± SE.

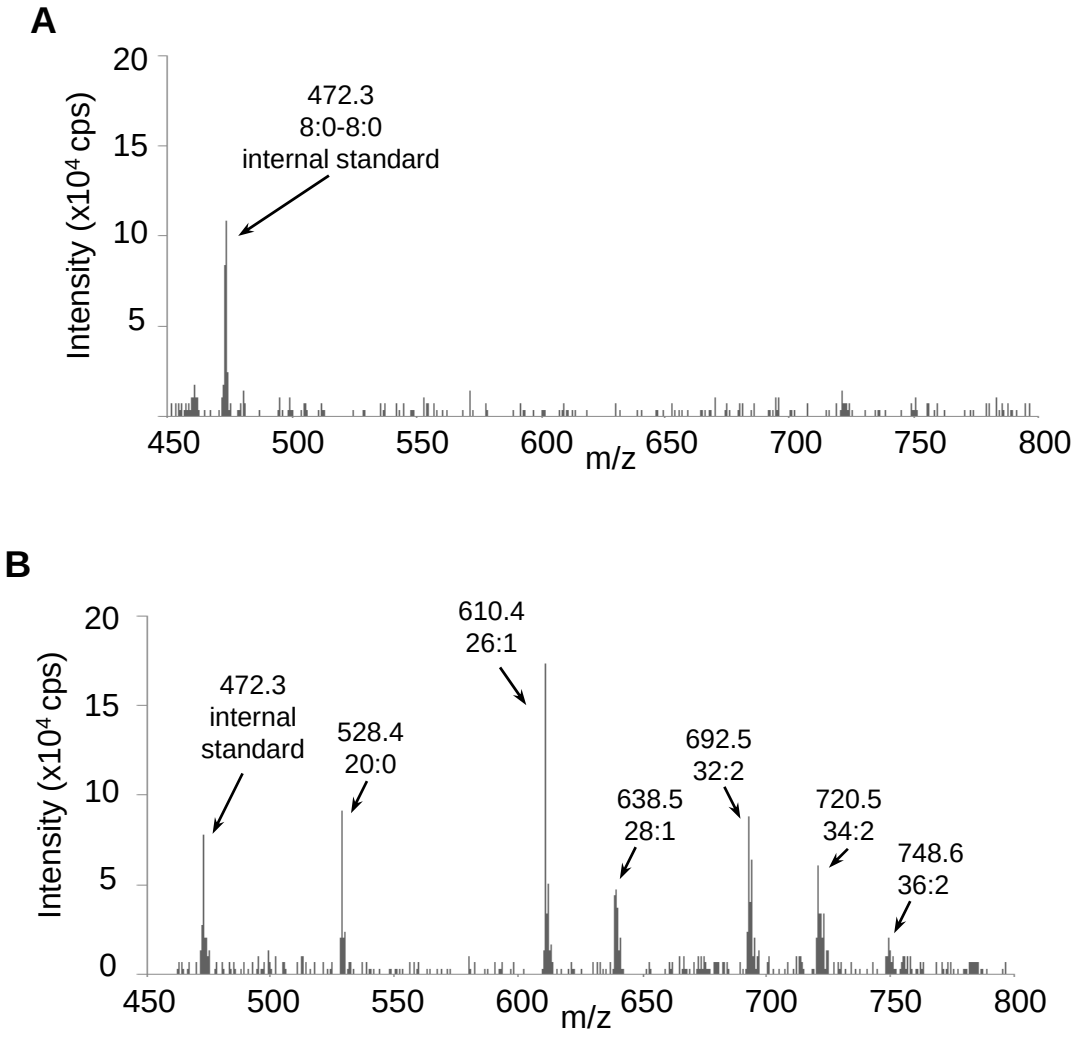


Fig. 2.6 Remodeling of deuterium-labeled diC10PE in TKY12Ga

TKY12Ga was precultured as in Fig. 2.1, then was shifted to SD medium containing non-labeled diC10PE at a starting OD_{600}=5 x 10^{-3} and cultured for 24h. After 24 h incubation in the non-labeled PE-containing medium, the cells were pulse-labeled with 20 μM deuterium-labeled diC10PE for 15 min. Before (A) and after (B) labeled, lipids were extracted and subjected to ESI-MS/MS. Deuterium-labeled PEs were detected with neutral loss scan for *m/z* 145 in positive ion mode. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

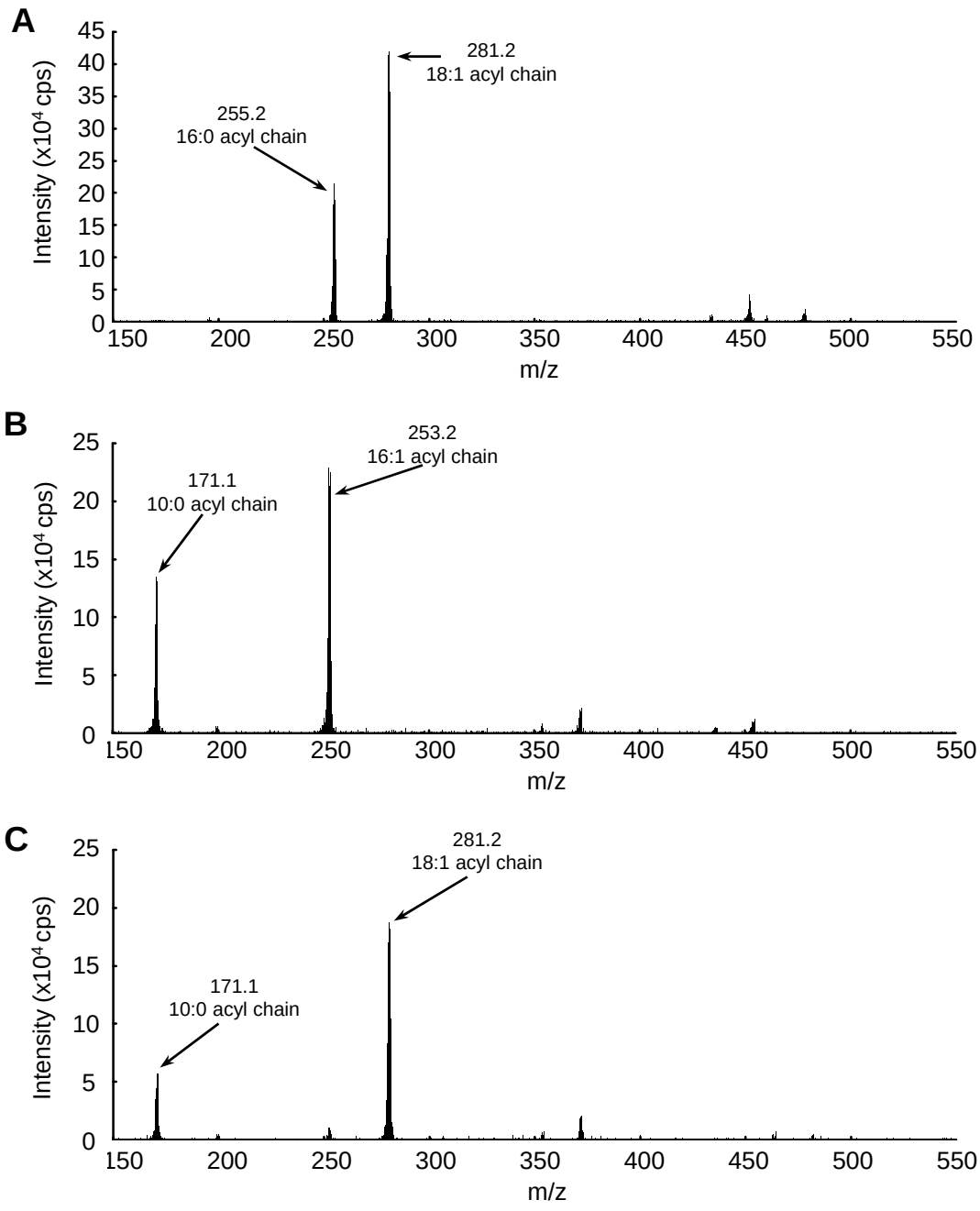


Fig. 2.7 Fatty acyl chain profiles in the remodeling intermediates

Fatty acyl chains fragmented from POPE (A) and the remodeling intermediates, 26:1-PE (B) and 28:1-PE (C) in the phospholipid extracts prepared from cells grown in the presence of deuterium-labeled diC10PE, were analyzed using ESI-MS/MS with product ion scan in negative ion mode for *m/z* 716, 608, and 636, respectively. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

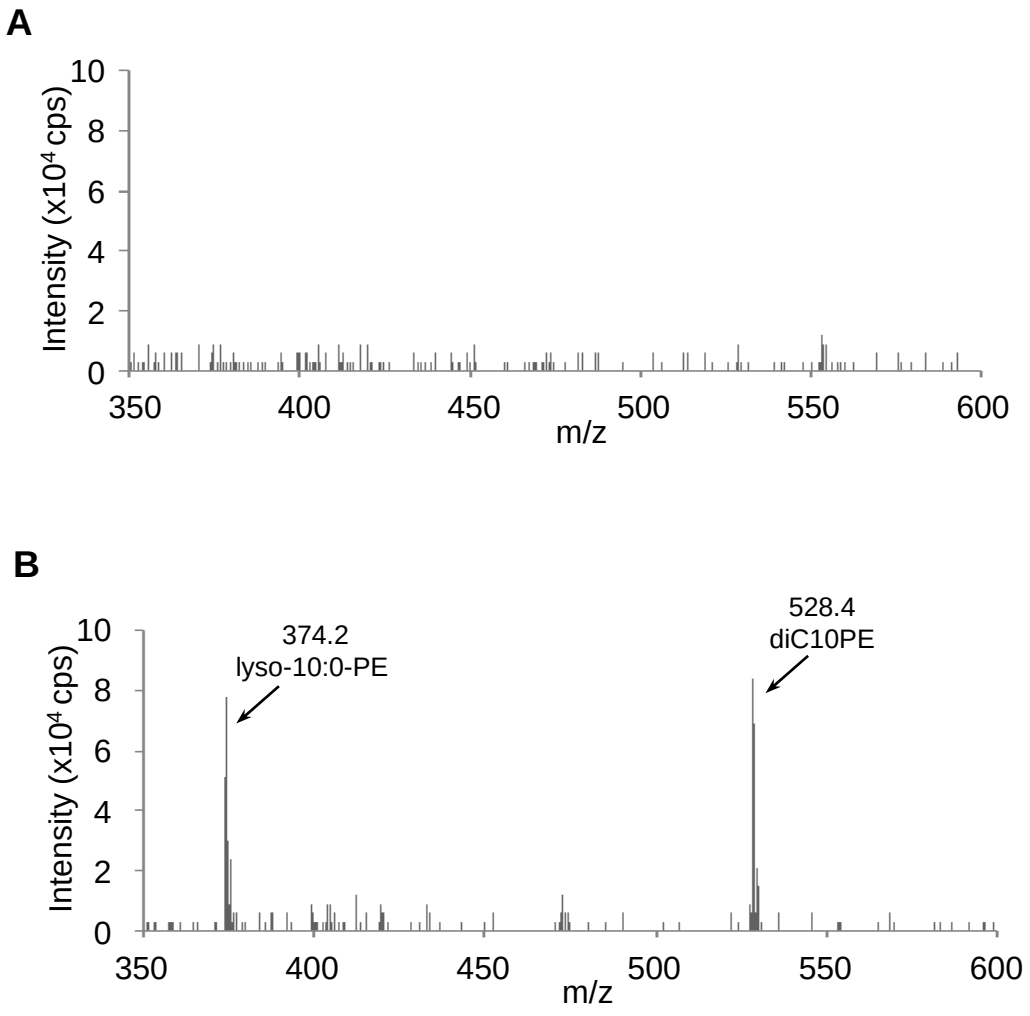


Fig. 2.8 Analysis of water-soluble metabolites of deuterium-labeled diC10PE

Water-soluble species of PE and lyso-PE were extracted from cells incubated with deuterium-labeled diC10PE for 0 (A) and 15 min (B) and subjected to ESI-MS/MS analysis with neutral loss scan for *m/z* 145 in positive ion mode. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

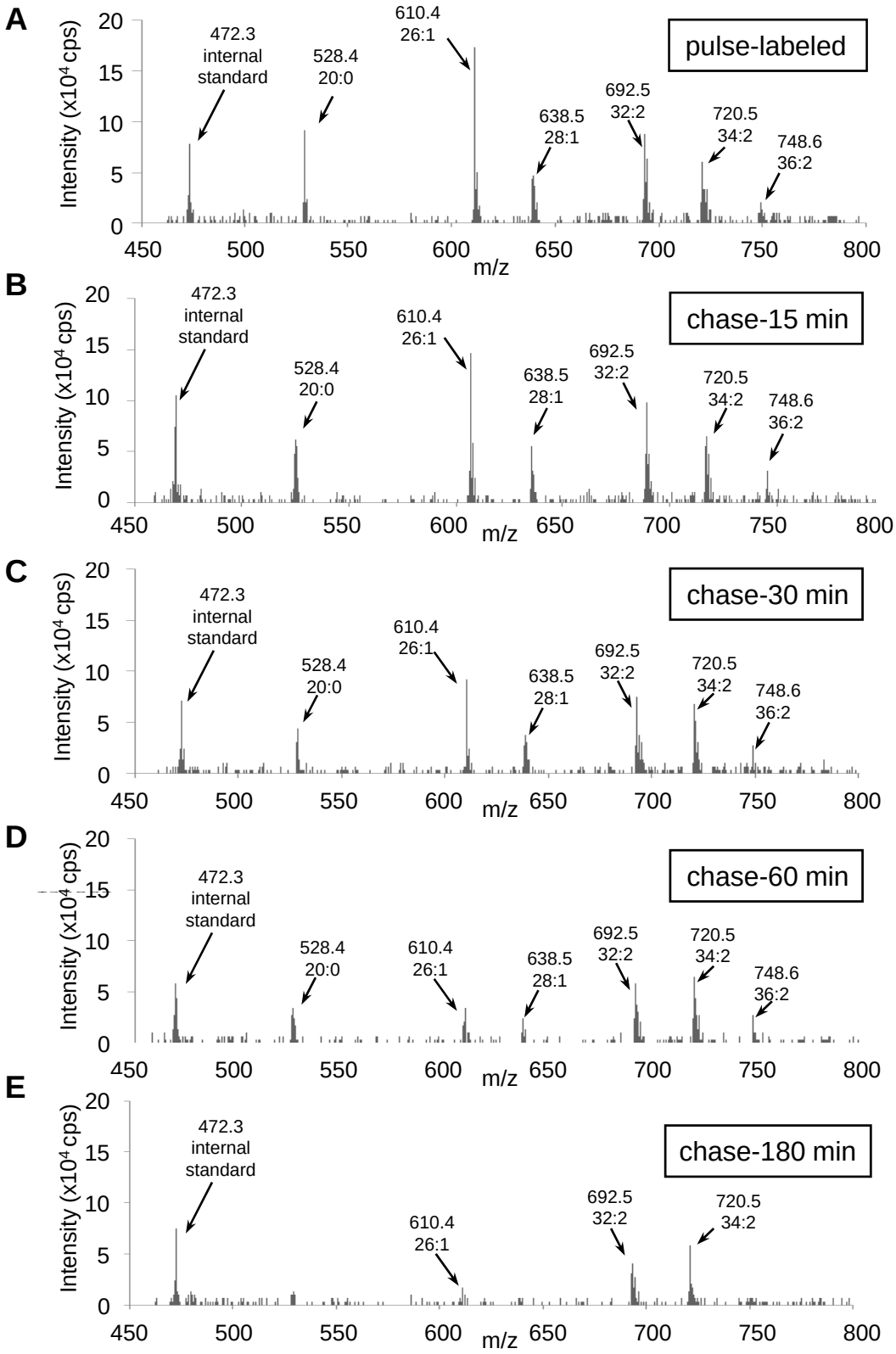


Fig. 2.9 Pulse-chase analysis of the metabolish of deuterium-labeled diC10PE in TKY12Ga. After pulsed-labeled as that in Fig. 2.6, TKY12Ga was chased with non-labeled diC10PE for 15, 30, 60, and 180 min. Then Lipids were extracted and subjected to ESI-MS/MS. Similar results were obtained in three independent experiments.

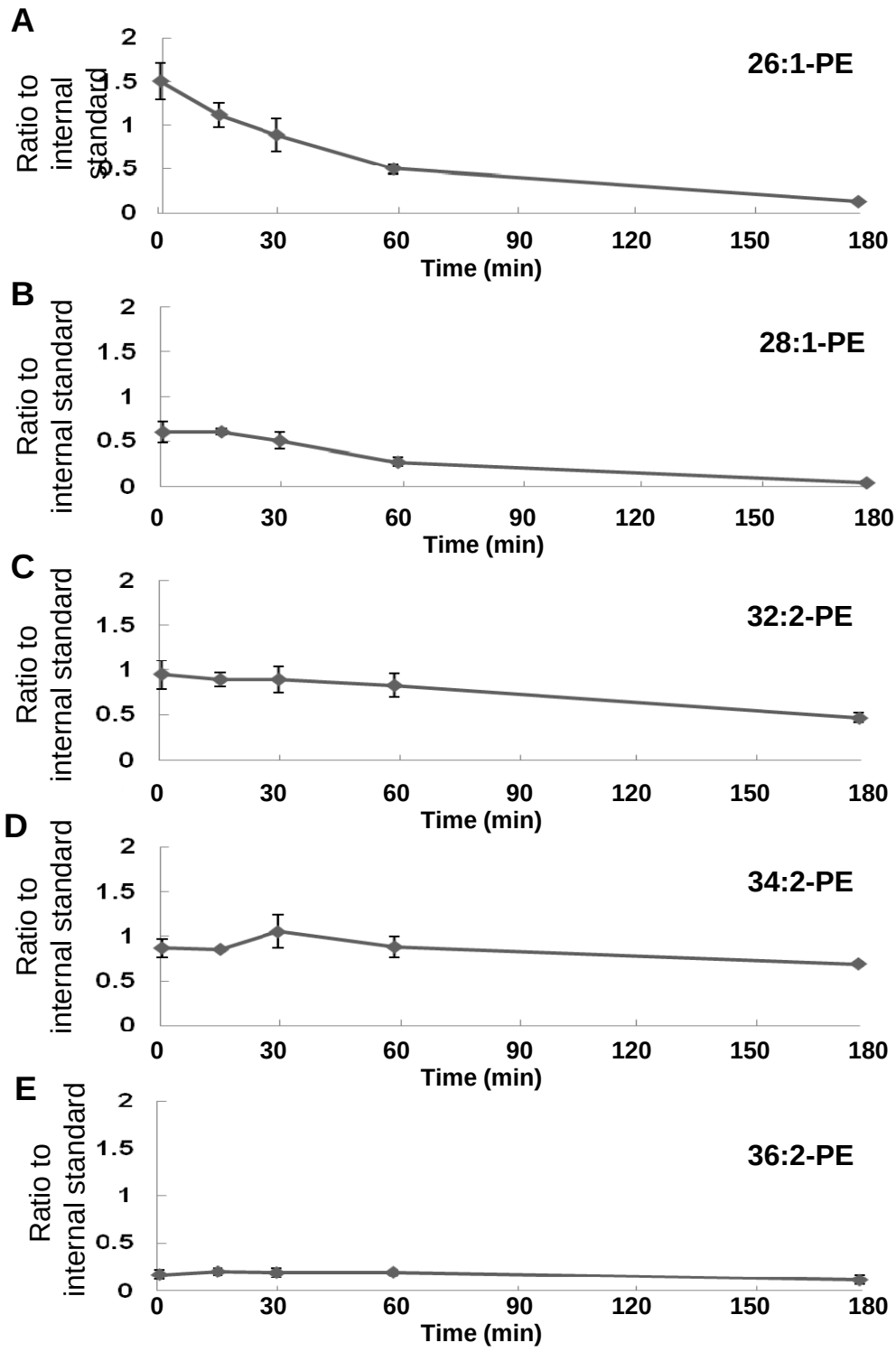


Fig. 2.10 Quantification of the pulse-chase analysis in Fig.2-9

The PE species in Fig.2.9 was quantified and amounts of those species were expressed as ratios to that of internal standard, deuterium-labeled diC8PE. Results represent an average of three independent experiments ± S.E.

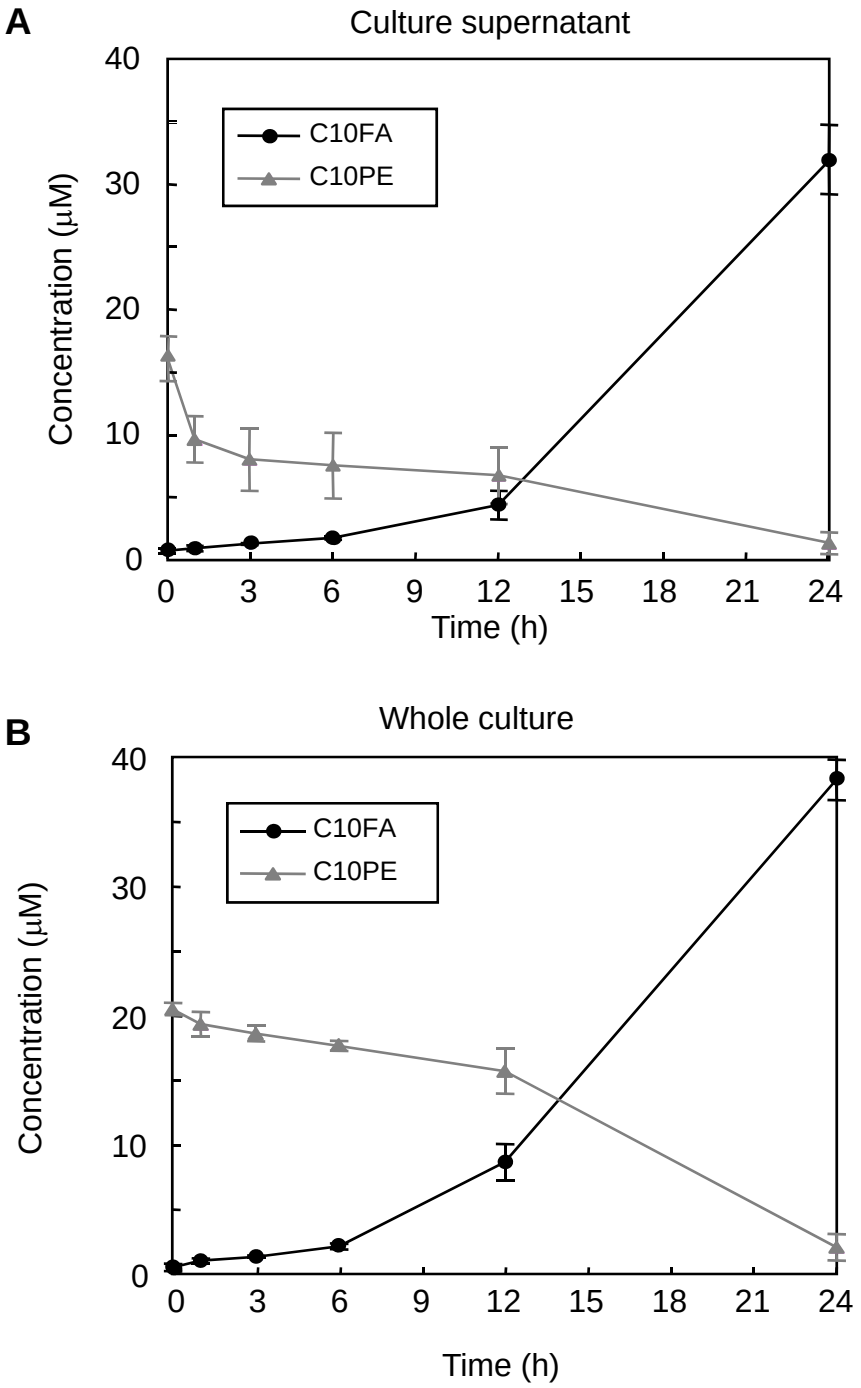


Fig. 2.11 Excretion of C10FA into culture medium under diC10PE-supported growth

TKY12Ga cells precultured as in Fig.2.1 were cultured in SD medium containing diC10PE. The culture supernatant (A) and whole culture (B) were collected. Fatty acids and PE were extracted and quantified by LC/MS. Results represent an average of three independent experiments ± S.E..

