

Differential Regulation of *Saccharomyces cerevisiae* Phospholipase D in Sporulation and Sec14-Independent Secretion

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ABSTRACT

Saccharomyces cerevisiae Spo14, a phosphatidylcholine-specific, phosphatidylinositol (4,5) bisphosphate-activated phospholipase D (PLD), is essential for meiosis and spore formation. Spo14 is also required for secretion in the absence of the phosphatidylinositol/phosphatidylcholine transfer protein Sec14 (*i.e.*, Sec14-independent secretion). In sporulating cells Spo14 is phosphorylated and relocalized within the cell. In contrast, Spo14 does not relocalize and is not phosphorylated in Sec14-independent secretion. Analysis of a partially phosphatidylinositol (4,5) bisphosphate-activated Spo14 mutant, spo14^{RS94G}, revealed that Spo14 function in Sec14-independent secretion, unlike the situation in meiosis, requires fully stimulated PLD activity. Consistent with the differential regulation of Spo14 function during sporulation and secretion, we isolated a mutant allele, *spo14-S251P*, the product of which is improperly phosphorylated and fails to relocalize and rescue the sporulation phenotype of homozygous *spo14* diploids, but supports Sec14-independent secretion. Furthermore, we show that the N-terminal domain of Spo14 is both phosphorylated and sufficient for prospore membrane localization during sporulation. These data indicate that Spo14 phosphorylation and relocalization are essential for the process of sporulation, but dispensable for Sec14-independent secretion. Finally, we demonstrate that Spo14 phosphorylation and relocalization are initiated by nitrogen and glucose limitation and occur independently of the process of meiosis.

THE *Saccharomyces cerevisiae* *SPO14* gene product encodes a phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P₂]-activated, phosphatidylcholine-specific phospholipase D (PLD) whose localized enzymatic activity is required for the successful completion of the meiotic divisions and is essential for spore formation (ROSE *et al.* 1995; RUDGE *et al.* 1998b; SCIORRA *et al.* 1999). Genetic analyses indicate that Spo14 is dispensable for mitotically dividing wild-type yeast cells (HONIGBERG *et al.* 1992; ROSE *et al.* 1995; ELLA *et al.* 1996; WAKSMAN *et al.* 1996). However, Spo14 enzymatic activity is essential for mutant yeast cells to bypass the normally essential requirement for Sec14 in growth and secretion (SREENIVAS *et al.* 1998; XIE *et al.* 1998).

Sec14, a phosphatidylinositol/phosphatidylcholine transfer protein, is required for Golgi secretory function (BANKAITIS *et al.* 1990). However, yeast cells mutated in at least one of seven genes are able to produce Golgi vesicles independently of Sec14, a process referred to as Sec14-independent secretion (LI *et al.* 2000b). These genes include *KESI* (a yeast homolog of a human oxysterol binding protein; FANG *et al.* 1996), *SAC1* (a phosphoinositide phosphatase; CLEVES *et al.* 1989), and genes encoding for enzymes of the cytidine 5'-diphos-

phate-choline route of phosphatidylcholine synthesis (*CKII*, *PCT1*, and *CPT1*; CLEVES *et al.* 1991). The dependency of Sec14-independent secretion on Spo14 most probably reflects the requirement of PLD activity to compensate for the role of Sec14 in regulating phosphatidylcholine and phosphatidic acid levels required for Golgi function.

During sporulation Spo14 function is dependent on cellular relocation. Failure of Spo14 to relocalize to the sites of prospore membrane formation, at the yeast spindle pole bodies, results in the complete absence of spores (RUDGE *et al.* 1998b). However, the dependency of Sec14-independent secretion on Spo14 intracellular relocalization is not known. We previously proposed that protein phosphorylation drives Spo14 movement during sporulation (RUDGE *et al.* 1998b); consequently, we were interested in determining whether Spo14 was regulated by these same mechanisms when required for secretion. In this report we demonstrate that Spo14 neither is phosphorylated nor undergoes relocalization in Sec14-independent secretion. Analyses of mutant alleles suggest that PtdIns(4,5)P₂ activation is the primary regulator of Spo14 PLD activity in secretion, while phosphorylation and relocalization, in addition to PtdIns(4,5)P₂, regulate the enzyme during meiosis.

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MATERIALS AND METHODS

Strains, genetic manipulation, and media: Genotypes of yeast strains are listed in Table 1. Yeast manipulations were

