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Identification of Ste4 as a Potential Regulator of Byr2 in the Sexual Response Pathway of *Schizosaccharomyces pombe*

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A conserved MAP kinase cascade is central to signal transduction in both simple and complex eukaryotes. In the yeast *Schizosaccharomyces pombe*, Byr2, a homolog of mammalian MAPK/ERK kinase kinase and *Saccharomyces cerevisiae* STE11, is required for pheromone-induced sexual differentiation. A screen for *S. pombe* proteins that interact with Byr2 in a two-hybrid system led to the isolation of Ste4, a protein that is known to be required for sexual function. Ste4 binds to the regulatory region of Byr2. This binding site is separable from the binding site for Ras1. Both Ste4 and Ras1 act upstream of Byr2 and act at least partially independently. Ste4 contains a leucine zipper and is capable of homotypic interaction. Ste4 has regions of homology with STE50, an *S. cerevisiae* protein required for sexual differentiation that we show can bind to STE11.

The MAP kinase (MAPK) module is a highly conserved intermediary in the transmission of signals from cell surface receptor molecules to the nucleus (2, 4, 5, 9, 13, 22). The module comprises a common trio of sequentially acting protein kinases (for reviews, see references 8, 16, and 19). A MAPK/ERK kinase kinase (MEKK) phosphorylates and activates a MAPK/ERK kinase (MEK), which in turn phosphorylates and activates a MAPK/ERK. MAPK modules are found ubiquitously in all eukaryotes examined and participate in a broad spectrum of regulatory pathways. For example, in *Saccharomyces cerevisiae* alone, four distinct MAPK signaling pathways have been identified as participating in at least four physiological processes: pheromone-induced sexual differentiation, osmoregulation, cell wall construction, and nutrient-regulated growth (for a review, see reference 2).

The pheromone-responsive MAPK pathway of *S. cerevisiae* has been extensively characterized (for a review, see reference 13). In response to pheromone binding to its receptor, the haploid budding yeast undergoes cellular changes that prepare the cell to mate. These pheromone-inducible responses include G_1 arrest, transcriptional activation of mating genes, and morphological changes. Upon pheromone receptor activation, an inactive heterotrimeric G protein dissociates, freeing the $G_{\beta\gamma}$ subunits from an inhibitory G_α subunit. $G_{\beta\gamma}$ is then able to activate the MAPK module through an unknown mechanism, possibly involving the STE20 kinase and STE5. Consequent to pheromone binding, STE11 phosphorylates and activates its downstream target, STE7, a MEK. STE7 in turn phosphorylates and activates the redundant MAPKs: FUS3 and KSS1. Both MAPKs activate nuclear targets including the transcriptional activator, STE12. STE12 is responsible for the regulation of genes involved in cell cycle arrest, cell fusion, and the pheromone response pathway itself. Another substrate for FUS3 is FAR1, which promotes cell cycle arrest by binding to cyclin-dependent kinases.

The fission yeast *Schizosaccharomyces pombe*, like *S. cerevisiae*, has two mating types, h^+ and h^-, two transmembrane-spanning pheromone receptors, encoded by *mam2* and *map3*, and a G_α subunit, encoded by *gpa1*, and pheromones that signal through a MAPK module (14). The Byr2, Byr1, and Spk1 protein kinases of *S. pombe* are structurally and functionally homologous to STE11, STE7, and FUS3/KSS1 of *S. cerevisiae*, respectively (22). Substantial differences between the budding and fission yeast mating pathways have been found. In *S. cerevisiae*, $G_{\beta\gamma}$ activates the pheromone response pathway, whereas in *S. pombe*, it is the G_α subunit of the heterotrimeric G protein that is the positive signal transducer (23). Additionally, in *S. pombe* Ras1 is necessary for sexual differentiation (14, 21) whereas no evidence for the involvement of *S. cerevisiae* RAS1 and RAS2 in pheromone response has been found. Most importantly, both mating pheromone and starvation are required to induce sexual differentiation in *S. pombe* (20). Therefore, multiple inputs may be needed to activate the *S. pombe* MAPK module and, in particular, Byr2.

To explore how Byr2 becomes activated, we have been utilizing the two-hybrid system of Fields and Song (11). Complex formation between Ras1 and Byr2 was demonstrated in this manner (30), confirming the placement of Byr2 as a downstream effector of Ras1 (32). We have now identified an additional component of the *S. pombe* mating MAPK pathway by this means. We show that a protein encoded by a previously cloned sterile gene, *ste4* (24), interacts with Byr2, and at a site distinct from Ras1. Our genetic findings, described in this work, suggest a regulatory role for Ste4 in the pheromone-responsive MAPK module of *S. pombe*. We also report that STE50 of *S. cerevisiae*, which shows limited homology to Ste4, interacts with members of the mating MAPK module in that organism as well.

