Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12151–12155, December 1995
Genetics

Phospholipase D signaling is essential for meiosis

KRISTINE ROSE, SIMON A. RUDGE, MICHAEL A. FROHMAN, ANDREW J. MORRIS, AND JOANNE ENGEBRECHT

Department of Pharmacology, State University of New York, Stony Brook, NY 11794-8651

Communicated by William J. Lennarz, State University of New York, Stony Brook, NY, September 15, 1995

ABSTRACT Phospholipid metabolism plays an important role in cellular regulation by generating second messengers for signal transduction. Many stimuli activate a phospholipase D, which catalyzes the hydrolysis of phosphatidylcholine, producing phosphatidic acid and choline. Here we report that the yeast *SPO14* gene, which is essential for meiosis [Honigberg, S. M., Conicella, C. & Esposito, R. E. (1992) *Genetics* 130, 703–716], encodes a phospholipase D. SPO14 RNA and protein activity are induced during late meiotic prophase, and the enzyme has properties similar to mammalian phosphatidylinositol 4,5-bisphosphate-regulated phospholipase D. Characterization of an unusual allele of *SPO14* defines regions of the protein important for enzyme catalysis and regulation. These results implicate phospholipase D signaling in regulating cellular differentiation.

Lipid metabolism plays a crucial role in cell signaling and differentiation (1). The role of phospholipases A_2 and C in signal transduction pathways is well characterized (2). In addition, many extracellular stimuli activate phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine (PC), producing phosphatidic acid (PA) and choline. PA is thought to be an effector in several physiological processes including secretion (3), DNA synthesis, and cell proliferation (4, 5). PA can also be converted to the second messengers, diacylglycerol (6) or lysophosphatidic acid (7).

At least two classes of membrane-associated PC-specific PLD enzymes have been described in mammalian tissues. One is stimulated by phosphatidylinositol 4,5-bisphosphate (PIP_2) (8), while the other is stimulated by oleate (9, 10). The PIP_2-dependent PLD activity in monocytic cells and brain can be activated by a number of monomeric GTP-binding proteins (8, 11). One class of these, the Arfs, are regulators of protein trafficking (12). This finding has led to the speculation that PLD functions in vesicular movement by altering the local structural characteristics of membranes (13). However, the function of PLD and the role that different isoforms play in signal transduction have remained elusive due to a lack of molecular definition.

Meiotic development in *Saccharomyces cerevisiae* culminates in the production of spores. Genetic analysis of meiosis and spore formation has revealed some of the regulatory mechanisms that control and coordinate these processes. Studies on meiotic initiation show that entry into meiosis is complex and is governed by the integration of both genetic and external signals, which induce a transcriptional program (14). However, the signals that govern meiotic progression are not well understood.

Herein we demonstrate that the yeast *SPO14* gene encodes a PLD. The requirement for PLD in meiosis implies a direct role for phospholipid signaling in regulating cellular differentiation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Yeast Strains. The following *S. cerevisiae* strains were used: KR1-20A, *HO/HO lys2/lys2 ura3-1/ura3-1 leu2/leu2 thr1-4/thr1-4 trp1-1/trp1-1 ade2/ade2 spo14-3/spo14-3*; KR52-3B, *HO/HO ura3-1/ura3-1 leu2/leu2 arg4-8,thr1-4/arg4-8,thr1-4 trp1-1/trp1-1 ade2/ade2*; KR52-3C, KR52-3B containing *spo14::URA3/spo14::URA3*. KR52-3B and KR52-3C are derived by transformation (15) of BR2171-7B (16).

Plasmids. pKR325 contains the *SPO14* complementing region on a 6.0-kb *Pvu* II–*Cla* I fragment at the *Sma* I–*Cla* I sites of pUN55 (ref. 17; see Fig. 2). The 6.0-kb fragment was cloned into Bluescript SK(+) by using *Xba* I and *Cla* I from the polylinker of pKR325 (pKR465). The *spo14::URA3* disruption plasmid KR466 was constructed by inserting a *Eco*RI-blunt-end *URA3* fragment (18) into *Eco*RI–*Eco*RV of *SPO14* in pKR465; this removes 80% (4014 bp) of the *SPO14* coding sequences. pKR466 was targeted for integration in yeast with *Xba* I–*Cla* I. For baculovirus expression, PCR amplification was used to introduce a unique *Not* I site 17 bases upstream of the *SPO14* ATG; a *Not* I-blunt-end *SPO14* fragment was moved into the *Not* I–*Sma* I sites of pVL1392 (Invitrogen) (pKR845). pKR337 contains the 800-bp *Sal* I fragment from pKR325 in Bluescript SK(+). The *PYK1* gene was amplified with *Taq* polymerase from yeast genomic DNA, restricted with *Bgl* II–*Eco*RI and inserted into the *Bam*HI–*Eco*RI sites of Bluescript SK(+) (pME817). The *spo14-3* mutation was cloned by gap repair (15) using pKR325 partially digested with *Bgl* II; pKR493 was recovered and sequenced. The wild-type and mutant sequences were moved into YEp352 (19) by digesting pKR325 and pKR493 with *Kpn* I–*Cla* I (pKR577 and pKR578, respectively). The N-terminal region from pKR493 was subcloned on a *Xba* I-*Hin*dIII fragment into the corresponding sites in pUN105 (17) (pKR842).

