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Roles of Phosphoinositides and of Spo14p (phospholipase D)-generated Phosphatidic Acid during Yeast Sporulation

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During yeast sporulation, internal membrane synthesis ensures that each haploid nucleus is packaged into a spore. Prospore membrane formation requires Spo14p, a phosphatidylinositol 4,5-*bis*phosphate [PtdIns(4,5)P_2]-stimulated phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PtdCho) to phosphatidic acid (PtdOH) and choline. We found that both meiosis and spore formation also require the phosphatidylinositol (PtdIns)/PtdCho transport protein Sec14p. Specific ablation of the PtdIns transport activity of Sec14p was sufficient to impair spore formation but not meiosis. Overexpression of Pik1p, a PtdIns 4-kinase, suppressed the *sec14-1* meiosis and spore formation defects; conversely, *pik1-ts* diploids failed to undergo meiosis and spore formation. The PtdIns(4)P 5-kinase, Mss4p, also is essential for spore formation. Use of phosphoinositide-specific GFP-PH domain reporters confirmed that PtdIns(4,5)P_2 is enriched in prospore membranes. *sec14*, *pik1*, and *mss4* mutants displayed decreased Spo14p PLD activity, whereas absence of Spo14p did not affect phosphoinositide levels in vivo, suggesting that formation of PtdIns(4,5)P_2 is important for Spo14p activity. Spo14p-generated PtdOH appears to have an essential role in sporulation, because treatment of cells with 1-butanol, which supports Spo14p-catalyzed PtdCho breakdown but leads to production of Cho and Ptd-butanol, blocks spore formation at concentrations where the inert isomer, 2-butanol, has little effect. Thus, rather than a role for PtdOH in stimulating PtdIns(4,5)P_2 formation, our findings indicate that during sporulation, Spo14p-mediated PtdOH production functions downstream of Sec14p-, Pik1p-, and Mss4p-dependent PtdIns(4,5)P_2 synthesis.

INTRODUCTION

Sporulation in *Saccharomyces cerevisiae* is a form of cellular differentiation analogous to gametogenesis in metazoans. In sporulation, the chromosomes in a *MAT***a**/*MAT*α diploid are duplicated and then segregated, via the meiotic divisions, into four haploid sets, which are then packaged into spores (reviewed in Kupiec *et al.*, 1997). Spore formation requires synthesis of a membrane, known as the prospore membrane, around each haploid nucleus (Byers, 1981). Prospore membrane formation is thought to require rerouting of exocytic post-Golgi vesicles to the spindle pole bodies (SPBs; Neiman, 1998). These vesicles then fuse, initiating formation of the prospore membrane, which then spreads, by further vesicle fusion, until it surrounds each haploid nucleus. How the secretory apparatus is modified to carry out these membrane trafficking events is not well understood, although considerable evidence suggests that signaling by phospholipids is involved.

SPO14 (also known as *PLD1*) encodes the major phospholipase D (PLD) activity in yeast. Although additional PLD activities have been detected and can hydrolyze phosphatidylcholine (PtdCho) as well as other phospholipids (reviewed in Rudge and Engebrecht, 1999), Spo14p is the only yeast member of the highly conserved eukaryotic PtdCho-specific PLD family; these enzymes hydrolyze PtdCho to phosphatidic acid (PtdOH) and choline. Spo14p action is essential for the formation of the prospore membrane (Rudge *et al.*, 1998b) and for increased PtdOH synthesis during sporulation (Rudge *et al.*, 2001). In contrast, under normal physiological conditions, Spo14p is dispensable for secretion and hence is not essential for vegetative growth (Sreenivas *et al.*, 1998; Xie *et al.*, 1998). Thus, Spo14p plays a pivotal role in distinguishing between constitutive and developmentally regulated membrane trafficking events (Rudge *et al.*, 2001; Rudge *et al.*, 2002). However, the specific function of Spo14p action in prospore membrane biogenesis has not yet been elucidated.

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It has been amply demonstrated that mammalian PLD is potently stimulated by phosphatidylinositol 4,5-*bis*phosphate [PtdIns(4,5)P_2] (Brown *et al.*, 1995). Likewise, PtdIns(4,5)P_2 stimulates Spo14p in vitro (Rose *et al.*, 1995), and genetic analyses indicate that PtdIns(4,5)P_2 is essential for activating the function of Spo14p in vivo (Sciorra *et al.*, 1999, 2002). These observations suggest that PtdIns(4,5)P_2 generation is necessary to activate Spo14p for its role in prospore membrane formation. However, in mammalian cells, PtdOH also stimulates PtdIns(4,5)P_2 production because PtdOH activates certain PtdIns(4)P 5-kinase isoforms (Honda *et al.*, 1999). The sole PtdIns(4)P 5-kinase in *S. cerevisiae*, Mss4p, is highly homologous to its mammalian counterparts (Desriviéres *et al.*, 1998; Homma *et al.*, 1998), and there is some evidence that it is also stimulated by PtdOH (Homma *et al.*, 1998). Hence it is possible that PtdIns(4,5)P_2 is a lipid critical for prospore membrane biogenesis and spore coat deposition, in analogy to the demonstrated role of phosphoinositides in serving as recognition determinants for the recruitment of many classes of proteins to biological membranes (Cullen *et al.*, 2001; Thorner, 2001) and that the essential role of Spo14p is to generate PtdOH to ensure that PtdIns(4,5)P_2 synthesis occurs at a sufficiently high rate. Thus, central to understanding Spo14p/PLD function is delineating the relationship between PtdOH and PtdIns(4,5)P_2 in vivo.

Exit of secretory proteins from the Golgi requires the function of Sec14p, a PtdIns/PtdCho transfer protein (Bankaitis *et al.*, 1989, 1990), and Pik1p, a PtdIns 4-kinase, which phosphorylates PtdIns to synthesize PtdIns(4)P (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). Consequently, *SEC14* and *PIK1* are essential genes in vegetatively growing cells. A second PtdIns 4-kinase, Stt4p (Yoshida *et al.*, 1994), generates a distinct and essential pool of PtdIns(4)P (Audhya *et al.*, 2000), whereas a third PtdIns 4-kinase (Lsb6p) has no essential function and is not required for sporulation (Han *et al.*, 2002). There is substantial evidence that Sec14p contributes significantly to the pool of PtdIns that is converted to PtdIns(4)P in the Golgi via the action of Pik1p (Hama *et al.*, 1999; Rivas *et al.*, 1999).

It has been postulated that phosphatidylinositol transfer proteins (PITPs) such as Sec14p stimulate PLD function by promoting synthesis of PtdIns(4,5)P_2 (Liscovitch and Cantley, 1995). Consistent with this suggestion, increased expression of Shf2p and Shf4p, yeast PITPs that display PtdIns- but not PtdCho- transfer activity in vitro, can rescue the growth and secretory defects of *sec14* mutants and requires the function of Spo14p to do so (Li *et al.*, 2000). However, Sec14p itself exhibits both PtdCho and PtdIns transfer activity in vitro (Bankaitis *et al.*, 1990), and the results of genetic analyses have not supported a model in which the essential vegetative function of Sec14p is to provide for activation of Spo14p (Sreenivas *et al.*, 1998; Xie *et al.*, 1998), because unlike *sec14*Δ mutants, *spo14*Δ mutants are viable (Honigberg *et al.*, 1992; Rose *et al.*, 1995). Moreover, homozygous *sfh2*Δ and *sfh4*Δ diploids sporulate at wild-type frequencies (Rabitsch *et al.*, 2001). Because Spo14p does have an essential function in sporulation, we wanted to determine if, under these circumstances, Sec14p might be required to supply the PtdIns necessary for PtdIns(4,5)P_2 production and Spo14p/PLD activation. Our analysis supports a model whereby Spo14p-generated PtdOH acts downstream of Sec14p-, Pik1p-, and Mss4p-dependent PtdIns(4,5)P_2 synthesis during sporulation.

MATERIALS AND METHODS

Yeast Strains and Media

Routine growth and manipulation of *S. cerevisiae* strains were performed as described by Rose *et al.* (1990). Yeast strains used in this study are listed in Table 1. CTY159 (*sec14-1^{ts}*) (obtained from Vytas Bankaitis, University of North Carolina, Chapel Hill; Xie *et al.*, 1998), YES47 (*pik1-11*) and YES102 (*pik1-83*) (Hendricks *et al.*, 1999), and SD102 (*mss4-2*) (obtained from Michael Hall, University of Basel; Desrivieres *et al.*, 1998) were backcrossed six times against the SK-1 strain background (Fast, 1973). Pairs of haploid segregants of opposite mating type from the final backcrosses were mated to generate homozygous *sec14-1* (Y3242), *pik1-11* (Y3637), *pik1-83* (Y3732), and *mss4-2* (Y4350) diploids. Derivatives of these strains were used for the experiments described here (Table 1).

