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RESEARCH ARTICLE

Ssp2 Binding Activates the Smk1 Mitogen-Activated Protein Kinase

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ABSTRACT Smk1 is a meiosis-specific mitogen-activated protein kinase (MAPK) in *Saccharomyces cerevisiae* that couples spore morphogenesis to the completion of chromosome segregation. Similar to other MAPKs, Smk1 is controlled by phosphorylation of a threonine (T) and a tyrosine (Y) in its activation loop. However, it is not activated by a dual-specificity MAPK kinase. Instead, T207 in Smk1's activation loop is phosphorylated by the cyclin-dependent kinase (CDK)-activating kinase (Cak1), and Y209 is autophosphorylated in an intramolecular reaction that requires the meiosis-specific protein Ssp2. In this study, we show that Smk1 is catalytically inert unless it is bound by Ssp2. While Ssp2 binding activates Smk1 by a mechanism that is independent of activation loop phosphorylation, binding also triggers autophosphorylation of Y209 in Smk1, which, along with Cak1-mediated phosphorylation of T207, further activates the kinase. Autophosphorylation of Smk1 on Y209 also appears to modify the specificity of the MAPK by suppressing Y kinase and enhancing S/T kinase activity. We also found that the phosphoconsensus motif preference of Ssp2/Smk1 is more extensive than that of other characterized MAPKs. This study therefore defines a novel mechanism of MAPK activation requiring binding of an activator and also shows that MAPKs can be diversified to recognize unique phosphorylation motifs.

KEYWORDS MAPK, autophosphorylation, meiosis, yeast, mitogen-activated protein kinases

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Cells must adapt to changing environments and respond to extracellular signals, such as mitogens, developmental cues, and stress signals. Mitogen-activated protein kinases (MAPKs) are signal transducers that couple a broad array of extracellular signals to cellular responses by phosphorylating regulatory proteins (1). MAPKs are often activated by conserved kinase cascades composed of MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) family members. In these canonical signaling pathways, the activated MAPKK phosphorylates a threonine (T) and a tyrosine (Y) in the activation loop, a flexible loop of the MAPK that, upon phosphorylation, structurally locks the kinase into an active confirmation.

MAPKs can also be activated noncanonically by kinases outside the MAPKK family and by autophosphorylation. Some MAPKs, such as extracellular signal-regulated kinase 1 (ERK1) and ERK2, appear to be activated only by a canonical mechanism. Others, such as ERK7/8, appear to be activated only by a noncanonical mechanism (2, 3). However, the line separating canonical and noncanonical signaling is not always distinct. A MAPK can be activated by a canonical mechanism in one cell type and a noncanonical mechanism in another. For example, in many cells, the human p38α MAPK is activated by a MAPKK, while in T cells, p38α is activated by autophosphorylation (4, 5). Noncanonical and canonical mechanisms can also coregulate a MAPK in a single pathway. For example, the yeast Fus3 MAPK is canonically activated in response to mating pheromone, yet the Ste5 scaffold also stimulates Fus3 autophosphorylation of its activation

loop Y to shape the output properties of the system (6). Despite the widespread role of autophosphorylation in noncanonical MAPK signaling, relatively little is known about how the reaction is regulated.

Smk1 is a meiosis-specific MAPK in the yeast *Saccharomyces cerevisiae* that is activated noncanonically. In this pathway, the cyclin-dependent kinase (CDK)-activating kinase Cak1 phosphorylates Smk1 on its activating T (residue T207), while the sporulation-specific protein Ssp2 activates Smk1 autophosphorylation of its activating Y (residue Y209) (7–10). Activated Smk1 controls the postmeiotic program of spore morphogenesis (9, 11).

SMK1 and *SSP2* are transcriptionally induced during meiosis I (MI) as tightly regulated middle meiotic genes (both are activated by the Ndt80 transcription factor). *SMK1* mRNA is translated shortly after it is transcribed, and the newly translated Smk1 protein is phosphorylated on T207 by Cak1 (8, 10). In contrast, *SSP2* mRNA is translationally repressed until MII, as nuclear segregation is being completed. Thus, Smk1 is monophosphorylated on T207 during MI and dually phosphorylated on T207 and Y209 only as nuclear segregation is drawing to a close. Smk1 activation is also spatially regulated. Prior to MII, Smk1 is present throughout the cytoplasm. As cells progress through MII, Ssp2 is translated, and both Smk1 and Ssp2 localize to prospore membranes (PSMs) (8), the double membranous structures that grow around the meiotic products in anaphase II and serve as platforms for spore wall assembly (12, 13).

Ssp2 contains two functional domains, namely, a targeting domain (TD; residues 1 to 100) and a kinase-activating domain (KAD; residues 162 to 371) (8). The TD is required to target Ssp2 to the PSM, while the KAD is required to activate the autophosphorylation of Smk1 on Y209. Interestingly, $Ssp2^{KAD}$ contains two segments that show similarity to motifs termed RNA recognition motifs (RRMs). While RRMs typically bind specific segments of RNA to regulate RNA processing, translation, and other processes in the cell, RRMs have also been shown to bind protein (14, 15). Whether the RRMs in Ssp2 bind protein or RNA has not been established.

Genotype/phenotype studies show that an *smk1* phosphosite mutant encoding a Y209F change (*smk1-Y209F* mutant) has a partial-function terminal phenotype (10). The phenotype of the *ssp2*Δ mutant is more severe than that of the *smk1-Y209F* mutant and similar to that of the *smk1*Δ mutant. One interpretation of these data is that in addition to triggering the autophosphorylation of Smk1's activation loop Y, Ssp2 activates Smk1 through one or more additional mechanisms.

In this study, we show that Ssp2 activates Smk1 through two mechanisms. First, Ssp2 binding directly activates the kinase. Second, Ssp2 binding also triggers the autophosphorylation of Y209, which further enhances kinase activity and modulates specificity. Cak1 also increases catalytic activity by phosphorylating T207. This study also challenges the notion that all MAPKs share the same phosphorylation consensus motif by demonstrating that in addition to the classic MAPK phosphoconsensus, Smk1 also prefers a Y at the −4 position relative to the phosphorylation site.

RESULTS

Ssp2-dependent Smk1 autophosphorylation does not require additional meiotic factors. Ssp2 and Cak1 were previously identified as positive regulators of Smk1 (7, 9, 16). Although Cak1 is present in both meiotic and mitotic cells, Ssp2 and Smk1 are meiosis specific. In order to determine whether additional meiosis-specific proteins are required for activation of Smk1 autophosphorylation, we tested whether the ectopic expression of Ssp2 is sufficient to activate ectopically expressed Smk1 in mitotic yeast cells by using estrogen-inducible promoters. Smk1-8His-HA (Smk1-HH), purified using a nickel affinity resin (NTA) and tested with a Y209p phospho-specific antiserum, showed that Smk1 was phosphorylated on Y209 when glutathione *S*-transferase (GST) fused to Ssp2 lacking the TD ($Ssp2^{KAD}$-GST) was coexpressed with Smk1 but not when Smk1 was expressed by itself (Fig. 1A, NTA panels). $Ssp2^{KAD}$-GST purified using a reduced glutathione (GSH) affinity matrix enriched for the coexpressed Smk1 protein (Fig. 1A, GSH

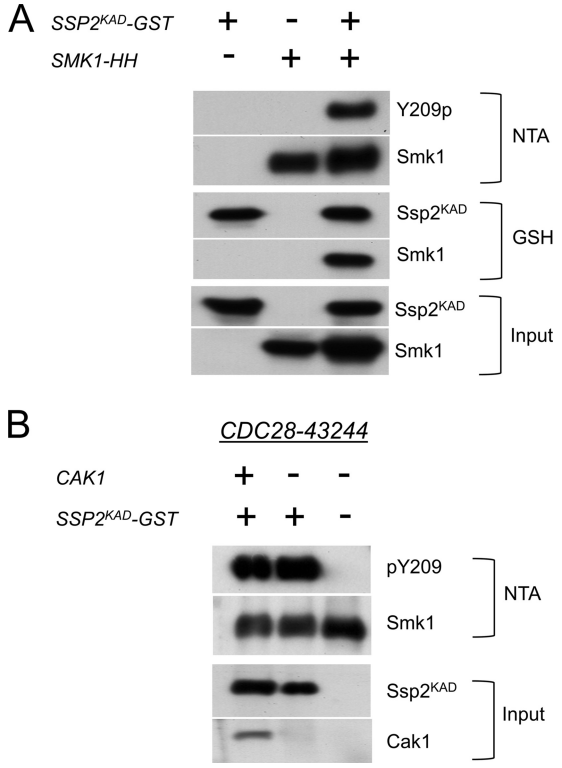


FIG 1 Ssp2-Smk1 complex formation and Smk1 autophosphorylation do not require additional meiosis-specific factors or Cak1. (A) Mitotic yeast cells expressing an estradiol-inducible form of the Gal4 transcription factor (Gal4-ER), with or without the indicated genes driven by *GAL1* promoters, were harvested at 6 h post-estradiol addition, when Ssp2^{KAD}-GST was at its maximal level. Total cellular extracts (input), protein that bound a GST-affinity resin (GSH), and protein that bound a Ni^{+} resin (NTA) were analyzed by immunoblotting using antibodies specific for GST (Ssp2), Smk1, or pY209, as indicated. (B) *GAL-Smk1-HH GAL4-ER CDC28-43244* haploid mitotic cells with or without the indicated genes were harvested at 6 h post-estradiol addition. Proteins were analyzed as described for panel A, using the indicated antibodies.

panels). These data demonstrate that Ssp2 is the only meiosis-specific protein required to activate Smk1 Y209 autophosphorylation.