TABLE 1
Yeast Strains

Strain	Genotype	Source
Y524	<i>HO ura3-1 leu2 arg4-8 thr1-4 trp1-1 ade2</i>	ROSE <i>et al.</i> (1995)
Y494	<i>HO ura3-1 leu2 arg4-8 thr1-4 trp1-1 ade2</i>	ROSE <i>et al.</i> (1995)
Y522	Y524 but homozygous <i>spo14::URA3</i>	ROSE <i>et al.</i> (1995)
Y1520	Y524 but homozygous <i>spo14::LEU2</i>	SCIORRA <i>et al.</i> (1999)
NH144	<i>MATa ura3-52, his3-200, lys2-801, sec14-3ts, kes1-1, spo14::HIS3</i> <i>MATa leu2-kHIS4 ho::LYS2 ura3-1 lys2 art4-Nsp</i>	SCIORRA <i>et al.</i> (1999)
Y2053	<i>MATα leu2::hisGhis4-x ho::LYS2 ura3-1 lys2 ARG4</i>	HOLLINGSWORTH <i>et al.</i> (1995)
Y2455	NH144 but homozygous <i>spo14::LEU2</i>	RUDGE <i>et al.</i> (2001)
Y2844	Y524 + <i>HA-spo14-313 CEN URA3</i> (pME 1686)	This study
Y1099	NH144 + <i>GFP-spo14-313 2μ URA3</i> (pME1759)	This study
Y2366	Y522 + <i>HA-spo14-S251P CEN URA3</i> (pME1155)	This study
Y3846	Y522 + <i>HA-SPO14 CEN URA3</i> (pME986)	SCIORRA <i>et al.</i> (1999)
Y2159	Y2053 + <i>GFP-spo14-S251P 2μ URA3</i> (pME1163)	This study
Y3845	Y2053 + <i>GFP-SPO14 2μ URA3</i> (pME1095)	RUDGE <i>et al.</i> (2001)
Y2371	NH144 + <i>GFP-spo14-S251P 2μ URA3</i> (pME1163)	This study
Y2274	Y1520 + <i>HA-SPO14 CEN URA3</i> (pME986)	SCIORRA <i>et al.</i> (1999)
Y2441	Y1520 + <i>CEN URA3</i> (pUN55)	SCIORRA <i>et al.</i> (1999)
Y1531	Y1520 + <i>HA-spo14-S251P CEN URA3</i> (pME1155)	This study
Y1530	Y1520 + <i>GFP-SPO14 2μ URA3</i> (pME1095)	SCIORRA <i>et al.</i> (1999)
Y500	Y1520 + <i>2μ URA3</i> (Yep352)	SCIORRA <i>et al.</i> (1999)
Y568	Y494 + <i>CEN LEU2</i> (pUN105)	RUDGE <i>et al.</i> (1998b)
Y1977	Y494 + <i>HA-SPO14 CEN LEU2</i> (pME940)	RUDGE <i>et al.</i> (1998b)
Y1981	Y522 + <i>HA-spo14-R894G 2μ URA3</i> (pME1576)	SCIORRA <i>et al.</i> (1999)
Y2079	Y1520 + <i>HA-spo14-R894G 2μ URA3</i> (pME1576)	SCIORRA <i>et al.</i> (1999)
Y2080	Y522 + <i>HA-spo14-R894G CEN URA3</i> (pME1597)	This study
Y805	Y1520 + <i>HA-spo14-R894G CEN URA3</i> (pME1597)	This study
Y814	<i>MATa leu2-27his4-260 ura3-1 trp1-1 thr1-4 ADE2 lys2 SPO14</i> <i>MATα leu2-3,112his4-280 ura3-1 trp1-1 THR1 ade2 lys2 spo14::LEU2</i> + <i>HA-SPO14 CEN URA3</i> (pME986)	This study
Y814	<i>MATa leu2-27his4-260 ura3-1 trp1-1 thr1-4 ADE2 lys2 SPO14</i> <i>MATa leu2-3,112his4-280 ura3-1 trp1-1 THR1 ade2 lys2 spo14::LEU2</i> + <i>HA-SPO14 CEN URA3</i> (pME986)	This study

performed and media were prepared using standard procedures (ROSE *et al.* 1990). Yeast transformations were carried out by the lithium acetate procedure (ITO *et al.* 1983).

Cells were initiated to sporulate in 2% potassium acetate after transfer from synthetic complete medium without uracil (SC-ura). Sporulation frequencies were determined after 48 hr by examining a minimum of 600 cells. Strains were grown in SD(-N) (0.17% yeast nitrogen base without ammonium sulfate or amino acids containing 2% glucose; TAKESHIGE *et al.* 1992) for 15 hr after transfer from SC-ura for analysis of Spo14 phosphorylation.

Plasmid construction: *spo14-S251P* was generated by amplifying the nucleotide sequence corresponding to the phosphorylation domain of *SPO14* open reading frame (RUDGE *et al.* 1998b), using the polymerase chain reaction (PCR) and *in vivo* recombination with a gapped plasmid (MUHLRAD *et al.* 1992). PCR was performed using standard conditions with *SPO14* centromere plasmid ME325 as template (ROSE *et al.* 1995). *In vivo* recombination was achieved by cotransformation of the PCR product and the *EcoRI*-gapped *spo14ΔN* centromere plasmid ME419 (ROSE *et al.* 1995).

The construction and characterization of fully functional triple hemagglutinin (HA)-tagged and green fluorescent protein (GFP)-tagged Spo14 proteins have previously been described in full detail elsewhere (RUDGE *et al.* 1998b; SCIORRA

et al. 1999). HA- and GFP-tagged derivatives of *spo14*³¹³ (ROSE *et al.* 1995) and *spo14*^{S251P} were constructed by insertion of HA and GFP sequences (excised from *HA-* and *GFP-SPO14* using *SphI*; RUDGE *et al.* 1998b) into the unique *SphI* site of *spo14-313* (pME1686 and pME1759, respectively) and *spo14-S251P* (pME1153 and pME1163, respectively).

Immunoprecipitation of HA-Spo14 and HA-spo14^{S251P}: HA-Spo14 proteins were immunoprecipitated using mAb 12CA5 (BabCO, Richmond, CA) as previously described in detail by RUDGE *et al.* (1998b). After 15 hr in sporulation medium at 25° or 33.5°, homozygous *spo14* diploids containing *HA-SPO14* or *HA-spo14-S251P* in low-copy *CEN* vectors were collected by centrifugation and Nonidet P-40-soluble fractions prepared for immunoprecipitation as previously described (RUDGE *et al.* 1998b).

Treatment of immunoprecipitated HA-Spo14 with shrimp alkaline phosphatase: Immune complexes of HA-Spo14 and HA-spo14^{S251P} subjected to treatment with shrimp alkaline phosphatase (SAP) were suspended in 15 μl phosphatase buffer (40 mM HEPES, pH 8.0, 10 mM magnesium chloride) and incubated for 30 min at 30° with 1.5 units of SAP (United States Biochemical, Cleveland) in the presence or absence of phosphatase inhibitors (10 mM sodium pyrophosphate, 5 mM EGTA, 5 mM EDTA; RUDGE *et al.* 1998b). For immunoblot analysis, the phosphatase reaction was terminated with the

addition of 5 μ l of 4 \times Laemmli buffer, and the beads were boiled for 5 min.

Preparation of whole cell extracts: Extracts of yeast proteins were prepared from frozen cell pellets by glass-bead homogenization (RUDGE *et al.* 1998a). The homogenates were assayed for protein content (BRADFORD 1976) and an appropriate volume of 4 \times Laemmli buffer was added. The samples were then boiled for 3 min and centrifuged at 16,000 \times *g* for 10 min prior to SDS-PAGE analysis.