Baculovirus Expression. Monolayers of Hi5 cells (3×10^7 cells in a 225-cm^2 flask) were infected with recombinant baculoviruses containing pKR845 at a multiplicity of infection of 10 and cultured at 27°C for 48 h (20). Infected Hi5 cells were washed in ice-cold PBS, scraped into lysis buffer (25 mM Tris·HCl, pH 7.5/2 mM EDTA/1 mM dithiothreitol/0.1 mM benzamidine/0.1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. After an initial centrifugation at 2000 × *g* for 10 min, the supernatant was centrifuged at $10^5 \times g$ for 1 h at 4°C. The supernatant was removed to give the cytosolic fraction and the pellet resuspended in lysis buffer and centrifuged at 2000 × *g* for 5 min to generate the membrane fraction.

PLD Enzyme Assays. PLD activity was assayed by using a ^{32}P-labeled lipid substrate and the formation of [^{32}P]PA was monitored. [^{32}P]PC, [^{32}P]phosphatidylinositol ([^{32}P]PI), and [^{32}P]phosphatidylethanolamine ([^{32}P]PE), with specific radioactivities of ≈10,000 dpm/nmol, were purified by two-dimensional TLC of lipid extract from U937 cells labeled overnight with [^{32}P]PO_4^{-2}. Lipid vesicles of [^{32}P]PC and [^{32}P]PI contained 50 μM ^{32}P-labeled substrate (≈50,000 dpm),

Abbreviations: PLD, phospholipase D; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; Spo14p, Spo14 protein.

95 μM PE (Avanti Polar lipids), and 5 μM PIP_2 (21) and were prepared by bath-sonicating lipid films in 10 mM Hepes (pH 7.0). [^{32}P]PE lipid vesicles contained 50 μM [^{32}P]PE and 5 μM PIP_2. The assay buffer contained 32 mM Hepes (pH 7.0), 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, 40 mM β-glycerophosphate, 1 mM dithiothreitol, and 20 μl of extract (20–30 μg of protein) in 100 μl. Assays were incubated for 30 min at 37°C and terminated by adding 125 μl of methanol. Chloroform (125 μl) was added and the lower phase was collected and dried under vacuum. Lipids were analyzed by TLC on oxalate-impregnated Whatman model 60A glass-backed silica gel plates in a solvent system of chloroform/methanol/acetic acid, 13:3:1 (vol/vol).

RNA Isolation and Analysis. Strains were cultured and assessed as described (16). RNA was prepared by the method of Brill and Sternglanz (22). Northern blot analysis was performed (23) with radioactive RNA probes synthesized by *in vitro* transcription (24) of plasmids pKR337, digested with *Xho* I, and pME817, digested with *Xba* I, using T3 and T7 polymerase (Boehringer Mannheim), respectively.

Preparation of *S. cerevisiae* Cell Homogenates. Cell pellets were resuspended in 400 μl of ice-cold lysis buffer [25 mM Hepes, pH 7.0/150 mM NaCl/5 mM EGTA/1 mM EDTA/40 mM β-glycerophosphate/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/0.2 mM benzamidine (Sigma)]. The cells were disrupted by glass bead (425–600 μm, acid-washed; Sigma) agitation. Protein concentrations were determined (25) by using bovine serum albumin as standard.