MATERIALS AND METHODS

Yeast and *Escherichia coli* strains, media, and genetic manipulations. The genotypes of all *S. cerevisiae* and *S. pombe* strains used in this study are listed in Table 1. *S. cerevisiae* YPB2, HF7c, and L40 were used as reporter strains for two-hybrid experiments. YPB2 and HF7c are GAL4-based two-hybrid reporter strains. The LexA-based two-hybrid system which utilizes the bacterial LexA DNA-binding domain (LBD) in combination with an acidic activation domain to drive transcription from a *lexA-lacZ* reporter gene was also employed (31). In some instances, this system is more sensitive than the GAL4-based system (18). L40 is a LexA-based two-hybrid reporter strain. In the following experiments, both the LexA-based and GAL4-based two-hybrid systems were utilized.

S. cerevisiae cultures were grown in YPD (2% peptone, 1% yeast extract, and 2% glucose) or in synthetic minimal medium (0.67% yeast nitrogen base, 2% glucose, and appropriate auxotrophic supplements) (17). *S. pombe* strains were

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TABLE 1. Strains used in this study

Strain	Relevant genotype	Source or reference
S. cerevisiae		
YPB2	*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can*^{r} *gal4-542 gal80-538 LYS*::*GAL1*UAS-*LEU2*TATA-*HIS3 URA3*::(*GAL1* 17-mers)$_3$-*CYC1*TATA-*lacZ*	P. Bartel and S. Fields
HF7c	*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 URA3*::(*GAL4* 17-mers)$_3$-*CYC-lacZ*	H. Feilotter, G. Hannon, and D. Beach
L40	*MAT***a** *ade2 his3 leu2 trp1 LYS*::*lexA-HIS3 URA3*::*lexA-lacZ*	31
S. pombe		
SP870	h^{90} *leu1-32 ade6-210 ura4-D18*	D. Beach
SP525	h^{90} *leu1-32 ade6-216 ura4 ras1*::*LEU2*	D. Beach
SP565	SP525 diploid	D. Beach
SPRU	h^{90} *leu1-32 ade6-210 ura4-D18 ras1*::*ura4*	32
SPRUD	SPRU diploid	32
SPSL	h^{90} *leu1-32 ade6-210 ura4-D18 sir1*(*byr2*)::*LEU2*	32
SPSL-D	SPSL diploid	32
SP4A	h^{90} *leu1-32 ade6-210 ura4-D18 ste4*::*ADE2*	This study
SP4A-D	SP4A diploid	This study

grown in the rich medium (YEA) or synthetic minimal medium (PM) with appropriate auxotrophic supplements (21). Standard yeast genetic methods were followed (28). The lithium acetate method was used for transformations of yeast cells (15).

Sporulation of *S. pombe* cells was detected by the iodine vapor staining method as described previously (12). Microscopic observation was used to calculate the conjugation and sporulation efficiencies. *S. pombe* mating assays were performed as described previously (22). For the induction of mating and sporulation, PM lacking NH_4Cl (PM-N) was used (21). The homozygous diploid strains used in this study were generated during the transformation process and isolated from plates containing phloxin B (1). Ploidy was confirmed by microscopic examination of cell size and the presence of azygotic sporulation.

A *ste4*^{null} strain (SP4A) was created by transforming SP870 with pBSII*ste4*::*ADE2* (see below) digested with *Eco*RI and *Xho*I and plating transformants on a synthetic minimal medium lacking adenine (PMLU) (21). After 5 days, isolated Ade$^+$ colonies were examined microscopically and were found to be sterile. Gene deletions were confirmed by PCR (26).

The *E. coli* strain DH10B (Bethesda Research Laboratories) was used for plasmid propagation and isolation. pGAD plasmids in *S. cerevisiae* cells were recovered by transforming *E. coli* MH4 (3) with crude DNA extracts prepared from transformed yeast cells. *E. coli* BL21(DE3) (Novagen) was used for expressing bacterial fusion proteins.

Nucleic acid manipulation and analysis. Manipulation of DNA was carried out according to standard procedures (26). DNA restriction endonucleases, polymerases, and ligases were used under the conditions recommended by the suppliers (New England Biolabs, Bethesda Research Laboratories, Perkin-Elmer Cetus, and Boehringer Mannheim). DNA sequencing was performed by using the dideoxynucleotide chain-termination method (27) and Sequenase (United States Biochemical).

