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Shk1, a homolog of the *Saccharomyces cerevisiae* Ste20 and mammalian p65^{PAK} protein kinases, is a component of a Ras/Cdc42 signaling module in the fission yeast *Schizosaccharomyces pombe*

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ABSTRACT **We describe a protein kinase, Shk1, from the fission yeast *Schizosaccharomyces pombe*, which is structurally related to the *Saccharomyces cerevisiae* Ste20 and mammalian p65^{PAK} protein kinases. We provide genetic evidence for physical and functional interaction between Shk1 and the Cdc42 GTP-binding protein required for normal cell morphology and mating in *S. pombe*. We further show that expression of the *STE20* gene complements the *shk1* null mutation and that Shk1 is capable of signaling to the pheromone-responsive mitogen-activated protein kinase cascade in *S. cerevisiae*. Our results lead us to propose that signaling modules composed of small GTP-binding proteins and protein kinases related to Shk1, Ste20, and p65^{PAK}, are highly conserved in evolution and participate in both cytoskeletal functions and mitogen-activated protein kinase signaling pathways.**

ras genes are highly conserved in evolution and encode small GTP-binding proteins that regulate cell growth and differentiation in a broad spectrum of eukaryotic organisms (1). The fission yeast *Schizosaccharomyces pombe* possesses a single known *ras* homolog, *ras1*, the product of which is required for at least two distinct cellular functions. First, Ras1 is required for sexual differentiation—namely, conjugation and sporulation—which is induced by starvation and by peptide mating pheromones that bind to serpentine receptors (2–4). Ras1 functions upstream of a mitogen-activated protein (MAP) kinase (MAPK) module composed of the Byr2 [MAPK kinase kinase (MAPKKK)], Byr1 [MAPK kinase (MAPKK)], and Spk1 (MAPK) protein kinases (5). Data suggest that Ras1 acts in concert with a G protein α subunit homolog, Gpa1, and that both signal to the MAPK module (6). The yeast two-hybrid system was used to show that Ras1 physically associates with the N-terminal regulatory domain of the Byr2 component of the module (7). Numerous studies have demonstrated a role for Ras proteins in the activation of MAPK cascades induced by receptor tyrosine kinases in mammalian cells (for review, see ref. 8). However, recent studies have suggested that Ras proteins are also key elements of MAPK cascades induced by certain mammalian heterotrimeric G protein-coupled serpentine receptors (for review, see ref. 9), suggesting that signal-transduction pathways analogous to the Ras-dependent peptide mating factor response pathway of *S. pombe* are conserved in evolution.

A second and distinct function of Ras1 in *S. pombe* is the control of cell morphology. Wild-type *S. pombe* cells are rod-like in morphology, whereas *ras1*^{null} mutants are spherical in shape (2, 3). This aspect of Ras1 function is separable from its functions on the MAPK module, as *byr1, byr2, spk1*, and *gpa1* null mutants are normal in morphology (10–13). We recently reported that a member of the Rho subfamily of Ras-related GTP-binding proteins, Cdc42 (14), acts downstream of Ras1 in the regulation of morphology in *S. pombe*, and that Ras1 and Cdc42 form a multiprotein complex with two other proteins, Scd1 and Scd2 (15). Interestingly, Ras proteins also participate in the morphologic transformation of mammalian cells. Stimulation of mammalian cells with growth factors or with oncogenic Ras proteins rapidly induces actin reorganization and membrane ruffling (for review, see ref. 16). As for *S. pombe*, Ras-dependent cytoskeletal control in mammalian cells is regulated by small GTP-binding proteins belonging to the Rho subfamily (17, 18).

In this report, we describe a protein kinase, Shk1,¶ and provide evidence that it mediates functions of the Ras1/Cdc42 signaling complex in *S. pombe*. Shk1 is highly related in structure to the Ste20 kinase, which is required for sexual response in *Saccharomyces cerevisiae* (19, 20), and to the mammalian Cdc42/Rac1-binding kinase, p65^{PAK} (Pak) (21). Our results lead us to propose that signaling pathways mediated by small GTP-binding proteins and protein kinases related to Shk1 are conserved in evolution and participate in regulation of the cytoskeleton and MAPK modules.

