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The CDK-activating kinase *CAK1* can dosage suppress sporulation defects of *smk1* MAP kinase mutants and is required for spore wall morphogenesis in *Saccharomyces cerevisiae*

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Mitogen-activated protein (MAP) kinase pathways are evolutionarily conserved kinase cascades that are required for the response of eukaryotic cells to a wide variety of environmental stimuli. MAP kinase pathways are also required for the execution of developmental and differentiative programs in a variety of cell and tissue types. *SMK1* encodes a developmentally regulated MAP kinase in yeast that is required for spore wall morphogenesis. Cyclin-dependent kinase-activating kinases (CAKs) phosphorylate a conserved threonine residue in the activating loop of cyclin-dependent kinases. *CAK1* encodes the major CAK activity in yeast and is required for cell cycle progression. The work presented here demonstrates that *CAK1* functions positively in the spore wall morphogenesis pathway. First, *CAK1* has been isolated as a dosage suppressor of a conditional *smk1* mutant that is defective for spore wall morphogenesis. Second, *CAK1* mRNA accumulates during spore development contemporaneously with *SMK1* mRNA. Third, *cak1* mutant strains have been isolated that are able to complete meiosis I and II but are specifically defective in assembly of the spore wall. These results show that cell cycle progression and morphogenetic pathways can be regulated by a single gene product and suggest mechanisms for coordinating these processes during development.

Keywords: CAK/development/MAP kinase/sporulation/yeast

Introduction

Mitogen-activated protein (MAP) kinases comprise a family of signal transducing enzymes that phosphorylate regulatory molecules in response to a broad array of stimuli (Blenis, 1993; Blumer and Johnson, 1994; Marshall, 1994; Waskiewicz and Cooper, 1995). MAP kinases are activated by dual specificity MAP kinase kinases (MEKs) that phosphorylate both a tyrosine and threonine residue in the active site lip of the enzyme. MEKs are activated via serine and threonine phosphorylation by upstream MEK kinases. This sequential activation of MAP kinase by MEK through MEK kinase constitutes what has been defined as the MAP kinase module (Neiman *et al.*, 1993). These modules have been evolutionarily conserved throughout eukaryotes in the biochemical mechanism and amino acid sequence of the individual kinase members. Activated MAP kinases phosphorylate regulatory molecules, such as transcription factors and components of the cell cycle machinery, which effect cellular responses appropriate to the input signal. These cellular responses can include adaptation to environmental stress, changes in proliferative activity, and induction and execution of developmental programs. The roles of MAP kinase pathways in development have been studied in a wide range of cell and tissue types and a diverse set of organisms. MAP kinase pathways have been shown to be required for neural differentiation in mammalian PC12 cells (Marshall, 1994), mesoderm induction in *Xenopus* (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995; Umbhauer *et al.*, 1995), eye development in *Drosophila* (Perrimon, 1994), vulval development in *Caenorhabditis elegans* (Eisenmann and Kim, 1994) and cell fate decisions in *Dictyostelium* (Firtel, 1995).

In the yeast *Saccharomyces cerevisiae*, there are a number of MAP kinase pathways that elicit appropriate responses to particular stimuli (Ammerer, 1994; Herskowitz, 1995; Kron and Gow, 1995; Levin and Errede, 1995). The mating pheromone response pathway is the most thoroughly understood and represents a paradigm for the molecular mechanisms that mediate MAP kinase signaling. Additional MAP kinase pathways in yeast include the osmoregulatory pathway, the protein kinase C/cell wall integrity pathway, the pseudohyphal differentiation pathway and the *SMK1* spore wall morphogenesis pathway. *SMK1* (sporulation MAP kinase 1) encodes a developmentally regulated MAP kinase in yeast that is essential for the completion of sporulation (Krisak *et al.*, 1994).

Sporulation in yeast provides a model system in which to study developmental processes. Similarly to differentiation programs in higher eukaryotic cells, induction of sporulation is controlled by a combination of cell type and environmental signals (Esposito and Klapholz, 1981). Yeast spore development is restricted to the **a**/α diploid and occurs in response to starvation for nitrogen and a fermentable carbon source. Upon initiation, cells will exit the mitotic cell cycle and enter meiosis. The four main phases or landmark events of sporulation are: (i) meiotic prophase during which DNA synthesis, meiotic recombination and formation of synaptonemal complexes occur; (ii) meiosis I or the reductional division where homologous chromosomes segregate from each other; (iii) meiosis II or the equational division when sister chromatids segregate; and (iv) spore wall morphogenesis and spore maturation. The end-product of development is an ascospore which contains four haploid spores, two **a** and two α, which are distinct cell types from the starting diploid cell. Spore development is tightly regulated at the level of transcription (Mitchell, 1994). Upon initiation, a cascade of sporulation-specific gene expression ensues with genes

being classified as early genes expressed at the onset of meiotic prophase, middle genes expressed during the meiotic divisions and late genes expressed during spore wall morphogenesis. Thus, sporulation in yeast is an example of a developmental process that is intimately coupled to a transcriptional program.

SMK1 is a tightly regulated middle sporulation gene required for morphogenesis of the spore wall and completion of the sporulation program (Krisak *et al.*, 1994). Spore wall morphogenesis is characterized by growth of a bimembranous prospore wall which nucleates at each meiosis II spindle pole body and eventually encloses each meiotic product. Subsequently, the four-layered spore wall is assembled from within/around the prospore wall (Byers, 1981; Esposito and Klapholz, 1981). The two innermost layers are made primarily of glucan; the next layer contains chitin and chitosan; and the outermost layer is rich in dityrosine (Briza *et al.*, 1986, 1988, 1990b). These layers are vital to the integrity of the spore as they render it resistant to environmental stresses. *smk1* null mutants initiate sporulation and progress through meiosis I and II normally. However, functional and ultrastructural assays show that subsequent developmental events are defective. Electron microscopy of *smk1* null homozygous asci reveals a variety of aberrant spore wall assembly patterns. Even within a single *smk1* mutant ascospore, varied aberrant patterns are observed. Spore wall layers are either missing, extranumerary or improperly ordered. These data indicate that *SMK1* is required for the coordination of spore wall morphogenesis. Additionally, transcription of late but not early or middle sporulation genes is significantly reduced in *smk1* null mutants, suggesting that *SMK1* is also required for subsequent steps in spore development and maturation. *SPS1* encodes a protein kinase homolog which is also required for spore wall assembly (Friesen *et al.*, 1994). *SPS1*, like *SMK1*, is expressed as a middle sporulation gene. Also, *sps1* null homozygous asci exhibit analogous defects in spore wall morphogenesis and transcription of late sporulation-specific genes. These data and the homology of Sps1p to the Ste20p/Pak family of MAP kinase cascade activators have led to the proposal that *SPS1* is an upstream kinase in the *SMK1* spore wall morphogenesis pathway. The *SMK1*/*SPS1* pathway and sporulation affords an excellent opportunity to study how a MAP kinase signaling pathway can coordinate complex processes during development.

Here we describe the isolation of a collection of temperature-sensitive *smk1* alleles. One of these alleles, *smk1-2*, has been characterized in detail and is defective for spore wall morphogenesis and subsequent developmental events. A dosage suppressor of the *smk1-2* mutant phenotype has been identified. This dosage suppressor is allelic to *CAK1* (cyclin-dependent kinase-activating kinase 1) which encodes an essential protein kinase that has been shown by others to activate Cdc28p (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996; Thuret *et al.*, 1996). Activated Cdc28p is required for and regulates mitotic as well as meiotic cell divisions. An allele of *cak1* which allows initiation of sporulation and completion of meiosis I and II, but not spore wall morphogenesis, has been isolated. Thus, *CAK1* plays a positive regulatory role in and is required for spore wall morphogenesis. These results suggest that *CAK1* may play a role in coordinating meiotic cell divisions with spore wall morphogenesis during spore development.

Results

Isolation of smk1 conditional alleles

The spore wall contains high levels of insoluble dityrosine, which fluoresces naturally in the visible spectrum when excited by UV light. The fluorescence of sporulated colonies, hereafter referred to as the fluorescence assay, provides a sensitive method by which spore wall assembly may be monitored (see Materials and methods). When subjected to this assay, sporulated wild-type diploids fluoresce while *smk1*-Δ, *sps1*-Δ and *smk1*-Δ*sps1*-Δ homozygous diploids do not. In order to isolate a collection of *smk1* conditional alleles, an *smk1*-Δ homozygous diploid was transformed with an *smk1* mutant plasmid library which had been generated *in vitro* by hydroxylamine mutagenesis. Transformants were sporulated at the permissive (26°C) and non-permissive (34°C) temperatures and scored by the fluorescence assay. Twenty *smk1* temperature-sensitive alleles have been identified in this manner, and *smk1* mutant diploid strains generated by replacing the chromosomal *SMK1* locus via standard homologous recombination techniques. All alleles isolated are recessive with respect to the fluorescence phenotype. Further characterization of one of these alleles, *smk1-2*, is described below. Analyses and detailed phenotypic characterization of other *smk1* conditional mutants will be described elsewhere.