Two-hybrid fusion plasmids. PCR was used to generate fragments of *ras1*(*C216R*), *ras1*(*G17V C216R*), *byr2*Δ*C* (encoding residues 1 to 392), *byr2*Δ*N* (encoding residues 392 to 652), *byr1*, *spk1*, *STE50*, and *GPA1* coding sequences. These fragments were inserted into the polylinker of GAL4 transcriptional activation domain (GAD), GAL4 binding domain (GBD), or LBD two-hybrid expression vectors to make in-frame protein fusions. pGADGHste4ΔN (encoding amino acids 161 to 264) was created by excising a *Spe*I fragment from pGADGHste4 (thus removing *ste4*Δ*C*) and religating the vector. pGADGH is a GAL4 activation domain fusion vector (30). pGADGHste4ΔC (encoding amino acids 1 to 160) was created by cloning the *ste4*Δ*C* fragment (excised from the pGADGHste4 cDNA clone) into the *Spe*I multicloning site of pGADGH. pADESte4 was made by inserting a *Bam*HI-*Kpn*I fragment of *ste4* in the vector pAA (7). pADEbyr2ΔC was made by replacing the *ste4* insert of pADEste4 with a *byr2*Δ*C* fragment. pHIS3byr2 was made by cloning the *Bam*HI-*Sac*I fragment of pAD5byr2 (22) into pRS423, a *HIS*-based 2μm vector (29). The *S. pombe* GAD cDNA fusion library (available through Clontech) was kindly provided by G. Hannon.

Construction of mutant *byr2*Δ*C* library. *byr2*Δ*C* was randomly mutagenized by PCR as described previously (34) and ligated into the vector pVJL11 (18) to create in-frame fusions with the LexA DNA-binding domain. The primer pair 5′-CACCCACTTTCC***GGATCC***TATGGAATATTA-3′ and 5′-TATCCATTTG ***GTCGAC***TGATCATCAG-3′ was used.

These primers contain, respectively, *Bam*HI and *Sal*I sites (in italics and boldface type) for directional cloning into pVJL11. pUC119byr2 (32) was used as a template. A library of more than 10^6 clones was generated from two separate PCRs.

To make full-length clones of Byr2 mutants, a *Sal*I-*Pst*I *byr2*Δ*N* fragment (from pVJL11byr2ΔN) was cloned into the *Sal*I-*Pst*I site of the pVJL11byrΔC mutant, creating full-length pVJL11byr2 mutants. These full-length pVJL11byr2 mutants exhibited binding properties identical to those of the original pVJL11byr2ΔC isolates. To generate constructs for expression analysis in *S. pombe*, the full-length *Bam*HI-*Sac*I mutant *byr2* fragment (excised from pVJL11byr2) was cloned into the *Bam*HI-*Sac*I site of pART1. 5′ to the *Pst*I site following byr2ΔN of pVJL11byr2ΔN is an engineered *Sac*I site.

***S. pombe* deletion and expression constructs.** The following strategy was followed to create the *ste4* disruption vector, pBSIIste4::ADE2. An *Eco*RI-*Xho*I fragment was excised from pGADste4 and cloned into pBSII (26). The resulting pBSIIste4 was digested with *Bbs*I-*Sph*I, deleting 607 bp of the *ste4* coding region (763 bp of the genomic *ste4* which contains a small intron) and creating pBSIIste4Δ71-701. A *Sma*I-*Sph*I *ADE2* fragment was cloned into pBSIIste4Δ71-701, creating the *ste4* disruption vector. pAAUCM1ste4 was constructed by cloning a *Sal*I-*Sac*I *ste4* fragment from pBSIIste4 into the multicloning site of pAAUCM1. pAAUCM1 allows for the expression of a c-*myc* (10)-tagged protein driven from the *S. pombe adh* promoter and contains the selectable URA3 marker. pARTICMIste4 plasmid, which also contains an *adh* promoter fused to a c-*myc* epitope tag preceding the multicloning site, was made by cloning the *Bam*HI-*Sac*I *ste4* fragment of pBSIIste4 into pART1CM1, a *leu2*-selectable plasmid (7). pARTICMIste4ADE2 was created by cloning a *Sac*I *ADE2* fragment into the *Sac*I site of pARTICMIste4. pAAUCM1ste4ΔC was created by cloning a *Bam*HI *ste4*Δ*C* fragment of pGADste4ΔC into pAAUCM1. pART1CM1ras1, pAIS1, and pART1MC1byr1 have been described previously (32, 33). pAUR contains *ras* in pIRT5 which is derived from pUC118, with an autonomously replicating sequence and *ura4*-selectable marker (32).

Detection of protein complex formation by the two-hybrid system. To identify proteins that interact with Byr2, a pGAD *S. pombe* cDNA fusion library (available from Clontech) and pGBDbyr2 were cotransformed into the *S. cerevisiae* reporter strain HF7c. Transformants were plated on synthetic medium lacking histidine, leucine, and tryptophan (dropout [DO]-His-Leu-Trp), thereby selecting for plasmids encoding proteins capable of two-hybrid interaction as evidenced by transactivation of a *GAL1-HIS3* reporter gene and histidine prototrophy. The plates were incubated at 30°C for 5 days, and these His$^+$ colonies were patched on plates lacking leucine and tryptophan (DO-Leu-Trp). After 2 days at 30°C, these patches were replica plated on a Whatman no. 50 filter paper placed onto DO-Leu-Trp plates. After 24 h, β-galactosidase filter assays were performed as described previously (30). Library pGADGH plasmids were recovered and retransformed into HF7c in combination with pGBDbyr2 or control plasmids to test for plasmid dependency. Clones exhibiting specific interaction with Byr2 were sequenced.

