Distinct Steps in Yeast Spore Morphogenesis Require Distinct *SMK1* MAP Kinase Thresholds

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ABSTRACT

The *SMK1* mitogen-activated protein kinase is required for spore morphogenesis in *Saccharomyces cerevisiae*. In contrast to the multiple aberrant spore wall assembly patterns seen even within a single *smk1* null ascus, different *smk1* missense mutants block in a coordinated fashion at intermediate stages. One *smk1* mutant forms asci in which the four spores are surrounded only by prospore wall-like structures, while another *smk1* mutant forms asci in which the spores are surrounded by inner but not outer spore wall layers. Stepwise increases in gene dosage of a hypomorphic *smk1* allele allow for the completion of progressively later morphological and biochemical events and for the acquisition of distinct spore-resistance phenotypes. Furthermore, *smk1* allelic spore phenotypes can be recapitulated by reducing wild-type *SMK1* expression. The data demonstrate that *SMK1* is required for the execution of multiple steps in spore morphogenesis that require increasing thresholds of *SMK1* activity. These results suggest that quantitative changes in mitogen-activated protein kinase signaling play a role in coordinating multiple events of a single cellular differentiation program.

MITOGEN-activated protein kinases (MAPKs) participate in signal transduction pathways that couple myriad extracellular stimuli to specific biological responses (Blenis 1993; Blumer and Johnson 1994; Guan 1994; Marshall 1994; Waskiewicz and Cooper 1995; Madhani and Fink 1998). The essential role of MAPKs during development has been demonstrated for a multitude of morphogenetic and differentiative programs and in a wide range of eukaryotes that span the evolutionary gamut from yeast to humans (Eisenmann and Kim 1994; Marshall 1994; Perrimon 1994; Firtel 1995; Gotoh *et al.* 1995; LaBonne *et al.* 1995; Umbhauer *et al.* 1995; Glise and Noselli 1997).

Cellular differentiation programs are characterized by multiple steps that must be coordinated properly. It is not clear how a specific sequence of events is established within a cell. While it is known that MAPKs are required for differentiation of many (and perhaps most) cell types, their role in coordinating events during differentiation remains to be elucidated. For example, the MAPK may be required for only a single step of the differentiation program. Alternatively, it could be required for the execution of multiple independent steps. Furthermore, the MAPK may have an instructive role in specifying the proper sequence of events during a differentiation program.

Within the MAPK gene family, there are examples where a single MAPK has been shown to regulate multiple downstream processes in a single differentiation program. For example, the yeast mating pheromone response MAPK phosphorylates both a transcription factor to cause altered gene expression and a cell cycle regulatory component to cause growth arrest (Elion *et al.* 1993; Peter *et al.* 1993). In mammalian cells, MAPK has been shown to be required at both early and late stages of skeletal muscle differentiation (Bennett and Tonks 1997).

There are also examples where a single MAPK can specify distinct developmental outcomes. For instance, in mammalian PC12 cells, although nerve growth factor (NGF)- and epidermal growth factor (EGF)-generated signals are transduced through the same MAPK, NGF causes terminal differentiation into parasympathetic neurons, while EGF induces proliferation. Interestingly, in this system, durational thresholds of MAPK activity appear to determine response specificity (Marshall 1995). During Xenopus embryogenesis, fibroblast growth factor (FGF)-generated signals transduced through a single MAPK induce equipotent animal cells in the blastula to differentiate into either ventral, medial, or lateral mesoderm. Strikingly, FGF induces the different cell fates in a dose-dependent manner, and manipulation of activated MAPK levels can effect the same dose-dependent response (Gotoh *et al.* 1995; LaBonne *et al.* 1995; Umbhauer *et al.* 1995). These studies indicate that threshold levels of MAPK signaling in a single cell can be important in specifying alternative cell fates. However, they do not address whether MAPK

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thresholds are important in coordinating multiple events of a single cellular differentiation program.

In the yeast *Saccharomyces cerevisiae*, diploid cells deprived of nitrogen and a fermentable carbon source initiate sporulation (Kupiec *et al.* 1997). Analogous to metazoan differentiation programs, sporulation is induced in a specific cell type in response to specific environmental signals, and it is characterized by the ordered progression of morphogenetic stages that lead to a differentiated state. Upon induction, the cell exits the mitotic cell cycle at G1, and the four landmark events of spore development ensue: (1) meiotic prophase, during which DNA synthesis, meiotic recombination, and formation of synaptonemal complexes occur; (2) meiosis I; (3) meiosis II; and (4) spore wall morphogenesis and spore maturation. The end product is an ascospore that contains four dormant haploid spores, two each of the **a**- and α-mating types. Also, similar to metazoan differentiation, progression through sporulation is tightly linked to the transcriptional program. A cascade of gene expression accompanies sporulation, with sporulation-specific genes generally classified as early, middle, or late, depending on when they are expressed (Mitchell 1994).

The *SMK1* MAPK is a middle sporulation-specific gene required for postmeiotic events, including spore wall morphogenesis (Krisak *et al.* 1994). At the end of meiosis II, fusion of targeted secretory vesicles gives rise to bimembranous prospore walls that nucleate at the outer plaque of each spindle pole body and grow to enclose each meiotic product (Neiman 1998). The four-layered spore wall is subsequently assembled from within/around each prospore wall (Byers 1981; Esposito and Klapholz 1981). The two innermost layers are made primarily of glucan, the next layer consists of chitosan and residual chitin, and the outermost layer is proteinaceous and rich in dityrosine (Briza *et al.* 1986, 1988, 1990b). The spore wall protects against environmental stresses and is vital to spore integrity.

smk1 null mutants initiate sporulation and progress through meiosis normally, but they are defective in subsequent developmental events. Electron microscopy reveals a variety of aberrant spore wall assembly patterns, with layers that are missing, extranumerary, or improperly ordered. It is important to note that even among the four spores contained within a single *smk1* null ascus, multiple and distinct aberrant spore wall patterns are observed. This ability to assemble certain spore-specific structures, although in a disorganized and haphazard manner, indicates that *SMK1* is required for coordination of this morphogenetic program. Furthermore, the magnitude of late sporulation-specific gene transcription is significantly reduced in *smk1* null mutants, suggesting that *SMK1* is also required for additional steps of spore development.

Two other protein kinases that are expressed as middle sporulation genes, *SPS1* and *CAK1*, also function positively in and are required for spore wall morphogenesis. Sps1p, which bears homology to the Ste20p/Pak family of MAPK module activators, has been proposed to be an upstream kinase in the *SMK1* pathway (Friesen *et al.* 1994). *CAK1*, the Cdk-activating kinase in yeast, is an essential gene required for mitotic cell cycle progression (Espinoza *et al.* 1996; Kaldis *et al.* 1996; Thuret *et al.* 1996). We previously showed that overexpression of *CAK1* suppresses the multiple defects of a weakened *smk1* mutant, and that certain *cak1* mutant backgrounds that progress normally through meiosis make defective spore walls (Wagner *et al.* 1997). The involvement of *CAK1* in the *SMK1* pathway raises the possibility that transit through the cell cycle (meiosis) may activate spore morphogenesis. We have used *SMK1* and sporulation as a model system to study MAPKs and differentiation.

In this manuscript, we describe the isolation and characterization of conditional and partial-function missense mutants in the *SMK1* MAPK. In contrast to the uncoordinated developmental phenotype of *smk1* null asci, *smk1* missense mutants block at distinct intermediate stages of spore wall morphogenesis. Also, different *smk1* mutants show distinct defects in executing biochemical steps of spore development. Biochemical, morphological, and functional assays revealed that small increases in dosage of a hypomorphic *smk1* allele have qualitative developmental consequences and allow for the completion of progressively later events of spore morphogenesis. We also show that terminal blocks at discrete intermediate morphogenetic stages, as seen in the *smk1* missense mutants, can be recapitulated by reducing wild-type *SMK1* expression. These results demonstrate that the *SMK1* MAPK is required for multiple events during spore morphogenesis that require increasing *SMK1* activity levels. A model is proposed in which regulated changes in MAPK activity thresholds serve to temporally coordinate the complex sequence of events that characterizes cellular differentiation.