Characterization of the smk1-2 allele

The fluorescence phenotype of a sporulated *smk1-2*/*smk1*-Δ diploid is shown in Figure 1A. At the permissive temperature, the *smk1-2* strain fluorescence is comparable with that of the wild-type, while at the non-permissive temperature *smk1-2* fluorescence is comparable with that of the *smk1* null mutant. *smk1* null mutant spores are known to be hypersensitive to heat shock and exposure to glusulase or ether. Additionally, *smk1*-Δ null mutants are deficient in transcription of late sporulation genes, such as *SPS100*. The resistances of *smk1-2*, *smk1*-Δ and wild-type spores formed at 26°C or 34°C to heat shock, glusulase or ether treatments were compared. Additionally, comparisons of *SPS100–lacZ* reporter gene expression in these sporulated strains were made (see Materials and methods). The *smk1-2* spores exhibited temperature-senstive defects in all of these phenotypes (Figure 1B). However, the resistance to the environmental stresses was not completely wild-type at the permissive temperature. This suggests that, despite the ability to assemble birefringent spore walls (see below) and to fluoresce to wild-type levels, *smk1-2* spores formed at the permissive temperature may be deficient in subtle aspects of spore wall morphogenesis or maturation. Interestingly, *smk1-2* spores formed at the non-permissive temperature are still less sensitive to heat shock, glusulase or ether treatments than are *smk1*-Δ spores. This suggests that certain developmental events that lead to these resistance phenotypes may be executed in the *smk1-2* mutant under these conditions.

Phase-contrast and fluorescence microscopy of DAPI-stained cultures sporulated at the permissive temperature revealed that *smk1-2* ascospores are indistinguishable from

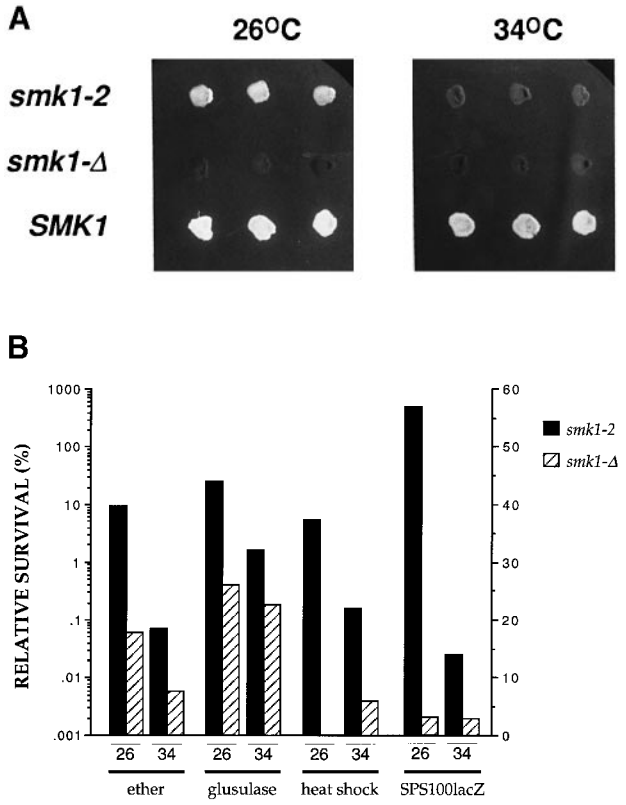

Fig. 1. Sporulation phenotypes of *smk1-2* mutants. (**A**) Fluorescence assay of *smk1* strains. Patches of wild-type, *smk1-Δ3* null and *smk1-2* temperature-sensitive strains were grown on solid YPD medium, replica plated to nitrocellulose filters, which were incubated on solid sporulation medium at the permissive (26°C) and non-permissive (34°C) temperatures, and fluorescence assays carried out as described in Materials and methods. (**B**) Heat shock, ether and glusulase resistance, and *SPS100lacZ* expression levels of *smk1* strains. Resistance is presented on a logarithmic scale (left *y*-axis) relative to wild-type. Expression of *SPS100lacZ* is shown on a linear scale (right *y*-axis) relative to wild-type. All values for wild-type control strain (LNY150) are taken as 100%, and numbers for the mutant strains normalized accordingly. The value for heat shock resistance of the *smk1*Δ strain at 26°C is 0.001%.

wild-type (Figure 2). In both wild-type and *smk1-2* mutant cultures there were four distinct nuclei, each encapsulated by a refractile spore wall, in >85% of the ascospores. *smk1-2* cultures sporulated at the non-permissive temperature initiated sporulation and completed meiosis I and II with the same efficiency as wild-type, as evidenced by the presence of four distinct DAPI-staining foci. However, in contrast to both the *smk1-2* spores formed at 26°C and the wild-type spores formed at 34°C, <1% of *smk1-2* spores formed at the non-permissive temperature were surrounded by birefringent spore walls.

Nucleotide sequence analysis of the *smk1-2* gene revealed that it contains a single missense mutation in codon 169 which results in a serine for proline substitution. This altered residue lies in subdomain VI, which is highly conserved in all protein kinases and is 14 residues amino-terminal to the catalytic aspartate in the presumed activation loop of Smk1p (Hanks *et al.*, 1988). The analogous amino acid substitution in the *Schizosaccharomyces pombe* Cdc2, *Drosophila* MEK and *Dictyostelium* Erk2 kinases also confers a temperature-sensitive phenotype (Carr *et al.*, 1989; Hsu and Perrimon, 1994; Gaskins *et al.*, 1996). Western analysis of an epitope-tagged Smk1-2 protein indicates that the P169S substitution does not affect protein stability, and thus the mutation must hinder either the ability of Smk1-2p to be activated by upstream signaling components or its ability to interact with or phosphorylate downstream effectors required for spore development.

Isolation of CAK1 as a dosage suppressor of the smk1-2 fluorescence defect

A genomic library of yeast DNA contained on a high-copy (2μ-based) vector was used to identify sequences able to suppress the *smk1-2* fluorescence defect at the non-permissive temperature. Three classes of plasmid were recovered. Class I transformants fluoresced to levels indistinguishable from that seen in the wild-type control strain. Diagnostic restriction enzyme analysis showed that this class of plasmid contains the *SMK1* gene. Class II transformants fluoresced to an intermediate level at the non-permissive temperature, and all class II plasmids share a common set of restriction enzyme fragments. Characterization of the class II suppressor is described in detail below. Class III suppressors, which yielded a relatively weak but reproducible increase in fluorescence, will be described elsewhere.

Plasmid pHCS12-5 is the class II plasmid that contains the smallest DNA fragment capable of suppressing the *smk1-2* fluorescence defect. Nucleotide sequence analysis of the pHCS12-5 insert revealed that it contains a 3.4 kb genomic fragment from chromosome VI with two complete putative open reading frames (YFL029c and YFL030w). Fluorescence assays of transformants harboring subcloned derivatives of pHCS12-5 revealed that the YFL029c open reading frame is necessary and sufficient for dosage suppression of the *smk1-2* fluorescence defect (Figure 3). YFL029c did not increase fluorescence of the *smk1*-Δ null mutant or the wild-type control strain. The YFL029c gene product is a 368 residue protein kinase homolog most similar to the cyclin-dependent kinase family and 54% similar and 30% identical to Cdc28p. The YFL029c protein contains most of the amino acid residues conserved in practically all serine/threonine protein kinases, with the noted exception of the glycine-rich nucleotide-binding fold which is typically located in subdomain I (Hanks *et al.*, 1988; Hanks and Quinn, 1991). During the preparation of this manuscript it was reported that YFL029c encodes *CAK1* (*CIV1*), the major CAK in yeast (Espinoza *et al.*, 1996; Kaldis *et al.*, 1996; Thuret *et al.*, 1996). YFL029c is hereafter referred to as *CAK1*.

