SPO14 Separation-of-Function Mutations Define Unique Roles for Phospholipase D in Secretion and Cellular Differentiation in *Saccharomyces cerevisiae*

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ABSTRACT

In *Saccharomyces cerevisiae*, phospholipase D (PLD), encoded by the *SPO14* gene, catalyzes the hydrolysis of phosphatidylcholine, producing choline and phosphatidic acid. *SPO14* is essential for cellular differentiation during meiosis and is required for Golgi function when the normal secretory apparatus is perturbed (Sec14-independent secretion). We isolated specific alleles of *SPO14* that support Sec14-independent secretion but not sporulation. Identification of these separation-of-function alleles indicates that the role of PLD in these two physiological processes is distinct. Analyses of the mutants reveal that the corresponding proteins are stable, phosphorylated, catalytically active *in vitro*, and can localize properly within the cell during meiosis. Surprisingly, the separation-of-function mutations map to the conserved catalytic region of the PLD protein. Choline and phosphatidic acid molecular species profiles during Sec14-independent secretion and meiosis reveal that while strains harboring one of these alleles, *spo14S-11*, hydrolyze phosphatidylcholine in Sec14-independent secretion, they fail to do so during sporulation or normal vegetative growth. These results demonstrate that Spo14 PLD catalytic activity and cellular function can be differentially regulated at the level of phosphatidylcholine hydrolysis.

PHOSPHOLIPASE D (PLD) catalyzes the hydrolysis of phospholipids and is believed to play important roles in signal transduction pathways and the regulation of membrane trafficking events (reviewed in Exton 1998). The linkage of PLD activation to receptor-coupled pathways in mammalian cells led to the hypothesis that PLD is not required for constitutive vesicular transport but regulates several different aspects of membrane trafficking during cell signaling (Jones *et al.* 1999). The specific role for PLD in these pathways has not been elucidated; however, recent evidence suggests that the direct product of PLD catalysis, phosphatidic acid (PA), acts as a lipid second messenger that is required for full activation of phosphatidylinositol 4-phosphate 5-kinase, which is essential for phosphatidylinositol 4,5-bisphosphate (PIP_2) biosynthesis (Honda *et al.* 1999). PIP_2 is itself a lipid signal that has been established to play a critical role in membrane trafficking events (Liscovitch and Cantley 1995; Matsuoka *et al.* 1998). In addition, the formation of PA may also alter the structural characteristics of cellular membranes thereby facilitating vesicle formation and/or fusion. Finally, PA can serve as a substrate for a number of different lipid modifying enzymes; thus PLD-generated PA may be an intermediate step in the formation of other biologically active lipids such as diacylglycerol or lyso-phosphatidic acid.

The view that PLD plays a role in regulated membrane trafficking events, as opposed to constitutive vesicular transport, is supported by the analysis of PLD function in the yeast *Saccharomyces cerevisiae*. *SPO14* encodes the major phosphatidylcholine (PC)-specific PLD in yeast (Rose *et al.* 1995; Ella *et al.* 1996; Waksman *et al.* 1996) and as the name implies was originally identified in a mutant that failed to undergo the developmental process of sporulation (Honigberg *et al.* 1992). Genetic and cytological analyses have demonstrated that Spo14 is required for the formation of the prospore membrane (Rudge *et al.* 1998b), which is created by a developmentally regulated branch of the secretory pathway (Neiman 1998).

In addition to its role in sporulation, PLD is required for survival under conditions of *sec14* bypass (Sreenivas *et al.* 1998; Xie *et al.* 1998). Under normal circumstances the *SEC14* gene product plays an essential role in protein exit from the Golgi and represents the major phosphatidylinositol and phosphatidylcholine transfer protein of yeast; hence deletion of the gene is lethal (Bankaitis *et al.* 1989, 1990). In contrast, deletion of *SPO14* in an otherwise wild-type genetic background has no effect on constitutive secretion and viability. However, mutations that bypass the requirement for *SEC14* in cell viability and secretion are dependent on PLD activity for survival (Sreenivas *et al.* 1998; Xie *et al.* 1998). These secondary

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mutations suppress the requirement for Sec14 in several ways. For example, mutations that inactivate the cytidine 5′-diphosphate (CDP)-choline pathway for PC biosynthesis (Cleves *et al.* 1991) as well as mutation of a proposed phosphoinositide binding protein (*i.e.*, *kes1*; Fang *et al.* 1996; Rivas *et al.* 1999) bypass *sec14* mutations. Thus, Spo14 is required for vesicular transport only when phospholipid metabolism is altered through the inactivation of *Sec14*.

Structure/function studies on PLD gene family members have delineated conserved regions of the protein that are important for catalytic activity (Sung *et al.* 1997) and responsiveness to PIP_2 (Sciorra *et al.* 1999). In addition, mutational analysis of the nonconserved N-terminal extension in Spo14 indicates that these sequences define important regulatory domains required for correct cellular targeting and phosphorylation (Rudge *et al.* 1998b). To date, analyses of mutants perturbed for the different domains of Spo14 similarly impair both sporulation and Sec14-independent secretion (Rudge *et al.* 1998b; Xie *et al.* 1998; Sciorra *et al.* 1999).

In this study we show that single missense mutations can delineate PLD function in sporulation and Sec14-independent growth. Surprisingly, these separation-of-function mutations map to the catalytic core of the protein. We demonstrate that one of the separation-of-function proteins hydrolyzes PC only under conditions of *sec14* inactivation but not during sporulation or normal vegetative growth. Our findings indicate that PLD plays distinct roles in secretion and cellular differentiation.

MATERIALS AND METHODS

Yeast strains and media: Routine growth and manipulation of *S. cerevisiae* strains were performed as described (Rose *et al.* 1990). Yeast strains used in this study are listed in Table 1. Yeast strains were transformed using the lithium acetate procedure (Ito *et al.* 1983). All integrative transformants were verified by polymerase chain reaction (PCR) analysis. Gene disruptions were performed by one-step gene replacement (Rothstein 1983).

Mutagenesis strategy: Random mutagenesis of complementing sequences was achieved by amplifying the *SPO14* open reading frame (ORF) using PCR and *in vivo* recombination (Muhlrad *et al.* 1992). PCR was carried out using standard conditions with the *SPO14* centromere plasmid KR325 (Rose *et al.* 1995) as template and primers P48 (5′-CAAAGAGA AAAACGAAG-3′), which binds at position +163 in the *SPO14* gene, and P41 (5′-TGAGCATACTCCATTTG-3′), which binds at position +4216. The resulting DNA was cotransformed with *Eco*RI- and *Sal*I-linearized pME472, which resulted in a gapped plasmid deleted for sequences between +387 and +4390 in the *SPO14* ORF. Recombination between the gapped plasmid and the PCR product *in vivo* resulted in yeast transformants harboring plasmids containing randomly mutagenized *SPO14* sequences. Approximately 5000 transformants were screened from three individual pools of PCR amplification for growth at 35° (*sec14* bypass) and sporulation by dityrosine fluorescence (Esposito *et al.* 1990). Approximately 5% of transformants represented plasmid molecules that did not undergo a recombination event on the basis of the frequency of transformation obtained when linearized plasmid was introduced alone. A total of 115 transformants were able to support either Sec14-independent growth or sporulation. Of those, 66 retested for the phenotype, of which 47 supported sporulation but not *sec14* bypass and 19 supported *sec14* bypass but not sporulation. The former class most likely represented temperature-sensitive alleles of *SPO14*, as *sec14* bypass was assayed at 35° and when strains harboring these plasmids were sporulated at 33.5° (maximum temperature of sporulation in the strain used for mutagenesis), function was impaired. Consequently, these were not further studied. DNA was isolated from the 19 transformants that failed to undergo sporulation and introduced into *Escherichia coli*, and individual plasmids were introduced into Y2053 (Δ*spo14*/Δ*spo14*), CTY1128 (*sec14-1^{ts} pct1-2* Δ*spo14*), and CTY1130 (*sec14-1^{ts} kes1-1* Δ*spo14*). Of those, 10 conferred the mutant phenotype when a single plasmid was reintroduced into the yeast strains. The mutation responsible for the mutant phenotype was determined for five alleles by a combination of subclone and sequence analyses (*spo14S-11*, *-18*, *-55*, *-29*, and *-41*). The remaining five mutations (*spo14S-8*, *-23*, *-26*, *-28*, and *-52*) map outside the region determined for the first five; the specific lesions have not been determined.

Plasmids: Plasmids KR325 and KR577 have been described (Rose *et al.* 1995). Plasmid ME472 was constructed by filling in the *Sal*I site in the polylinker of pUN55 (Elledge and Davis 1988) with the Klenow fragment of DNA polymerase and inserting the *Pvu*II-*Cla*I fragment from pME314 (original complementing plasmid harboring *SPO14* sequences; Rose *et al.* 1995). This was subsequently digested with *Sal*I and religated. The plasmid was digested with *Eco*RI and *Sal*I for gap repair in yeast (Rothstein 1991). The *TRP1 CEN4 SPO14* plasmid was constructed by inserting the *Spe*I-*Cla*I fragment from pME986 (Rudge *et al.* 1998b) into the corresponding sites of pUN15 (Elledge and Davis 1988) to generate pME1761.