MATERIALS AND METHODS

Strains and culture conditions: Genotypes and sources of strains are shown in Table 1. Vegetative cultures were propagated in YEPD (1% yeast extract, 2% peptone, 2% glucose), SD [0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI), 2% glucose], or SA (0.67% yeast nitrogen base without amino acids, 1% potassium acetate, 1% pthallic acids, pH 5.5) supplemented with nutrients essential for auxotrophic strains at the levels specified by Sherman *et al.* (1986). Synchronous sporulation in liquid culture was achieved by inoculating logarithmic cells into YEPA (1% yeast extract, 2% peptone, 2% potassium acetate), expanding the culture to a density of 10^7 cells/ml (allowing for at least four to five doublings), collecting cells by centrifugation, washing with 2% potassium acetate, and resuspending 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

TABLE 1

Yeast strains

Strain[a]	Genotype	Source
LNY150	*MAT***a**/*MAT*α *leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	L. Neigeborn
LAKY70	*MAT***a**/*MAT*α *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 can1-100/can1-100*	Wagner *et al.* (1997)
MDPY10	*MAT***a**/*MAT*α *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*	Wagner *et al.* (1997)
MWY16	*MAT***a**/*MAT*α *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*	Wagner *et al.* (1997)
MWY12	*MAT***a**/*MAT*α *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*	Wagner *et al.* (1997)
MWY15	*MAT***a**/*MAT*α *smk1-4/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
MWY43	*MAT***a**/*MAT*α *smk1-4/smk1-4 leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2-lys2 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2*	This study
MWY61	MWY43 + *URA3*::pRS406*smk1-4/ura3-SK1*	This study
MWY63	MWY43 + *URA3*::pRS406*smk1-4/URA3*::pRS406*smk1-4*	This study
MDPY34	MDPY10 + *URA3*::pRS406 / URA3-SK1	This study
MDPY48	MDPY10 + *URA3*::pMDP199 / *ura3-SK1*	This study
MDPY46	MDPY10 + *URA3*::pMDP187 / *ura3-SK1*	This study
MWY90	MDPY10 + *URA3*::pMDP199 / *URA3*::pMDP187	This study
MWY93	MDPY10 + *URA3*::pMDP187 / *URA3*::pMDP187	This study
MWY95	MWY93 + pJS20	This study
MWY99	MDPY10 + pJS19	This study

[a] All strains are SK1 background (Alani *et al.* 1990).

with vigorous aeration for 24–36 hr. Sporulation on solid media was performed by patching or replica plating colonies to YEPD, incubating for 12–18 hr, and then replica plating directly to solid SM (liquid SM with 2% agar, 0.1% yeast extract, 0.05% glucose) or a nitrocellulose filter that was placed on solid SM, colony side up. Sporulation was allowed to proceed at the appropriate temperature for 48–72 hr.

Plasmids, libraries, and genetic screen: Plasmid names, markers, and sources are detailed in Table 2. Construction of the mutagenized *SMK1* plasmid library has been described (Wagner *et al.* 1997). For isolation of *smk1* conditional alleles, the mutant *SMK1* library in pLAK40 was transformed into yeast strain LAKY70 (*smk1*-Δ/*smk1*-Δ). Roughly 500,000 independent transformants were pooled and frozen in multiple

TABLE 2

Plasmids

Plasmid	Markers	Source
pRS316	*CEN URA3*	Sikorski and Hieter (1989)
pRS414	*CEN TRP1*	Sikorski and Hieter (1989)
pRS314	*CEN TRP1*	Sikorski and Hieter (1989)
pLAK40	pRS316 + *SMK1*	Krisak *et al.* (1994)
pRS406	*URA3*	Sikorski and Hieter (1989)
YEp352	2μ *URA3*	Hill *et al.* (1986)
pLAK51	pRS314 + *SMK1*	Krisak *et al.* (1994)
pMDPFlu55	pRS414 + *HA-SMK1*	This study
pMDP71	pRS414 + *HA-smk1-2*	This study
pMDP74	pRS414 + *HA-smk1-4*	This study
YIP*smk1-4*	pRS406 + *smk1-4*	This study
pMDP199	pRS406 + *SMK1*	This study
pMDP187	pMDP199 + *smk1-mse*^{s} *urs1*^{s}	This study
pJS19	YEp352 + *smk1-mse*^{s} *urs1*^{s}	This study
pJS20	pRS414 + *smk1-mse*^{s} *urs1*^{s}	This study
p152-SPS100TB	2μ *TRP1 URA3 SPS100-lacZ*	J. Segall

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 26° and 34° and scored by the fluorescence assay (see below). The sequence of the entire open reading frame and 200 bp of promoter of 12 independently isolated *smk1* conditional alleles in pLAK40 was determined by standard dideoxy-chain termination methods (Ausubel *et al.* 1987). *smk1* diploid strains were made by replacing *SMK1* in *MAT***a** and *MAT*α haploids and mating two conditional *smk1* haploids to each other or to an *smk1*-Δ or *SMK1* strain of the opposite mating type. For chromosomal integrations of *smk1* missense alleles, the *Kpn*I-*Xho*I *smk1*-containing fragment of pLAK40 was subcloned into pRS406 to create an integrating construct, which was then linearized with *Bgl*II, and *smk1* conditional strains were selected by standard gene replacement techniques (Rothstein 1991). For the three- and four-copy *smk1-4* strains, the YIP *smk1-4* vector was linearized with *Stu*I, generating a single cut within *URA3*, to target *smk1-4* integration to the chromosomal *ura3* locus. For the 2μ-based *smk1* vectors, the *Kpn*I-*Xho*I *smk1*-containing fragment was subcloned into the *Kpn*I-*Sal*I site of YEp352.

To test for mutant *smk1* promoter phenotypes, an integrating *SMK1* plasmid (pMDP199) was constructed that contained the *SMK1* gene from 140 bp upstream of the initiator ATG to 953 bp downstream of its terminator TAG in the *Kpn*I-*Xho*I sites of pRS406. Mutations in the URS1 consensus site, starting at position −92 relative to the initiator ATG (TCGGCGCCA), and in the MSE consensus site, starting at position −80 (ATTT GTGAC), were introduced by PCR to generate *smk1-urs1*^{s} *mse*^{s} in pMDP187. The urs^{s} mutation removed base pairs −84 to −90, and the mse^{s} mutation changed the sequence TTTG at positions −79 to −76 to CCCA. All promoter mutations were confirmed by DNA sequence analysis. pMDP187 and pMDP199 were integrated at the *ura3* locus using standard methods after linearizing the plasmids with *Stu*I. The *smk1-urs1*^{s} *mse*^{s} mutant promoter, unlike the wild-type control promoter, is derepressed in vegetative cells and is not activated during middle sporulation. A detailed analysis of the *SMK1* promoter will be described elsewhere.

Microscopy: For light microscopy, cells were fixed in ethanol and stained with DAPI (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). Specimens were postfixed in 1% osmium tetroxide for 1.5 hr, 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 fluorescence assay was modified from the method of Esposito *et al.* (1991). Nitrocellulose filters with sporulated colonies or patches were placed colony side up 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 (NEE-154, crude solution; Dupont, Wilmington, DE), 15 μl β-mercaptoethanol], incubated at 37° for 4 hr, briefly blotted on 3M Whatman paper, and then placed in a petri plate containing 300 μl concentrated NH_4OH. Fluorescence was viewed with a 304-nm UV light source and photographed through a blue filter (Wratten no. 98; Kodak, Rochester, NY).

Spore viability after heat shock (40 min at 55°) or treatment with glusulase (1 hr at 26°) 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 (gift from 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 strain (LNY150) harboring p152-SPS100TB expressed β-galactosidase activity starting at 12 hr after transfer to SM, with maximal levels being reached by 20 hr and then remaining constant for the next 48 hr. *SPS100lacZ* activities in the various homozygous conditional mutant strains were determined at 24 hr after transfer to SM. *SPS100lacZ* activity levels in the *smk1-4* gene dosage series were determined during a sporulation time course with samples taken 0, 5, 10, 15, 20, 25, and 30 hr after transfer to SM. Preparation of cell lysates and β-galactosidase assays were done as described by Rose *et al.* (1990).

Western analysis: Immunoblot analysis of mutant Smk1p was carried out using a construct that contains the 12-residue hemagglutinin (HA) epitope inserted between the initiator ATG and the second *SMK1* codon in pLAK51. Diploids transformed with either wild-type (pMDPFlu55), *smk1-2* (pMDP71), or *smk1-4* (pMDP74) in this backbone were synchronously sporulated, and samples were collected by centrifugation at the indicated times, immediately resuspended in sample loading buffer, boiled for 2 min, and stored at −80°. Cell lysates prepared from 10^7 cells were electrophoretically resolved for 16 hr at 13 mA on a 12% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose and probed for HA immunoreactivity using a 1:5000 dilution of the HA.11 monoclonal antibody (Berkeley Antibody Company, Richmond, CA). Immunoreactivity was detected by chemiluminescence using alkaline phosphatase-conjugated goat anti-mouse IgG.