RESULTS

Isolation, Cloning, and Mapping of the *SPO14* Gene. The *spo14-3* mutant was isolated in a screen for mutants defective in viable spore production (16), with the additional requirement of proficiency in meiotic recombination (K.R. and J.E., unpublished data). Diploids homozygous for *spo14-3* entered meiosis and completed meiotic prophase but most cells were unable to progress through the meiosis II division and none formed spores (Fig. 1). The wild-type gene was cloned; restriction map and complementation analyses demonstrated that it was identical to the *SPO14* gene (26). Sequences encoding amino acids 130–1467 of the *SPO14* gene were deleted, marked with *URA3* (*spo14::URA3*), and integrated into the yeast genome. The *spo14::URA3* mutant behaved similarly to previously described *spo14* mutants (26). When induced to undergo meiosis, strains homozygous for these mutations displayed a reduction in the number of cells that were able to progress to the meiosis II division. In addition, many cells failed to complete meiosis II chromosome segregation as evidenced by trailing DNA, which appears as cross structures when nuclei are examined cytologically (26). In contrast to the other alleles described above, a smaller fraction of cells progressed to the second meiotic division in *spo14-3* mutants (Fig. 1). The more severe phenotype of the *spo14-3* mutant, compared to the null mutant, suggests that the *spo14-3* gene product interferes with processes necessary for meiotic progression. *spo14-3*/*SPO14* heterozygotes sporulated, indicating that the *spo14-3* allele is recessive.

The *SPO14* Sequence Reveals Homology to Plant PLD. The *SPO14* gene contains a 5049-bp open reading frame encoding a putative protein of 1683 amino acids with a predicted molecular mass of 195 kDa. The *SPO14* sequence matched a hypothetical open reading frame, YKR031c, identified in the chromosome *XI* sequencing project (27). In addition, a region of 440 amino acids in the middle of the Spo14 protein (Spo14p) is 21% identical to the predicted protein sequence of a PLD identified from *Ricinus communis* (ref. 28 and Fig. 2).

Recombinant Spo14p Hydrolyzes PC and Requires PIP_2. The similarity to a PLD prompted us to investigate whether *SPO14* encoded a functional enzyme. *SPO14* was expressed in Hi5 insect cells by using a baculovirus vector (20). The infected cells were separated into membrane and cytosolic fractions and PLD activity was measured by using ^{32}P-labeled lipids