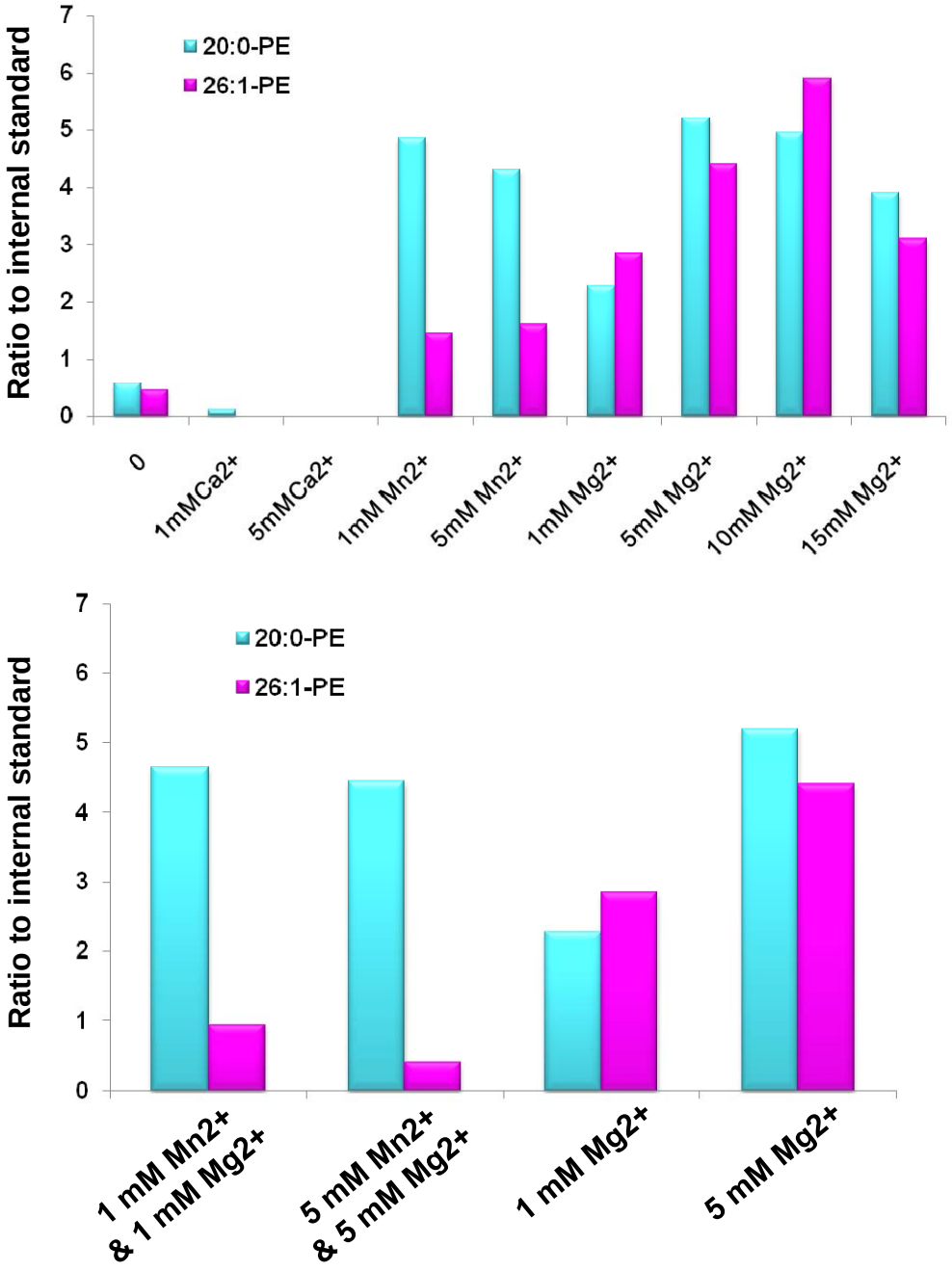


Fig. 2.12 Screening for optimum divalent cation concentration for *in vitro* PE remodeling

Cell lysate of TKY12Ga was incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA in the present of Ca^{2+}, Mn^{2+},or Mg^{2+} at the indicated concentrate for 60 min as described in Materials and Method. Lipids were extracted and subjected to ESI-MS/MS. Quantitative data was expressed as ratio of the peak intensity to that of the internal standard. Results represent the means of two experiments

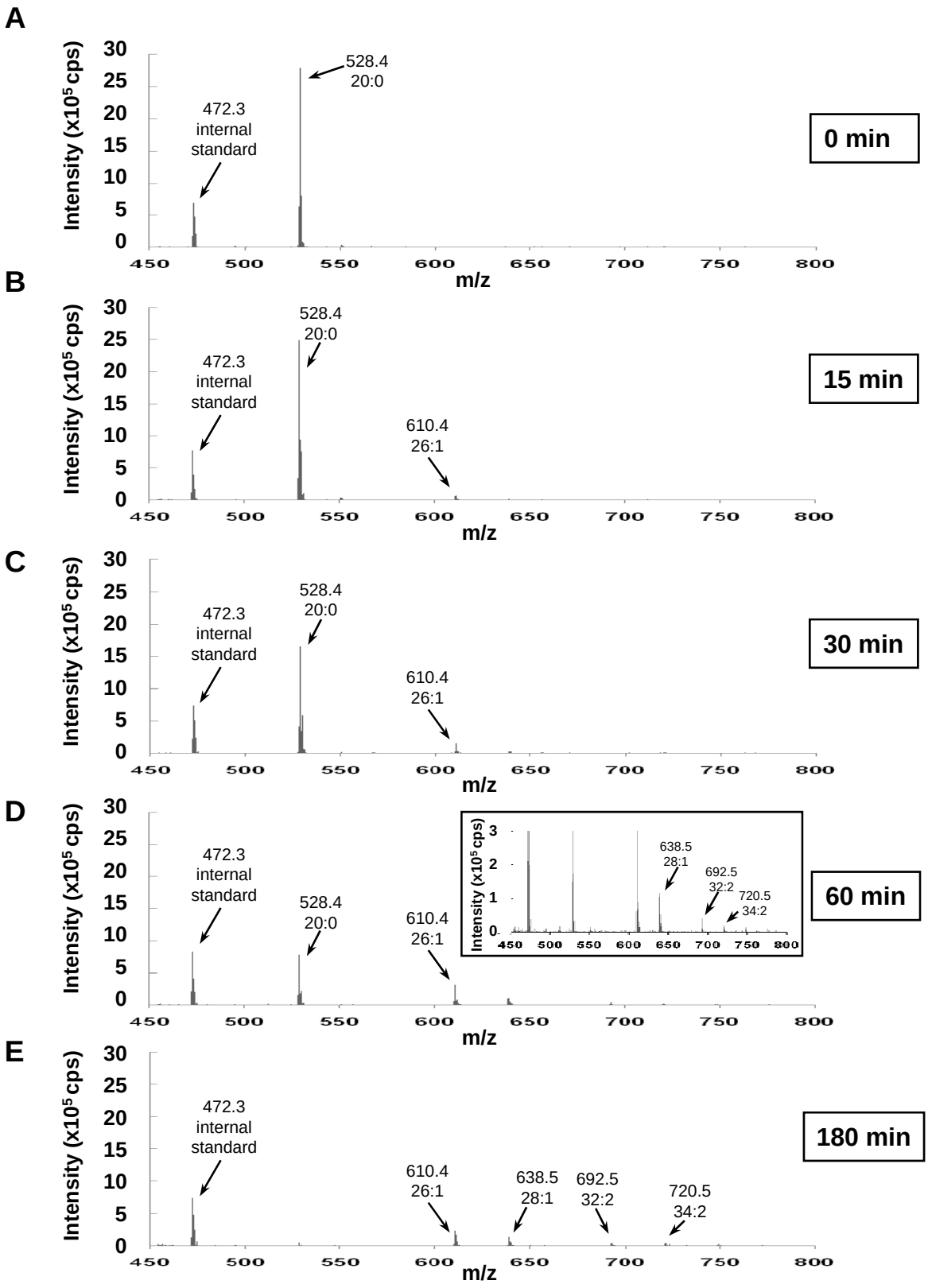


Fig. 2.13 Deuterium-labeled diC10PE was remodeled *in vitro*

Cell lysate of TKY12Ga was incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA for 0 (A), 15 (B), 30 (C), 60 (D), and 180 min (E) as described in Materials and Method. Lipids were extracted and subjected to ESI-MS/MS. Similar results were obtained in three independent experiments.

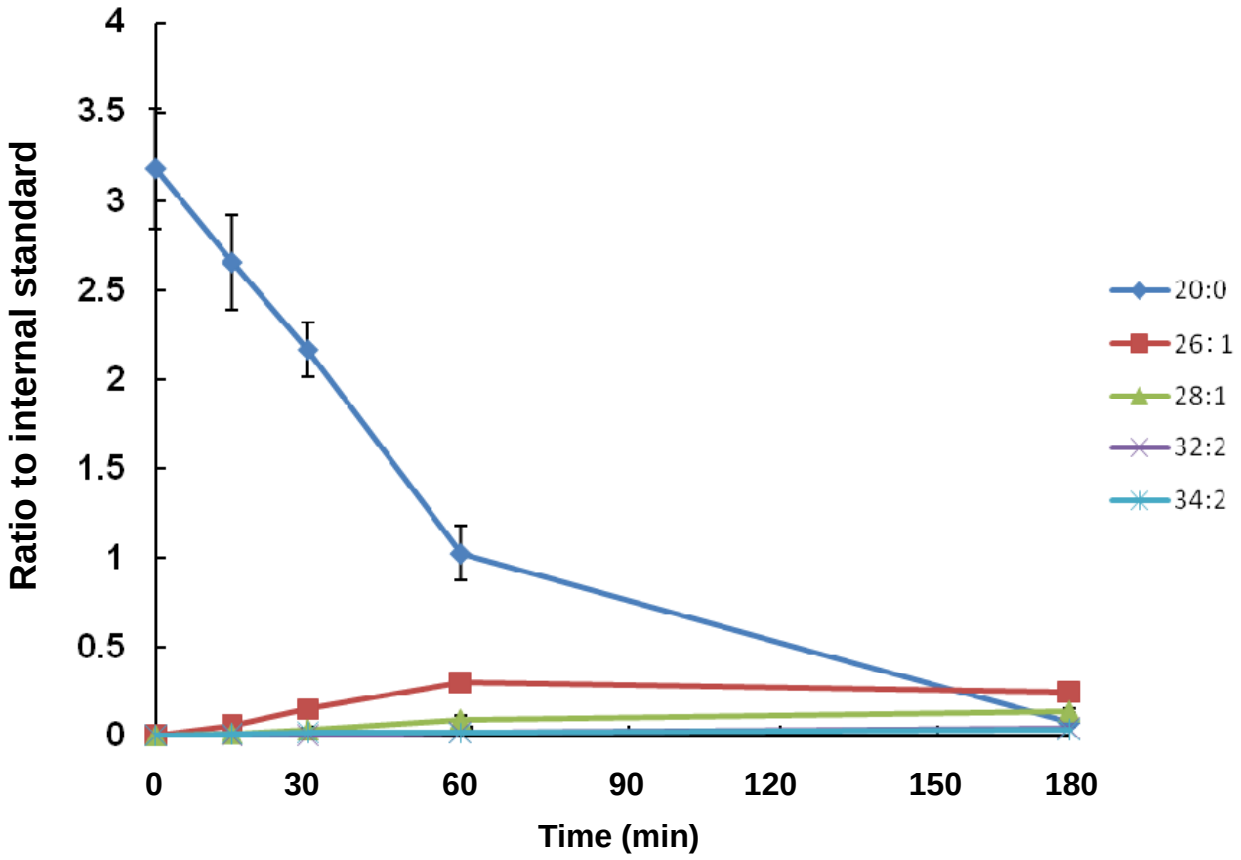


Fig. 2.14 Quantification of the *in vitro* PE remodeling analysis in Fig. 2.13

The PE species in Fig. 2.13 was quantified and amounts of those species were expressed as ratios to that of internal standard, deuterium-labeled diC8PE. Results represent an average of three independent experiments ± S.E.

Chapter 3

Analysis of genes involved in remodeling of PE

3.1 Introduction

As shown in chapter 2, remodeling intermediates, lyso-10:0-PE, 10:0-16:1-PE, and 10:0-18:1-PE, were detected when TKY12Ga was incubated with diC10PE. Presence of these intermediates suggests that the remodeling can be dissected into at least two reactions, the cleavage of acyl chains by phospholipase activities and reacylation by acyltransferase activities.

Yeast genome contains five genes encoding phospholipase B, *PLB1, PLB2, PLB3, SPO1,* and *NTE1* (Lee et al., 1994; Merkel et al., 1999; Tevzadze et al., 2000; Zaccheo et al., 2004). *PLB1*, *PLB2*, and *PLB3* genes code phospholipase Bs which were reported to have transacylase activities that can form PC from lysoPC, and the activities to hydrolyze the *sn*-1 and *sn*-2 ester bond of phospholipids and lysophospholipids *in vitro* with different substrate preference (Lee et al., 1994; Merkel et al., 1999). Base on the analysis of the glycerophosphodiesters produced in strains bearing deletion mutations in those genes, it was shown that deletion of *PLB1*, extracellular GroPEtn and GroPCho levels were reduced to 14% and 5% of wild-type levels and deletion of *PLB3* resulted in a 50% reduction of PI breakdown. In contrast, deletion of *PLB2* had no effect upon the production of extracellular glycerophospholipid, but overexpression of it results in resistance to lyso-PC (Lee et al., 1994; Merkel et al., 1999).

Product of *SPO1* is a meiosis-specific protein with similarity to phospholipase B. The product of *NTE1* gene, homologue of neuropathy target esterase, degrades PC derived from the CDP-choline pathway, to glycerophosphocholine in living cells (Zaccheo et al., 2004). In addition, *YOR022C* encodes a protein that has 20% identity to bovine phosphatidic acid-preferring phospholipase A1 that preferentially hydrolyze the *sn*-1 ester bond of PA (Higgs et al., 1998). These genes are all non-essential for growth (Lee et al., 1994; Merkel et al., 1999; Winzeler et al., 1999; Zaccheo et al., 2004). It was speculated that some of these genes are involved in the remodeling of PE. In this chapter, multiple deletion mutants of these genes were constructed in TKY12Ga by using Cre-*lox*P system which allows repeated use of marker gene (Fig. 3.1). Their growth in SD medium containing diC10PE and remodeling of deuterium-labeled

diC10PE in the sextuple mutant were analyzed.

On the other hand, several genes have been reported encode acyltransferase in *S. cerevisiae*. As shown in Fig. 1.2, *GAT1/GPT2* and *GAT2/SCT1* genes code for the vast majority of the Gly-3-P and DHAP acyltransferase activities found in yeast, catalyzing the acylation at the *sn*-1 position and producing either lyso-PA and 1-acyldihydroxyacetone phosphate. The latter compound is reduced subsequently by Ayr1p to lyso-PA. Then lyso-PA is acylated by Ale1p or Slc1p at the *sn*-2 position to form PA (Benghezal et al., 2007) (Tamaki et al., 2007). Genetic inactivation of either *GAT1* or *GAT2* did not alter cell growth but inactivation of both resulted in growth cessation (Zaremberg and McMaster, 2002). Similarly, null mutation in *ALE1* is synthetic lethal with the deletion of *SLC1*. Ale1p was also reported to have acyltransferase activity against the *sn*-2 position in various lysophospholipids, including lyso-PE, lyso-PI, lyso-PS and lyso-PA (Benghezal et al., 2007) (Jain et al., 2007) (Riekhof et al., 2007b) (Tamaki et al., 2007). To analyze the involvement of these genes in the remodeling of PE, individual deletion mutants in these genes were constructed under TKY12Ga background, and their growth in SD medium containing diC10PE and remodeling of deuterium-labeled diC10PE were examined.

3.2 Materials and Methods

3.2.1 Strains

Escherichia.coli

JA221	*recA1, leuB6, trpE5, hsdR-, hsdM-, lacI, thr, thi*

Saccharomyces cerevisiae

W3031A	*Mat a, his3, leu2, ura3, trp1, ade2*
TKY12Ga	*Mat a, P_{GAL1}ECT1::HIS3, Δpsd2::LEU2, ura3, trp1, ade2, Δpsd1::KAN^r* (Kakihara et al., 2004)
W3031A*Δspo1*	W3031A*Δspo1::loxP-KlURA3-loxP* (This study)
TKY12Ga*Δspo1*	TKY12Ga *Δspo1::loxP-KlURA3-loxP* (This study)

W3031A*Δplb1*	W3031A*Δplb1::loxP-KlURA3-loxP* (This study)
TKY12Ga*Δplb1*	TKY12Ga*Δ plb::loxP-KlURA3-loxP* (This study)
W3031A*Δspo1Δplb1*	W3031A *Δspo1::loxP*, *Δplb1::loxP-KlURA3-loxP* (This study)
TKY12Ga*Δspo1Δplb1*	TKY12Ga *Δspo1::loxP*, *Δplb1::loxP-KlURA3-loxP* (This study)
W3031A *Δplb1Δplb2*	W3031A *Δplb1::loxP-KlURA3-loxP, Δplb2::loxP-hph-loxP* (This study)
TKY12Ga*Δplb1Δplb2*	TKY12Ga *Δplb1::loxP-KlURA3-loxP, Δplb2::loxP- hph -loxP* (This study)
W3031A*Δspo1Δplb1Δplb2*	W3031A *Δspo1::loxP*, *Δplb1::loxP-KlURA3-loxP, Δplb2::loxP-hph-loxP* (This study)
TKY12Ga*Δspo1Δplb1Δplb2*	TKY12Ga *Δspo1::loxP*, *Δplb1::loxP-KlURA3-loxP, Δplb2::loxP-hph-loxP* (This study)
W3031A*Δspo1Δplb1Δplb2Δplb3*	W3031A *Δspo1::loxP, Δplb1::loxP, Δplb2::loxP, Δplb3::loxP-KlURA3-loxP* (This study)
TKY12Ga*Δspo1Δplb1Δplb2Δplb3*	TKY12Ga *Δspo1::loxP, Δplb1::loxP, Δplb2::loxP, Δplb3::loxP-KlURA3-loxP* (This study)
W3031A*Δspo1Δplb1Δplb2Δplb3Δnte1*	W3031A *Δspo1::loxP*, *Δplb1::loxP, Δplb2::loxP, Δplb3::loxP-KlURA3-loxP,Δnte11::loxP-hph-loxP* (This study)

TKY12Ga*Δspo1Δplb1Δplb2Δplb3Δnte1* (**DLY08**)
TKY12Ga*Δspo1::loxP, Δplb1::loxP, Δplb2::loxP, Δplb3::loxP-KlURA3-loxP, Δnte11::loxP-hph-loxP* (This study)

TKY12Ga*Δspo1Δplb1Δplb2Δplb3Δnte1Δyor022c* (**DLY09**)
TKY12Ga *Δspo1::loxP*, *Δplb1::loxP, Δplb2::loxP,Δplb3::loxP-KlURA3-loxP, Δnte11::loxP-hph-loxP, Δyor022c::ADE2* (This study)

TKY12Ga*Δale1* TKY12Ga *Δale1::loxP-KlURA3-loxP* (This study)

TKY12Ga*Δslc1* TKY12Ga *Δslc1::loxP-KlURA3-loxP* (Narita, unpublished)

TKY12Ga*Δgat1* TKY12Ga *Δgat1::loxP-KlURA3-loxP,* (Narita, unpublished)

TKY12Ga*Δgat2* TKY12Ga *Δgat2::loxP-KlURA3-loxP,* (Narita, unpublished)

3.2.2 Media

SD medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-glucose (Wako)	2%

SG medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-galactose (Wako)	2%

YPD medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-glucose (Wako)	2%

YPG medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-galactose (Wako)	2%

For adding exogenous PE to medium, PE is dissolved in ethanol by a 30 min sonication and then added to medium with a final ethanol concentration of 1% in the medium.

For solid medium, 2% agar (Wako) was added.

When required, add the amino acid and antibiotic as the following concentration:

L-Histidine (NACALAI)	2.4 μg/ml
L-Leucine (Kanto Kagaku)	10 μg/ml
Uracil (Kanto Kagaku)	2.4 μg/ml
L-Tryptophan (Kanto Kagaku)	4 μg/ml
Adenine Hydrochloride	2.4 μg/ml
Ampicillin Sodium (Wako)	50 μg/ml

When TKY12Ga or the stains derived from it were cultured, choline chloride (Kanto Kagaku) was always supplied to the glucose containing medium at the final concentration of 1 mM; 2-Aminoethanol (NACALAI) and choline chloride (Kanto Kagaku) were always supplied to the galactose containing medium both at the final concentration of 1 mM.

3.2.3 Plasmid

Plasmids used in this chapter were shown in the following,

pUG72	Euroscarf, Frankfurt, Germany
pAG32	Euroscarf, Frankfurt, Germany
pUG66	Euroscarf, Frankfurt, Germany
pUG-hph	This study
pSH63	Euroscarf, Frankfurt, Germany
YCplac22	Given by Dr.Gietz (Iwamoto, 2002)

pALE1 This study

3.2.4 Cultivation

If there was not specially designated, for the liquid cultivation, yeast was cultured at 30°C, *E.coli* was cultured at 37 °C on a shaking incubator. The growth was determined by measuring OD_{600} with the spectrophotometer U-2000.

To determined the growth of the multiple genes deleted stains in PE medium, those stains were first seeded from SG to SD medium and precultured for 24 h to consume Ect1p and/or PE, after the consumption of Ect1p and/or PE, they were seeded to SD medium containing diC10PE and SG medium (as a control) at a starting OD_{600}=5 x 10^{-3} respectively, cultured for 48 h. After 48 h, OD_{600} was measured.

3.2.5 Methods

3.2.5.1 Construction of pUG-hph

For repeated gene deletion and marker rescue with Cre-loxP system, it was advantageous to have another marker gene with two flanking *loxP* sequences, besides *KlURA3* which is available using pUG72. Here, the plasmid containing *hph*-expression unit, which is derived from *Klebsiella pneumoniae* and confers resistance to the antibiotic hygromucin B, between two tandem *loxP* sequences, was constructed. This second deletion cassette allows to remove two marker gene at the same time after successive deletion of two genes. Two restriction endonucleases, *Sac*I and *Bgl*II, were used to digest both of pAG32 and pUG66. After ligation of the 1.66-kb fragment derived from pAG32 and the 2.57-kb fragment derived from pUG66, the plasmid that carrying the *loxP-hph-loxP* expression unit sequence was obtained and named as pUG-hph.

3.2.5.2 Construction of gene disruption cassette

Construction of gene disruption cassette of Cre-*lox*P system was performed by a one-step PCR reaction using the Takara Ex Taq™ kit. The reaction conditions and program parameters were as indicated in the manufacturer's instruction.

Cre-*lox*P system was used in deletion of *SPO1, PLB1, PLB2, PLB3, NTE1,* and *ALE1* genes. To construct the disruption cassette of *SPO1, PLB1, PLB3,* and *ALE1,*The

template used in the PCR reaction was pUG72 (containing the *KlURA3* marker gene); for the *PLB2* and *NTE1* gene deletion cassettes, pUG-hph was used as PCR reaction template (containing the *hph* gene as marker gene), and the primers were purchased oligonucleotides comprising 19-bp or 23-bp 3' nucleotides complementary to sequences in the template flanking the disruption cassette and 45-bp 5' nucleotides that anneal to sites upstream or downstream of the genomic target sequence to be deleted.

Construction of YOR022c disruption cassette was performed by fusion PCR as described previously (Szewczyk et al., 2006).

The primers used in construction of gene disruption cassette were shown as follows:

SPO1-DEL-U	5'-TATACAAGTAAAAAACCTAAGTAAAGACATAAATATCCCAAAATACA GCTGAAGCTTCGTACGC-3'
SPO1-DEL-L	5'-AGGTGAACAAAAACTCATTAAATTTCACAGAAAAAGTCTACTCTCGCA TAGGCCACTAGTGGATCTG-3'
PLB1-DEL-U	5'-GCTATCCATCCTAAGGGCAAACGGGAAAGAAAATAAAACAAAACCCA GCTGAAGCTTCGTACGC-3'
PLB1-DEL-L	5'-AAACTATTTGTATGTTGATATTTTTGCGCTTTTCTCACTCTCGTAGCATA GGCCACTAGTGGATCTG-3'
PLB2-DEL-U	5'-GAGTTAAAATTCACTCGCGCTGGTAAAGTAACACTTCTTACGCAGCAG CTGAAGCTTCGTACGC-3'
PLB2-DEL-L	5'-TCTACCAAAAATAGCGGAAAGAGGAGTGATAATCGGGAAAAAAATGC ATAGGCCACTAGTGGATCTG-3'
PLB3-DEL-U	5'-AAGAGGACTAAAAGCAAGGAAATTACACACATAATAATATAAGTACA GCTGAAGCTTCGTACGC-3'
PLB3-DEL-L	5'-GATTTTTATTGCCTCATTTTGTATACCGTTATTACGCAAGAGATAGCAT AGGCCACTAGTGGATCTG-3'
NTE1-DEL-U	5'-ATAAAATAAACAAGAAAAGAATAGAAAGGCTTGGCTCTAGTTTTTCAG CTGAAGCTTCGTACGC-3'
NTE-DEL-L	5'-AAAACAATGCAATACCTAAATCTATCTACAAAAAACGGTCACCAAGC ATAGGCCACTAGTGGATCTG-3'
ALE1-DEL-U	5'-CAAACCGCATACGCCAAGACAAACCGTGGTGATTTAATTCTGCTGCAG CTGAAGCTTCGTACGC-3'
ALE1-DEL-L	5'-GAACTGGAAAATAAGACAACAAGACTGTGACTTCCACACGCATCTGC ATAGGCCACTAGTGGATCTG -3'
YOR022-Af	5'-CAATTAAGATATCAAAAGG-3'

YOR022-Ar	5’-CAAGTAATTGTTCACTGTGAG-3’
YOR022-bf	5’-CTGGTTGATCAGTAAACGCAAGG-3’
YOR022-br	5’-CTCTGCCATCTTTTAACAG-3’
YOR022-ade2-f	5’-CTCACAGTGAACAATTACTTGGCCGTATCGTGATTAACG-3’
YOR022-ade2-r	5’-CCTTGCGTTTACTGATCAACCAGGCAGGTAATTATTCCTTGC-3’
YOR022-nest-f	5’-CAAATCTGATGACGAAGC-3’
YOR022-nest-r	5’-GTAGTCCATCTTAAACCAAG-3’

3.2.5.3 Construction of plasmid pALE1

The *ALE1* ORF with 700-bp 5’ flanking region and 206-bp 3’ flanking region was amplified by PCR from W3031A genomic DNA using primers, ALE1-complementary-u (5’-AACTGCAGCTAAATTTGTCCAAATAG-3’) and ALE1-complementary-l (5’-GCTCTAGACGACTTCTAGTTGCAGTG-3’). The amplified fragment was digested by *Pst*I and *Xba*I, and then inserted into vector YCplac22, which had been digested by the same restriction endonucleases.

The PCR reaction of DNA cloning was performed using iProof^{TM} kit (BIO-RAD, CA), the reaction conditions and program parameters were as indicated in the manufacturer’s instruction.