Plasmid ME1451 is full-length *SPO14* in pUN55, containing the S11 mutation (nucleotide 2267 G → A; amino acid 756 G → E). This was generated in two steps. First, a three-way ligation with the 2.3-kb *Hpa*I-*Cla*I fragment from pME1403, harboring the original mutagenized plasmid, the 3.2-kb *Xba*I-*Hpa*I fragment from pKR325, and the 6-kb *Xba*I-*Cla*I fragment of pUN55 was performed to create pME1444. Next, a three-way ligation with the 3.5-kb *Cla*I-*Dra*I fragment from pME1444, the 2.1-kb *Xba*I-*Dra*I fragment from pKR325, and the 6-kb *Xba*I-*Cla*I fragment of pUN55 was performed to generate pME1451. The 1.15-kb *Dra*I-*Hpa*I fragment derived from pME1403 was sequenced to determine the mutation responsible for the mutant phenotype. The 1.5-kb *Sac*I fragment from pME940 (*HA-SPO14*; Rudge *et al.* 1998b) was substituted for the corresponding *Sac*I fragment of pME1451 to create the hemagglutinin (HA; Wilson *et al.* 1984) epitope-tagged version of this mutant protein in pME1531. The green fluorescent protein (GFP; Chalfie *et al.* 1994)-tagged mutant was generated by insertion of the *Sph*I GFP fragment from pME1086 (Rudge *et al.* 1998b) into the *Sph*I site of pME1451 to create pME1527. This fusion was subcloned into the *Xba*I and *Kpn*I sites of the 2μ plasmid YEp352 (Hill *et al.* 1986) on a 6-kb *Xba*I-*Kpn*I fragment, generating pME1528. The *TRP1 CEN4* plasmid was constructed by inserting the *Spe*I-*Cla*I fragment from pME1531 into the corresponding sites of pUN15 to generate pME1753.

pME1568 is full-length *HA-SPO14* in pUN55, containing the S18 mutation (nucleotide 3878 T → C; amino acid 1293 F → S). A three-way ligation with the 2.3-kb *Hpa*I-*Cla*I fragment from pME1650, the original mutagenized plasmid, the 3.2-kb *Xba*I-*Hpa*I fragment from pME986 (*HA-SPO14*), and the 6-kb *Xba*I-*Cla*I fragment of pUN55 was performed to create pME1568. The 2.3-kb *Hpa*I-*Cla*I fragment derived from

TABLE 1

S. cerevisiae strains used in this study

Strain	Genotype
Y1694	*MAT***a** *his4 lys2 trp1-1 ura3-1 arg4-8 spo14::HIS3 sec14-1ts ADE2 kes1-1* *MAT*α *his4 lys2 TRP1 ura3-1 arg4-8 spo14::HIS3 sec14-1ts ade2 kes1-1*
CTY102[a]	*MAT***a** *ura3-52 lys2-801* Δ*his3-200 sec14-1^{ts} pct1-2*
Y2281	CTY102, plus YEp352 (*URA3* 2μ)
Y2283	CTY102, plus pKR577 (*SPO14 URA3* 2μ)
CTY1128[b]	CTY102 *spo14::HIS3*
Y2262	CTY1128, plus pUN55 (*URA3 CEN4*)
Y2263	CTY1128, plus Yep352 (*URA3* 2μ)
Y2264	CTY1128, plus pKR325 (*SPO14 URA3 CEN4*)
Y2265	CTY1128, plus pKR577 (*SPO14 URA3* 2μ)
Y2266	CTY1128, plus pME1451 (*spo14S-11 URA3 CEN4*)
Y2421	CTY1128, plus pME1568 (*HA-spo14S-18 URA3 CEN4*)
Y2188	CTY1128, plus pME1560 (*HA-spo14S-41 URA3 CEN4*)
Y2194	CTY1128, plus pME1593 (*HA-spo14S-55 URA3 CEN4*)
CTY159[a]	*MAT***a** *ura3-52 lys2-801* Δ*his3-200 sec14-1^{ts} kes1-1*
CTY1130[b]	CTY159 *spo14::HIS3*
Y2274	CTY1130, plus pUN55 (*URA3 CEN4*)
Y2275	CTY1130, plus Yep352 (*URA3* 2μ)
Y2276	CTY1130, plus pKR325 (*SPO14 URA3 CEN4*)
Y2277	CTY1130, plus pKR577 (*SPO14 URA3* 2μ)
Y2278	CTY1130, plus pME1451 (*spo14S-11URA3 CEN4*)
Y2138	CTY1130, plus pME1568 (*HA-spo14S-18 URA3 CEN4*)
Y2178	CTY1130, plus pME1560 (*HA-spo14S-41 URA3 CEN4*)
Y2184	CTY1130, plus pME1593 (*HA-spo14S-55 URA3 CEN4*)
NH144[c]	*MAT***a***leu2-k HIS4 ho::LYS2 ura3 lys2 arg4-Nsp* *MAT*α *leu2::hisG his4-x ho::LYS2 ura3 lys2 ARG4*
Y2170	NH144, plus pME1095 (*GFP-SPO14 URA3* 2μ)
Y2171	NH144, plus pME1528 (*GFP-spo14S-11 URA3* 2μ)
Y2545	NH144, plus pME1697 (*GFP-spo14S-18 URA3* 2μ)
Y2172	NH144, plus pME1561 (*GFP-spo14S-41 URA3* 2μ)
Y2175	NH144, plus pME1594 (*GFP-spo14S-55 URA3* 2μ)
Y2053	NH144 but homozygous *spo14::LEU2*
Y2581	Y2053, plus pUN55 (*URA3 CEN4*)
Y2302	Y2053, plus pME986 (*HA-SPO14 URA3 CEN4*)
Y2159	Y2053, plus pME1095 (*GFP-SPO14 URA3* 2μ)
Y2162	Y2053, plus pME1531 (*HA-spo14S-11 URA3 CEN4*)
Y2163	Y2053, plus pME1528(*GFP-spo14S-11 URA3* 2μ)
Y2288	Y2053, plus pME1568 (*HA-spo14S-18 URA3 CEN4*)
Y2544	Y2053, plus pME1697 (*GFP-spo14S-18 URA3* 2μ)
Y2160	Y2053, plus pME1560 (*HA-spo14S-41 URA3 CEN4*)
Y2161	Y2053 plus pME1561 (*GFP-spo14S-41 URA3* 2μ)
Y2168	Y2053, plus pME1593 (*HA-spo14S-55 URA3 CEN4*)
Y2169	Y2053, plus pME1594 (*GFP-spo14S-55 URA3* 2μ)
Y3420	*MAT*α *leu2 sec14-1 trp1::hisG ura3* Δ*his3-200 cki1::LEU2 spo14::URA3*
Y3426	Y3420, plus pUN15 *(TRP1 CEN4)*
Y3430	Y3420, plus pME1761 *(SPO14 TRP1 CEN4)*
Y3431	Y3420, plus pME1753 *(spo14S-11 TRP1 CEN4)*
Y3428	Y3420, plus pME1781 *(spo14S-18 TRP1 CEN4)*
Y3427	Y3420, plus pME1784 *(spo14S-41 TRP1 CEN4)*
Y3429	Y3420, plus pME1779 *(spo14S-55 TRP1 CEN4)*

[a] Described in Xie *et al.* (1998).
[b] Obtained from V. Bankaitis.
[c] Described in Hollingsworth *et al.* (1995).

pME1650 was sequenced to determine the mutation responsible for the mutant phenotype. The GFP-tagged mutant protein was generated by insertion of the *Sph*I GFP fragment from pME1086 into the *Sph*I site of pME1568 to create pME1694. This fusion was subcloned into the *Xba*I and *Kpn*I sites of the 2μ plasmid YEp352 on a 6-kb *Xba*I-*Kpn*I fragment, generating pME1697. The *TRP1 CEN4* plasmid was constructed by inserting the *Spe*I-*Cla*I fragment from pME1568 into the corresponding sites of pUN15 to generate pME1781.