Suppression of other smk1-2 phenotypes by multicopy CAK1

In order to characterize the dosage suppression phenotype further, *smk1-2* strains harboring the high-copy control vector, YEp352 or YEp352+*CAK1* were assayed for the ability to form birefringent spore walls as viewed by phase-contrast microscopy. At the non-permissive temperature, both strains underwent meiosis with >80% efficiency (Table I). Of those cells which completed meiosis, 68% of the asci harboring the *CAK1*-containing plasmid formed birefringent spore walls, in contrast to <0.5% for the negative control plasmid. The *smk1-2* diploid harboring this dosage suppressor yielded asci indistinguishable from wild-type at this microscopic level

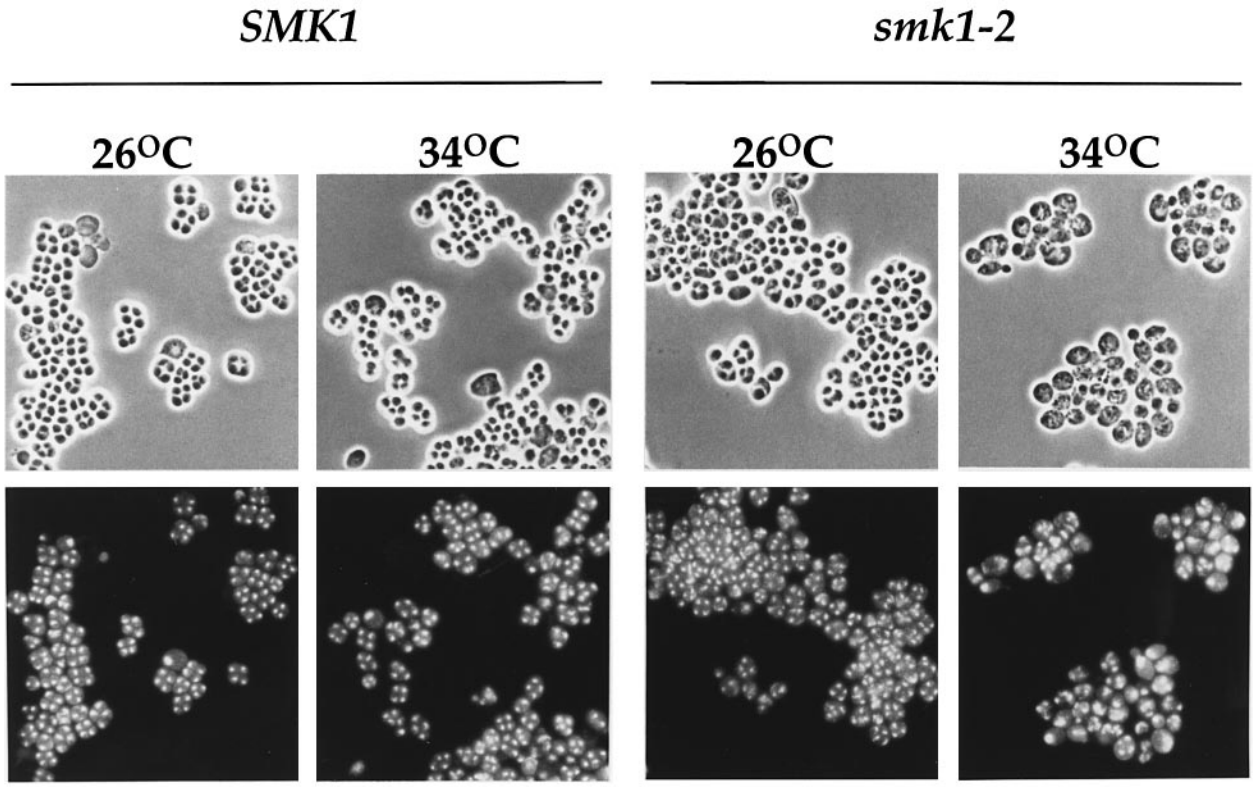

Fig. 2. Light microscopy of wild-type and *smk1-2* strains. Indicated strains were sporulated at 26°C or 34°C in liquid medium and then aliquots fixed in ethanol, stained with the DNA-specific dye DAPI, and photographed under visible (upper) or UV (lower) illumination.

(Figure 4) and at the electron microscopic level (data not shown). Multicopy *CAK1* had no discernible effect on spore formation in the *smk1* null mutant.

Spores of the *smk1-2* strain carrying the *CAK1*-multicopy plasmid or the negative control plasmid formed at either 26 or 34°C were tested for sensitivity to heat shock, glusulase or ether treatment. In all cases, *CAK1* dosage suppressed the resistance defect of the *smk1-2* mutant (Figure 5). A large contribution to heat shock resistance occurs relatively early in sporulation and does not require assembly of the outer spore wall layer (M.Wagner, unpublished results); glusulase resistance presumably reflects assembly of the spore-specific wall layers, while ether resistance is established later during sporulation in a period termed maturation (Law and Segall, 1988). Thus the overexpression of *CAK1* can suppress multiple *smk1* defects. *CAK1* was also able to dosage suppress mutant phenotypes of other *smk1* conditional alleles which contain missense mutations in different subdomains of the Smk1p protein kinase (M.Wagner, unpublished results).

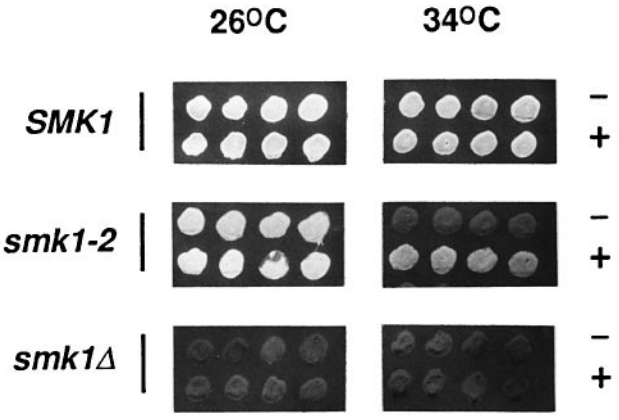

Fig. 3. Fluorescence assay of *smk1* strains harboring high-copy YFL029c (*CAK1*) plasmids. Indicated strains were transformed with a subcloned derivative of pHCS12-5 that contains only the *CAK1* open reading frame (+), or with a negative control plasmid (YEp352) lacking any genomic insert (–). Transformants were assayed by the fluorescence assay at the *smk1-2* permissive and non-permissive temperatures as described in Figure 1 and in Materials and methods. Four independently derived transformants of each strain were tested in parallel.

Expression of CAK1 during sporulation

Northern blot hybridization analysis of *CAK1* expression showed that it is transcribed in vegetatively growing cells (Figure 6, lane 0). Interestingly, Northern blot hybridization analysis of RNA isolated during a sporulation time-course revealed that there is a sporulation-specific peak of *CAK1* mRNA accumulation. This peak of expression occurs concomitantly with the peaks of both *SPS1* and *SMK1* transcript accumulation which are known to occur shortly after meiosis II (Figure 6). Consistent with these observations, the *CAK1* promoter region contains two T_4C consensus sequences (both have six out of seven matches to the consensus) centered at positions –292 and –128 relative to the initiator ATG. T_4C promoter elements have been implicated in positive regulation of sporulation-specific gene expression (Mitchell, 1994). *CAK1* mRNA levels during a sporulation time-course are not altered in a *smk1*-Δ mutant background, indicating that *SMK1* is not

Table I. *CAK1* dosage suppresses *smk1-2* morphological defect

	1[a] (%)	Multi[a] (%)	Asci[b] (%)
2μ	13	87	<0.5
2μ+*CAK1*	18	82	68

The *smk1-2* strain (MWY12) harboring YEp352 vector alone (2μ) or containing *CAK1* (2μ+*CAK1*) was sporulated at the non-permissive temperature, and aliquots taken for DAPI staining followed by visible and epifluorescence microscopy. In either case, a total of 500 cells (250 cells each from two independent transformants) were scored for completion of meiosis and presence of birefringent spore walls.
[a]Number of DAPI staining foci per cell was classified as '1' (cells which either had not initiated sporulation or not undergone any meiotic divisions) or 'Multi' (cells which had undergone meiotic divisions).
[b]Of cells which went through meiosis, percentage in which a birefringent spore wall surrounded each DAPI-staining focus.

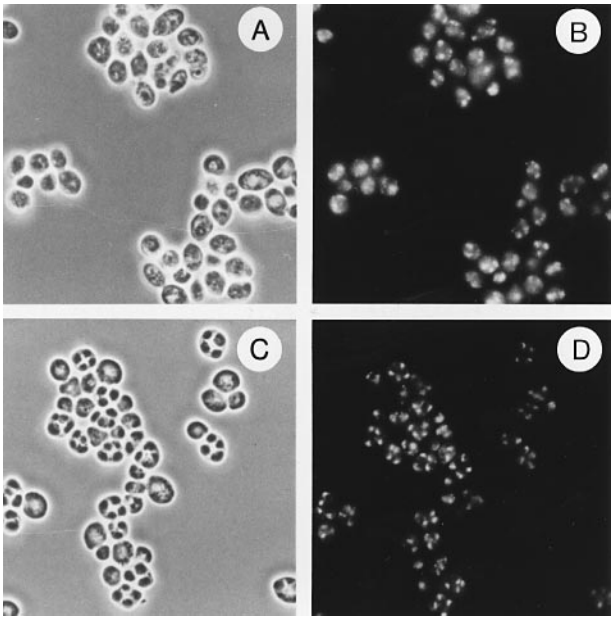

Fig. 4. Light microscopy of sporulated *smk1-2* diploids harboring high-copy *CAK1* plasmids. *smk1-2* strain MWY12 harboring the negative control plasmid YEp352 (**A** and **B**), or the high-copy *CAK1* plasmid (**C** and **D**) were sporulated in liquid at the non-permissive temperature (34°C) for 36 h before aliquots were prepared for visible (A and C) and UV epifluorescence (B and D) microscopy.

required for transcriptional regulation of *CAK1* during sporulation.