Biochemical analysis of spores: Homogenates of sporulated cultures were prepared, hydrolyzed, and analyzed for dityrosine as described in Briza *et al.* (1990b). Cells were disrupted by shaking with glass beads, and wall and soluble fractions were separated by differential centrifugation. Dried samples were hydrolyzed in 12 n HCl at 95°, and the residue was dissolved in water and analyzed by isocratic reversed-phase HPLC [column, Waters Nova-Pak C_{18} (3.9 × 150 mm); eluent, 5% CH_3CN in 0.1% trifluoroacetic acid]. Dityrosine was detected using a fluorescence detector set at 285 nm as excitation and 425 nm as emission wavelengths. Quantitative estimation of glucosamine in hydrolysates of wall fractions was performed by HPLC after derivatization with phenylisothiocyanate (Bidlingmeyer *et al.* 1984) using the Waters Pico-Tag system. Analyses were done on a Hewlett Packard 1100 HPLC system with a Waters Nova-Pak C_{18} column (3.9 × 300 mm), according to the Waters Pico-Tag manual, using the elution conditions for amino sugars. To discriminate between chitin and chitosan, wall fractions were treated with HNO_2 before hydrolysis as described in Briza *et al.* (1988). Chitin deacetylase activity was measured by the method of Araki and Ito (1988), using radioactive labeled chitin as a substrate.

RESULTS

Isolation of conditional SMK1 MAPK mutants: The outer layer of the spore wall in *S. cerevisiae* contains insoluble dityrosine, which fluoresces in the visible spectrum under ultraviolet illumination. *smk1*-Δ asci do not form fluorescent spore wall structures. This assay was used as the criterion in the isolation of conditional (*ts*) *smk1* alleles. A mutagenized *smk1* plasmid library was

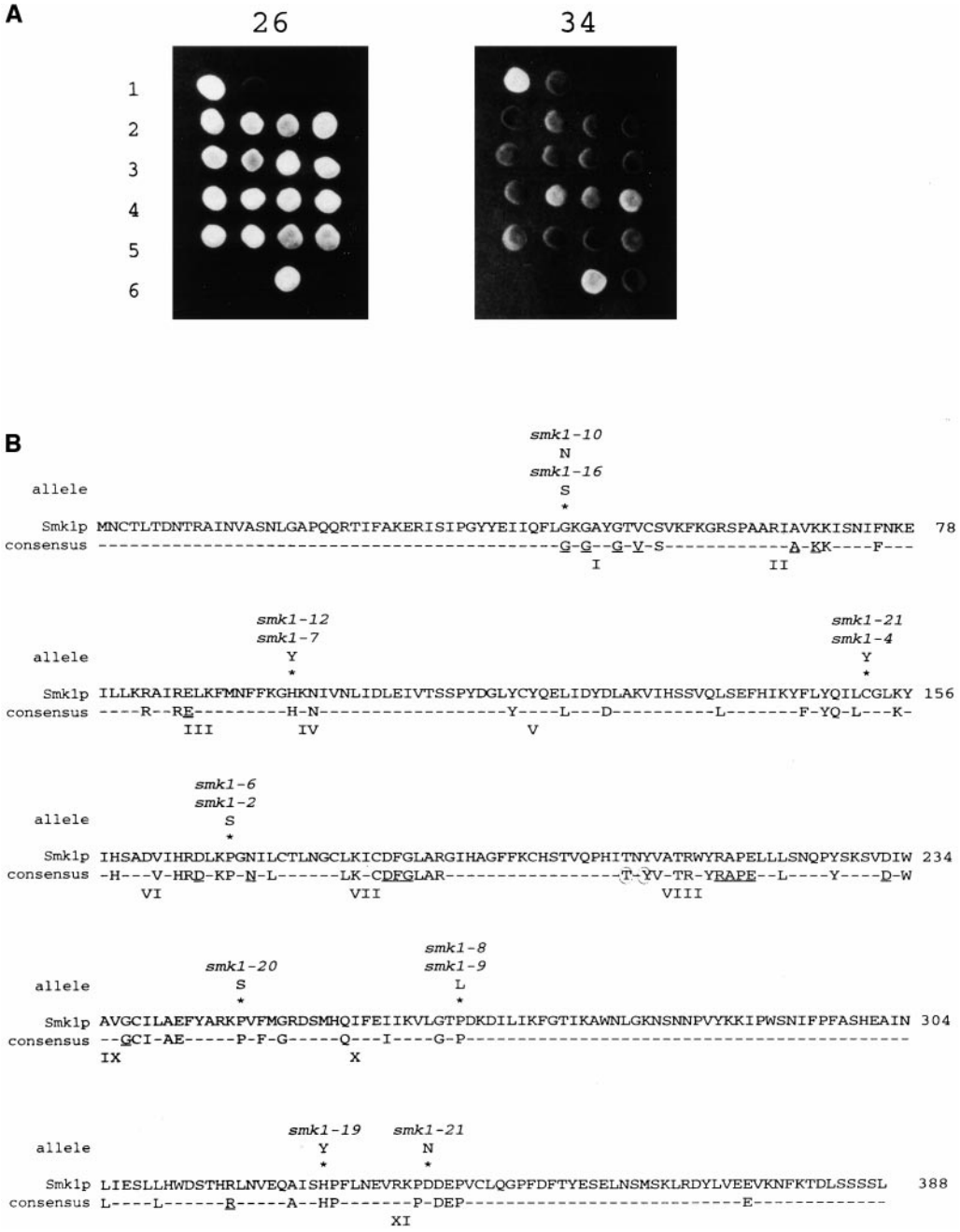

Figure 1.—Isolation of conditional *smk1* mutants. (A) Fluorescence phenotypes of *smk1* missense mutant strains. Patches of *smk1* conditional mutants were sporulated at the permissive (26°) or nonpermissive (34°) temperature and assayed for the fluorescence of insoluble dityrosine. The *SMK1* allelic designation of each strain from left to right is as follows: row 1, wt/wt, Δ/Δ; row 2, 2/Δ, 2/2, 4/Δ, 4/4; row 3, 6/6, 7/Δ, 8/8, 9/9; row 4, 10/10, 12/12, 16/Δ, 16/16; row 5, 19/Δ, 19/19, 20/20, 21/21; and row 6, wt/wt, Δ/Δ. All *smk1* mutants were derived from the wild-type LNY150 strain by standard gene replacement methods. (B) Sequence analysis of *smk1* missense alleles. The wild-type Smk1p amino acid sequence is shown with the missense changes of the 12 independently isolated *smk1* conditional alleles indicated above. The consensus sequence indicates the amino acid residues that are absolutely conserved in all MAPKs, with the activating threonine and tyrosine circled; residues that are also conserved in most protein kinases are underlined. Protein kinase domains as defined by Hanks *et al.* (1988) are indicated below.

generated by hydroxylamine treatment *in vitro* and then transformed into an *smk1*-Δ diploid. The transformants were sporulated, assayed for fluorescence, and *smk1*-containing plasmids were recovered from colonies that fluoresced when sporulated at 26° but not at 34°. A total of 12 independently isolated *smk1-ts* alleles were sequenced, revealing that 10 contain single missense mutations and 2 contain double missense mutations. All the single missense changes are in or adjacent to codons that specify highly conserved residues in kinases or MAPKs (Figure 1B).

Eight of the *smk1-ts* alleles were used to make conditional diploid strains by standard gene replacement techniques. Figure 1A shows the temperature-sensitive fluorescence phenotypes of the sporulated *smk1* MAPK mutants. *smk1-ts*/*SMK1* spores were indistinguishable from *SMK1*/*SMK1* spores in the fluorescence assay, indicating that all the *smk1-ts* alleles isolated are recessive. The different *smk1-ts* haploids were mated to each other in all pairwise combinations to generate heterozygotes. The resulting diploids were sporulated at the nonpermissive temperature and assayed for fluorescence. None of the *smk1-ts* alleles exhibited intragenic complementation with respect to this assay, suggesting that the different missense lesions do not affect distinct functions (such as recognition of distinct substrates) of the Smk1p enzyme.