Screening for mutations that affected binding of LBDbyr2ΔC to GADste4 in yeast strain L40 was performed. Approximately 10^4 clones were assayed for abolished or decreased β-galactosidase activity in a β-galactosidase filter assay. Mutant LBDbyr2ΔC plasmids were isolated and retransformed into L40 with either GADras1(C216R) or GADste4. Those mutants that retained the ability to interact with GADras1(C216R) but not with GADste4 were sequenced.

Bacterial expression of Ste4 and Byr2ΔC proteins. The constructs pRP-ste4 and pTrcHis-byr2ΔC were used for expression of glutathione *S*-transferase (GST)-ste4 and oligohistidine-, T7-tagged byr2ΔC (HT-byr2ΔC) fusion proteins, respectively, in *E. coli*. pRP-ste4 was made by cloning *ste4* into the *Bam*HI-*Sal*I sites of pRP259, a derivate of GEX-3 (Pharmacia) obtained from M. Gebbink. pTrcHis-byr2ΔC was created by cloning the *Bam*HI-*Sal*I *byr2*Δ*C* fragment in frame into the polylinker of pTrcHisB (Invitrogen). This vector contains also the leader peptide of the T7 major capsid protein. pRP-scd2 (GST-scd2) has been described elsewhere (7). BL21(DE) cells (Novagen) containing the lysozyme-

expressing plasmid pLysS (Novagen) were transformed with either pRP-ste4 or pTrcHis-byr2ΔC and were grown overnight at 37°C in Luria broth containing 50 μg of ampicillin per ml. Overnight cultures were used to inoculate fresh 500-ml Luria broth-ampicillin cultures. Cultures were grown to an optical density at 600 nm of 0.5 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and harvested 4 h later. Pellets were resuspended in 10 ml of sonication buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, and 2 μg of aprotinin per ml) and sonicated. Cellular debris was cleared by two successive 30-min spins at 12,000 × *g* at 4°C.

Lysates containing GST-ste4 and GST-scd2 were incubated with glutathione-Sepharose for 2 h at 4°C. Coupled proteins were washed extensively with sonication buffer to remove nonspecific proteins. Aliquots (300 μl) of lysates containing HT-byr2ΔC were mixed and incubated with glutathione-Sepharose coupled to GST-ste4 or GST-scd2. Mixtures were incubated for 2 h at 4°C while rotating. Beads were washed extensively and resuspended in 50 μl of sodium dodecyl sulfate (SDS) sample buffer; 5 μl of this sample was resolved by SDS–12% polyacrylamide gel electrophoresis and then transferred to nitrocellulose (Schleicher and Schuell).

All blots were preincubated in blocking solution (3% bovine serum albumin prepared in TBST [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween]). Monoclonal antibodies to GST (Santa Cruz) and T7 (Novagen) were used for the detection of GST-ste4 and HT-byr2ΔC, respectively. All antibodies were diluted in the blocking agent, and this was followed by detection with a chemiluminescence kit (ECL kit; Amersham).

RESULTS

Isolation of a protein that interacts specifically with Byr2. A two-hybrid system was used to identify proteins that interact with Byr2. Byr2 was fused to the GBD to generate GBDbyr2. *S. cerevisiae* HF7c containing GBDbyr2 was transformed with DNA from a library of *S. pombe* cDNAs fused to the GAD. Interactions between the GBD fusions and the GAD fusions result in histidine prototrophy and synthesis of β-galactosidase (see Materials and Methods). Roughly 10^6 transformants were obtained, and about 100 were able to grow on medium lacking histidine. Ten of these His^+ colonies also tested positive for β-galactosidase activity as indicated by blue color in a filter assay. GAD plasmids were isolated from these colonies and were transformed into the reporter strain HF7c containing GBDbyr2. Out of 10 clones, 8 could reproducibly transactivate both the *lacZ* and *HIS3* reporter genes. Furthermore, none interacted with GBDSNF4 (6), an unrelated fusion protein used as a negative control. These eight clones were sequenced. All contained full-length *ste4*, a previously cloned gene. We did not isolate Ras1 in this round of screening, so our screen was not exhaustive.

ste4 was originally cloned by complementation of a *ste4* mutant strain (24). *ste4* encodes a 264-amino-acid protein with a typical leucine zipper motif homologous to that found in the jun family of transcription factors but lacks a putative DNA binding domain. Amino acids 204 to 262 show 32% identity and 64% homology to residues 267 to 325 of *STE50*, an *S. cerevisiae* gene involved in mating responses (Fig. 1). STE50 does not contain a leucine zipper domain.