Plasmids ME1646 (S29, nucleotide 2555 A → G; amino acid 853 H → R), ME1647 (S41, nucleotide 2555 A → G; amino acid 853 H → R), and ME1648 (S55, nucleotides 2260 A → G, 2545 G → A; amino acids 754 N → D, 849 A → T) were sequenced between the *Dra*I and *Hpa*I sites to determine the mutations responsible for the mutant phenotypes. Site-directed mutagenesis was performed to generate the single (S29/S41) and double mutations (S55) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended. The primers used were: S29/S41 P126, 5′-CAG AAT TGC TGA TTT TC**G** TTT AGA TAA ACC ATT C, and P127, 5′-G AAT GGT TTA TCT AAA TCA **C**GA AAA TCA GCA ATT CTG; S55 (nucleotide 2260 A → G) P135, 5′-TTG TGA TTT ATA GG**G** ATG TGG GGA ATA TTG, and P136, 5′-CAA TAT TCC CCA CAT **C**CC TAT AAA TCA CAA; and S55 (nucleotide 2545 G → A) P132, 5′-TAT TCA AAT GCC AGA ATT **A**CT GAT TTT CAT GAT TTA G, and P133, 5′-C TAA ATC ATG AAA ATC AG**T** AAT TCT GGC ATT TGA ATA (the boldface nucleotides represent the introduced mutation). Plasmids ME986 and ME1095 were used as template for the mutagenesis. The resulting plasmids, pME1560 (*HA-spo14S-41 URA3 CEN4*), pME1561 (*GFP-spo14S-41 URA3* 2μ), pME1593 (*HA-spo14S-55 URA3 CEN4*), and pME1594 (*GFP-spo14S-55 URA3* 2μ) were sequenced to confirm that only the desired mutations were generated. The *TRP1 CEN4* plasmids were constructed by inserting the *Spe*I-*Cla*I fragments from pME1560 and pME1593 into the corresponding sites of pUN15 to generate plasmids ME1784 and ME1779, respectively.

The *CKI1* gene (pDO254) on a yeast-replicating vector was obtained from Dr. G. Carman. The *Xba*I-*Hin*dIII *CKI1* fragment from pDO254 was inserted into the corresponding sites of SK+ (Stratagene) to generate pME1824. The *Hpa*I-*Bam*HI *LEU2* fragment from YEp351 (Hill *et al.* 1986) was inserted into the *Nde*I, whose ends had been filled in with the Klenow fragment of DNA polymerase, and *Bgl*II sites of *CKI1* in pME1824 to generate the *cki1::LEU2* deletion construct in pME1827. Plasmid ME1827 was targeted for integration to the *CKI1* locus by digesting with *Xho*I and *Bam*HI.

Analysis of meiosis and sporulation: Cells were grown in YPAcetate medium and sporulated as previously described (Krisak *et al.* 1994). A minimum of 600 cells was examined for the determination of sporulation frequency.

Invertase assays: Invertase secretion was assayed essentially as described (Novick *et al.* 1980). Briefly, after growth to early logarithmic phase in YPAD at 30°, 1 A_{600} unit of cells was pelleted and washed with water. The pellets were resuspended in 2 ml of YP + 0.1% glucose medium and incubated with shaking for 120 min at 37°. The cells were sedimented, and the pellets were washed three times with cold 10 mm NaN_3 and then resuspended in 75 μl 0.1 m NaOAc. Each sample was subsequently split into two equal parts. One was adjusted to 75 μl with 0.1 m NaOAc (external pool), while the other was adjusted with 0.1 m NaOAc, 0.1% Triton X-100 (final) and subjected to dry ice for 5 min and then thawed (total cellular pool). A total of 25 μl of 0.5 m sucrose was added to each and incubated 10 min at room temperature (RT). The reaction was stopped by addition of 100 μl 0.5 m KH_2PO_4, followed by boiling for 2 min, and was subjected to centrifugation. Glucose levels were determined using the Sigma glucose diagnostic kit. The invertase secretion index is the percentage of invertase activity measured in the extracellular pool divided by the total cellular invertase activity. Each value represents a minimum of three invertase assays; standard deviations were calculated with secretion index values obtained from individual experiments.

Immunoblot analysis: Cell extracts were prepared by vortexing cells at 4° in lysis buffer (10 mm triethanolamine, 300 mm sorbitol, 2 mm EDTA, 50 mm sodium fluoride, 40 mm β-glycerophosphate, 1 mm DTT, 2 mm PMSF, 2 mm benzamidine, 0.057 units/ml aprotinin) with glass beads and removing the resulting supernatant after centrifugation at 16,000 × *g* for 10 min. A total of 10 μg of the resulting protein extracts was subjected to SDS-PAGE on 5% SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45 mm; Bio-Rad Laboratories, Hercules, CA) for 1–2 hr. Antibody detection was performed as previously described (Rudge *et al.* 1998b). Proteins on immunoblots were visualized by enhanced chemiluminescence detection on preflashed film. The resulting films were quantitated on an imaging densitometer (model GS-670; Bio-Rad).

PLD assays of immunopurified HA-Spo14: Immunoprecipitation of HA-Spo14 and mutant variants was performed as previously described using the 12CA5 monoclonal antibody (Rudge *et al.* 1998b). PLD activity was measured using 2-dec-anoyl-1-(*O*-[11-{4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s indacene-3-propionyl}amino]undecyl)-*sn*-glycero-3-phospha-tidylcholine (BODIPY-PC) as substrate and 2-decanoyl-1-(*O*-[11-{4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl}amino]undecyl)-*sn*-glycero-3-phosphatidate (BODIPY-PA) quantified as a percentage of BODIPY-PC and normalized for protein recovery (Rudge *et al.* 1998b). All assays were performed in triplicate a minimum of two times.

***In vivo* BODIPY-PC analysis:** BODIPY-PC (in ethanol) was added directly (4 μm final concentration) to cultures inactivated for *SEC14* or induced to sporulate. After 3 hr at the semipermissive temperature of 33.5° for Sec14-independent growth or midway through the sporulation program (at the time of the meiotic divisions) cells were harvested. Phospholipids were extracted from frozen cell pellets by vigorous mixing with methanol (2 ml) followed by chloroform (4 ml). After standing for 15 min, phases were split by addition of 2 ml 0.1 m HCl in 0.88% KCl and the upper aqueous phase was discarded. The lower organic phase was washed with 2 ml methanol/0.1 m HCl in 0.88% KCl (1:1 v/v) and then dried under a stream of nitrogen before resuspending in a small volume of chloroform/methanol (2:1 v/v). Lipids were analyzed by thin-layer chromatography (TLC) as described (Rose *et al.* 1995). BODIPY-labeled lipids were identified by comigration of known standards.

All assays were performed in triplicate. The percentage conversion of intracellular BODIPY-PC to BODIPY-PA was determined from arbitrary fluorescent units obtained from TLC plates using a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA) operating at excitation and emission wavelengths of 488 and 530 nm, respectively. Data values of BODIPY-PC and BODIPY-PA were recorded using ImageQuant 5.1 software (Molecular Dynamics). The amount of BODIPY-PA measured was an underestimation, since PLD-derived BODIPY-PA was further metabolized to lyso-BODIPY-PA (by deacylation) and BODIPY-diacylglycerol (by dephosphorylation).

Quantification of choline release: Choline was chemically measured in samples from medium obtained from the appropriate yeast strains essentially as described (Li *et al.* 2000). Yeast strains harboring the *cki1* mutation, which prevents reutilization of choline, and various alleles of *spo14* were grown

overnight in minimal defined Wickerham's medium supplemented with inositol and lacking choline. Cells were pelleted, washed several times with water, and resuspended in fresh choline-free medium at 30° or 33.5°. After 3 hr, cells were pelleted by centrifugation, and 200 μl of the culture supernatant was removed. Choline content of the supernatant was determined by a coupled reaction in which H_2O_2, produced from choline oxidase, was reacted with aminoantipyrine and phenol to form quinoneimine (Li *et al.* 2000). To measure choline release from sporulating cultures, cells were sporulated as described above and choline was measured in the medium after 24 hr.

HPLC analysis: Total lipid extracts, prepared as described above for the *in vivo* BODIPY-PC analysis, were separated and characterized by liquid chromatography-mass spectrometry (LC-MS; QP8000alpha; Shimadzu, Tokyo) using a Luna silica column (3 μm, 2.0 × 150 mm; Phenomenex, UK) with a solvent gradient of chloroform/methanol/water/ammonia (90:9.5:0.5:0.32 by volume) changing to chloroform/ methanol/water/ammonia (50:48:2:0.32 by volume) over 40 min at 0.35 ml/min. Detection in negative electro spray ionization mode allowed characterization of PA, phosphatidylethanol (PEtOH), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) while detection in positive electro spray ionization mode allowed characterization of PC. No exogenous ethanol was added to the cultures for the measurement of PEtOH as yeast generate ethanol as a consequence of fermentation of intracellular glucose and hence naturally produce low levels of PetOH, which could be analyzed by LC-MS. The retention times of the acidic phospholipids, particularly PA, were very dependent on the ammonia concentration. Zwitterionic lipids such as PC and PE were largely unaffected by the ammonia concentration. A total of 0.32% ammonium solution (60 mm) proved optimum for the Luna silica column used, with most phospholipid classes resolving essentially to baseline.