3.2.5.4 Introduction of disruption cassette into yeast

Introduction of gene disruption cassette into yeast was performed as describe in 2.2.4.5. When the marker in the gene disruption cassette was *hph* gene, after step ⑧ （42^{o}C water bath for 40 min）, the cells were incubated with 2 ml YPG liquid medium for 4 h, and then plate to YPG solid medium that containing 300 μg/ml hygromycin B.

3.2.5.5 Verification of gene deletion

When the transformants grew on the selected medium after the transformation, they were replicated to a same new medium for two times. Those transformants that showed stable growth on the medium were selected, the total DNA of them and their parent stains were extracted as described in 2.2.4.6 and used as template in the verification PCR reaction. The PCR reaction was performed with the Takara Ex Taq^{TM} kit. The reaction conditions and program parameters were as described in 2.2.4.1. The primers that used in the verification PCR reaction were showed as follows:

SPO1-confirm-U	5’-GATGCTATGGGTAACAAG-3’
SPO1-confirm-L	5’-GAATGATGACTTTGAATGG-3’
PLB1-confirm-U	5’-CATGTCCGCTTATTCCCG-3’
PLB1-confirm-L	5’-CTGATCATTTTCATGAAC-3’
PLB2-confirm-U	5’-CCTACAAAGGGACGCGTC-3’
PLB2-confirm-L	5’-GTTATGCCAAGATCAGAG-3’
PLB3-confirm-U	5’-CATCCGTTTTCGTTTCGC-3’
PLB3-confirm-L	5’-GGTAATTCTGACATCCTC-3’
NTE-confirm-U	5’-TGCTGATGTGGTTTGTAG-3’
NTE-confirm-L	5’-TCAACCTAGAGCTCGACC-3’
YOR022C-confirm-u	5’- CAATTAAGATATCAAAAGG -3’
YOR022C-confirm-l	5’- CTCTGCCATCTTTTAACAG -3’
ALE1-confirm-u	5’-CCAGCATCCTCTTTCATG-3’
ALE1-confirm-L	5’-GTACTGCTAAAGAAGTTG-3’

After the PCR verification, the correct ones and their parent stains were performed the Southern blotting analysis as described in 2.2.4.7.

3.2.5.6 DNA purification

All of the DNA fragment that would be used as the probe of Southern blotting or for the ligation, were purified by electrophoresed them in agarose gel and recovered them from the gel as described in 2.2.4.2.

3.2.5.7 Plasmid rescue

Yeast stains harboring pSH63 (carrying *TRP1* as marker gene) were cultured in 2 ml YPD or YPG medium to the stationary phase. Diluted the cell culture properly, and plated the dilution to YPD or YPG solid medium. About 30 colonies were simultaneously replicated to two selected media, one containing tryptophan, the other one did not. Colonies that could growth only in the tryptophan containing medium were derived from the cells that had lost the plasmid.

3.2.5.8 DNA ligation

DNA ligation was performed by the utilization of Ligation Kit Ver.2.1 (TAKARA), according to the manufacturer's instruction.

3.2.5.9 DNA sequencing

As described in 2.2.4.3

3.2.5.10 Lipid extraction

As described in 2.2.4.8

3.2.5.11 Mass spectrometry

As described in 2.2.4.13

3.2.5.12 Analysis of the remodeling of deuterium-labeled diC10PE *in vivo*

Cells were first seeded from SG to SD medium and precultured for 24 h to consume Ect1p and/or PE, and then was shifted to SD medium containing 20 μM non-labeled diC10PE at a starting OD_{600}=5 x 10^{-3}, and cultured till OD_{600} reached 1. Then cells were collected, washed with SD medium for 2 times, and incubated with 20 μM deuterium-labeled diC10PE for 1 h. Then lipids were extracted from the labeled cells after they were washed with 0.15 M KCl for 2 times. PE species of these incubations were analyzed by ESI-MS/MS

3.2.5.13 *In vitro* remodeling reaction

As described in 2.2.4.16

3.3 Results

3.3.1 Construction of sextuple deletion mutant of phospholipase encoding genes

Deletion of *SPO1* gene

The gene disruption cassette for *SPO1* gene was constructed and introduced into TKY12Ga and W3031A as described in Materials and Methods, resulting in

TKY12Ga*Δspo1* and W3031A*Δspo1*, respectively. The deletion of *SPO1* gene was verified by PCR (data not shown) and Southern blotting (Fig. 3.2B). The primers used in PCR for confirmation of *SPO1* gene deletion were SPO1-confirm-U and SPO1-confirm-L that were shown in Materials and Methods.

Because the disruption construct had only 45-bp sequences homologous to the target region in *S. cerevisiae*, it was too short to use as a probe in the Southern blotting analysis. Thus the PCR product that contained 372-bp 5' non-coding region of *SPO1*, *KlURA3*, and 336-bp 3' non-coding region of *SPO1*, was used as a probe, after confirmation of nucleotide sequence. Southern blotting was performed as described in 2.2.4.7. Restriction enzymes *Xba*I was used. Its restriction sites were shown in Fig. 3.2A. *Xba*I treatment resulted in a 4.4-kbp detectable band for parent stains, in contrast, a 1.5-kbp and a 2.5-kbp detectable band for *spo1* null mutants (Fig. 3.2B).

For the subsequent deletion of the other genes, marker gene from *Δspo1* stains was removed with expression of Cre recombinase by introduction of the pSH63 into those strains. The removal of the marker was verified by PCR (data not shown). After confirming that the marker was removed, rescue of plasmid pSH63 from those strains was performed as described in Materials and Methods.

Deletion of *PLB1* gene

PLB1 gene was deleted as *SPO1* gene using *KlURA3* in TKY12Ga, TKY12Ga*Δspo1*, W3031A, W3031A*Δspo1*, resulting in TKY12Ga*Δplb1*, TKY12Ga*Δspo1Δplb1*, W3031A*Δplb1*, and W3031A*Δspo1Δplb1*, respectively. Deletion of *PLB1* was verified with PCR (data not shown) and Southern blotting analysis (Fig. 3.2D). The primers used in the verification of *PLB1* gene deletion were PLB1-confirm-U and PLB1-confirm-L that were shown in Materials and Methods.

For Southern blotting analysis, the probe was prepared as that for *SPO1*. The restriction enzyme used was *Hin*dIII. Its restriction sites were shown in Fig. 3.2C. *Hin*dIII treatment resulted in a 1.2-kbp and a 2.4-kbp detectable band for parent stains, in contrast, a 0.48-kbp and a 2.7-kbp detectable band for *plb1* null mutants (Fig. 3.2D).

Deletion of *PLB2* gene

PLB2 was deleted similarly with *SPO1* and *PLB1*, but using the *hph* gene as a selectable marker, in TKY12aG*Δplb1*, TKY12Ga*Δspo1Δplb1*, W3031A*Δplb1*, W3031A*Δspo1Δplb1* strains, resulting in TKY12Ga*Δplb1Δplb2*, TKY12Ga*Δspo1Δplb1Δplb2*, W3031A*Δplb1Δplb2*, and W3031A*Δspo1Δplb1Δplb2*, respectively. Deletion of *PLB2* was verified with PCR (data not shown) and Southern

blotting analysis (Fig. 3.3B). The primers used in the confirmation PCR of *PLB2* gene deletion were PLB2-confirm-U and PLB2-confirm-L as shown in Materials and Methods.

For Southern blotting analysis, the probe was prepared as that for *SPO1* and *PLB1*. The restriction enzyme *Xho*I was used, and its restriction sites were shown in Fig. 3.3A. *Xho*I treatment resulted in a 6.6-kb detectable band for parent stains, in contrast, a 0.7-kbp and a 3.0-kbp detectable band for *plb2* null mutants (Fig. 3.3B).

Before the next deletion, two marker genes, *KlURA3* for *PLB1* deletion and *hph* for *PLB2* deletion, were removed as described above.

Deletion of *PLB3* gene

After rescue of the marker used in the deletion of *PLB1* gene and *PLB2* gene, *PLB3* genes were deleted in TKY12Ga*Δplb1Δplb2*, TKY12Ga*Δspo1Δplb1Δplb2*, W3031A*Δplb1Δplb2*, W3031A*Δspo1Δplb1Δplb2* using KlURA3 marker, resulting in TKY12Ga*Δplb1Δplb2Δplb3*, TKY12Ga*Δspo1Δplb1Δplb2Δplb3*, W3031A*Δplb1Δplb2Δplb3*, and W3031A*Δspo1Δplb1Δplb2Δplb3*, respectively. Deletion of *PLB3* was verified with PCR (data not shown) and Southern blotting analysis (Fig. 3.3D). The primers used in the confirmation of *PLB3* gene deletion were PLB3-confirm-U and PLB3-confirm-L as shown in Materials and Methods.

For Southern blotting analysis, the probe was prepared as that for *SPO1*. The restriction enzymes used was *Xba*I. Its restriction sites were shown in Fig. 3.3A, *Xba*I treatment resulted in a 7.8 -kbp detectable band for parent stains, in contrast, a 3.2-kbp and a 4.0-kbp detectable band for *plb3* null mutants (Fig. 3.3D).

Deletion of *NTE1* gene

The *NTE1* gene disruption was performed similarly as those of the genes mentioned above, with the *hph* gene as a selectable marker. *NTE1* genes were deleted in TKY12Ga*Δspo1Δplb1Δplb2Δplb3* and W3031A*Δspo1Δplb1Δplb2Δplb3*, resulting in TKY12Ga*Δspo1Δplb1Δplb2Δplb3Δnte1* (denominated DLY08) and W3031A*Δspo1Δplb1Δplb2Δplb3Δnte1*, respectively. Deletion of *NTE1* was confirmed with PCR (data not shown) and Southern blotting analysis (Fig.3.4B). The primers used in the confirmation of *NTE1* gene deletion were NTE1-confirm-U and NTE1-confirm-L as shown in Materials and Methods.

For Southern blotting analysis, the probe was prepared as that for *SPO1*. The restriction enzyme used was *Xba*I. Its restriction site were shown in Fig. 3.4A, *Xba*I treatment resulted in a 4.3-kbp and a 6.3-kbp detectable band for parent stains, in

contrast, a 2.6-kbp and a 4.6-kbp detectable band for *nte1* null mutants (Fig.3.4B).

Deletion of *YOR022c* gene

The disruption cassette of *YOR022c* was constructed using fusion PCR as described previously (Szewczyx, 2006), which contained the *ADE2* gene as a selectable marker. *YOR022c* genes were deleted in TKY12Ga*Δspo1Δplb1Δplb2Δplb3Δnte1*strain (DLY08), yielded the sextuple deletion mutant, TKY12Ga*Δspo1Δplb1Δplb2Δplb3Δnte1Δyor022c* strain, which was named DLY09. The primers used in the confirmation of *YOR022c* gene deletion were *YOR022c*-confirm-U and *YOR022c* -confirm-L as shown in Materials and Methods. By the confirmation PCR reaction, the *YOR022c* gene-containing template yielded a DNA fragment of 3.1-kbp, and template prepared from *YOR022c*-deleted strain yielded a DNA fragment of 3.6-kbp (Fig.3-4C & D).

3.3.2 Growth of multiple phospholipase defective mutants in SD containing diC10PE

Growth of multiple phospholipase defective mutants in SD medium containing diC10PE and SG medium was examined. As shown in Fig. 3.5A, the indicated null mutants in W3031A background grew similarly to their parent strain both in SG medium and in SD medium containing diC10PE. As shown in Fig. 3.5B and 3.6, those null mutants in TKY12Ga background also grew similarly to their parent strain in SG medium. And no defect was detected in the growth of those null mutants in SD medium containing diC10PE. Rather, TKY12Ga*Δplb1Δplb2Δplb3,* TKY12Ga*Δspo1Δplb1Δplb2Δplb3,* DLY08 and DLY09 grew better than their parent strain, TKY12Ga, in the same medium. These results suggested that other enzyme(s) is/are involved in the removal of acyl chains in diC10PE.

3.3.3 Analysis of the remodeling of deuterium-labeled diC10PE in DLY09 stain

3.3.3.1 Remodeling of deuterium-labeled diC10PE in DLY09 *in vivo*

After precultured and cultured as described in 3.2.5.12, cells of TKY12Ga and DLY09 were incubated with 20 μM deuterium-labeled diC10PE for 1 h. Then lipids were extracted and analyzed by ESI-MS/MS (Fig. 3.7). Quantitative data was expressed as ratio of the peak intensity of each PE molecular species to that of the internal

standard.

As shown in Fig. 3.7, deuterium-labeled diC10PE was remodeled in both TKY12Ga and DLY09, and a larger amount of the deuterium-labeled substrate, remodeling intermediates (26:1-PE and 28:1-PE), and remodeling products (32:2-PE and 34:2-PE), was detected in DLY09 than that in TKY12Ga.

Lipids in the culture supernatant were also extracted and were analyzed by ESI-MS/MS. As shown in Fig. 3.8, the lyso-10:0-PE produced in DLY09 was not less than that of TKY12Ga. These results suggest that acyl chains in diC10PE are remodeled in DLY09 and yeast has other enzyme(s) having phospholipase activity that is/are involved in removal of acyl chains in diC10PE, besides the reported six phospholipases.

3.3.3.2 Remodeling of deuterium-labeled diC10PE in DLY09 *in vitro*

Cell lysate of TKY12Ga and DLY09 were incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA for 1 h. Lipids were extracted from the reaction mixture and subjected to ESI-MS/MS (Fig. 3.9). Quantitative data of the *in vitro* experiment was also expressed as ratios of the peak intensity of each PE molecular species to that of the internal standard.

As shown in Fig. 3.9, deuterium-labeled diC10PE was remodeled at the same level in both TKY12Ga and DLY09. These results suggested again that other enzyme(s) is/are involved in the removal of acyl chains in diC10PE again.

3.3.4 Deletion of *ALE1* gene

Ale1p was reported to have an acyltransferase activity against the *sn*-2 position in various lysophospholipids, including lyso-PA, lyso-PE, lyso-PI, and lyso-PS. It is interesting to examine whether Ale1p is involved in the remodeling of PE with short acyl chains. *ALE1* gene was deleted in TKY12Ga using *KlURA3* as marker gene. Deletion of *ALE1* was verified by PCR (data not shown) and Southern blotting analysis (Fig. 3.10). The primers used in the verification of *ALE1* gene deletion were ALE1-confirm-U and ALE1-confirm-L that were shown in Materials and Methods.

For Southern blotting analysis, the probe was prepared as that for *SPO1*. The restriction enzyme used was *Eco*0109I. Its restriction sites were shown in Fig. 3.10A. *Eco*0109I treatment resulted in a 1.8-kbp and a 2.2-kbp detectable band for parent stains, and a 3.7-kbp detectable band for *ale1* null mutants (Fig. 3.10B).

The growth of *ale1* null mutant in SD medium containing diC10PE and in SG

medium was examined (Fig. 3.10C). As shown in Fig. 3.10C, growth of TKY12Ga*Δale1* in SG medium was similar with its parent strain TKY12Ga, but was impaired in SD medium containing diC10PE, and introduction of the plasmid pALE1 which contained *ALE1* gene, complemented the growth defect.

Narita in our laboratory constructed an *slc1* null mutant in TKY12Ga background. The growth of TKY12Ga*Δslc1* in SG medium was similar to that of TKY12Ga, but also partially impaired in SD medium containing diC10PE (Narita, unpublished). These results suggest that Ale1p and Slc1p were involved in the remodeling of diC10PE.

3.3.5 Analysis of the remodeling of deuterium-labeled diC10PE in *ale1*, *slc1* null mutant

3.3.5.1 Remodeling of deuterium-labeled diC10PE in *ale1* and *slc1* null mutants *in vivo*

Remodeling of deuterium-labeled diC10PE in TKY12Ga*Δale1,* TKY12Ga*Δslc1* was examined *in vivo* as that of DLY09 in 3.3.3.1. After TKY12Ga, TKY12Ga*Δale1* and TKY12Ga*Δslc1* were incubated with 20 μM deuterium-labeled diC10PE for 1 h, lipids were extracted from the cells. PE species of these incubations were analyzed by ESI-MS/MS (Fig. 3.11) and quantified (Fig. 3.12). Quantitative data was expressed as ratio of the peak intensity of each PE molecular species to that of the internal standard.

As shown in Fig. 3.11 and Fig. 3.12, the remodeling of deuterium-labeled diC10PE was impaired in both TKY12Ga*Δale1* and TKY12Ga*Δslc1,* and the defect was much more serious in TKY12Ga*Δale1* than in TKY12Ga*Δslc1,* consistent with their growth defects in SD medium containing diC10PE.

Structural analysis of the remodeling intermediates was performed in *ale1*, *slc1* null mutant as in 2.3.4.3, in TKY12Ga, acyl chains fragmented from 10:0-16:1-PE were detected by product ion scan for m/z 608.4 (Fig. 3.13A), and the intensity of the peak at m/z 253.2, which corresponded to 16:1 acyl chain, was higher than that of m/z 171.1, which corresponded to 10:0 acyl chain. Similar result was obtained by the analysis of 10:0-18:1-PE (Fig. 3.13B), suggesting that the majority of the remodeling intermediates contained C16:1 or C18:1 acyl chain at *sn*-2 position. However, in TKY12Ga*Δale1,* the difference in the intensity between the peak derived from 16:1 or 18:1 acyl chain and the peak derived from 10:0 acyl chain was much smaller than that in TKY12Ga (Fig. 3.13C, D), suggesting the remodeling of diC10PE at *sn*-2 position was decreased in *ale1*

null mutant. In contrast, the fragment pattern of remodeling intermediates in TKY12Ga*Δslc1* was similar with that in TKY12Ga (Fig. 3.13E, F).

These results strongly suggest that Ale1p and Slc1p were involved in the remodeling of diC10PE, and Ale1p account for the majority of the remodeling of PE at the *sn*-2 position.

3.3.5.2 Remodeling of deuterium-labeled diC10PE in *ale1*, *slc1* null mutant *in vitro*

Remodeling of deuterium-labeled diC10PE in *ale1* and *slc1* null mutants were investigated *in vitro* as in 3.3.3.2 (Fig. 3.14). Quantitative data of the *in vitro* experiment was also expressed as ratios of the peak intensity of each PE molecular species to that of the internal standard (Fig. 3.15).

As shown in Fig. 3.14 and Fig. 3.15, amount of the remodeling intermediates (the 10:0-16:1-PE and 10:0-18:1-PE) was decreased in the *ale1* and *slc1* null mutant, especially in the *ale1* null mutant, comparing to that in TKY12Ga, suggesting remodeling of deuterium-labeled diC10PE *in vitro* was also impaired in both TKY12Ga*Δale1* and TKY12Ga*Δslc1,* and the defect was more serious in TKY12Ga*Δale1* than in TKY12Ga*Δslc1*. These results were consistent with the *in vivo* experiment.

3.3.6 Analysis of the remodeling of deuterium-labeled diC10PE in *gat1*, *gat2* null mutant

GAT1 and *GAT2* encode two Gly-3-P acyltransferase which synthesized lyso-PA by transfer of a fatty acid from fatty acyl-CoA to the *sn*-1 position of Gly-3-P and DHAP. Genetic inactivation of both *GAT1* and *GAT2* resulted in synthetic lethal phenotype of yeast cell. Narita in our laboratory constructed individual null mutant of these two genes in TKY12Ga and examined their growth in SD medium containing diC10PE. However, no growth defect of TKY12Ga*Δgat1* and TKY12Ga*Δgat2* was observed (Narita, unpublished). Further analysis was performed using deuterium-labeled diC10PE in these two mutants.

Remodeling of deuterium-labeled diC10PE in TKY12Ga*Δgat1* and TKY12Ga*Δgat2* was examined i*n vivo* as in 3.3.3.1. Cells of TKY12Ga, TKY12Ga*Δgat1* and TKY12Ga*Δgat2* were incubated with 20 μM deuterium-labeled diC10PE for 1 h. Lipids were extracted and analyzed by ESI-MS/MS (Fig. 3.16).

Quantitative data were expressed as ratio of the peak intensity of each PE molecular species to that of the internal standard (Fig. 3.17).

As shown in Fig.3-16 and Fig.3-17, TKY12Ga*Δgat1* and TKY12Ga*Δgat2* remodeled deuterium-labeled diC10PE at the same level with TKY12Ga, consistent with their growth in SD medium containing diC10PE, suggesting that other enzymes are involved in the remodeling of diC10PE or *GAT1* and *GAT2* genes have redundant function in this reaction.

Remodeling of deuterium-labeled diC10PE in *gat1*, *gat2* null mutant was also investigated *in vitro* as in 3.3.3.2. As shown in Fig.3.18, no defect was detected in the remodeling of deuterium-labeled diC10PE *in vitro* in both TKY12Ga*Δgat1* and TKY12Ga*Δgat2,* consistent with the experiment *in vivo* (Fig.3.18).

3.4 Discussion

3.4.1 Phospholipase involved in remodeling

It was reported that in the *in vitro* assay, phospholipases, Plb1p and Plb2p, showed high phospholipase B and lysophospholipase activity with PS as the substrate. Their phospholipase B activity toward the major phospholipid classes were in the order of PS > PI > PC > PE. Plb3p was selective for PS and PI, whereas PC and PE were not cleaved by it. When using lysoPC as the substrate for Plb1p, and using lysoPI as the substrate for Plb2p and Plb3p, they all showed transacylase activity, converting lysoPC or lysoPI to PC or PI (Lee et al., 1994; Merkel et al., 1999; Merkel et al., 2005). Yon and Ono showed that when all of *PLB1*, *PLB2*, and *PLB3* were deleted in *cho1* or *Δpem1Δpem2* mutant, it still grew in diC8PC-containing medium, although the growth was partially impaired (Yon et al., 1998) (Ono, 2008). These results raised the possibilities that Plb1p, Plb2p, and Plb3p are involved in remodeling of PC and that other enzymes also has a significant role in remodeling of PC.

DLY09 can grow in SD medium containing diC10PE and remodel the deuterium-labeled diC10PE. In addition, lyso-10:0-PE was detected in the culture supernatant. These results suggest other enzyme(s) is/are involved in the removal of acyl chains in diC10PE. In *S. cerevisiae*, product of *PER1* gene possesses the GPI-phospholipase A2 activity, and function in the lipid remodeling from normal PI to a C26 fatty acid-containing PI in the GPI anchor (Fujita et al., 2006). There has been no information available on substrate of Per1p except PI, PE might be one of its substrates. It is also possible that the acyl chain of diC10PE is removed by triacylglycerol lipase(s),

ester(s) or the enzyme(s) not homologous to any lipase. In *S. cerevisiae*, products of four genes, *TGL2, TGL3, TGL4* and *TGL5*, were reported to have triacylglycerol lipase activity (Athenstaedt and Daum, 2003, 2005; Van Heusden et al., 1998). *TGL1, YLL012/YEH1, YLR020/YEH2* encode lipases that are required for steryl ester hydrolysis (Koffel et al., 2005). It would be of interest to examine the remodeling of diC10PE in their individual or multiple null mutants in TKY12Ga or DLY09.

The growth of DLY09 in SD medium containing diC10PE was better than its parent strain TKY12Ga (Fig. 3.6). In addition, when TKY12Ga and DLY09 were incubated with deuterium-labeled diC10PE, a larger amount of deuterium-labeled PE species was detected in DLY09 than in TKY12Ga (Fig. 3.7). These results indicate that degradation of exogenous diC10PE in DLY09 is decreased, which makes it easier to get sufficient remodeling substrate to maintain the PE level required for the growth when it was cultured in SD medium containing diC10PE.

3.4.2 Remodeling of PE in acyltransferase disrupted mutant

Defect of TKY12Ga*Δale1* and TKY12Ga*Δslc1* in the remodeling of deuterium-labeled diC10PE *in vivo* and *in vitro* suggests Ale1p and Slc1p are involved in the reacylation of diC10PE metabolites. Ale1p catalyzes the transfer of acyl chains to various lysophospholipids, including lyso-PE, at *sn*-2 position (Benghezal et al., 2007) (Jain et al., 2007) (Riekhof et al., 2007b) (Tamaki et al., 2007). Slc1p also acylates various lysophospholipids *in vitro*, although the acyltransferase activity at the *sn*-2 position of lyso-PE is less prominent compared to those of lyso-PA, lyso-PS, and lyso-PI (Benghezal et al., 2007). These reports are consistent with the result that remodeling defect in TKY12Ga*Δale1* was more serious than in TKY12Ga*Δslc1*. Since lyso-10:0-PE was one of the remodeling intermediates in the diC10PE remodeling process, Ale1p and Slc1p probably transfer fatty acid to the *sn*-2 position of lyso-10:0-PE.

It was shown that TKY12Ga*Δgat1* and TKY12Ga*Δgat2* remodeled deuterium-labeled diC10PE at the same level with TKY12Ga both *in vivo* and *in vitro*. One possibility is that these acyltransferase are not involved in remodeling of PE. These enzymes might not prefer diC10PE or lyso-10:0-PE as a substrate for transacylation, since lyso-PE and Gly-3-P are quite different in structure. Another possibility is that both of these enzymes are involved in the remodeling of diC10PE and have an overlapping function in the reaction. The enzymes involved in the introduction of acyl

chains at *sn*-1 position of phospholipid remain to be determined. Intramolecular transacylation of acyl chain from *sn*-2 position to *sn*-1 is possible. However, since the ratio of remodeling intermediate that contains 16:1 or 18:1 acyl chain at *sn*-1 position was increased in the *ale1* null mutant, there appear to exist enzymes that have acyltransferase activities at *sn*-1 position of lyso-PE or GroPEtn in yeast.

Fig. 1.4 summarized characterized and deduced acyltransferases in MBOAT, LPAAT and DGAT2 families in yeast and mammalian cell. Three uncharacterized genes, *YBR042c*, *YDR018c* and *YOR298w*, are in LPAAT family. It would be of interest to examine the remodeling of diC10PE in their individual or multiple null mutants in TKY12Ga.