CAK1 protein kinase activity is required for vegetative growth and dosage suppression of *smk1-2*

A *TRP1*-disrupted null allele of *CAK1* was introduced into a diploid by standard gene replacement techniques. *CAK1* wild-type/null heterozygotes were sporulated, tetrads microdissected, and individual spores germinated. In 15 out of 15 tetrads analyzed, 2 to 2 segregation for growth was seen. The growth of wild-type and null haploids was indistinguishable for the first several generations. However, longer incubation gave normal-sized colonies for the wild-type and microcolonies (~50 cells) which grew no further for the null mutant. Cells in these microcolonies exhibited elongated morphologies,

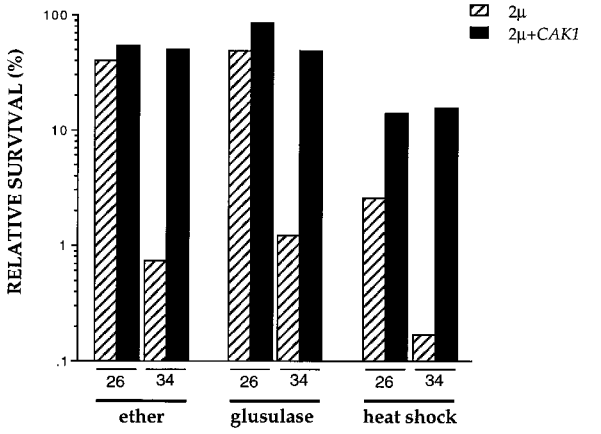

Fig. 5. Resistance assays of *smk1-2* diploids harboring high-copy *CAK1* plasmids to environmental stresses. *smk1-2* strain MWY12 harboring the 2μ negative control plasmid YEp352 or the YEp352 plasmid containing *CAK1* was sporulated in liquid at the permissive (26°C) or non-permissive (34°C) temperature for 36 h and aliquots assayed for resistance to ether, glusulase or heat shock as described in Materials and methods. In each case, the value for the wild-type control strain (LNY150) harboring the indicated plasmid and treated in parallel is taken as 100% and the value for the mutant normalized accordingly. All values represent the average of three or four independent experiments.

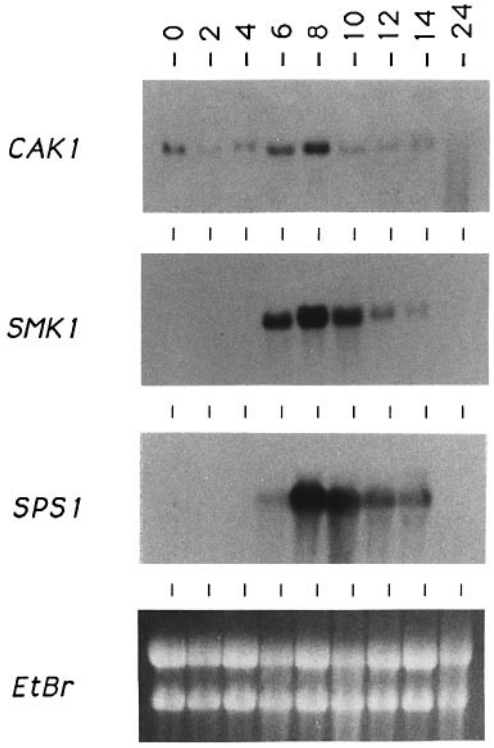

Fig. 6. Expression of *CAK1* during sporulation. Wild-type strain LNY150 was sporulated in liquid for the indicated number of hours (0–24) before total RNA was prepared. Northern hybridizations of a single filter were carried out sequentially with sequence-specific hybridization probes as described in Materials and methods. EtBr represents the ethidium bromide-stained agarose gel prior to transfer. Maximal accumulation of the early *SPO13* transcript in these samples occurs between 4 and 6 h while maximal accumulation of the late *SPS100* transcript in these samples occurs between 12 and 14 h (Krisak *et al.*, 1994).

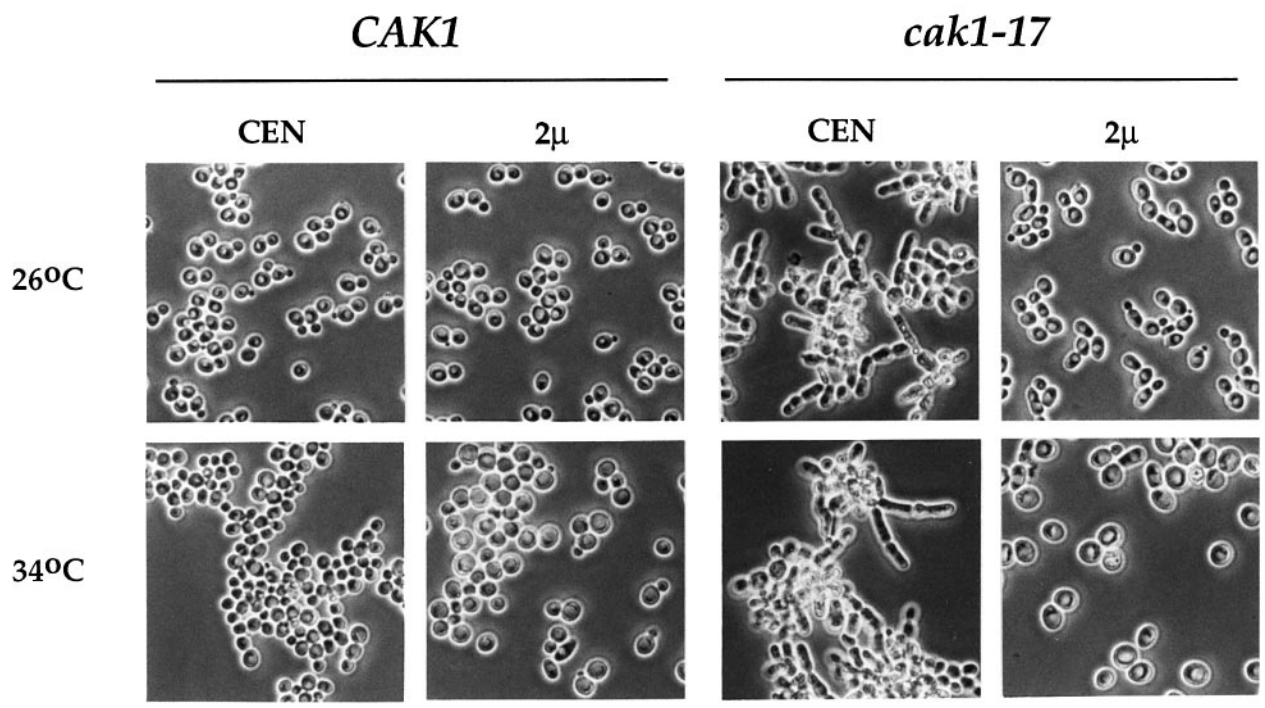

Fig. 7. Light microscopy of vegetative growth in *cak1* mutants. *cak1::TRP1* haploids harboring wild-type *CAK1* on a low-copy (CEN) or high-copy (2μ) vector or *cak1-17* on a low-copy (CEN) or high-copy (2μ) vector (strains MWY1079, MWY1081, MWY5501 and MWY5601, respectively) were grown in selective medium at the *cak1-17* permissive (26°C) or non-permissive temperature (34°C) as indicated. Cells were photographed using phase optics.

reminiscent of pseudohyphal filamentous growth (Gimeno *et al.*, 1992).

Several mutant alleles of the *CAK1* gene were constructed and their vegetative and sporulation phenotypes assessed. Three of these *cak1* alleles were made by site-directed mutagenesis. The aspartate in the DFG peptide motif of protein kinase subdomain VII is absolutely required for enzymatic activity in protein kinases tested and has been proposed to play a crucial role in phosphotransfer (Hanks *et al.*, 1988). The codon for the analogous aspartate residue (D179) in *CAK1* was mutated to encode arginine. This substitution has been shown to ablate *in vivo* and *in vitro* kinase activity in all protein kinases examined. The *cak1-D179R* mutant allele does not support vegetative growth and also does not suppress the sporulation defect of *smk1-2* strains when present in high (2μ) copy. These results indicate that Cak1p protein kinase activity is essential for vegetative growth, and for the suppression of the *smk1-2* sporulation defect. The amino-terminal region of protein kinases, including subdomains I and II, typically contains a conserved glycine-rich nucleotide-binding fold and phosphotransfer lysine (Hanks *et al.*, 1988). This is the region in Cak1p which is most divergent from typical protein kinases. A deletion mutation, which removes the 31 amino-terminal codons 8–38, was constructed. This *cak1-Δ31* mutant allele did not support mitotic growth or suppress the *smk1-2* sporulation defect. Additionally, the residue predicted to be the phosphotransfer lysine residue by spatial alignment, K31, was mutated to arginine. This conservative substitution is known to cripple kinase activity severely in almost all protein kinases tested. The *cak1-K31R* mutant allele does support vegetative growth and does suppress the *smk1-2* sporulation defect, indicating that this lysine residue is not required for the *in vivo* function of Cak1p. The vegetative and sporulation phenotypes of these three mutant alleles indicate that Cak1p is indeed a kinase, but with some unusual properties.