***smk1* mutants fall into distinct morphological classes:**

Phase-contrast microscopy of sporulated cultures revealed that the *smk1* mutants fall into two classes. Wild-type asci contain four spore compartments surrounded by birefringent spore walls. Class I conditional mutants assembled birefringent spore walls at 26° that appeared to be indistinguishable from the wild type, and they failed to form birefringent spore walls at 34°. Class II mutants failed to form recognizable spore walls at either temperature. Both classes of mutants were positive for fluorescence of insoluble dityrosine when sporulated at 26°. Thus, at 26°, class II mutants execute one *smk1*-dependent event (accumulation of insoluble dityrosine), but they fail to execute a second *smk1*-dependent event (assembly of birefringent spore walls). Of the *smk1* conditional mutants for which fluorescence assays are shown in Figure 1, the *smk1-4*/*smk1*-Δ and *smk1-7*/*smk1*-Δ strains are class II, while the remainder are class I mutants. The *smk1-2* and *smk1-4* heterozygotes were chosen as representatives of each class for further analysis.

The *SMK1*, *smk1-2*, and *smk1-4* diploids were placed in sporulation medium at 26° or 34°, and the terminal spore wall structures were examined by electron microscopy (Figure 2, C and D). Wild-type spore walls consist of two inner electron-lucent (glucan) layers (see arrow in Figure 2D) surrounded by a more diffuse (chitosan-containing) layer of intermediate electron density, as well as an outermost electron-dense (dityrosine-containing) coat. The *smk1-2* spore walls made at the permissive temperature appeared similar to the wild type in that each spore within an ascus was surrounded by the four spore wall layers, which were in the appropriate order. The inner glucan-containing layer, however, consistently appeared thinner than in the wild type. In contrast, the *smk1-4* asci formed at the permissive temperature showed little evidence of the structures typical of the mature spore wall. Instead, all the visible meiotic products in 55% (121/221) of these asci were surrounded by a double-membranous structure reminiscent of what others have described as the prospore wall (Esposito and Klapholz 1981). *smk1*-Δ spores, even those found within a single ascus, always exhibit multiple abnormal and random spore wall patterns at all temperatures tested (Krisak *et al.* 1994; data not shown). This lack of intra-ascal coordination is a hallmark of the *smk1* null phenotype. The intra-ascal coordination exhibited by the *smk1-4* subpopulation indicates that there is some residual *SMK1* activity that allows for coordination but not completion of morphogenesis. The remainder of the *smk1-4* asci had spore walls that were heterogeneous, even within a single ascus, as are *smk1*-Δ asci. None of the *smk1-4* spore walls appeared to be wild type.

When sporulated at the nonpermissive temperature, 30% (26/71) of the *smk1-2* asci had spores surrounded by electron-lucent layers, but not the outer, more diffusely staining or thin, osmiophilic layers that are characteristic of wild-type spore walls. The intra-ascal coordination of this subpopulation of *smk1-2* asci is again consistent with residual *SMK1* activity, which allows for coordination but not completion of morphogenesis. The remainder of the *smk1-2* asci were heterogeneous and *smk1*-Δ-like, with no normal spore walls. The *smk1-4* asci made at the nonpermissive temperature were indistinguishable from *smk1*-Δ asci.

Electron microscopy of the end-stage class I *smk1-2* (34°) mutant asci revealed densely staining vesicles at the periphery of spore boundaries (see Figure 2, C and D). Others have noted the coalescence of similar vesicles during spore development, and it has been hypothesized that these are an intermediate to spore wall assembly (Esposito and Klapholz 1981). It was unexpected that the *smk1-4* spores made at 26°, which were positive for the presence of insoluble dityrosine in the fluorescence assay, were not surrounded by the electron-dense spore wall layer thought to be rich in dityrosine.

In summary, the *smk1-2* and the *smk1-4* mutants exhibit distinct phenotypes that are suggestive of intermediate stages in morphogenesis. This demonstrates that the *SMK1* MAPK is required for the completion of multiple events during spore wall assembly. Furthermore, the coordinated intermediate blocks seen in the *smk1-ts* mutants, which are in contrast to the *smk1* null uncoordinated phenotype, indicate that *SMK1* can also negatively regulate certain aspects of spore morphogenesis. For example, incompletion of an early event may activate an *SMK1*-dependent checkpoint function that prevents onset of a subsequent event.

***smk1-2* and *smk1-4* encode stable proteins:** One possible explanation for the coordinated intermediate blocks seen in the *smk1-2* and *smk1-4* asci is that the Smk1p mutant enzymes are unstable or destroyed before the next step in the pathway is executed. The *smk1-2* missense mutation occurs at an absolutely conserved residue in the catalytic core of the enzyme (Figure 1B). The analogous amino acid substitution (P169S) also confers conditional MAPK activity to the *Schizosaccharomyces pombe* Cdc2, Drosophila MEK, and Dictyostelium Erk2 kinases (Carr *et al.* 1989; Hsu and Perrimon 1994; Gaskins *et al.* 1996). The *smk1-4* lesion (C152Y) lies just N-terminal to the predicted catalytic core at a residue that is always cysteine or arginine in yeast MAPKs. Western analysis was performed to detect epitope-tagged *SMK1*, *smk1-2*, or *smk1-4* encoded proteins in extracts prepared from diploids sporulated at either 26° or 34°. We have previously shown that upon transfer of wild-type diploid cells to sporulation medium, *SMK1* mRNA levels peak at ~8 hr and then sharply decline (Krisak *et al.* 1994; Pierce *et al.* 1998). The peak of Smk1p expression was also seen at ~8 hr (Figure 3). In contrast to the rapid disappearance of *SMK1* mRNA, Smk1p levels remained relatively constant; they were still present at a high level at 24 hr. The timing and levels of Smk1-2p (not shown) and Smk1-4p expression during sporulation at 34° were comparable to the wild type, indicating that the missense changes do not alter pro-