Binding properties of Ste4. As a first step in the characterization of the Ste4 protein, the physical interactions between Ste4 and components of the *S. pombe* mating response pathway were examined. As previously noted, Ste4 has a leucine zipper motif. Leucine zippers have been shown to mediate protein-protein interactions. Leucine zipper interactions may be either homotypic or heterotypic. To determine whether Ste4 interacts homotypically, Ste4 was expressed as both GBD and GAD fusion proteins in the reporter strain HF7c. As shown in Table 2, GBDste4 and GADste4 form a protein complex. Ste4 did not form a complex with Ras1, Gpa1, Byr1, or Spk1.

To determine which region of Ste4 is necessary for homotypic interaction and for interaction with Byr2, truncations of Ste4 were made. *ste4ΔC* encodes amino acids 1 to 160 and possesses the leucine zipper. *ste4ΔN* encodes amino acids 161 to 264 and contains the region homologous to STE50 (Fig. 1). It is the N terminus of Ste4 (Ste4ΔC), containing the leucine zipper, that is necessary and sufficient for two-hybrid interaction with both itself and Byr2 (Table 2).

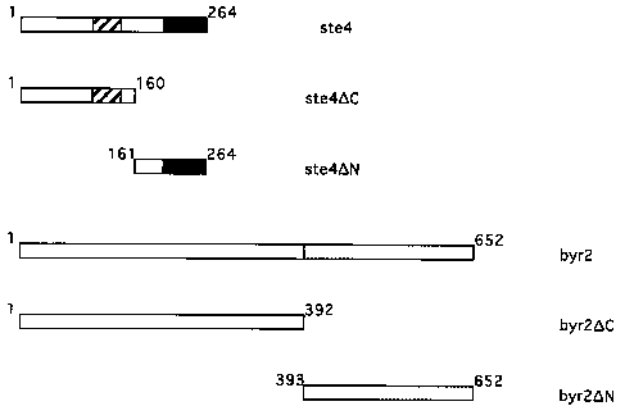

FIG. 1. Schematic representation of Ste4 and Byr2 fusion proteins tested in the two-hybrid system. *byr2ΔC* and *byr2ΔN* encode amino acids 1 to 392 and 393 to 652, respectively. The shaded box represents the catalytic domain of the Byr2 protein kinase. *ste4ΔC* and *ste4ΔN* encode residues 1 to 160 and 161 to 264, respectively. The striped box represents the leucine zipper (residues 97 to 139), and the black box represents the region of STE50 homology (residues 204 to 262).

To obtain biochemical evidence for complex formation between Ste4 and Byr2, epitope-tagged fusion proteins were expressed in *E. coli*. Byr2ΔC was expressed as an oligohistidine, T7-tagged fusion protein, HT-byr2ΔC. The truncated protein, Byr2ΔC, which lacks the kinase domain (see Materials and Methods), was used, because the full-length Byr2 fusion protein was insoluble. Ste4 was expressed as a GST fusion protein, GST-ste4. *E. coli* lysates containing GST-ste4 were coupled to glutathione-Sepharose. Lysates from *E. coli* expressing Byr2ΔC were mixed with GST-ste4 beads. The beads were split into two aliquots, and both were analyzed by Western blotting (immunoblotting). Blots were probed with a monoclonal antibody directed to GST, which detected GST-ste4, or with a monoclonal antibody directed to the T7 tag, which detected HT-byr2ΔC. As shown in Fig. 2, HT-byr2 complexed with GST-ste4 beads. As controls, no complex formation between GST-scd2 beads and HT-byr2ΔC or between GST-ste4 beads and HT-scd1ΔN was observed (see reference 7 for the preparation of GST-scd2 and HT-scd1ΔN). These results indicate that Ste4 and Byr2ΔC interact directly and specifically.

Physical interactions of Ras1 and Ste4 with the regulatory domain of Byr2. The studies of Wang et al. (32) suggest that the N terminus of Byr2 contains regulatory elements. Expression of a truncated Byr2 lacking amino acids 389 to 606 of the carboxy terminus dominantly interferes with wild-type Byr2 function, while a deletion mutant lacking the first 320 residues of Byr2 retains Byr2 activity. Truncations of Byr2 were made in order to define the region of Ste4 association (Fig. 1). It was found that Ste4 binds to the putative regulatory domain of Byr2 (Byr2ΔC, encoding amino acids 1 to 392) but not to the catalytic region of Byr2 (Byr2ΔN, encoding residues 393 to 652). Ras1 also interacts with Byr2ΔC (reference 30 and Table 2). This suggests that, like Ras1, Ste4 could be a regulator of Byr2 (see below).