DNA-mediated transformation of yeast cells used the lithium acetate procedure (Ito *et al.*, 1983). Gene replacement and disruptions (see below) were performed by the one-step method (Rothstein, 1983) and were confirmed using the PCR and appropriate synthetic oligonucleotide primers.

Plasmids

Low-copy number (*CEN*) plasmids (pUN series; Elledge and Davis, 1988) expressing wild-type *SPO14* (pME1761) or the mutant allele *spo14-S11* (pME1753) are described in detail elsewhere (Rudge *et al.*, 2001). pKR577 (*URA3*) and pME936 (*TRP1*) are high-copy number plasmids (2 μ) containing *SPO14* (Rose *et al.*, 1995). *SPO14* tagged with the influenza virus hemagglutinin (HA) epitope and expressed from its own promoter (pME940) is fully functional and was characterized previously (Rudge *et al.*, 1998b). pKR466 (*spo14::URA3*, which removes 4014 base pairs of the 5052-base pair ORF) and pME913 (*spo14::LEU2*, which removes 4187 base pairs) were used to generate chromosomal *spo14* disruption/deletions and have been described (Rose *et al.*, 1995; Rudge *et al.*, 1998a).

pME1878 was isolated by complementation of the *sec14-1* mutation from a genomic library in a *CEN* vector (Rose *et al.*, 1987) and contains *SEC14* coding sequences. Site-directed mutagenesis was performed to generate the double mutant *sec14(K66A K239A)* (pME1946), using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. The mutagenic primers were identical to those described by Phillips *et al.* (1999). *SEC14* and *sec14(K66A K239A)* were each subcloned into a multicopy (2 μ DNA-based) yeast vector, YEp352 (Hill *et al.*, 1986), as ~3.5-kb *Xba*I-*Hin*dIII fragments to generate pME1881 and pME2062, respectively.

The *Bam*HI-*Sac*I fragment containing the *PIK1* gene from pRS314*PIK1* (Flanagan *et al.*, 1993) was moved into the corresponding sites of pUN55 (Elledge and Davis, 1988) to create pME1963. The *Bam*HI-*Xho*I fragment containing the *MSS4* gene from pYO1966 (Homma *et al.*, 1998; generous gift of Yoshikazu Ohya, University of Tokyo, Tokyo, Japan) was moved into the *Bam*HI and *Xho*I sites of pUN55 and YEp352 to generate pME2188 and pME1308, respectively. The *Cla*I−, whose ends had been filled in with the Klenow fragment of DNA polymerase, *Sac*I fragment of *PDR17/SFH4* from pBVH1402 (van den Hazel *et al.*, 1999; generous gift of Maria Adelaide do Valle Matta, Universite Catholique de Louvain, Louvain-la-Neuve, Belgium), was moved into the *Sma*I and *Sac*I sites of YEp352 to generate pME1854. YEp352 *PIK1* (Flanagan *et al.*, 1993), pGALHA-STT4–8 (Cutler *et al.*, 1997; generous gift of Maria Cardenas, Duke University Medical Center, Durham, NC), and YEp24-*CSR1/SFH2* (Santos and Snyder, 2000; generous gift of Beatriz Santos, Universidad de Salamanca, Salamanca, Spain) are all described in detail in the references cited. pRE352 (*kes1::URA3*, which removes the N-terminal 262 amino acids of Kes1p; Fang *et al.*, 1996), and pME1827 (*cki1::LEU2*, which removes 757 base pairs in the middle of the 1749-base pair ORF; Rudge *et al.*, 2001) were used to generate chromosomal disruption/deletions of *kes1* and *cki1*, respectively. Finally, plasmids encoding in vivo phosphoinositide-specific reporters, pRS426GFP-PH(FAPP1) and pRS426GFP-2xPH(PLCδ), are described in detail elsewhere (Stefan *et al.*, 2002; generous gift of Scott Emr and Christopher Stefan, University of California at San Diego, La Jolla, CA).

Analysis of Meiosis and Sporulation

Cells were grown in YP-acetate medium and induced to undergo sporulation as previously described (Krisak *et al.*, 1994). In experiments in which *sec14*, *pik1*, or *mss4* conditional mutants were shifted to restrictive temperature, the culture was induced for 1 h at room temperature to ensure initiation of the sporulation program before the shift to 34.5°C. To drive expression of pGALHA-STT4, 0.03% galactose was added to the sporulation medium (McCarroll and Esposito, 1994). For analysis of meiotic division, samples from triplicate cultures were removed and fixed with 3.7% formaldehyde. Fixed cells were then stained with 4′6-diamindino-2-phenyl-indole (DAPI), examined under a fluorescence microscope, and scored as described before (Rose *et al.*, 1995). For each time point, a minimum of 300 cells was examined from each of three independent cultures. The frequency of spore formation was monitored as the formation of two-, three-, or four-spored asci by phase microscopy; a minimum of 600 cells from each culture was examined.

Table 1. Genotypes of yeast strains

Strain	Genotype
NH144[a]	*MATa leu2-k HIS4 ho :: LYS2 ura3 lys2 arg4-Nsp* / *MATα leu2 :: hisG his4-x ho :: LYS2 ura3 lys2 ARG4*
Y433	NH144 homozygous *spo14::URA3*
Y598	Y433, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4048	NH144, plus pRS426 GFP-PH(FAPP1)
Y4049	NH144, plus pRS426 GFP-2xPH(PLCδ)
Y3242	*MATa leu2 his4 sec14-1 trp1 :: hisG ura3 ho :: LYS2 HIS3* / *MATα leu2 HIS4 sec14-1 trp1 :: hisG ura3 ho :: LYS2 Δhis3-200*
Y3247	Y3242, plus pUN55 (*URA3 CEN4*)
Y3248	Y3242, plus pME1878 (*SEC14 URA3 CEN4*)
Y3351	Y3242 homozygous *kes1::URA3*
Y3352	Y3242 homozygous *cki1::LEU2*
Y3404	Y3242, plus Yep24-*CSR1/SFH2* (*SFH2 URA3* 2μ)
Y3408	Y3242, plus pME1308 (*MSS4 URA3* 2μ)
Y3437	Y3351 homozygous *spo14::LEU2*
Y3449	Y3437, plus pUN15 (*TRP1 CEN4*)
Y3450	Y3437, plus pME1753 (*spo14S-11 TRP1 CEN4*)
Y3451	Y3437, plus pME1761 (*SPO14 TRP1 CEN4*)
Y3528	Y3242, plus pME1946 (*sec14(K66A K239A) URA3 CEN4*)
Y3553	Y3242, plus pME2062 (*sec14(K66A K239A) URA3* 2μ)
Y3587	Y3242, plus pGALHA-STT4-8 (*HA-STT4 URA3* 2μ)
Y3645	Y3242, plus pME1854 (*SFH4 URA3* 2μ)
Y3663	Y3242, plus YEp352 *PIK1* (*PIK1 URA3* 2μ)
Y3753	Y3242 homozygous *spo14::LEU2*
Y3756	Y3753, plus pUN55 (*URA3 CEN4*)
Y3757	Y3753, plus pME1878 (*SEC14 URA3 CEN4*)
Y4278	Y3247, plus pUN105 (*LEU2 CEN4*)
Y4279	Y3247, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4281	Y3248, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4297	Y3528, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4509	Y3753, plus pME1854 (*SFH4 URA3* 2μ)
Y4510	Y3753, plus YEp352 *PIK1* (*PIK1 URA3* 2μ)
Y4513	Y3528, plus pME936 (*SPO14 TRP1* 2μ)
Y4514	Y3528, plus pYO1966 (*MSS4 TRP1* 2μ)
Y4515	Y3753, plus Yep24-*CSR1/SFH2* (*SFH2 URA3* 2μ)
Y315[b]	*MATa leu2-27his4-260 ura3-1 trp1-1 ADE2 thr1-4 lys2* / *MATα leu2-3,112his4-280 ura3-1 trp1-1 ade2 THR1 lys2*
Y1483	Y315 homozygous *kes1::URA3*
Y3537	Y1483 homozygous *sec14::LEU2*
Y1838	Y315 homozygous *spo14-S11*
Y1925	Y315 homozygous *spo14::LEU2*
Y3825	Y1838, plus YEp352 (*URA3* 2μ) and pME856 (*TRP1* 2μ)
Y3826	Y1838, plus Yep352 (*URA3* 2μ) and pYO1966 (*MSS4 TRP1* 2μ)
Y3827	Y1838, plus YEp352 *PIK1* (*PIK1 URA3* 2μ) and pME856 (*TRP1* 2μ)
Y3828	Y1838, plus YEp352 *PIK1* (*PIK1 URA3* 2μ) and pYO1966 (*MSS4 TRP1* 2μ)
Y4516	Y1925, plus YEp352 (*URA3* 2μ) and pME856 (*TRP1* 2μ)
Y4517	Y1925, plus YEp352 *PIK1* (*PIK1 URA3* 2μ) and pME856 (*TRP1* 2μ)
Y4518	Y1925, plus pGALHA-STT4-8 (*HA-STT4 URA3* 2μ) and pME856 (*TRP1* 2μ)
Y4519	Y1925, plus Yep352 (*URA3* 2μ) and pYO1966 (*MSS4 TRP1* 2μ)
Y4520	Y1925, plus YEp352 *PIK1* (*PIK1 URA3* 2μ) and pYO1966 (*MSS4 TRP1* 2μ)
Y4521	Y1925, plus pGALHA-STT4-8 (*HA-STT4 URA3* 2μ) and pYO1966 (*MSS4 TRP1* 2μ)
Y4522	Y1838, plus pGALHA-STT4-8 (*HA-STT4 URA3* 2μ) and pME856 (*TRP1* 2μ)
Y4523	Y1838, plus pGALHA-STT4-8 (*HA-STT4 URA3* 2μ) and pYO1966 (*MSS4 TRP1* 2μ)
Y3637	*MATa leu2 his4 ura3 ho :: LYS2 pik1-11* / *MAT α leu2 HIS4 ura3 ho :: LYS2 pik1-11*
Y3654	Y3637, plus pUN55 (*URA3 CEN4*)
Y3655	Y3637, plus pME1963 (*PIK1 URA3 CEN4*)
Y3890	Y3637 homozygous *spo14::LEU2*
Y4076	Y3637, plus pME1881 (*SEC14 URA3* 2μ)
Y4077	Y3637, plus pKR577 (*SPO14 URA3* 2μ)
Y4288	Y3654, plus pUN105 (*LEU2 CEN4*)
Y4289	Y3654, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4291	Y3655, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y3732	*MATa leu2 his4 ura3 ho :: LYS2 pik1-83* / *MAT α leu2 his4 ura3 ho :: LYS2 pik1-83*
Y3723	Y3732, plus pUN55 (*URA3 CEN4*)
Y3724	Y3732, plus pME1963 (*PIK1 URA3 CEN4*)
Y3907	Y3732 homozygous *spo14::LEU2*
Y4078	Y3732, plus pME1881 (*SEC14 URA3* 2μ)
Y4079	Y3732, plus pKR577 (*SPO14 URA3* 2μ)
Y4284	Y3723, plus pUN105 (*LEU2 CEN4*)
Y4285	Y3723, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4287	Y3724, plus pME940 (*HA-SPO14 LEU2 CEN4*)
Y4350	*MATa leu2 trp1 ura3 mss4 :: HIS3MX6 arg4-Nsp* / *MAT α leu2 TRP1 ura3 mss4 :: HIS3MX6 arg4-Nsp* plus *YCplac111::mss4-2^{ts}* (*LEU2 CEN*)
Y4354	Y4350, plus pUN55 (*URA3 CEN4*)
Y4355	Y4350, plus pME2188 (*MSS4 URA3 CEN4*)
Y4359	Y4350, plus pKR577 (*SPO14 URA3* 2μ)
Y4360	Y4350, plus YEp352 *PIK1* (*PIK1 URA3* 2μ)
Y4361	Y4350, plus pME1881 (*SEC14 URA3* 2μ)
Y4409	Y4350 homozygous *spo14::URA3*

