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Schizosaccharomyces pombe Spk1 is a Tyrosine-Phosphorylated Protein Functionally Related to *Xenopus* Mitogen-Activated Protein Kinase

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Mitogen-activated protein kinase (MAPK) and its direct activator, MAPK kinase (MAPKK), have been suggested to play a pivotal role in a variety of signal transduction pathways in higher eukaryotes. The fission yeast *Schizosaccharomyces pombe* carries a gene, named *spk1*, whose product is structurally related to vertebrate MAPK. Here we show that Spk1 is functionally related to *Xenopus* MAPK. (i) *Xenopus* MAPK partially complemented a defect in the *spk1⁻* mutant. An *spk1⁻* diploid strain could not sporulate, but one carrying *Xenopus* MAPK could. (ii) Both Spk1 and *Xenopus* MAPK interfered with sporulation if overexpressed in *S. pombe* cells. (iii) Spk1 underwent tyrosine phosphorylation as does *Xenopus* MAPK. Tyrosine phosphorylation of Spk1 appeared to be dependent upon mating signals because it occurred in homothallic cells but not in heterothallic cells. Furthermore, this phosphorylation was diminished in a *byr1* disruptant strain, suggesting that *spk1* lies downstream of *byr1*, which encodes a MAPKK homolog in *S. pombe*. Taken together, the MAPKK-MAPK cascade may be evolutionarily conserved in signaling pathways in yeasts and vertebrates.

Mitogen-activated protein kinases (MAPKs) are a group of protein serine-threonine kinases that are activated in various signal transduction systems, such as the growth factor-stimulated signaling process (23, 28, 50, 51), nerve growth factor-induced neural differentiation (23, 39), and M-phase-promoting factor-induced meiotic signal transduction (15, 21, 22, 49). In higher eukaryotes, MAPK is thus thought to be a key molecule that functions in a wide variety of intracellular signal transductions (8, 45, 48, 55). However, the functional aspects of MAPK and its relationship with other signaling molecules are poorly understood. A system which is amenable to genetic analysis would help us approach these problems.

Extensive studies have been focused on the activation mechanism of MAPK. It has been established that activation of MAPK requires dual phosphorylation on tyrosine and threonine (2). A factor responsible for the phosphorylation and activation of MAPK was identified and purified to homogeneity as a 45-kDa band from mature *Xenopus* oocytes (37). This MAPK activator is a serine-threonine-tyrosine kinase which is activated by serine-threonine phosphorylation (33). Thus, we call this factor MAPK kinase (MAPKK). In *Xenopus* oocytes, MAPKK is activated downstream of the M-phase-promoting factor (37). In growth factor- or nerve growth factor-stimulated mammalian cells, a MAPKK(s) similar or identical to M-phase-promoting factor-stimulated MAPKK is activated (1, 11, 20, 43, 52, 54, 66). Recently, it was reported that activated *ras* p21 causes activation of the MAPKK-MAPK cascade (27, 36, 53, 59, 65). In addition, studies with a dominant negative *ras* mutant (59, 65) have shown that *ras* is essential for tetradecanoyl phorbol acetate-, insulin- or nerve growth factor-induced signaling pathways leading to the activation of MAPK, suggesting the importance of *ras* in the MAPKK-MAPK cascade.

In yeasts, several gene products are reported to be structurally homologous to MAPK, i.e., those of *FUS3* (13), *KSS1* (9) *HOG1* (7) and *MPK1-SLT2* (35, 62) in *Saccharomyces cerevisiae* and that of *spk1* (60) in *Schizosaccharomyces pombe*. All of these gene products show ~50% identity to vertebrate MAPK and contain the TEY (TGY) sequence corresponding to the regulatory phosphorylation sites of vertebrate MAPK (47). cDNA cloning and sequencing of MAPKK revealed that *STE7* (58), *PBS2* (5), and *MKK1-MKK2* (31) in *S. cerevisiae* and *wis1* (64) and *byr1* (40) in *S. pombe* encode homologs of MAPKK, each showing about 40% sequence identity (10, 34). Ste7 is a kinase that acts in the mating signal transduction pathway that involves Fus3 and Kss1. Ste7 was recently shown to phosphorylate and activate Fus3 by in vitro reconstitution experiments (14, 19). Hog1, involved in osmotic sensitivity, was reported to undergo PBS2-dependent tyrosine phosphorylation (7). Thus, MAPKK-MAPK cascades also exist in *S. cerevisiae.*

S. pombe contains a single *ras* gene, *ras1* (16, 42), which is required for normal cell morphology, conjugation, and efficient sporulation (17, 18, 41). The *byr1* (40) and the *byr2* (63) genes were isolated because of their ability to suppress the sporulation defect of a *ras1⁻* strain when maintained in high copy number. All of the *spk1⁻* (60), *byr1⁻* (40), *byr2⁻* (63), and *ras1⁻* (18, 41) mutants are sterile. It thus appears likely that *spk1*, *byr1*, *byr2*, and *ras1* function in the same signaling pathway required for sexual differentiation. However, the relationship between *spk1* and the other genes is not fully understood. Also, it would be interesting to see whether Spk1 is not only structurally but also functionally related to vertebrate MAPKs.