Immunoblot analysis: Immunoblot analysis was conducted essentially as described (RUDGE *et al.* 1998a,b, 2001). Proteins were electrophoretically transferred from SDS-polyacrylamide gels onto nitrocellulose membranes (pore size: 0.45 μ m; Bio-Rad Laboratories, Hercules, CA) for 2 hr and HA-Spo14 proteins were detected using mAb 12CA5 and horseradish peroxidase-conjugated anti-mouse antiserum (Amersham International, Buckinghamshire, England) and visualized by enhanced chemiluminescence detection on Kodak BIOMAX MR film (Eastman Kodak, Rochester, NY; RUDGE *et al.* 1998a,b, 2001).

Cytology: For the visualization of GFP-Spo14 and variants, yeast cells were fixed for 15 min at 25 $^{\circ}$ with 3.7% formaldehyde as described (RUDGE *et al.* 1998b). Samples were mounted in Prolong mounting media (Molecular Probes, Eugene, OR) and viewed and images were captured with a Nikon Eclipse E600 confocal microscope.

RESULTS

PLD-mediated Sec14-independent secretion is more sensitive to changes in the specific activity of Spo14: To better understand the physiological roles of PLD in cells, we sought to differentiate the regulatory properties of Spo14 in yeast meiosis and Sec14-independent Golgi function. The demonstration that Spo14 drives Sec14-independent secretion (SREENIVAS *et al.* 1998; XIE *et al.* 1998) prompted us to examine how Spo14 is activated to generate phosphatidic acid under these conditions. In theory, the increase in Spo14 activity observed in Sec14-independent secretion (SREENIVAS *et al.* 1998; XIE *et al.* 1998) could be achieved through several nonexclusive mechanisms: increased protein expression, phosphorylation, relocalization, and increased exposure to substrate (*i.e.*, phosphatidylcholine) and/or activator [PtdIns(4,5)P₂]. Analyses of Spo14 protein in Sec14-independent secretion revealed that protein expression, phosphorylation, and localization are not significantly altered in the absence of Sec14 (data not shown). Thus, changes in these properties cannot account for the increase in PLD activity observed in Sec14-independent secretion.

To address whether changes in enzymatic activity contributed to activation in Sec14-independent secretion, we examined in more detail its regulation by PtdIns(4,5)P₂. We previously demonstrated that PtdIns(4,5)P₂-mediated activation of Spo14 is essential for both sporulation and Sec14-independent secretion (SCIORRA *et al.* 1999). This was achieved through mutagenesis of a newly identified PtdIns(4,5)₂-activation domain in eukaryotic PLDs (SCIORRA *et al.* 1999). One mutation in particular, spo14^{R894G},

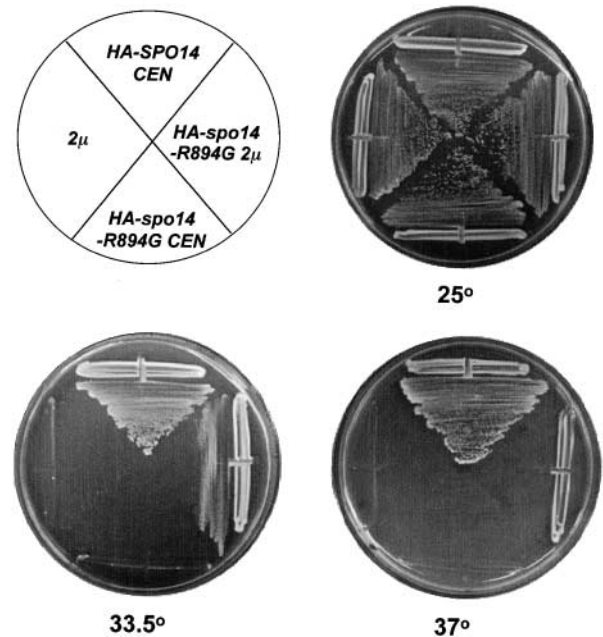


FIGURE 1.—Sec14-independent secretion is more sensitive to changes in the specific activity of Spo14. *spo14-R894G CEN* fails to support secretion in *sec14-1 kes1 spo14* mutants at the restrictive temperatures of 33.5 $^{\circ}$ and 37 $^{\circ}$.

resulted in a partial loss of Spo14 PtdIns(4,5)P₂ activation (SCIORRA *et al.* 1999) and was only partially functional for sporulation and secretion when expressed from a high-copy vector. Surprisingly, *spo14-R894G* expressed from a low-copy vector is incapable of supporting secretion in *sec14-1 kes1 spo14* mutants as measured by growth at 33.5 $^{\circ}$ or 37 $^{\circ}$ (Figure 1). However, the same construct enables homozygous *spo14* deletion mutants to sporulate to a similar degree as those cells expressing *spo14-R894G* on a high-copy vector (*spo14-R894G CEN* 35 \pm 3%, *spo14-R894G 2 μ* 36 \pm 5%). Since spo14^{R894G} is not activated to the extent to which PtdIns(4,5)P₂ activates wild-type Spo14 (SCIORRA *et al.* 1999), Spo14 function in Sec14-independent secretion appears more sensitive to loss of PLD activity. Moreover, this result reiterates our previous conclusion that PtdIns(4,5)P₂ activation of Spo14 drives Sec14-independent secretion (SCIORRA *et al.* 1999).

The failure of spo14^{R894G} to sustain Sec14-independent secretion at 33.5 $^{\circ}$ and 37 $^{\circ}$ (Figure 1) could simply reflect that this mutation rendered the protein temperature sensitive. However, spo14^{R894G} retains its ability to partially rescue the sporulation defect of homozygous *spo14* diploids at elevated temperatures of up to 35 $^{\circ}$ (the maximal permissible temperature for sporulation in the SK-1 strain background). At 25 $^{\circ}$ the sporulation frequencies were 90 \pm 4% for *SPO14* *vs.* 61 \pm 5% for *spo14-R894G*, and at 35 $^{\circ}$ the frequencies were 53 \pm 6% for wild type *vs.* 34 \pm 4% for *spo14-R894G*. Therefore, spo14^{R894G} is not a temperature-sensitive protein.