MATERIALS AND METHODS

Microbial Manipulation and Analysis. *S. pombe* strains SP870 (*h*^{90} *ade6-210 leu1-32 ura4-D18*) and SP66 (*h*^{90} *ade6-216 leu1-32*) were provided by D. Beach (Cold Spring Harbor Laboratory). SP870D (*h*^{90} *ade6-210/ade6-210 leu1-32/leu1-32 ura4-D18/ura4-D18*) is a spontaneous diploid derived from SP870 (V. Jung, personal communication). SP206U (*h*^{90} *ade6-210/ade6-210 leu1-32/leu1-32 ura4-D18/ura4-D18 shk1::ura4/shk1*$^{+}$) was constructed by transformation of SP870D with an *Ecl*136II–*Msc* I fragment of *shk1::ura4* from plasmid pBSSHK1::URA4. SP206UA (*h*^{90} *ade6-210/ade6-210 leu1-32/leu1-32 ura4-D18/ura4-D18 shk1::ura4::ADE2/shk1*$^{+}$) was constructed by transforming SP206U with a *Not* I fragment of *ura4::ADE2* obtained from pVIN (22). SP42N17 (*h*^{90} *ade6-210 leu1-32 ura4::adh1-cdc42[T17N]-ADE2*) was constructed by transforming the *S.*

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Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; GST, glutathione *S*-transferase.

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¶The sequence has been deposited in the GenBank data base (accession no. L41552.

pombe strain SP66 with a *Not* I fragment of *ura4::adh1-cdc42-[T17N]-ADE2* from pVINCECDC42[T17N]. The *S. cerevisiae* two-hybrid tester strain L40 (*MAT***a** *ade2 his3 leu2 trp1 LYS2::lexA-HIS3 URA3::lexA-lacZ*) has been described (23). *S. cerevisiae* strains AN43-5A (*MAT***a** *ade1 arg4 leu2-3, 112 trp1 ura3-52 mfa1::FUS1::lacZ his3::FUS1::HIS3*) and AN1016 (*MAT***a** *leu2-3, 112 ura3-52 trp1 his4 FUS1::lacZ::LEU2*) were from A. Neiman and I. Herskowitz (University of California, San Francisco). *S. pombe* cultures were grown on either rich medium, yeast extract/dextrose/adenine (YEA), or synthetic minimal medium (PM) with appropriate auxotrophic supplements (3). *S. cerevisiae* cultures were grown on either rich medium, yeast extract/peptone/dextrose, or drop-out medium (DO) with auxotrophic supplements (24). Standard yeast genetic methods were followed (24, 25).