We have examined whether Spk1 belongs to the MAPK

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype
JY246	h^{90} *ade6-M210*
JY333	h^{-} *ade6-M216 leu1*
JY362	h^{+}/h^{-} *ade6-M210/ade6-M216 leu1/leu1*
JY450	h^{90} *ade6-M216 leu1*
JY878	h^{90} *ade6-M216 leu1 ura4-D18*
JZ840	h^{90}/h^{90} *ras1/ras1 ade6-M210/ade6-M216 leu1/leu1*
JZ944	h^{90} *spk1::ura4⁺ ade6-M216 leu1 ura4-D18*
JZ945	h^{90} *spk1::ura4⁺ ade6-M210 leu1 ura4-D18*
JZ966	h^{90}/h^{90} *ste1/ste1 ade6-M210/ade6-M216 leu1/leu1*
JZ967	h^{90}/h^{90} *ste8/ste8 ade6-M210/ade6-M216 leu1/leu1*
JZ968	h^{90}/h^{90} *spk1::ura4⁺/spk1::ura4⁺ ade6-M210/ade6-M216 leu1/leu1 ura4-D18/ura4-D18*
JX1	h^{90} *ste1::ura4⁺ ade6-M216 leu1 ura4-D18*
JX3	h^{90} *ste8::ura4⁺ ade6-M216 leu1 ura4-D18*
114-9C	h^{90} *spk1::ura4⁺ leu1 ura4*

family in terms of function and activation mechanism. In this report, we show that *spk1* is necessary for sporulation and that this function can be replaced by *Xenopus* MAPK. Neiman et al. also reported recently that mammalian MAPK suppressed the sporulation defect in *spk1⁻* (44). We found that overexpression of either *spk1* or *Xenopus* MAPK interfered with the sexual differentiation process, again suggesting the functional similarity of the two kinases. Furthermore, we demonstrated tyrosine phosphorylation of the Spk1 protein, which is dependent upon *byr1* gene function. The functional relatedness of *STE11* (a gene upstream of *STE7*) and *byr2* (44, 56), of *STE7* and *byr1* (44), and of *FUS3*, *KSS1*, and *spk1* (44) has been reported. Therefore, it can be assumed that the kinase cascade that includes MAPKK-MAPK may be conserved in *S. pombe*, *S. cerevisiae*, and vertebrates.

MATERIALS AND METHODS

Yeast strains and microbial methods. The *S. pombe* strains used in this study are listed in Table 1. Rich medium YPD (1% yeast extract, 2% polypeptone, 2% glucose), minimal medium SD (0.67% yeast nitrogen base without amino acids, 1% glucose), and SSA for nitrogen starvation (12) were used. Transformation of *S. pombe* was done as previously described (46). The diploid *spk1⁻* mutant strain JZ968 was constructed by protoplast fusion between JZ944 and JZ945. Diploid colonies were selected on sorbitol-containing minimal medium plates lacking adenine by use of intragenic complementation between *ade6-M210* and *ade6-M216* (26).

Plasmids. The whole coding sequence of *spk1* (60) was amplified by polymerase chain reaction with primers 5′-TTCAATTTGGATCCTAATGGCGAGCG-3′ and 5′-ATTCTAGTGGATCCTTTTTATCTGAA-3′. The fragment obtained by cleavage of this polymerase chain reaction product with *Bam*HI was cloned into the *Bam*HI sites of two *S. pombe* vectors, namely, pDB248′ (3) and pART1 (38). pART1 contains the *adh* promoter, which is stronger than the cryptic promoter residing in pDB248′, and the copy number of pART1 in *S. pombe* is higher than that of pDB248′. DNA encoding either wild-type *Xenopus* MAPK or kinase-negative (Lys-57→Asp) MAPK (KN-MAPK) was prepared as previously described (21, 34). Coding regions for these MAPK were amplified by polymerase chain reaction with primers 5′-CCGGATCCCCATGGCAGCGGCAGGAGCTGCGTCT-3′ and 5′-CGGGATCCGTCAGTACCCTGGCTGGAATCTAGCG-3′. The amplified MAPK fragments were cloned into the *Bam*HI sites of pDB248′ and pART1. A fragment that encodes *Xenopus* MAPKK (34) was also amplified with primers 5′-ACTCAGATCTAACATGCCTAAAAAGAAG-3′ and 5′-GCCAAGATCTCTCACACTCCGGCGGCAT-3′ and subcloned into the *Bam*HI site of pART1. Both the *byr1* (40) and *byr2* (63) genes used in this study were obtained in a genetic screening of genes involved in the *ras1* pathway (30a). pART1-byr1, carrying the *byr1* gene, was isolated from a gene library in which *Sau*3AI partial digests of *S. pombe* genomic DNA were inserted into the *Bam*HI site of pART1. pART1-byr2 was constructed by insertion of a 4.0-kb *Bam*HI-*Bgl*II fragment carrying the *byr2* gene into the *Bam*HI site of pART1.