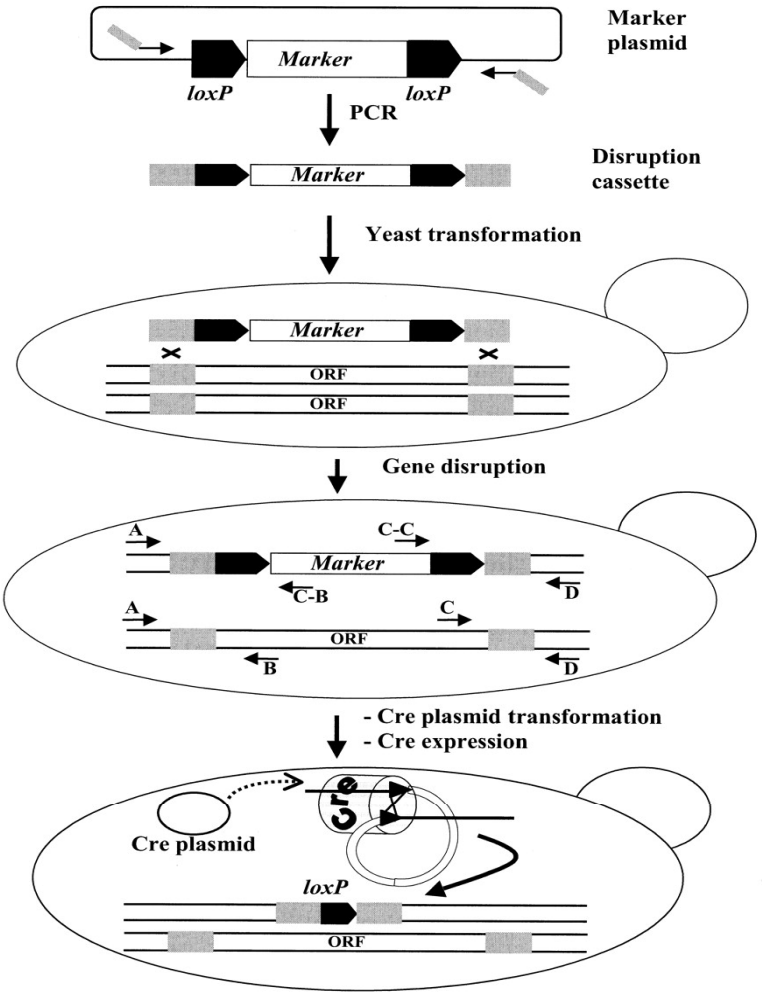


Fig. 3.1 The Cre-*loxP* gene disruption and marker rescue procedure.

[Gueldener et al., 2002]

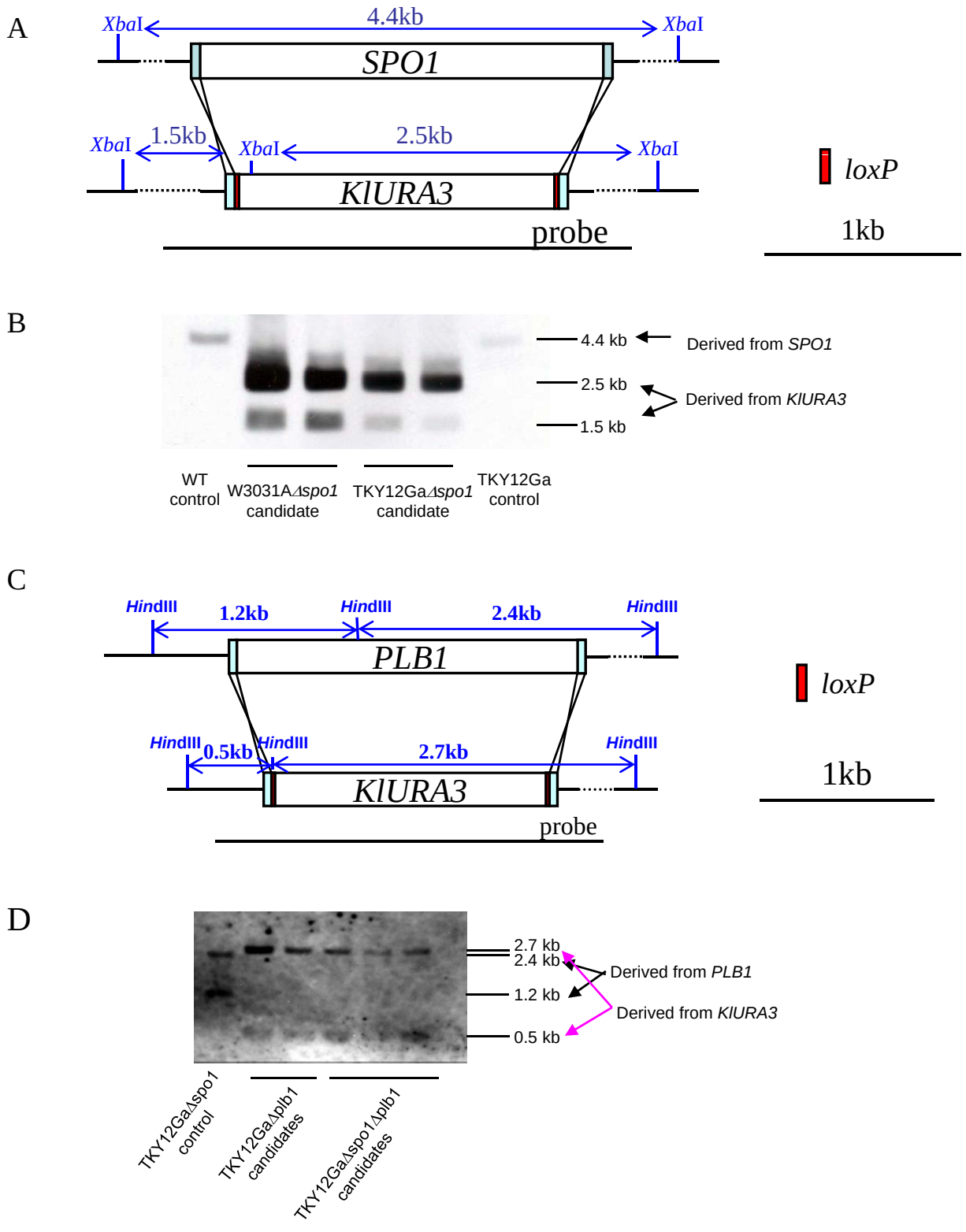


Fig. 3.2 (A) strategy of the *SPO1* gene deletion
(B) *Δspo1* verification by Southern blotting analysis
(C) strategy of the *PLB1* gene deletion
(D) *Δplb1* verification by Southern blotting analysis

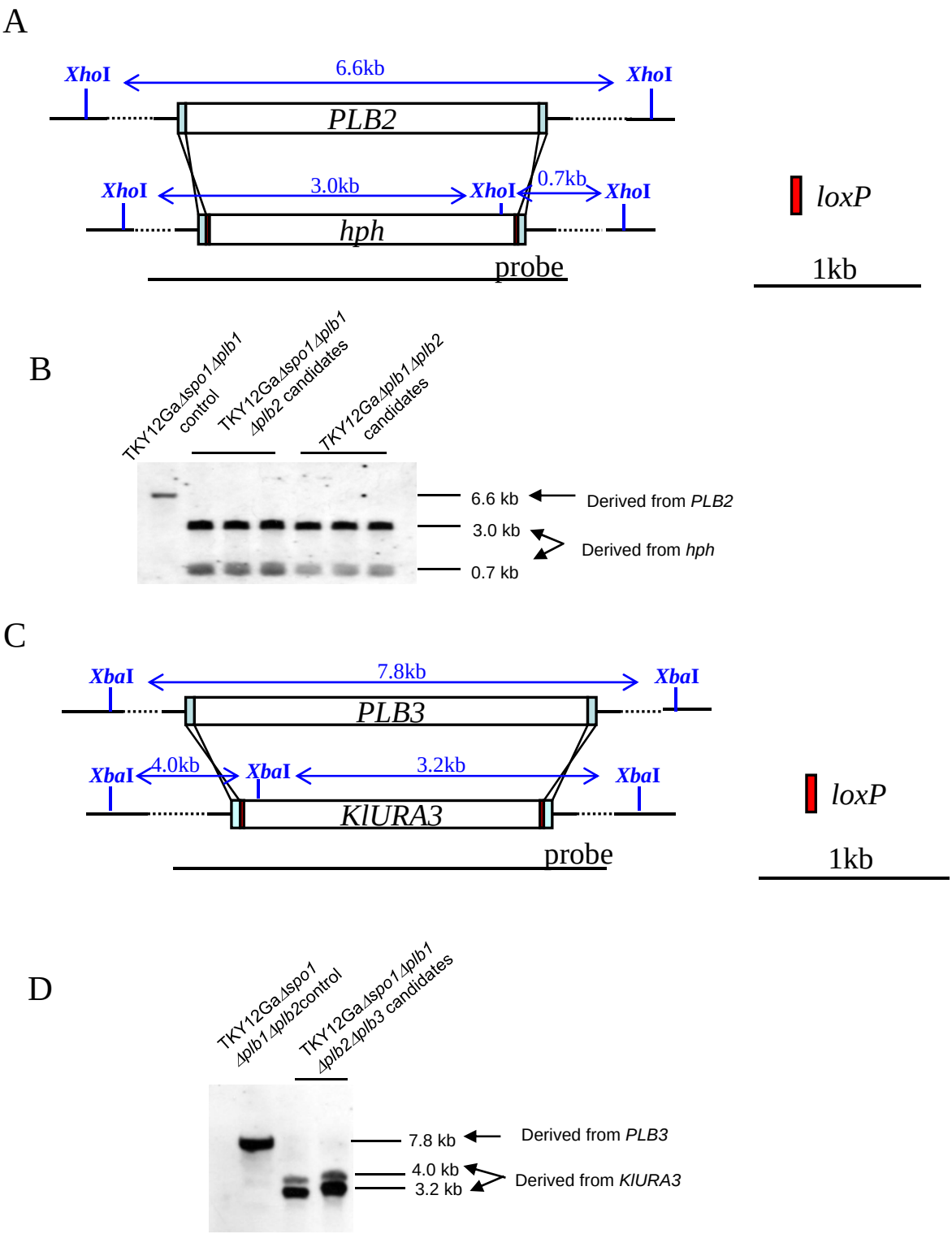


Fig. 3.3 (A) strategy of the *PLB2* gene deletion
(B) *Δplb2* verification by Southern blotting analysis
(C) strategy of the *PLB3* gene deletion
(D) *Δplb3* verification by Southern blotting analysis

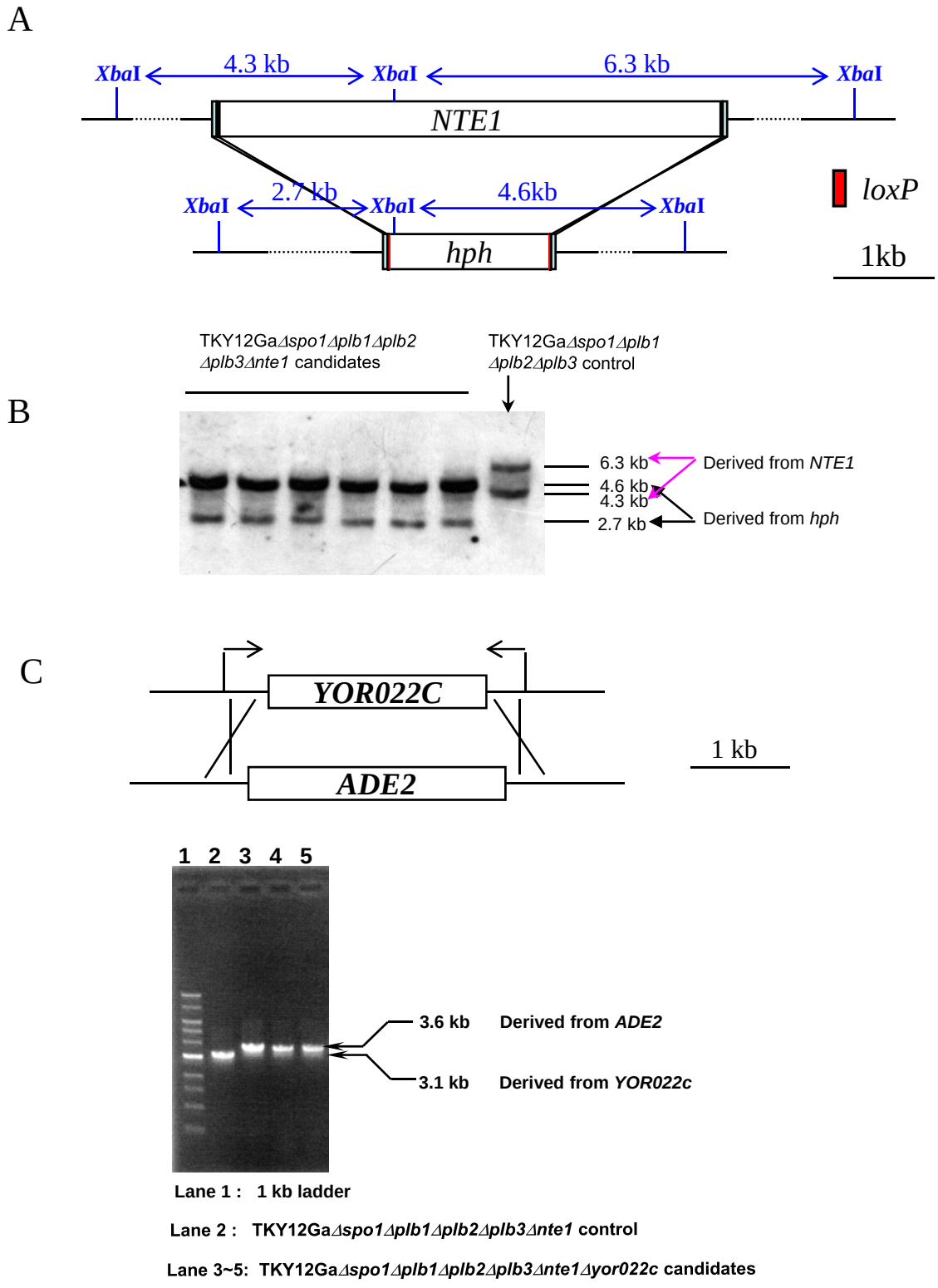


Fig. 3.4 (A) strategy of the *NTE1* gene deletion
(B) *⊿nte1* verification by Southern blotting analysis
(C) *⊿yor022c* verification by PCR

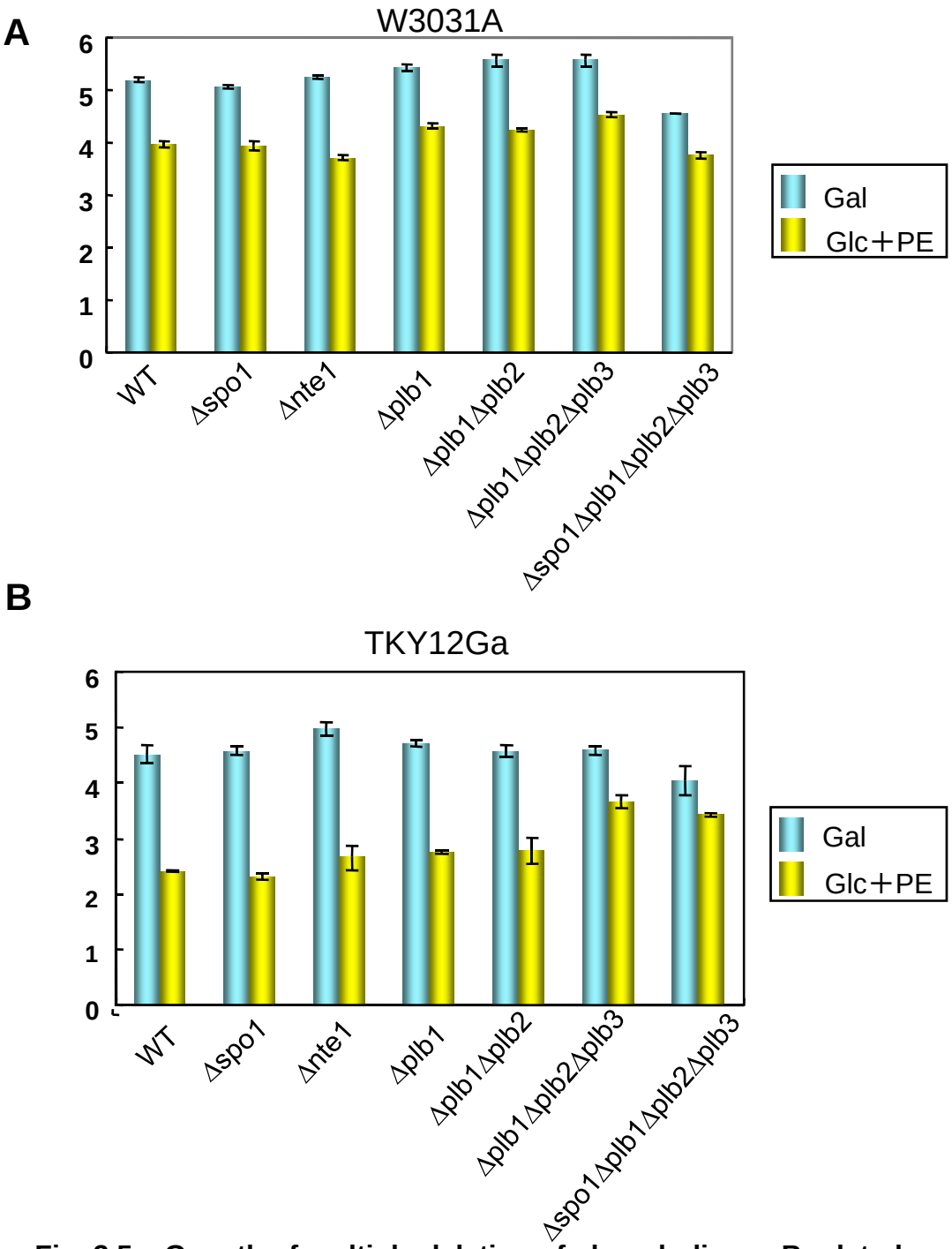


Fig. 3.5 Growth of multiple deletion of phospholipase B related genes in TKY12Ga in SD medium containing diC10PE.

The mutants were first seed from SG medium to SD medium and precultured 24 h to consume Ect1p and/or PE, then was seeded at a starting OD_{600}=5 x 10^{-3} to SD medium containing diC10PE and SG medium (as control) and cultured for 48 h. After 48 h, OD_{600} of the cultures were measured. Results represent the means of three independent experiments ± SE.

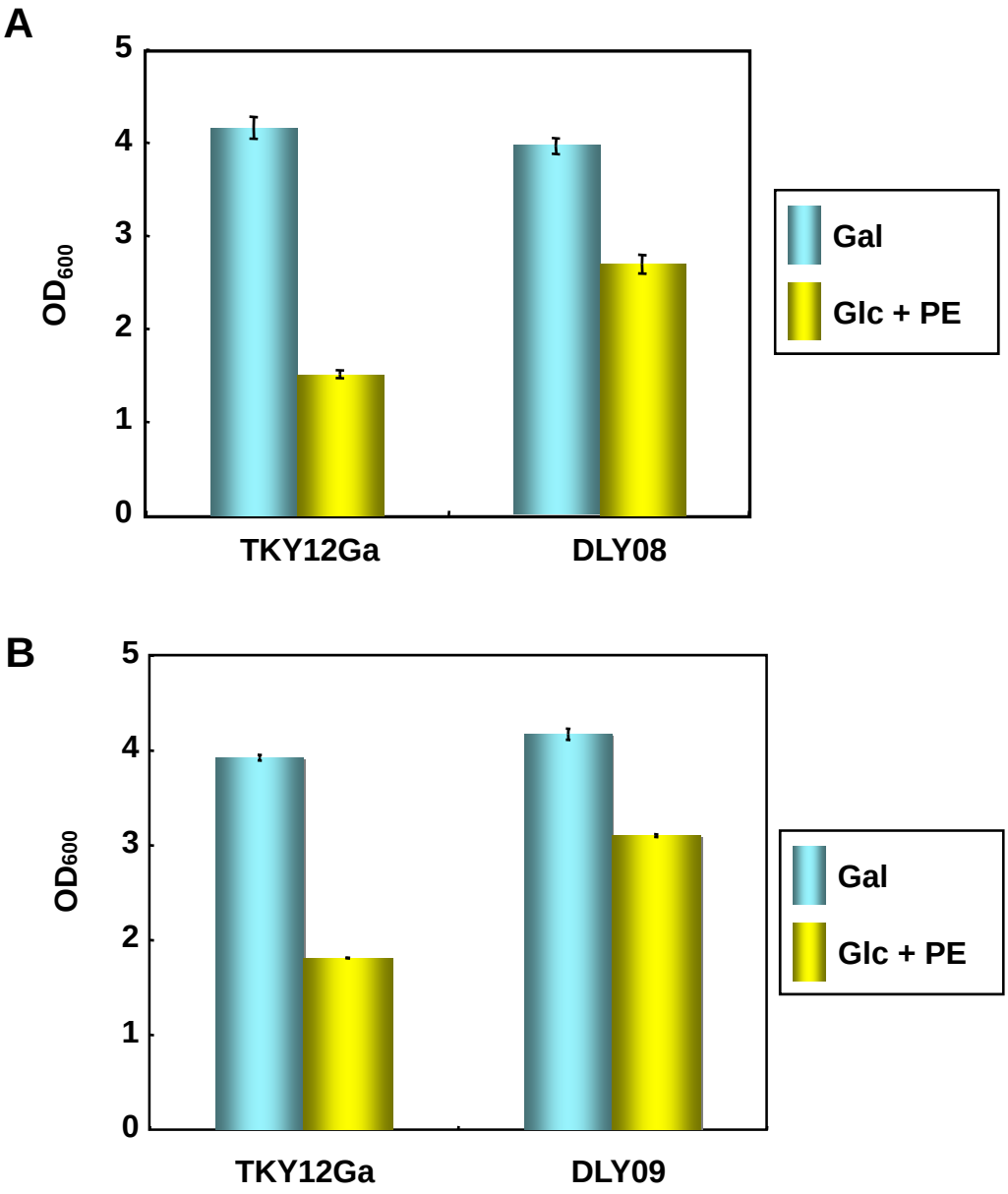


Fig. 3.6 DLY08 and DLY09 grow better than their parent strain TKY12Ga in SD medium containing diC10PE.

The mutants were first seed from SG medium to SD medium and precultured 24 h to consume Ect1p and/or PE, then was seeded at a starting OD_{600}=5 x 10^{-3} to SD medium containing diC10PE and SG medium (as control) and cultured for 48 h. After 48 h, OD_{600} of the cultures were measured. Results represent the means of three independent experiments ± SE.