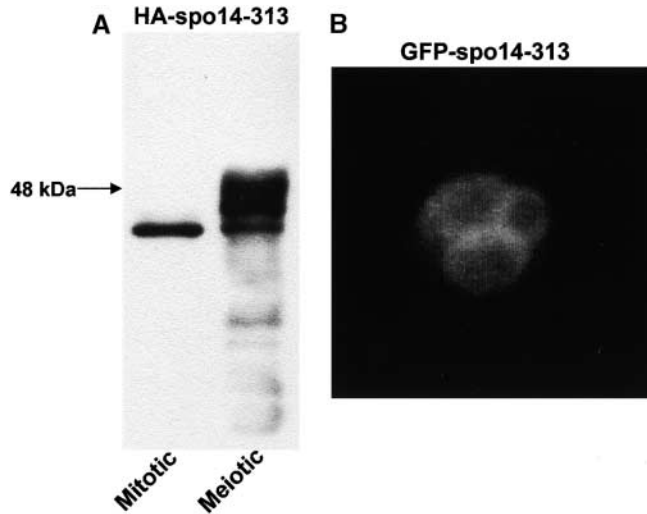


FIGURE 2.—The N terminus of Spo14 is phosphorylated during meiosis and is sufficient to localize PLD protein to the prospore membrane. (A) Wild-type homozygous diploids containing *HA-spo14-313* 2μ were grown in SC-ura or sporulation medium for 15 hr at 30°. Whole cell lysates were prepared and the mobility of HA-spo14³¹³ was determined by SDS-PAGE and immunoblotting. (B) Cellular fluorescence of GFP-spo14³¹³ within sporulating cells.

The N terminus of Spo14 is phosphorylated during meiosis and is sufficient to localize PLD protein to the prospore membrane: Fungal members of the PLD gene family are unique in that they have an extended N terminus (MORRIS *et al.* 1996), which is adjacent to the conserved PX and PH domains (PONTING 1996; STEED *et al.* 1998). We previously showed that the Spo14 N-terminal domain is the major site of phosphorylation and is necessary for localization of the enzyme to the prospore membrane during sporulation (RUDGE *et al.* 1998b). To determine if this region alone is sufficient for mediating localization, a truncated mutant, *spo14-313*, which encodes for the first 313 amino acids but does not include the PX and PH domains of Spo14 (ROSE *et al.* 1995), was epitope tagged separately with both HA and GFP. The phosphorylation state and localization of the corresponding proteins were examined.

Wild-type diploid cells containing *HA-spo14-313* on a high-copy vector were grown vegetatively and induced to sporulate. Protein extracts were made, and the mobility of HA-spo14³¹³ was determined by SDS-PAGE. The immunoblot shown in Figure 2A reveals that additional slower-migrating species of HA-spo14³¹³ were detected only in samples prepared from sporulating cells. This result suggests that HA-spo14³¹³ is phosphorylated during meiosis and reaffirms our previous data that demonstrated that the N terminus of Spo14 is the major site of phosphorylation (RUDGE *et al.* 1998b).

To investigate whether the N terminus of Spo14 is sufficient to localize Spo14 to the prospore membrane, *GFP-spo14-313* on a high-copy vector was introduced into

wild-type diploid cells. Using confocal microscopy, the cellular localization of GFP-spo14³¹³ was determined in formaldehyde-fixed sporulating cells. Figure 2B shows that, in common with GFP-Spo14 (Figure 5C and RUDGE *et al.* 1998a,b, 2001), GFP-spo14³¹³ localizes to the prospore membrane. This result indicates that the N terminus of Spo14 is sufficient for localizing PLD activity to the prospore membrane during meiosis.

Spo14 phosphorylation and relocation are essential for sporulation, but dispensable for Sec14-independent secretion: The essential role for Spo14 in Sec14-independent secretion is dependent on both PLD catalytic activity and the extended N-terminal domain (XIE *et al.* 1998). However, analysis of Spo14 in the absence of Sec14 revealed that it is neither phosphorylated nor relocated, while this domain alone is sufficient for these events (see above). To differentiate the role of the N-terminal extension in Sec14-independent secretion and meiosis, we isolated conditional mutations within this domain of *SPO14* by targeted PCR mutagenesis (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 sporulate at both 25° and 33.5°. Two mutants were identified that exhibited the conditional sporulation phenotype. DNA sequencing revealed that both contained three base changes within a single codon in the N-terminal domain at nucleotides 751–753 (TCT mutated to CTC), generating a serine-to-proline change at position 251. The resulting protein is designated spo14^{S251P}.

At the permissive temperature (25°) spo14^{S251P} expressed from a low-copy (CEN) yeast plasmid allowed homozygous *spo14* diploids to sporulate at near wild-type frequency (*spo14-S251P* 50 ± 4% *vs.* *SPO14* 62 ± 3%). However, at the restrictive temperature (33.5°) no spores were formed in *spo14-S251P* mutants (<0.1%). In contrast, homozygous *spo14* mutants containing *SPO14* sporulated at reasonable efficiency at 33.5° (28 ± 2%). Elevated temperatures are known to impede the process of meiosis and sporulation (BYERS and GOETSCH 1982); consequently, the efficiency to which wild-type diploids can sporulate is affected at 33.5°.

Although Sec14-independent secretion is normally assayed by examining growth at 37°, the report that *sec14-1 kes1 spo14* mutants are unable to grow at 33.5° (RIVAS *et al.* 1999) enabled us to test the ability of spo14^{S251P} grown at the nonpermissive temperature to support secretion. Interestingly, spo14^{S251P} restored growth of the *sec14-1 kes1 spo14* mutants to the same degree as the wild-type protein at 33.5° (Figure 3).