Nucleic Acid Manipulation and Analysis. Plasmids pRD56, pGADGH; pVJL11, pLBDCDC42SP, and pALUCDC42 have been described (7, 15, 23, 26). The *shk1* gene was cloned from a *S. pombe* genomic bank constructed in plasmid pWH5 (13, 27). pAAUCM is a *ura4*-based *S. pombe–E. coli* shuttle vector used for expressing c-Myc epitope-tagged (28) proteins from the strong *S. pombe adh1* promoter (S.M., unpublished work). pART1CM is similar to pAAUCM but contains the *S. cerevisiae LEU2* gene as a selectable marker in place of *ura4* (15). pSL1508 is a YCp50-based plasmid containing the hyperactive *ste11[P279S]* mutant gene (from B. Stevenson and G. Sprague, University of Oregon). A *Bam*HI–*Kpn* I fragment of the *STE20* gene was isolated from pSTE20-5 (from E. Leberer, National Research Council, Montreal) and cloned into the corresponding sites of pAAUCM. The resulting plasmid, pAAUCMSTE20, expresses an N-terminal c-Myc epitope-tagged protein lacking the first 69 amino acids of the Ste20 regulatory domain. A *Xho* I–*Ssp* I fragment of the *shk1* open reading frame was isolated from pSP206A and cloned into the *Xho* I–*Eco*RV sites of pBluescriptII SK (Stratagene). A *Kpn* I–*Sac* I fragment of *shk1* was then isolated from the resulting plasmid, pBSSHK1, and cloned into the corresponding sites of pAAUCM, producing pAAUCMSHK1, and into pUC119, to produce pUC119SHK1. A *Bam*HI–*Eco*RI fragment of *shk1* was isolated from pUC119SHK1 and cloned into the corresponding sites of pGADGH to produce pGADSHK1. pGADSTE20 was constructed by cloning a *Bam*HI–*Sma* I fragment of the *STE20* gene into the corresponding sites of pGADGH. pRDSTE20RI, which was used for expression of a Ste20ΔN (residues 496–939) glutathione *S*-transferase (GST) fusion protein in yeast, was provided by A. Neiman and I. Herskowitz. pGSTSHK1ΔN was constructed by cloning an *Xmn* I–*Eco*RI fragment of the *shk1* gene, from pBSSHK1, into pRD56, and allows for expression of a Shk1ΔN (residues 183–540) GST fusion protein in *S. cerevisiae.* pLBDCDC42N17 was constructed by cloning a *Bam*HI–*Xho* I PCR-derived fragment of the dominant negative *cdc42[T17N]* coding sequence into pVJL11. A *Sal* I–*Sac* I fragment of *cdc42[T17N]* was isolated from pALUT17N (15) and cloned into the corresponding sites of pVINCE1 (22) to produce pVINCECDC42T17N. PCR was used to generate a *Bam*HI–*Sac* I deletion fragment of the *shk1* gene corresponding to the putative Shk1 regulatory domain (amino acid residues 1–262). This fragment was cloned into the corresponding sites of pART1CM to produce the plasmid pART1CMSHK1ΔC. The oligonucleotide primer pair 5′-AAGGATCCGAGCTCGACACAATCCCAATGA and 5′-GTATTCCAAGATCTTCCACAACTCTGATT were used to generate a 5′ fragment of the *shk1* sequence, and the primer pair 5′-ACCGTGAGATCTAAGCTTACAATATCCTTC and 5′-CGACGGTACCTTGGATGGCCATAGTAGCAA were used to generate a 3′ fragment of *shk1.* The 5′ *shk1* PCR fragment was digested with *Sac* I and *Bgl* II, and the 3′ fragment was digested with *Bgl* II and *Kpn* I; then the two fragments were ligated together into a *Sac* I–*Kpn* I fragment of pBluescriptII SK, producing pBSSHK1ΔKD. A *Hin*dIII fragment of the *S. pombe ura4* gene was then cloned into the corresponding site of the *shk1* fragment in pBSSHK1ΔKD. The resulting plasmid, pBSSHK1::URA4, contains a *ura4* replacement of the DNA sequence corresponding to amino acid residues 337–391 of the Shk1 kinase (see Fig. 1).

β-Galactosidase Assays. The filter assay for testing two-hybrid interactions was done as described (7). LexA two-hybrid experiments were conducted using LexA DNA-binding domain and Gal4-activating domain pairs of fusion proteins. Liquid β-galactosidase assays were done as described (29).

Mating Assay. Mating assays were done as described (30). Briefly, transformants were grown on PM agar for 6 days to induce sexual activity. Zygotes, asci, and unmated cells within individual clones were quantitated by microscopy.

RESULTS

Cloning and Sequence Analysis of *shk1*. A fragment of the *shk1* coding sequence was cloned in a screen for *STE20*-related genes from fission yeast and rat using the PCR with degenerate oligonucleotide primers based on peptide sequences within the *S. cerevisiae* Ste20 catalytic domain (MEYMEG/HRDIKSDN). The PCR-derived fragment was used as a probe to screen a *S. pombe* genomic library constructed in plasmid pWH5 (27). Six strongly hybridizing transformants were isolated from among ≈30,000 transformed bacterial colonies screened. Plasmids were isolated from two of these transformants. pSP206A carried an insert of ≈10.5 kb, whereas pSP206B contained an insert ≈8 kb in size (data not shown). Dideoxynucleotide chain-termination sequencing indicated that pSP206A and pSP206B inserts each contained identical *STE20*-related sequences, which we named *shk1*, for Ste20 homologous kinase. The nucleotide sequence of *shk1* revealed an intronless open reading frame of 1818 bp that encodes a predicted protein of 540 amino acids. Shk1 was most closely related structurally to the *S. cerevisiae* Ste20 (49% identity) (19, 20) and mammalian Pak (41% identity) (21) protein kinases (Fig. 1). These kinases shared significant identity within their catalytic domains (57% identity). In addition, Shk1 contains, in its putative regulatory domain, a peptide motif similar in structure to the Cdc42/Rac1-binding domain of Pak (Fig. 1). A similar motif is found in the regulatory domain of the *S. cerevisiae* Ste20 kinase (Fig. 1; ref. 21). Ste20 and Shk1 share additional regions of limited homology within their regulatory domains not found in Pak.