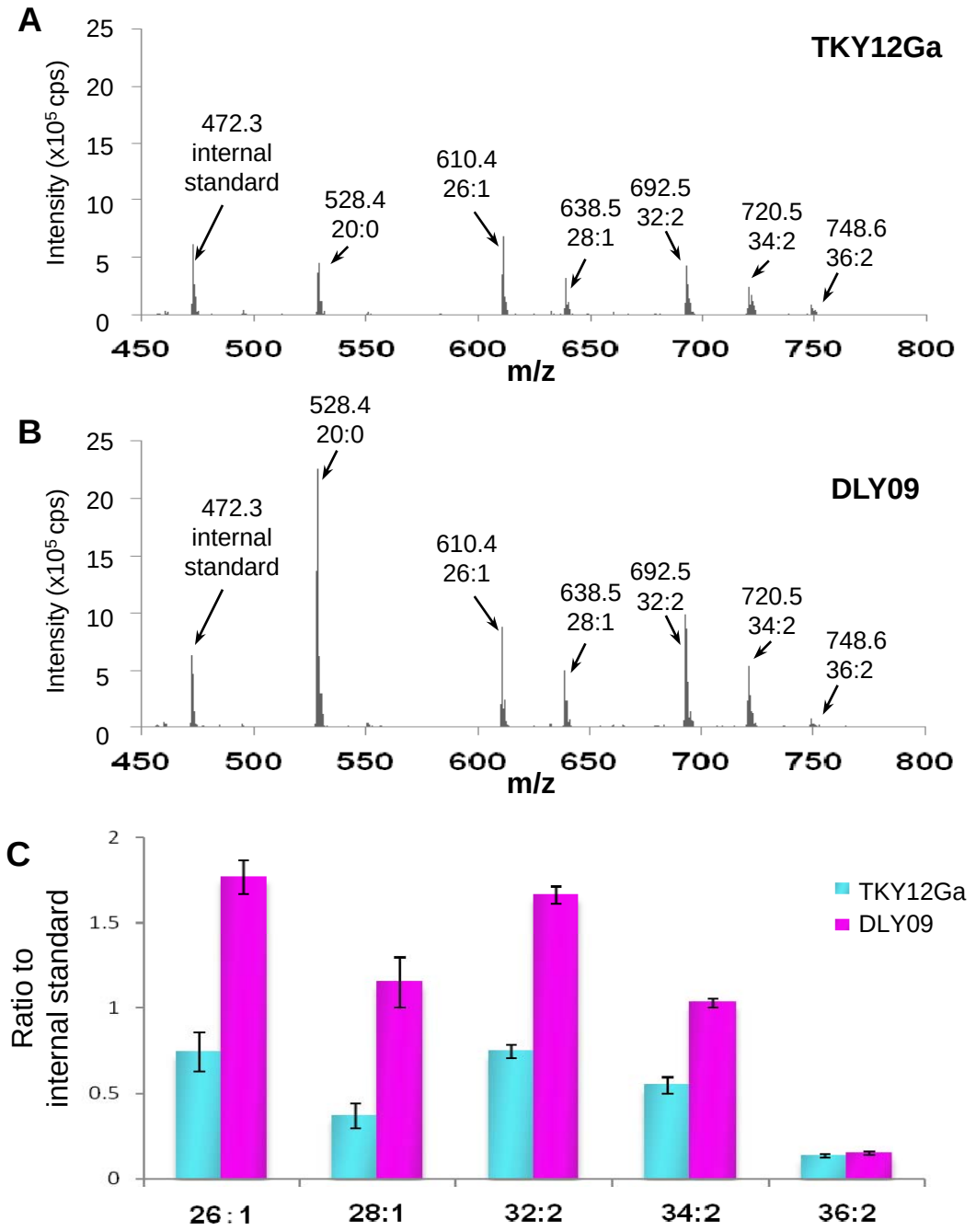


Fig. 3.7 Remodeling of deuterium-labeled diC10PE in TKY12Ga and DLY09

Cells of TKY12Ga (A) and DLY09 (B) were precultured as in Fig. 3.6, then was shifted to SD medium containing non-labeled diC10PE at a starting OD_{600}=5 x 10^{-3} and cultured till OD_{600} reached 1.Then cells were incubated with 20 μM deuterium-labeled diC10PE for 1 h. Lipids were extracted and subjected to ESI-MS/MS. Deuterium-labeled PEs were detected with neutral loss scan for *m/z* 145 in positive ion mode. Quantitative data (C) was expressed as ratio of the peak intensity of each PE molecular species to that of the internal standard. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

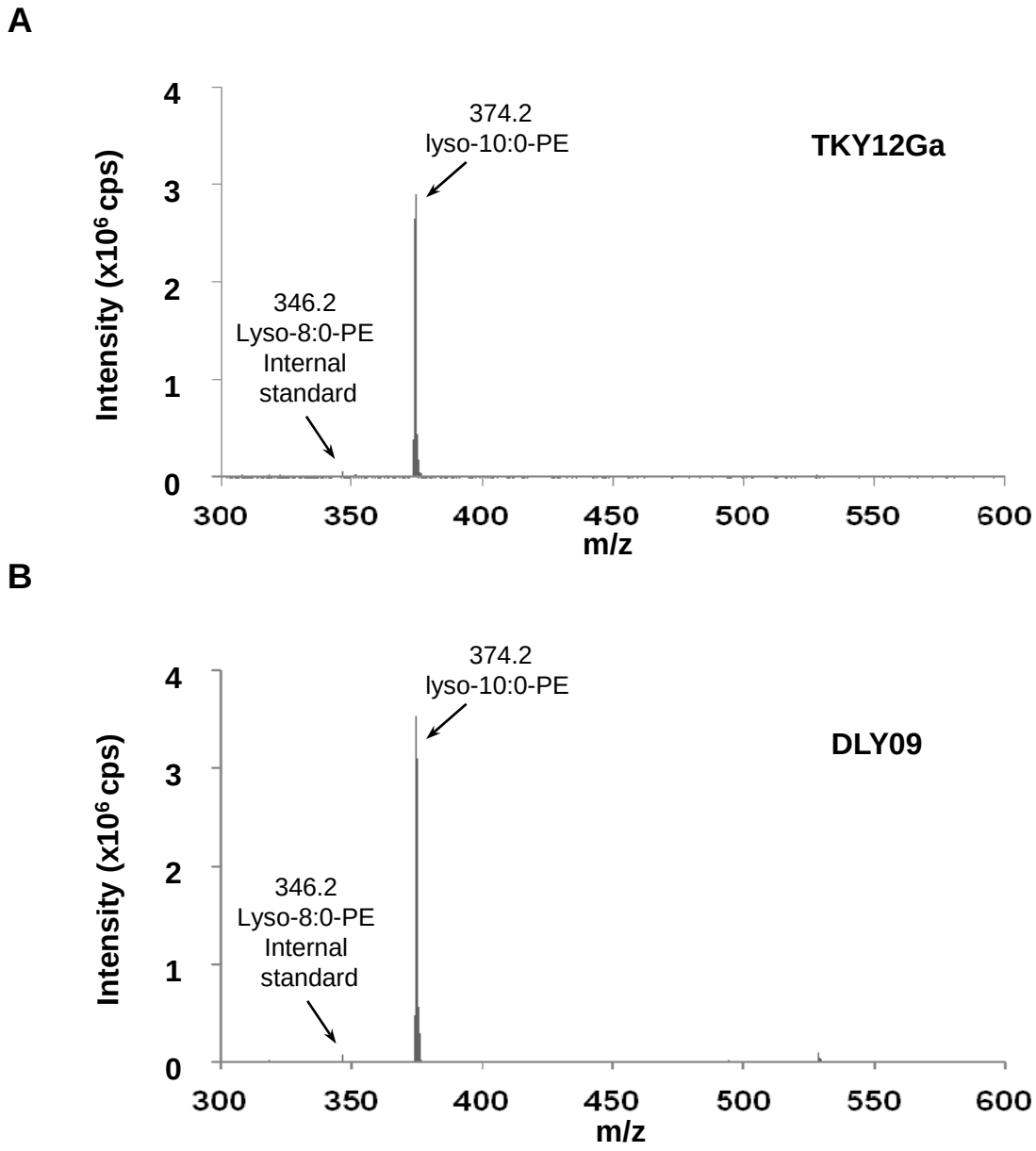


Fig. 3.8 DLY09 hydrolyzes exogenous PE at the same level with TKY12Ga

TKY12Ga and DLY09 were incubated with deuterium-labeled diC10PE as in Fig. 3.7. Then lipids in the culture supernatant were extracted and analyzed by ESI-MS/MS. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

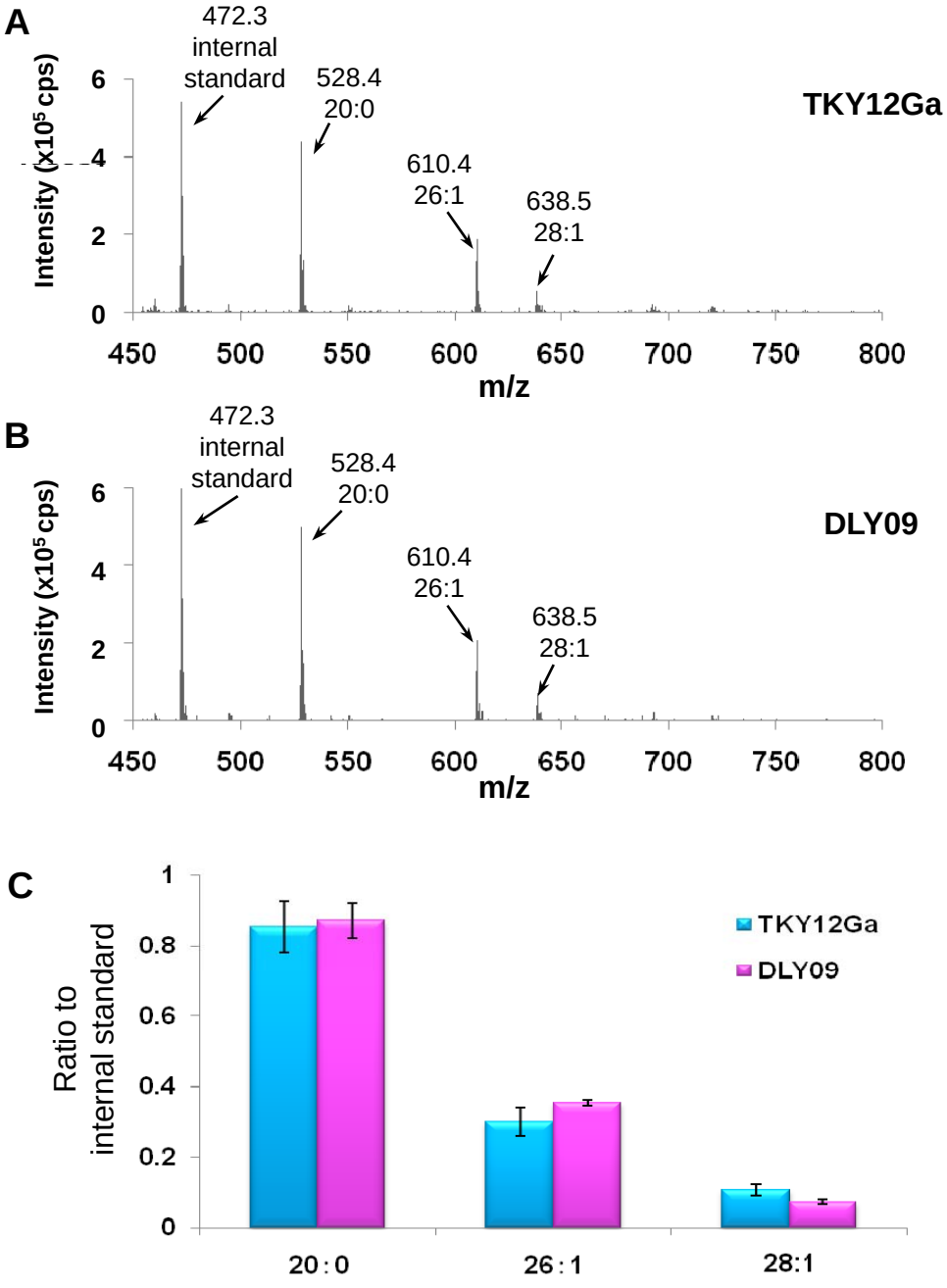


Fig. 3.9 DLY09 remodeled diC10PE *in vitro* similar as TKY12Ga

Cell lysate of TKY12Ga (A) and DLY09 (B) were incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA for 1 h as described in Materials and Method. Lipids were extracted and subjected to ESI-MS/MS. Quantitative data (C) was expressed as ratio of the peak intensity of each PE molecular species to that of the internal standard. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

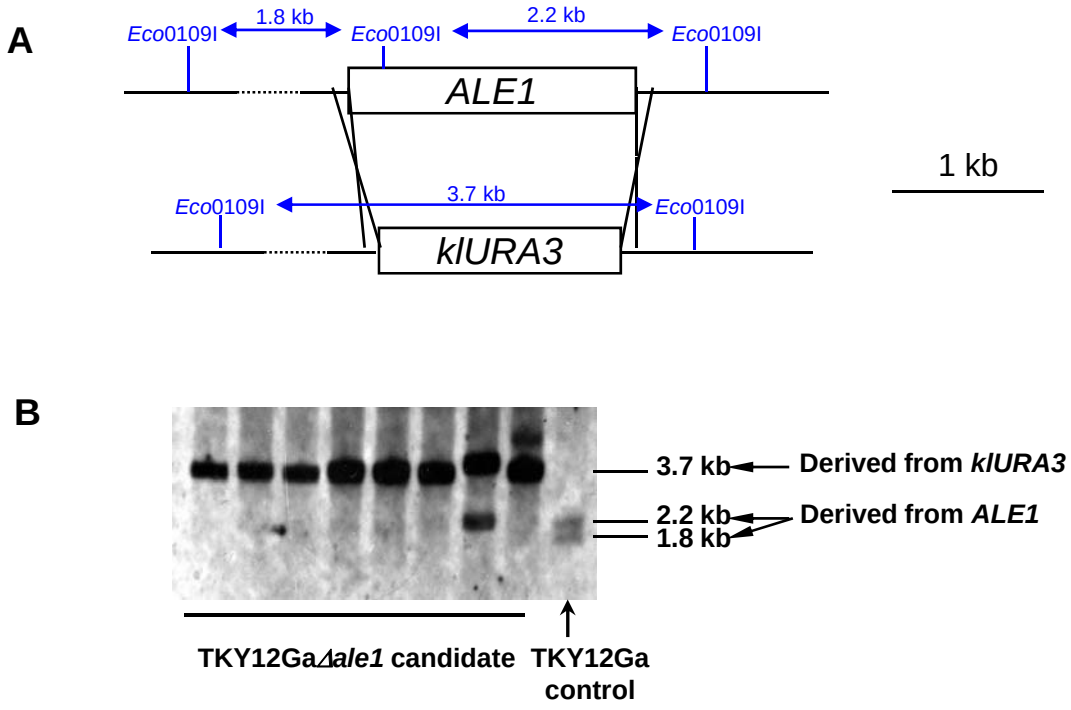


Fig. 3.10 (A) strategy of the *ALE1* gene deletion
(B) *Δale1* verification by Southern blotting analysis
(C) Growth of ale1 null mutant is impaired in SD medium containing diC10PE Analysis was performed as in Fig. 3.5. Results represent the means of three independent experiments ± SE.

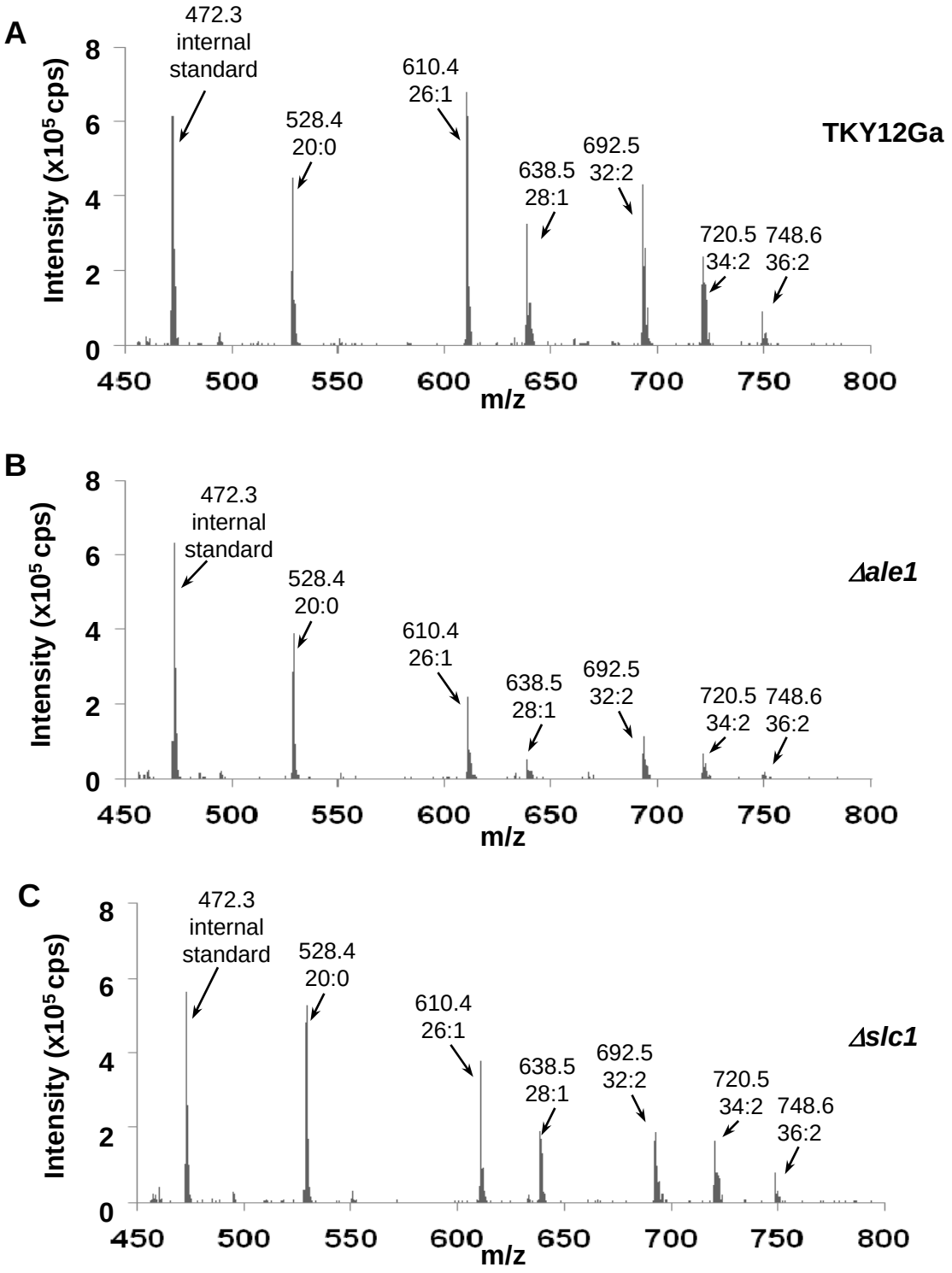


Fig.3.11 Remodeling of deuterium-labeled diC10PE was partially impaired in *Δale1* and *Δslc1* mutant *in vivo*

Analysis was perform as in Fig. 3.7. After cells were incubated with deuterium-labeled diC10PE for 1 h, lipids were extracted from TKY12Ga (A), *Δale1* (B) and *Δslc1* (C) cells and subjected to ESI-MS/MS. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

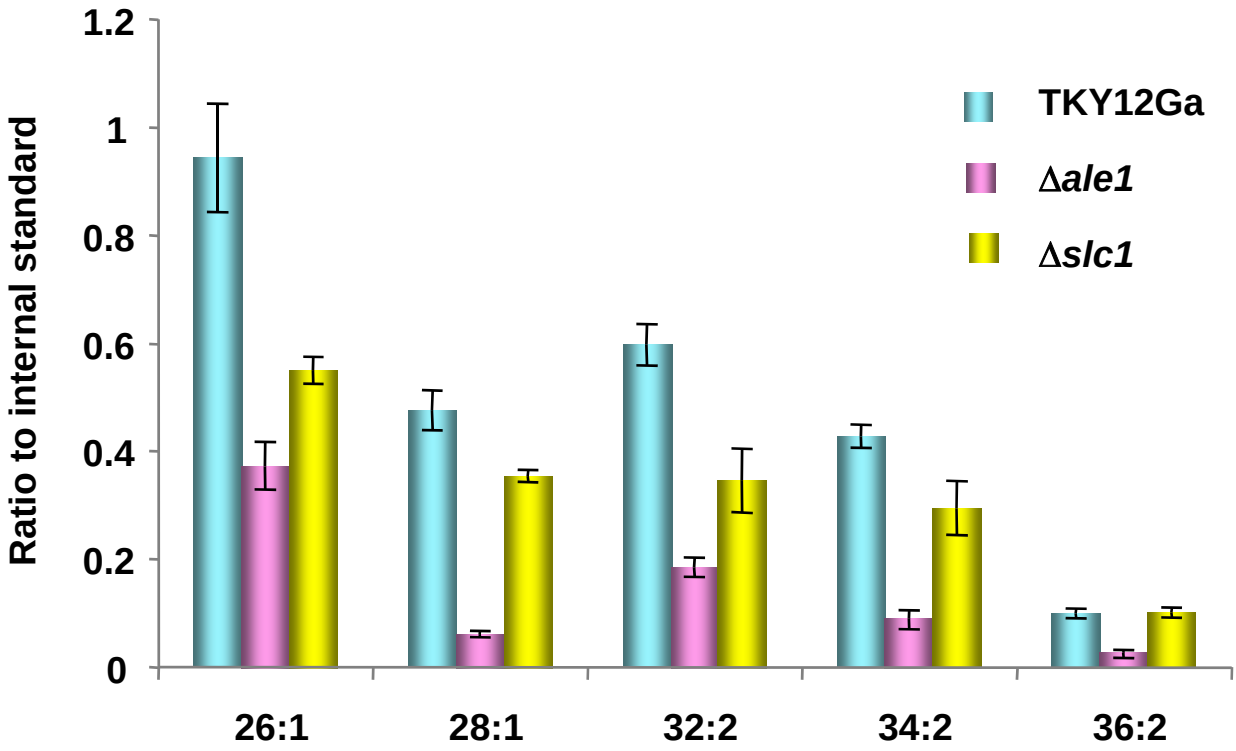


Fig.3.12 Quantification of *in vivo* remodeling of deuterium-labeled diC10PE in *Δale1* and *Δslc1* mutant

Quantitative data was expressed as ratio of the peak intensity of each PE molecular species in Fig. 3.11 to that of the internal standard. Results represent the means of three independent experiments ± SE.

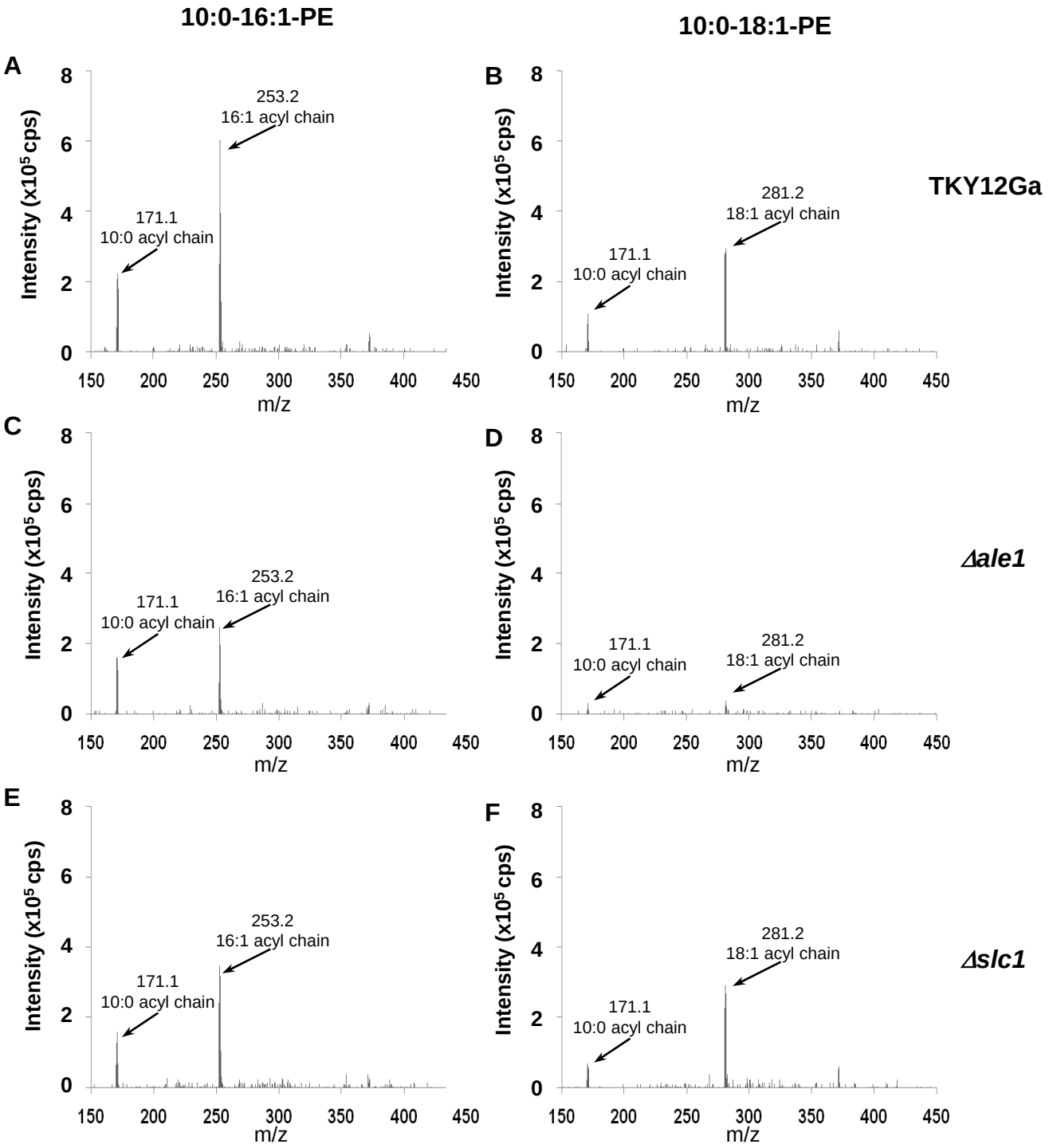


Fig. 3.13 Analysis of remodeling intermediates in *Δale1* and *Δslc1*

Fatty acyl chains fragmented from the remodeling intermediates, 26:1-PE (A, C & E) and 28:1-PE (B, D & F) in the phospholipids extracted from TKY12Ga (A & B), *Δale* (C & D) and *Δslc1* (E & F) cells after they were incubated with deuterium-labeled diC10PE for 1h were analyzed by ESI-MS/MS. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

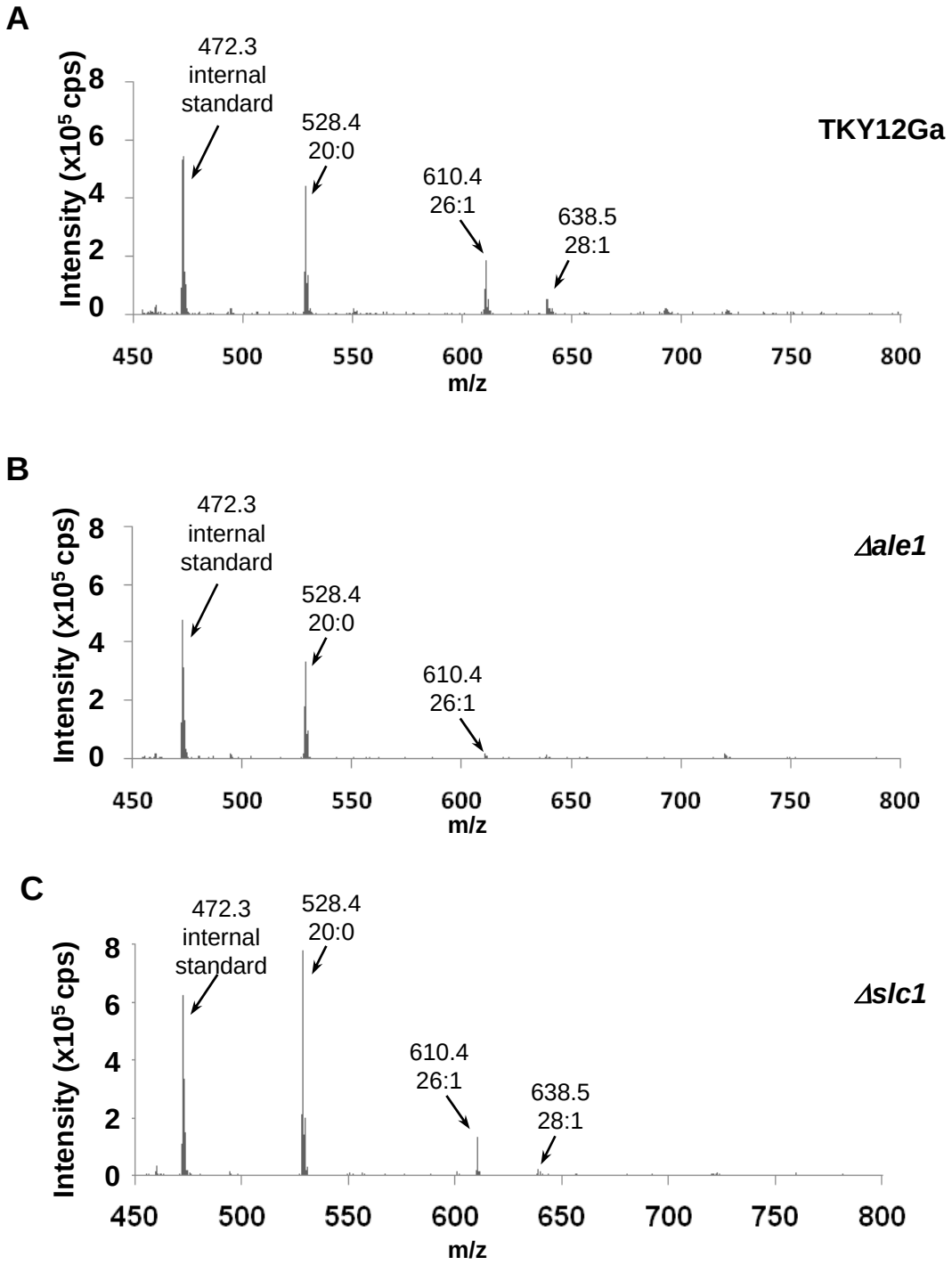


Fig.3.14 *Δale1, Δslc1* defected in remodeling of PE *in vitro*

Cell lysate of TKY12Ga (A) *Δale1* (B) and *Δslc1* (C) were incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA for 1 h as described in Materials and Method. Lipids were extracted and subjected to ESI-MS/MS. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