Ectopic expression of full-length Ssp2-GST also activated Smk1 autophosphorylation, yet the fraction of Smk1 that was autophosphorylated on Y209 was lower than that when Ssp2^{KAD}-GST was expressed. When full-length Ssp2-GST and Ssp2^{KAD}-GST were coexpressed in mitotic cells by use of the estrogen-inducible promoter system, the full-length protein accumulated to only 17% of the level seen for the truncated Ssp2^{KAD}-GST protein. These data suggest that the TD, which is predicted to be disordered, can destabilize Ssp2. Because Ssp2^{KAD} is more stable than full-length Ssp2, we used Ssp2^{KAD} to activate Smk1 in the experiments described below.

Cak1, which is expressed in both mitotic and meiotic cells, positively regulates Smk1 by phosphorylating T207. We next asked whether Cak1 is required for Smk1 to autophosphorylate Y209. Cak1 activates the CDK Cdc28 by phosphorylating a T residue in its activation loop, and *CAK1* is therefore required for vegetative growth. It was previously shown that an allele of Cdc28 that contains a T $\rightarrow$ E substitution of the Cak1 phosphoacceptor as well as additional hyperactivating substitutions (*CDC28-43244*) bypasses the requirement of *CAK1* for mitotic growth (17). To test whether Cak1 is required for Ssp2-dependent Smk1 autophosphorylation, Smk1-HH was purified from estrogen-induced mitotic *CDC28-43244* cells containing and lacking *CAK1*. These experiments showed that Ssp2^{KAD} can trigger Smk1 autophosphorylation in the absence of Cak1 (Fig. 1B).

Ssp2 binds Smk1 and activates Smk1 autophosphorylation directly. To test whether the Ssp2-Smk1 complex can be formed *in vitro*, we incubated Ssp2^{KAD}-GST

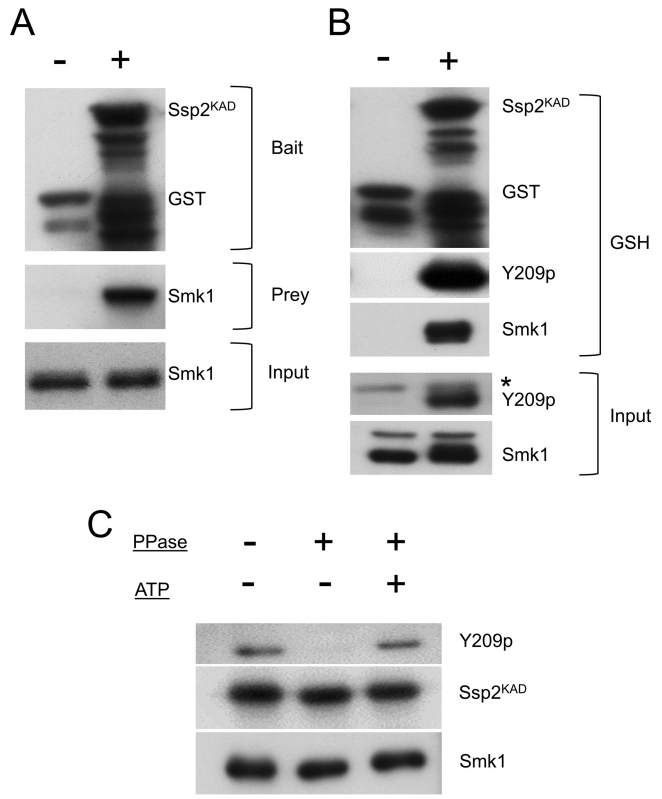


FIG 2 Ssp2 binds Smk1 and activates Smk1 autophosphorylation. (A) Purified GST (−) or Ssp2^{KAD}-GST (+) was incubated with yeast extracts containing Smk1-HH. GST and Ssp2^{KAD}-GST were repurified using GSH resin, and the material was assayed by immunoblot analyses with antibodies specific for GST (migration of Ssp2^{KAD}-GST and GST is indicated) or HA (Smk1). (B) Extracts of *E. coli* cells expressing Smk1 and GST (−) or Smk1 and Ssp2^{KAD}-GST (+) were purified using glutathione beads (GSH) and assayed by immunoblot analyses using the indicated antibodies. Equivalent fractions of the total extracts were also analyzed (input). For the input panels, the band observed above the Y209p signal (*) in both lanes is an unidentified cross-reacting *E. coli* protein. (C) The Ssp2^{KAD}-GST/Smk1 complex purified from bacteria was incubated with λ phosphatase (PPase). The phosphatase-treated complex was repurified using GSH resin and incubated in the presence (+) or absence (−) of ATP.

purified from *Escherichia coli* (bait) with a yeast mitotic extract containing ectopically expressed Smk1-HH (prey). Ssp2^{KAD}-GST was subsequently repurified from the mixtures and assayed by immunoblotting (Fig. 2A). Enrichment of Smk1 was observed with Ssp2^{KAD}-GST but not with GST.

To further study the Ssp2-Smk1 interaction, Smk1 was coexpressed in bacterial cells with Ssp2^{KAD}-GST or the negative control (GST). Smk1 enrichment was observed when Ssp2^{KAD}-GST was purified but not when GST was purified. Furthermore, Smk1 in the Ssp2^{KAD}/Smk1 complex was immunoreactive with the Y209p phospho-specific antiserum (Fig. 2B). Together, these data indicate that Ssp2 directly binds Smk1 and activates Smk1 autophosphorylation.

There are multiple examples of CDK/MAPK/glycogen synthase kinase (GSK)/CDK-like kinase (CMGC) group protein kinases that autophosphorylate activation loop Y residues. In some cases, autophosphorylation is associated with translation of the kinase. Based on these observations, it has been proposed that autophosphorylation of CMGC group kinases can require a transitional form of the protein that exists cotranslationally (DYRK1a) (18) or in association with chaperones that act shortly after a polypeptide is released from polyribosomes (GSK3β) (19). To investigate whether Ssp2-dependent Smk1 autophosphorylation requires a transitional intermediate state associated with translation, the Ssp2^{KAD}/Smk1 complex purified from *E. coli* was treated with phosphatase and subsequently incubated with ATP (Fig. 2C). This experiment showed that dephosphorylated Smk1 can autophosphorylate its activation loop Y after it has formed

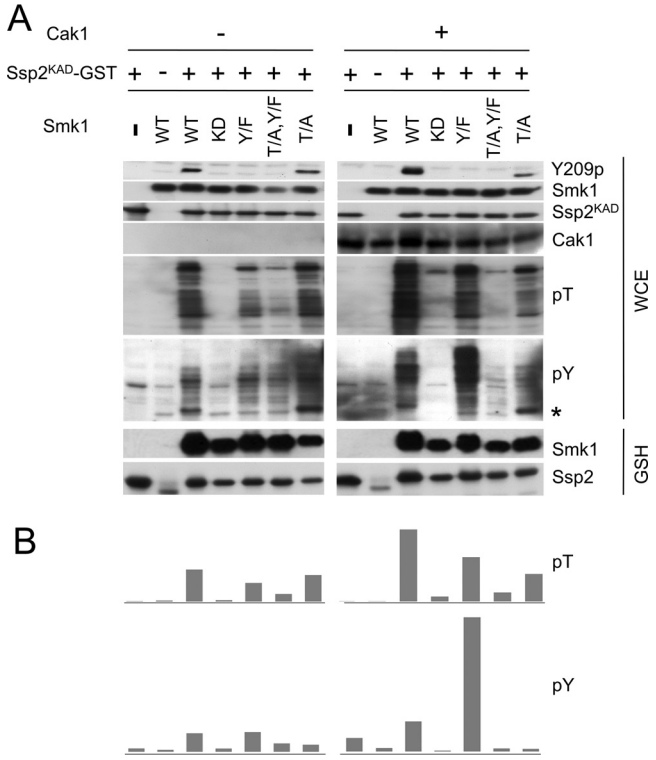


FIG 3 Reconstitution of Smk1 activation in bacteria. (A) *E. coli* cells harboring plasmids expressing Ssp2^{KAD}-GST, Cak1, and wild-type Smk1 (WT), kinase-dead Smk1 (KD), or Smk1 mutated at Y209F (Y/F), T207A/Y209F (T/A,Y/F), or T207A (T/A), as indicated, were harvested, boiled in SDS-containing sample buffer, and assayed by immunoblot analyses with the indicated antisera/antibodies (WCE). Ssp2^{KAD}-GST was purified from a fraction of the same samples, and the association of Smk1 was assayed by immunoblot analyses using a GST antibody (Ssp2) or an Smk1 antiserum, as indicated (GSH). The pY immunoreactive band indicated with an asterisk comigrated with Smk1. (B) Quantitation of the relative intensities of pT and pY immunoreactivities.