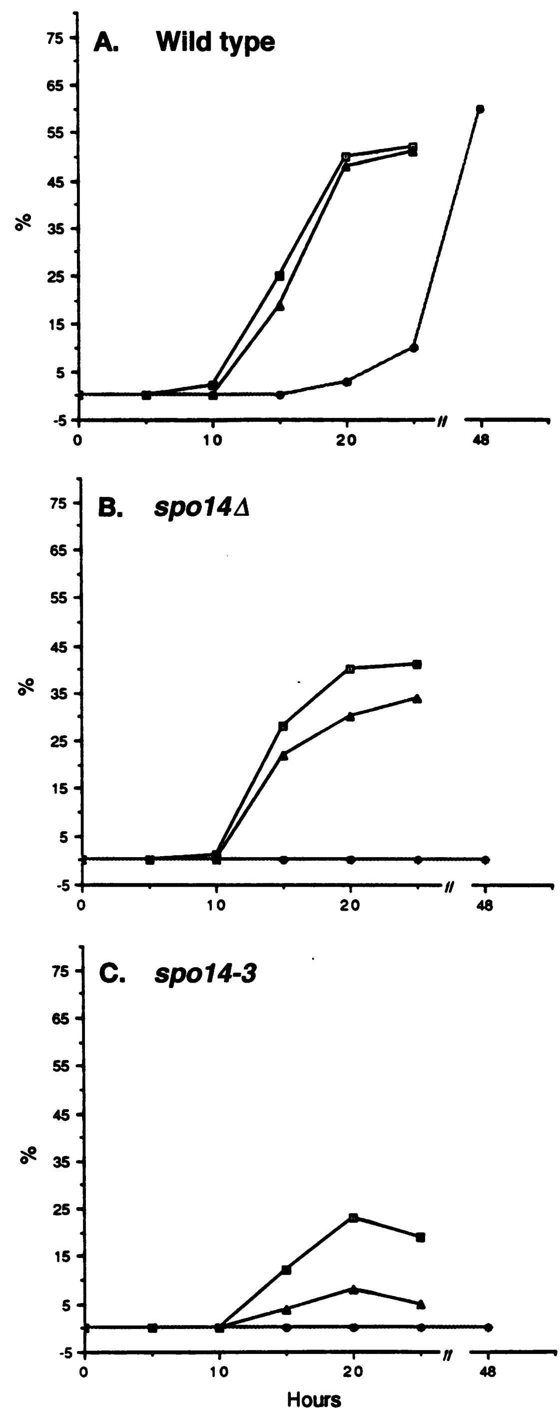

FIG. 1. Effect of *spo14* mutations on the meiotic divisions and sporulation. (*A*) *SPO14*/*SPO14* (KR52-3B). (*B*) *spo14::URA3*/*spo14::URA3* (KR52-3C). (*C*) *spo14-3*/*spo14-3* (KR1-20A). The percentage of cells completing meiosis I (open squares) or meiosis II (solid triangles) were monitored by 4′,6-diamidino-2-phenylindole fluorescence microscopy, and ascus formation (solid circles) was monitored by phase-contrast microscopy. The percentage of cells having completed meiosis I was calculated by dividing the sum of bi-, tri-, and tetranucleated cells and asci by the total number of cells. The percentage of cells having completed meiosis II was similarly calculated by dividing the sum of tri- and tetranucleated cells and asci by the total number of cells. At least 300 nuclei were counted at each time point for each strain.

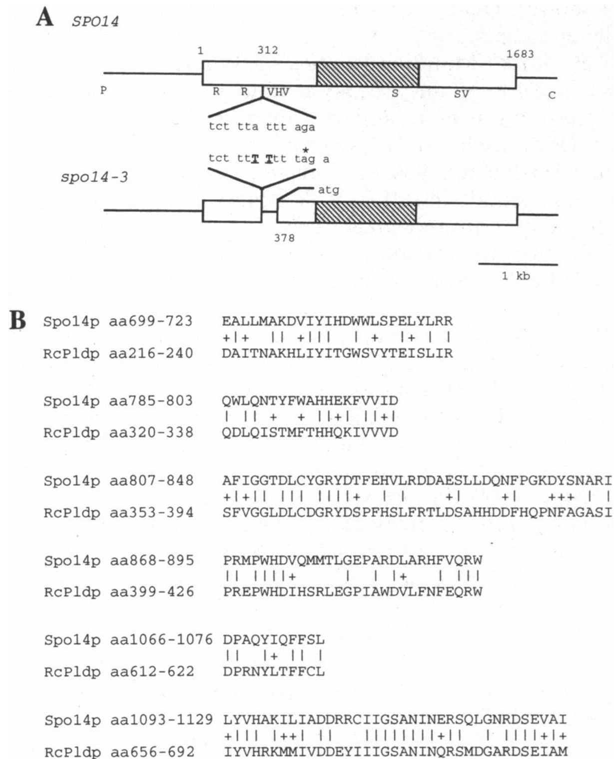

FIG. 2. *SPO14* gene structure (*A*) and similarity to plant PLD (*B*). Numbers refer to amino acid positions. The nucleotide sequence surrounding the *spo14-3* mutation (and the corresponding region in *SPO14*) is shown. The underlined bases represent the base change and insertion in the *spo14-3* sequence; * indicates the introduced stop codon. The ATG at amino acid 378 denotes a likely site of translation reinitiation; there are three additional ATGs, which would encode for very short peptides, between the termination sequence and the ATG at amino acid 378. The hatched box delineates the region with similarity to plant PLD (28). P, *Pvu* II; R, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; S, *Sal* I; C, *Cla* I; Rc, *Ricinus communis*; aa, amino acid; |, identical amino acids; +, conservative changes.

followed by TLC of the products. Membranes from Hi5 cells uninfected or infected with an unrelated viral construct contained very little PLD activity, while cells infected with the *SPO14* baculovirus vector exhibited substantial membrane-associated PLD activity (Fig. 3*A*).

Phospholipases are classified according to the bond they hydrolyze as well as their phospholipid substrate specificity. Hydrolysis of PC by the Spo14p resulted in the simultaneous production of PA and choline, indicative of PLD activity, and was determined by analyzing the water-soluble products by HPLC (data not shown). The specificity of Spo14p was examined by substituting the labeled PC with [^{32}P]PI or [^{32}P]PE. Essentially no hydrolysis was observed with PI and PE (Fig. 3*A*). PC-specific PLDs have been shown to catalyze the transfer of the substrate phosphatidyl group to a primary alcohol, generating a phosphatidylalcohol (29). In the presence of 2% ethanol, Spo14p catalyzed the formation of both PA and PE (Fig. 3*A*). Thus, these results show that Spo14p is a PC-specific PLD.