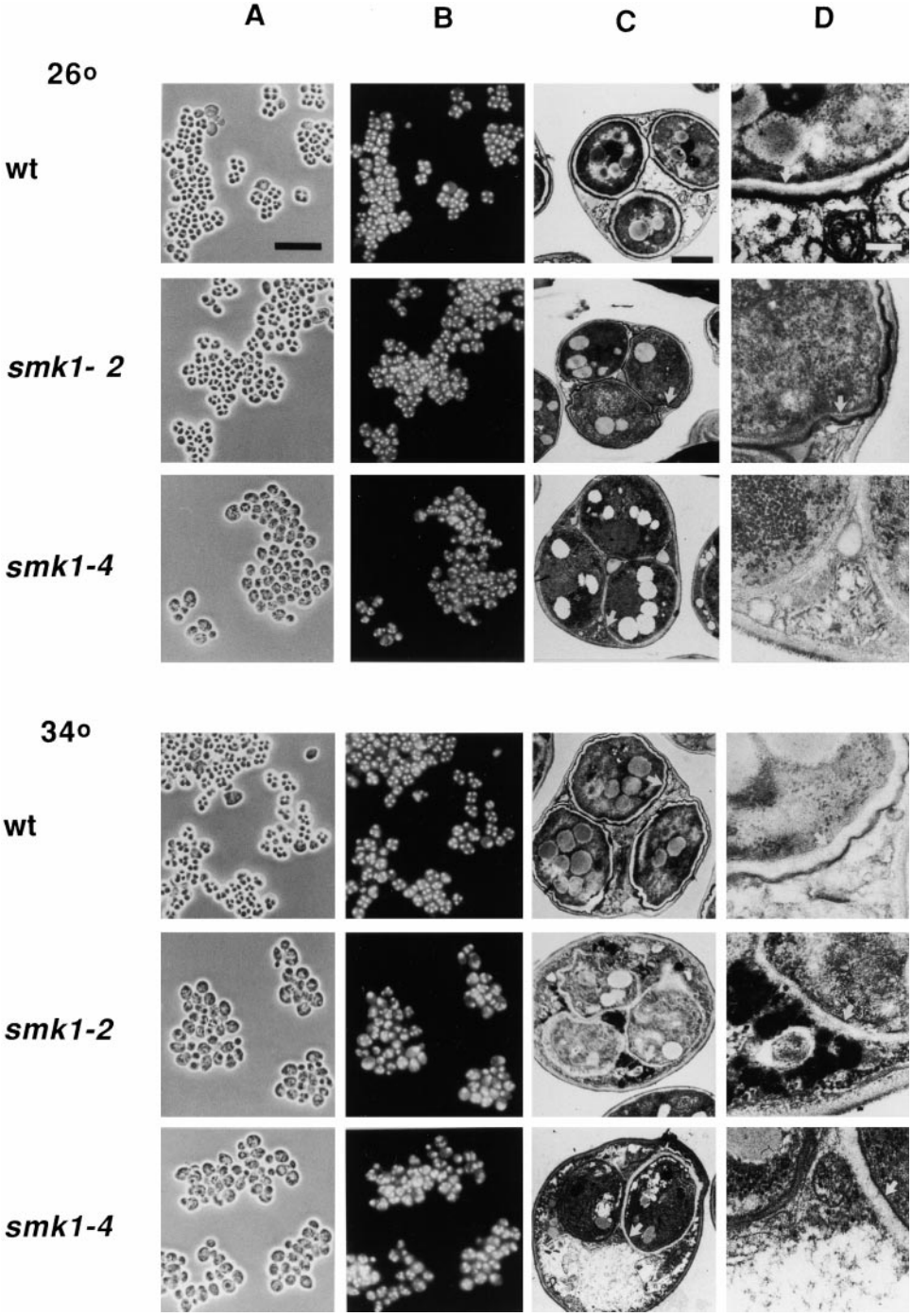

Figure 2.—*smk1-2* (class I) and *smk1-4* (class II) asci block at distinct intermediate stages of spore wall morphogenesis. Class I *smk1-2* and class II *smk1-4* mutants were sporulated at 26 or 34° for 36 hr, and asci were fixed in ethanol, stained with the DNA-specific dye DAPI, and viewed by phase contrast (A) and epifluorescent (B) microscopy. These same sporulated samples were also examined by electron microscopy at low (C) and high (D) magnification. The *smk1-2* and wild-type samples are identical to those described previously (Wagner *et al.* 1997). Although both the *smk1-2* and *smk1-4* strains are positive in the fluorescence assay when sporulated at 26°, *smk1-2* asci exhibit birefringent spore walls, as the wild type do, while in contrast, there is no evidence of birefringent spore walls in the *smk1-4* asci (A). When sporulated at 34°, neither missense mutant produced birefringent spore walls. Electron microscopy of *smk1-2* spore walls made at 26° and wild-type spore walls made at either temperature shows the characteristic electron-lucent inner (glucan) layers (arrows) surrounded by the diffusely staining (chitosan-rich) layer and the tightly juxtaposed electron dense (dityrosine-rich) outermost layer (C and D). Most of the *smk1-4* asci made at 26° were blocked at an intermediate stage of spore wall morphogenesis in which each of the visible spores within a given ascus was surrounded by a bimembranous prospore wall but no mature spore wall layers. Most of the *smk1-2* asci produced at 34° exhibited a distinct intermediate morphogenetic block in which each of the visible spores within a given ascus was surrounded by the inner electron-lucent (glucan) layers but not the outer spore-specific layers. Note the coalescence of densely staining vesicular structures at the periphery of the immature spore walls in the *smk1-4* (26°) and *smk1-2* (34°) mutants. At 34°, *smk1-4* asci were indistinguishable from *smk1*-Δ asci (lack of intra-ascal coordination). Bars in A, C, and D are 10, 1, and 0.2 μm, respectively.

tein stability. These data are consistent with *smk1-2* and *smk1-4* both encoding stable but conditionally weakened MAPKs.

***smk1* mutants exhibit distinct biochemical defects in spore wall assembly:** The two outer layers of the spore wall are unique to the spore, with no structural equivalent in vegetative cells (Briza *et al.* 1986, 1988, 1990b). The outermost layer is comprised of a highly cross-linked insoluble macromolecule, a major component of which is the dimerized amino acid dityrosine [2,2′-bishydroxy-5,5′-bis(α-amino propionyl)biphenyl]. This spore-specific molecule is synthesized from l-tyrosine by the activities of Dit1p and Dit2p, which are encoded by sporulation-specific genes expressed shortly after Smk1p (Briza *et al.* 1990a). Free l,l-dityrosine is incorporated into insoluble (spore wall) material and subse-

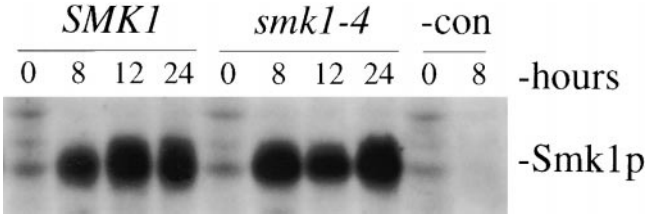

Figure 3.—The *smk1-4* missense mutation does not affect protein stability. Diploids expressing an epitope-tagged *SMK1* or *smk1-4*, or untagged *SMK1* (-con) were sporulated at the nonpermissive temperature (34°). Total protein extracts were prepared at 0, 8, 12, and 24 hr after transfer to sporulation medium and analyzed by immunoblot analysis as described in materials and methods.

quently epimerized to the d,l-form. Epimerization occurs late in spore development, and a higher ratio of d,l- to l,l-dityrosine is associated with more mature spore walls (Briza *et al.* 1994). Closely juxtaposed is the next inner layer, which is made of insoluble chitosan and residual chitin (polymerized forms of glucosamine). Chitin synthase converts glucosamine to chitin, an essential component of the vegetative cell wall. In spores, chitin is subsequently deacetylated by chitin deacetylase, giving rise to the spore-specific molecule chitosan (Christodoulidou *et al.* 1996).

smk1-Δ and *SMK1* asci were assayed for biochemical indicators of spore wall synthesis (Table 3). Incorporation of dityrosine into insoluble material (wall fraction) was greatly reduced in *smk1*-Δ asci made at both 26° and 34°, which is consistent with results of the fluorescence assay. At 26°, *smk1*-Δ levels of soluble dityrosine were indistinguishable from the wild type, indicating that the fluorescence phenotype results from the failure of *smk1*-Δ asci to incorporate the soluble dityrosine precursor into insoluble material. At 34°, however, the level of soluble dityrosine precursor in the null mutant was reduced, suggesting that Smk1p also positively regulates soluble dityrosine biosynthesis, and that the regulatory step is rate limiting at 34° but not at 26°. We previously showed that *DIT1* mRNA is expressed at reduced levels (25% of wild type) in *smk1*-Δ asci, which provides one possible explanation (Krisak *et al.* 1994). The null mutant also exhibited defects in dityrosine epimerization. Chitosan was undetectable in *smk1*-Δ spores despite only a twofold reduction in chitin deacetylase activity. This suggests that the defect in chitosan accumulation is not simply a result of decreased deacetylase activity. These data indicate that in the *smk1*-Δ background, there is a failure to incorporate spore wall precursors into insoluble structures. The data also suggest that *SMK1* can directly or indirectly positively regulate precursor biosynthesis. Thus, Smk1p positively regulates multiple biochemical steps of spore wall synthesis.

smk1-2 and *smk1-4* mutant asci were analyzed for the same biochemical markers (Table 3). Consistent with the qualitative results of the fluorescence assay, the *smk1-2* and *smk1-4* mutants do exhibit quantitative temperature-sensitive defects in insoluble dityrosine accumulation. The lack of a dityrosine-rich layer in the *smk1-4* spores made at 26° despite the near-wild-type levels of insoluble dityrosine, as evidenced by electron microscopy, suggests that this mutant cannot incorporate this spore wall component into a recognizable layer. Both mutants are less efficient at dityrosine epimerization than the wild type, with the *smk1-4* defect being most severe. At 26°, chitosan accumulation was slightly reduced for *smk1-2* and undetectable for *smk1-4* compared to the wild type. This is consistent with the ultrastructural analysis in which the *smk1-2* spores exhibit a thinner chitosan-rich layer compared to the wild type, and the *smk1-4* spores appear to lack this layer. Chitosan was undetectable in both mutants sporulated at 34°. In asci formed at 26°, both mutants had wild-type chitin deacetylase activity levels, which is consistent with Smk1p's role in chitosan synthesis being more complex than simple regulation of deacetylase activity. Both mutants demonstrated a temperature-sensitive defect in chitin deacetylase activity. These data show that *smk1-2* is able to complete more of the biochemical events required for spore wall assembly than *smk1-4*, which is consistent with ultrastructural observations suggesting that *smk1-2* can produce morphologically more mature terminal spore wall structures. In summary, the terminal

TABLE 3

Biochemical analysis of sporulated cultures of *SMK1*, *smk1-2*, *smk1-4*, and *smk1*-Δ

	Dityrosine in wall fraction (nmol/10^8 cells)		Dityrosine in soluble fraction (nmol/10^8 cells)		d,l-Dityrosine in wall fraction (%)		Total glucosamine in wall fraction (nmol/10^8 cells)		Chitosan in wall fraction (nmol/10^8 cells)		Chitin deacetylase activity (10^{-3} units/mg protein/min)	
	26°	34°	26°	34°	26°	34°	26°	34°	26°	34°	26°	34°
Wild type	2.08	1.64	0.20	0.29	40.0	41.5	44.3	43.5	36.1	31.7	0.23	0.24
smk1-2	0.66	0.07	0.27	0.04	37.9	12.3	36.5	5.1	13.1	ND	0.26	0.12
smk1-4	0.32	0.04	0.32	0.03	33.7	2.9	8.7	5.1	ND	ND	0.23	0.09
smk1-Δ	0.06	0.03	0.25	0.04	5.6	0.0	12.8	6.0	ND	ND	0.12	0.11

All values are from cultures 24 hr after transfer to sporulation medium. ND, not detected.

asci of null, class I, and class II *smk1* mutants display distinct spectra of biochemical defects.