RESULTS

Sporulation and secretion are differentially sensitive to PLD levels: *SPO14* encodes the major PC-PLD in yeast (Rose *et al.* 1995; Ella *et al.* 1996; Waksman *et al.* 1996) and is required for sporulation (Honigberg *et al.* 1992; Rose *et al.* 1995) and suppression of phosphatidylinositol transfer protein defects (*sec14* bypass) in secretion and cell viability (Sreenivas *et al.* 1998; Xie *et al.* 1998). Genetic analyses to investigate the requirement for PLD in both of these cellular processes have revealed that Spo14 must be properly targeted within the cell, enzymatically competent (Rudge *et al.* 1998b; Xie *et al.* 1998), and responsive to the lipid activator, PIP_2 (Sciorra *et al.* 1999). However, PLD function in sporulation and Sec14-independent secretion is not equivalent. Examination of the effect of overexpressing *SPO14*, which results in increased PLD activity (Rudge *et al.* 1998b), on cell growth in *sec14* bypass strains revealed that more PLD activity improved growth during Sec14-independent growth (Figure 1, A and B) but did not affect sporulation (*SPO14*-CEN = 72 ± 12%, *SPO14*-2μ = 55 ± 12%; Rudge *et al.* 1998b). As expected, no growth of *sec14* bypass strains (at the restrictive temperature) or sporulation was seen in the absence of Spo14 (Δ*spo14*). Vegetative growth (but not sporulation) does occur in Δ*spo14* strains provided *SEC14* is fully functional. These results suggest that PLD activity is limiting for Sec14-independent growth but not for sporulation.

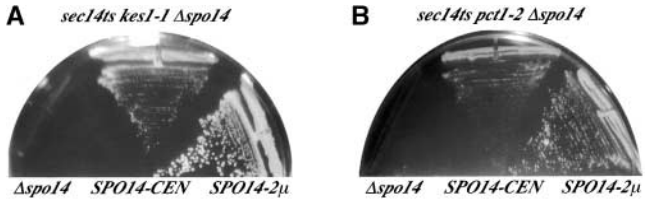

Figure 1.—Sporulation and secretion are differentially sensitive to PLD levels. (A) *sec14* bypass suppression is improved with increasing PLD activity. *sec14-1^{ts} kes1-1* Δ*spo14* strains harboring either low-copy-number plasmid (Δ*spo14*), low-copy-number plasmid with *SPO14* (*SPO14 CEN*), or high-copy-number plasmid with *SPO14* (*SPO14* 2μ) were incubated on solid medium lacking uracil at 37° for 72 hr. Growth at this temperature is indicative of *sec14* suppression. (B) *sec14-1^{ts} pct1-2* Δ*spo14* harboring the same plasmids were incubated at 37° for 7 days.

Identification of separation-of-function alleles of *SPO14*: To investigate the molecular basis for this differential PLD requirement in Sec14-independent growth and sporulation, we undertook a genetic screen to identify mutant PLDs that support Sec14-independent secretion but not sporulation. We constructed a diploid yeast strain (Y1649) homozygous for Δ*spo14, sec14^{ts}*, and *kes1*. This strain fails to grow at temperatures above 35° where Sec14 is inactivated and cannot sporulate at any temperature because of the absence of Spo14. Introduction of *SPO14* on a centromere plasmid rescued both growth above 35° and sporulation. We introduced mutated *SPO14* sequences and a gapped plasmid into this strain (see materials and methods). *In vivo* recombination (Muhlrad *et al.* 1992) generated a library of replicating plasmids harboring mutant *spo14* sequences; the resultant transformants were screened for their ability to support growth above 35° and to sporulate. Ten mutants were identified that exhibited the separation-of-function phenotype (*spo14S*) characterized by a total defect in sporulation but nearly wild-type growth under *sec14* suppression in Y1649. The lesions responsible for the mutant phenotype in five of these (*spo14S-11*, *spo14S-18*, *spo14S-29*, *spo14S-41*, *spo14S-55*) were determined; *spo14S-29* and *spo14S-41* have the same lesion and hence, of these two, only *spo14S-41* was analyzed further.

The ability of the four mutant alleles to support growth and secretion during *sec14* bypass was determined. Strains harboring these *spo14* alleles combined with *sec14* bypass mutations obtained either through inactivation of a proposed phosphoinositide binding protein (Figure 2A, *kes1*; Fang *et al.* 1996; Rivas *et al.* 1999) or by inactivating the CDP-choline PC pathway (Figure 2B, *pct1*; Cleves *et al.* 1991) suppressed *sec14-1^{ts}*-associated growth defects to varying extents. While growth of *sec14* bypass strains harboring *spo14S-18*, *spo14S-41*, and *spo14S-55* was essentially equivalent to

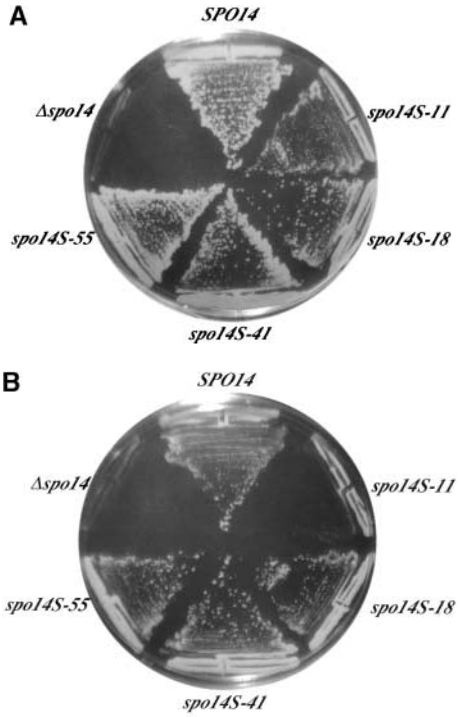

FIGURE 2.—Phenotypes of *spo14* separation-of-function mutants. (A) A *sec14-1*^{ts} *kes1-1* Δ*spo14* strain harboring low-copy-number plasmids with either no *SPO14* sequences (Δ*spo14*) or wild-type *SPO14*, *spo14S-11*, *spo14S-18*, *spo14S-41*, or *spo14S-55* incubated at 37°. (B) A *sec14-1*^{ts} *ptc1-2* Δ*spo14* strain harboring low-copy-number plasmids with either no *SPO14* (Δ*spo14*) or wild-type *SPO14*, *spo14S-11*, *spo14S-18*, *spo14S-41*, or *spo14S-55* incubated at 37°.

SPO14 strains, *spo14S-11* grew less well. This effect was more pronounced in *pct1 sec14* strains than in *kes1 sec14* strains. The functionality of these alleles in supporting Sec14-independent secretion was assessed by measuring trafficking of invertase through the secretory pathway to the cell surface, which is a reliable measure of Golgi secretory function (NOVICK *et al.* 1980). As shown in Table 2, the secretion index of invertase in *sec14* bypass strains harboring these alleles was equivalent to the same strains harboring wild-type *SPO14*. As reported previously, the secretion competency of Δ*spo14 sec14*^{ts} *kes1* strains is greater than that of Δ*spo14 sec14*^{ts} *pct1* strains (XIE *et al.* 1998). Thus, although secretion and viability were both improved in the presence of these alleles, as noted previously (LI *et al.* 2000) there is not always a direct correlation between growth and secretion. In contrast to their ability to support Sec14-independent secretion, diploid strains harboring these alleles as the only source of *SPO14* were either severely restricted (*spo14S-55*; 6 ± 2%) or completely failed to sporulate (*spo14S-11*, *-18*, *-41*; <0.01%).

Separation-of-function alleles produce stable proteins that are phosphorylated during meiosis, catalytically active, and properly localized: We previously showed that both catalytic activity and relocalization of Spo14

TABLE 2

Secretion efficiency of *spo14* separation-of-function alleles

SPO14 genotype	Invertase secretion index[a]	
	sec14-1^{ts} *kes1-1*	*sec14-1*^{ts} *pct1*
Δ*spo14*	0.71 ± 0.02	0.31 ± 0.08
SPO14	0.93 ± 0.04	0.82 ± 0.05
spo14S-11	0.93 ± 0.05	0.79 ± 0.02
spo14S-41	0.90 ± 0.05	0.82 ± 0.05
spo14S-18	0.91 ± 0.07	0.85 ± 0.05
spo14S-55	0.94 ± 0.05	0.83 ± 0.02

[a] Invertase secretion was measured at 37° as previously described (NOVICK *et al.* 1980).

were essential for PLD function in meiosis and that phosphorylation of the enzyme correlated with protein movement (RUDGE *et al.* 1998b). Thus, the failure of strains harboring these mutations to sporulate could be due to protein instability in meiosis, altered phosphorylation, reduced catalytic activity, and/or mislocalization. To examine Spo14 protein, sequences encoding three epitopes from the influenza virus HA protein were introduced into the mutant sequences at the same location where we had previously tagged wild-type protein without affecting function (RUDGE *et al.* 1998b). Western blot analysis of meiotic whole cell extracts revealed that all of the mutant proteins were synthesized and stable (Figure 3A). Quantification of steady-state Spo14 levels indicated there was only a twofold difference in relative protein levels among the different mutants (HA-Spo14 = 1.0; HA-spo14S-11 = 0.52; HA-spo14S-41 = 0.90; HA-spo14S-18 = 0.87; HA-spo14S-55 = 0.69).