[a] Hollingsworth *et al.* (1995); unless otherwise indicated, all strains are congenic to this SK-1 derivative.

[b] Engebrecht *et al.* (1998); derived from S288C.

In Vivo BODIPY-PtdCho Analysis

BODIPY-PtdCho (4 μM final concentration; Molecular Probes, Eugene, OR) was added directly to cultures (~2×10^8 cells) 4 h after induction of sporulation (and thus 3 h after shift to 34.5°C). Cells were harvested 3 h later, and lipids were extracted as previously described (Rudge *et al.*, 2001). All assays were performed in triplicate. The percentage conversion of intracellular BODIPY-PtdCho to BODIPY-PtdOH was determined from arbitrary fluorescence 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 were recorded using ImageQuant 5.1 software (Molecular Dynamics). The amount of BODIPY-PtdOH measured was an underestimation, because Spo14p-derived BODIPY-PtdOH was further metabolized to lyso-BODIPY-PtdOH and BODIPY-diacylglycerol (Rudge *et al.*, 2001).

Immunoblot Analysis

Cell extracts were prepared as described (Rudge *et al.*, 2001). A total of 10 μg of each protein extract was subjected to SDS-PAGE on 5% SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45 μm; Bio-Rad Laboratories, Hercules, CA), and antibody detection was performed as previously described (Rudge *et al.*, 1998b) using enhanced chemiluminescence detection on preflashed film.

Assays of PLD Activity in Immunoprecipitates

To measure PLD activity in vitro, HA-Spo14p was expressed and immunoprecipitated from lysates of sporulating cells using the mAb 12CA5 (BabCO, Richmond, CA) as described previously (Rudge *et al.*, 1998b). PLD activity was then measured using vesicles containing BODIPY-PtdCho as the substrate, and the product BODIPY-PtdOH was quantitated as a percentage of the input BODIPY-PtdCho, as described before (Rudge *et al.*, 1998b). At least two independent trials of all assays, each performed in triplicate, were carried out.

Fluoresence Microscopy

To visualize the distribution of PtdIns(4)P and PtdIns(4,5)P_2 in live cells in real time during sporulation, diploids expressing GFP-2xPH(PLCδ) or GFP-PH(FAPP1) were induced to sporulate, concentrated, and examined by fluorescence microscopy using an Axiovert S1002TV inverted fluorescence microscope (Carl Zeiss, Thornwood, NY). Images were acquired and stored digitally using a Delta Vision deconvolution system (Applied Precision, Seattle, WA).

Table 2. Meiotic progression and spore formation by *sec14* mutants

Strain	Relevant genotype[a]	% Meiotic division[b]	% Spore formation[c]	
			25°C	34.5°C
1. Y3247	*sec14-1^{ts} + CEN*	3 ± 1	55 ± 5	0.2 ± 0.5
2. Y3248	*sec14-1^{ts} + SEC14 CEN*	75 ± 8	81 ± 12	71 ± 10
3. Y3757	*sec14-1^{ts} spo14Δ + SEC14 CEN*	42 ± 5	<0.1	<0.1
4. Y3756	*sec14-1^{ts} spo14Δ + CEN*	2 ± 1	<0.1	<0.1
5. Y3351	*sec14-1^{ts} kes1Δ*	62 ± 6	75 ± 12	55 ± 8
6. Y3352	*sec14-1^{ts} cki1 Δ*	45 ± 8	73 ± 9	41 ± 7
7. Y3449	*sec14-1^{ts} kes1Δ spo14Δ + CEN*	4 ± 2	<0.1	<0.1
8. Y3451	*sec14-1^{ts} kes1Δ spo14Δ + SPO14 CEN*	58 ± 9	76 ± 12	56 ± 6
9. Y315[d]	*SEC14 KES1*	65 ± 6	59± 4	
10. Y1483[d]	*SEC14 kes1Δ*	68 ± 6	63± 6	
11. Y3537[d]	*sec14Δ kes1Δ*	63 ± 5	55± 5	
12. Y3450	*sec14-1^{ts} kes1Δspo14Δ + spo14S-11 CEN*	37 ± 4	<0.1	<0.1
13. Y3528	*sec14-1^{ts} + sec14(K66A K239A)CEN*	48 ± 8	59 ± 7	5 ± 1
14. Y3553	*sec14-1^{ts} + sec14(K66A K239A) 2μ*	68 ± 7	77 ± 1	64 ± 5
15. Y3404	*sec14-1^{ts} + SFH2 2μ*	ND	78 ± 5	65 ± 3
16. Y4515	*sec14-1^{ts} spo14Δ + SFH2 2μ*	2 ± 1	<0.1	<0.1
17. Y3645	*sec14-1^{ts} + SFH4 2μ*	ND	75 ± 4	56 ± 4
18. Y4509	*sec14-1^{ts} spo14Δ + SFH4 2μ*	3 ± 1	<0.1	<0.1
19. Y3663	*sec14-1^{ts} + PIK1 2μ*	52 ± 6	77 ± 6	52 ± 4
20. Y4510	*sec14-1^{ts} spo14Δ + PIK1 2μ*	3 ± 2	<0.1	<0.1
21. Y3587	*sec14-1^{ts} + STT4 2μ*	6 ± 2	55 ± 5	<0.1
22. Y3408	*sec14-1^{ts} + MSS4 2μ*	2 ± 1	55 ± 5	<0.1
23. Y4514	*sec14-1^{ts} + sec14(K66A K239A)CEN + MSS4 2μ*	ND	62 ± 6	4 ± 1
24. Y4513	*sec14-1^{ts} + sec14(K66A K239A)CEN + SPO14 2μ*	ND	58 ± 7	6 ± 2

[a] *CEN* refers to a low-copy-number plasmid, whereas 2μ refers to a high-copy-number plasmid.