Phenotype Conferred by Disruption of *shk1*. A *shk1* disruption was made by replacing a fragment of *shk1*, encoding part of the kinase domain, with *ura4* (see *Materials and Methods*). The *shk1::ura4* DNA fragment was used to transform the wild-type *S. pombe* diploid strain SP870D. Diploid transformants carrying a single disrupted copy of *shk1* were identified by Southern blot analysis of genomic DNA digests. Two independent *shk1::ura4/shk1*$^+$ diploids were sporulated, and asci containing four spores were dissected. Spores were incubated on rich medium for 4 days, and the resulting colonies were replica-plated onto selective medium supplemented with or not supplemented with uracil. A representative tetrad analysis is shown in Fig. 2*A*. Of 46 tetrads analyzed, 40 produced two viable spores, whereas 6 produced only one viable spore. None of the tetrads analyzed produced more than two viable spores, and viable spores were never Ura$^+$. These results demonstrate that the Shk1 kinase is required for at least one essential cellular function.

We examined further the *shk1*$^-$ phenotype by microscopic analysis. Wild-type *S. pombe* cells are rod-like in morphology (Fig. 2*B*). *shk1*$^-$ spores typically germinated but arrested as spherical cells after one to several rounds of cell division (Fig. 2*C*). This phenotype is similar to that observed for *S. pombe* strains carrying *cdc42* null mutations (14). Because Shk1 contains a motif structurally similar to the Cdc42/Rac1-binding domain of the mammalian Pak protein kinase, we