Spo14 is modified by phosphorylation during meiosis. This is easily monitored by its retarded migration relative to the nonphosphorylated protein on SDS-PAGE (RUDGE *et al.* 1998b). The mutant proteins migrated similarly to wild-type Spo14 under sporulating conditions, suggesting that phosphorylation occurred normally (Figure 3A).

PLD activity was measured using immunoprecipitates isolated from cells harboring the different Spo14 proteins induced to undergo meiosis. The immunoprecipitates were examined by immunoblot analysis (data not shown) and assayed for PLD activity. As shown in Table 3, each of the mutant proteins had significant PLD specific activity ranging from 35 to 100% of that obtained with the wild-type enzyme.

To determine if the failure of these mutant proteins to support sporulation was a result of reduced PLD activity, we expressed each *spo14S* gene from either a 2μ plasmid, which is retained at high copy number within yeast cells (for review, see BROACH and VOLKERT 1991), or a low-copy-number CEN plasmid. In contrast to the situation when expressed from the CEN plasmid, cells expressing *spo14S-41* and *-55* from the high-copy-

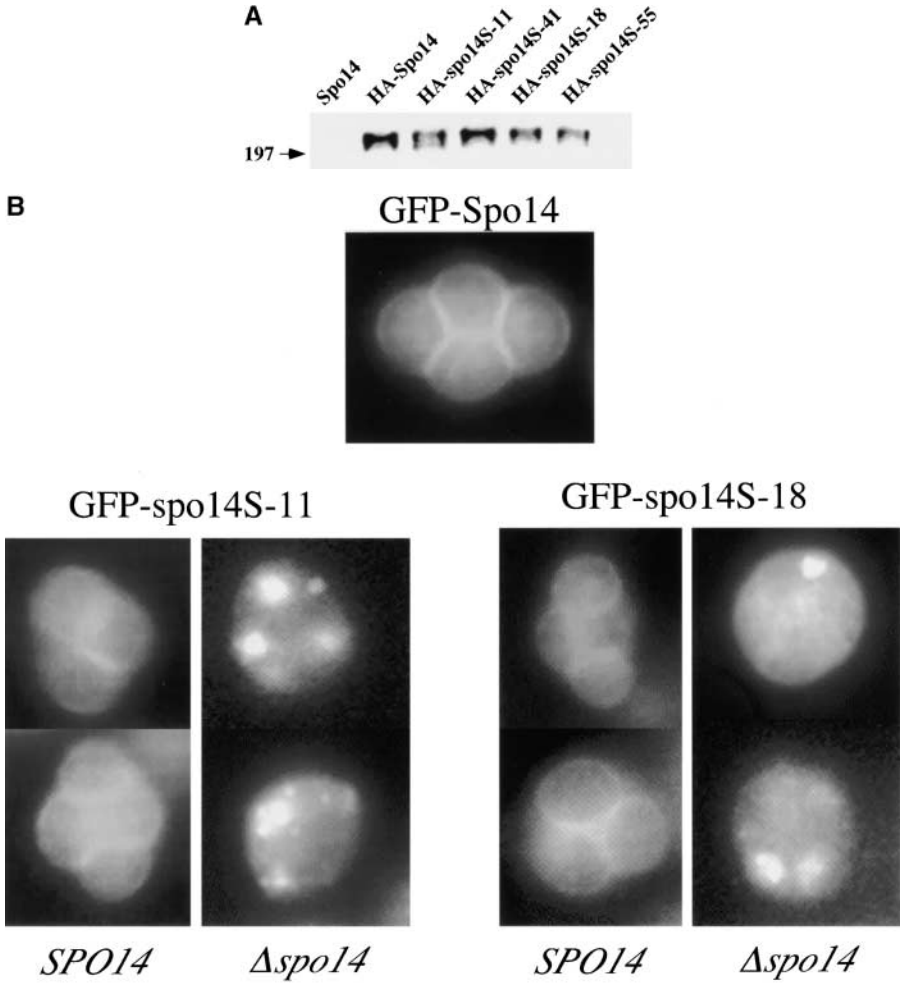

FIGURE 3.—spo14 separation-of-function protein and localization. (A) Immunoblot analysis of meiotic whole cell extracts (10 μg) from cells harboring *HA-SPO14*, *HA-spo14S-11*, *HA-spo14S-18*, *HA-spo14S-41*, or *HA-spo14S-55* expressed from low-copy-number plasmids. The number on the left of the immunoblot indicates the position of molecular mass standard (kilodaltons). (B) GFP-Spo14, GFP-spo14S-11, and GFP-spo14S-18 staining in yeast cells induced in meiosis. (Top) The characteristic rings of GFP-Spo14 staining late in sporulation; (left) GFP-spo14S-11; (right) GFP-spo14S-18 staining in wild-type (*SPO14*) and deletion (Δ*spo14*) cells late in the sporulation process.

number plasmid as the only source of Spo14 were able to sporulate at close to wild-type levels (Table 4), indicating that these alleles are partially functional for meiosis provided sufficient PLD is expressed. However, cells expressing *spo14S-11* and *-18* from either plasmid completely failed to sporulate, indicating that the inability of these proteins to support sporulation was not due simply to weakened PLD activity.

TABLE 3

Relative specific PLD activity of separation-of-function proteins

Protein	Relative PLD specific activity[a]
HA-Spo14	1.0 ± 0.01
HA-spo14S-11	1.03 ± 0.03
HA-spo14S-41	0.69 ± 0.01
HA-spo14S-18	0.58 ± 0.01
HA-spo14S-55	0.36 ± 0.01

[a] PLD assays were performed with immunopurified proteins and vesicles containing 100 μM BODIPY-PC and 5 μM PIP_2 as described in MATERIALS AND METHODS. The mean values ± SD from three independent experiments are shown.

Spo14 relocalizes to the developing membrane during meiosis and this relocalization is essential for membrane formation (RUDGE *et al.* 1998b). GFP-Spo14 fusions were constructed with each of the mutant sequences to

TABLE 4

Sporulation proficiency of *spo14* separation-of-function alleles

Strain	*SPO14* genotype	% sporulation[a]
Y2581	Δ*spo14*	<0.1
Y2302	*SPO14 CEN*	71 ± 12
Y2159	*SPO14* 2μ	55 ± 12
Y2162	*spo14S-11 CEN*	<0.1
Y2163	*spo14S-11* 2μ	<0.1
Y2288	*spo14S-18 CEN*	<0.1
Y2544	*spo14S-18* 2μ	<0.1
Y2160	*spo14S-41 CEN*	<0.1
Y2161	*spo14S-41* 2μ	44 ± 6
Y2168	*spo14S-55 CEN*	5 ± 1
Y2169	*spo14S-55* 2μ	51 ± 14

[a] Percentage sporulation was determined by phase-contrast microscopy. At least 600 cells were counted from three independent cultures; standard deviations were calculated with sporulation values obtained from individual experiments.