[b] The meiotic divisions at 34.5°C were monitored by DAPI fluorescence (see MATERIALS AND METHODS); cells that had completed Meiosis I (2 DAPI-staining bodies) or Meiosis II (3 or 4 DAPI-staining bodies) were scored. No significant differences in success in completing Meiosis I vs. Meiosis II were observed. Values are means ± SD.

[c] Frequencies of spore formation at the indicated temperatures were determined as described in MATERIALS AND METHODS; values are means ± SD.

[d] S288C strain background. Meiosis and spore formation were monitored at 30°C because strains of this background do not sporulate well at 34.5°C.

In Vivo Phosphoinositide Analysis

Analysis of phosphoinositide levels in wild-type and homozygous *spo14* diploids was performed by labeling cells with 100 μCi myo-[2-^{3}H]inositol (Nycomed Amersham, Princeton, NJ) for 6 h after transfer to sporulation medium. Cells were harvested by centrifugation and resuspended in 1 ml ice-cold 4.5% perchloric acid (Whiteford *et al.*, 1996). After 15 min on ice, 0.5 g of acid-washed glass beads were added and the cells were lysed by vortexing for 5 min at maximum speed. The cell extracts were centrifuged at 14,000 rpm for 10 min at 4°C. The pellets were washed with 1 ml of 100 mM EDTA, centrifuged as described above, and resuspended in 50 μl of sterile distilled deionized water.

The phospholipids contained within each cell pellet were deacylated by treatment with methylamine (Hawkins *et al.*, 1986). Briefly, 0.5 ml of methylamine reagent (10.7% methylamine, 45.7% methanol, 11.4% 1-butanol) was added to each cell pellet and samples were incubated in a 53°C heat block for 50 min. Unreacted methylamine was then removed in vacuo, and the dried pellet was resuspended in 300 μl of sterile water. After a second sequence of drying in vacuo and resuspension in 300 μl of sterile water, an equal volume of 1-butanol/ethyl ether/formic acid ethyl ester (20:4:1) was added to the pellet suspension. Each sample was vortexed for 5 min and centrifuged at 14,000 rpm for 2 min. The aqueous phase containing the [^{3}H]glycero-phosphoinositides was transferred to a new tube, and the extraction was repeated once more with 1-butanol/ethyl ether/formic acid ethyl ester (20:4:1). Finally, the aqueous phase was collected and dried in vacuo.

Samples were resuspended in sterile water, and equal cpm quantities of wild-type and *spo14* diploid [^{3}H]glycero-phosphoinositides were analyzed using an anion exchange PartisphereSAX (Whatman Inc., Clifton, NJ) column coupled to a System Gold HPLC (Beckman Coulter, Fullerton, CA) system and an on-line radiomatic detector (Packard Instruments, Meriden, CT). The column was developed with a gradient of 1 M $(NH_4)_2HPO_4$, pH 3.8 (pH adjusted with phosphoric acid): 1% for 5 min, 1–20% >44 min, 20–50% >3.75 min, and 50% for 8 min more; the flow rate used was 1.0 ml/min (Stack *et al.*, 1995).

The total cpm values of the [^{3}H]glycero-phosphoinositides extracted from wild-type and *spo14* diploids were comparable, and for comparison of the levels of the individual species within each sample, the raw cpms in each peak were expressed as a percentage of the total cpms eluted.

RESULTS

Sec14p Is Required for Meiosis and Spore Formation

To determine if Sec14p might play a role in delivering lipids that contribute to the ability of Spo14p to carry out its essential function during sporulation, we generated a *sec14-1/sec14-1* homozygous diploid in the SK-1 strain background, in which sporulation is ordinarily rapid and efficient, even at high temperatures (Fast, 1973). This strain was sporulation proficient at the permissive temperature (25°C) but was unable to form spores at the restrictive temperature of 34.5°C (Table 2, line 1). This defect was due solely to the lack of Sec14p function, because it was fully rescued by a *SEC14* plasmid (Table 2, line 2). To determine the stage at which sporulation stalled, we monitored the meiotic divisions. In the absence of Sec14p function, very few cells completed the meiotic divisions (Table 2, lines 1 and 2). In contrast, a *spo14* mutant, although totally unable to form spores, did undergo the meiotic divisions, albeit with reduced efficiency (Table 2, line 3; Honigberg *et al.*, 1992; Rose *et al.*, 1995). As expected, a *sec14-1 spo14* double mutant was severely defective in both meiosis and spore formation (Ta-

ble 2, line 4). These data indicate that the Sec14p-dependent processes required for sporulation are not all mediated by Spo14p.

The failure of the *sec14-1* cells to undergo the meiotic divisions might mean that meiosis was not initiated in these strains. To address this issue, we examined the expression of *HOP1*, a gene that is not expressed in vegetative cells but is specifically induced in prophase of Meiosis I (Hollingsworth *et al.*, 1990; Vershon *et al.*, 1992). Using a plasmid-borne *HOP1-lacZ* gene fusion as a reporter (Vershon *et al.*, 1992), we found that no activity was expressed in vegetatively growing *sec14-1* cells, as expected, but that expression was induced upon transfer to sporulation medium at both permissive (25°C) and restrictive (34.5°C) temperatures (unpublished data). Thus, the *sec14-1* cells do appear to initiate the sporulation program.

The Requirement for Sec14p in Meiosis Can Be Bypassed

In vegetative cells, the requirement for Sec14p can be alleviated by the inactivation of any of several genes, including *CKI1,* an enzyme in the CDP-choline pathway for PtdCho biosynthesis (Cleves *et al.*, 1991), and *KES1*, a PtdIns-binding protein that antagonizes Sec14p function (Li *et al.*, 2002). This phenomenon is referred to as "Sec14 bypass" (Cleves *et al.*, 1991); under these circumstances, Spo14p is essential (Sreenivas *et al.*, 1998; Xie *et al.*, 1998; Li *et al.*, 2002). To determine whether the requirement for Sec14p in sporulation could also be relieved by such mutations, we constructed the appropriate double mutants. The *sec14-1 kes1*Δ and the *sec14-1 cki1*Δ cells underwent meiosis and sporulated at essentially wild-type efficiency (Table 2, lines 5–6), and, as in vegetative cells, this suppression required the presence of functional Spo14p (Table 2, lines 7–8; unpublished data). Because the 34.5°C used in the sporulation experiments is close to the minimum restrictive temperature for the *sec14-1* allele (Bankaitis *et al.*, 1989), it seemed possible that *kes1* and *cki1* mutations permit *sec14-1* diploids to sporulate simply by reducing the severity of the *sec14-1* allele. However, a *sec14*Δ *kes1*Δ strain was both viable, as reported previously (Cleves *et al.*, 1991), and sporulated well (Table 2, lines 9–11).

We previously identified the *spo14S* (*S* for separation-of-function) mutant alleles, which permit vegetative growth of *sec14* cells carrying bypass mutations but fail to support spore formation in an otherwise wild-type background (Rudge *et al.*, 2001). When these *spo14S* alleles were expressed as the sole source of Spo14p in *sec14-1 kes1*Δcells, they again were unable to support spore formation (Table 2, line 12; our unpublished results). However, these strains were able to complete meiosis with reasonable efficiency. Thus, these findings suggest that Sec14p, or under Sec14-bypass conditions Spo14p, is adequate for supporting meiosis, but that Spo14p function satisfies an additional requirement in prospore membrane formation.

PtdIns Binding/Transport Activity of Sec14p is Required for Maximal Spo14p Activity and for Spore Formation

To determine whether inactivation of Sec14p had any effect on Spo14p function in sporulating cells, we monitored Spo14p PLD activity by measuring conversion of internalized BODIPY-PtdCho to BODIPY-PtdOH. As previously reported, hydrolysis of BODIPY-PtdCho occurs almost exclusively by the action of Spo14p, as *spo14*Δ strains did not generate any appreciable BODIPY-PtdOH (Table 3, lines 1–2; Rudge *et al.*, 2001). Inactivation of Sec14p resulted in a decrease in Spo14p-dependent generation of BODIPY-PtdOH (Table 3, lines 3–4). This did not appear to be due to reduced synthesis of Spo14p, because steady state levels of HA-Spo14p were not significantly altered in the *sec14-1* mutant (Figure 1; lanes 2 and 4). Thus, in contrast to what is observed in vegetative cells, where Spo14p activity is stimulated in the absence of Sec14p (Sreenivas *et al.*, 1998), Spo14p activity in sporulating cells appears to be Sec14p dependent. From these data alone, it is not clear whether this effect reflects a direct role of Sec14p in promoting Spo14p activity or the early blockage of the sporulation program (at a point before Spo14p activity is maximal) by the *sec14-1* lesion.