***smk1-4* is hypomorphic:** Two general models can be invoked to explain the distinct biochemical and morphological phenotypes seen in the different *smk1* mutants. The first model posits that the different *smk1* gene products are defective in executing specific subsets of biochemical functions. For example, perhaps the Smk1p protein kinase recognizes and phosphorylates multiple downstream targets, and the different mutant enzymes exhibit different spectra of defects in substrate recognition. Our finding that the *smk1* missense alleles identified in this study do not exhibit intraallelic complementation is inconsistent with this model. The second model posits that the different *smk1* gene products are defective in a single biochemical activity, and that the distinct phenotypes are related to quantitative and not qualitative defects. For example, the Smk1p mutant enzymes may be catalytically crippled to different extents because of mutations that affect either the enzyme's ability to be activated or to complete a catalytic cycle. Such mutants would be predicted hypomorphs. Muller (1932) defined a hypomorph as a mutant that encodes a gene product with similar but weaker function than the wild type. In a true hypomorph, the wild-type phenotype can be restored by increasing the gene dosage of the mutant allele.

To determine if the *smk1-ts* alleles are hypomorphic, each of the eight alleles (for which *smk1-ts* conditional strains had been constructed) was subcloned into a high-copy, 2μ-based plasmid. The end-stage spore phenotypes for each *smk1-ts* strain containing either its cognate *smk1-ts* overexpression plasmid or a negative control plasmid were assessed. In all cases, increased gene dosage of *smk1-ts* correlated with an increased signal in the fluorescence assay. Furthermore, as evidenced by phase-contrast microscopy, the increased gene dosage caused class I mutants (which normally make birefringent spore walls only at the permissive temperature) to make birefringent spore walls at the nonpermissive temperature, and it caused class II mutants (which normally do not make birefringent spore walls at either temperature) to make birefringent spore walls at the permissive temperature. Asci that overexpress wild-type *SMK1* via a 2μ-plasmid appear wild type in all respects.

To more precisely define the effects of gene dosage on the execution of multiple events in a *smk1* mutant background, diploids that contained zero, one, two, three, or four copies of *smk1-4* were generated. These strains were constructed such that chromosomal copies of the *smk1-4* allele were present at either the endogenous locus and/or the *ura3* locus in all possible combinations. Resistance and morphological assays demonstrated that the sporulation phenotypes were independent of the chromosomal context of the *smk1-4* allele. As a result, an *smk1-4* allelic series of five strains was used for the studies described below.

Diploids containing either zero, one, two, three, or four copies of *smk1-4* were sporulated at 27.5°, and the terminal asci were viewed by phase-contrast microscopy (Figure 4A). This temperature was chosen to maximize the range of observed phenotypes. Neither the null mutant nor the single-copy *smk1-4* strain produced birefringent spore walls. In the asci of the two-copy *smk1-4* strain, a hint of birefringent structure was infrequently evident, and these asci mostly resembled the null mutant. When the *smk1-4* allele was present in three or four copies, the terminal asci did contain birefringent spore walls and were morphologically indistinguishable from the wild type. A small increase in *smk1-4* gene dosage (going from two to three copies per cell) resulted in a large shift of the percentage of morphologically normal spores (from <1 to 95%, respectively), confirming that *smk1-4* is hypomorphic. Subsequently, this series of strains was used to analyze the progression of spore development as a function of increasing *smk1-4* gene dosage.

Completion of different developmental events requires distinct *smk1-4* gene dosage thresholds: A number of indicators of spore development were assessed for the *smk1-4* hypomorphic strains (Figure 4B). Quantitative results of the phase-contrast morphologies are shown as the percentage of asci that contain mature (birefringent) spore walls. Electron microscopy (not shown) confirmed that the three- and four-copy asci appeared to be wild type. The spores from the *smk1-4* hypomorphic series were also tested for the acquisition of functional characteristics of wild-type spores, including resistance to glusulase, heat shock, and ether. Asci made from the single-copy *smk1-4* strain were as hypersensitive to these assaults as the null mutant. In all cases, increasing the *smk1-4* copy number resulted in spores that were more resistant. The acquisition of different resistance phenotypes required different *smk1-4* allelic thresholds. For example, the lower level of *SMK1* activity found in the two-copy strain allowed for wild-type-like glusulase resistance. However, the slightly higher level of *SMK1* activity in the three-copy strain was required to achieve wild-type-like heat shock resistance.

Levels of insoluble glucosamine and dityrosine were assessed for the *smk1-4* hypomorphic series. These spore wall components were undetectable in the zero- or single-copy strains. However, doubling the *smk1-4* copy number (two-copy strain) allowed for their accumulation to wild-type levels. Consistent with the quantitative analysis of insoluble dityrosine, only the two-, three-, and four-copy strains were positive in the fluorescence assay when sporulated at 27.5° (data not shown). In the electron micrographs of the terminal asci of the *smk1-4* allelic series, the coalescence of densely staining vesicles at the periphery of spore boundaries was not evident in the null mutant, rarely seen with single copy, predominant with two copies, and absent with three or four copies. While insoluble dityrosine/glucosamine

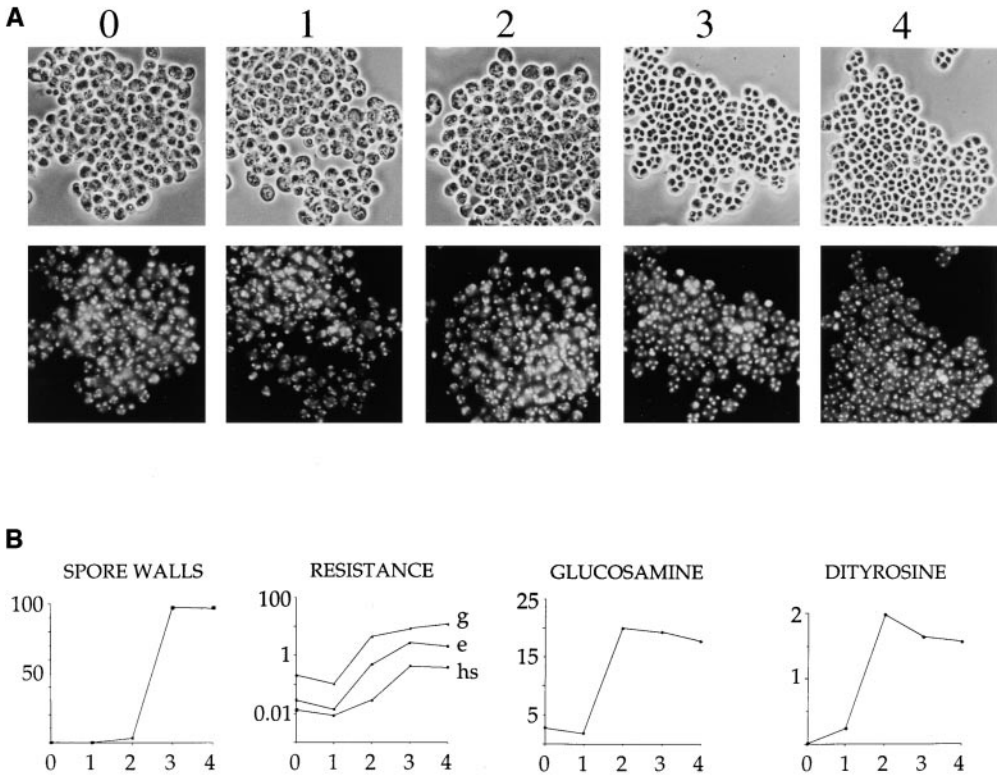

Figure 4.—Stepwise increases in *smk1-4* gene dosage allow the execution of more events; different events require distinct allelic thresholds. (A) Phase-contrast and DAPI epifluorescent microscopy of *smk1-4* asci. Strains that contain zero, one, two, three, or four chromosomal copies of the *smk1-4* allele were sporulated at 27.5° and examined by microscopy. Note the appearance of birefringent spore walls that accompanies the increase from two to three copies of the *smk1-4* allele (see quantitation in B). (B) Quantitative analysis of asci formed at 27.5° that contain zero, one, two, three, or four copies of *smk1-4*. Shown is the effect of stepwise increases in *smk1-4* gene dosage (*x*-axis) on the ability of spores to produce mature spore walls (*y*-axis plots the percentage of asci with birefringent spore walls), resistance to glusulase (g), ether (e), or heat shock (hs; *y*-axis plots survival relative to untreated asci) and on the ability to accumulate spore-specific glucosamine and dityrosine in insoluble fractions (*y*-axis plots nanomoles per 10^8 cells).