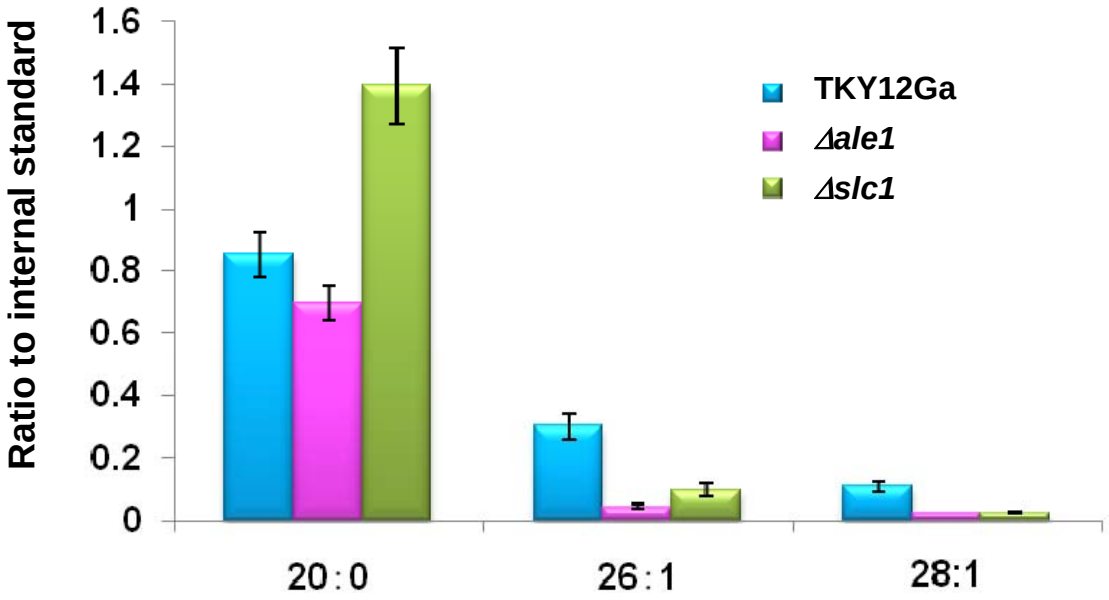


Fig.3.15 Quantification of *in vitro* remodeling of deuterium-labeled diC10PE in *Δale1* and *Δslc1* mutant

Quantitative data was expressed as ratio of the peak intensity of each PE molecular species in Fig. 3.14 to that of the internal standard. Results represent the means of three independent experiments ± SE.

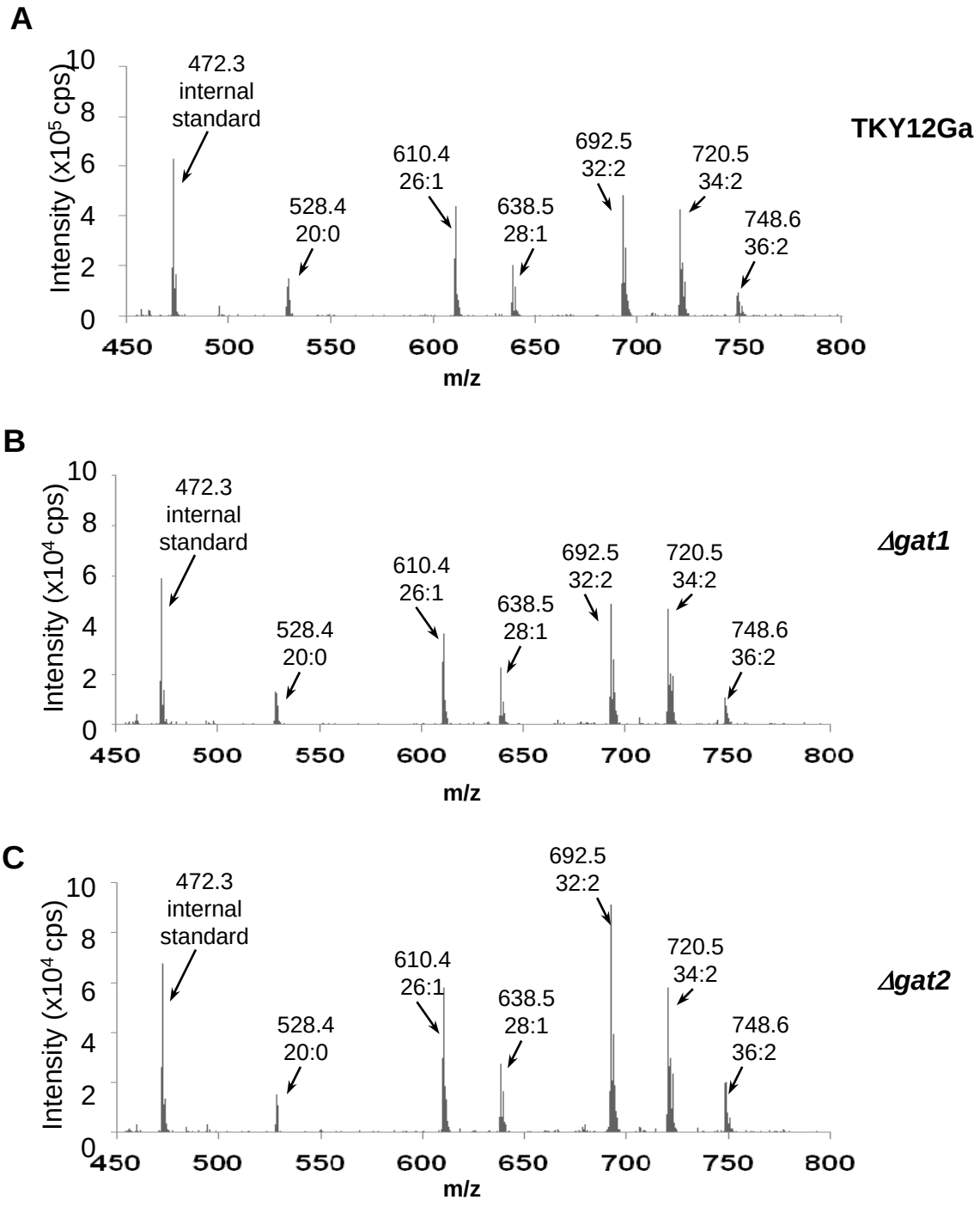


Fig.3.16 Remodeling of deuterium-labeled diC10PE in *Δgat1* and *Δgat2* mutant was similar with TKY12Ga *in vivo*

Analysis was perform as in Fig. 3.7. After cells were incubated with deuterium-labeled diC10PE for 1 h, lipids were extracted from TKY12Ga (A), *Δgat1* (B) and *Δgat2* (C) cells and subjected to ESI-MS/MS. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

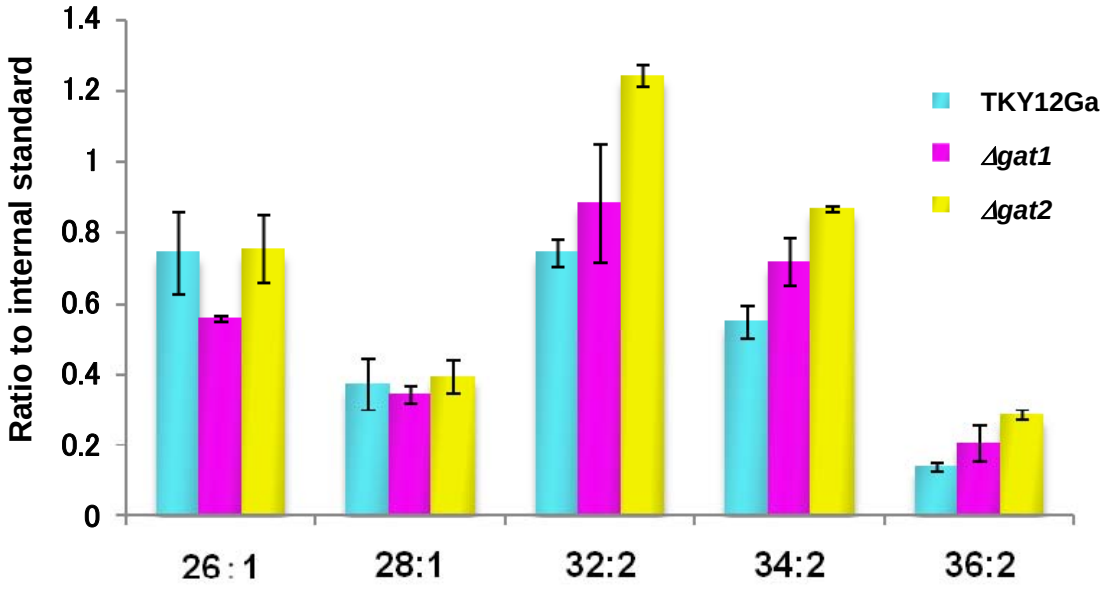


Fig.3.17 Quantification of *in vivo* remodeling of deuterium-labeled diC10PE in *Δgat1* and *Δgat21* mutant

Quantitative data was expressed as ratio of the peak intensity of each PE molecular species in Fig. 3.16 to that of the internal standard. Results represent the means of three independent experiments ± SE.

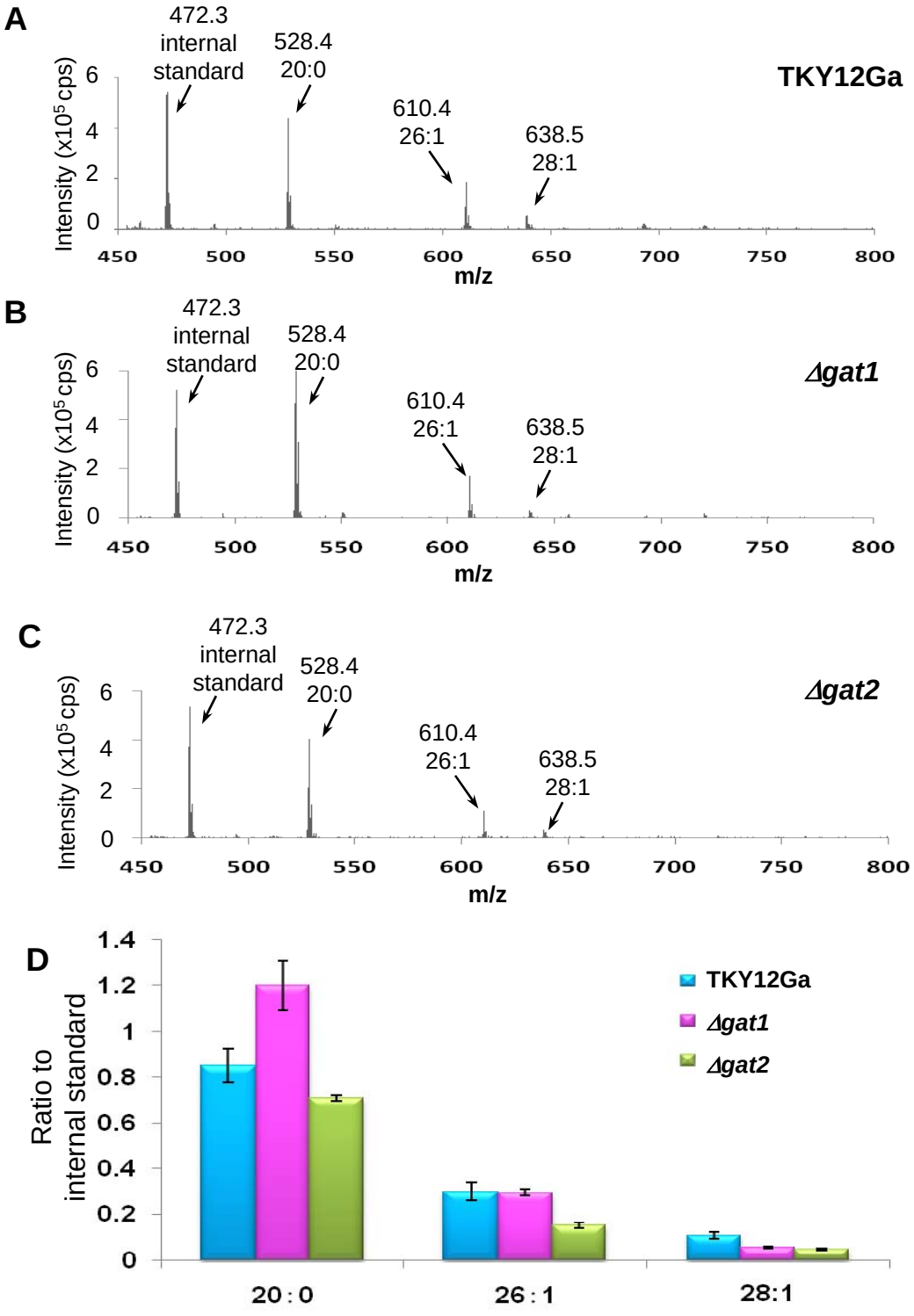


Fig.3.18 Quantification of *in vitro* remodeling of deuterium-labeled diC10PE in *Δgat1* and *Δgat21* mutant

Cell lysate of TKY12Ga (A) *Δgat1* (B) and *Δgat* (C) were incubated with deuterium-labeled diC10PE and palmitoleoyl-CoA for 1 h as described in Materials and Method. Lipids were extracted and subjected to ESI-MS/MS. Quantitative data (C) was expressed as ratio of the peak intensity of each PE molecular species to that of the internal standard. The theoretical *m/z* values are indicated, and the experimental errors were within 0.3 mass unit. Similar results were obtained in three independent experiments.

Chapter 4

Analysis of mutant defective in utilization of exogenous PE

4.1 Introduction

In chapter 3, analysis of deletion mutants of the genes encoding acyltransferases suggested that *ALE1* and *SLC1* genes were involved in the remodeling reaction of PE. To identify the enzymes involved in the remodeling reaction, in addition to these acyltransferases, analysis of mutants defective in utilization of exogenous PE will be effective.

Kakihara mutagenized TKY12Ga with mutagen ethyl methanesulfonate (EMS) and isolated 33 mutants that could grow in the SG media but have defects in growth in the SD medium containing diC10PE (Kakihara, 2004). M25 is one of these mutants. M25 grows at a wild type rate in the SG medium, in which PE can be synthesized through Kennedy pathway, but cannot grow in SD medium containing diC10PE. In addition, M25 has the phenotype of temperature sensitivity in growth in SG medium at 37℃. It was reported that fluorescently-labeled PE (NBD-PE) can be internalized across the plasma membrane to the nuclear envelope/ER and mitochondia, in *S. cerevisiae* (Grant et al., 2001). Kakihara showed that the uptake of NBD-PE by M25 was similar to that by TKY12Ga and the internalized NBD-PE was also localized in the same organelles as wild type, suggesting that M25 does not have defects in the uptake of PE (Kakihara, 2004). Furthermore, the mutation(s) that caused growth defects in PE-containing medium and temperature sensitivity was/were recessive. These results raised the possibility that M25 has defects in remodeling of diC10PE.

In this chapter, M25 was analyzed and the gene that complements both the defect in growth in PE-containing medium and temperature sensitivity of it was isolated.

4.2 Materials and Methods

4.2.1 Strains

Escherichia.coli

JA221	*recA1, leuB6, trpE5, hsdR-, hsdM-, lacI, thr, thi*

Saccharomyces cerevisiae

TKY12Ga	*Mat a*, P_{GAL1}*ECT1::HIS3, Δpsd2::LEU2, ura3, trp1, ade2, Δpsd1::KAN*^{r} (Kakihara, 2004)
TKY12Gα	*Mat* α, P_{GAL1}*ECT1::HIS3, Δpsd2::LEU2, ura3, trp1, ade2, Δpsd1::KAN*^{r} (Kakihara, 2004)
M25	Mutant derived from TKY12Ga, defective in the utilization of exogenous PE in SD medium

4.2.2 Media

SG medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-galactose (Wako)	2%

SD medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-glucose (Wako)	2%

YPG medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-galactose (Wako)	2%

Spore medium

Yeast extract (DIFCO)	0.1%
Pottasium acetate (NIHON SEIYAKU)	1%
D-galactose (Wako)	0.05%

For adding exogenous PE to medium, PE is dissolved in ethanol by a 30 min sonication and then added to medium with a final ethanol concentration of 1% in the medium.

For solid medium, 2% agar (Wako) was added.

When required, add the amino acid and antibiotic as the following concentration:

L-Histidine (NACALAI)	2.4 μg/ml
L-Leucine (Kanto Kagaku)	10 μg/ml
Uracil (Kanto Kagaku)	2.4 μg/ml
L-Tryptophan (Kanto Kagaku)	4 μg/ml
Adenine Hydrochloride (Kanto Kagaku)	2.4 μg/ml
Ampicillin Sodium （Wako）	50 μg/ml

When cultured TKY12Ga or the stains derived from it, choline chloride (Kanto Kagaku) was always supplied to the glucose containing medium at the final concentration of 1 mM; 2-Aminoethanol (NACALAI) and choline chloride (Kanto Kagaku) were always supplied to the galactose containing medium both at the final concentration of 1 mM

4.2.3 Plasmids

YCplac22	Given by Dr.Gietz (Iwamoto, 2002)
YCplac33	Given by Dr.Gietz (Iwamoto, 2002)
YCplac50	Given by Dr.Gietz (Iwamoto, 2002)
pM25-66	This study
YCpYOL153C	This study
YCpFRE7	This study
YCpGRE2	This study
YCpYOL150	This study
YCpDCP1	This study
YCpSPT20	This study

4.2.4 Chemicals

As described in 2.2.3

4.2.5 Methods

4.2.5.1 Tetrad analysis

① Transform M25 with YCplac50 (carrying *URA3* as marker gene) and TKY12Gα with YCplac22 (carrying *TRP1* as marker gene)
② Overnight pre-culture M25/ YCplac50 and TKY12Gα/ YCplac22 in 2ml SG medium at 30℃ shaking incubator, respectively.
③ mix 300 μl of each culture and 300 μl YPG medium in a microtube, incubate for 6 h in an immobile incubator.
④ Collect cells by centrifuge, plate to Ura-Trp- SG medium.
⑤ Replicate the colonies of the diploid to the Ura-Trp- SG medium again.
⑥ Pre-culture the diploid in YPG medium, overnight.
⑦ Harvest the cells and wash with the spore medium for 2 times.
⑧ Incubate the cells in spore medium at 25℃ on shaking incubator for 3 days.
⑨ Harvest the cells and resuspend in 200~500 μl 0.5mg/ml zymolyase solution.
⑩ Incubate for 10 min in a 37℃ water bath
⑪ Add 1 ml water and place on ice.
⑫ Aliquot 50~200 μl of spores onto a YPG medium, allow the solution to dry.
⑬ Under a microscope, try to find nice 4-spore tetrads, pick up with the needle without avoid also picking up surrounding cells. Move to a predefined position on the plate and deposit the tetrad.
⑭ Incubate the plate at 30℃.

4.2.5.2 Plasmid extraction from yeast

Plasmid extraction from yeast was followed the method described by Robzyk *et al*., (Robzyk and Kassir, 1992).

4.2.5.3 Plasmid extraction from *E.coli*

Plasmids were extracted by the rapid alkaline extraction method according to Sambrook *et al.* (Sambrook *et al,* 1989).

4.2.5.4 Transformation of *E.coli*

Transformation of E.coli was followed the CaCl2/RbCl method described by Sambrook *et al*. (Sambrook *et al*, 1989).

4.2.5.5 DNA ligation

The DNA fragments that were recovered from the gel were mix in the molar ratio roughly insert :vector＝2:1. Same volume of DNA ligation solution I (Ligation kit version 2, TaKaRa) was add and mixed thoroghly. The reaction mixture was incubated at 16℃ for 30 min to overnight and used for transformation into *E.coli* competent cells.

4.2.5.6 Polymerase chain reaction (PCR)

In this chapter, KOD –plus- DNA polymerase (TOYOBO) was used in all the PCR reactions, the reaction mixture composition and program parameters were set according to the manufacturer's instruction..

4.2.5.7 DNA sequencing

As described in 2.2.4.3.

4.3 Results

4.3.1 Tetrad analysis of M25

To determine whether single mutation caused both of growth defects in SD medium containing diC10PE and temperature sensitivity, tetrad analysis was performed as described in Materials and Methods with the diploid obtained by mating M25 and TKY12Gα. The growth of spores from 14 asci on SD medium containing diC10PE and SG medium at 37℃ was examined. The growth defects in SD medium containing diC10PE segregated in 2:2, and all the spores that could not grow in SD medium containing diC10PE were temperature sensitive. These results suggest that single mutation caused both growth defect in SD medium containing diC10PE and temperature sensitivity.

4.3.2 Introduction of genomic library to M25

To isolate the gene that complements the growth of M25 in SD medium containing diC10PE, the *S. cerevisiae* genomic DNA library, which was constructed in

low-copy vector, YCp50, was introduced into M25 to isolate a gene that complements the growth defects of M25 in utilization of exogenous diC10PE. In approximately 6,300 transformants, 52 could grow on SD medium containing diC10PE. Plasmids were isolated from these 52 transformants and reintroduced into M25. Among those, one transformant reproducibly showed positive growth on SD medium containing diC10PE. This transformant was named as M25-66 and the plasmid derived from genomic library in M25-66 was denominated pM25-66.

4.3.3 pM25-66

The nucleotide sequences of approximately 500 bp regions at both ends of the insert in pM25-66 were analyzed and the corresponding sequence was searched in *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). The insert of pM25-66 was a 12-kb fragment on chromosome XV from 35975 to 48125, containing six ORFs of *YOR153C, FRE7, GRE2, YOL150C, DCP1* and *SPT20,* besides a part of *PEX11* (Fig. 4.1).

To identify which ORF in the insert of pM25-66 was responsible for the complementation of growth defect of M25 in SD medium containing diC10PE, the insert of pM25-66 was digested with restriction endonucleases and the fragments were cloned into the low copy vector YCplac33. For details, a 4.7-kb fragment containing *YOL153C* after *Hin*dⅢ and *Sal*I digestion, a 6.4-kb fragment containing *FRE7* after *Pvu*Ⅱ digestion, a 1.9-kb fragment containing *GRE2* and *YOL150C* after *Pst*I and *Spe*I digestion, a 0.77-kb fragment containing *YOL150C* after *Hin*dⅢ and *Xba*I digestion, a 1.5-kb fragment containing *DCP1* after *Stu*I and *Eco*RV digestion, and a 3.4-kb fragment containing *SPT20* after *Hin*dⅢ digestion, were cloned into YCplac33 to construct plasmid YCpYOL153C, YCpFRE7, YCpGRE2, YCpYOL150, YCpDCP1, YCpSPT20, respectively (Fig. 4.1A).

The constructed plasmids were introduced to M25 and the growth of the transformants in SD medium containing diC10PE and in SG medium at 37℃ were examined. As shown in Fig. 4.1B-D, only transformant containing YCpDCP1 showed positive growth either in SD medium containing diC10PE or in SG medium at 37℃. This result suggested the product of *DCP1* gene was involved in the growth in SD medium containing diC10PE.

4.3.4 Mutation site in the *DCP1* gene of M25

DCP1 encodes an mRNA decapping enzyme in *S. cerevisiae*, and *dcp1* mutant is defective in 5' to 3' mRNA degradation and has impaired growth (Beelman et al., 1996) (Hajji et al., 1999). *DCP1* is essential for growth in certain genetic background. We next analyzed the nucleotide sequence of *DCP1* region of M25, to identify the mutation site. Total DNA of M25 was prepared and used as a template to amplify the *dcp1* ORF, 260 bp of 5'-non-coding region, and 28 bp of 3'-non-coding region in three independent PCR reactions, and the nucleotide sequences were determined. It was found that the *dcp1* gene of M25 had a point mutation from guanine to adenine at nucleotide residue 421 in the *DCP1* ORF. This mutation leads to a substitution of Gly to Arg at amino acid residue 141 within the highly conserved region (Fig. 4.2) (She et al., 2004). This result suggests that M25 likely has an aberrant Dcp1p and this aberrant Dcp1p makes the yeast cells defective in utilizing exogenous PE when the synthesis of PE is suppressed.