Disruption of the *byr1* and *byr2* genes. For disruption of the *byr1* gene, a 0.4-kb *Nde*I-*Cla*I fragment was eliminated from the cloned *byr1* open reading frame and a 1.8-kb *S. pombe ura4⁺* cassette (25) was inserted. A linearized DNA fragment carrying this disrupted *byr1* gene was used to transform homothallic haploid strain JY878. For disruption of the *byr2* gene, a 1.6-kb *Xho*I-*Spe*I fragment was eliminated from the cloned *byr2* open reading frame and the *ura4⁺* cassette was inserted. A linearized fragment carrying this disrupted *byr2* gene was used to transform JY878. In each case, transformants showing the Ura⁺ and sterility phenotypes were selected and proper replacement of the chromosomal gene was confirmed by Southern blot analysis of genomic DNA (data not shown).

Mating and sporulation assays. To examine mating efficiency, a strain to be tested carrying *ade6-M216* and the mating partner carrying *ade6-M210*, or vice versa, were crossed and the number of adenine prototrophs, i.e., diploid cells generated by mating, was determined. Qualitative assay for sporulation was done by iodine vapor staining (26). Sporulation efficiency was examined by counting the asci under a microscope.

Preparation of cell extracts, Western blotting (immunoblotting), and immunoprecipitation. Strains (JY450, JY333, JX1, and JX3) carrying pART1, pART1-MAPK, or pART1-spk1 were grown to a concentration of 10^7 cells per ml in PM medium (4) containing adenine. The cells were collected by centrifugation, and 0.2 g of each cell pellet was suspended in 0.6 ml of extraction buffer (50 mM Tris-Cl [pH 7.5], 5 mM EDTA, 5 mM PP_i, 50 mM NaF, 1 mM Na_3VO_4, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml). After addition of 0.8 ml of glass beads, the cell suspension was vigorously vortexed five times for 1 min each time at 4°C. The supernatant obtained after centrifugation at 100,000 × *g* for 3 min at 4°C was boiled in the presence of Laemmli's sample buffer. The samples (10 μl of each) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred electrophoretically to nitrocellulose filters, and probed with anti-phosphotyrosine monoclonal antibody PY20 (ICN), with an affinity-purified polyclonal anti-Spk1 antibody (60), or with an affinity-purified polyclonal anti-*Xenopus* MAPK antibody. Anti-*Xenopus* MAPK serum was raised in a rabbit against a synthetic peptide (DMELDDLPKERLKE) as previously described (21). Immunoreactive bands were detected by horseradish peroxidase-conjugated second antibodies and the ECL Western blotting detection system (Amersham). For immunoprecipitation experiments, cells were incubated with 5% trichloroacetic acid for 10 min on ice, washed with excess acetone, and dried before cell extracts were prepared. Cell extracts (100 μl, ~10^8 cells) were mixed in the presence of 0.15% SDS with 20 μl of a 1:1 slurry of protein A-Sepharose beads that had been bound to anti-Spk1 serum

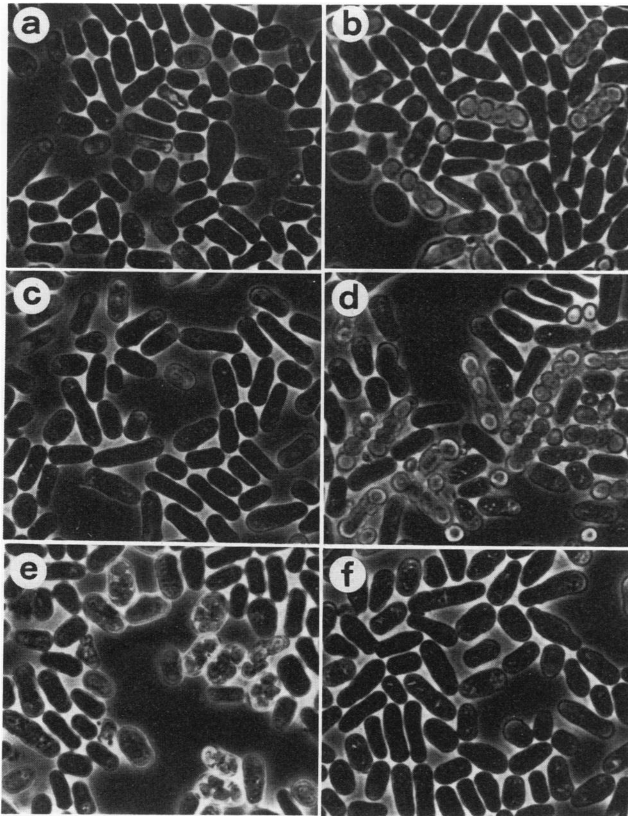

FIG. 1. Effect of overexpression of *spk1*$^+$ or *Xenopus* MAPK on the phenotype of *spk1*-disrupted diploid strain JZ968. JZ968 was transformed with pART1 (a), pART1-spk1 (b), pDB248′ (c), pDB248′-spk1 (d), pART1-*Xenopus* MAPK (e), or pART1-*Xenopus* KN-MAPK (f). Cells were grown on SSA plates which contained only a limited amount of nitrogen. Phase-contrast micrographs were taken after 2 days of incubation at 30°C.

(5 μl) (60) or to anti-*Xenopus* MAPK serum (5 μl) (21). After incubation at 0°C for 1.5 h, the immune complexes were pelleted and washed four times with 0.5 ml of the extraction buffer. The immune complexes were boiled in Laemmli's buffer and subjected to immunoblotting with anti-phosphotyrosine antibody, anti-*Xenopus* MAPK antibody, or anti-Spk1 antibody as described above. For the last two blottings, the upper half of each nitrocellulose filter was cut off to eliminate the reaction between the electrophoresed, transferred rabbit immunoglobulin G and the second antibody.