To determine if binding of Ste4 and Ras1 to Byr2 is mutually exclusive, Byr2 was overexpressed in the presence of Ras1 and Ste4. We found that Byr2 can bridge an interaction between

TABLE 2. Interactions between components of the *S. pombe* MAPK module in transformed reporter cells[a]

Transformant	β-Galactosidase activity[b]			
	Ras1	Ste4	Byr2	Byr2ΔC
Ras1(C216R)	− (0.2 ± 0.12)	−	+ (220.9 ± 64.5)	+ (104.8 ± 6.2)
Gpa1	−	−	−	− (1.2 ± 0.01)
Ste4	− (0.1 ± 0.21)	+	+ (199.5 ± 86.5)	+ (202.4 ± 75.8)
Ste4ΔC	−	+	+	+
Ste4ΔN	−	−	−	−
Byr2	+ (44.1 ± 9.2)	+	− (0.7 ± 0.13)	−
Byr2ΔC	+ (289.40 ± 28.2)	+	− (10.8 ± 0.12)	−
Byr2ΔN	− (1.65 ± 0.3)	−	− (10.6 ± 0.12)	−

[a] The proteins in the table stub were fused to the GAD, and the proteins in the subheads were expressed as LBD (Ste4) or LBD and GBD (Ras1, Byr2, and Byr2ΔC) fusion proteins. (Byr1 and Spk1 were expressed as LBD fusion proteins, Ste4ΔN was expressed as a GBD fusion protein, and Gpa1 was expressed as both [data not shown].) GBD-GAD combinations were expressed in the GAL4 two-hybrid reporter strain, HF7c. LBD-GAD combinations were expressed in the LexA two-hybrid reporter strain, L40. At least 16 transformants were tested for each determination. No inconsistencies between reporter strains were observed.

[b] Liquid β-galactosidase assays were performed for some of the interactions using the LexA-based system (numbers in parentheses). Transformants were grown in selective synthetic medium, and β-galactosidase activity was assayed with *O*-nitrophenyl β-D-galactosidase; values (means ± standard deviations of triplicate determinations) are given in Miller units (20). The expression of Ste4ΔN LexA fusion proteins was confirmed by Western blotting. + and −, transformants that did and did not express detectable levels of β-galactosidase activity, respectively. The Gpa1, Ste4ΔN, Byr1, and Spk1 binding domain fusions all failed to express detectable levels of β-galactosidase activity when combined with any of the activation domain fusions (data not shown).

Ras1 and Ste4, which otherwise do not interact (Fig. 3). Furthermore, overexpression of the regulatory domain of Byr2 is necessary and sufficient for bridging the interaction between Ste4 and Ras1, indicating that catalytic activity of Byr2 is not required for multiprotein complex formation. These results suggest that Ste4, Byr2, and Ras1 can form a complex and indicate that Ste4 and Ras1 bind to different regions of the N terminus of Byr2. (The latter conclusion is supported by mutational studies; see below). These observations indicate that Ste4 and Ras1 might act, in part, independently upon Byr2 (see below).

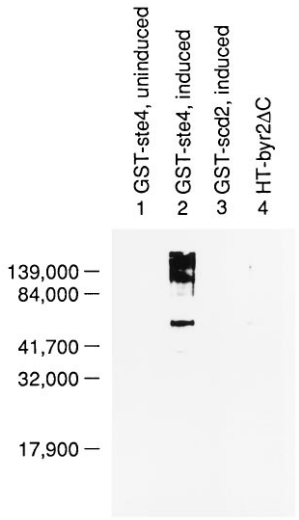

FIG. 2. Ste4 and Byr2ΔC form a complex in vitro. GST-ste4 and GST-scd2 fusion proteins were purified from bacterial lysates and bound to glutathione-Sepharose beads. Induced or uninduced HT-byr2ΔC lysates were incubated with equal amounts, as determined by Coomassie-blue staining, of the above-mentioned glutathione-Sepharose-coupled proteins. After a 2-h incubation at 4°C, the beads were washed extensively, resuspended in a small volume of SDS sample buffer, and boiled for 10 min. Samples were electrophoresed in 12.5% polyacrylamide gels, and proteins were transferred to nitrocellulose. Monoclonal antibody to GST (Santa Cruz) was used to detect GST-ste4 (predicted size, 46 kDa) and GST-scd2 (data not shown). Monoclonal antibody to T7 (Novagen) was used to detect Byr2ΔC (at a molecular mass of 46 kDa). Lane 1, GST-ste4 beads + uninduced HT-byr2ΔC lysate; lane 2, GST-ste4 beads+ induced HT-byr2ΔC lysate; lane 3, GST-scd2 beads + induced HT-byr2ΔC lysate; lane 4, partially purified HT-byr2ΔC (eluted from a nickel resin by boiling). Induced HT-scd1ΔN lysates complex with GST-scd2 (7) beads but not GST-ste4 beads (not included on this blot). The bands with extra-high molecular weights, seen in lanes 2 and 4, accompany HT-byr2ΔC during its partial purification, whether by immunoprecipitation or nickel column chromatography.