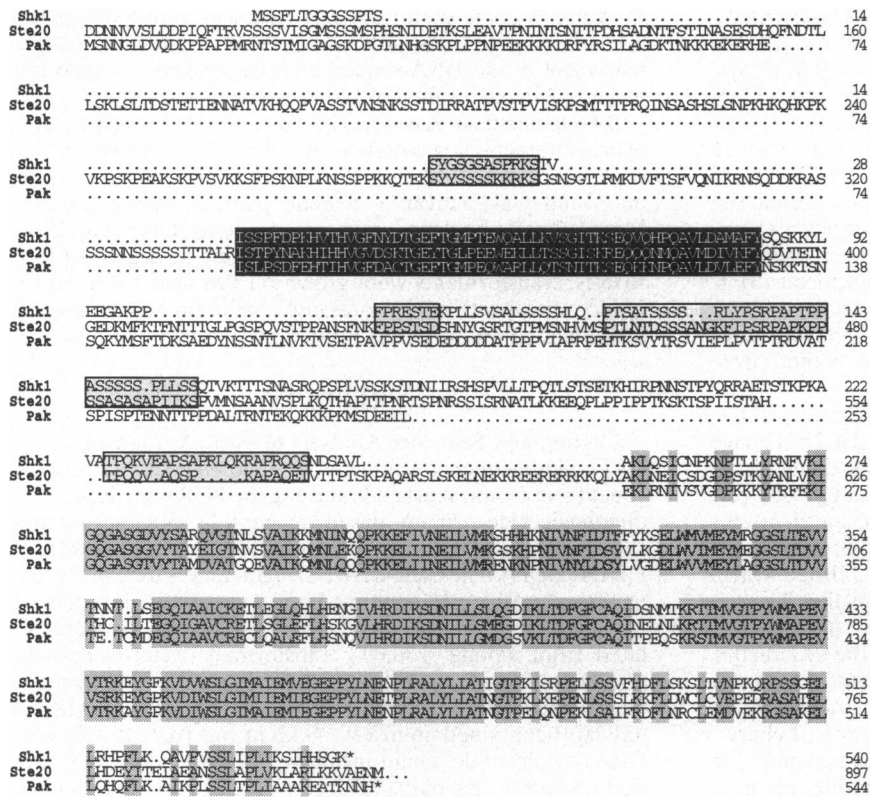

FIG. 1. Sequence alignments of *S. pombe* Shk1, *S. cerevisiae* Ste20, and mammalian Pak protein kinases. Identical and similar amino acid residues in the highly homologous catalytic domains of these kinases are indicated by dark gray boxes. Domains homologous to the Cdc42/Rac1-binding domain of Pak are highlighted by white letters in the black box. Additional regions of limited homology between the N-terminal putative regulatory domains of Shk1 and Ste20 are highlighted by outlined shaded boxes. The Shk1 and Pak sequences are complete, whereas residues 61–897 of the 939-residue Ste20 kinase are shown.

examined whether Shk1 and Cdc42 interact physically and functionally.

Complex Formation Between Shk1 and Cdc42. We used the yeast two-hybrid system to determine whether Shk1 and Cdc42 interact physically. Shk1 was expressed as a Gal4-activating domain fusion protein, and Cdc42 and Cdc42[T17N] were expressed as fusions to the LexA DNA-binding domain. We detected complex formation between Shk1 and wild-type Cdc42 but not between Shk1 and the dominant negative Cdc42[T17N] mutant protein. The T17N mutation is analogous to mutations identified at the corresponding positions of yeast and mammalian Ras proteins that result in defective guanine nucleotide exchange (31–33). Our failure to detect an interaction between Shk1 and Cdc42[T17N] in the two-hybrid system may indicate that interaction between the two proteins is GTP-dependent and confirms the results of Manser *et al.* (21), who showed that binding between mammalian Cdc42 and the mammalian Shk1 homolog Pak is GTP-dependent. Like Shk1, the structurally similar *S. cerevisiae* Ste20 kinase also interacted with Cdc42 but not with Cdc42[T17N] (data not shown). Neither Shk1 nor Ste20 showed detectable interactions with Ras1, Gpa1, Byr2, Scd1, or Scd2 in the two-hybrid system.

Functional Interaction Between Shk1, Cdc42, and Ras1 in *S. pombe*. Genetic experiments were conducted to establish a functional relationship between Shk1 and Cdc42. First, we expressed the N-terminal putative regulatory domain of Shk1 (Shk1ΔC) in wild-type *S. pombe* cells. Fig. 2*D* shows that Shk1ΔC caused cells to become spherical in morphology. This phenotype was indistinguishable from that resulting from overexpression of the dominant inhibitory Cdc42 mutant protein Cdc42[T17N] (Fig. 2*E*) and further supports the notion that the N-terminal domain of Shk1 is regulatory in function. Additional genetic evidence suggesting a functional interaction between Cdc42 and Shk1 came from overexpression of Shk1 in a *S. pombe* strain overexpressing Cdc42[T17N]. Cells expressing this dominant inhibitory GTP-binding protein mate at a frequency of about one-fiftieth that of wild-type cells. Expression of Shk1 partially suppressed this mating defect (Table 1).

Cdc42 appears to act downstream of Ras1 in *S. pombe*, and data from two-hybrid experiments suggest that the two proteins are part of a multiprotein complex (15). Unlike Cdc42, Ras1 is not required for cell viability, possibly because Cdc42 has sufficient basal activity to sustain cell viability in the absence of Ras1. Although high-copy expression of Shk1 failed to measurably suppress the sporulation or conjugation defects of a *ras1* null mutant, expression of the N-terminal regulatory domain of Shk1 markedly attenuated the hypersexual responses (elevated levels of agglutination and projection of conjugation tubes) of a *S. pombe* strain carrying an activated *ras1[G17V]* mutant gene (data not shown). These results are consistent with a role for Shk1 in the sexual functions of Ras1. Further evidence for this comes from the functional homology between Shk1 and Ste20, as described below.