a stable complex with Ssp2^{KAD} and is presumably folded into its mature form. This indicates that although Smk1 autophosphorylation may occur in association with polyribosomes, it can also occur after translation and folding have taken place.

Reconstitution of Smk1 activation in bacteria. We next tested whether Smk1 activation can be reconstituted in bacteria by coexpressing various Smk1 mutants with Ssp2^{KAD}-GST in the presence or absence of Cak1 (Fig. 3A). Similar to that expressed in yeast, bacterially expressed Ssp2^{KAD}-GST activated the autophosphorylation of Y209 in the presence or absence of Cak1. A catalytically inactivating substitution in Smk1-K69R (KD) eliminated Ssp2-dependent autophosphorylation. Thus, the bacterial system accurately reflects many properties of the yeast meiotic pathway. These findings are consistent with a model in which binding of Ssp2 to Smk1 directly activates Smk1 autophosphorylation on Y209.

To investigate whether Smk1 is catalytically active toward endogenous bacterial proteins, total extracts were probed with an antibody specific for phosphothreonine (pT) (Fig. 3A). pT immunoreactivity was observed only when Ssp2^{KAD} and Smk1 were coexpressed. The further coexpression of Cak1 increased pT immunoreactivity toward bacterial proteins, but only in the samples in which T207 had not been mutated, and only when Ssp2^{KAD} was present. Smk1 catalytic activity was reduced when either T207 or Y209 was changed to a nonphosphorylatable residue (A or F, respectively [T/A or Y/F]), and Smk1 catalytic activity was reduced to a level near the background level when both the T and Y were mutated (Fig. 3B). These findings indicate that phosphorylation of either T207 or Y209 increases Smk1 catalytic activity, but only in the presence of Ssp2. These data also indicate that the doubly phosphorylated form of Ssp2/Smk1 is more active than either singly phosphorylated form of the enzyme.

We also tested the bacterial extracts by using an antiserum specific for phosphotyrosine (pY). These experiments showed that Ssp2/Smk1 not only autophosphorylates Smk1 on Y209 (the pY immunoreactive band corresponding to Smk1 is indicated with an asterisk in Fig. 3A) but also has the ability to phosphorylate bacterial proteins on Y. Nevertheless, the follow-up studies described below showed that activated Smk1 has a preference for T and S in target proteins. Notably, the Y/F substitution of Ssp2/Smk1 produced in the presence of Cak1 increased pY while decreasing pT in substrate proteins (Fig. 3B). This indicates that autophosphorylation of Smk1 on Y209 decreases its ability to function as a Y kinase while increasing its ability to function as an S/T kinase.

We also tested whether Smk1 and Ssp2^{KAD} formed a stable complex in these experiments by purifying Ssp2^{KAD}-GST with a GST affinity matrix and assaying the purified preparations with an Smk1 antiserum (Fig. 3A, GSH panels). The results of these experiments are consistent with Ssp2 binding being essential for Smk1 catalysis. These data also indicate that complex formation is not detectably affected by a mutation that reduces Smk1's ability to bind ATP (KD) and show that the complex is impervious to changes to the T and/or Y in Smk1's activation loop or to the presence of Cak1.

Ssp2 binding activates Smk1 catalytic activity *in vitro*. We purified Smk1-6His, Smk1-KD-6His, Smk1-6His/Ssp2^{KAD}, and Smk1-KD-6His/Ssp2^{KAD} proteins from bacteria that also expressed Cak1 and incubated these proteins with myelin basic protein (MBP) in the presence of radiolabeled ATP. Incorporation of radioactivity into MBP was observed in reaction mixtures containing Smk1-6His/Ssp2^{KAD} and not in companion reaction mixtures containing Smk1-6His alone (Fig. 4A). These data are consistent with the phospho-specific immunoassays of bacterial extracts described above and indicate that Smk1 is active only when it is bound to Ssp2. These findings indicate that phosphorylation of Smk1 on T207 by Cak1 is insufficient to activate Smk1.

Ssp2 may activate Smk1 exclusively by promoting the autophosphorylation of Smk1's activation loop Y, or it may also activate Smk1 through another mechanism. To address this issue, Ssp2-Smk1 activation loop mutants and kinase-dead mutants were purified from bacterial cells that also expressed Cak1, and the preparations were incubated with MBP in the presence of radiolabeled ATP. MBP was phosphorylated to the maximal level when it was incubated with Ssp2^{KAD}/Smk1. A moderate decrease in MBP phosphorylation was observed when MBP was incubated with Smk1-Y/F, whereas a more substantial decrease in MBP phosphorylation was observed with Ssp2^{KAD}/Smk1-T/A. No MBP phosphorylation was observed when MBP was incubated with Ssp2^{KAD} complexed with either Smk1-KD or Smk1-T/A,Y/F (Fig. 4B). Subsequent immunoblot analyses showed that the phosphorylated MBP from these reactions was recognized by the pT antibody but not the pY antibody. These data suggest that Ssp2 binding activates Smk1 independently of Smk1 Y209 phosphorylation and that Smk1 T207 and Y209 phosphorylation modulates Smk1 catalytic output.

Smk1 possesses a low level of tyrosine kinase activity. MAPKs are well-characterized S/T kinases, yet our previously published data demonstrate that Smk1 can transfer phosphate to itself on Y209, and the experiments presented above indicate that it can also transfer phosphate to Y residues in bacterial proteins during expression in *E. coli* (Fig. 3A and B). To further characterize the proteins phosphorylated by Smk1 in bacterial cells, lysates of bacteria expressing Ssp2, Cak1, and either wild-type (WT) Smk1 (in which the ratio of pY to pT immunoreactivity is low) or Smk1-Y/F (in which the ratio of pY to pT immunoreactivity is higher) were digested with protease (trypsin), phosphopeptides were enriched by a metal affinity procedure, and phosphosites were analyzed by mass spectrometry (MS). We identified 4,136 and 1,839 phosphopeptides in the wild-type and Smk1-Y/F samples, respectively. For the wild-type sample, only 0.9% of peptides were phosphorylated on Y, while 99.1% were phosphorylated on S or T. For the Smk1-Y/F sample, 3.5% of peptides were phosphorylated on Y, while 96.5% of the peptides were phosphorylated on S or T (Fig. 5A; see Tables S1 and S2 in the supplemental material). Statistical analysis of peptides phosphorylated on T or Y of

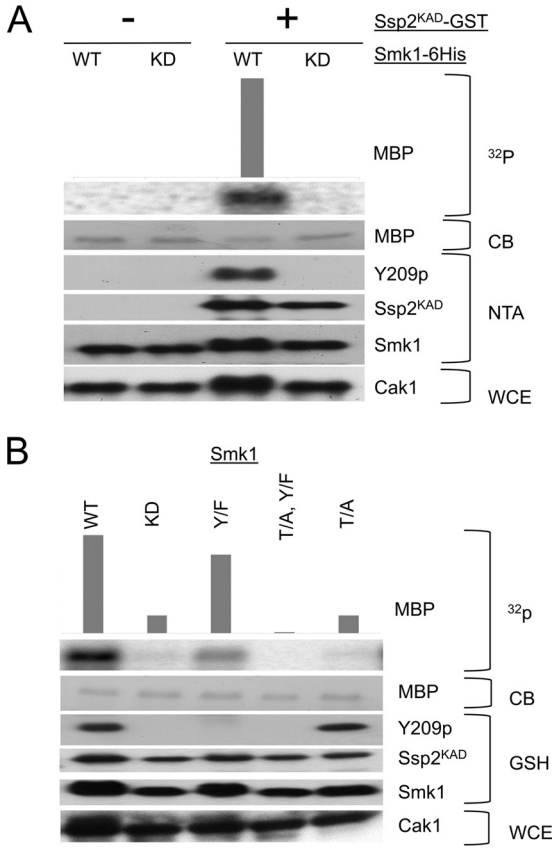
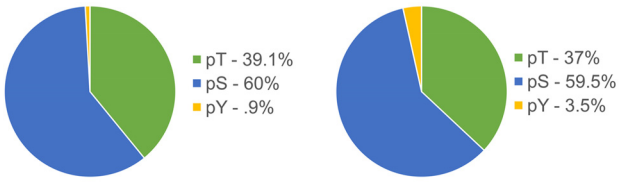