To examine protein phosphorylation and localization of spo14^{S251P} at the permissive and restrictive temperatures, HA- and GFP-tagged derivatives were constructed and introduced into yeast cells. SAP treatment of immunoprecipitated HA-Spo14 results in the removal of phosphate groups from Spo14 and a decrease in the appar-

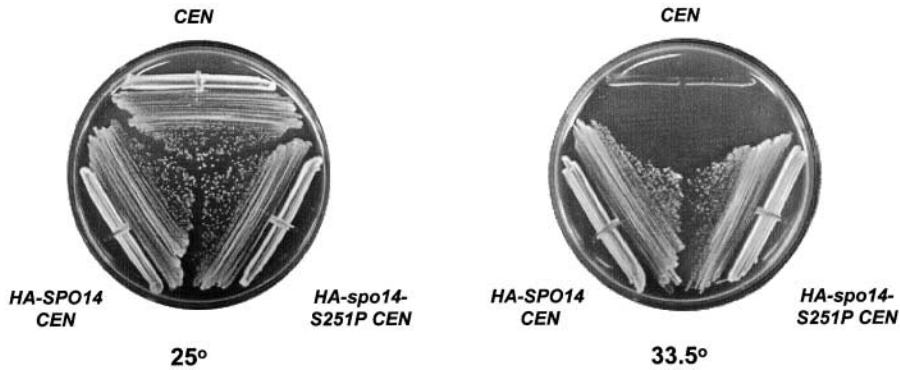


FIGURE 3.—Spo14 phosphorylation is essential for sporulation, but dispensable for Sec14-independent secretion. A *sec14-1 kes1 spo14* strain harboring no *SPO14* sequences (*CEN*), *HA-SPO14* (*HA-SPO14 CEN*), or *HA-spo14-S251P* (*HA-spo14-S251P CEN*) was incubated on solid SC-ura medium at 25° and 33.5° for 48 hr.

ent molecular weight of the protein on SDS-PAGE (RUDGE *et al.* 1998b). To determine whether HA-spo14^{S251P} was phosphorylated correctly, both HA-Spo14 and HA-spo14^{S251P} were immunoprecipitated from extracts prepared from homozygous *spo14* diploids sporulated at 25° and 33.5°. At both temperatures HA-Spo14 is detected exclusively as a single phosphorylated species, whose electrophoretic mobility is sensitive to treatment with SAP (Figure 4, A and B; RUDGE *et al.* 1998b). In contrast, immunoprecipitated HA-spo14^{S251P} was found to exist in three states at 25° (Figure 4A). The electrophoretic mobility of the two least-mobile species of HA-spo14^{S251P} was sensitive to SAP treatment (Figure 4A). However, the electrophoretic mobility of the third, and most migratory and prominent, species of HA-spo14^{S251P} was insensitive to treatment with SAP (Figure 4, A and B). Interestingly, only this third species of HA-spo14^{S251P} was detected at 33.5° (Figure 4B), a temperature at which Sec14-independent secretion occurs but not sporulation (Figure 3 and above). Moreover, since HA-spo14^{S251P} and HA-Spo14 are both expressed to the same levels at 25° and 33.5° (Figure 4, A and B), we can eliminate the possibility that the sporulation phenotype associated with HA-spo14^{S251P} is due to this protein being uniquely labile at high temperature.

One possible explanation for this data is that the serine-to-proline mutation perturbs the folding of the N terminus such that the SDS-PAGE mobility of spo14^{S251P} is altered independently of protein phosphorylation. To test this, the electrophoretic mobility of HA-spo14^{S251P} isolated from mitotically dividing cells was determined. During vegetative growth Spo14 is not phosphorylated to the extent to which the protein is phosphorylated during sporulation (RUDGE and ENGBRECHT 1999). Figure 4C shows that the electrophoretic mobility of HA-spo14^{S251P} is significantly diminished during vegetative growth both at 25° and 33.5°. Furthermore, only one species of HA-spo14^{S251P} is detected in mitotically dividing cells, consistent with the notion that the mobility of the mutant protein is altered independently of phosphorylation. Taken together, these results suggest that a fraction of the protein is phosphorylated at the permissive temperature, as monitored by the slower-mobility species;

however, at the restrictive temperature the enzyme is not phosphorylated and hence fails to support sporulation.

When expressed from a high-copy yeast (2 μ) plasmid, GFP-spo14^{S251P} remains incapable of rescuing the sporulation phenotype of homozygous *spo14* diploids at 33.5° (<0.1% sporulation *vs.* 30% \pm 4% for *SPO14*). Thus, we examined the intracellular localization of GFP-spo14^{S251P} 2 μ within sporulating *spo14* and *SPO14* diploids. In *spo14* cells propagated at the permissive temperature, GFP-spo14^{S251P} localized to the prospore membrane (Figure 5A); however, at the restrictive temperature GFP-spo14^{S251P} was unable to localize to sites of prospore membrane synthesis (Figure 5B). GFP-Spo14 staining looked normal at 33.5° in this strain background (Figure 5C), indicating that the temperature did not affect GFP-Spo14 localization and subsequent PLD-mediated prospore membrane synthesis and spore formation. Furthermore, in *SPO14* cells where the prospore membrane is formed, GFP-spo14^{S251P} failed to localize (Figure 5D). These results demonstrate that spo14^{S251P} perturbs both phosphorylation and relocalization of the enzyme to sites of prospore membrane assembly during sporulation.

Spo14 phosphorylation does not influence *in vitro* PLD activity: spo14^{S251P} is capable of supporting at least one process absolutely dependent on Spo14 PLD activity (*i.e.*, Sec14-independent secretion; XIE *et al.* 1998; RIVAS *et al.* 1999), but not sporulation. These results are consistent with our previous observation that Spo14 phosphorylation does not influence the protein's *in vitro* PLD catalytic activity (RUDGE *et al.* 1998b). To further test this prediction, HA-Spo14 and HA-spo14^{S251P} were immunoprecipitated from homozygous *spo14* diploids cultured in sporulation medium at 33.5°. Quantification of PLD assays conducted with immunoprecipitated HA-Spo14 and HA-spo14^{S251P} revealed that the catalytic activity of Spo14 was not significantly altered by the introduction of the serine-to-proline mutation in the N terminus. The specific activity of immunoprecipitated HA-Spo14 was 410 \pm 22 pmol/min compared with 373 \pm 31 pmol/min for HA-spo14^{S251P}. Thus, perturbation of phosphorylation and localization does not influence PLD activity *per se*.