A number of *cak1* temperature-sensitive mutant alleles were selected from a library of randomly mutagenized *CAK1* alleles. The *cak1* library in a *URA3*–centromere-based plasmid was generated *in vitro* by hydroxylamine mutagenesis. The library was transformed into a *CAK1/cak1::TRP1* heterozygote. Transformants were pooled in liquid culture, sporulated *en masse*, the ascal sacs digested and the spores separated. The resulting haploid spores were plated on media lacking uracil and tryptophan to select for both the *cak1::TRP1* null allele and the plasmid-borne mutagenized *CAK1*. Isolates containing temperature-sensitive alleles of *CAK1* were identified as those which grew at the permissive (26°C) but not the non-permissive (34°C) temperature. At both 26°C and 34°C, all of the *cak1* mutant isolates examined exhibited highly elongated cells which remained attached to each other after budding, as viewed by phase-contrast microscopy (see *CEN/cak1-17* cells in Figure 7). These vegetative phenotypes are consistent with observations of *cak1-22* mutant background described by Kaldis *et al.* (1996). The position of bud scars revealed by calcofluor staining showed that the *cak1* mutant haploids manifest a bipolar budding pattern at both 26°C and 34°C. Actin staining with rhodamine phalloidin showed punctate foci at the cell periphery often concentrated at the end of elongated cells at both temperatures, suggesting the occurrence of polarized growth. DAPI staining showed that some cells within the mutant *cak1* population lacked nuclear material, suggesting defects in fidelity of nuclear segregation.

One of the *cak1* temperature-sensitive mutant alleles,

cak1-17, was shown to confer wild-type vegetative growth morphology at the permissive temperature while retaining a temperature-sensitive growth defect when expressed on a 2μ-based plasmid (Figure 7). At the non-permissive temperature this *cak1-17* genetic background caused cells to arrest as large unbudded cells, similar to what has been seen in another *cak1* (*civ1-4*) mutant strain (Thuret *et al.*, 1996). That different terminal phenotypes are exhibited when the *cak1-17* allele is expressed in low (centromere) versus high (2μ) copy number suggests that different mitotic requirements for *CAK1* may be met by different threshold levels of *CAK1* activity.

CAK1 is required for spore wall morphogenesis

cak1::TRP1 homozygous diploids harboring either the 2μ-*CAK1* or 2μ-*cak1-17* plasmid were sporulated at 26°C or 34°C and scored by the fluorescence assay. The *cak1-17* mutant strain showed a temperature-sensitive defect for fluorescence at the non-permissive temperature, indicating that *CAK1* is required for completion of sporulation (Figure 8). These same *CAK1* and *cak1-17* strains were sporulated in liquid at the permissive or non-permissive temperature and the morphology of the ascospores examined by phase contrast and fluorescence microscopy (Figure 9). DAPI staining for nuclei indicates that both strains initiate meiosis and complete meiosis I and II with the same efficiency, ~80%, at both temperatures (Table II). However, at the non-permissive temperature, only 9% of *cak1-17* ascospores form refractile spore walls as compared with 87% in the *CAK1* control. That the *cak1-17* allele causes temperature-sensitive spore wall defects is corroborated by the observation that while the heat shock resistance of the *CAK1* and *cak1-17* spores formed at 26°C and the *CAK1* spores formed at the 34°C are all comparable, the *cak1-17* strain sporulated at 34°C is >10-fold more sensitive to heat shock.

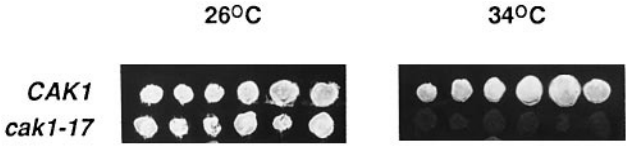

Fig. 8. Fluorescence assay of sporulated *cak1* mutants. The *cak1-Δ1* homozygous diploid harboring a 2μ-*CAK1* plasmid (yeast strain MWY55) or a 2μ-*cak1-17* plasmid (yeast strain MWY56) were sporulated at 26°C or 34°C as indicated for 36 h, and fluorescence assays carried out as described in Materials and methods. Six independently isolated transformants are shown in each case.

These ascospores were also examined by electron microscopy (Figure 10). The spore walls found in wild-type asci formed at 26°C and 34°C were indistinguishable and characterized by the two inner electron-lucent glucan layers (see bracket in Figure 10C), surrounded by the more diffuse spore-specific chitosan-containing layer, which is in turn surrounded by the electron-dense coat (see arrow in Figure 10C). The ultrastructure of the *cak1-17* mutant spore walls formed at 26°C were indistinguishable from those seen in the wild-type (Figure 10D). In striking contrast, a variety of ultrastructural defects are observed in the *cak1-17* mutant asci formed at the non-permissive temperature (Figure 10E–I). Of 200 asci scored, 10% appeared similar to wild-type when superficially examined at low magnification. However, careful examination of these asci whose spore wall assembly patterns most closely resembled wild-type (Figure 10E and F) revealed multiple interruptions in the outer electron-dense coat (Figure 10E)

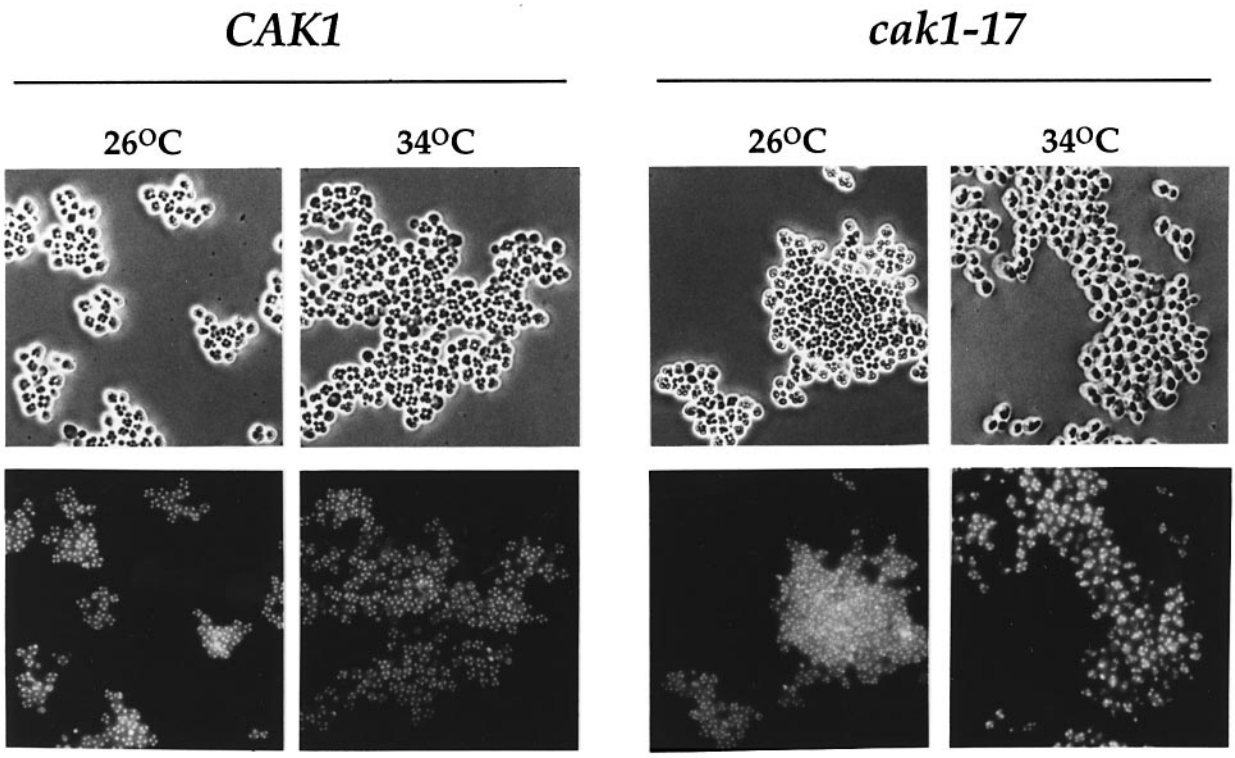

Fig. 9. Light microscopy of sporulated wild-type and *cak1* mutant cultures. The *CAK1* and *cak1-17* diploid strains (MWY55 and MWY56) were propagated at 26°C prior to sporulation in liquid medium at 26°C or 34°C. Aliquots were stained after 36 h with DAPI and photomicrography carried out under visible (upper) or UV (lower) illumination.

Table II. *cak1-17* asci are deficient in formation of birefringent spore walls

		1 (%)	2 (%)	3/4 (%)	SW (%)
CAK1	26°C	12 (0/58)	8 (32/38)	81 (391/404)	85 (423/500)
	34°C	10 (0/50)	6 (24/28)	84 (412/440)	87 (435/500)
cak1-17	26°C	5 (0/27)	7 (29/33)	90 (412/440)	88 (441/500)
	34°C	13 (0/67)	10 (4/52)	76 (41/381)	9 (45/500)

The MWY55 (*CAK1*) and MWY56 (*cak1-17*) yeast strains were sporulated at the permissive (26°C) or non-permissive (34°C) temperature and aliquots prepared for visible and epifluorescence microscopy. For each sample, 500 cells were viewed (250 cells each of two independent transformants). Cells were scored for number of DAPI-staining foci (1, 2 or 3/4) as an indication of meiotic progression and presence of birefringent spore walls (SW). Numbers in parentheses represent numbers of SW/total for each subclass.