FIG 4 Ssp2 binding activates Smk1 catalytic activity. (A) Wild-type Smk1-6His (WT) and Smk1-K69R-6His (KD) were purified using Ni affinity (NTA) from bacteria that coexpressed Cak1 and Ssp2^{KAD}-GST (+) or expressed Cak1 alone (−), as indicated, and the purified material was analyzed by immunoblot analyses of the indicated proteins and of Y209p immunoreactivity. A fraction of the *E. coli* cells was also assayed by immunoblot analysis with Cak1 antiserum (WCE). The purified material was incubated with myelin basic protein (MBP) and radiolabeled ATP. Kinase reactions were resolved in an acrylamide gel, transferred to a membrane, and assayed for catalytic activity (^{32}P). The phosphorylation of MBP was quantitated with ImageJ. A companion acrylamide gel was stained with Coomassie blue (CB) to control for MBP loading. (B) Ssp2^{KAD}-GST/Smk1 complexes with the indicated Smk1 mutant proteins were purified using reduced glutathione beads and then analyzed by immunoblot analyses and quantitated as described for panel A (GSH). A fraction of the total *E. coli* cell extracts was also analyzed with a Cak1 antibody as for panel A (WCE). Purified preparations were incubated with myelin basic protein (MBP) and radiolabeled ATP. Kinase reactions were analyzed as described above. The WT, KD, Y/F, T/A,Y/F, and T/A forms of Smk1, shown from left to right, are designated as described in the legend to Fig. 3.

wild-type and Smk1-Y/F samples indicated that the increase in Y-phosphorylated peptides observed in Smk1-Y/F samples is statistically significant (Fig. 5A). These data are consistent with the pT and pY immunoblot analyses of bacterial whole-cell extracts and suggest that autophosphorylation of Smk1 on its activation loop Y enhances specificity for S/T versus Y residues in substrate proteins. We also note that the majority of the proteins that were phosphorylated on Y in lysates containing Smk1 or Smk1-Y/F are chaperones, transcriptional factors, translational factors, and ribosomal proteins (Fig. 5B; Table S1 and S2), which may have the tendency to physically form a complex with Ssp2^{KAD}/Smk1, thus overcoming K_m barriers that could otherwise limit the reaction. Collectively, these data suggest that the mature Ssp2/Smk1 enzyme is predominantly an S/T kinase but can also phosphorylate Y residues at a low rate in a reaction that is partially suppressed once autophosphorylation of Y209 takes place.

Smk1 phosphorylates S/T residues within a Y-X-P-X-S/T-P consensus motif. Most members of the CMGC group of protein kinases are proline-directed kinases, requiring a P at position +1 relative to the S/T phosphoacceptor. Individual family members in this group have additional preferences of various strengths, and MAPKs

A



	pS/pT	pY	Total
Smk1	4849	34	4883
Smk1-Y/F	1774	65	1839

Chi-Square
$X^2 = 74.2$
$P < 0.0001$

B Y-phosphorylated proteins

WT

%	Protein Functions
33	Ribosomal proteins/Translation factors/Transcription factors/Chaperones
67	Other

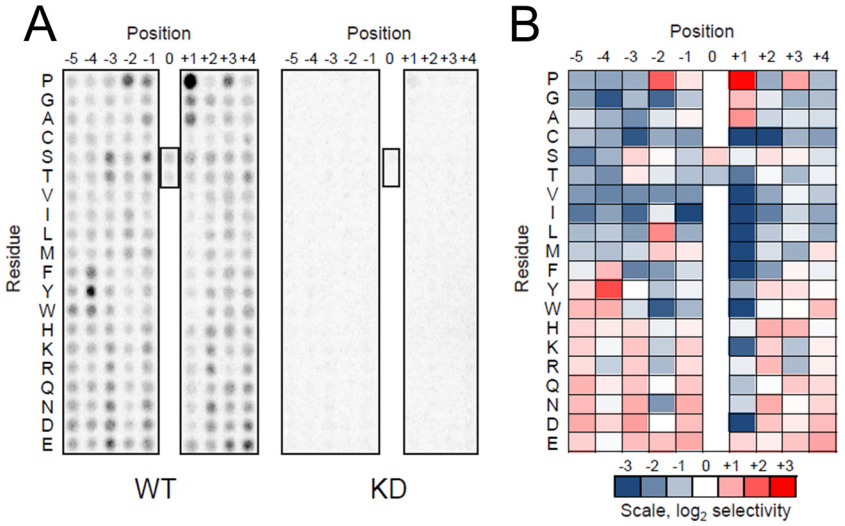
Y/F

%	Protein Functions
30	Ribosomal proteins/Translation factors/Transcription factors/Chaperones
70	Other

FIG 5 Smk1 prefers to phosphorylate substrates on serine and threonine but can also phosphorylate tyrosine. *E. coli* BL21(DE3) cells expressing Cak1, Smk1 or Smk1-Y/F, and Ssp2^{KAD} were lysed with denaturing buffer. Total cellular extracts were analyzed by mass spectrophotometry to identify peptides that were phosphorylated on threonine (T), serine (S), and tyrosine (Y). (A) (Top) Percentages of phosphorylated T, S, and Y peptides in Smk1- and Smk1-Y/F-containing extracts. (Bottom) Table showing the numbers of peptides that were phosphorylated on T, S, and Y in Smk1- and Smk1-Y/F-containing extracts. The *P* value was computed using the chi-square test. (B) Y-phosphorylated proteins identified by BLAST were sorted into the indicated sets based on annotations in the UniProt *E. coli* database.

have a modest preference for P at position −2. To define the Smk1 phosphorylation consensus motif, the Ssp2^{KAD}/Smk1 complex purified from bacteria expressing Cak1 was assayed with a combinatorial peptide library designed for S/T kinases (Fig. 6A and B). These analyses showed that Ssp2^{KAD}/Smk1 most strongly selects a P at +1, but like some other CMGC kinases, it appeared to tolerate a G or A at that position as well (20–22). Similar to other MAPKs, Ssp2^{KAD}/Smk1 preferred a P and, to a lesser extent, other aliphatic residues at position −2. Ssp2^{KAD}/Smk1 also showed an apparently unique strong preference for Y at position −4. The clearly defined pattern of phosphorylation observed in the context of peptides harboring a fixed S/T phosphoacceptor sequence suggests that Ssp2^{KAD}/Smk1 is predominantly an S/T kinase and that Y kinase activity on random peptide substrates is undetectable. We also compared the sequences surrounding the phosphorylated peptides identified in the bacterial phosphoproteomic analysis by using a program that normalizes amino acid occurrence in the set of pT- and pS-containing phosphopeptides to the relative abundance in the proteome (pLogo) (23, 24) (Fig. 6C; Table S1). The Ssp2^{KAD}/Smk1 phosphorylation consensus generated from this analysis is similar to the consensus generated from the combinatorial peptide arrays (compare Fig. 6C and D).

To interrogate whether Smk1-Y209 phosphorylation affects its phosphorylation site consensus, we also analyzed Ssp2^{KAD}/Smk1-Y/F by using the combinatorial peptide and bacterial phosphoproteomic approaches. While this analysis revealed the selectivity for P at the +1 position and for Y at the −4 position seen with the WT kinase, the lower activity of the mutant resulted in a low signal-to-noise ratio, precluding more in-depth analysis of its specificity (data not shown). The phosphoproteomic approach did reveal a modest preference for aspartate (D) at position +1 that was not observed with wild-type Ssp2^{KAD}/Smk1. One possible explanation for this observation is that the acidic property of Y209p prevents peptides containing D at position +1 from productively engaging the active site of the enzyme, raising the possibility that autophosphorylation influences the spectrum of substrates that are phosphorylated in the cell. Besides the additional preference for D at position +1, Smk1-Y/F showed the same −4Y, −2P, and +1P preferences as WT Smk1 (Fig. 6C). Together, these data define the Smk1 phosphorylation consensus motif as Y-X-P-X-S/T-P (Fig. 6D).