3.4 Discussion

It is known that mRNA turnover contributes to the control and regulation of gene expression. In *S. cerevisiae*, the major pathway of mRNA decay is the deadenylation-dependent decapping pathway (Kushner, 2004) (Meyer et al., 2004). Decapping of mRNA by the combined action of Dcp1p and Dcp2p is a key step in this pathway because it induces degradation of the mRNA, and is also critical in an aspect of mRNA surveillance in which aberrant mRNA containing nonsense mutant codons are decapped without a requirement for deadenylation and are degraded in a 5' to 3' direction (Muhlrad and Parker, 1994) (Hagan et al., 1995) (Cao and Parker, 2003). Containing such an important function, the Dcp1p is highly conserved in eukaryotes (She et al., 2004). It was reported that a *DCP1* null mutation caused defect in 5' to 3' mRNA degradation and stabilized unstable transcripts that were degraded *via* deadenylation-dependent decapping pathway (Beelman et al., 1996).

For the engagement of Dcp1 in the metabolism of PE, two possibilities exist. One is that Dcp1 is involved in the degradation process of the transcript(s) which is/are essential for utilization of exogenous PE and that abnormal Dcp1p in M25 is hyper-activated or constitutively activated because of the mutation and therefore the amount of the essential transcript(s) for the utilization of exogenous PE is decreased in M25. The other is that Dcp1p participates in the turnover of transcripts(s) whose translation product(s) negatively regulates the utilization of exogenous PE and that the amount of those transcript(s) is increased and therefore the utilization of exogenous PE

is suppressed in M25. Analysis of transcript profile in M25, in addition to the effect of the mutation on the Dcp1p activity in M25, may provide valuable information on the metabolism of PE in eukaryotic cells.

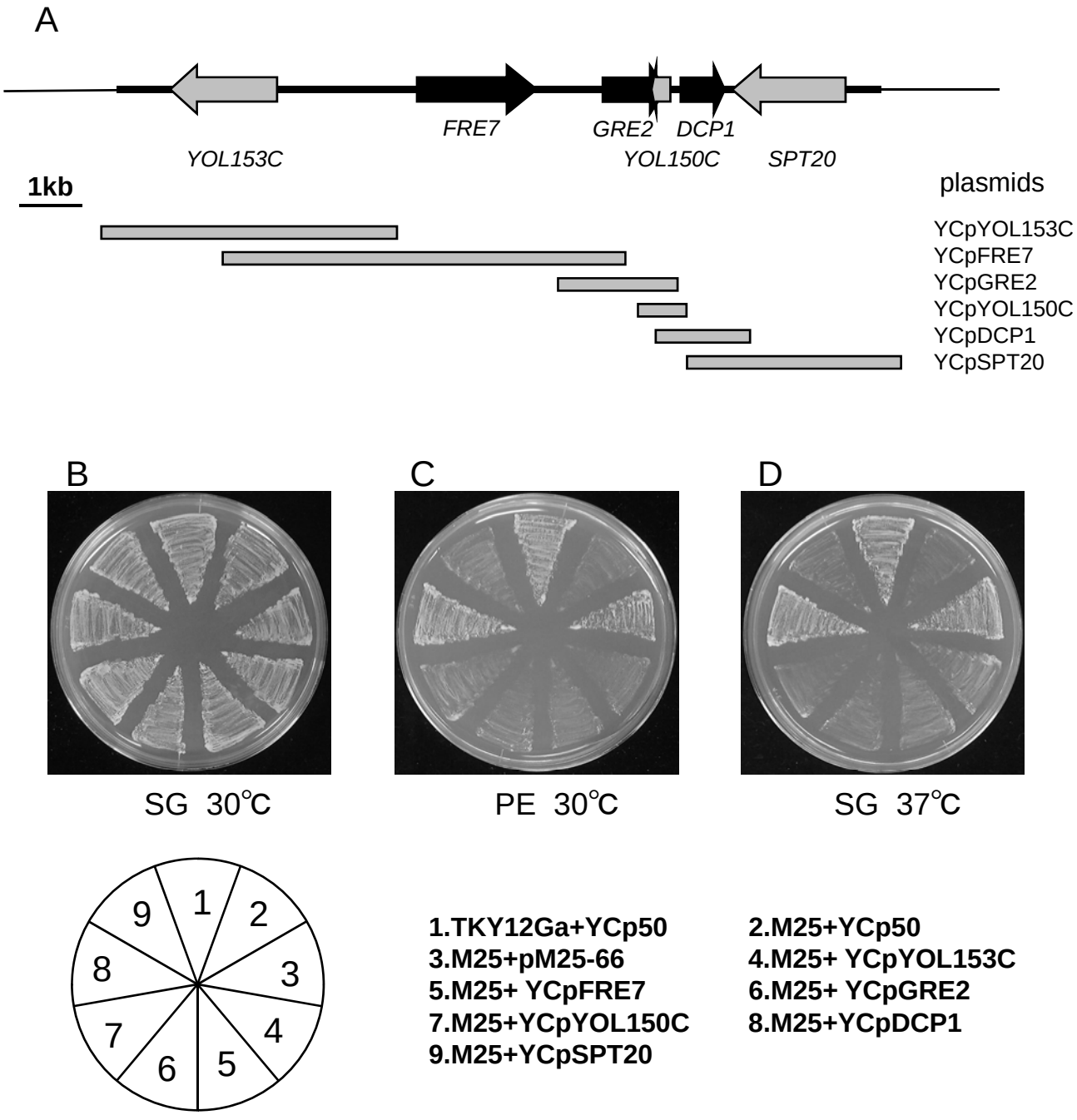
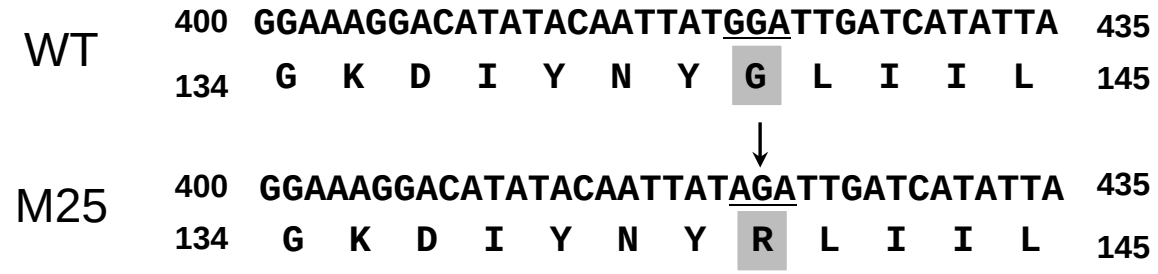


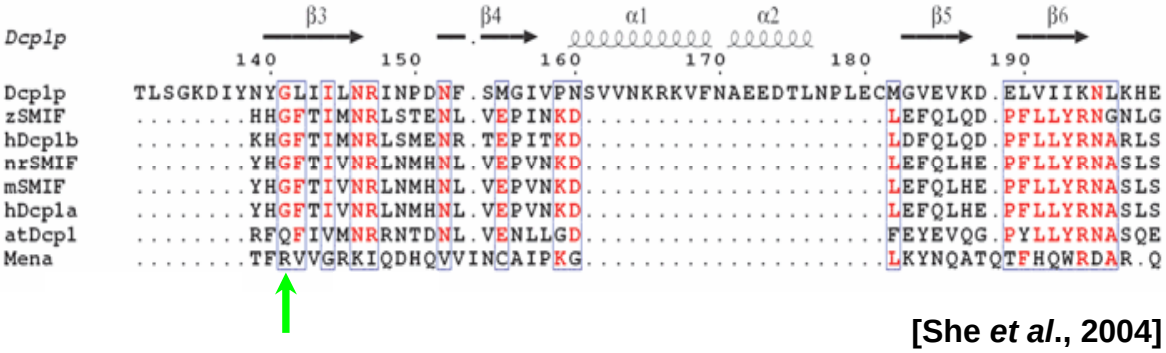
Fig. 4.1 *DCP1* complements the growth defect of M25 in SD medium containing diC10PE.

(A) Inserts of the constructed plasmids, which were obtained by digestion of pM25-66. (B-D) M25 cells containing indicated plasmids were plated on SD medium, and incubated for 2-3 days to consume ECT1p and/or PE in TKY12Ga. Cells were then plated on SG medium (B, D) or SD medium containing 20 mM diC10PE (C). Incubation temperature was 30°C (B, C) or 37°C (D).

(A)



(B)



[She *et al*., 2004]

Fig. 4.2 M25 contain a mutation in the high conserved region of *DCP1*

(A) The mutation site of the *dcp1* gene in M25 strain

(B) One of the high conserved regions of *DCP1* in eukaryotic cells. The mutation point of M25 was indicated by the green arrow.

Chapter 5

Requirement of *de novo* synthesis of PE and PC in yeast sporulation

5.1 Introduction

Nitrogen deprivation in the continuous presence of a non-fermentable carbon source, such as acetate, causes the diploid cells of *Saccharomyces cerevisiae* to exit the mitotic cycle, undergo meiosis, and sporulate. The process of spore formation requires *de novo* synthesis of prospore membranes, which initiates at the spindle pole bodies during meiosis II. The prospore membranes grow by SNARE-dependent post-Golgi vesicle fusion, and closure of the membranes is accompanied by completion of meiosis (Neiman, 2005). Spo20p, an SNARE protein, mediates the fusion of the vesicle with the prospore membrane and is required for formation of the spore (Neiman, 1998) (Neiman et al., 2000).(Neiman, 1998; Neiman et al., 2000) When it is first synthesized, Spo20p is targeted to the nucleus, but its nuclear localization is lost as cells enter meiosis II. Thereafter, Spo20p localizes to the prospore membrane by virtue of its lipid-binding domain, whose *in vivo* ligand is likely PA (Nakanishi et al., 2004). PA also stimulates Spo20p-mediated membrane fusion *in vitro* (Liu et al., 2007). Spo14p, a known phospholipase D that hydrolyzes PC to PA and choline in vegetative cells, is required for sporulation (Rose et al., 1995) (Ella et al., 1996) (Waksman et al., 1996). It was suggested that Spo14p-generated PA (and/or products derived from it, such as diacylglycerol) plays an important role in prospore membrane formation (Rudge et al., 2004). In addition, Spo1p, a phospholipase B homolog, is required for spindle pole body duplication during meiosis (Tevzadze et al., 1996) (Tevzadze et al., 2000). A recent study suggested that Spo1p acts on PI or its derivatives in a meiosis-specific signaling pathway (Tevzadze et al., 2007). Sec14p, the PI/PC transfer protein, has also been reported to be required for sporulation (Rudge et al., 2004).

It was reported that amount of various lipids increased in yeast during spore formation. Therefore, it is of great interest to test whether the *de novo* synthesis of certain species of lipids is necessary for spore formation in yeast. In this chapter, requirements of PE and PC in the sporulation of yeast were analyzed using strains in which the synthesis of these lipids could be controlled.

5.2 Materials and Methods

5.2.1 Strains

Strain	Genotype	Source
W303	*MAT*a/α *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	ATCC
KUY503	*MAT*a/α *his3-*Δ*200 leu2-3,112 ura3-52 trp1-1 lys2-801*	Laboratory strain
TKY12Ga	*MAT*a *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100* Δ*psd1*::*kanMX4* Δ*psd2*::*LEU2 ect1*::*HIS3-GAL1p-ECT1*	kakihara, 2004
TKY12Gα	*MAT*α *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100* Δ*psd1*::*kanMX4* Δ*psd2*::*LEU2 ect1*::*HIS3-GAL1p-ECT1*	kakihara, 2004
TKY12G	derivative of W303 Δ*psd1*::*kanMX4*/Δ*psd1*::*kanMX4* Δ*psd2*::*LEU2*/Δ*psd2*::*LEU2* *ect1*::*HIS3-GAL1p-ECT1*/ *ect1*::*HIS3-GAL1p-ECT1*	this study
KEY503	derivative of MHY503, Δ*pem1*::*HIS3*/Δ*pem1*::*HIS3* Δ*pem2*::*LEU2*/Δ*pem2*::*LEU2*	Laboratory strain

5.2.2 Media

SD medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-glucose（Wako）	2%

SG medium:

Yeast Nitrogen Base (w/o amino acids and ammonium sulfate, DIFCO)	0.17%
Ammonium sulfate (Kanto Kagaku)	0.5%
D-galactose (Wako)	2%

YPD medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-glucose（Wako）	2%

YPG medium:

Yeast extract (DIFCO)	1%
Polypeptone (NIHON SEIYAKU)	2%
D-galactose (Wako)	2%

Spore medium (SM)

Potassium acetate (Kanto kagaku)	1%
Yeast extract (DIFCO)	0.1%
D-glucose (Wako)	0.05%

(SM-Gal contained 0.05% D-galactose instead of D-gluctose)

For solid medium, 2% agar (Wako) was added.

5.2.3 Plasmid

YCplac22	Given by Dr.Gietz (Iwamoto, 2002)
YCplac33	Given by Dr.Gietz (Iwamoto, 2002)

5.2.4 Methods

5.2.4.1 Construct of TKY12G by yeast mating

① Transform TKY12Ga with YCplac50 (carrying *URA3* as marker gene) and TKY12Gα with YCplac22 (carrying *TRP1* as marker gene)

② Overnight pre-culture TKY12Ga / YCplac50 and TKY12Gα/ YCplac22 in 2ml SG medium at 30^{o}C shaking incubator, respectively.

③ mix 300 μl of each culture and 300 μl YPG medium in a microtube, incubate for 6 h in an immobile incubator.

④ Collect cells by centrifuge, plate to Ura-Trp- SG medium.
⑤ Replicate the colonies of the diploid, TKY12G, to the Ura-Trp- SG medium again, and incubate the plate at 30°C.

5.2.4.2 Determination of spore formation efficiency.

Yeast cells were fixed for 30 min in 70% ethanol, washed twice with H_2O, and stained in a solution of 625 ng/ml DAPI. After 10 min of incubation, the cells were washed twice with H_20, and then observed under Nomarski optics and fluorescence microscopy using appropriate filters. The percentages of cells without spores, with incomplete spores (one or two spores), and with three or four spores were determined from both characteristic asci morphology of Nomarski image and DAPI stained nuclei.

5.2.4.3 Phospholipid quantification

As described in 2.2.4.11

5.3 Results

5.3.1 *de novo* synthesis of PE is necessary for yeast sporulation

To determine whether *de novo* synthesis of PE would be required for sporulation, TKY12G was shifted to an SM-Gal with and without ethanolamine or choline after precultured in YPG medium overnight, and incubated for 72 h. As shown in Fig. 5.1, when TKY12G was incubated in SM-Gal with exogenous ethanolamine, sporulation efficiency was approximately 21%. However, in SM-Gal without ethanolamine, no sporulation was observed. Furthermore, TKY12G sporulated very poorly in SM-Gal in the presence of exogenous choline, with a sporulation efficiency of about 1.3%, although TKY12G grew in a galactose-containing medium even in the absence of exogenous ethanolamine when the medium was supplemented with choline, probably because the cells synthesized enough PE for growth through Kennedy pathway with phosphoethanolamine derived from the degradation of dihydrosphingosine-1-phosphate or phytosphingosine-1-phosphate by Dpl1p (Deng et al., 2007). The PE content of TKY12G increased during incubation in SM-Gal supplemented with ethanolamine, whereas it decreased in SM-Gal and in SM-Gal with choline (Fig. 5.2A). In contrast, no significant difference in phospholipid contents was

observed among the cells incubated in the presence or absence of ethanolamine or choline (Fig. 5.2B). Based on these results, it was suggested that the yeast cells required *de novo* PE synthesis for efficient spore formation.

5.3.2 *de novo* synthesis of PC is required for yeast sporulation

The requirement of PC for sporulation using diploid strain KEY503, in which both alleles of *PEM1* and *PEM2* are deleted was also examined. KEY503 synthesizes PC only via the CDP-choline branch of the Kennedy pathway and is therefore auxotrophic for choline. When KEY503 was incubated in SM containing 1 mM choline, spore formation was efficient (Fig. 5.3). In contrast, the spore formation efficiency of KEY503 was lower than that of wild-type cells in the absence of choline, and instead cells with incomplete spores increased in number, although the effect was less drastic than that of repression of PE synthesis. The PC content of KEY503 was elevated during incubation in SM supplemented with choline, while no increase was observed in SM without choline (Fig. 5.4A). No significant difference in the phospholipid contents was observed among cells incubated in the presence or absence of choline (Fig. 5.4B). These results suggest that *de novo* synthesis of PC was also required for efficient spore formation by yeast.

5.4 Discussion

In this chapter, it was shown that in the absence of exogenous ethanolamine, no spore was formed in TKY12G, suggesting *de novo* synthesis of PE was required for proper spore formation.

Since most of the TKY12G cells did not contain any spores in the absence of ethanolamine, PE might be required in the early stages of sporulation. PE tends to form a non-bilayer hexagonal-II structure under physiological conditions, due to its small polar head group. This property perhaps affects the dynamics of the biological membrane, for instance, membrane fission and fusion, which are also involved in prospore membrane formation. In addition, it is known that mitochondrial activity is essential for sporulation (Olempska-Beer, 1987). Mitochondrial Psd1p provides for approximately 90% of total cellular PS decarboxylase activity (Greenberg and Lopes, 1996). A *psd1* null mutant does not grow on nonfermentable carbon sources without supplementation with ethanolamine, choline, or serine (Birner et al., 2001). These data suggest that PE plays an essential role in mitochondrial function, and raise the

possibility that TKY12G is unable to sporulate in the absence of ethanolamine due to a defect in mitochondrial function. It is interesting to perform further analysis about in which stage sporulation is stopped in TKY12G in the absence of exogenous ethanolamine, that would help to understand the function of *de novo* synthesis of PE in spore formation.

On the other hand, in the absence of exogenous choline, sporulation in KEY503 was partially impaired, suggesting *de novo* synthesis of PC was also required for proper spore formation.

Although there are slight differences in lipid composition due to strain backgrounds and growth conditions, PC is one of the most abundant bilayer-forming phospholipids in yeast vegetative cells under most conditions. It is also a major structural component of the spore membrane (Illingworth et al., 1973). As shown in Fig. 5.4A, PC was a major phospholipid in the membrane of the wild-type cells incubated in SM, and the PC content of KEY503 increased to 50% during incubation in SM supplemented with choline. *De novo* synthesis of PC may be required to maintain the structural integrity of the spore membrane, in addition to its role in the supply of a substrate for Spo14p, PC-specific phospholipase D, which is required for sporulation (Howe and McMaster, 2001).

In addition to PE and PC, Nagasawa in our laboratory examined the requirement of ergosterol for yeast sporulation, using a yeast strain, ISY01, in which sterol synthesis can be controlled with the carbon sources in the medium, by replacement of the promoters of both alleles of *ERG7*, encoding lanosterol synthase catalyzing the reaction to form a sterol structure, with *GAL1* promoters (Nagasawa, 2008). Expression of *ERG7* was induced in a galactose-containing medium, so ISY01 grew as well as and had similar free and total ergosterol contents with the wild-type strain. When ISY01 was incubated in a glucose-containing medium for 24 h, both free and total ergosterol contents decreased remarkably as compared with those of the wild-type strain and the viability of ISY01 remained 56% of that of the wild-type cells. The sporulation efficiency of the cells after preculture in a galactose- and a glucose-containing medium was determined. When ISY01 was incubated in SM-Gal for 72 h after preculture in a galactose-containing medium, sporulation efficiency was almost the same as that of the wild-type cells, but after preculture in a glucose-containing medium for 24 h, the sporulation efficiency of ISY01 was significantly impaired. This suggests that the cellular sterol content was important for sporulation (Deng et al., 2008).

All together, the analysis of the requirement of lipid synthesis for yeast

sporulation shown that *de novo* synthesis of PE and PC was required for proper spore formation; furthermore, the cellular sterol content was also found to be important for sporulation. Currently, it remains to be determined whether synthesis of PE, PC, and sterol is required for prospore membrane formation *per se* or for some other step. It is also possible that these lipids are required to maintain the viability of yeast in SM, although cellular proliferation does not occur in this medium.

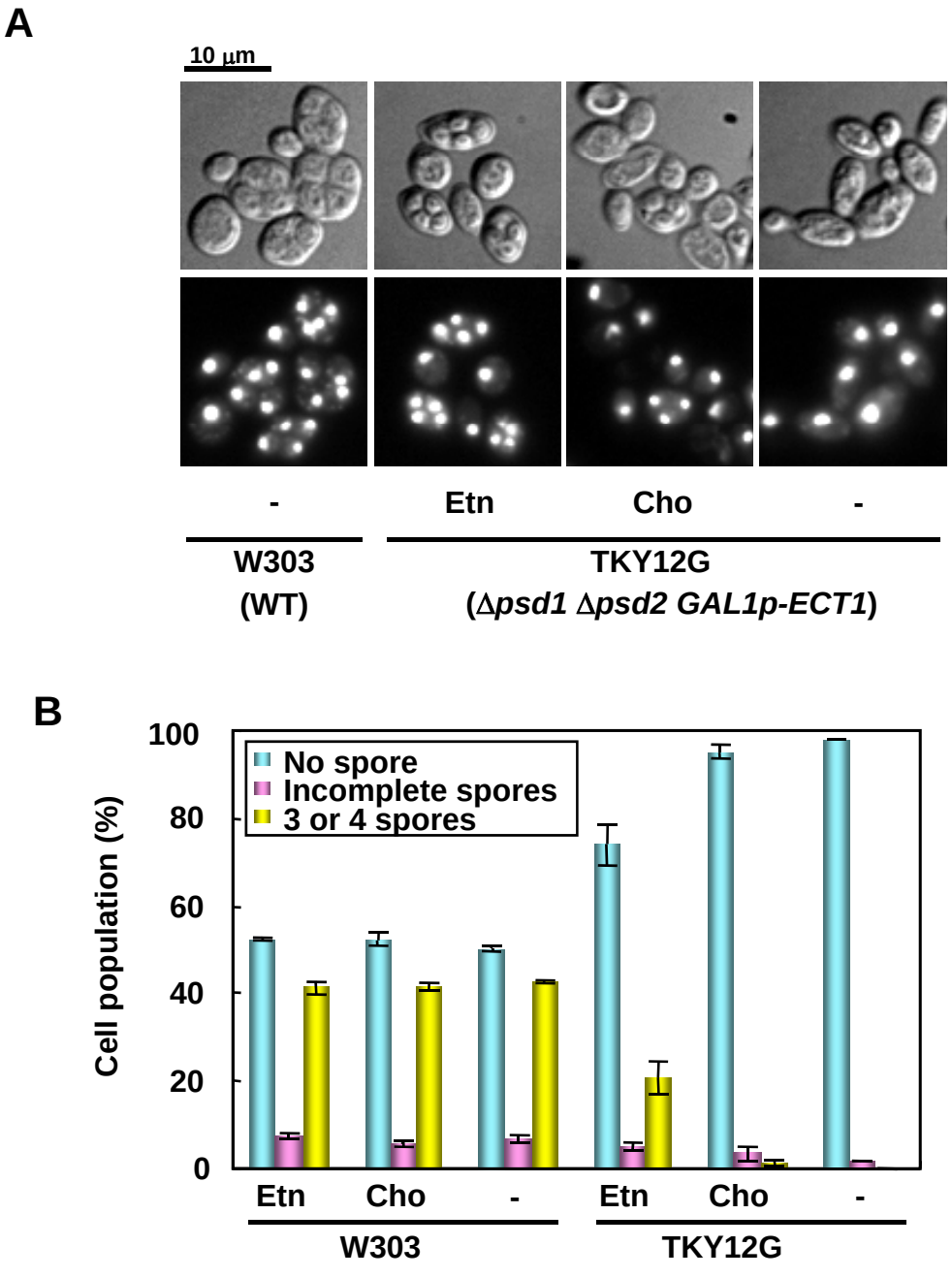


Fig.5.1 *de novo* synthesis of PE is required for Spore Formation.

TKY12G and W303 were precultured in YPG medium overnight and then inoculated into 1 ml SM-Gal at an initial OD_{600} of 1.0, and incubated at 25∓C for 72 h. (A) Cells were stained with DAPI and observed under Nomarski optics and by fluorescence microscopy. (B) The percentages of cells without spores, those with incomplete spores, and with three or four spores, are indicated. Results represent the mean and S.E. of three independent experiments, in which at least 200 cells were scored for each strain.