accumulation correlated with the coalescence of these vesicles and required the *SMK1* activity level found in the two-copy strain, the appearance of normal spore wall structures was coincident with the disappearance of these vesicles and required the slightly higher *SMK1* activity levels found in the three- or four-copy strains. These observations are consistent with the vesicles being intermediates in spore wall morphogenesis, which require increasing threshold levels of Smk1p activity for their accumulation and subsequent processing. The biochemical, morphological, and functional phenotypes of the *smk1-4* hypomorphic series show that *SMK1* is required for the execution of multiple developmental processes during spore morphogenesis. Furthermore, these results indicate that different biochemical and morphological events require distinct thresholds of *SMK1* activity.

The effect of *smk1-4* gene dosage thresholds on late gene expression was tested by examining *SPS100-lacZ* reporter gene activity during a sporulation time course. *SPS100-lacZ* activity in *smk1*-Δ asci is reduced fivefold compared to the wild type. The single-copy *smk1-4* strain expressed near-wild-type levels of reporter gene activity at the appropriate time, and this level was not further affected by increasing the *smk1-4* gene dosage. This suggests that expression of *SPS100* (which occurs after spore wall formation) may require a low threshold level of *SMK1* activity, which is met with a single copy of *smk1-4*.

Reduction of wild-type *SMK1* expression levels recapitulates distinct morphogenetic blocks: The dependence of different phenotypes on MAPK activity that we inferred from our studies of *smk1* hypomorphs suggested that different steps in spore wall morphogenesis require distinct levels of Smk1p enzyme activity. We therefore tested this hypothesis by varying the amount of wild-type *SMK1* and examining the effects on spore morphology. If the hypothesis is correct, then it should be possible to recapitulate certain *smk1-2* and *smk1-4* phenotypes by reducing wild-type *SMK1* expression levels.

We have characterized the *cis*-acting promoter elements that control the timing and magnitude of *SMK1* gene expression (Pierce *et al.* 1998). One particular promoter mutant, which contains substitutions in two *cis*-acting DNA elements, termed MSE^s and URS1^s, expresses *SMK1* mRNA constitutively, with severely reduced levels during middle sporulation. A single copy of the wild-type *SMK1* coding sequence under the control of this mutant promoter (*smk1-mse^s urs1^s*) in an otherwise *smk1*-Δ background does not allow for assembly of birefringent spore walls or formation of fluorescent spore wall structures (Figure 5, A and B). Consis-

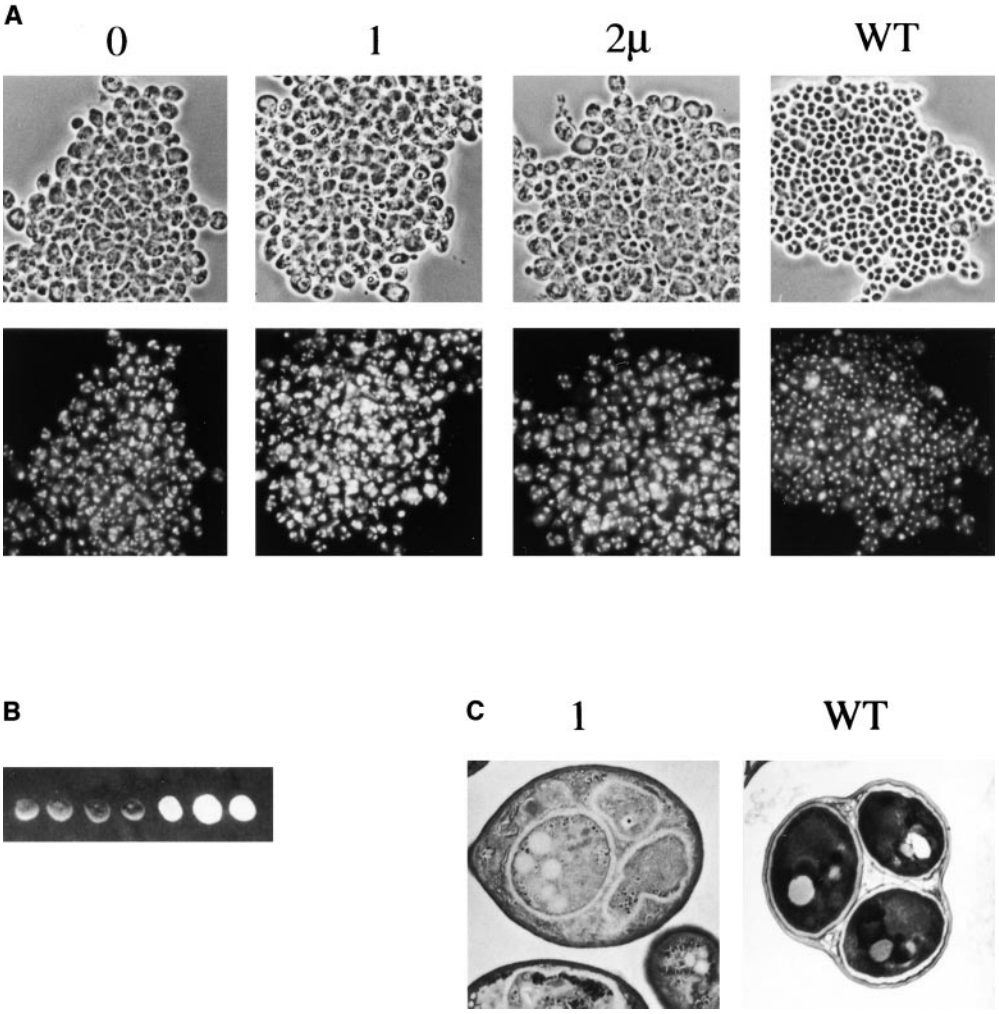

Figure 5.—A mutant promoter that reduces wild-type *SMK1* expression (*smk1-urs1*^{s} *mse*^{s}) recapitulates *smk1* missense mutant phenotypes. (A) Phase-contrast and DAPI epifluorescent microscopy of *smk1-urs1*^{s} *mse*^{s} asci. Strains that contain a single copy (1) of the *smk1-urs1*^{s} *mse*^{s} allele in an otherwise *smk1*-Δ background appear indistinguishable from *smk1*-Δ asci, with no evidence of birefringent spore walls. Also, for the most part, strains expressing *smk1-urs1*^{s} *mse*^{s} from a 2-μm high-copy-expression plasmid (2μ) do not exhibit birefringent spore wall morphology compared to the *SMK1* wild-type control (WT). (B) Fluorescence phenotypes of sporulated *smk1-urs1*^{s} *mse*^{s} strains. From left to right, zero-, one-, or two-copy strains of the *smk1-urs1*^{s} *mse*^{s} allele do not form fluorescent spore wall structures. The two-copy *smk1-urs1*^{s} *mse*^{s} strain, which harbors a low-copy centromeric *smk1-urs1*^{s} *mse*^{s}-plasmid, also does not fluoresce (middle patch). The final three (fluorescent) patches, from left to right, are a strain expressing *smk1-urs1*^{s} *mse*^{s} on a high-copy 2μ plasmid, as well as the *SMK1*/*SMK1* and *SMK1*/*smk1-urs1*^{s} *mse*^{s} controls. (C) Electron microscopy. Terminal asci of the single-copy *smk1-urs1*^{s} *mse*^{s} strain (1) predominantly appear with a distinct intermediate block in spore wall morphogenesis, with each of the visible spores within an ascus surrounded by the inner glucan layers and not by the outer spore-specific layers similar to those seen in the *smk1-2* asci formed at 34°. The ultrastructural phenotype of the wild-type control (WT) is shown for comparison.

tent with these observations, these mutant spores are hypersensitive to environmental stresses (Pierce *et al.* 1998). Reminiscent of the *smk1-2* phenotype, electron microscopic examination revealed that 49% (98/201) of the *smk1-mse*^{s}*urs1*^{s} asci block at a discrete stage of spore wall morphogenesis, with each of the four spores in a given ascus surrounded by an electron-lucent layer, but not the outer, more diffusely staining or thin, osmiophilic layers that are characteristic of wild-type spore walls (Figure 5C). In contrast, a single *smk1-mse*^{s} *urs1*^{s} allele in a wild-type *SMK1* background allows for production of asci that appear wild type in all respects, indicating that misexpression of *SMK1* does not cause a dominant phenotype.