RESULTS

***spk1*$^-$/*spk1*$^-$ diploids are defective in sporulation.** The construction and characteristics of haploid *spk1*$^-$ strains have been previously described by Toda et al. (60). The *spk1* null mutant can grow apparently normally but has very low mating ability. To investigate the function of *spk1* in sporulation, which was not examined before, we made a diploid strain homozygous for *spk1*. Under nitrogen starvation, *spk1*$^-$/*spk1*$^-$ diploid strain JZ968 did not sporulate (Fig. 1a and c, strains carrying vector plasmids) whereas a wild-type diploid strain sporulated efficiently (more than 90%). This result confirms the finding of Neiman et al. (44).

***Xenopus* MAPK partially suppresses the sporulation defect in *spk1*$^-$.** To estimate the functional relationship between Spk1 and MAPK, we first examined whether *Xenopus* MAPK could replace the function of *spk1*. The mating defect of *spk1*$^-$ haploid strain JZ945 (with JZ450 as a mating partner) was not rescued by *Xenopus* MAPK carried on multicopy plasmid pDB248′ or pART1 (data not shown). However, the sporulation defect of JZ968 (*spk1*$^-$/*spk1*$^-$) was partially rescued by pART1-MAPK (*Xenopus* MAPK in pART1) (Fig. 1e; Table 2). pART1-MAPK was almost as efficient as pART1-spk1 in this rescue (Fig. 1b; Table 2). The complementation ability of *Xenopus* MAPK required its kinase activity, since KN-MAPK did not rescue the sporulation defect (Fig. 1f; Table 2). Thus, *Xenopus* MAPK could replace part of the function of Spk1. JZ968 transformed with pART1-MAPK formed slightly round asci (Fig. 1e), whereas

TABLE 2. Efficiency of sporulation in *ras1*, *ste8* (*byr2*), *ste1* (*byr1*), and *spk1* mutants transformed with various plasmids[a]

Plasmid	% Sporulation			
	ras1$^-$/*ras1*$^-$	*ste8*$^-$/*ste8*$^-$	*ste1*$^-$/*ste1*$^-$	*spk1*$^-$/*spk1*$^-$
pART1	<0.1	<0.1	<0.1	<0.1
pART1-MAPK	<0.1	<0.1	<0.1	13.6
pART1–KN-MAPK	<0.1	<0.1	<0.1	<0.1
pART1-MAPKK	<0.1	<0.1	<0.1	<0.1
pART1-spk1	<0.1	<0.1	<0.1	14.5
pDB248′-spk1	<0.1	<0.1	<0.1	39.1

[a] *S. pombe* JZ840 (*ras1*$^-$/*ras1*$^-$ diploid), JZ967 (*ste8*$^-$/*ste8*$^-$ diploid), JZ966 (*ste1*$^-$/*ste1*$^-$ diploid), and JZ968 (*spk1*$^-$/*spk1*$^-$ diploid) were transformed with each plasmid indicated and grown on SSA plates for 2 days at 30°C. Sporulation in more than 10 independent clones was examined for each transformant. The average ratio of the number of asci to the total number of cells is presented as a percentage.

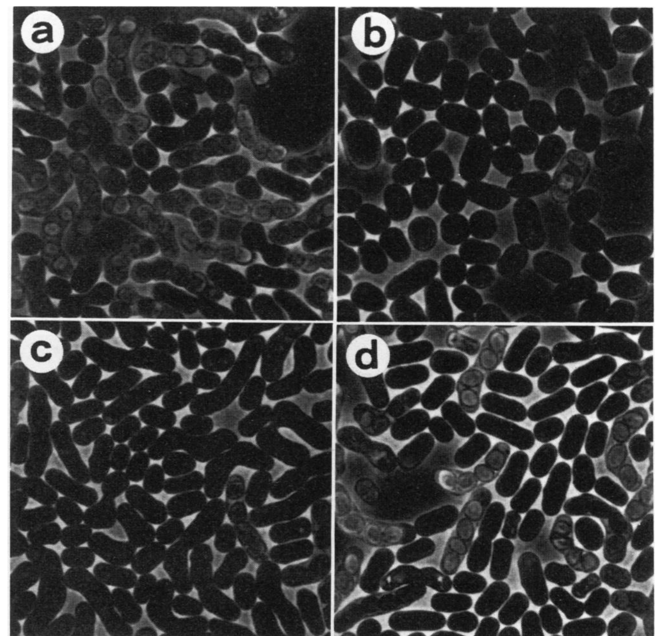

FIG. 2. Effect of overexpression of *spk1*$^+$ or *Xenopus* MAPK in homothallic haploid strain JY450 (*spk1*$^+$). JY450 was transformed with pART1 (a), pART1-*Xenopus* MAPK (b), pART1-spk1 (c), or pART1-*Xenopus* KN-MAPK (d) and grown on SSA plates containing adenine for 2 days at 30°C.