Table 3. Spo14p-catalyzed hydrolysis of internalized BODIPY-PtdCho[a]

Strain	Relevant genotype	% BODIPY-PtdOH
1. Y3757	*spo14*Δ	0.41 ± 0.05
2. Y3248	*SPO14 SEC14*	5.1 ± 0.7
3. Y3247	*sec14-1*	2.0 ± 0.5
4. Y3756	*sec14-1 spo14* Δ	0.51 ± 0.10
5. Y3528	*sec14(K66A K239A)*	1.5 ± 0.2
6. Y4283	*sec14(K66A K239A) spo14* Δ	0.43 ± 0.08
7. Y3655	*PIK1*	5.0 ± 0.6
8. Y3654	*pik1-11*	2.0 ± 0.4
9. Y3890	*pik1-11 spo14*Δ	0.48 ± 0.10
10. Y3724	*PIK1*	5.9 ± 0.7
11. Y3723	*pik1-83*	1.9 ± 0.1
12. Y3907	*pik1-83 spo14*Δ	0.45 ± 0.08
13. Y4355	*MSS4*	5.3 ± 1.0
14. Y4354	*mss4-2*	1.9 ± 0.4
15. Y4409	*mss4 spo14*Δ	0.43 ± 0.10

[a] The percentage conversion of intracellular BODIPY-PtdCho to BODIPY-PtdOH was determined in cells sporulating at 34.5°C as described in MATERIALS AND METHODS. Values are means ± SD from three independent experiments.

Sec14p possesses both PtdCho and PtdIns transfer activity (Bankaitis *et al.*, 1990). Thus, the reduction in Spo14p activity observed in *sec14-1* cells could be due to the loss of Sec14p-dependent transport of the substrate, PtdCho, to the cellular compartment(s) where Spo14p functions, to loss of Sec14p-dependent transport of PtdIns and the consequent failure to synthesize the PLD activator PtdIns(4,5)P_2, or both. To explore the relative contributions of PtdIns and PtdCho binding/transport by Sec14p, we took advantage of a mutant, *sec14(K66A K239A*), that retains the ability to bind and transport PtdCho but is specifically defective in PtdIns binding and transport (Phillips *et al.*, 1999). This mutant supports the

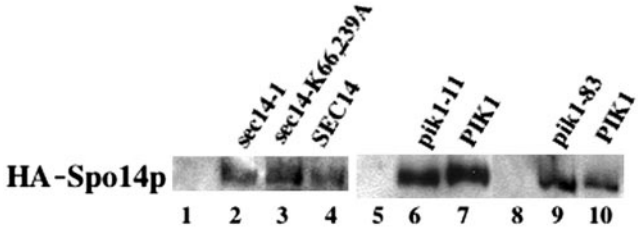

Figure 1. HA-Spo14p in *sec14* and *pik1* mutants. Immunoblot of HA-Spo14p in (1) *sec14-1* (Y4278); (2) *sec14-1* + *HA-SPO14* (Y4279); (3) *sec14(K66A K239A)* + *HA-SPO14* (Y4297); (4) *SEC14* + *HA-SPO14* (Y4281); (5) *pik1-11* (Y4288); (6) *pik1-11* + *HA-SPO14* (Y4289); (7) *PIK1* + HA-SPO14 (Y4291); (8) *pik1-83* (Y4284); (9) *pik1-83* + *HA-SPO14* (Y4285); and (10) *PIK1* + *HA-SPO14* (Y4287).

essential function of Sec14p for growth and secretion in vegetative cells (Phillips *et al.*, 1999). However, when expressed in *sec14-1* cells at near-normal levels from a low-copy-number plasmid, this mutant supported only very low levels of spore formation at the nonpermissive temperature (Table 2, line 13). Remarkably, however, the mutant restored the ability to complete meiosis to 64% of the level seen in cells expressing wild-type *SEC14* (Table 2, lines 2 and 13). These observations suggest that Sec14p function (and its PtdIns-transport role specifically) plays an essential role in spore formation that is independent of the Sec14p role in meiosis.

Moreover, when we assayed Spo14p activity in the *sec14(K66A K239A)* mutant, it was reduced as in the *sec14-1* strain (Table 3, lines 5 and 6), even though the *sec14(K66A K239A)* mutation also did not appear to affect Spo14p protein levels (Figure 1, lanes 3 and 4). These data suggest strongly that the reduction in Spo14p activity in the absence of Sec14p function does not simply reflect an early block in the sporulation program. Instead, the PtdIns transport activity of Sec14p appears to be necessary to obtain maximal Spo14p activity, perhaps explaining, at least in part, its importance for spore formation. A plausible hypothesis is that PtdIns transport by Sec14p is necessary for maximal synthesis of the Spo14p activator PtdIns(4,5)P_2.

The observation that *sec14(K66A K239A)* increased the sporulation proficiency of a *sec14-1* strain significantly (from ~0.2 to ~5%) might mean that the mutant protein possesses residual PtdIns transport activity and/or the delivery or removal of PtdCho to or from a membrane compartment also contributes to the efficiency of sporulation. Consistent with either alternative, expression of *sec14(K66A K239A)* from a high-copy-number plasmid increased sporulation proficiency to near normal levels at both permissive and restrictive temperatures (Table 2, line 14).

Suppression of sec14 Sporulation Defect by Overexpression of Sfh2p, Sfh4p, or Pik1p, but not Mss4p or Spo14p

A number of Sec14p homologues exist in yeast that possess PtdIns, but not PtdCho, transport activities. In vegetative cells, two of these, Sfh2/Csr1p (Li *et al.*, 2000; Santos and Snyder, 2000) and Sfh4/Pdr17p (van den Hazel *et al.*, 1999; Li *et al.*, 2000), potently suppress the *sec14-1* lethality when overexpressed, and this suppression is dependent on Spo14p (Li *et al.*, 2000). Likewise, these proteins suppressed the *sec14-1* meiosis and spore-formation defects (Table 2, lines 15 and 17), and the suppression of the meiotic defect was dependent on Spo14p function (Table 2, lines 16 and 18). These results support the conclusions that Spo14p can compensate for loss of Sec14p function in meiosis and that PtdIns transport is important for spore formation.

In vegetative *sec14(K66A K239A)* cells, the synthesis of PtdIns(4)P is reduced (Phillips *et al.*, 1999), and overexpression of the PtdIns 4-kinase Pik1p suppresses the growth defect of *sec14-1* cells at semirestrictive temperature (Hama *et al.*, 1999). Similarly, we found that overexpression of *PIK1* could restore the sporulation of *sec14-1* diploids to near wild-type frequency, and suppression of the meiotic defect was dependent on Spo14p (Table 2, lines 19–20). In contrast, overexpression of the other PtdIns 4-kinase isoform, Stt4p, which supplies PtdIns(4)P primarily at the plasma membrane (Audhya *et al.*, 2000), was unable to suppress the sporulation defect of *sec14-1* cells (Table 2, line 21). These results support the hypothesis that PtdIns delivery is an essential function of Sec14p for spore formation and suggest that it is specifically the pool of PtdIns(4)P available at the Golgi that is critical.

In contrast, overexpression of the PtdIns(4)P 5-kinase Mss4p was unable to rescue the *sec14-1* or *sec14(K66A K239A)* sporulation defect (Table 2, lines 22–23). Superficially, these results suggest that the critical lipid for spore formation is PtdIns(4)P and not PtdIns(4,5)P_2. However, in the absence of an elevated supply of its immediate substrate, overexpressed Mss4p may be unable to generate a marked elevation in PtdIns(4,5)P_2, as suggested by the observations of Desrivières *et al.* (1998). We favor this interpretation because our other observations (see below) suggest that PtdIns(4,5)P_2 does have an important role(s) in spore formation. Alternatively, both PtdIns(4)P and PtdIns(4,5)P_2 could be important for sporulation.

Finally, overexpression of Spo14p was unable to rescue the *sec14(K66A K239A)* spore-formation defect (Table 2, line 24). This result suggests that when phosphoinositides are limiting, additional copies of Spo14p do not provide more PLD activity, that phosphoinositides play roles in spore formation in addition to activating Spo14p, or both.

PIK1 Function Is Required for Sporulation

Like *sec14-1* cells, diploids homozygous for either of two *pik1-ts* alleles (Hendricks *et al.*, 1999) were defective for both meiosis and spore formation at the nonpermissive temperature (Table 4, line 1, and unpublished data). These defects were fully rescued by a low-copy-number *PIK1* plasmid (Table 4, line 2). As expected, *pik1 spo14*Δ double mutants were severely defective in both meiosis and spore formation (Table 4, line 3). Consistent with the hypothesis that Sec14p provides precursor for Pik1p (see above), overexpression of *SEC14* could not suppress the *pik1* defect (Table 4, line 4). These observations suggest that PtdIns(4)P synthesis by Pik1p is required for meiosis for reasons independent of any possible PtdIns(4,5)P_2-mediated stimulation of Spo14p. Consistent with this conclusion, overexpression of *SPO14* also could not suppress the sporulation defect of *pik1-ts* cells (Table 4, line 5). Nonetheless, as measured by the BODIPY-PtdCho assay, Spo14p activity was reduced when Pik1p was inactivated (Table 3, lines 7–12), although Spo14p levels were not significantly altered (Figure 1, lanes 6–10). Thus, although Pik1p appears to have a role in meiosis that is independent of Spo14p, it also appears to be necessary for full activation of Spo14p.