PC-specific PLDs have been classified based on cofactors required for activity; PIP_2- and oleate-dependent PLDs have been characterized. Spo14p is a member of the former class as it was stimulated by PIP_2 and inhibited by oleate (Fig. 3*A*). Additionally, Spo14p was inhibited by Mg^{2+}. The enzymatic properties of the recombinant protein were identical to the enzyme activity measured in meiotic yeast cell homogenates (Fig. 3*B*).

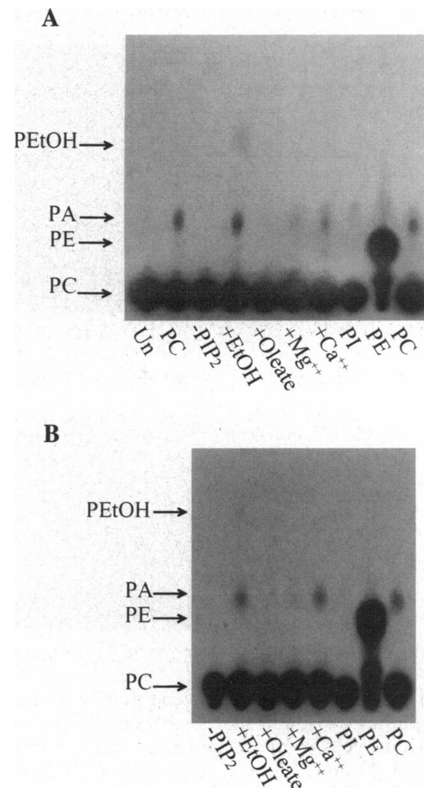

FIG. 3. Properties of Spo14p in Hi5 and meiotic yeast cell homogenates. (*A*) Autoradiograph of a TLC plate demonstrating PLD activity from Hi5 membranes expressing Spo14p. (*B*) Autoradiograph of a TLC plate demonstrating PLD activity from meiotic yeast cell homogenates. Un, uninfected Hi5 cells; PC, standard assay conditions using PC as substrate; $-PIP_2$, without the addition of PIP_2; + EtOH, in the presence of 2% ethanol; + Oleate, in the presence of 4 mM oleate; + Mg^{++}, in the presence of 1 mM Mg^{2+}; + Ca^{++}, in the presence of 1 mM Ca^{2+}; PI, with PI as substrate; PE, with PE as substrate. [^{32}P]PI and [^{32}P]PE vesicles were analyzed by TLC prior to the addition of enzyme and were identical to the samples shown. PA, PC, PE, and phosphatidylethanol (PEtOH) are indicated and were determined by the migration of standards. Equivalent amounts of extracts were used in each assay as determined by protein concentration.

SPO14 RNA and Activity Are Induced Late in Meiotic Prophase. Northern blot analysis was performed on RNA from wild-type, *spo14* deletion, and *spo14-3* strains in mitosis and at various times throughout meiosis (Fig. 4*A*). A 5.5-kb RNA molecule was detected with a radiolabeled *SPO14*-specific antisense RNA but was absent in *spo14*::*URA3* strains. In addition, a less-abundant *SPO14*-specific transcript was observed (2.8 kb) that is probably a degradative product of the large transcript. *SPO14* RNA was present in mitotically dividing cells (0 h) and levels increased 7-fold late in prophase (15 h; RNA amounts were normalized by probing with the constitutively expressed gene *PYK1*; ref. 30). SPO14 RNA levels remained high during the two meiotic divisions (15–20 h) and declined at the completion of sporulation (25 h). The *SPO14* induction profile was similar to genes expressed midway through meiosis (14), but in contrast to these genes, *SPO14* expression was not meiosis-specific. The *SPO14* transcript level was increased in response to meiosis and not starvation as no RNA induction was observed when RNA from a *MATa*/*MATa* strain, which is unable to initiate meiosis, was analyzed (data not shown). RNA from *spo14-3* strains was also maximally expressed at 15 h, although at lower levels (Fig. 4*A*).

PLD activity was measured during mitosis and meiosis by using an *in vitro* assay with cell homogenates. Enzyme activity