The effects of increasing *smk1-mse*^{s} *urs1*^{s} gene dosage in an otherwise *smk1*-Δ background were examined. Asci with two or three copies of this mutant allele did not form fluorescent spore wall structures (Figure 5B), and they were morphologically indistinguishable from single-copy or null asci in phase-contrast microscopy. Strikingly, when the gene dosage was increased via expression from a 2μ vector, the asci did test positive in the fluorescence assay, but they did not have birefringent spore walls (Figure 5, A and B). Thus, the class II phenotype as seen in the *smk1-4* missense mutant is observed in the 2μ *smk1-mse*^{s} *urs1*^{s} genetic background. These data demonstrate that reducing expression of wild-type *SMK1* below certain thresholds can recapitulate distinct terminal phenotypes exhibited by the *smk1* missense mutants.

DISCUSSION

The distinct spectra of functional, biochemical, and morphological phenotypes exhibited by different *smk1* mutants demonstrate that *SMK1* is required for the execution of multiple steps during spore morphogenesis. The *SMK1*-dependent phenotypes characterized in this

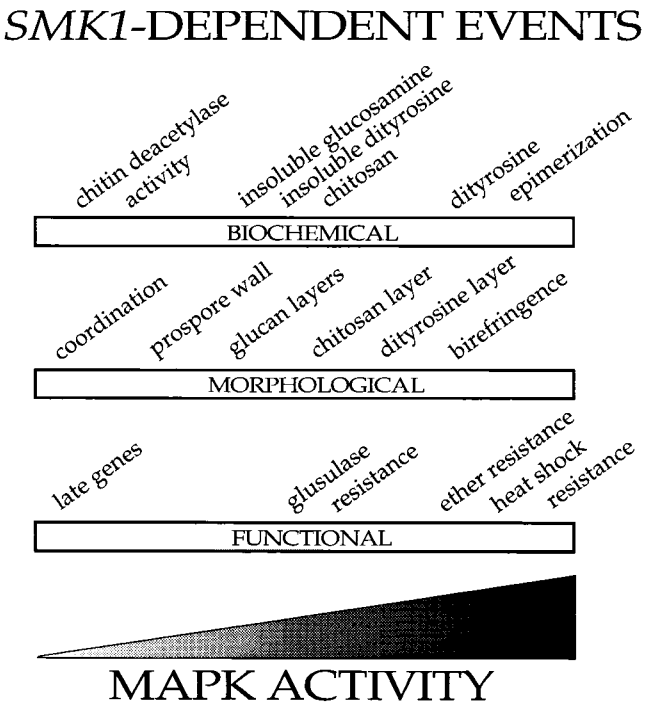

Figure 6.—Distinct steps in spore morphogenesis directly correlate with the magnitude of *SMK1* activity. The distinct *SMK1*-dependent biochemical, morphological, and functional phenotypes are shown relative to each other as a function of increasing *SMK1* activity levels. These phenotypes are ordered on the basis of dosage and allelic comparisons (see text for details). Higher *SMK1* activity thresholds allow for the completion of more and later events. Execution of these events may be directly or indirectly regulated by the Smk1p MAP kinase.

article are summarized in Figure 6. The number of steps that *smk1* asci can complete directly correlates with *SMK1* activity. In the *smk1* missense mutants, permissive conditions allow for the execution of more events than do restrictive conditions. Small increases in the dosage of a hypomorphic *smk1* allele result in the completion of more steps of the differentiation program, and distinct allelic thresholds are required for the acquisition of different wild-type-like phenotypes. Additionally, severe reduction of wild-type *SMK1* expression can recapitulate *smk1* missense mutant phenotypes, with small increases in expression levels allowing progression to more advanced intermediate stages of sporulation. These results demonstrate that the execution of distinct sporulation events requires distinct *SMK1* activity thresholds.

In a *smk1* null mutant, some semblance of morphogenetic processes can occur, but in a random, uncoordinated order, with each of the four spores in a single ascus exhibiting a distinct aberrant spore wall assembly pattern. The presence of some low threshold level of *SMK1* activity, provided by either a missense allele or reduced expression of the wild-type gene, restores the ability to coordinate morphogenetic events; however, the developmental program blocks at discrete intermediate stages. These mutants are unable to progress to the next stage, and yet they do not randomly assemble additional layers as the *smk1*-Δ do, suggesting the existence of *SMK1*-dependent checkpoint-like controls in spore wall morphogenesis.

A model for how distinct sporulation phenotypes are generated when *SMK1* activity is reduced to different levels must incorporate two fundamental concepts. The first concept involves dependency relationships among the multiple steps that characterize spore formation, and the second concept involves *SMK1* activity threshold requirements for some of these steps. *A priori*, certain events of spore morphogenesis must be dependent upon completion of a previous event. For example, spore wall precursors must accumulate to critical levels before they are assembled into recognizable spore wall structures, and certain layers of the spore wall must be assembled before others to generate the appropriate order of spore wall layers. A model based purely on dependency relationships would posit that *SMK1* is required for the completion of a single early step, and the efficiency with which this step is executed determines whether a subsequent morphogenetic event occurs, which in turn determines the execution of even later events. It is unlikely that a model based exclusively on dependency relationships can account for the diverse nature and number of *smk1*-dependent phenotypes, which include multiple and distinct homogeneous blocks in the differentiation program.

In a threshold model, distinct *SMK1* activity levels directly regulate multiple and distinct molecular events during the developmental program. Assuming that certain intermediate blocks in spore morphogenesis exhibited by different *smk1* mutants correlate with distinct execution points, it then follows that these threshold-dependent phenotypes reflect different quantitative requirements for *SMK1* during sporulation. Mutants with decreased *SMK1* activity progress only through those steps whose execution can be supported by the expressed activity threshold. Threshold-dependent control by protein kinases may be important for the coordination of morphogenetic programs whose inherent complexity has surpassed that which can be ordered by simple dependency relationships. A threshold model for the role of MAPKs in cellular differentiation provides a unifying principle for how complex morphogenetic processes might be temporally coordinated by a protein kinase.

How could functionally relevant *SMK1* thresholds be generated in the wild type? One might think that the tight transcriptional control of *SMK1* during spore development plays a significant role. However, we have demonstrated in other studies that this transcriptional regulation is not required for the progression of spore development as long as some critical amount of *SMK1* transcript is present during the middle sporulation window (Pierce *et al.* 1998). In fact, in wild-type asci, *SMK1* is transcribed to levels at least 10-fold higher than what is

necessary for normal spore development. Smk1p levels peak concomitantly with *SMK1* mRNA and then remain relatively constant during subsequent stages of development. These data suggest that *SMK1* thresholds are regulated by upstream signaling molecules. We have previously shown that *CAK1* positively regulates and is required for spore morphogenesis, thus implicating meiotic cell cycle events in Smk1p activation (Wagner *et al.* 1997). It is possible that there are multiple signals that impinge on Smk1p. Furthermore, different signals could have different quantitative effects on Smk1p enzyme activity.

The *smk1* allelic dosage experiments demonstrate that a twofold increase in MAPK catalytic activity can have qualitative effects on development. How could such a modest increase in *SMK1* activity serve as a switch to allow completion of the next event? If *SMK1* phosphorylates multiple substrates, then different substrates may have different affinities for the activated MAPK. One might imagine that if a high-affinity substrate is present in concentrations that approach that of the activated enzyme, then small changes in MAPK activity could have dramatic effects on its ability to interact with a lower-affinity substrate. The concentration of activated MAPK at which such a switch in substrate interaction occurs could define a threshold level of activity. Thus, the dynamics of substrate competition, in conjunction with regulated changes in the concentration of activated MAPK, might provide a mechanism by which distinct *SMK1* thresholds can specify different events during development. Additional layers of complexity that could be applied to this model include positive or negative regulatory feedback loops initiated by a particular Smk1p-substrate interaction or shifts in substrate availability. *SMK1* and spore morphogenesis may well provide the first documented example that MAPK thresholds can play an instructive role in organizing distinct morphogenetic events during a single cellular differentiation program.

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