Spo14 is phosphorylated independently of meiosis in

response to nitrogen and carbon starvation: As a first step toward identifying the kinase(s) responsible for Spo14 phosphorylation during sporulation, the external and genetic requirements for Spo14 phosphorylation were examined. Since catalytically deficient mutants of Spo14 are phosphorylated even when meiosis and sporulation are impaired (RUDGE *et al.* 1998a; SCIORRA *et al.* 1999), we hypothesized that the kinase(s) would not be one whose activity was intimately regulated by the proficiency of the meiotic divisions and/or spore packaging. Instead, we investigated the idea that the kinase(s) responsible for Spo14 phosphorylation would be activated prior to the meiotic divisions in response to the nutrient conditions that initiate yeast sporulation,

i.e., nitrogen limitation and the presence of a nonfermentable carbon source.

The immunoblot displayed in Figure 6A shows the apparent molecular weight of immunoprecipitated HA-Spo14 proteins extracted from yeast cell cultures grown in either sporulation medium (2% potassium acetate) or SD(-N). SD(-N) does not contain a nitrogen source; however, the presence of glucose in the medium prevents the initiation of sporulation in diploid yeast (KUPIEC *et al.* 1997). The extent of HA-Spo14 phosphorylation was detectable but reduced when cells were placed in SD(-N) medium (compare the electrophoretic mobility of HA-Spo14 isolated from cells grown in SD(-N) *vs.* 2% potassium acetate; Figure 6A). Treatment with SAP confirmed that HA-Spo14 was phosphorylated during exposure to SD(-N) (Figure 6A). These results demonstrate that Spo14 phosphorylation is acutely sensitive to both extracellular nitrogen and glucose levels and suggests that the kinase (or kinases) responsible for Spo14 phosphorylation during sporulation are activated by nutrient limitation.

Only diploids of opposite mating types (*a/α*) are capable of sporulating, while diploids of the same mating type (*a/a* or *α/α*) are incapable of initiating the meiotic program (KUPIEC *et al.* 1997). Taking advantage of this genetic requirement for meiosis and sporulation, the phosphorylation state of Spo14 was determined in diploids homozygous at the mating-type locus. The electrophoretic mobility of HA-Spo14 extracted from *a/a* diploids exposed to SD(-N) and 2% potassium acetate was similarly altered as HA-Spo14 extracted from *a/α* diploid cells exposed to the same medium (Figure 6B). The only noticeable difference between the two diploid strains was a decrease in the total levels of HA-Spo14 detected in the *a/a* diploids. This most probably reflects the fact that diploids homozygous at the *MAT* locus are unable to initiate meiosis and sporulation and *SPO14* is not transcriptionally induced (ROSE *et al.* 1995). Similar to what is observed in meiosis (Rudge *et al.* 1998a), HA-Spo14 is solubilized by detergent in *a/a* cells propagated in sporulation medium (data not shown), indicating that phosphorylation and relocalization are coupled under these conditions. Thus, Spo14 is phosphorylated and relocalizes independently of meiosis.