PtdIns(4,5)P_2 Synthesis is Essential for Sporulation

If *sec14* and *pik1* mutants display decreased Spo14p activity because of failure to generate adequate levels of PtdIns(4,5)P_2, then the PtdIns4(P) 5-kinase Mss4p should also be required for spore formation and maximal Spo14p PLD activity. Indeed, an *mss4-2* strain was defective for spore formation at the nonpermissive temperature although many cells progressed through meiosis (Table 4, lines 6–7). As expected, deletion of *SPO14* eliminated the residual sporulation of the *mss4-2* mutant (Table 4; line 8).

Overexpression of *SEC14* or *PIK1* did not suppress the *mss4-2* defect (Table 4, lines 9–10), suggesting that increasing the Mss4p substrate, PtdIns(4)P, did not increase PtdIns(4,5)P_2 levels. Furthermore, as measured by the BODIPY-PtdCho assay, Spo14p activity was reduced when Mss4p was inactivated (Table 3, lines 13–15), suggesting that Mss4p function might be required for spore formation at least in part through activation of Spo14p. However, a complication for this simple model is that overexpression of *SPO14* did not suppress the *mss4-2* defect (Table 4, line 11).

Table 4. Meiotic progression and sporulation proficiency of *pik1* and *mss4* mutants[a]

Strain	Relevant genotype	Meiotic division[b]	% Spore formation[b]	
			25°C	34.5°C
1. Y3654	*pik1-11 + CEN*	4 ± 2	75 ± 3	1 ± 1
2. Y3655	*pik1-11 + PIK1 CEN*	72 ± 8	80 ± 5	72 ± 3
3. Y3890	*pik1-11 spo14Δ*	2 ± 1	<0.1	<0.1
4. Y4076	*pik1-11 + SEC14 2μ*	2 ± 1	75 ± 3	1 ± 1
5. Y4077	*pik1-11 + SPO14 2μ*	2 ± 1	75 ± 3	1 ± 1
6. Y4354	*mss4-2 + CEN*	30.3 ± 6.3	75 ± 3	8.3 ± 1.5
7. Y4355	*mss4-2 + MSS4 CEN*	80 ± 4.4	80 ± 5	72 ± 3
8. Y4409	*mss4-2 spo14Δ*	35 ± 5	<0.1	<0.1
9. Y4361	*mss4-2 + SEC14 2μ*	37 ± 5	75 ± 3	9.1 ± 1
10. Y4360	*mss4-2 + PIK1 2μ*	29 ± 5	75 ± 3	7.8 ± 1
11. Y4359	*mss4-2 + SPO14 2μ*	35 ± 5	79 ± 6	8.6 ± 1

[a] Data for *pik1-11* are shown; data for *pik1-83* were essentially identical.

[b] Meiotic divisions at 34.5°C and spore formation were monitored as described in Table 2.

PtdIns(4,5)P_2 Is a Specific Activator of the PLD Activity of Spo14p

Given the effects of the *sec14(K66A K239A)* and *pik1* mutations on spore formation and Spo14p activity, it seemed possible that PtdIns and/or PtdIns(4)P, in addition to PtdIns(4,5)P_2 (Rose *et al.*, 1995; Sciorra *et al.*, 1999, 2002), might directly modulate Spo14p activity. However, when epitope-tagged Spo14p was expressed in sporulating cells, immunoprecipitated, and assayed for PLD activity, only PtdIns(4,5)P_2 had a significant effect on PLD activity (Table 5). Thus, PtdIns and PtdIns(4)P would presumably need to be converted in the cell to PtdIns(4,5)P_2 to influence the PLD activity of Spo14p.

PtdIns(4,5)P_2 Localizes to the Prospore Membrane during Sporulation

To explore further the apparent requirement for PtdIns(4,5)P_2 during sporulation, we used fluorescent probes for localizing specific phosphoinositides to individual cellular membranes in yeast (Stefan *et al.*, 2002). In these probes, green fluorescent protein (GFP) is fused to pleckstrin homology (PH) domains specific for either PtdIns(4)P [PH(FAPPI)] (Dowler *et al.*, 2000) or PtdIns(4,5)P_2 [PH(PLCδ)] (Kavran *et al.*, 1998). In vegetatively growing cells, these probes indicate that PtdIns(4)P is enriched at the Golgi, whereas PtdIns(4,5)P_2 is concentrated at the plasma membrane (Figure 2, top panels; Levine and Munro, 2002; Stefan *et al.*, 2002). In sporulating cells, the PtdIns(4)P-specific probe decorated defined patches within each spore, similar to the pattern seen in vegetative cells (Figure 2, left panels). In striking contrast, the PtdIns(4,5)P_2-specific probe was confined essentially to the plasma membrane and/or prospore membranes depending on the stage of sporulation examined (Figure 2, right panels). Thus, both Spo14p (Rudge *et al.*, 1998b) and PtdIns(4,5)P_2 localize to the prospore membrane, consistent with the hypothesis that production of PtdIns(4,5)P_2 for activation of Spo14p is a key role of Sec14p, Pik1p, and Mss4p in spore formation. A complication is that we did not observe a defined intracellular signal for the PtdIns(4,5)P_2-specific probe before prospore membrane closure, although a *spo14Δ* mutant blocks early in prospore membrane formation. However, this may be because most of the PtdIns(4,5)P_2 is in the plasma membrane of the mother cell and a certain critical mass of prospore membrane is required to observe the internal pools of PtdIns(4,5)P_2. In addition, we cannot exclude the possibility that there are small pools of PtdIns(4)P at the prospore membranes that are not detectable by the PtdIns(4)P-specific probe, perhaps because they are rapidly phosphorylated to PtdIns(4,5)P_2 by Mss4p.

Table 5. Phosphoinositide specificity of Spo14p activation

Phosphoinositide	BODIPY-PtdOH[a]
No phosphoinositide	30 ± 7
PtdIns	36 ± 11
PtdIns(4)P	35 ± 6
PtdIns(4,5)P_2	246 ± 17

[a] BODIPY-PtdOH formation is expressed as nM per input of HA-Spo14p. HA-Spo14p was immunoprecipitated from extracts of sporulating cells of strain Y598 and its PLD activity was assayed as described in MATERIALS AND METHODS. PtdIns, PtdIns(4)P, or PtdIns(4,5)P_2 was added at 5 μM, the concentration of PtdIns(4,5)P_2 known to produce maximum stimulation of Spo14p activity (Rose *et al.*, 1995). Values are means± SD of three independent trials, each performed in duplicate.

Dependence of Spore Formation on Spo14p-derived PtdOH

Spo14p action simultaneously reduces PtdCho and increases PtdOH and choline. To determine which of these effects represents the essential role of Spo14p in spore formation, we made use of an enzymatic property of the PtdCho-specific PLDs, the transphosphatidylation reaction, which is specific to Spo14p in yeast (Rose *et al.*, 1995; Rudge and Engebrecht, 1999; Rudge *et al.*, 2001). In the presence of primary alcohols, these enzymes cleave PtdCho and release choline, but generate a biologically inert phosphatidylalcohol lipid rather than PtdOH. Thus, we monitored the effect of addition of either 1-butanol (which is a substrate for the transphosphatidylation reaction) or of 2-butanol (which is not a substrate) on sporulation. Spore formation, but not meiosis, was significantly inhibited by 1-butantol at concentrations at which 2-butanol had little effect (Figure 3), suggesting that PtdOH is the essential product of Spo14p action during sporulation.

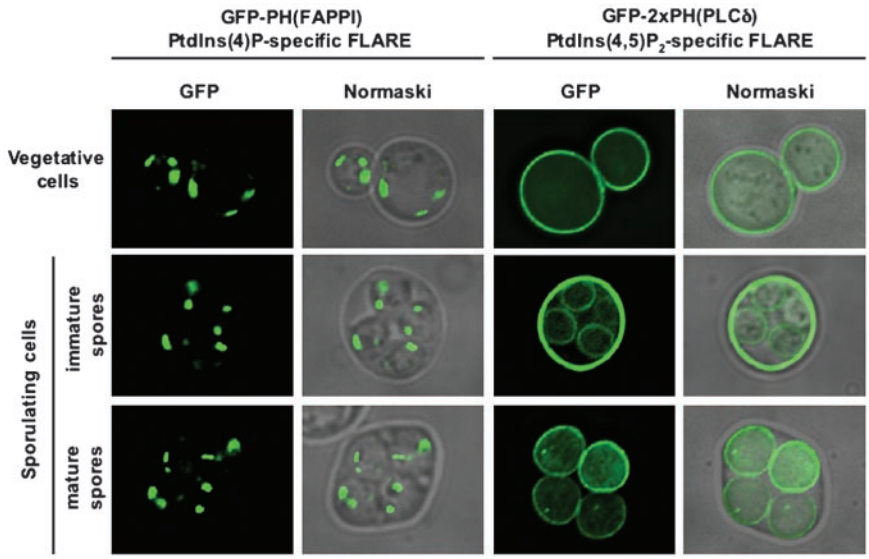

Figure 2. Prospore membranes are enriched in PtdIns(4,5)P_2. The steady state localizations of PtdIns(4)P and PtdIns(4,5)P_2 were visualized in vegetative and sporulating wild-type cells (strains Y4048 and Y4049) as described in MATERIALS AND METHODS. Samples were taken 8 and 12 h after the induction of sporulation. Fluorescence (left panels) and Normarski (right panels) images of the same cells are shown.