TABLE 3. Effects of overexpression of various genes on the sporulation of wild-type haploid and diploid strains[a]

Plasmid	% Sporulation	
	JY450	JY362
pART1	56.9	91.3
pART1-MAPK	2.1	49.5
pART1–KN-MAPK	21.7	67.4
pART1-spk1	8.8	37.5
pART1-MAPKK	34.2	ND[b]
pART1-byr1	53.3	ND
pART1-byr2	58.6	ND
pDB248′	58.2	ND
pDB248′-MAPK	52.6	ND
pDB248′-spk1	65.2	ND

[a] Homothallic haploid strain JY450 or diploid strain JY362, carrying the plasmid indicated, was grown on SSA plates, and the number of asci produced was counted. More than 10 independent clones were examined for each transformant.

[b] ND, not determined.

the same strain carrying pART1-spk1 formed normal asci in which four spores were arranged linearly (Fig. 1b).

Overproduction of *spk1*$^+$ and *Xenopus* MAPK prevents sexual differentiation. The *spk1* gene in vector pDB248′ complemented the sporulation defect of *spk1*$^-$/*spk1*$^-$ diploids more efficiently than when introduced on vector pART1, which expresses *spk1* at a higher level (Fig. 1d; Table 2). This suggested that *spk1* overproduction has an inhibitory effect on sporulation. We then examined the effect of overexpression of the *spk1*$^+$ gene on the sexual differentiation of wild-type (homothallic haploid) strain JY450. pART1-spk1 inhibited the formation of asci in this strain (Fig. 2c; Table 3). Interestingly, pART1-MAPK also inhibited spore formation (Fig. 2b; Table 3). Thus, *Xenopus* MAPK is functionally similar to *spk1* in this respect, too. The inhibitory effect of MAPK on sexual differentiation was partly dependent on its kinase activity (Fig. 2d; Table 3; see Discussion).

The sexual differentiation of haploid cells consists of two major events, conjugation and sporulation. The sporulation efficiency of diploid wild-type strain JY362 carrying either pART1-spk1 (Fig. 3c; Table 3) or pART1-MAPK (Fig. 3b; Table 3) was lower than that of JY362 carrying only the vector (Fig. 3a; Table 3). Thus, overexpression of either *spk1*$^+$ or MAPK could inhibit sporulation. The conjugation

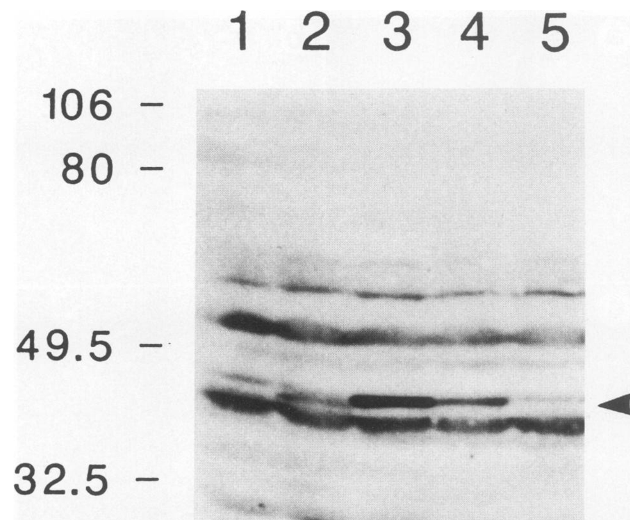

FIG. 4. Tyrosine phosphorylation of Spk1 and *Xenopus* MAPK in *S. pombe*. Homothallic wild-type strain JY450 was transformed with pART1 (lanes 1 and 5), pART1-spk1 (lane 2), pART1-*Xenopus* MAPK (lane 3), or pART1-*Xenopus* KN-MAPK (lane 4). Cell extracts were prepared as described in Materials and Methods and subjected to immunoblotting with anti-phosphotyrosine antibody PY20. The arrowhead indicates the position of the Spk1 and *Xenopus* MAPK expressed. Prestained SDS-PAGE standards (Bio-Rad) were used as molecular weight markers. The numbers on the left are molecular sizes in kilodaltons.

efficiency of wild-type haploid strain JY450 was reduced by overexpression of *Xenopus* MAPK to 26% and by that of *spk1*$^+$ to 90%, relative to the control (JY450 carrying pART1). Thus, *spk1*$^+$ overexpression was less inhibitory of conjugation than of sporulation. This result was consistent with the finding that JY450 carrying pART1-spk1 formed many zygotes without spores (Fig. 2c). The morphology of wild-type cells (JY450 and JY362) carrying pART1-MAPK (Fig. 2b and 3b) was rounder than that of cells carrying any other plasmid.

Tyrosine phosphorylation of Spk1. As tyrosine phosphorylation is critical for the activation of vertebrate MAPK (2), it is important to know whether Spk1 undergoes tyrosine phosphorylation or not. To test this, extracts were prepared from wild-type homothallic (h^{90}) cells carrying various plas-