C

Smk1 S/T phosphorylation consensus (pLogo)								
-4	-3	-2	-1	0	1	2	3	4
Y	E	**P**/L	P/H/D	S	**P**/A/G	X	P/E	D/E
Y	N/E	**P**/L/A	P/D	T	**P**/A/G	X	P	K

Smk1-Y/F S/T phosphorylation consensus (pLogo)								
-4	-3	-2	-1	0	1	2	3	4
Y	E/R	**P**/L	D/N	S	**P**/A/D	D	P/E	X
Y	X	**P**	X	T	**P**/G/D	X	X	X

D

Smk1 S/T phosphorylation consensus (peptide array)								
-4	-3	-2	-1	0	1	2	3	4
Y	X	P	X	S/T	P	X	X	X

FIG 6 Identification of Smk1 phosphorylation consensus motifs. (A) Purified Ssp2^{KAD}-GST/Smk1 preparations were incubated with radiolabeled ATP and a combinatorial peptide library with the general sequence Y-A-X-X-X-X-X-S/T-X-X-X-X-A-G-K-K-biotin, where the indicated amino acid was present at the indicated position relative to the phosphoacceptor site and all other ("X") positions were equimolar mixtures of the 17 amino acids (excluding S, T, or C). Reaction mixtures were transferred to a streptavidin-coated membrane and analyzed with a phosphorimager. Data from a representative experiment ($n = 3$) are shown. (B) Spot intensities from panel A were quantified and normalized such that the average value within a position was equal to 1. The heat map shows average normalized data for 3 separate experiments with WT Ssp2^{KAD}/Smk1. (C) Sequences of peptides surrounding phosphorylated S or T residues generated from mass spectrometry of bacterial proteins were analyzed with pLogo software to generate S and T phosphorylation consensus motifs for WT Ssp2^{KAD}/Smk1 and Ssp2^{KAD}/Smk1-Y/F. Amino acids predicted to have a 4.08-log-odds probability or higher ($P > 0.05$) were included in the WT Smk1 and Smk1-Y/F phosphorylation consensus motifs for S and T. (D) Smk1 S/T phosphorylation consensus motif generated from the combinatorial peptide data shown in panel B.

To further test the influence of −4Y in Ssp2^{KAD}/Smk1 substrates, biotinylated peptides designed to be phosphorylated optimally based on the combinatorial peptide array data and that contained either a Y (Y-tide) or an A (A-tide) at position −4 were tested in Ssp2^{KAD}/Smk1 phosphotransferase reactions. We also assayed these peptides by using mammalian Erk2 purified from bacterial cells coexpressing constitutively active MEK1 (Fig. 7A). Activated Erk2 phosphorylated the A- and Y-tides to equivalent extents, whereas Ssp2^{KAD}/Smk1 phosphorylated Y-tide 5-fold more efficiently than A-tide at subsaturating peptide concentrations when reactions were terminated within the linear interval of the reaction (15 min).

We also assayed various concentrations of the Y- and A-tides in Ssp2^{KAD}/Smk1 phosphotransferase reactions. Fitting to the Michaelis-Menten equation produced a K_m value of 64.5 ± 2.7 μM for Y-tide (Fig. 7B). This value is significantly lower than the K_m

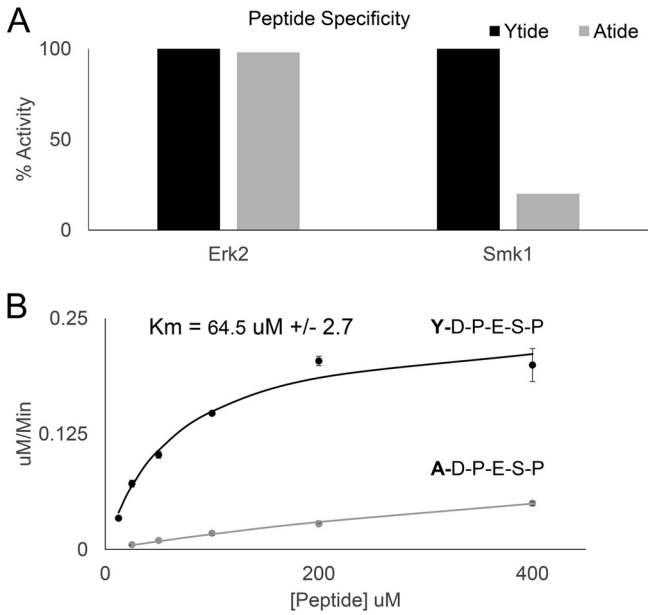


FIG 7 Kinetic analysis of Smk1. (A) Purified Erk2 and Ssp2^{KAD}-GST/Smk1 were incubated with a streptavidin-coupled peptide, either GDYDPESPHPEA (Y-tide) or GDADPESPHPEA (A-tide), in kinase buffer in the presence of radiolabeled ATP. Reaction mixtures were transferred to a streptavidin-coated membrane and quantitated. (B) Purified Smk1 was incubated in kinase buffer with radiolabeled ATP and various peptide concentrations ranging from 0 to 400 μM A-tide or Y-tide. Reactions were terminated, and reaction mixtures were transferred to a streptavidin-coated membrane and quantitated. A Michaelis-Menten curve was fit to the data to compute the K_m for Y-tide.

values for activated MAPK/substrate peptides reported in the literature (25). In contrast to the K_m for Y-tide, the K_m for A-tide was too high to be measured. Given that Smk1 bound Y-tide with such a high affinity, it is conceivable that Smk1 also binds its substrates with a high affinity.

An "early pool" of Smk1 is not required for spore morphogenesis. Although dual phosphorylation of the TXY motif is required for full activation of a variety of different MAPKs, a MAPK can also be partially activated by monophosphorylation on either the T or the Y (26). Our published genetic data demonstrate that thresholds of Smk1 activity are required for different steps in spore morphogenesis (27). We have also shown that Smk1 that is present early in meiosis is monophosphorylated on T207, while Smk1 that is present later in the program, when exit from MII occurs, is predominantly phosphorylated on both T207 and Y209 (8, 10). Based on these findings, we previously hypothesized that Smk1 exists in two states: a low-activity state (meiosis I) and a high-activity state (meiosis II). However, the data presented in this study indicate that Ssp2 binding is essential for any Smk1 activity and that Smk1 is therefore inactive until Ssp2 is translated during MII.

Ssp2 is not translated until MII because *SSP2* mRNA is translationally repressed by the Rim4/Ime2 pathway until MII (8). *CLB3* is the founding member of the set of Rim4/Ime2-responsive meiotic mRNAs, and its translational repression is controlled by elements in its 5′ untranslated region (5′UTR) (28). Thus, placing other mRNAs under the control of the *CLB3* promoter generates mRNAs that are not translated until MII. Several other members of the Rim4/Ime2-regulated set of mRNAs are also controlled through their 5′UTRs (29). We replaced the *SMK1* promoter with the *SSP2* promoter to investigate whether *SSP2* translational repression may be controlled by its 5′UTR, similar to that of other members of the Rim4/Ime2 set of mRNAs, since if it were, this *SSP2pr-SMK1* allele would eliminate Smk1 translation until MII, thus providing a tool for assessing the functional significance of the early (MI) pool of Smk1.