Functional Homology Between Shk1 and the *S. cerevisiae* Ste20 Protein Kinase. To examine the functional relatedness of Shk1 and Ste20, we constructed the high-copy plasmid pAAUCMSTE20 for expressing Ste20 in *S. pombe*. A *shk1::ADE2/shk1*$^+$ diploid strain was transformed with pAAUCMSTE20, and the resulting transformants were subjected to tetrad analysis. Expression of Ste20 restored not only viability to *shk1::ADE2* haploid cells, as determined by the recovery of *ADE2*$^+$ spores, but also mating and normal morphology (Fig. 2*F*). These results demonstrate that Shk1 and Ste20 are functionally related.

We also examined the function of Shk1 in *S. cerevisiae*. In *S. cerevisiae*, the Ste20 kinase is required for signaling from heterotrimeric G protein-coupled peptide mating pheromone receptors to a MAPK module composed of the Ste11 (MAPKKK), Ste7 (MAPKK), and Fus3/Kss1 (MAPK) protein kinases (19). Deletion of the Ste20 N-terminal regulatory domain results in a kinase capable of constitutively activating the mating factor response pathway in the absence of mating pheromone (ref. 20; Table 2). Similarly, we found that high-copy expression of a Shk1 kinase lacking its N-terminal regulatory domain was capable of inducing the *S. cerevisiae*

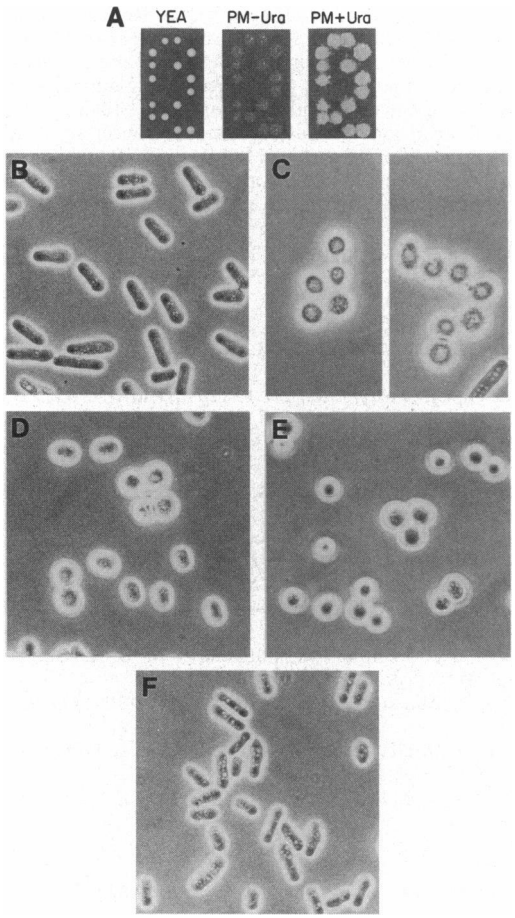

FIG. 2. Functional characterization of Shk1. (*A*) *shk1* is an essential gene. The *shk1*$^+$/*shk1D::ura4* diploid strain SP206U was grown on PM medium to induce sporulation. Asci containing four spores were dissected by micromanipulation, placed on YEA, and incubated for 4 days at 30°C. The resulting colonies were replica-plated onto PM supplemented with (PM+Ura) or without (PM−Ura) uracil. No more than two viable spores were isolated from any four-spore ascus, and no viable spores were ever Ura$^+$. (*B*) Photomicrograph of the wild-type *S. pombe* strain SP870. (*C*) Photomicrograph of *shk1*$^-$ *S. pombe* cells from culture of sporulated SP206U diploids. (*D*) Photomicrograph of *S. pombe* cells transformed with plasmid pART1CMSHK1ΔC, which overexpresses the Shk1 N-terminal regulatory domain. Note similarity to cells in *D*. (*E*) Photomicrograph of *S. pombe* strain SP42N17, which overexpresses the dominant inhibitory *cdc42[T17N]* gene. (*F*) Photomicrograph of *shk1*^{null} cells transformed with pAAUCMSTE20, which expresses the *S. cerevisiae STE20* gene. *shk1*^{null} *STE20*$^+$ cells are viable, competent for mating, and normal in morphology. (×500.)