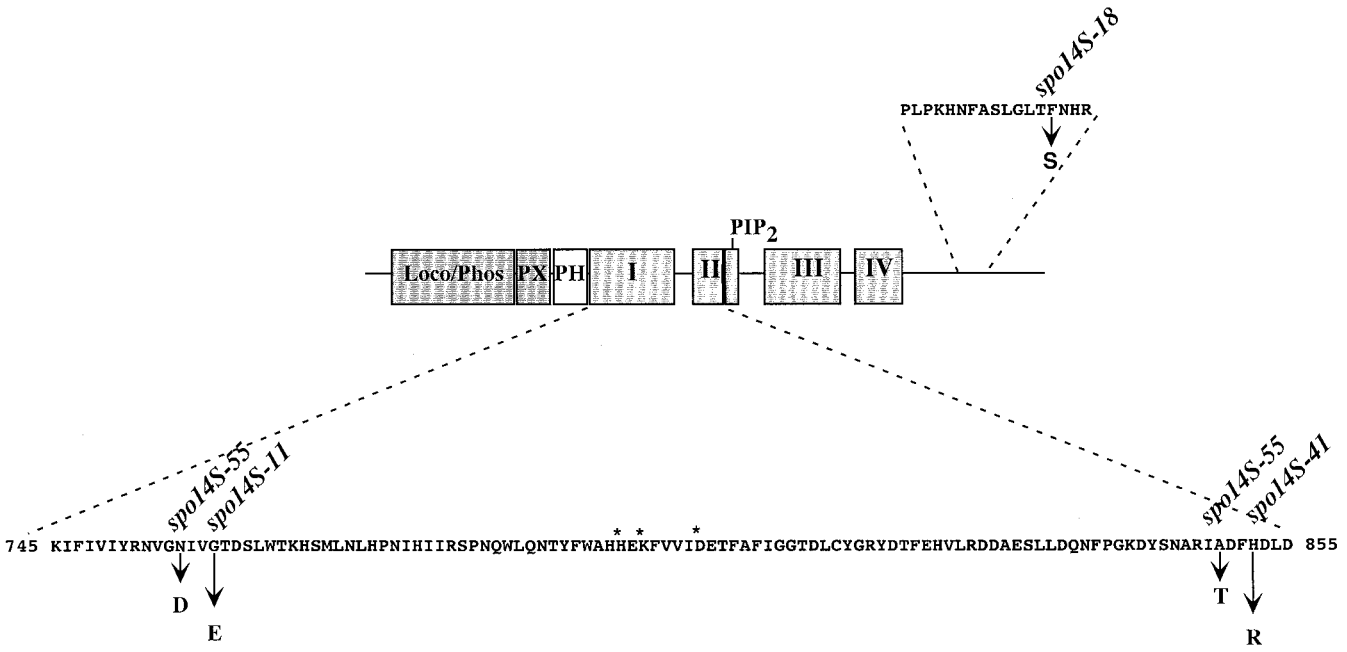

FIGURE 4.—Separation-of-function mutations map to the catalytic domain of PLD. Boxed regions denote domains in Spo14 based on experimental and sequence alignment analyses. Loco/Phos, localization and phosphorylation (RUDGE *et al.*1998a,b); PX, phox domain (PONTING 1997); PH, Pleckstrin homology (STEED *et al.* 1998); catalytic regions I–IV (MORRIS *et al.* 1996; SUNG *et al.* 1997); PIP_2, PIP_2 binding domain (SCIORRA *et al.* 1999). The first putative catalytic triad is denoted by asterisks. The amino acid changes in each of the separation-of-function alleles are indicated with arrows.

examine localization of PLD in living yeast cells. As previously reported, the introduction of GFP into *SPO14* sequences does not impair function of the wild-type protein; however, a signal is observed only when this fusion is expressed from a high-copy-number plasmid (RUDGE *et al.* 1998b). Examination of GFP-spo14S-41 and GFP-spo14S-55 revealed that they were properly localized to the developing yeast cell membrane (data not shown), which agrees with their ability to support sporulation when expressed from a high-copy-number plasmid. However, while GFP-spo14S-11 and GFP-spo14S-18 appeared to move to specific locations during meiosis, they failed to form rings characteristic of the spore membrane when expressed as the only source of PLD (Figure 3B). These fusions did stain the spore membrane when expressed in wild-type cells (Figure 3B), indicating that the localization signal was not impaired in the mutant proteins.

Separation-of-function mutations map to the catalytic domain of PLD: Sequence analyses of the mutant alleles showed that, in all but one case (*spo14S-55*), a single amino acid change was responsible for the mutant phenotype (Figure 4). Both of the point mutations identified in *spo14S-55* were required to obtain the mutant phenotype (data not shown). Surprisingly, all but one of the mutations (*spo14S-18*) map to the catalytic core of the enzyme. These mutations flank the first HKD motif that, in conjunction with the second HKD motif in domain IV, has been shown to be essential for catalytic activity (SUNG *et al.* 1997). *spo14S-18* maps C-terminal to the catalytic region.

***In vivo* PLD activity of the separation-of-function mutants:** The finding that all but one of the separation-of-function alleles mapped to the catalytic core of the enzyme, yet were competent to cleave PC *in vitro*, prompted us to examine their activities *in vivo*. To achieve this aim, we took advantage of the finding that yeast cells readily internalize fluorescent derivatives of PC (KEAN *et al.* 1993). Yeast cells were grown in the presence of the fluorescent-labeled substrate, BODIPY-PC, under *sec14* bypass conditions and when cells were induced to sporulate. Cellular lipids were extracted and separated by TLC to examine BODIPY-PA formation. Hydrolysis of BODIPY-PC occurred almost exclusively by the action of Spo14, as Δ*spo14* strains did not generate any appreciable levels of BODIPY-PA or other products (Figure 5). We observed the same pattern and extent of hydrolysis for wild type and *spo14S-11*, *-41*, and *-55* during both Sec14-independent secretion (Table 5) and sporulation (Figure 5; Table 5). However, *spo14S-18* exhibited an approximately twofold decrease in BODIPY-PA formation under both conditions (Table 5). These results indicated that, consistent with the *in vitro* results, exogenous substrate was also cleaved *in vivo* by the separation-of-function PLD enzymes.

To examine *in vivo* PLD activity from endogenous substrate we took advantage of the choline excretion phenotype that has been described for *sec14*^{ts} strains

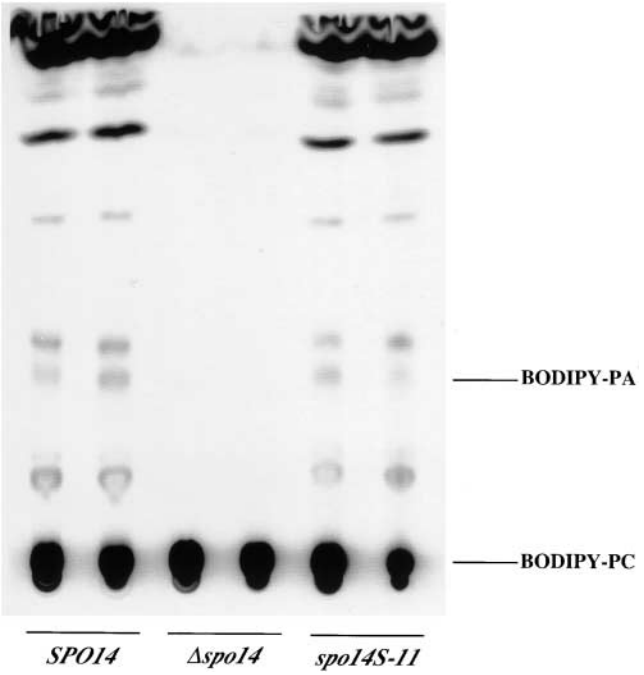

FIGURE 5.—PLD-catalyzed hydrolysis of internalized BODIPY-PC. TLC plate of fluorescent lipids extracted from wild type (*SPO14*), deletion (Δ*spo14*), and separation-of-function mutant (*spo14S-11*) after induction of meiosis in the presence of BODIPY-PC. BODIPY-PC and BODIPY-PA are denoted. The remaining fluorescent molecules are derived principally from the cellular metabolism of PLD-derived BODIPY-PA and include BODIPY-diacylglycerol, BODIPY-monoacylglycerol, and free BODIPY.

carrying CDP-choline pathway mutations when elevated to the *sec14*^{ts} restrictive temperature. The excretion of choline results from accelerated turnover of PC under circumstances where choline reutilization is blocked (*i.e.*, in the presence of a CDP-choline pathway mutation; PATTON-VOGT *et al.* 1997) and is due in part to activation of Spo14 (SREENIVAS *et al.* 1998). We used an enzyme-coupled reaction to measure choline release as described by LI *et al.* (2000). *CKI1* encodes the first enzyme in the CDP-choline PC biosynthetic pathway and its inactivation, together with *sec14* inactivation, results in the *sec14* bypass phenotype. Furthermore, inactivation of *CKI1* alone did not affect meiosis and sporulation (data not shown). Choline released under *sec14* bypass conditions (Table 6) was approximately eightfold higher in the wild-type, *spo14S-18*, *-41*, and *-55* mutants (2.3–2.5 μmol/OD_{600}) relative to the control Δ*spo14* cells (0.3 μmol/OD_{600}). In cells harboring *spo14S-11* there was a smaller, although still significant, increase in choline release (approximately fourfold). During meiosis (Table 6), there was a higher, PLD-independent, basal release of choline (2.0 μmol/OD_{600}; Δ*spo14*) and the increase over this level to ~4.5 μmol/OD_{600} was similar for wild-type, *spo14S-18*, *-41*, and *-55* mutants. However, under these conditions the *spo14S-11* mutants showed little increase in choline release.

TABLE 5

PLD-catalyzed hydrolysis of internalized BODIPY-PC

	% BODIPY-PA[a]	
SPO14 genotype	Sec14-independent growth	Sporulation
Δ*spo14*	0.33 ± 0.05	0.45 ± 0.07
SPO14	3.60 ± 0.10	2.60 ± 0.14
spo14S-11	2.90 ± 0.14	2.20 ± 0.14
spo14S-18	1.35 ± 0.21	1.15 ± 0.07
spo14S-41	2.45 ± 0.20	3.30 ± 0.02
spo14S-55	2.70 ± 0.14	2.9 ± 0.21

[a] The percentage conversion of intracellular BODIPY-PC to BODIPY was determined as described in MATERIALS AND METHODS. The mean values ± SD from three independent experiments are shown.