The *SSP2pr-SMK1/SSP2 smk1*Δ*/smk1*Δ strain and an *ssp2*Δ*/SSP2 SMK1/smk1*Δ control strain were sporulated, protein samples were collected every 30 min post-MI (5 h

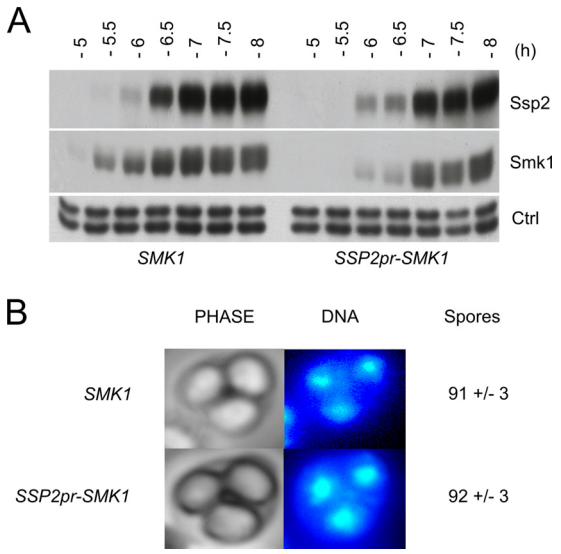


FIG 8 "Early Smk1" is not required for spore morphogenesis. (A) Cells expressing the *SMK1* open reading frame from its natural promoter (*SMK1*) or from the *SSP2* promoter (*SSP2pr-SMK1*) were collected at the indicated times after sporulation was induced and analyzed by electrophoresis and immunoblotting for Ssp2, Smk1, and Cdc28 (Ctrl), as indicated. (B) Sporulating cells from panel A were collected at 24 h postinduction, stained with DAPI, and examined by phase-contrast (PHASE) and fluorescence (DNA) microscopy. The strains were sporulated for 48 h, and the fraction of cells that formed spores was quantified by phase-contrast microscopy (100 cells counted per culture; $n = 3$).

postinduction), and Ssp2 and Smk1 proteins were assayed by electrophoresis and immunoblot analyses. The production of Smk1 in *SSP2pr-SMK1/SSP2* cells was delayed compared to that in the control strain and coincided with the production of Ssp2, which accumulated only in cells that were completing MII (6 h) (Fig. 8A). These data suggest that *SSP2* translational repression is mediated by its 5′UTR, similar to that of other Ime2/Rim4-regulated mRNAs. The *SSP2pr-SMK1/SSP2* strain completed meiosis and sporulated indistinguishably from the control cells (Fig. 8B). Moreover, the fractions of *SSP2pr-SMK1/SSP2* and control spores that produced viable colonies after extended enzymatic (glusulase) digestion and microdissection were indistinguishable (32/48 and 31/48 spore colonies, respectively). Together, these microscopic and functional assays of spore wall assembly suggested that the early pool of Smk1 is not required for sporulation and led to a revision of the model of Smk1 function in coordinating spore morphogenesis.

DISCUSSION

In previous studies, we demonstrated that Smk1 is activated in a developmentally regulated noncanonical pathway in which Cak1 phosphorylates the activation loop T of Smk1 during MI and Ssp2 induces Smk1 to autophosphorylate its activation loop Y as cells exit MII. This study demonstrates that the C-terminal half of Ssp2 directly binds Smk1 and triggers Smk1 autophosphorylation. In addition, this study shows that Ssp2 binding activates Smk1 catalytic output through a mechanism that is independent of activation loop autophosphorylation. In previous studies, we showed that Ssp2 is tightly localized to the PSM and that Ssp2 translation is triggered specifically as cells are completing meiosis. Together these findings show that Ssp2 functions as a modular integrator of noncanonical MAPK signaling that spatiotemporally controls Smk1 activation in this developmental program.

Model of *SMK1* function in spore morphogenesis. *SMK1* is transcribed slightly earlier than most middle genes (it has been classified as an early/middle gene) (30), and unlike that of *SSP2*, its mRNA is almost immediately translated. Shortly after Smk1 is produced, it is phosphorylated on T207 by Cak1. Thus, Smk1-T207p is present significantly before Ssp2/Smk1-T207p,Y209p (the "early" and "late" pools of Smk1, respec-

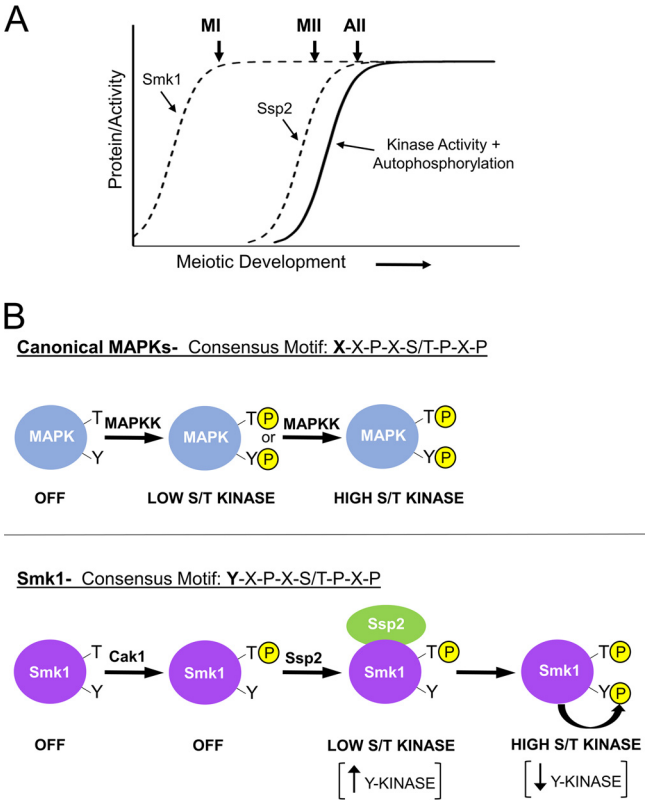


FIG 9 Models for Smk1 pathway regulation and function. (A) Model illustrating the timing of Smk1 and Ssp2 expression and Smk1 activation. (B) Comparison of canonical versus Smk1 MAPK activation. See the text for details.

tively). Moreover, the early pool of Smk1 is distributed throughout the cell, while the late pool of Smk1 is localized to the PSM. These properties of the system raised the possibility that early Smk1 has a distinct role in meiotic development. However, this study shows that Smk1-T207p that is not complexed to Ssp2 is catalytically inert *in vitro*. Furthermore, delaying the production of Smk1 by use of the *SSP2* promoter, thereby eliminating early Smk1 from the system, does not detectably affect the sporulation program. Together these findings led to a revision of our model of Smk1 function. This revised model posits that early Smk1 is nonfunctional and that Smk1-T207p may be best understood as a "primed" form of Smk1 that is awaiting activation in a switch-like manner (Fig. 9). It follows that the threshold-dependent properties of the system revealed by genotype/phenotype studies (27) are not established through a mechanism involving differential phosphorylation of the TXY activation motif of the MAPK. One possibility is that variations in the concentration of the Ssp2/Smk1 complex, which increases following exit from MII in conjunction with PSM closure, play a role in the threshold-dependent outputs that regulate this morphogenetic program.

Mechanism of Smk1 activation by Ssp2. Activation loop phosphorylation is sufficient to activate many MAPKs (e.g., ERK1/2) in the absence of additional binding proteins. In those cases, activation loop phosphorylations induce multiple structural alterations to the MAPK. These changes not only reposition the activation loop so that it no longer blocks the active site but also can reorient ATP, increase the affinity for ATP, induce changes to the active site, and modify the positions of segments of the enzyme that interact with substrates through allosteric mechanisms (31). Our data demonstrate that Ssp2 binding is an essential step that activates Smk1 but that either the T or the Y also needs to be phosphorylated for the Ssp2/Smk1 complex to have detectable catalytic activity. These findings suggest that Ssp2 and phosphorylation of the TXY motif activate Smk1 through distinct mechanisms. There are 2 examples of MAPKs

whose activation loops are noncanonically activated by MAPK-binding proteins that have been studied using structural approaches. The first is p38α, which is activated in cardiac myocytes by transforming growth factor beta-activated protein kinase binding protein 1 (Tab1) (32). Similar to that of Ssp2, Tab1 binding activates *cis* autophosphorylation, yet unlike Ssp2, Tab1 activates the autophosphorylation of both the activation loop T and the Y. Also similar to the proposed Ssp2/Smk1 mechanism, Tab1 activation of p38α precedes activation loop phosphorylation. This mechanism has been shown to allosterically increase the affinity of p38α for ATP. The second example of a structurally defined MAPK-binding protein interaction that triggers activation loop autophosphorylation is the yeast Ste5-Fus3 interaction (6, 33). While the *cis* autophosphorylation of Fus3's activation loop Y is activated by the binding of a segment from Ste5, this interaction does not appear to increase Fus3's affinity for ATP. Instead, Ste5 appears to activate Fus3 autophosphorylation by inducing a structural change that aligns Fus3's N-terminal (ATP binding) lobe with its C-terminal lobe. Further structural studies of the Ssp2/Smk1 complex are required to elucidate whether Ssp2 activates Smk1 by a Tab1/p38α-like mechanism, an Ste5/Fus3-like mechanism, or a completely different mechanism.