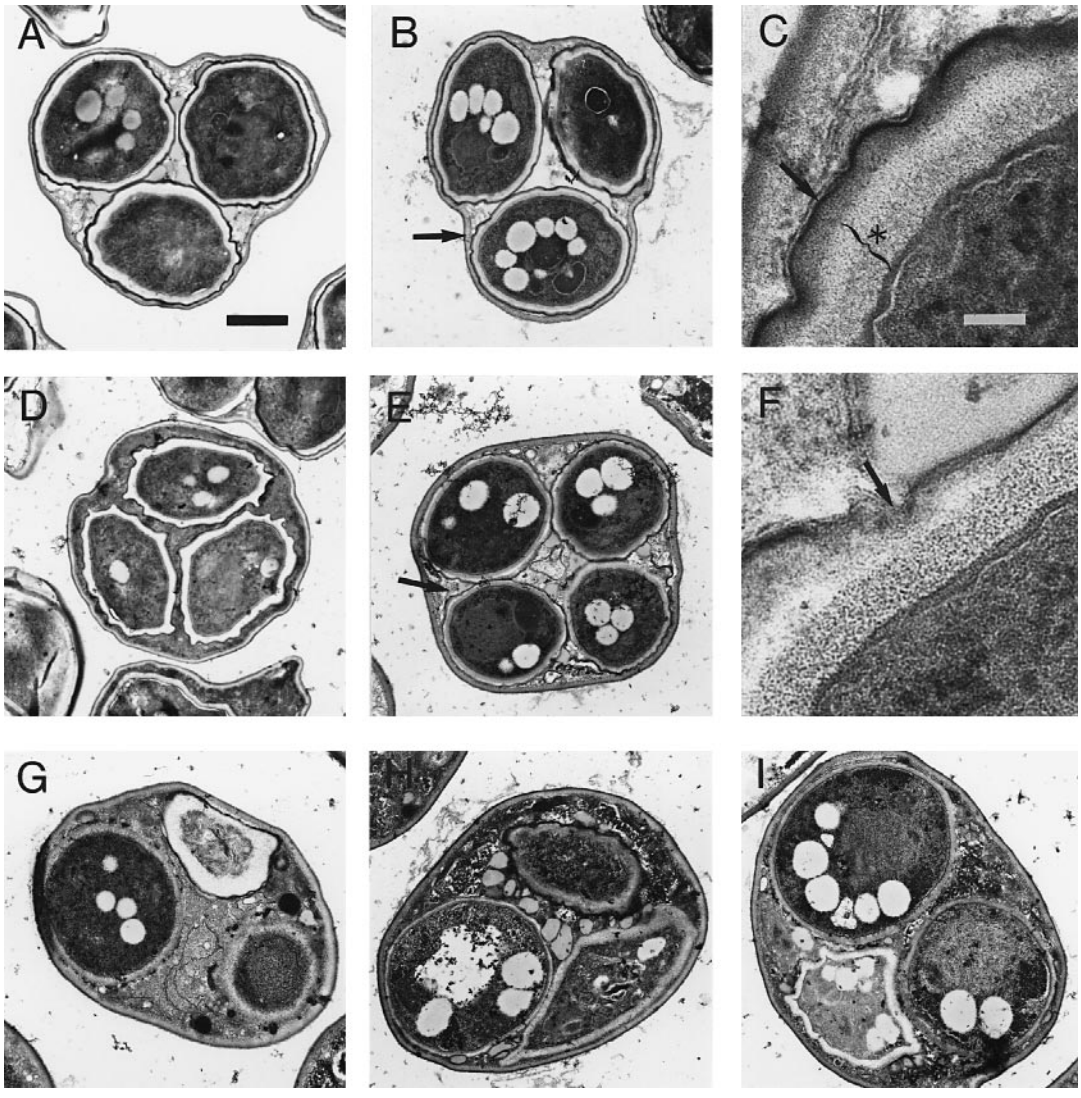

Fig. 10. Electron microscopy of wild-type and *cak1* mutant spores. Wild-type strain (MWY55) sporulated at 26°C (**A**) or 34°C (**B** and **C**), and *cak1-17* strain (MWY56) sporulated at 26°C (**D**) or at 34°C (**E–I**) for 36 h were fixed in glutaraldehyde, post-fixed with osmium tetroxide, sectioned and stained with uranyl acetate. The high-magnification images in (C) and (F) correspond to the region of the spore wall indicated by an arrow in the wild-type (B) and *cak1* mutant (E) spores, respectively. In (C) the two inner electron-lucent glucan layers (bracketed) have slightly different electron densities (demarcated with an asterisk). The outer glucan layer is surrounded by the more diffuse chitosan-rich, spore-specific layer, which is in turn surrounded by the relatively thin and electron-dense spore coat (arrow). In the high-magnification image of the *cak1* spore walls that most resemble wild-type (F), the glucan layers appear identical to wild-type. However, the chitosan layer appears to be diminished or missing, and the outer coat appears to be discontinuous in multiple areas (see arrow in F, with larger discontinuities apparent in all spores in E). Multiple examples of the *cak1-17* spore wall phenotype are shown in low magnification (E and G–I) to demonstrate the variety of abnormal spore wall assembly patterns formed. Note the severe discontinuities or complete absence of spore-specific layers in most spores, and that several spores appear to have decreased deposition of glucan. The samples analyzed are the same as those analyzed by light microscopy in Figure 9. Scale bar, 1 μm (A, B, D, E and G–I) and 0.1 μm (C and F).

and under high magnification the chitosan layer appeared to be diminished in thickness or even missing (Figure 10F). The spore walls in the remaining 90% of the *cak1-17* mutant asci (80% of which completed meiosis II as determined by DAPI staining of the culture) were heterogeneous (Figure 10G–I). Each spore within the mutant asci appeared to have characteristic defects in which layers were missing. In addition, structures that appeared similar to prospore wall structures were seen in 10–20% of the asci. The phenotype of the *cak1-17* asci formed at the non-permissive temperature thus appears similar to that seen in a *smk1* or in an *sps1* mutant.

Discussion

This work demonstrates that *CAK1*, which encodes the major CAK in yeast, plays a positive role in the spore wall morphogenesis pathway. The evidence in support of this conclusion is 3-fold. First, *CAK1* was isolated in a dosage suppression screen of the fluorescence defect of a *smk1-2* conditional strain. High-copy (2μ) expression of *CAK1* suppresses both multiple *smk1* developmental defects and multiple *smk1* mutant alleles. Second, *CAK1* mRNA accumulates during sporulation concomitant with *SMK1* and *SPS1* mRNA accumulation. Third, conditional mutants in *CAK1* have been identified that complete meiosis I and II, but that are defective for spore wall assembly. Thus, an essential regulator of cell cycle progression is also required for spore wall morphogenesis.

How does *CAK1* regulate spore wall morphogenesis? The genetic interaction demonstrated between *SMK1* and *CAK1* and the similarity in sporulation phenotypes of *smk1* and *cak1* mutants suggest that *CAK1* functions in the *SMK1* MAP kinase pathway. *CAK1* may exert its effects upstream or downstream of *SMK1*. The ability of *CAK1* to dosage suppress multiple *smk1* MAP kinase defects suggests that the mechanism of dosage suppression may be a general increase in pathway activity, and thus that *CAK1* functions upstream of *SMK1* in the spore morphogenesis pathway. Cak1p phosphorylates Thr169 in the activating loop of Cdc28p, a modification essential for cell cycle progression. One possibility is that *CAK1* positively regulates the *SMK1* pathway through *CDC28*. Shuster and Byers (1989), using temperature-sensitive *cdc28* mutant backgrounds, have demonstrated that *CDC28* is required for meiosis I and II. In addition, it has been shown that the *CLB* genes, whose products are involved in *CDC28* activation, are required for the execution of meiotic events and that different *CLB*s play specialized roles in this process (Grandin and Reed, 1993; Dahmann and Futcher, 1995). Nevertheless, it appears that *CDC28* activity is not required for spore wall assembly since *cdc28* temperature-sensitive strains are fully competent to assemble spore walls at the non-permissive temperature (Shuster and Byers, 1989). These results may suggest that *CAK1* functions in the *SMK1* pathway by activating substrates other than Cdc28p. In vertebrates, CAK has been shown to activate multiple substrates, including distinct CDKs involved in diverse cellular functions. In addition, many protein kinases exist which contain a threonine in the presumed activating loop and thus could be substrates for positive regulation by CAK (Johnson *et al.*, 1996). At present, two kinases have been described that function directly in the spore wall morphogenesis pathway: *SPS1*, a sporulation-specific *STE20* (*PAK1*) homolog, and *SMK1*. Each of these gene products contains a threonine in its activating loop that could serve as a phosphorylation site for positive regulation. Thus, in principle, either *SPS1*, *SMK1* or as yet unidentified kinases that function in this signaling pathway could be direct targets of *CAK1*. Epistasis experiments using partial function mutants in other members of this pathway and direct biochemical approaches may resolve this issue.