The apparent disparity between the ability of spo14S-11 to hydrolyze BODIPY-PC but not endogenous PC during sporulation suggested that while spo14S-11 was catalytically competent, it was incapable of metabolizing the endogenous substrate *in vivo*. To confirm the choline data, we used LC-MS techniques to examine changes in phospholipid composition and molecular species profiles during Sec14-independent secretion (*kes1 sec14*), sporulation, and vegetative growth. A particular lipid molecular species is defined by its head group and its precise acyl chain substituents. After 3 hr of growth under *sec14* bypass, the PA profile was examined for cells expressing wild-type PLD (*SPO14*), cells deleted for PLD (Δ*spo14*), and cells expressing the *spo14S-11* separation-of-function allele. While *SPO14* and *spo14S-11* strains showed essentially the same PA profile, Δ*spo14* cells contained significantly lower proportions of 30:1 PA (12:0/18:1 + 14:0/16:1 PA), 32:1 PA (14:0/18:1 + 16:0/16:1 PA), and, in particular, 32:2 PA (14:1/18:1 + 16:1/16:1 PA; Figure 6A), suggesting that these species are specifically generated by Spo14. The other PA species such as 34:1 PA (16:0/18:1 + 18:0/16:1 PA) and 34:2 PA (16:1/18:1 PA) found as major components in the Δ*spo14* strain were probably generated through alternative pathways such as sequential acylation of glycerol-3-phosphate (reviewed in ATHENSTAEDT and DAUM 1999) or hydrolysis of PE/PS by ScPLD2 (MAYR *et al.* 1996; WAKSMAN *et al.* 1997). Positive assignment of precise acyl structures to these PA species is not possible with the LC-MS approach used so subsequent notation in the main text does not assign individual fatty acids but gives the summation of all the acyl carbons and double bonds in the structures on the basis of the molecular ions detected.

Confirmation that 30:1, 32:1, and 32:2 PA were indeed primarily products of Spo14 came from analysis of PEtOH. PEtOH is the product of the PLD transphosphatidylation reaction when ethanol is used as a nucleophile

TABLE 6

***In vivo* PLD activity of separation of function alleles**

SPO14 genotype	Choline (μmol/OD_{600})[a]	
	Sec14-independent growth	Sporulation
Δ*spo14*	0.31 ± 0.09	2.0 ± 0.8
SPO14	2.31 ± 0.25	4.5 ± 0.1
spo14S-11	1.17 ± 0.31	2.7 ± 0.4
spo14S-18	2.32 ± 0.23	4.5 ± 0.6
spo14S-41	2.17 ± 0.15	4.4 ± 0.9
spo14S-55	2.47 ± 0.23	4.5 ± 0.8

[a] Choline was measured using a coupled reaction as described in MATERIALS AND METHODS from culture supernatants after 3 hr at 33.5° for Sec14-independent growth and after 20 hr in sporulation medium. The mean values ± SD from three independent experiments are shown.

instead of water (VAN BLITTERSWIJK and HILKMANN 1993). This reaction is unique to this class of enzymes and thus is an excellent marker for PLD activity. Yeast cells generate ethanol as a consequence of fermentation of intracellular glucose and hence naturally produce low levels of PetOH; thus this analysis could be done in the absence of exogenous ethanol. This lipid was completely absent in the Δ*spo14* strain, whereas the *SPO14* and *spo14S-11* strains both contained particularly high levels of 32:2 PEtOH (~60 mol%), with lower proportions of 30:1 and 32:1 PEtOH.

We also examined PA profiles in meiotic (Figure 6B) and vegetative cells that were otherwise wild type (Sec14 proficient; Figure 6C). The PA profiles differed between the meiotic and vegetative cells, with the former having greater proportions of 34:1 PA species in all three strains. However, consistent with the choline measurements, 32:2 PA, the major product of PLD hydrolysis, was approximately twofold lower in the Δ*spo14* and *spo14S-11* strains. PEtOH (32:2) was formed only in the wild-type cells, indicating that this molecular species most likely reflects *in vivo* PLD activity. Taken together, these results show that, although spo14S-11 behaves similarly to the wild-type enzyme under *sec14* bypass conditions in its ability to hydrolyze PC, it is unable to do so during meiosis and vegetative growth where it behaves like the PLD deletion (Δ*spo14*) mutant. We interpret these results to imply that PLD hydrolysis occurs differently during Sec14-independent secretion and meiosis. Furthermore, the defect in spo14S-11 blocks the ability of the enzyme to cleave endogenous PC during meiosis and vegetative growth when Sec14 is present. As the PLD-dependent PEtOH profile was the same under all conditions, the same PC species are cleaved during Sec14-dependent and -independent growth and in meiosis. These results also indicate that substrate specificity is unaltered in spo14S-11. PC species profiles were very similar under sporulating or normal vegetative conditions: 6–10 mol% 32:2 PC in all cultures. Furthermore, no obvious differences between the three PLD strains under these conditions were noted for PE, PI, or PS species profiles (data not shown). However, under *sec14* bypass ~18 mol% was 32:2 PC and the Δ*spo14* strain had reduced proportions of 32:2 PC (4 mol%) as well as 34:2 PC, 32:2 PI, and 34:2 PI and greatly increased proportions of 34:2 PS (data not shown). The total mass of PI and PS also increased substantially in this strain; however, all these changes may be related to cell death since in the absence of Spo14 the temperature shift to inactivate Sec14 for *sec14* bypass is ultimately fatal.

DISCUSSION

In this article we have identified separation-of-function alleles of *SPO14* that support growth and secretion in the absence of Sec14 but that fail to function in meiosis. The identification of such alleles indicates that PLD function in these two physiological processes is distinct. The defect of one of these alleles, *spo14S-11*, perturbs the ability of the enzyme to cleave PC under one condition (meiosis) but not the other (Sec14-independent secretion), indicating that PLD hydrolysis of PC occurs differently during meiosis and Sec14-independent secretion.

Unlike the situation in Sec14-independent secretion (XIE *et al.* 1998; RIVAS *et al.* 1999), no change in the bulk levels of PLD-generated PA is detected in sporulating cells (S. RUDGE and J. ENGEBRECHT, unpublished results). However, analysis of molecular PA species has revealed that there exists a twofold increase in PLD-derived 32:2 PA in meiosis. Surprisingly, the same small changes in 32:2 PA species between wild-type, Δ*spo14*, and *spo14S-11* mutants was also detected in vegetative cells that were proficient for Sec14 (compare Figure 6B and 6C). However, while inactivation of *SPO14* has no effect on vegetative growth and secretion in an otherwise wild-type strain, it is absolutely essential for meiosis. Thus, the small change in 32:2 PA observed in meiosis must have a profound effect on the ability of the cell to mediate the developmentally regulated trafficking events that occur during sporulation (NEIMAN 1998). Our existing model of Spo14 regulation and function during meiosis can explain these results. We previously demonstrated that relocalization of Spo14 activity from a detergent insoluble cell fraction in vegetative cells to a detergent soluble cell fraction in meiotic cells (RUDGE *et al.* 1998a) is essential for the formation of the internal spore membrane (RUDGE *et al.* 1998b). Consequently, the small change in 32:2 PA most likely reflects much larger local increases in this PA species at different cellular locations in vegetative and sporulating cells. Thus, only changes in the levels of 32:2 PA at the site of new membrane formation in meiosis are essential for cell function.