mating factor response pathway to a level nearly equal to that induced by a Ste20 N-terminal deletion mutant (Table 2). Shk1 failed to induce *FUS1–lacZ* in a *ste11* null mutant. These results indicate that Shk1 has a conserved ability to interact with the *S. cerevisiae* mating factor-induced MAPK cascade. Expression of the full-length Shk1 sequence failed to provide detectable suppression of the mating defect of a Ste20 deletion mutant and only weakly restored FUS1 expression. We conclude from these experiments that Shk1 is not properly regulated when expressed in *S. cerevisiae* but that a constitutively activated form of the kinase is fully capable of signaling to the pheromone response cascade.

DISCUSSION

In *S. cerevisiae*, the Ste20 protein kinase is required for mating pheromone-induced signal transduction (19, 20). Ste20 appears to function downstream from the mating pheromone receptor-coupled G protein and upstream of the pheromone-responsive MAPK module (19). Establishing a similar role for Shk1 in *S. pombe* is complicated by the fact that *shk1* is an essential gene. However, two results suggest a possible role for Shk1 in mating: (*i*) Shk1 partially suppresses the mating defect of a *S. pombe* strain expressing the dominant inhibitory Cdc42[T17N] mutant protein; and (*ii*) expression of the Shk1 N-terminal putative regulatory domain attenuates the exaggerated sexual responses of a *S. pombe* strain carrying the activated *ras1[G17V]* mutant gene.

Although it remains to be demonstrated conclusively, several results lead us to speculate that Shk1 may also participate in the Ras1-regulated MAPK module. Shk1 is capable of signaling to the pheromone-responsive MAPK module in *S. cerevisiae* (this report) and is also capable of inducing MAPK activation in cell-free extracts of *Xenopus laevis* oocytes (S.M. and T.P., unpublished work). Moreover, Ste20, which also signals to the MAPK module in *S. cerevisiae*, can functionally replace Shk1 in *S. pombe*. Interestingly, recent biochemical studies by Wu and coworkers‖ and by A. Neiman and I. Herskowitz (personal communication) indicate the Byr2 homolog of *S. cerevisiae*, Ste11, is a Ste20 substrate *in vitro*.

Table 1. Overexpression of *shk1* partially suppresses the mating defect of a *S. pombe* strain expressing *cdc42[T17N]*

Strain	Overexpressed gene*	Mating,† %
SP66	None	53.3 ± 1.9
SP42N17	*cdc42[T17N]*	1.2 ± 0.2
	cdc42[T17N], shk1	5.6 ± 0.5
	cdc42[T17N], cdc42	10.1 ± 0.7

SP66 is a wild-type (*cdc42*$^+$) *S. pombe* strain. SP42N17 is isogenic to SP66 but carries an *adh1-cdc42[T17N]* sequence integrated at the *ura4* locus.

*Plasmids used for transformation of SP66 and SP42N17 were pAAUCM (empty vector); pALUCDC42SP, for *cdc42* overexpression; and pAAUCMSHK1, for *shk1* overexpression. pAAUCM contains the *adh1* promoter sequence, which is also used for overexpression of *cdc42[T17N]*, and its use as a control plasmid provides assurance that the effects observed from *shk1* overexpression are not the result of promoter competition.

†The quantitative mating assay was done as described (30). Values represent the average of three determinations.

‖Wu, C., Dignard, D., Whiteway, M., Thomas, D. Y. & Leberer, E., Yeast Genetics and Molecular Biology Meeting, Aug., 1994, Seattle, abstr. no. 167A.

Table 2. *S. pombe* Shk1 kinase activates the *S. cerevisiae* mating pheromone-responsive MAPK cascade

Overexpressed gene	*FUS1–lacZ* induction,* β-galactosidase units	
	STE11$^+$	*ste11*$^-$
None	4.3 ± 1.4	0.6 ± 0.2
Ste20ΔN	26.9 ± 3.3	0.8 ± 0.2
Shk1ΔN	23.9 ± 6.8	1.0 ± 0.4
Ste11[P279S]	8.8 ± 1.9	14.7 ± 1.1

Ste20ΔN and Shk1ΔN were expressed as GST fusion proteins from the galactose-inducible *GAL1* promoter in plasmids pRDSTE20RI and pRD56SHK1ΔN, respectively. Plasmid pSL1508 was used for expression of the Ste11[P279S] hyperactive mutant protein.