Spo14p Does Not Appear to Function Upstream of Mss4p during Spore Formation

Given the apparent stimulation of Mss4p by PtdOH (Homma *et al.*, 1998) and the observation that the *mss4-2* mutation affected spore formation more strongly than it did meiosis (Table 4, line 6), it seemed possible that a major role of Spo14p in sporulation is to ensure that PtdIns(4,5)P_2 synthesis occurs at a sufficiently high rate. In this case, PtdIns(4,5)P_2 synthesis would presumably be compromised in sporulating *spo14*Δ cells, and overexpression of the phosphoinositide kinases might rescue *spo14* mutants. To explore these possibilities, we first analyzed the glycero-phosphoinositides derived from [^{3}H]inositol-labeled sporulating cells. The results indicated that the levels of PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P_2 were similar in *spo14*Δ and normal cells (Figure 4). (PtdIns(3,5)P_2 was undetectable in all [^{3}H]inositol-labeled sporulating cells tested.) We then examined whether overexpression of *PIK1*, *STT*, and/or *MSS4* could suppress the spore formation defect of *spo14*Δcells or of cells containing either the *spo14S-11* or conditional *spo14(S251P)* allele (Rudge *et al.*, 2002). No one plasmid, or pair of plasmids, allowed *spo14* mutants to produce spores (Table 6 and unpublished data).

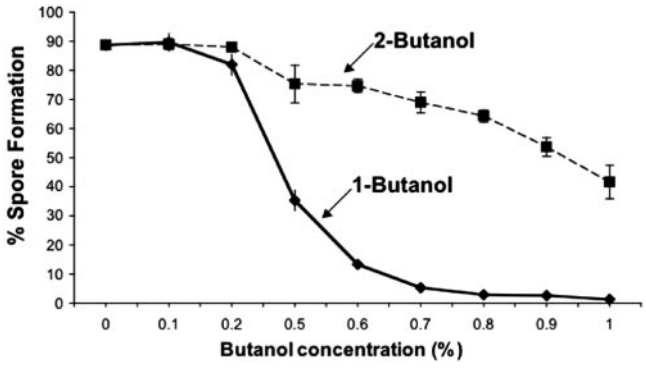

Figure 3. Dependence of spore formation on Spo14p-generated PtdOH. Spore formation by wild-type strain NH144 in the presence of various concentrations of 1-butanol or 2-butanol. 75± 7 and 62± 5% of the cells underwent the meiotic divisions in the presence of 0.6 and 0.7% of 1-butanol, respectively.

Taken together, these data argue against the hypothesis that a major role of Spo14p in sporulation is to generate PtdOH to stimulate PtdIns(4,5)P_2 synthesis. Instead, it appears that Spo14p-generated PtdOH (and/or products derived from it, such as diacylglycerol) plays an essential role in prospore membrane formation that is distinct from the role(s) of phosphoinositides.

DISCUSSION

Phosphoinositide Function during Meiosis

In this study, we have demonstrated that the PtdIns/PtdCho transfer protein Sec14p and the PtdIns 4-kinase Pik1p are both required for meiotic progression. In contrast, although the PLD Spo14p is required for spore formation, its activity is not necessary for reasonably efficient initiation and completion of Meiosis I and Meiosis II (Honigberg *et al.*, 1992; Rose *et al.*, 1995). Thus, although our data suggest that Sec14p-regulated synthesis of PtdIns(4,5)P_2 is essential for activation of Spo14p, as discussed further below, phosphoinositides clearly play additional roles during sporulation (Figure 5). In particular, it seems likely that PtdIns(4)P is important for meiosis because *pik1* mutants are severely defective in meiotic division, and overexpression of Pik1p, but not of the PtdIns(4)P 5-kinase, Mss4p, potently suppresses the *sec14-1* meiotic defect. In contrast to the *sec14-1* and *pik1* mutants, the *mss4-2* mutant was able to undergo meiosis with reasonable efficiency but failed to form spores. This indicates either that the synthesis of PtdIns(4)P, but not PtdIns(4,5)P_2, is important for meiosis or that the synthesis of PtdIns(4,5)P_2 is important but that the *mss4-2* allele is not a null mutation at the temperature sporulation was examined (34.5°C). In support of the latter possibility, the restrictive temperature for the *mss4-2* allele in vegetative cells is 37°C (Desrivières *et al.*, 1998), and even at 34.5°C there was a partial defect in the completion of meiosis.

Although Sec14p function is important for meiotic progression, its exact role(s) remain unknown. The ability of the *sec14(K66A K239A)* mutant, which is specifically defective in PtdIns transport (Phillips *et al.*, 1999), to undergo the meiotic divisions at first sight suggests that it is only the PtdCho transport activity, and not the PtdIns transport activity, of Sec14p that is important for meiosis. However, the finding

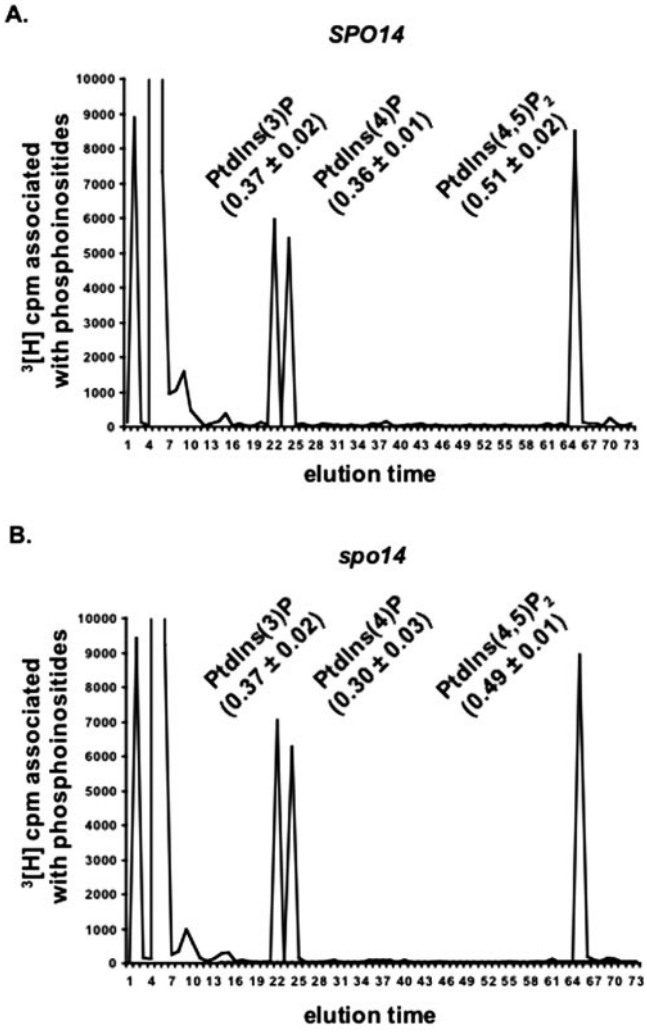

Figure 4. Spo14p does not regulate PtdIns(4,5)P_2 levels during sporulation. Phosphoinositide levels in wild-type (A) and homozygous *spo14*Δ diploids (strains NH144 and Y433; B) were determined by HPLC analysis of sporulating cells as described in MATERIALS AND METHODS. The numbers in parentheses represent the total cpm in that peak expressed as a percentage of the total cpm eluted from the column; means± ranges of two independent experiments are shown. (Note that glycero-phosphatidylinositol [the material eluted at 4–7 min] represents ~97% of the total glycero-phosphoinositides extracted.)

that overexpression of Sfh2p or Sfh4p, PITPs that lack PtdCho transport activity, suppresses the *sec14-1* sporulation defect, implies that PtdIns transport is indeed important for meiosis. Thus, presumably, either Sec14(K66A K239A)p has residual PtdIns transport activity or normal levels of Sfh2p and Sfh4p can provide sufficient PtdIns transport for meiosis in a *sec14(K66A K239A)* background. That the suppression by *SFH2* or *SFH4* is dependent on Spo14p indicates that Spo14p PLD activity can substitute for Sec14p under these conditions. This may be due to activation of Spo14p through enhanced PtdIns(4,5)P_2 generation, as observed in Sec14 bypass conditions (Li *et al.*, 2000), and may be a consequence of Spo14p-dependent PtdCho cleavage mimicking Sec14p-PtdCho transport function.