It should be pointed out that our experiments do not address the requirement of *CAK1* for the completion of meiosis I and II. We suspect that *CAK1* is required for the completion of meiosis since *CDC28* is required for meiosis and since CAK activity appears to be absolutely required for mitotic *CDC28* function. The isolation of a *cak1* mutant background that is able to complete meiosis with wild-type efficiency, but that is defective for spore wall morphogenesis, may suggest either that there is a higher critical threshold requirement for the function of *CAK1* in spore wall morphogenesis than in meiosis, or that pre-existing pools of *CAK1*-phosphorylated Cdc28p are able to support the meiotic function but not the spore wall morphogenesis function. In support of this latter possibility, we note that all of the eight independently derived *cak1* temperature-sensitive strains isolated in the course of this work continue to undergo normal mitosis for at least three or four divisions after they are shifted to the non-permissive temperature. These results are consistent with the idea that a pool of Cdc28p may exist that is phosphorylated on Thr169 and that this pool can interact with the appropriate regulatory subunits (Clns and Clbs) to achieve progression through the cell cycle. If meiosis can be completed using such a pre-existing pool of activated Cdc28p, this would suggest that regulated changes in *CAK1* activity are not required for progression through meiosis. However, the possibility that Cdc28p does not require CAK activation for its role in meiosis or that there exists a CAK activity that is specialized for meiotic progression cannot be ruled out at this time.

What is the signal which leads to activation of the *SMK1* MAP kinase pathway? The initiating signal is not the completion of meiosis I or II, *per se* (Klapholz and Esposito, 1980; Shuster and Byers, 1989; McCarroll and Esposito, 1994). Nor is the mere duplication and separation of the spindle pole bodies a prerequisite to spore wall morphogenesis (Schild and Byers, 1980; Shuster and Byers, 1989). Given that *smk1*-Δ strains do progress through meiosis II with wild-type efficiency, the initiating signal may require execution of a specific event that normally occurs after meiosis II but before assembly of the spore wall layers. Furthermore, the tight transcriptional regulation of this MAP kinase may generate a defined temporal window during which the developing ascospore is competent to respond to a *SMK1* pathway signaling event. The ultrastructural events that normally occur between the completion of meiosis II and spore wall assembly include a thickening of the outer plaque of the spindle pole bodies, outgrowth of the prospore wall from these plaques, and subsequent deposition of spore wall material from within and/or around the prospore wall (Moens, 1971; Moens and Rapport, 1971; Byers, 1981). It is tempting to speculate that the spindle pole body and

Table III. Yeast strains

Strain[a]	Genotype	Source
LNY150	MAT**a**/MATα *leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	L.Neigeborne
LAKY70	MAT**a**/MATα *smk1::LEU2/smk1::LEU2 ade2-1/ade2-1 his3-11-15/his3-11-15 leu2-3,115/leu2-3,115 trp1-1/trp1-1 ura3-1/ura3-1 canr1-100/canr1-100*	this study
MDPY10	MAT**a**/MATα *smk1::LEU2/smk1::LEU2 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	this study
MWY16	MAT**a**/MATα *smk1-Δ3/smk1-Δ3 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	this study
MWY12	MAT**a**/MATα *smk1-2/smk1::LEU2 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	this study
MWY30	MAT**a**/MATα *cak1::TRP1/CAK1 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	this study
MWY45	MAT**a**/MATα *cak1::TRP1/CAK1 ade2-1/ade2-1 his3-11-15/his3-11-15 leu2-3,115/leu2-3,115 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100*	this study
MWY1079	MAT**a** *cak1::TRP1 leu2-hisG trp1-hisG lys2 his4-G ura3-SK1 ho::LYS2 + pRS316CAK1*	this study
MWY1081	MAT**a** *cak1::TRP1 leu2-hisG trp1-hisG lys2 his4-G ura3-SK1 ho::LYS2 + pRS316cak1-17*	this study
MWY5501	MAT**a** *cak1::TRP1 leu2-hisG trp1-hisG lys2 his4-G ura3-SK1 ho::LYS2 + YEp352CAK1*	this study
MWY5601	MAT**a** *cak1::TRP1 leu2-hisG trp1-hisG lys2 his4-G ura3-SK1 ho::LYS2 + YEp352cak1-17*	this study
MWY55	MAT**a**/MATα *cak1::TRP1/cak1::TRP1 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2 + YEp352CAK1*	this study
MWY56	MAT**a**/MATα *cak1::TRP1/cak1::TRP1 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2 + YEp352cak1-17*	this study

[a]All strains are SK1 background (Alani *et al.*, 1990) with the exception of LAKY70 and MWY45 which are W303 background.

its associated morphological modification or perhaps the cellularization event that occurs as the prospore wall encompasses each of the haploid spores participates in the *SMK1* signal. It is possible that changes in the enzymatic activities of particular gene products associated with the execution or completion of such upstream events could directly activate the spore wall morphogenesis pathway.

What are the implications of *CAK1*'s role in spore wall morphogenesis? Once sporulation has been induced, a sequence of developmental events ensues in a pre-programmed and highly coordinated fashion (Esposito and Klapholz, 1981; Honigberg *et al.*, 1992; Honigberg and Esposito, 1994). The *SMK1* MAP kinase pathway may provide a developmental checkpoint to ensure that early events (meiosis) are completed before the onset of later events (spore wall morphogenesis). The requirement of *CAK1* for both cell cycle progression and spore wall morphogenesis suggests that Cak1p may serve as a regulatory nexus which coordinates these processes. It is possible that feedback regulatory interactions between Cdc28p and Cak1p may provide a mechanistic basis for activation of the *SMK1* morphogenesis pathway.

Materials and methods

Strains and culture conditions

The genotypes and sources of strains utilized in this study are shown in Table III. Vegetative cultures were propagated in either YEPD (1% yeast extract, 2% peptone, 2% glucose), SD (0.67% Difco yeast nitrogen base without amino acids, 2% glucose) or SA (0.67% yeast nitrogen base without amino acids, 1% potassium acetate, 1% phthalic acids, pH 5.5) supplemented with nutrients essential for auxotrophic strains at the levels specified by Sherman *et al.* (1986). Synchronous sporulation of diploids in liquid culture was achieved by inoculating logarithmic cells into YEPA (1% yeast extract, 2% peptone, 2% potassium acetate), expanding the culture for at least 7 h and to a density of 1×10^7 cells/ml, collecting cells by centrifugation, washing with 2% potassium acetate, and resuspending the cell pellet at 4×10^7 cells/ml in SM (2% potassium acetate, 10 μg/ml adenine, 5 μg/ml histidine, 30 μg/ml leucine, 7.5 μg/ml lysine, 10 μg/ml tryptophan, 5 μg/ml uracil). Sporulating cultures were maintained with vigorous aeration for 30–36 h. Sporulation of diploids on solid media was performed by patching or replica plating colonies to YEPD, allowing 12–18 h pregrowth, and then replica plating either directly to an SM plate (liquid SM with 2% agar, 0.1% yeast extract, 0.05% glucose) or a nitrocellulose filter which was then placed on an SM plate colony-side facing upward. Sporulation was allowed to proceed at the appropriate temperature for 48–72 h.

Table IV. Plasmids

Plasmid	Markers	Source
pRS316	*CEN URA3*	Sikorski and Hieter (1989)
pLAK40	pRS316 + *SMK1*	Krisak *et al.* (1994)
pMDP13	pRS316 + *smk1-2*	this study
pRS406	*URA3*	Sikorski and Hieter (1989)
YIPsmk1-2	PRS406 + *smk1-2*	this study
Yep352	2μ *URA3*	Hill *et al.* (1986)
pHCS12-5	YEp352 + genomic insert	this study
pMWB76	YEp352 + YFL030w	this study
pMWB77	YEp352 + YFL029c	this study
pMWB106	YEp352 + *CAK1*	this study
pMWB105	pRS316 + *CAK1*	this study
pMWB174	pRS316 + *cak1-D169R*	this study
pMWB176	YEp352 + *cak1-D169R*	this study
pMWB173	pRS316 + *cak1-Δ31*	this study
pMWB175	YEp352 + *cak1-Δ31*	this study
pMWB144	pRS316 + *cak1-K31R*	this study
pMWB146	YEp352 + *cak1-K31R*	this study
pMWB133	pRS316 + *cak1-17*	this study
pMWB167	YEp352 + *cak1-17*	this study
YDp-W	*TRP1*	Berben *et al.* (1991)
pSPS1-URA3	CEN *URA3 SPS1*	Friesen *et al.* (1994)
p152-SPS100TB	2μ *TRP1 URA3 SPS100/lacZ*	J.Segall

Plasmid constructs and libraries and genetic screens

Plasmid names, markers and sources are detailed in Table IV. The mutagenized plasmid libraries for *SMK1* and *CAK1* were made in the pLAK40 (*URA3*/*CEN*/*SMK1*) and pMWB105 (*URA3*/*CEN*/*CAK1*) yeast shuttle vectors, respectively. In either case, the vector was hydroxylamine-mutagenized *in vitro* for increasing times (Busby *et al.*, 1982). The

mutation frequency of the mutagenized plasmid pools was assessed by scoring transformants of *Escherichia coli* that are mutant at *pyrF* (the functional counterpart of the yeast *URA3* gene) for the inactivation of the plasmid-linked *URA3* (Sikorski and Boeke, 1991). Using this approach, a plasmid library representing in excess of 500 000 independent primary *E.coli* transformants with an average *URA3* inactivation frequency of 3.5% was generated. A high-copy yeast genomic library contained in the YEp352 yeast shuttle vector was kindly provided by Dr Shelly Berger.