Evidence that Ste4 acts upstream of Byr2. A *ste4*^{null} strain, SP4A, was constructed with the homothallic (*h*^{90}) wild-type strain SP870 (*S. pombe* strains used in this study are listed in Table 1). SP4A had a normal cell shape and grew at rates similar to those of the parental strain. As previously reported for *ste4*^{null} cells, SP4A cells are totally incapable of mating (24), and additionally we find that the diploid strain, SP4AD, lacking both copies of *ste4*, is incapable of azygotic sporulation. Transformation of the haploid and diploid *ste4*^{null} strains with plasmids overexpressing a c-*myc*-tagged Ste4 protein from the *S. pombe adh1* promoter, pAAUCM1ste4 (containing the *S. pombe ura3*-selectable marker) or pART1CM1ste4 (containing the *S. cerevisiae LEU2*-selectable marker), restored conjugation and sporulation. Therefore, Ste4, like Byr2, is required for both conjugation and sporulation but not for growth rate or cell shape (Table 3). Moreover, we have observed that disruption of *ste4* abolishes the hypersensitive pheromone response induced by activation of Gpa1(E244L) or Ras1(G17V) (data not shown), as we have found for disruption of *byr2* (32, 33). Hence, disruption of *ste4* results in a phenotype that is virtually indistinguishable from that resulting from disruption of *byr2.*

To explore further the relationship of Ste4 and Byr2, we next expressed Byr2 in a *ste4*^{null} strain, SP4A. Expression of full-length Byr2 (pAIS1) is able to restore both conjugation and

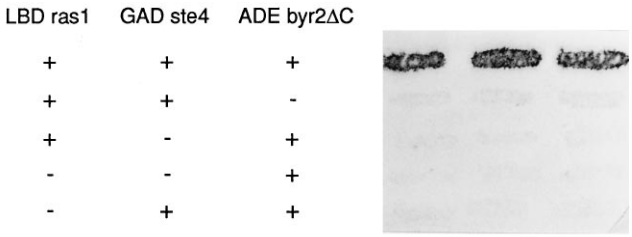

FIG. 3. The regulatory domain of Byr2 is necessary and sufficient for bridging complex formation between Ste4 and Ras1. The LEXA two-hybrid tester strain, L40, was transformed (+) or not (−) with plasmids expressing Ras1 fused to LBD, Ste4 fused to GAD, and Byr2ΔC expressed from pADE and assayed for β-galactosidase expression (shown to the right).

TABLE 3. Suppression of mating defects in an SP870 h^{90} *ste4*^{null} strain with overexpression of genes involved in mating response[a]

Expressed gene(s)	% Wild-type mating efficiency
ste4	100.0
*ste4*Δ*C*	<0.01
ras1$^{(a,b)}$	<0.01
byr2	1.8
byr1	<0.01
byr2 byr1	5.8
ras1$^{(a)}$ *byr2*	15.7
ras1$^{(b)}$ *byr1*	<0.01
Vector	<0.01

[a] The quantitative *S. pombe* mating assay was performed by microscopy. Three colonies of each transformant were inoculated and grown in liquid minimal medium (PM) and subsequently starved for nitrogen. In each culture, 1,000 cells were examined and mating efficiency was scored by the presence of zygotic asci. All values are expressed as percent mating efficiency relative to that of SP4A transformed with pAAUCM1ste4. (In absolute terms, the mating efficiency of the latter was 40%.) Plasmids used for transformation of SP4A were pAAUCM1ste4 and pAAUCM1ste4ΔC for Ste4 and Ste4ΔC expression, pART1CM1ras1 or pAUR for Ras1 expression (the former was used with *ras1 byr2*, the latter was used with *ras1 byr1*, and both were used with *ras1*), pAIS1 for Byr2 expression, pART1MC1byr1 for Byr1 expression, and pART1CM1 and pAAUCM1 for negative vector controls. All plasmids, except pAUR, contain an *adh* promoter.

sporulation to *ste4*^{null} strains, although conjugation of these strains is much less efficient than that of the wild type (Tables 3 and 4). Expression of both Byr1 and Byr2 enhances the suppression of the *ste4*^{null} strain (Table 3). On the other hand, overexpression of Ste4 does not rescue the conjugation or sporulation defects of a *byr2*^{null} strain (data not shown). Together, these results suggest that Ste4 acts upstream and through Byr2.

Multiple regulatory influences on Byr2. We observed that overexpression of Ras1 enhances the ability of Byr2 to rescue the defects of a *ste4*^{null} strain (Table 3). Moreover, we observed that overexpression of Ste4 enhances the ability of Byr2 to rescue the defects of a *ras1*^{null} strain (data not shown). These observations support a model in which Ste4 and Ras1 can each activate Byr2 and can do so in a manner that is at least partially independent of the other.