*The congenic *STE11*$^+$ and *ste11*$^-$ strains used for this experiment were AN43-5A and AN1016, respectively. Transformants were grown in DO medium lacking uracil (DO-Ura) at 30°C to ≈5 × 10^6 cells per ml, then transferred to DO-Ura-glucose containing 2% (wt/vol) galactose, 2% (wt/vol) glycerol, and 1% ethanol, and incubated overnight at 30°C. β-Galactosidase activity was measured as described (29). Units of β-galactosidase were calculated as [1000 × OD_{420}]/[OD_{600} × time (min) × vol of cells (ml)]. Values represent the averages of at least four determinations.

As we have shown that Shk1 and Cdc42 functionally interact and that Ras1 and Cdc42 functionally interact, we propose that Ras1 has two distinct inputs into the MAPK module: one by direct physical interaction with Byr2 (7, 13) and the second through Shk1, which Ras1 regulates via Cdc42. Our results have led us to propose the model depicted in Fig. 3, in which the Ras1/Cdc42-regulated morphology control pathway in *S. pombe* also participates in regulation of the Ras1-dependent MAPK cascade, the Byr2 component of which interacts with both Ras1 and Shk1.

As in *S. pombe*, small GTP-binding proteins have been implicated in morphological regulation in *S. cerevisiae*. A homolog of Cdc42 is required for normal polarized cell growth in *S. cerevisiae*, as is a Ras-related GTP-binding protein, Rsr1/Bud1 (for review, see ref. 34). Although Ste20 has not as yet been implicated in morphological control in *S. cerevisiae*, it binds to Cdc42 (this study) and to a mammalian Rho-related GTP-binding protein Rac1 (S.M. and L. Van Aelst, unpublished work) and functionally replaces Shk1 in *S. pombe*. These results suggest a potential role for Ste20 and/or Ste20-related proteins in the morphological regulation of *S. cerevisiae*. In this regard, a new gene, *CLA4*, encoding a protein kinase structurally related to Ste20, has recently been identified in *S. cerevisiae*. Although deletion of either *CLA4* or *STE20* alone does not affect cell viability, deletion of both genes is lethal, suggesting that Cla4 and Ste20 perform overlapping, essential cellular functions (35).

Whether Pak or related protein kinases play roles in mammalian cells analogous to the roles of their homologs in yeast—namely, regulation of MAPK modules and cellular morphology—remains to be seen.

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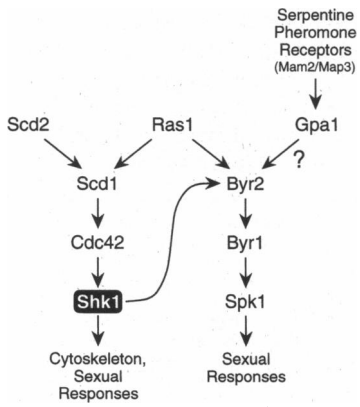

FIG. 3. Model for signal-transduction pathways mediated by Ras1 and Shk1 in fission yeast. The Ras1 protein regulates two genetically separable signaling pathways in *S. pombe*, one involved in regulation of a MAPK module composed of the Byr2, Byr1, and Spk1 protein kinases and the other involved in cytoskeletal control (see text for details). Genetic and biochemical data suggest that Cdc42 and its putative exchange factor Scd1 function downstream of Ras1 to modulate cytoskeletal regulation and that all three proteins are components of a multiprotein complex that also contains Scd2, a protein that has two SH3 domains (15). Genetic data presented in this report suggest that Shk1 links the Ras1/Cdc42 complex to control of the cytoskeleton in *S. pombe*. We propose that Shk1 may act on Byr2 because the *S. cerevisiae* Shk1 homolog Ste20 phosphorylates Ste11, the *S. cerevisiae* homolog of Byr2, and *S. pombe* Shk1 and *S. cerevisiae* Ste20 are functionally homologous when expressed in the respective hosts.

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