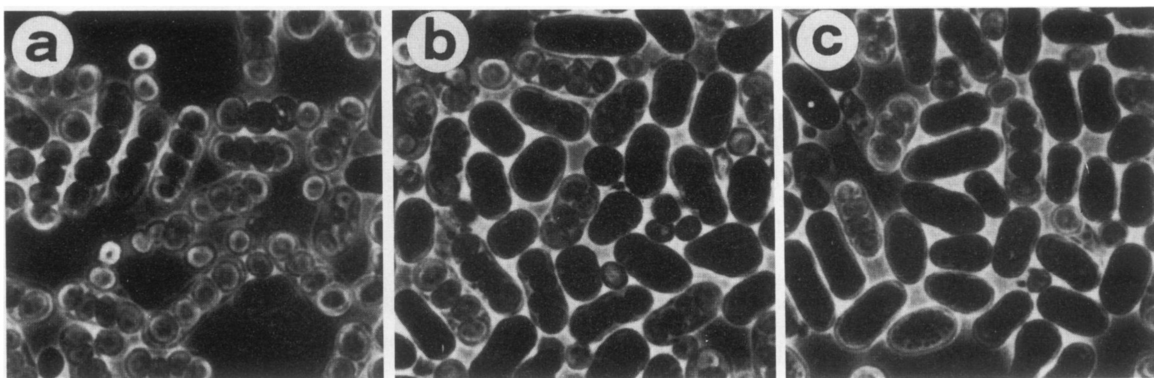

FIG. 3. Effect of overexpression of *spk1*$^+$ or *Xenopus* MAPK in wild-type diploid strain JY362. JY362 was transformed with pART1 (a), pART1-*Xenopus* MAPK (b), or pART1-spk1 (c) and grown on SSA plates for 2 days at 30°C.