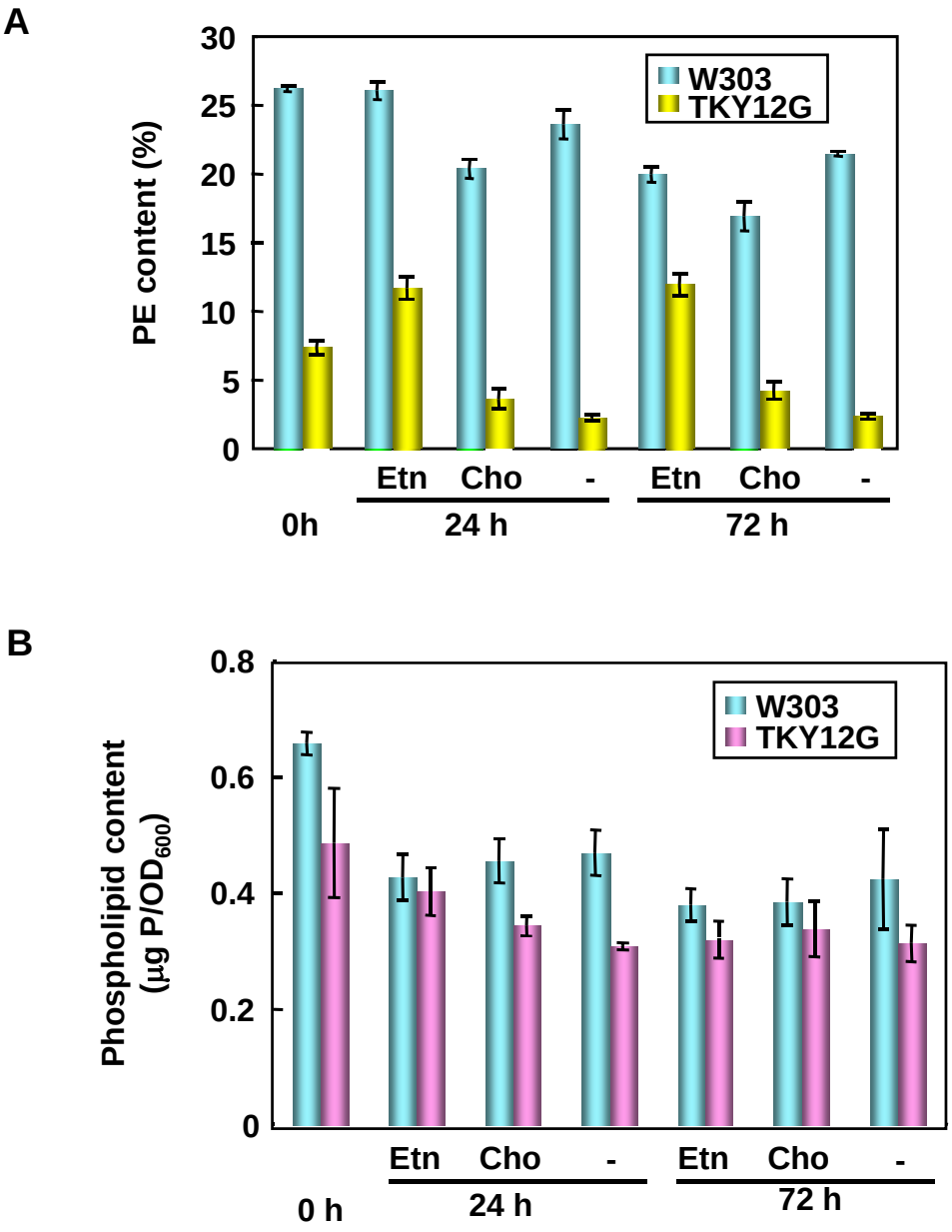


Fig.5.2 The PE amount was decreased in TKY12G in SM-Gal medium without exogenous ethanolamine

Total lipids were extracted from the cells before and during incubation in SM-Gal with and without ethanolamine and choline. The PE content (A) and the amount of total phospholipids (B) were determined as described in Materials and Methods. PE content was expressed as a ratio of the amount of PE to that of total phospholipids.

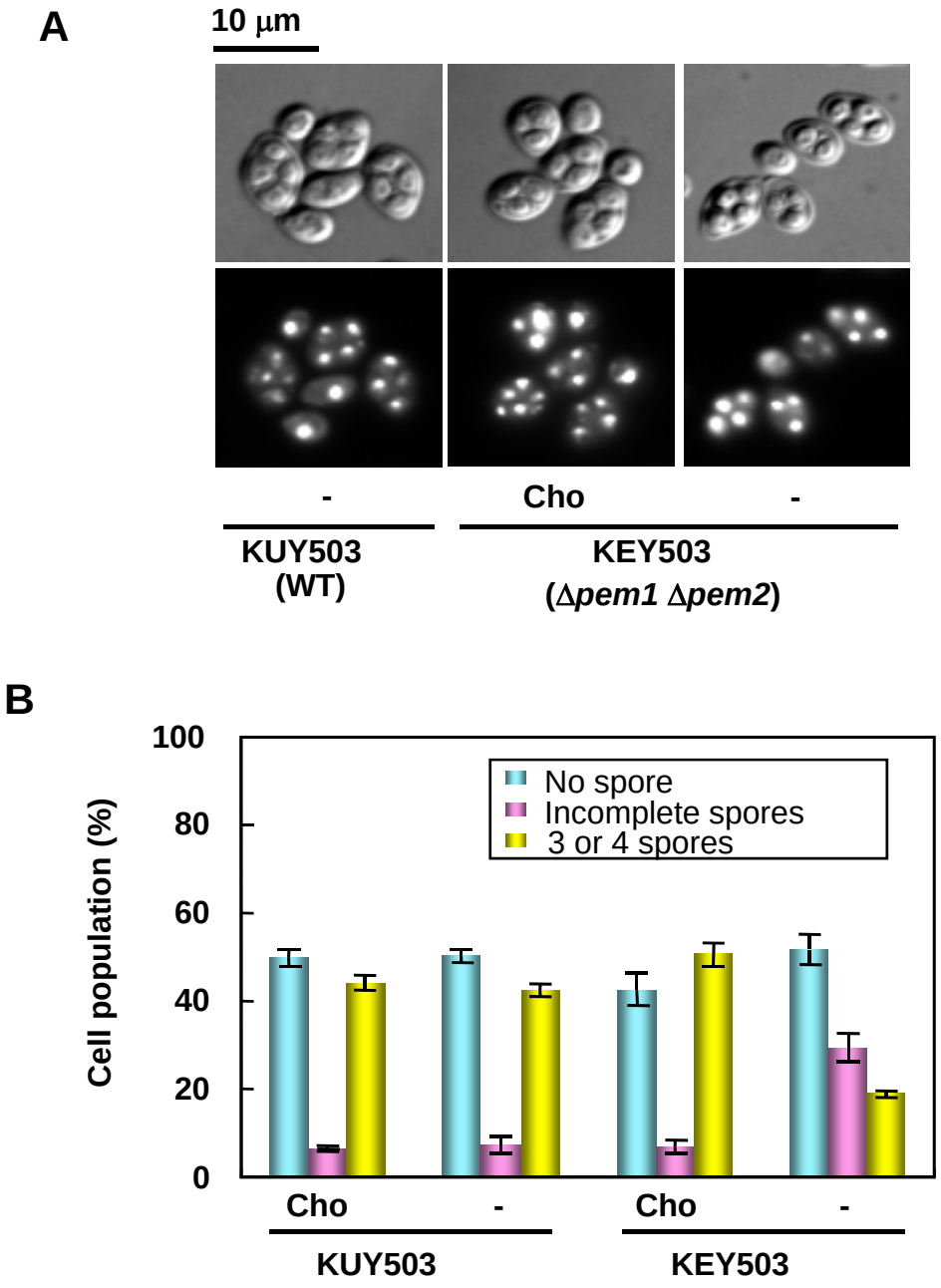


Fig.5.3 *de novo* synthesis of PC is required for Spore Formation.

KEY503 and KUY503 were precultured in YPD medium overnight and then inoculated into 1 ml SM at an initial OD_{600} of 1.0, and incubated at 25∓C for 120 h. (A) Spore formation was observed as in Fig. 5.1A. (B) The efficiency of spore formation was determined as in Fig. 5.1B. Results represent the mean and S.E. of three independent experiments, in which at least 300 cells were scored for each strain.

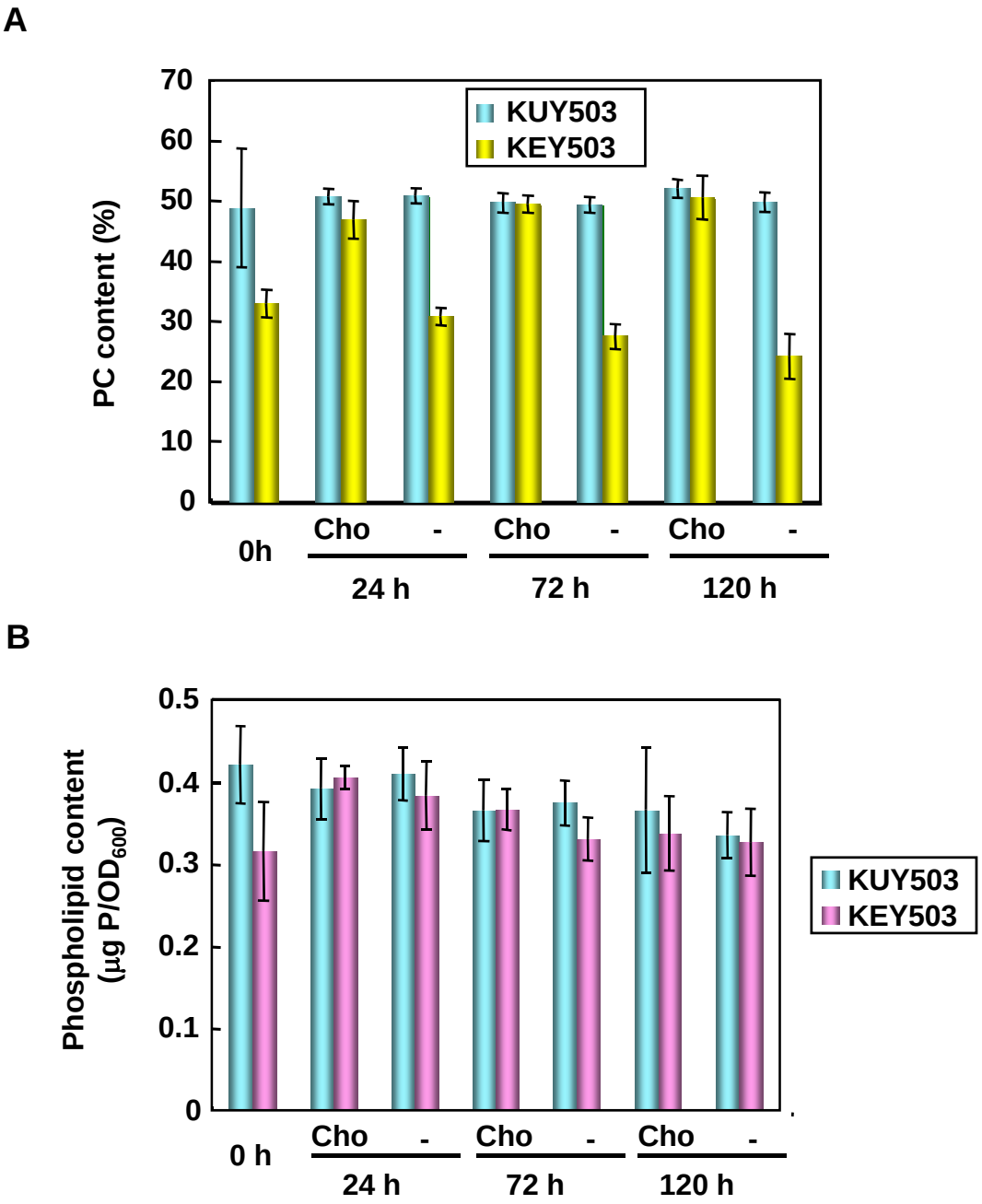


Fig.5.4 Repression of PC synthesis in SM medium without exogenous choline

Total lipids were extracted from cells before and during incubation in SM with and without choline, and the PC content (A) and the amount of total phospholipids (B) were determined as described in Materials and Methods. PC content was expressed as a ratio of the amount of PC to that of total phospholipids.

Chapter 6

Conclusion and perspective

PE is the ubiquitous phospholipid comprising ~20% of total phospholipids in eukaryotic cells. It is considered that, besides as a structural component of the membrane, PE also plays an essential function in cells. Although remodeling of phospholipid is thought to be important in maintenance of homeostasis in cells through generation and maintenance of the complexity of phospholipids, its mechanism has not been well elucidated. In this study, remodeling of diC10PE in TKY12Ga was analyzed to illuminate the mechanism of remodeling of PE.

In chapter 2, growth of TKY12Ga with *lem3*, *dnf1*, or *dnf2* null mutation singly or in combination in SD medium containing diC10PE was examined and it was suggested that Lem3p, Dnf1p, and Dnf2p, are involved in uptake of PE with short acyl chains. In addition, it was demonstrated that deuterium-labeled diC10PE was remodeled to PE containing fatty acyl chains of normal carbon number *in vivo* and *in vitro*. These results suggested that remodeling machinery of PE exists in yeast.

In chapter 3, remodeling of deuterium-labeled diC10PE in the individual or multiple deletion mutants of phospholipase-related or acyltransferase-related genes was examined. It was suggested that Ale1p and Slc1p are involved in the remodeling of *sn*-2 position of diC10PE, and other acyltransferase(s) is/are involved in the reacylation of sn-1 position of diC10PE than Gat1p and Gat2p. In addition, diC10PE was remodeled in the sextuple deletion mutant of *PLB1, PLB2, PLB3, SPO1, NTE1* and *YOR022c*, suggesting that other enzyme(s) with phospholipase activity are involved in removal of acyl chains in diC10PE.

In chapter 4, the mutant, M25, defective in growth in SD medium containing diC10PE was analyzed. It was indicated that M25 has a mutation in *DCP1* gene, which encodes the mRNA decapping enzyme.

In chapter 5, requirement of *de novo* synthesis of PE and PC for proper spore formation was shown.

The homeostasis of biological membrane is maintained by synthesis, transport, degradation, and remodeling of phospholipids. However, the study of metabolism and

transport of phospholipids have been hampered because they are ubiquitous and basically exist as a mixture in abundance in every membrane, because they can potentially affect various biological processes in a given cell, and because they have relatively simple structure and it is not easy to visualize *in vivo*.

Estimation of phospholipid remodeling is experimentally difficult since its physiological role is not clear. The system, in which the remodeling process of stable isotope-labeled phospholipids containing short acyl chains in yeast is monitored by ESI-MS/MS, will be applicable to the analysis of each reaction in the acyl chain remodeling of various phospholipid.

The mechanism of phospholipid transport also remains to be established. It was proposed that some phospholipids are transported between organelles in a vesicle-independent manner (Holthuis and Levine, 2005). For instance, PS synthesized by Cho1/Pss1p in the endoplasmic reticulum (ER) membrane is transported to mitochondrial and decarboxylated to PE by Psd1p in yeast. PE is then transported back to ER and methylated to PC by the sequential reaction of Pem1p and Pem2p. Vesicle transport pathway is not known to exist between ER and mitochondria. Therefore, it is speculated that these phospholipids are transported via membrane contact sites between ER and mitochondria, and/or by putative lipid transport proteins which extract phospholipids from one organelle and inserts it into the others, although the molecular detail is not clear. Such vesicle-independent transport machinery also proposed to exist between other organelles. Recently, it was reported that lipids are directly transferred from the ER to peroxisomes by a nonvesiclar pathway (Raychaudhuri and Prinz, 2008). It is supposed that diC10PE is transported to the organelle(s) in which it is remodeled and remodeled PE have to be transported to other organelles. It might also be possible to isolate mutants defective in transport of PE by screening mutants that cannot grow in SD medium containing diC10PE, using TKY12Ga as a parent strain.

Thus, this study will contribute to elucidating the mechanism of remodeling and transport of PE and to understanding how biological membrane is formed and maintained, and how functional organelles are built in eukaryotic cells.

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論文の内容の要旨

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論文題目

Study on molecular remodeling and function of phosphatidylethanolamine in *Saccharomyces cerevisiae*

（酵母*Saccharomyces cerevisiae*におけるホスファチジルエタノールアミンの分子リモデリングと機能に関する研究）

Biological membranes are composed of a wide variety of phospholipids with respect to their hydrophilic head moieties and hydrophobic acyl chains. The generation and maintenance of the diversity in phospholipid molecular species are accomplished by the process of synthesis, degradation, and remodeling of each phospholipid. The fatty acyl chains in phospholipids are considered to be remodeled in cells to maintain membrane homeostasis. Despite its potential importance for the structure and function of the biological membranes, the molecular mechanisms underlying the remodeling of acyl chains in phospholipids are poorly understood

Phosphatidylethanolamine (PE) is one of the abundant phospholipids, comprising 20 to 30% of total phospholipids in most eukaryotic cells. Due to its small polar head group, PE tends to form non-bilayer hexagonal-II structure under physiological condition. This property has been considered to affect dynamics of biological membrane, for instance, membrane fission and fusion.

In *Saccharomyces cerevisiae*, PE is biosynthesized through two pathways. In *de novo* pathway, phosphatidylserine (PS) is decarboxylated to PE by Psd1p or Psd2p. In CDP-ethanolamine branch of the Kennedy pathway, PE is synthesized from exogenous ethanolamine or ethanolamine formed endogenously through a lipid turnover process. In *S. cerevisiae*, PE is essential for growth and deletion of both *PSD1* and *PSD2* is lethal without a PE supply through the Kennedy pathway

Kakihara in our laboratory constructed a yeast mutant, TKY12Ga, in which both *PSD1* and *PSD2* were deleted and the promoter of *ECT1* encoding a key enzyme in PE on the Kennedy pathway, was replaced with the galactose-inducible and glucose-repressible *GAL1* promoter. In a medium containing galactose as a carbon source, the expression of *ECT1* was induced and the mutant grew using PE synthesized via the Kennedy pathway in the presence of ethanolamine. In contrast, the expression of *ECT1* was repressed and cell growth was arrested in a glucose-containing medium, and PE content was reduced to 1%. However, when didecanoyl PE (diC10PE) was added to the medium containing glucose, TKY12Ga grew despite the inability to synthesize PE. Since a 10-carbon-fatty acyl residue appears to be too short to maintain the structure and function of the biological membrane,

diC10PE was assumed to be remodeled according to the ones containing acyl chains of normal length as endogenous PE to support the growth of the mutant.

This study is aimed at elucidating the mechanism of PE remodeling and the physiological role of PE in *S. cerevisiae*.

Incorporation and remodeling of PE with short fatty acyl chains in yeast

It was reported that Lem3p/Ros3p and two P-type ATPase, Dnf1p and Dnf2p were involved in the inward-directed trans-bilayer transport of fluorescent-labeled PE and lyso-PE at the plasma membrane in yeast. Therefore the involvement of these three proteins in the uptake of PE with short acyl chains was examined. These proteins encoding genes were deleted in TKY12Ga and the growth of the null mutants in SD medium containing diC10PE was examined. Deletion of *DNF2* caused slight growth impairment in the PE-containing medium, while deletion of *DNF1* did not. However, deletion of both *DNF1* and *DNF2* conferred severer growth defect, suggesting their redundant functions in the uptake of diC10PE. In contrast, deletion of *LEM3* caused most significant growth impairment. These results indicated that the growth of TKY12Ga in SD medium containing diC10PE required uptake of PE with short acyl chains or its metabolites from the medium and the Lem3p and two P-type ATPases, Dnf1p and Dnf2p, were involved in this process.

The metabolism of diC10PE, in which ethanolamine moiety was labeled with deuterium, was analyzed using the ESI-MS/MS. Deuterium-labeled diC10PE was synthesized by the base exchange transphosphatidylation by phospholipase D from the didecanoyl PC and ethanol-1,1,2,2-d_4-amine.

TKY12Ga was pulse-labeled with deuterium-labeled diC10PE for 15 min, and chased with non-labeled diC10PE for another 15, 30, 60 and 180 min. Then, the molecular species profile of PE was determined by the neutral loss scan of *m/z* 145 to detect PEs containing ethanol-1,1,2,2,-d_4-amine phosphate headgroup in positive ion mode. After 15 min incubation with deuterium-labeled diC10PE, a portion of the deuterium-labeled diC10PE had been remodeled, as one fatty acyl chain was replaced with a longer monounsaturated C16:1 and C18:1 acyl chains, respectively. And these remodeling intermediates decreased quickly during chased incubation. In addition, PEs in which two fatty achy chains were replaced with C16:1 or C18:1 acyl chain (16:1-16:1-PE, 16:1-18:1-PE and 18:1-18:1-PE) were also detected after 15 min incubation with deuterium-labeled diC10PE. These results suggested that PE with short acyl chains was remodeled to PEs containing normal acyl chains.

Analysis of structures of the remodeling intermediates in detail using product ion scan by MS/MS showed that the majority of the remodeling intermediates contained C16:1 or C18:1 acyl chain at *sn*-2 position. These results suggested that diC10PE was first remodeled at *sn*-2 position and then at *sn*-1 position. The water-soluble metabolites of diC10PE, which were partitioned in the aqueous phase were also extracted, and their molecular profiles were analyzed. After a 30-min incubation with deuterium-labeled diC10PE, the peak corresponding to monoC10PE was observed. These results suggest that lyso-10:0-PE was also an intermediate of remodeling of diC10PE.

Quantification of diC10PE and decanoic acid in the whole culture and the culture supernatant using LC-MS showed that amount of decanoic acid increased in both of the whole culture and the culture

supernatant during the incubation of TKY12Ga with diC10PE, accompanied by the decrease of diC10PE. The amount of released decanoic acid in the culture supernatant was approximately twofold of the consumed diC10PE. These results suggested that most of C10 fatty acid cleaved from diC10PE were excreted into culture medium and it could be concluded that yeast remodeled diC10PE by cleaved C10 fatty acid and replaced it with normal length acyl chain, but not by elongation of the acyl chains.

On the other hand, *in vitro* remodeling reaction experiment was performed by incubated cell lysate of TKY12Ga with deuterium-labeled diC10PE and palmitoleoyl-CoA for 15, 30, 60 and 180 min. After 15 min incubation, a small portion of deuterium-labeled 10:0-16:1-PE was detected, and increased with the prolonged incubation. In addition, deuterium-labeled 10:0-18:1-PE, 16:1-16:1-PE and 16:1-18:1-PE were detected after 60 min incubation and 18:1-18:1-PE was detected after 180 min incubation. These results suggest the PE remodeling reaction can be occurred *in vitro*.

Analysis of genes involved in remodeling of diC10PE

Presence of monoC10PE, 10:0-16:1-PE, and 10:0-18:1-PE as remodeling intermediates of the diC10PE suggests that the remodeling can be dissected into at least two reactions, the cleavage of acyl chains by phospholipase activities and reacylation by acyltransferase activities. Yeast genome contains five genes encoding phospholipase B, *PLB1, PLB2, PLB3, SPO1,* and *NTE1,* and a gene, *YOR022c*, homologous to bovine phospholipase A1 coding gene. A mutant was constructed by deleted these six genes from TKY12Ga, and its growth in SD medium containing diC10PE and remodeling of deuterium-labeled diC10PE *in vivo* and *in vitro* were examined. No defect of the sextuple mutant in growth and remodeling was observed. In addition, monoC10PE was detected in the cell extract when this mutant was incubated with diC10PE. These data suggested that other enzyme(s) is/are involved in the removal of acyl chains in diC10PE.

Ale1p was reported to have an acyltransferase activity against the *sn*-2 position in various lysophospholipids. Null mutation in *ALE1* is synthetically lethal with the deletion of *SLC1*, which encodes a lyso-PA acyltransferase. Null mutants of *ALE1* or *SLC1* under TKY12Ga background were constructed. Their growth in SD medium containing diC10PE and their remodeling ability of deuterium-labeled diC10PE *in vivo* and *in vitro* were examined. Both mutants were partially impaired in the growth in SD medium containing diC10PE, and also impaired in remodeling of deuterium-labeled diC10PE *in vivo* and *in vitro*, suggesting that Ale1p and Slc1p were involved in the remodeling of diC10PE. Since yeast remodels diC10PE at both *sn-1* and *sn-2* positions, to identity genes involved in the remodeling at *sn-1* position, Same experiments were performed using null mutants of *GAT1* and *GAT2*, which encode glycerol-3-phosphate (Gly-3-P) acyltransferase that were reported to transfer fatty acid from acyl-CoA to the sn-1 position of Gly-3-P. But no growth and remodeling defect was observed in these two mutants.

Analysis of mutant defective in utilization of exogenous PE

To discover novel enzymes involved in PE remodeling reaction, analysis of mutants defective in utilization of exogenous PE will be an effective way. Kakihara mutagenized TKY12Ga with mutagen

ethyl methanesulfonate (EMS) and isolated 33 mutants that have defects in growth in SD medium containing diC10PE. M25 is one of these mutants. Analysis of M25 showed that introduction of a plasmid containing the *DCP1* gene to M25 complemented its growth defect in SD medium containing diC10PE. *DCP1* gene encodes an mRNA decapping enzyme on the major pathway of mRNA decay in *S. cerevisiae.* Nucleotide sequence analysis showed a point mutation within the highly conserved region of *DCP1* gene in M25, suggesting the possibility that Dcp1p is involved in the mRNA degradation process of the transcript(s) that is/are responsible for the utilization of exogenous PE in yeast.

Requirement of *de novo* synthesis of PE and PC in yeast sporulation

During the sporulation process of *S. cerevisiae*, meiotic progression is accompanied by *de novo* formation of the prospore membrane inside the cell. However, it remains to be determined whether *de novo* synthesis of certain species of lipids is necessary for spore formation. Therefore, requirements of PE and another major phospholipid, phosphotidylcholine (PC), in the sporulation of yeast were examined. When TKY12G, diploid of TKY12Ga, was incubated in spore medium (SM) with exogenous ethanolamine, sporulation efficiency was approximately 21%. However, when there is no exogenous ethanolamine, no sporulation was observed, suggested that *de novo* PE synthesis was essential to yeast sporulation. On the other hand, when KEY503, a strain synthesizes PC only via the CDP-choline branch of the Kennedy pathway and is therefore auxotrophic for choline because of the deletion of both *PEM1* and *PEM2* genes, was incubated in SM in the present of choline, spore formation was efficient. In contrast, spore formation efficiency of KEY503 was lower than that of wild-type cells in the absence of choline, but the effect was less drastic than that of repression of PE synthesis. These results suggest that *de novo* synthesis of PC was also required for efficient spore formation by yeast.

Conclusion

In this study, the incorporation and metabolism of diC10PE in yeast was analyzed. Results suggest that diC10PE was remodeled to PEs containing normal acyl chains and Ale1p and Slc1p were involved in this reaction. This system, in which the remodeling process of stable isotope-labeled phospholipids containing short acyl chains in yeast is monitored by ESI-MS/MS, will be applicable to the analysis of each reaction in the acyl chain remodeling of various phospholipid, provides a valuable tool to study the transport and metabolism of PE in yeast. In addition, an essential requirement of *de novo* PE synthesis during yeast spore formation was reported, giving information about the physiological role of PE in yeast sporulation.

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