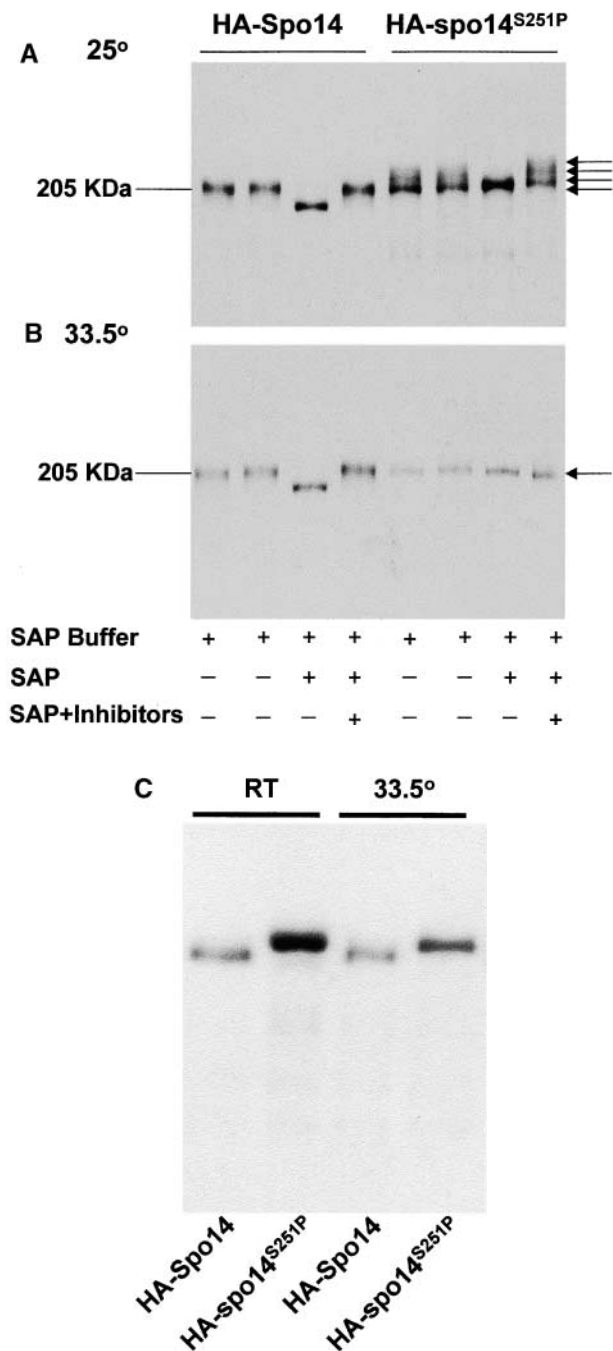


FIGURE 4.—Spo14^{S251P} is improperly phosphorylated. Homozygous *spo14* diploids containing either *HA-SPO14 CEN* or *HA-spo14-S251P CEN* were grown in sporulation medium for 15 hr at (A) 25° and (B) 33.5°. HA-Spo14 and HA-spo14^{S251P} were subsequently immunoprecipitated from Nonidet-P40-solubilized cell extracts, and immunocomplexes were subjected to SAP treatment. The electrophoretic mobility of HA-Spo14 was determined by SDS-PAGE and immunoblot analysis. Arrows denote discrete Spo14 phosphorylated species. (C) Homozygous *spo14* diploids containing either *HA-SPO14 CEN* or *HA-spo14-S251P CEN* were grown in SC-ura for 15 hr at 25° and 33.5°. Whole cell extracts were prepared and the mobility of the proteins determined by SDS-PAGE and immunoblotting.

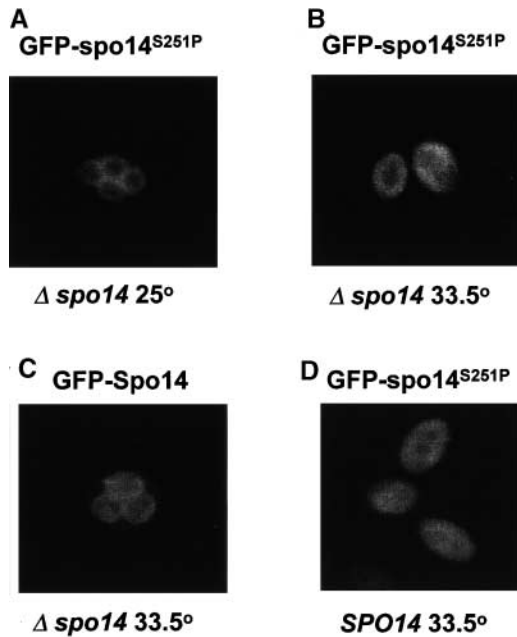


FIGURE 5.—Spo14^{S251P} fails to relocalize to the prospore membrane at the restrictive temperature. Cellular fluorescence of GFP-spo14^{S251P} within fixed homozygous *spo14* diploid cells initiated to sporulate at (A) 25° and (B) 33.5°. (C) Cellular fluorescence of GFP-Spo14 in *spo14* cells sporulated at 33.5°. (D) GFP-spo14^{S251P} fluorescence in wild-type cells sporulated at 33.5°.

DISCUSSION

Spo14 regulation in Sec14-independent secretion:

Spo14 function during spore formation and Sec14-independent secretion are absolutely dependent on PtdIns(4,5)P₂ activation of PLD catalytic activity (RUDGE *et al.* 1998b; SCIORRA *et al.* 1999). Moreover, the correct intracellular location of Spo14 is also essential for PLD function (RUDGE *et al.* 1998b; XIE *et al.* 1998). In this article we provide further evidence that Spo14 function during sporulation is dependent on protein phosphorylation and movement within the cell. In contrast, Spo14 phosphorylation and relocalization do not occur and are indeed both dispensable for Sec14-independent secretion. These results argue against the idea that Spo14 is activated in the absence of Sec14 by relocalizing to regions within the cell necessary for Sec14-independent secretion or that are perhaps rich in PLD substrate (phos-

phatidylcholine) or activator [PtdIns(4,5)P₂] and suggest that Spo14 executes its function within a cellular compartment, presumably endosomal membranes, normally occupied by the protein during Sec14-dependent growth (LI *et al.* 2000a). However, we cannot eliminate the possibility that a fraction of Spo14 resides in another compartment such as the Golgi and that production of phosphatidic acid there drives Sec14-independent secretion. As we discuss below, PtdIns(4,5)P₂ activation of PLD activity does play a major role in regulating Spo14 in secretion.

Further characterization of a partially PtdIns(4,5)P₂-responsive allele of *SPO14*, *spo14-R894G* (SCIORRA *et al.* 1999), indicates that Sec14-independent secretion is more acutely sensitive to loss of maximal Spo14 PLD activity. Moreover, we conclude that PtdIns(4,5)P₂ activation of PLD catalytic activity is perhaps a major regulatory mechanism of Spo14 function in Sec14-independent secretion. In support of this idea, LI *et al.* (2000a) have shown that Spo14 function in secretion is dependent on the presence of members of a novel family of phosphatidylinositol transfer proteins (*SFH* proteins). One of these proteins, Sfh2, co-localizes with Spo14 in the endosome (LI *et al.* 2000a) and is presumably responsible for maintaining phosphoinositide levels at sites of PLD-mediated phosphatidylcholine hydrolysis (LI *et al.* 2000b; RUDGE *et al.* 2001).

SREENIVAS *et al.* (1998) suggested that Sec14 is a negative regulator of Spo14 catalytic activity and that loss of Sec14 results in activation of Spo14. However, overexpression of *SPO14* fails to suppress *sec14-1* in an otherwise wild-type background (XIE *et al.* 1998). In contrast, increased gene dosage of *SPO14* improves Sec14-independent growth (RUDGE *et al.* 2001). To accommodate these findings we propose that Spo14 is activated in Sec14-independent secretion as a consequence of simultaneous loss of Sec14 inhibition and maximal PtdIns(4,5)P₂ activation. However, the precise mechanism of how and where Spo14-mediated hydrolysis of phosphatidylcholine drives Sec14-independent secretion remains elusive.