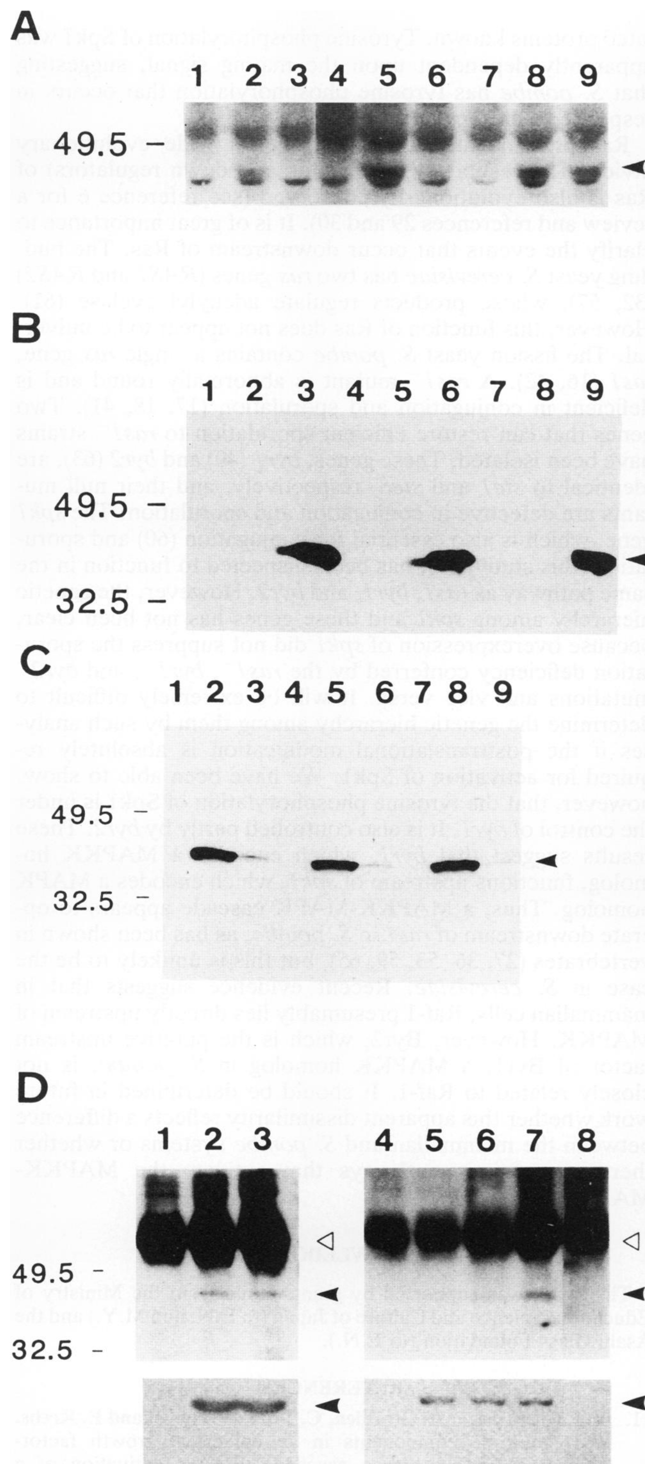

FIG. 5. Tyrosine phosphorylation of Spk1 depends on *byr1*. (A to C) h^- strain JY333 (lanes 1 to 3), *byr1* disruptant h^{90} strain JX1 (lanes 4 to 6), and h^{90} strain JY450 (lanes 7 to 9) were transformed with pART1 (lanes 1, 4, and 7), pART1-*Xenopus* MAPK (lanes 2, 5, and 8), or pART1-spk1 (lanes 3, 6, and 9). Cell extracts (10 μl of each) were subjected to immunoblotting with anti-phosphotyrosine antibody PY20 (A), anti-Spk1 antibody (B), or anti-*Xenopus* MAPK antibody (C). (D) Cell extracts obtained from JY450 transformed with pART1 (lane 1), JY450 transformed with pART1-*Xenopus* MAPK (lane 2), or JX1 transformed with pART1-*Xenopus* MAPK (lane 3) were subjected to immunoprecipitation with anti-*Xenopus* MAPK antibody. Cell extracts obtained from JY450 transformed with pART1 (lanes 4 and 8), JY333 transformed with pART1-spk1 (lane 5), JX1 transformed with pART1-spk1 (lane 6), and JY450 transformed with pART1-spk1 (lane 7) were subjected to immunoprecipitation with anti-Spk1 antibody. The immunoprecipitates were then subjected to immunoblotting with PY20 (lanes 1 to 8, upper panel), anti-*Xenopus* MAPK antibody (lanes 1 to 3, lower panel), or anti-Spk1 antibody (lanes 4 to 8, lower panel). Filled arrowheads indicate the position of the Spk1 or *Xenopus* MAPK expressed. Open arrowheads denote the immunoglobulin G heavy chain. The numbers on the left are molecular sizes in kilodaltons.

mids and subjected to immunoblotting with anti-phosphotyrosine monoclonal antibody PY20. As shown in Fig. 4, Spk1 (lane 2) and *Xenopus* MAPK (lane 3) overexpressed in a wild-type strain were detected by PY20. Spk1 migrated slightly faster than *Xenopus* MAPK in SDS-PAGE. Immunoprecipitation with anti-Spk1 or anti-*Xenopus* MAPK antibody, followed by the PY20 blot of the immunoprecipitated proteins (Fig. 5D, upper panel, lanes 2 and 7), confirmed that both Spk1 and *Xenopus* MAPK were tyrosine phosphorylated in wild-type homothallic cells. *Xenopus* MAPK has been shown to be capable of undergoing autophosphorylation on tyrosine (37). However, a kinase-negative mutant of *Xenopus* MAPK was also tyrosine phosphorylated (Fig. 4, lane 4), showing that a tyrosine kinase which phosphorylates MAPK exists in *S. pombe*.

Tyrosine phosphorylation of Spk1 depends on *byr1*. The homothallic (h^{90}) cell population used in the above-described phosphorylation analysis, or at least a certain fraction of it, was likely to be exposed and responding to the mating signal at a low level. To examine whether or not tyrosine phosphorylation of Spk1 is dependent upon the mating signal, phosphorylation in heterothallic (h^-) cells was analyzed. The expression level of Spk1 protein in h^- cells (Fig. 5B, lane 3) was almost equal to that in h^{90} cells (Fig. 5B, lane 9), but tyrosine phosphorylation of Spk1 was greatly diminished in h^- cells (Fig. 5A, lane 3, and 5D, upper panel; lane 5). This suggests that tyrosine phosphorylation of Spk1 depends on the mating signal. Tyrosine phosphorylation of *Xenopus* MAPK in h^{90} cells (Fig. 5A, lane 8) also appeared to be enhanced by the mating signal, although it still occurred to a lesser extent in h^- cells (Fig. 5A, lane 2). The expression levels of *Xenopus* MAPK in h^{90} and h^- cells did not differ (Fig. 5C, lanes 2 and 8).

The *byr1* gene is likely to function directly upstream of *spk1*, because the *ste1*$^-$ (*byr1*$^-$) mutant is sterile (40), like the *spk1*$^-$ mutant, and the *byr1* gene product is structurally homologous to vertebrate MAPKK (10, 33, 34). However, overexpression of *spk1*$^+$ did not suppress the sporulation deficiency of *byr1*$^-$, *ras1*$^-$, or *byr2*$^-$ cells (Table 2). The sporulation deficiency of *spk1*$^-$ cells was not suppressed by overexpression of *ras1*, *byr2*, and *byr1* either (data not shown). To examine the relationship between *byr1* and *spk1* further, we constructed a *byr1* disruptant (JX1) and analyzed tyrosine phosphorylation of *spk1* in this strain. Although the expression level of the Spk1 protein (Fig. 5B) and the amount of Spk1 protein immunoprecipitated (Fig. 5D, lower panel) were unaffected, the extent of tyrosine phosphorylation of Spk1 was very low in *byr1* disruptant cells (Fig. 5A, lane 6, and 5D, upper panel, lane 6); it was estimated to be about 10% of that in wild-type cells (Fig. 5A, lane 9, and 5D, upper panel, lane 7). Therefore, the mating signal that leads to tyrosine phosphorylation of Spk1 is likely to be mediated by *byr1*, although further studies are necessary to determine whether the *byr1* gene product directly phosphorylates and

activates the *spk1* gene product. Tyrosine phosphorylation of *Xenopus* MAPK expressed in *S. pombe* was only slightly diminished in *byr1* disruptant cells (Fig. 5A, lane 5, and 5D, upper panel, lane 3). This might suggest the existence of another MAPKK homolog which can phosphorylate *Xenopus* MAPK in *S. pombe*.

By using a *byr2* disruptant (JX3), we tested for the possible involvement of *byr2* in tyrosine phosphorylation of Spk1. The level of tyrosine phosphorylation of Spk1 in *byr2* disruptant cells was lower than that in wild-type cells but was significantly higher than the basal level (data not shown), suggesting only a partial requirement of *byr2* for the tyrosine phosphorylation of Spk1.