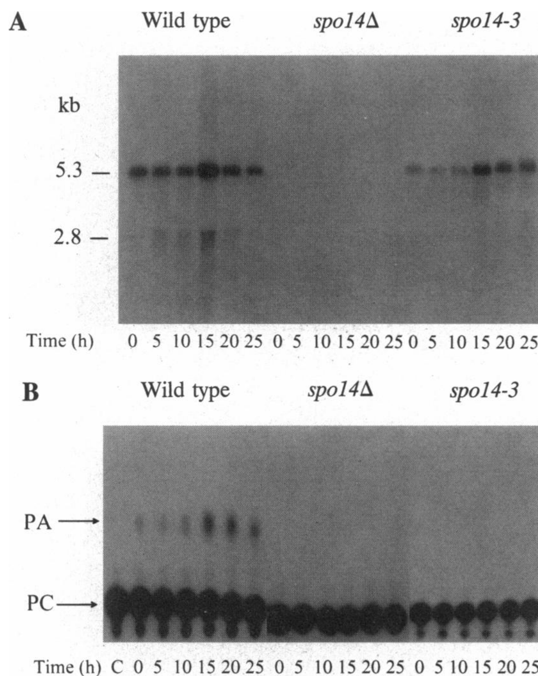

FIG. 4. Expression of the *SPO14* gene and enzyme activity. (*A*) Autoradiograph of a Northern blot analysis of RNA extracted from mitotically dividing cells (0 h) and at various times after induction of meiosis (5, 10, 15, 20, and 25 h). Wild type, *SPO14/SPO14* (KR52-3B); *spo14Δ*, *spo14::URA3/spo14::URA3* (KR52-3C); *spo14-3*, *spo14-3/spo14-3* (KR1-20A). Positions of molecular size standards are to the left. (*B*) Autoradiograph of a TLC plate demonstrating PLD activity in yeast homogenates corresponding to the same time course analyzed by Northern blot analysis. PA and PC are indicated and were determined by the migration of standards. Lane C, the reaction products from a reaction terminated without addition of extract.

paralleled RNA levels, being maximally induced 5-fold at 15 h (Fig. 4*B*). No activity was detected in the *spo14* deletion or *spo14-3* strain, indicating that *SPO14* encodes the only PLD active under these assay conditions.

The finding that *SPO14* is expressed during mitosis prompted us to examine the effect of a *spo14* null mutation on mitotic growth and signaling pathways in yeast. Four functionally distinct vegetative signal transduction pathways utilizing mitogen-activated protein (MAP) kinases have been identified in yeast: cell wall integrity, response to high osmolarity, pseudohyphal development, and mating. Consistent with analysis of previously characterized *spo14* mutants (26), strains harboring the *spo14::URA3* deletion grew at rates indistinguishable from an isogenic wild-type strain at all temperatures examined. In addition, *spo14* deletion strains did not exhibit defects in any of the vegetative MAP kinase pathways (data not shown). Hence, it is likely that Spo14p functions specifically in meiosis.

The *spo14-3* Mutation Produces a Truncated Protein. Genetic analysis of the *spo14-3* mutant revealed that meiotic progression was more impaired than in *SPO14* deletion mutants (Fig. 1). The *spo14-3* sequence had two changes: an A → T transversion at nt 936, resulting in a Leu-312 → Phe change, and a nucleotide insertion at the transversion, introducing a stop codon 3 nt downstream (Fig. 2). The *spo14-3* mutation should, therefore, result in a truncated protein coding for the first 313 amino acids of the *SPO14* gene with a single amino acid change.

Wild-type cells containing sequences encoding the first 313 amino acids of *spo14-3* on a centromere plasmid (p*CENspo14-313*; pKR842) were assessed for meiosis and sporulation (Table 1). Meiosis was inhibited in these strains as demonstrated by the number of cells progressing through the meiotic divisions, and sporulation was reduced. The effect of p*CENspo14-313* on meiosis and sporulation was not due to inhibition of PLD activity. When expressed in a *spo14* deletion strain *pCENspo14-313* also inhibited meiosis, suggesting that *spo14-313* is interfering with another process important for meiosis.

Surprisingly, a *spo14* deletion strain carrying the entire *spo14-3* gene on a high-copy-number plasmid (pKR578) produced spores, albeit at low levels (8%), and contained low PLD activity (≈10% of wild-type activity). In contrast, no spores and no PLD activity were observed in the deletion strain carrying the *spo14-3* gene on a low-copy-number plasmid (pKR493). Since the N-terminal region of the spo14-3 protein does not contain PLD activity (see above), the most likely explanation is that translation reinitiation is occurring, presumably at Met-378, and the resulting 1306 amino acids contain the catalytic domain (Fig. 2).

DISCUSSION

SPO14 encodes a PLD activity that is essential for yeast meiosis. The yeast enzyme is similar to the PIP_2-dependent PLD activity characterized from mammalian cells (8). However, Spo14p is inhibited by Mg^{2+} and catalyzes the transphosphatidylation reaction poorly, in contrast to mammalian PIP_2-dependent PLD (8) and plant PLD (28).