Previous studies have shown that multiple CMGC group kinases autophosphorylate their activation loop Y residues in *cis*. In the case of the DYRK1 family of kinases, the *Drosophila* enzyme is activated by an extension of the kinase domain, and it has been proposed that this reaction occurs cotranslationally (18). While this reaction appears to require a transitional intermediate form of the enzyme that exists during translation, this has been reported not to be the case for DYRK1A from mammals (34). In the case of GSK3β, phosphorylation of the activation loop Y occurs shortly after translation and requires the Hsp90 chaperone (19). In this study, we have shown that Ssp2/Smk1 that has been dephosphorylated *in vitro* can undergo autophosphorylation of Y209. Moreover, Ssp2 from bacteria can form a complex with Smk1 in yeast extracts. Although it is not known whether these reactions are physically coupled to translation/folding in the cell, these findings do not support a cotranslational or ribosome-associated requirement for the reaction.

The ability of ectopically expressed Ssp2/Smk1 to phosphorylate bacterial proteins on Y residues (albeit at a low level relative to that on S and T residues) and the increase in pY compared to pT and pS when Y209 in Smk1 is mutated to a nonphosphorylatable F residue raise the possibility that Smk1 that has not undergone autophosphorylation of its activation loop Y has a low level of discrimination for phosphoacceptor residues. If this is so, then discrimination against Y may be enhanced by autophosphorylation of Y209. According to this line of reasoning, autophosphorylation of Smk1 on its activation loop Y may be part of a feedback-like mechanism that restricts the ability of Smk1 to engage Y in its catalytic site while increasing overall catalytic activity. Further studies are required to establish whether this is the case and whether this mechanism is shared by other CMGC kinases.

Ssp2 as a cyclin-like activating subunit. The finding that Ssp2 can activate Smk1 by direct binding draws parallels to cyclin-CDK interactions. Indeed, other Smk1/CDK parallels exist, including the activation of Smk1 by a CAK and the tight coupling of Ssp2 to specific stages of the cell cycle (in this case, exit from MII). In this regard, it is interesting that expression of the Clb3 cyclin and Ssp2 is controlled by the same transcription factor (Ndt80) and that the Rim4 translational repressor holds both *CLB3* and *SSP2* mRNAs inactive until translational derepression is triggered by Ime2 (35, 36). In addition, similar to that of cyclin-CDKs, maximal phosphorylation of substrates by Ssp2/Smk1 requires residues ($-$4Y for Smk1) in addition to the minimal S/T-P site. Whether the requirement of Y at the -4 position is a consequence of Ssp2 binding or an inherent property of the Smk1 MAPK remains an open question. Despite the Ssp2/Smk1 and cyclin/CDK parallels, it is important to note that the primary sequences of Ssp2 and cyclins are unrelated. Indeed, the two segments of Ssp2 that show significant similarities to other yeast proteins are an 80-residue segment at the N

TABLE 1 Yeast strains used in this study[a]

Strain	Genotype
JTY83	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 KAN::pGAL1-SMK1-8HIS-HA::LEU2/smk1::LEU2 ura3::pGPD1-GAL4(848)ER::URA3/ura3 SSP2-ΔN137-GST::TRP1/SSP2*
JTY84	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 KAN::pGAL1-SMK1-8HIS-HA::LEU2/smk1::LEU2 ura3::pGPD1-GAL4(848)ER::URA3/ura3 KAN::pGAL1-SSP2-ΔN137-GST::TRP1/SSP2*
JTY85	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 KAN::pGAL1-SMK1-8HIS-HA::LEU2/smk1::LEU2 ura3::pGPD1-GAL4(848)ER::URA3/ura3 KAN::pGAL1-SSP2-GST::TRP1/SSP2*
JTY86	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 smk1::LEU2/smk1::LEU2 ura3::pGPD1-GAL4(848)ER::URA3/ura3 KAN::pGAL1-SSP2-ΔN137-GST::TRP1/SSP2*
JTY97	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1/smk1::LEU2 ura3::pGPD1-GAL4(848)ER::URA3/ura3 KAN::pGAL1-SSP2-ΔN137-GST::TRP1/KAN::pGAL1-SSP2-GST::TRP*
JTY102	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 SMK1-3HA::HIS3/smk1::LEU2 SSP2-13MYC::KAN/ssp2Δ*
JTY103	*MAT***a***/MATα ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2 smk1::LEU2/smk1::LEU2 SSP2-13MYC::KAN/pSSP2-SMK1-3HA::KAN*
TPY1007	*MATα ura3 leu2::hisG trp1::hisG lys2 ho::LYS2 KAN::pGAL1-Smk1-8HIS-HA::LEU2 ura3::pGPD1-GAL4(848)ER::URA3 cak1Δ::TRP1 cdc28-43244*
TPY1010	*MATα ura3 leu2::hisG trp1::hisG lys2 ho::LYS2 KAN::pGAL1-Smk1-8HIS-HA::LEU2 ura3::pGPD1-GAL4(848)ER::URA3 cak1Δ::TRP1 cdc28-43244 KAN::pGAL1-SSP2ΔN137-GST::TRP1*
TPY1012	*MATα ura3 leu2::hisG trp1::hisG lys2 ho::LYS2 KAN::pGAL1-Smk1-8HIS-HA::LEU2 ura3::pGPD1-GAL4(848)ER::URA3 cdc28-43244 KAN::pGAL1-SSP2ΔN137-GST::TRP1*

[a]All strains were constructed as part of the present study.

terminus of Ssp2^{KAD} (amino acids 162 to 242) and a 90-residue segment at its C terminus (amino acids 281 to 371) that resemble RRMs. We previously showed that even relatively small truncations in either of these segments eliminate Ssp2's ability to activate Smk1 autophosphorylation and cause *ssp2*Δ-like phenotypes (8). Thus, one possibility is that Ssp2 interacts with Smk1 via its RRM-like segments. Further studies are required to test whether this is the case and whether protein kinases other than Smk1 can be activated by Ssp2.

MATERIALS AND METHODS

Yeast strains, bacterial strains, culture conditions, and plasmids. All yeast strains used in this study are in the SK1 background (Table 1). Vegetative cultures were maintained in YPD (1% yeast extract, 2% peptone, 2% glucose). For estradiol induction, cells were grown overnight in YPR (1% yeast extract, 2% peptone, 2% raffinose). Cells were collected by centrifugation, washed in YPR, and resuspended to 10^7 cells/ml in YPR medium containing 2 μM β-estradiol. Resuspended cells were incubated at 30°C on a roller drum. For sporulation experiments, cells were grown overnight in YPA (1% yeast extract, 2% peptone, 2% potassium acetate) to a density of 10^7 cells/ml (mid-log phase). Cells were washed in 2% potassium acetate, resuspended to 4×10^7 cells/ml in sporulation medium (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), and placed on a roller drum at 30°C.

The SK1 forms of yeast genes were used for all plasmids used in this study. For pET-DUET-derived expression plasmids, the internal NdeI and NcoI sites in *SSP2* and *SMK1* were mutated by site-directed mutagenesis. Subsequently, PCR products of *SSP2ΔN137-GST* and *GST* alone, containing a 5′ NcoI site at the initiator ATG and a NotI site at the 3′ end, introduced by the oligonucleotides used for amplification, were digested and inserted into the same restriction sites to generate pJT110 and pJT111, respectively. Untagged wild-type, catalytically inactive (K69R), and activation loop (T207A, Y209F, and T207A/Y209F) forms of *SMK1* containing 5′ NdeI sites and 3′ KpnI sites were inserted into the same sites in pJT110 and pJT111 to generate pJT114, pJT115, and pJT127 to pJT130. A *CAK1* PCR product with a 5′ BglII site and a 3′ XhoI site introduced with oligonucleotides was inserted into the same sites in pACYC-Duet to generate pJT122. *SMK1* and *smk1-K69R*, containing 5′ NdeI and 3′ SalI sites introduced by PCR, were inserted into pET-30b at the same sites, generating *SMK1-6HIS* (pJT66) and *smk1-K69R-6HIS* (pJT70) plasmids. *SMK1-6HIS* and *smk1-K69R-6HIS* fragments were released from pJT66 and pJT70 and inserted into pJT111 by use of NdeI and KpnI to generate pJT132 and pJT133, respectively. Plasmids are listed in Table 2.

Recombinant proteins were expressed in BL21(DE3) bacteria. For the expression of Smk1, Ssp2, and Cak1, cells were grown overnight in LB (10% tryptone, 10% NaCl, and 5% yeast extract) containing a selective antibiotic(s) (100 μg/ml for ampicillin, 25 μg/ml for chloramphenicol, and 50 μg/ml for kanamycin). Saturated cultures were diluted 1:100 in LB with corresponding antibiotics and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. Cells were chilled on ice for 30 min and then induced with 500 μM IPTG (isopropyl-β-D-thiogalactopyranoside) at 18°C for 20 h. Cells with plasmids expressing ERK2 and constitutively active MEK1 were induced as described previously (37).