For isolation of *smk1* conditional alleles, the mutant *SMK1* library in pLAK40 was transformed into yeast strain LAKY70 (*smk1-*Δ/*smk1-*Δ). Approximately 500 000 independent transformants were pooled and frozen in multiple aliquots for further analysis. Transformants were plated onto selective SD medium at a density of 100–200 colonies per 100 mm diameter Petri plate. Colonies were sporulated at the permissive (26°C) and non-permissive (34°C) temperatures and scored by the fluorescence assay as described below. The sequence of the entire open reading frame and 200 bp of promoter of *smk1-2* allele in pLAK40 was determined by standard dideoxy-chain termination methods (Ausubel *et al.*, 1987). *smk1-2* diploid strains were made by replacing *SMK1* in MAT**a** and MATα haploids and mating the two conditional *smk1* haploids to each other or to a *smk1*Δ or *SMK1* strain of opposite mating type. For *smk1-2* integrations, the *Kpn*I–*Xho*I *smk1* fragment of pLAK40 was subcloned into pRS406 to create the YIP*smk1-2* construct, which was then linearized with *Bgl*II, and *smk1* conditional strains selected by standard gene replacement techniques (Rothstein, 1991).

For the isolation of dosage suppressors of *smk1-2*, the yeast genomic library contained in YEp352 was transformed into yeast strain MWY12. Over 50 000 independent transformants were sporulated at 34°C and scored for fluorescence. Three classes of plasmid were isolated which suppressed the *smk1-2* fluorescence defect at 34°C: class I (*SMK1*-containing) plasmids were isolated nine times; class II (*CAK1*-containing) plasmids were isolated three times; class III plasmids were isolated twice (to be described later). Of the class II dosage suppressors, the pHCS12-5 plasmid contained the smallest genomic insert (chromosome VI, 3.4 kb *Sau*3AI fragment, bp designation 75 816–79 247). The two complete open reading frames contained in the insert (YFL030w and YFL029c) were separately amplified from pHCS12-5 by PCR and subcloned into YEp352 (pMWB76 and pMWB77). In order to obtain the entire presumed promoter region of YFL029c, the YFL029c open reading frame including 780 bp of 5′ non-coding sequence was amplified from genomic DNA (bp designation 77 917–79 940) and subcloned into YEp352 (pMWB106) or pRS316 (pMWB105). The dosage suppression phenotype of pMWB106 was indistinguishable from that of pMWB77 and the pMWB106/pMWB105 *CAK1* constructs were used in all subsequent studies. A *CAK1* disruption allele was made by replacing the 0.7 kb *Nsp*V fragment contained in the *CAK1* coding region with the *TRP1* open reading frame (0.8 kb *Bam*HI fragment of YDp-W) via Klenow/blunt-ended DNA ligation. The *cak1::TRP1* allele was excised from the plasmid backbone and used for one-step gene replacement in a diploid *CAK1* strain by standard methods (Rothstein, 1991). Integrations and subsequent tetrad analyses were done in both the SK1 (MWY30) and W303 (MWY45) genetic backgrounds.

The *cak1* mutant alleles *-K31R*, *-D169R*, and *-*Δ*31*, were made using PCR techniques and confirmed by DNA sequencing. The ability of these *cak1* alleles to support vegetative growth was determined by transforming pRS316 (*CEN URA3*) vector alone, or pRS316 containing a wild-type or mutant *CAK1* allele into yeast strain MWY30 (*cak1::TRP1*/*CAK1*). Single transformants were sporulated in liquid, the spores separated enzymatically and physically as described above, and spore density of each culture quantitated in a hemacytometer. An equal number of spores for each sample was plated on SD lacking tryptophan and uracil to select for *cak1::TRP1* haploid transformants. The number of Trp^+Ura^+ haploid survivors was determined for the mutants and compared with that of vector alone (0% survival) and wild-type (taken as 100% survival). The ability of the *cak1* mutant alleles contained in YEp352 to dosage suppress the *smk1-2* mutant sporulation phenotype was tested by fluorescence assay as previously described.

For the isolation of *cak1* conditional alleles, the mutant *CAK1* library in pMWB105 was transformed into yeast strain MWY30 (*cak1::TRP1*/*CAK1*). Approximately 5000 independent transformants were pooled as above. Pooled transformants were revived in SD media, and then sporulated *en masse* in liquid at 30°C. Glusulase (500 μl) and glass beads (2 ml) were added directly to a 5 ml sporulated culture which was incubated on a rolling drum until the ascal wall was digested and the spores physically separated as determined by microscopy. Spores were plated on SD lacking uracil and tryptophan to select for *cak1::TRP1* spores harboring pMWB105 with a mutagenized *cak1* allele and incubated at 26°C. The resulting colonies were replica plated to a fresh SD plate and incubated at 34°C. Those colonies which failed to grow at 34°C were chosen for further analysis.

Microscopy

For light microscopy, cells were fixed in ethanol and stained with DAPI (Sherman *et al.*, 1986). Fixation and staining with rhodamine phalloidin and calcofluor were carried out as described by Sherman *et al.* (1986). Samples were viewed and photographed as a wet mount under phase-contrast oil immersion optics using a Nikon Optiphot equipped for epifluorescence. For electron microscopy, cells were pelleted by centrifugation and fixed in 2.5% glutaraldehyde in 0.13 M cacodylate buffer, pH 7.4. The specimens were post-fixed in 1% osmium tetroxide for 1.5 h, dehydrated through a graded series of ethanol, and embedded in Spurr low viscosity resin. Ultrathin sections of 600 Å thickness were cut, mounted on copper grids, and stained with saturated aqueous uranyl acetate and Reynold's lead citrate. Sections were viewed and photographed using a JEOL 100B transmission electron microscope at 60 or 80 kV.

Assays for spore wall assembly

The procedure for the fluorescence assay is modified from the method of Esposito *et al.* (1991). Nitrocellulose filters with sporulated colonies or patches were placed colony-side facing upward in a Petri plate containing ascal wall lysis buffer [350 μl 0.1 M Na-citrate, 0.01 M EDTA, pH 5.8; 70 μl glusulase (Dupont NEE-154, crude solution); 15 μl β-mercaptoethanol], incubated at 37°C for 4 h, briefly blotted on 3MM Whatman paper to remove excess liquid, and then placed in a Petri plate containing 300 μl concentrated ammonium hydroxide. Fluorescence was viewed under a 304 nm UV light source and photography performed using a blue filter (Kodak, Wratten filter, #98).

Spore viability after heat shock (40 min at 55°C) or treatment with glusulase (1 h at 26°C) was determined as described by Briza *et al.* (1990a). Sensitivity of cells to ether exposure (3 min with constant gentle rocking) was assayed according to the method of Dawes and Hardie (1974). The level of *SPS100* expression was assessed as β-galactosidase activity in sporulating yeast strains harboring plasmid p152-SPS100TB (the gift of J.Segall) which contains the entire *SPS100* promoter and a portion of the coding sequence fused in-frame to the *lacZ* reporter gene. The wild-type diploid (LNY150) harboring p152-SPS100TB expressed β-galactosidase activity starting at 12 h after transfer to SM, maximal activity levels were reached by 20 h, and these levels remained constant for at least the next 48 h. β-Galactosidase activity levels in *smk1* mutant strains were determined at 24 h after transfer to SM. Preparation of cell lysates and β-galactosidase assays were carried out as described by Rose *et al.* (1990).

Northern blot hybridization

A single filter was used for all Northern analyses shown in Figure 6. These RNA samples were previously described and characterized in Krisak *et al.* (1994). DNA probes from the coding regions of the indicated genes were isolated from preparative agarose gels, ^{32}P-radiolabeled by random priming (Ausubel *et al.*, 1987), and used in hybridization analysis at 10^6 d.p.m./ml. DNA probes used were as follows: *CAK1*, 1.5 kb *Bam*HI–*Sal*I fragment of pMWB106; *SMK1*, 0.8 kb *Sty*I fragment of pLAK40; *SPS1*, *Bgl*II–*Eco*RI *SPS1*-specific fragment from p*SPS1-URA3*.

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