Another group (31) has reported regulated PLD activity in yeast cell extracts, but the assay used and the activity detected are different in several respects to those reported herein. (*i*) A synthetic fluorescent lipid (1-acyl-2-alkyl-glycerophosphocholine) in detergent was used as substrate. (*ii*) The enzyme activity was measured in the absence of PIP_2, a known activator of Spo14p (Fig. 3). (*iii*) The activity was induced immediately upon transfer to starvation conditions. Thus, it is likely that Ella *et al.* (31) were not measuring Spo14p activity, suggesting that there may be other PLD activities in yeast.

Table 1. Effect of *spo14-313* on meiotic progression in wild-type and *spo14::URA3* cells

Strain	Genotype	Meiosis I, %	Meiosis II, %	Relative PLD activity	% sporulation
KR52-3B (pUN100)	*SPO14* (p*CEN*)	34	29	1.0	30
KR52-3B (pKR842)	*SPO14* (p*CENspo14-313*)	10	6	0.96	23
KR52-3C (pUN100)	*spo14::URA3* (p*CEN*)	29	19	0	0
KR52-3C (pKR842)	*spo14::URA3* (p*CENspo14-313*)	10	4	0	0

Strains were grown and sporulated as described (16); samples were removed after 20 h and examined by 4′,6-diamidino-2-phenylindole fluorescence. The percentage of cells having completed meiosis I was calculated by dividing the sum of bi-, tri-, and tetranucleate cells by the total number of cells. The percentage of cells having completed meiosis II was calculated by dividing the sum of tri- and tetranucleated cells by the total number of cells. At least 300 cells of each genotype were evaluated. PLD activity was determined as described in the text. Densitometry scanning was performed and normalized for protein content. Activity from the wild-type extract was given a relative value of 1.0. To measure sporulation, cultures were examined by phase-contrast microscopy after 48 h in sporulation medium. The percent sporulation was calculated by dividing the number of asci containing two, three, or four spores by the total number of cells. p*CEN*, centromere-based plasmid; p*CENspo14-313*, the N-terminal fragment of *spo14-3* on a centromere-based plasmid.

The *spo14-3* allele is inhibitory to meiosis, demonstrating that the N-terminal region of Spo14p is deleterious in the absence of the catalytic domain. One apparent contradiction in the behavior of the truncated protein is that heterozygous *spo14-3/SPO14* strains sporulate close to wild-type levels while strains carrying the p*CENspo14-313* sporulate at reduced levels (Table 1). There are several possible reasons for this difference: (*i*) the truncated protein may be present at lower levels than expected in the heterozygote due to mRNA instability caused by the presence of an internal stop codon (ref. 32 and Fig. 4*A*); (*ii*) the C-terminal protein derived from reinitiation in the heterozygous mutant allele (Fig. 2) may interfere with the inhibitory action of the N-terminal portion; (*iii*) the centromere plasmid may exist in more than one copy per cell (33), effectively increasing the concentration of the truncated protein relative to the heterozygote.

Spo14p may function to coordinate meiosis and sporulation by generating a lipid messenger or by altering the local structural characteristics of membranes. The identification of the sporulation-specific kinases, *SMK1* and *SPS1*, with homology to MAP kinase and Ste20p kinase, respectively, implies that a signaling pathway is operating to coordinate spore wall assembly (30, 34). In addition, substantial membrane reordering occurs during meiosis and sporulation in yeast (35).

Meiotic arrest at either metaphase I or II before fertilization is a common component of oogenesis in many organisms. Release of the arrest is usually triggered by external cues and is mediated via a signal transduction pathway (36). Unlike higher eukaryotes, yeast do not normally arrest during meiosis. Once normal yeast cells execute the first meiotic division, they are committed and are no longer responsive to external stimuli (37). Since Spo14p appears to function after the point of commitment, it is unlikely that extracellular stimuli are activating Spo14p. Spo14p may be activated by an internal event, perhaps involving some aspect of DNA metabolism or transcription.

J.E. and A.J.M. were coadvisors on this project. We thank L. Davis, N. Hollingsworth, and S. Strickland for helpful discussions and comments on the manuscript and T. Kessel and K. Sanyal for expert technical help. This work was supported by National Institutes of Health Grants GM4863903 to J.E. and GM50388 to A.J.M. J.E. is a recipient of American Cancer Society Junior Faculty Research Award and S.A.R. has an Affiliate Fellowship from New York State Heart Association (Grant 950209). K.R. is supported by National Institutes of Health Training Grant 5T32GM07518 and by a Hoffmann–La Roche scholarship from the Institute for Cell and Developmental Biology.