Purification of proteins. For purification of GST-tagged Ssp2, 1×10^9 β-estradiol-induced yeast cells were collected by centrifugation, washed with yeast lysis buffer (YLB; 300 mM NaCl, 5 mM $MgCl_2$, 25 mM

TABLE 2 Plasmids used in this study

Plasmid	Description	Source or reference
pJT66	pET-30b + *SMK1-6HIS*	This study
pJT70	pET-30b + *SMK1-K69R-6HIS*	This study
pJT110	pET-Duet-1 + *GST*	This study
pJT111	pET-Duet-1 + *SSP2ΔN137-GST*	This study
pJT114	pET-Duet-1 + *GST* + *SMK1*	This study
pJT115	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1*	This study
pJT122	pACYC-Duet-1 + *CAK1*	This study
pJT127	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1-K69R*	This study
pJT128	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1-Y209F*	This study
pJT129	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1-T207A,Y209F*	This study
pJT130	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1-T207A*	This study
pJT132	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1-6HIS*	This study
pJT133	pET-Duet-1 + *SSP2ΔN137-GST* + *SMK1-K69R-6HIS*	This study
pJT168	pGEX4T-1 + *GST-ERK2*	37
pJT169	pET-28a(+) + *MKK1ΔN3*	37

Tris-Cl, pH 8.0, 1 mM dithiothreitol [DTT], 0.5% NP-40) supplemented with protease inhibitors at previously specified concentrations (38), and lysed in 1 ml of YLB. Recombinant GST-tagged proteins were purified as described previously (8). For the purification of HH-tagged Smk1 under denaturing conditions, 5×10^8 β-estradiol-induced cells were collected by centrifugation, lysed, and purified as previously described (39).

For purification of GST-tagged Ssp2-Smk1 complexes, GST-ERK2, and GST (negative control) proteins from BL21(DE3) cells, 200 ml of IPTG-induced cultures was centrifuged and cells were washed with bacterial lysis buffer (BLB; YLB without NP-40 and with 1 mM phenylmethylsulfonyl fluoride [PMSF] as a protease inhibitor) and lysed in BLB by sonication (model 60 Sonic Dismembrator; Fisher Scientific) with 4 10-s pulses at an intensity of 7, with intermittent cooling on ice. Lysed cells were incubated with 0.5% NP-40 at 4°C with end-over-end rotation for 10 min, and extracts were clarified by centrifugation at 22,000 × *g* for 30 min. Cleared lysates were incubated at 4°C for 2 h with 500 μl of glutathione-Sepharose 4B (GE Healthcare). Beads were washed with BLB and kinase buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$) and then eluted in 500 μl of kinase buffer containing 25 mM reduced glutathione (Acros Organics), pH 7.4. Glycerol was added to a final concentration of 20% before freezing the preparations at −80°C, and protein was quantitated by electrophoresis using bovine serum albumin as a standard, with Coomassie brilliant blue R-250 staining. HH-tagged monomeric Smk1 and Smk1-Ssp2 complexes were purified by use of a protocol similar to the one described above, with the following modifications: 1 mM DTT was replaced with 20 mM imidazole in the BLB, and 25 mM reduced glutathione was replaced with 200 mM imidazole in the elution buffer.

Kinase assays and combinatorial peptide library screening assay. For protein kinase assays using myelin basic protein (MBP) as a phosphoacceptor, 1 μg of eluted GST-tagged complexes or HH-tagged complexes was incubated with 2 μg of MBP in kinase buffer containing 10 μM ATP and 0.4 μCi [γ-^{32}P]ATP for 60 min at 30°C. Reactions were terminated with 2× Laemmli buffer. MBP kinase reaction products were separated by polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane, analyzed with a phosphorimager (Typhoon 9400), and quantitated with ImageJ.

For peptide kinase assays, the biotinylated Y-tide GDYDPESPHPEA and A-tide GDADPESPHPEA (Genscript) were suspended in dimethyl sulfoxide (DMSO) to a final stock concentration of 20 mM. For velocity-versus-time analyses of Ssp2-Smk1, 0.1 μg of eluted GST-Ssp2-Smk1 complex was incubated in kinase buffer with 150 μM Y-tide, 1 mM DTT, 2 μCi [γ-^{32}P]ATP, and 100 μM ATP in a 20-μl reaction mixture. Reaction mixtures were incubated for either 0, 5, 15, or 45 min at 30°C, terminated with 10 μl of 7.5 M guanidinium hydrochloride, and applied to SAM biotin capture membranes (Promega), which were subsequently washed with 2 M NaCl four times, 2 M NaCl and 1% H_3PO_4 four times, and water twice, for 3 min per wash. Membranes were dried, and the reactions were quantitated by scintillation spectrometry. For measurements of reaction rate versus ATP concentration, reactions were initiated and analyzed as described above, with the following modifications: reaction mixtures were incubated at 30°C for 15 min with various ATP and [γ-^{32}P]ATP concentrations. For K_m analyses, kinase reactions were carried out as described above, but in the presence of different peptide concentrations at 30°C for 15 min. Comparative analysis of Erk2 and Smk1 specificities was performed and analyzed as described above, with the following modifications: 0.05 μg of Erk2 or 0.1 μg of Smk1 was incubated with either 150 μM Y-tide or A-tide for 30 min at 30°C in kinase buffer with 100 μM ATP and 2 μCi [γ-^{32}P]ATP. Combinatorial peptide library screening assays of Smk1 were performed as described previously (40).

Electrophoresis and immunoblot analyses. For the ectopic expression of Ssp2 and Smk1 in mitotic yeast cells and for sporulation experiments, cells were lysed with NaOH and proteins were extracted with 8 M urea as described previously (41). Proteins were analyzed as described previously (8, 10). BL21(DE3) total protein extracts were prepared by resuspending 3×10^8 cells in 100 μl of Laemmli buffer and by boiling. Proteins were separated using polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane, and probed for phosphothreonine with a 1:2,000 dilution of pT monoclonal antibody (Cell Signaling), for phosphotyrosine with a 1:2,000 dilution of pY monoclonal antibody (Cell Signaling), for GST with a 1:500 dilution of GST monoclonal antibody (Santa Cruz Biotechnology), for Smk1-Y209p with

a 1:2,000 dilution of Y209 phospho-specific antiserum, and for total Smk1 with a 1:5,000 dilution of an Smk1 C-terminal peptide antibody raised against residues 349 to 370 of Smk1 (10). Alkaline phosphatase-conjugated goat anti-mouse (Promega) and anti-rabbit (Merck Millipore) IgGs diluted 1:5,000 were used to detect immunoreactivity. To quantitate the phosphorylation of bacterial proteins on T and Y residues in Ssp2/Smk1-expressing strains, pT and pY immunoreactivities of entire lanes of the pT and pY immunoblots shown in Fig. 3, exclusive of the regions corresponding to Smk1, were quantitated with ImageJ.

Miscellaneous assays and procedures. For the λ protein phosphatase reactions, the recombinant purified Ssp2^{KAD}-GST-Smk1 complex (2 μg) was incubated with λ protein phosphatase (800 U) (New England BioLabs) for 1 h at 30°C. The phosphatase-treated sample was subsequently repurified with glutathione beads and washed with kinase buffer to remove phosphatase. The sample was resuspended in kinase buffer with 250 μM ATP and incubated at 30°C for 45 min. GST pulldown assays were performed by incubating purified bead-bound recombinant GST (10 μg) or Ssp2-GST (10 μg) with a yeast mitotic extract containing ectopically expressed Smk1-HH, prepared from 1 × 10^9 cells, at 4°C for 2 h with end-over-end rotation. Beads were washed with YLB and eluted with YLB containing 25 mM reduced glutathione. Eluted samples were diluted with 2× Laemmli buffer and analyzed using gel electrophoresis. For proteomic mass spectrophotometry analysis, total extracts from *E. coli* BL21(DE3) cells expressing Cak1, Ssp2^{KAD}, and Smk1 or Smk1-Y/F were collected and lysed with denaturing buffer (9 M urea, 1 mM EDTA, 100 mM Tris-Cl, pH 8.0, PhosStop phosphatase inhibitors [Roche Life Science]). The sample was subsequently digested with trypsin, and peptides were purified by use of a C_{18} SepPak column. Phosphopeptides were enriched by use of a TiO_2 column, and enriched peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify phosphorylated residues. For phenotypic analyses of yeast cells, sporulating cells were fixed, stained with DAPI (4′,6-diamidino-2-phenylindole), and analyzed as described previously (11).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MCB.00607-16.

SUPPLEMENTAL FILE 1, XLSX file, 0.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.3 MB.

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