DISCUSSION

We have demonstrated here that *S. pombe* Spk1 is related to vertebrate MAPK not only structurally but also functionally. This was shown by three findings. (i) The sporulation deficiency of *spk1*$^-$ cells was overcome by expression of *Xenopus* MAPK. (ii) Both *spk1* and *Xenopus* MAPK interfered with sexual differentiation of wild-type strains if expressed from a high-copy-number plasmid. (iii) Spk1 underwent tyrosine phosphorylation, which is a characteristic of MAPK. Most recently, analyses of tyrosine phosphorylation of the *S. cerevisiae* MAPK homologs Fus3, Kss1, and Hog1 were reported (7, 14, 19). Furthermore, after submission of this report, Neiman et al. (44) reported genetic evidence that *byr2*, *byr1*, and *spk1*, the *S. pombe* genes that are structurally related to *S. cerevisiae* genes *STE11*, *STE7*, and *FUS3*/*KSS1*, respectively, are also functionally related and that the *spk1* function in *S. pombe* can be partially replaced by a mammalian MAPK. It is thus strongly suggested that signal transduction pathways involving MAPK are ubiquitous in eukaryotic cells from yeasts to vertebrates.

Overexpression of *Xenopus* MAPK or *spk1*$^+$ interfered with formation of asci in a homothallic haploid strain. This effect appears to be due to inhibition of both conjugation and sporulation processes, since overexpression of *Xenopus* MAPK (or *spk1*$^+$) inhibited both mating of haploid cells and sporulation of diploid cells, although the inhibitory effect of *spk1*$^+$ on mating was not prominent. At least two mechanisms can be assumed to account for these inhibitory effects. (i) *Xenopus* MAPK (or Spk1) may compete with another putative MAPK homolog for yeast MAPKK involved in the signal transduction pathways for sexual differentiation and block transmission of signals (competitive inhibition). (ii) *Xenopus* MAPK (or Spk1) may phosphorylate a target(s) involved in down regulation of signal transduction (negative feedback, for instance). Concerning these points, we examined the effect of a kinase-negative form of MAPK on sexual differentiation. KN-MAPK also inhibited both conjugation (~47% reduction) and sporulation (~24% reduction), although this inhibitory effect was weaker than that of intact MAPK (74 and 42% reduction, respectively). This result may suggest that both of the above-described mechanisms are responsible in combination.

Xenopus MAPK appeared to affect the cell shape of *S. pombe*. Its overexpression made both haploid and diploid cells rounder. This effect of MAPK required its kinase activity. These findings may mean that a MAPK homolog is involved in maintaining normal *S. pombe* cell shape.

Protein tyrosine phosphorylation is a key regulatory event in a variety of signal transduction pathways in higher eukaryotes. In *S. pombe*, the *cdc2* (24) and *spk1* (this study) gene products are the only examples of tyrosine-phosphorylated proteins known. Tyrosine phosphorylation of Spk1 was apparently dependent upon the mating signal, suggesting that *S. pombe* has tyrosine phosphorylation that occurs in response to extracellular stimuli.

Ras proteins are conserved over a wide evolutionary divide. The regulatory system (up and down regulators) of Ras is also evolutionarily conserved (see reference 6 for a review and references 29 and 30). It is of great importance to clarify the events that occur downstream of Ras. The budding yeast *S. cerevisiae* has two *ras* genes (*RAS1* and *RAS2*) (32, 57), whose products regulate adenylyl cyclase (61). However, this function of Ras does not appear to be universal. The fission yeast *S. pombe* contains a single *ras* gene, *ras1* (16, 42). A *ras1*$^-$ mutant is abnormally round and is deficient in conjugation and sporulation (17, 18, 41). Two genes that can restore efficient sporulation to *ras1*$^-$ strains have been isolated. These genes, *byr1* (40) and *byr2* (63), are identical to *ste1* and *ste8*, respectively, and their null mutants are defective in conjugation and sporulation. The *spk1* gene, which is also essential for conjugation (60) and sporulation (this study; 44), has been suspected to function in the same pathway as *ras1*, *byr1*, and *byr2*. However, the genetic hierarchy among *spk1* and these genes has not been clear, because overexpression of *spk1* did not suppress the sporulation deficiency conferred by the *ras1*$^-$, *byr1*$^-$, and *byr2*$^-$ mutations and vice versa. It will be extremely difficult to determine the genetic hierarchy among them by such analyses if the posttranslational modification is absolutely required for activation of Spk1. We have been able to show, however, that the tyrosine phosphorylation of Spk1 is under the control of *byr1*. It is also controlled partly by *byr2*. These results suggest that *byr1*, which encodes a MAPKK homolog, functions upstream of *spk1*, which encodes a MAPK homolog. Thus, a MAPKK-MAPK cascade appears to operate downstream of *ras1* in *S. pombe*, as has been shown in vertebrates (27, 36, 53, 59, 65), but this is unlikely to be the case in *S. cerevisiae*. Recent evidence suggests that in mammalian cells, Raf-1 presumably lies directly upstream of MAPKK. However, Byr2, which is the putative upstream factor of Byr1, a MAPKK homolog in *S. pombe*, is not closely related to Raf-1. It should be determined in future work whether this apparent dissimilarity reflects a difference between the mammalian and *S. pombe* systems or whether there are various pathways that activate the MAPKK-MAPK cascade.

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