1. Divecha, N. & Irvine, R. (1995) *Cell* **80,** 269–278.
2. Exton, J. H. (1994) *Biochim. Biophys. Acta* **1212,** 26–42.
3. Simmonds, A. C. & Halsey, M. J. (1985) *Biochim. Biophys. Acta* **813,** 331–337.
4. Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. & Laat, S. W. (1986) *Nature (London)* **323,** 171–173.
5. Yu, C.-L., Tsai, M.-H. & Stacey, D. W. (1988) *Cell* **52,** 63–71.
6. Nishizuka, Y. (1995) *FASEB J.* **9,** 484–496.
7. Moolenaar, W. H. (1995) *J. Biol. Chem.* **270,** 12949–12952.
8. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. & Sternweis, P. C. (1993) *Cell* **75,** 1137–1144.
9. Okamura, S. & Yamashita, S. (1994) *J. Biol. Chem.* **269,** 31207–31213.
10. Massenburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J. & Vaughan, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11718–11722.
11. Singer, W. D., Brown, H. A., Bokoch, G. M. & Sternweis, P. C. (1995) *J. Biol. Chem.* **270,** 14944–14950.
12. Boman, A. L. & Kahn, R. A. (1995) *Trends Biochem. Sci.* **4,** 147–150.
13. Liscovitch, M. & Cantley, L. C. (1995) *Cell* **81,** 659–662.
14. Mitchell, A. P. (1994) *Microbiol. Rev.* **58,** 56–70.
15. Rothstein, R. (1991) *Methods Enzymol.* **1994,** 281–301.
16. Engebrecht, J. & Roeder, G. S. (1989) *Genetics* **121,** 237–247.
17. Elledge, S. J. & Davis, R. W. (1988) *Gene* **70,** 303–312.
18. Siefert, H. S., Chen, E. Y., So, M. & Heffron, F. (1986) *Proc. Natl. Acad. Sci USA* **83,** 735–739.
19. Hill, J. E., Myers, A. M., Koerner, T. J. & Tzagoloff, A. (1986) *Yeast* **2,** 163–167.
20. O'Reilly, D. R., Miller, L. K. & Luckow, V. A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual* (Freeman, New York).
21. Morris, A., Rudge, S., Mahlum, C. & Jenco, J. (1995) *Mol. Pharmacol.* **48,** 532–539.
22. Brill, S. J. & Sternglanz, R. (1988) *Cell* **54,** 403–411.
23. Low, R. & Rausch, T. (1994) *BioTechniques* **17,** 1026–1030.
24. Krieg, P. A. & Melton, D. A. (1987) *Methods Enzymol.* **155,** 397–415.
25. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–252.
26. Honigberg, S. M., Conicella, C. & Esposito, R. E. (1992) *Genetics* **130,** 703–716.
27. Dujon, B., Alexandraki, D., André, B., Ansorge, W., Baladron, V., *et al.* (1994) *Nature (London)* **369,** 371–378.
28. Wang, X., Xu, L. & Sheng, L. (1994) *J. Biol. Chem.* **269,** 20312–20317.
29. Kobayashi, M. & Kanfer, J. N. (1987) *J. Neurochem.* **48,** 1597–1603.
30. Friesen, H., Lunz, R., Doyle, S. & Segall, J. (1994) *Genes Dev.* **8,** 2162–2175.
31. Ella, K. M., Dolan, J. W. & Meier, K. E. (1995) *Biochem. J.* **307,** 799–805.
32. Peltz, S. W., He, F., Welch, E. & Jacobson, A. (1994) *Prog. Nucleic Acid Res. Mol. Biol.* **47,** 271–298.
33. Clarke, L. & Carbon, J. (1980) *Nature (London)* **287,** 504–506.
34. Krisak, L., Strich, R., Winters, R. S., Hall, J. P., Mallory, M. J., Kreitzer, D., Tuan, R. S. & Winter, E. (1994) *Genes Dev.* **8,** 2151–2161.
35. Byers, B. (1981) in *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, eds. Strathern, J. N., Jones, E. W. & Broach, J. (Cold Spring Harbor, Lab. Press, Plainview, NY), pp. 59–96.
36. Murray, A. W. (1992) *Nature (London)* **359,** 599–604.
37. Simchen, G., Pinon, R. & Salts, Y. (1972) *Exp. Cell Res.* **75,** 297–318.