The role of the N-terminal extension in PLD function:

Analysis of spo14^{S251P} indicates that phosphorylation and relocalization are essential for PLD function in meiosis. These events are tightly coupled, raising the possibility that phosphorylation triggers relocalization of the en-

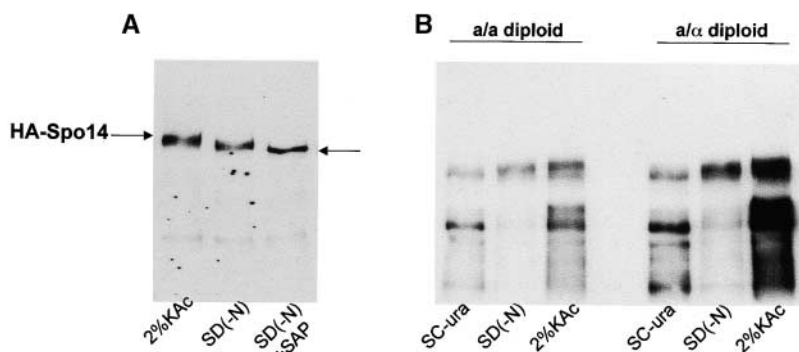


FIGURE 6.—Spo14 phosphorylation is regulated by nitrogen and glucose limitation and occurs independently of the meiotic divisions. (A) Immunoblot of immunoprecipitated HA-Spo14 from extracts of *a/α* diploid wild-type yeast grown in 2% potassium acetate and SD(-N) and treated with SAP. (B) Immunoblot of HA-Spo14 from whole cell extracts of *a/a* and *a/α* diploids grown in SC-ura, 2% potassium acetate, and SD(-N).

zyme as has been demonstrated for protein kinase C translocation (KERANEN *et al.* 1995). However, our data are also consistent with the idea that phosphorylation is a consequence of relocalization.

The finding that *spo14*^{S251P} but not *spo14*^{ΔN}, which harbors a deletion encompassing S251 (RUDGE *et al.* 1998b; XIE *et al.* 1998), supports Sec14-independent secretion indicates that the N terminus is important for more than phosphorylation and relocalization of the enzyme. We propose that the N-terminal domain plays a role in targeting the enzyme to endosomal membranes in vegetative cells and early in meiosis and its absence from endosomes results in loss of PLD activity in both secretion and sporulation. In contrast to deletion of this domain, mutation of serine 251 enables the enzyme to be targeted to endosomal membranes in vegetative cells and early in meiosis; however, introduction of a proline residue prevents phosphorylation and relocalization during meiosis. The inability to phosphorylate serine 251 in *spo14*^{S251P} alone is unlikely to account for the phenotypes associated with *spo14-S251P*, since this potentially phosphorylated residue cannot account for the multiple phosphorylation sites on Spo14 (RUDGE *et al.* 1998b). Thus, we favor the hypothesis that introduction of proline at 251 induces a conformational change in the N terminus of Spo14 such that the mobility of the protein is altered during SDS-PAGE analysis and the N terminus is not phosphorylated and fails to relocalize at elevated temperatures of $\geq 33.5^\circ$. We believe this is a relatively specific effect on phosphorylation and relocalization as the enzyme functions in Sec14-independent secretion and displays wild-type PLD activity. However, we cannot eliminate the possibility that introduction of a proline leads to gross alteration in the secondary structure of the protein and perturbs additional properties of the enzyme.

Alignment of the three fungal PLDs sequenced to date—Spo14, *CaPLD* (*Candida albicans* PLD; KANO *et al.* 1998), and SPAC2F7.16c (*Schizosaccharomyces pombe* PLD; RUDGE and ENGBRECHT 1999)—reveals a single region within the N terminus of Spo14 (amino acids 230–246; KANO *et al.* 1998) that shares significant homology to *CaPLD* (KANO *et al.* 1998) and SPAC2F7.16c (RUDGE and ENGBRECHT 1999). From results presented here, we hypothesize that the extended N termini of *CaPLD* and SPAC2F7.16c are also phosphorylated and serve to regulate the localization of these PLD enzymes to phospholipid membranes necessary for cellular function.

Spo14 kinases: Phosphorylation appears essential for Spo14 function during meiosis and sporulation. Here we report that the kinase(s) responsible for Spo14 phosphorylation are responsive to both nitrogen and glucose limitation. Since it is the absence of extracellular nitrogen and the presence of a nonfermentable carbon source that provokes *S. cerevisiae* to form spores, budding yeast appear to have harnessed a nutritional cue to acti-

vate a kinase (or kinases) responsible for regulating the localization of a PLD activity essential for meiosis and sporulation. The identity of the intracellular signaling pathways that respond to nitrogen and carbon source availability and the kinases responsible for phosphorylating Spo14 merit further study.

We have previously noted that overexpression of Spo14 during sporulation does not result in a concurrent increase in the number of asci formed (RUDGE *et al.* 2001). However, increased gene dosage of *SPO14* significantly improves Sec14-independent secretion (RUDGE *et al.* 2001). These data suggest that there exists a rate-limiting step of Spo14 function in sporulation, but not in secretion. When expressed from a low-copy vector, HA-Spo14 is detected exclusively as a single phosphorylated protein in meiotic cell extracts (RUDGE *et al.* 1998a,b, 2001; this report). Interestingly, expression of HA-Spo14 from a high-copy vector during sporulation results in detection of both a phosphorylated and faster-migrating species (RUDGE *et al.* 1998a). However, in Sec14-independent secretion only the faster-migrating species of Spo14 is detected. These observations indicate that Spo14 phosphorylation may be the rate-limiting step of PLD function during yeast sporulation.

In summary, Spo14 is differentially regulated during sporulation and Sec14-independent secretion. This result is in keeping with our recent work demonstrating that the function of Spo14 is different during sporulation and secretion (RUDGE *et al.* 2001). In Sec14-independent secretion the total cellular levels of phosphatidic acid are sensitive to Spo14 inactivation. In contrast, the total levels of phosphatidic acid within homozygous *spo14* mutants are equivalent to those levels measured in wild-type cells (RUDGE *et al.* 2001). Thus, Spo14 mediates small changes, which we propose are highly localized, of a specific molecular species of phosphatidic acid essential for sporulation (RUDGE *et al.* 2001). Taken together, our data indicate that Spo14 is regulated and functions differently during the processes of sporulation and secretion.

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