What is the role of phosphoinositides in meiosis? Substantial evidence demonstrates that both *sec14* (Bankaitis *et al.*, 1989) and *pik1* (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999) mutations block vesicle-mediated transport from the Golgi. That both *sec14-1* and *pik1-ts* diploids are unable to carry out meiosis suggests that trafficking through the secretory pathway is required for meiotic progression. Alternatively, these proteins and the phosphoinositides generated by their action may play additional roles during meiosis. Consistent with the idea that trafficking is important for meiosis, *gsg1* mutants are defective in meiotic division (Engebrecht *et al.*, 1998), and recent work has indicated that Gsg1p is the high-molecular-weight subunit of both the TRAPP I complex, involved in vesicle-mediated transport from the ER to the Golgi, and the TRAPP II complex, involved in intra-Golgi trafficking (Sacher *et al.*, 2000, 2001). Mutations in *SPO15/VPS1*, which encodes a dynamin-like protein required for sorting of proteins from the Golgi to the vacuole, also fail to undergo meiotic division (Yeh *et al.*, 1991). Thus, it seems that successful meiotic progression requires the function of much of the normal secretory machinery.

Table 6. Sporulation proficiency of *spo14* mutants

Strain	Relevant genotype	% Sporulation[a]
Y4516	*spo14*Δ + 2μ	<0.1
Y4517	*spo14*Δ + *PIK1* 2μ	<0.1
Y4518	*spo14*Δ + *STT4* 2μ	<0.1
Y4519	*spo14*Δ + *MSS4* 2μ	<0.1
Y4520	*spo14*Δ + *PIK1* 2μ + *MSS4* 2μ	<0.1
Y4521	*spo14*Δ + *STT4* 2μ + *MSS4* 2μ	<0.1
Y3825	*spo14-S11* + 2μ	<0.1
Y3827	*spo14-S11* + *PIK1* 2μ	<0.1
Y4522	*spo14-S11* + *STT4* 2μ	<0.1
Y3826	*spo14-S11* + *MSS4* 2μ	<0.1
Y3828	*spo14-S11* + *PIK1* 2μ + *MSS4* 2μ	<0.1
Y4523	*spo14-S11* + *STT4* 2μ + *MSS4* 2μ	<0.1

[a] Spore formation was monitored 30°C as described in Table 2.

Phosphoinositides and Spo14p-generated PtdOH in Spore Formation

In addition to their involvement in meiosis, phosphoinositides, and PtdIns(4,5)P_2 in particular, appear to have a distinct role(s) in spore formation (Figure 5). This conclusion is supported by several lines of evidence, including 1) the completion of meiosis but not spore formation by the *sec14(K66A K239A)* and *mss4-2* mutants; 2) the suppression of the *sec14-1* spore-formation (as well as meiosis) defect by overexpression of *PIK1*, *SFH2*, or *SFH4*; 3) the dependence of

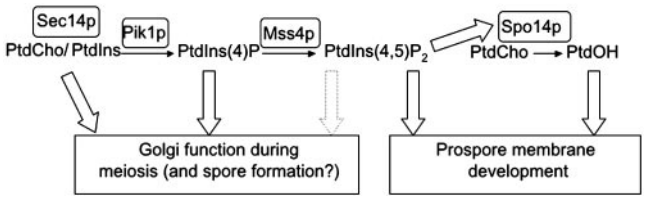

Figure 5. Model for the possible relationships between the phosphoinositides [PtdIns, PtdIns(4)P and PtdIns(4,5)P_2] and Spo14p-generated PtdOH and their functions in meiosis and spore formation. See text for details. The dashed arrow indicates uncertainty about the role of PtdIns(4,5)P_2 in meiosis, probably due to leakiness of the *mss4* allele used.

spore formation on Spo14p and its product PtdOH; and 4) the dependence of Spo14p activity on Sec14p-, Pik1p-, and Mss4p-dependent generation of PtdIns(4,5)P_2. Not surprisingly, it does not appear that activation of Spo14p is the only target of phosphoinositides in spore formation, because overexpression of Spo14p was unable to relieve the sporulation defect of either the *sec14(K66A K239A*) or *mss4-2* mutant.

What is the relationship between PtdIns(4,5)P_2 and PtdOH during spore formation? One possibility is that PtdOH derived from Spo14p-mediated hydrolysis of PtdCho is important for stimulating Mss4p (Homma *et al.*, 1998), thereby providing for a positive feedback loop that promotes optimal synthesis of PtdIns(4,5)P_2 and supports those processes that depend uniquely and vitally on this phosphoinositide. However, the stimulation of PtdIns(4)P 5-kinases by PtdOH has been observed in vitro, and the physiological relevance of these observations is not known. Because Spo14p action is a major pathway for regulated PtdOH formation in *S. cerevisiae* (which lacks any recognizable diacylglycerol kinase; Kearns *et al.*, 1997), this organism is a good system to investigate the relationship between PtdOH levels and PtdIns(4,5)P_2 synthesis. Our finding that PtdIns(4,5)P_2 levels are not detectably reduced in the absence of Spo14p, whereas PtdOH levels are reduced (Rudge *et al.*, 2001), indicates that PtdOH does not markedly stimulate PtdIns(4,5)P_2 synthesis. However, we cannot exclude the possibility that Spo14p-dependent PtdOH stimulates small and highly localized changes in PtdIns(4,5)P_2 concentration that are especially critical for sporulation.

Hence, it seems that both PtdIns(4,5)P_2 and PtdOH generation contribute to establishing a lipid environment critical to the function of proteins necessary for the membrane trafficking events required for prospore membrane synthesis. Recently, it has been reported that Gcs1p and Age2p, two Golgi-associated GTPase-activating proteins (GAPs) for the Arf family of small GTPases, which have an essential role in the formation of coated vesicles for both exocytic and endocytic transport, are activated by PtdOH and diacylglycerol (which can be derived from PtdOH), inhibited by PtdCho, and probably recruited to membranes via a PH domain that binds phosphoinositides (Yanagisawa *et al.*, 2002). These Arf-GAPs, and perhaps other proteins, may act as integrators that decipher the relative levels of PtdCho, PtdOH, and phosphoinositides and thereby adjust the rate of vesicle-mediated membrane trafficking accordingly. In support of these ideas is the requirement for Arf1p in sporulation, which occurs downstream of Spo14p-mediated PtdOH synthesis (Rudge *et al.*, 1998a) and perhaps explains why yeast do not contain an Arf-activated PLD (Rudge *et al.*, 1998a; Rudge and Engebrecht, 1999). Once vesicles are generated, there may be a specific requirement for Spo14p-generated PtdOH in vesicle fusion at the SPBs (our unpublished results).

Although our study was in progress, it was found that the *spo20*$^+$ gene of the fission yeast *Schizosaccharomyces pombe*, which was identified originally on the basis of its requirement for prospore membrane formation (Hirata and Shimoda, 1992), encodes a homolog of *S. cerevisiae* Sec14p (Nakase *et al.*, 2001). Unlike *sec14-1* cells, which we found were unable to carry out meiosis, the *spo20* mutant *S. pombe* cells progressed through meiosis. This difference is probably attributable to a partial retention of function by the *spo20* allele used, based on its other phenotypes. In addition, the PtdIns-binding–deficient Sec14(K66A K239A)p, when expressed from a strong inducible promoter on a high-copy plasmid, fully compensated for the loss of Spo20p function during *S. pombe* sporulation (Nakase *et al.*, 2001). However, these investigators did not examine lower levels of expression. As we have shown here, when expressed at a more physiological level, Sec14(K66A K239A)p is unable to support *S. cerevisiae* spore formation. It would be interesting to determine if the *S. pombe* homolog of Spo14p (Rudge and Engebrecht, 1999) is essential for prospore membrane formation and/or if Spo20p is required for the maximal activity of that PLD. Alternatively, the requirements for PtdIns transport activity in sporulation may be different in the two yeasts.

In conclusion, our results provide the first in vivo evidence that Sec14p positively contributes to Spo14p/PLD function through its role in the synthesis of PtdIns(4,5)P_2. Furthermore, our findings indicate that both phosphoinositides and Spo14p-dependent PtdOH synthesis are essential for sporulation in *S. cerevisiae*. We propose that the action of Spo14p in generating PtdOH lies downstream of a Sec14p-Pik1p-Mss4p–dependent pathway of PtdIns(4,5)P_2 synthesis that is essential for the formation and/or delivery of post-Golgi vesicles to, or their fusion at, the meiotic plaques of the SPBs, where prospore membrane formation begins.

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