Analysis of PA molecular species indicates that Spo14,

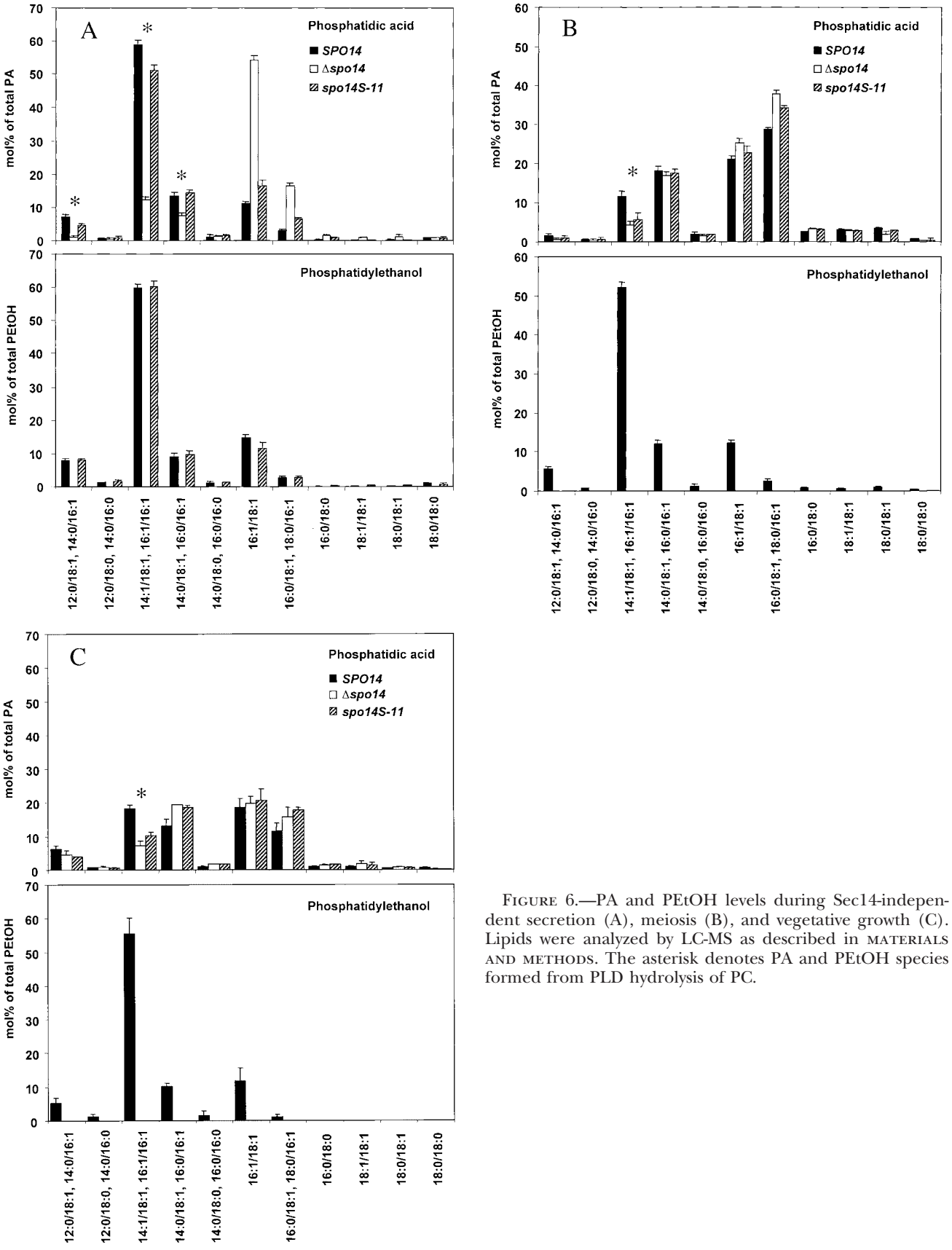

FIGURE 6.—PA and PEtOH levels during Sec14-independent secretion (A), meiosis (B), and vegetative growth (C). Lipids were analyzed by LC-MS as described in MATERIALS AND METHODS. The asterisk denotes PA and PEtOH species formed from PLD hydrolysis of PC.

like its mammalian counterpart, PLD1 (Pettitt *et al.* 1997), hydrolyzes specific PC species *in vivo* during both Sec14-independent growth and sporulation. Here we demonstrate for the first time a physiological requirement for the hydrolysis of a particular molecular species of PC. Furthermore, our data distinguish between two possible consequences of Spo14-catalyzed hydrolysis of PC: generation of a lipid signal *vs.* mass synthesis of a lipid. During Sec14-independent secretion, bulk PA levels are sensitive to PLD inactivation (Xie *et al.* 1998; Rivas *et al.* 1999), indicating that PLD activity grossly alters the lipid environment for survival in the absence of Sec14. In contrast, during meiosis the total cellular levels of PC and PA are essentially unaltered in the presence and absence of Spo14, hence PLD does not simply generate PA to drive the increased *de novo* phospholipid synthesis that occurs during sporulation (Henry and Halvorson 1973). Instead, our data indicate a signaling function for PLD-derived 32:2 PA during yeast meiosis.

S. cerevisiae readily internalize PC molecules labeled on one acyl chain with a fluorescent moiety (Kean *et al.* 1993; Grant *et al.* 2001). Internalization can occur via two pathways, one dependent on the process of endocytosis and a second process resembling phospholipid flip-flop (Kean *et al.* 1993). BODIPY-PC is metabolized almost exclusively by Spo14, since Δ*spo14* mutants fail to generate BODIPY-PA despite internalizing the fluorescent substrate. Within the cell, BODIPY-PC and its metabolites are transported by endocytosis to the vacuole during vegetative growth and to the prospore membrane in wild-type sporulating cells (S. Rudge and J. Engebrecht, unpublished data). These results demonstrate that at least a pool of Spo14 is localized within endosome membranes of vegetative cells and are in good agreement with previous reports of GFP-Spo14 residing in the endosome (Li *et al.* 2000). They are also consistent with our previous report that GFP-Spo14 localizes to the prospore membrane during meiosis (Rudge *et al.* 1998b).

All separation-of-function alleles of Spo14 have the capacity to hydrolyze internalized BODIPY-PC. This suggests that the separation-of-function alleles are active *in vivo*. Furthermore, TLC analysis of the PLD-dependent metabolites of BODIPY-PC revealed no difference in the profile of fluorescent lipids generated by the catalytic activity of wild-type or separation-of-function proteins. This demonstrates that the metabolism of PLD-derived PA is unhindered in the separation-of-function mutants.

Yeast cells have a second PLD activity, ScPLD2, which preferentially hydrolyzes PE (Mayr *et al.* 1996; Waksman *et al.* 1997). However, this activity fails to enable *spo14* mutants to sporulate (Rudge and Engebrecht 1999). Similar to Spo14 (Rose *et al.* 1995), the separation-of-function PLDs do not hydrolyze PE *in vitro* (S. Rudge and J. Engebrecht, unpublished data). This fits with the LC-MS data, which showed that Spo14 and spo14S-11 both generate identical PEtOH species profiles, making it highly unlikely that the spo14S-11 protein has an altered substrate specificity. In addition to PA, mammalian PLDs have the potential to catalyze the formation of bis-phosphatidic acid (bis-PA). In such catalytic reactions, diacylglycerol behaves as a primary alcohol for PC-PLDs (van Blitterswijk and Hilkmann 1993). Bis-PA is a potential precursor of semilysobisphosphatidic acid, an unusual phospholipid that is thought to regulate Golgi structure and function (Cluett and Machamer 1996). However, LC-MS failed to detect *in vivo* bis-phosphatidic acid formation under any condition examined here and more recent work has found that yeast synthesizes these lipids independently of Spo14 (S. Rudge and J. Engebrecht, unpublished data). Thus, we cannot attribute the phenotype of the separation-of-function mutants to a change in the enzymes' substrate specificity or an inability to catalyze an alternative PLD reaction.

The identification of separation-of-function alleles indicates that PLD function in Sec14-independent secretion and meiosis is distinct. Furthermore, the finding that the mutants do not have identical phenotypes suggests that multiple mechanisms exist to differentiate PLD function in these two physiological processes. One possible explanation for the differential requirement of PLD in these two physiological settings is that sporulation requires a higher threshold of PLD activity than Sec14-independent growth. However, we do not favor this hypothesis for the following reasons: (1) Sec14-independent growth, but not sporulation, is improved when more PLD activity is supplied to the cell (Figure 1); (2) bulk changes in PA can be measured in Sec14-independent growth but not during sporulation (Xie *et al.* 1998; Rivas *et al.* 1999; Figure 6); (3) spo14S-11 and spo14S-18, which display substantial PLD activity *in vitro* and *in vivo* (Tables 3 and 5), do not support sporulation when overexpressed (Table 4); and (4) a number of hypomorphic alleles of *SPO14* exist that have reduced PLD activity and show a corresponding reduction in spore formation, indicating that there is not a simple threshold level of PLD activity required to support sporulation (*e.g.*, Sciorra *et al.* 1999). Instead the characterization of spo14S-11 in particular has led us to favor the hypothesis that there must exist alternative mechanisms of PLD PC hydrolysis during Sec14-independent growth and sporulation. The defect in spo14S-11 may prevent the formation of an active enzymatic complex that is normally required for PA production in Sec14-proficient cells.

Taken together, our findings suggest that PA is the biologically active product of PLD hydrolysis. What then is PA doing? PA is either a specific activator of membrane regulators and/or its production in a localized region of the cell contributes to the biophysical properties of the membrane for vesicle formation or fusion. Both of these are hypothesized to occur in mammalian

cells; however, the precise role of PLD-generated PA in membrane trafficking has been controversial. The importance of PA in membrane structure has been illustrated by the finding that conversion of lysophosphatidic acid to PA is important for membrane curvature during synaptic vesicle endocytosis, although in this case PA is produced via the action of endophilin, a lysophosphatidic acid acyl transferase (Schmidt *et al.* 1999). PA synthesis by PLD has been shown to be necessary for secretion in endocrine cells (Siddhanta *et al.* 2000). However, the necessity to convert PLD-derived PA to diacylglycerol has also been proposed for PLD function in eukaryotes (Rivas *et al.* 1999; Sciorra and Morris 1999; Henneberry *et al.* 2001). Analysis of the specific defect in *spo14* mutants in the process of this novel membrane trafficking event should provide insight into the function of the PLD-derived PA signal.

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