TABLE 4. Suppression of mating and/or sporulation defects in *byr2*^{null}, *ras1*^{null}, and *ste4*^{null} strains by overexpression of *byr2* mutants[a]

Strain (gene)	% Asci in cells transformed with:		
	Byr2	Byr2(N28I)	Vector
SPSU (*byr2*^{null} haploid)	60	1	<0.01
SP4A (*ste4*^{null} haploid)	0.2	0.1	<0.01
SP4AD (*ste4*^{null} diploid)	5	2	<0.01
SPRUD (*ras1*^{null} diploid)	35	3.5	1.5

[a] *byr2*^{null} haploid, *ste4*^{null} haploid and diploid, and *ras1*^{null} diploid cells were transformed with pART1-byr2(WT), pART1byr2(N28I), or pART1. Transformants were selected in medium with the appropriate auxotrophic supplements on plates. After 5 days of incubation at 30°C, colonies were patched, and after another 3 days of incubation at 30°C, the patches were examined microscopically and the percentage of asci was determined. The percentages shown in the table represent averages from more than 10 transformant colonies. Values given for haploid cells represent zygotic asci, indicative of conjugation, while those for diploids represent azygotic asci, indicative of sporulation. We observed that the suppression of the mating defects in a *ste4*^{null} haploid strain by overexpression of Byr2 when the mating assay is performed in liquid (Table 3) is better than that when the assay is performed on plates.

TABLE 5. Interaction of STE50 with components of the *S. cerevisiae* pheromone response pathway[a]

GAD fusions	β-Galactosidase activity[b]				
	STE50	STE5	STE11	STE7	FUS3
GBDGPA1	−	−	−	−	−
GBDSTE20	−	−	−	−	−
GBDSTE5	+	−	+	+	+
GBDSTE11	+	+	+	−	+
GBDSTE7	−	+	−	−	+
GBDFUS3	−	+	+	+	−
LBDSTE11	+	+	+	+	+
LBDSTE11ΔC	+	+	NT	NT	NT
LBDSTE7	−	+	+	+	+
LBDSTE50[c]	+	+	+	−	−

[a] The proteins in the table stub were fused to LBD or GBD as indicated, and the proteins in the subheads were fused to the GAD. GBD-GAD combinations were expressed in HF7c, and LBD-GAD combinations were expressed in L40.

[b] At least 16 transformants were tested for the determination of β-galactosidase activity. + and −, transformants that did and did not express detectable levels of β-galactosidase activity, respectively; NT, not tested.

[c] LEXSTE50 gives higher-than-background levels if filter assays extend beyond a 1-h incubation.

Further evidence for this model was obtained with the identification of a mutant of Byr2 that is defective in binding Ste4. The two-hybrid system was utilized to identify mutants of Byr2ΔC that separate its Ras1-binding ability from its Ste4-binding ability. The *byr2ΔC* regulatory domain was randomly mutagenized using the polymerase chain reaction, exploiting the inherent error rate of *Taq* polymerase (34), and fused to the LBD. This resulting library was screened both with a GADras1(C216R) fusion and with a GADste4 fusion to identify clones that failed to interact with Ste4 but retained the ability to interact with Ras1(C216R). One such mutant, Byr2ΔC(N28I), failed to interact with Ste4 but retained an undiminished ability to bind to Ras1 and was studied further.

To determine the biological activity of Byr2(N28I), the mutant Byr2ΔC(N28I) was rejoined to an intact catalytic domain, expressed from pART1 (see Materials and Methods). Then, we compared the ability of Byr2(wt) and Byr2(N28I) to rescue *byr2*^{null}, *ras1*^{null}, and *ste4*^{null} defects. Byr2(wt) efficiently suppressed the inability of *byr2*^{null} mutants to conjugate, while Byr2(N28I) was at least 50-fold less effective, consistent with the idea that interaction with Ste4 is essential for wild-type activity. In keeping with this, both Byr2(wt) and Byr2(N28I) were only weakly able to suppress the conjugation and sporulation defects of *ste4*^{null} strains (Table 4). In the absence of *ste4*, Byr2 and the mutant protein that fails to bind Ste4 are essentially equivalent. Finally, while the wild-type Byr2 can effectively suppress the sporulation defects of the *ras1*^{null} strain, Byr2(N28I) cannot, indicating that Byr2 needs input from both Ras1 and Ste4 (Table 4).

Interactions of STE50 with components of the *S. cerevisiae* pheromone-responsive MAPK cascade. Amino acids 267 to 327 of STE50 (25) exhibit limited similarity to residues 202 to 262 of Ste4. The *S. cerevisiae* gene, *STE50*, was cloned through the genome project and has been suggested to be required for activation of conjugation at an early step in mating. The interactions of STE50 with components of the *S. cerevisiae* pheromone-responsive MAPK module and Byr2 were therefore examined. As shown in Table 5, STE50 can complex with STE11 but not with Byr2. Conversely, Ste4 does not bind to STE11. STE50 interacts weakly with STE5 (Table 5), another component of the *S. cerevisiae* mating pheromone pathway, but did not interact with STE20, STE7, FUS3, or GPA1 (Table 5),

other critical components of this pathway. STE50 bound to the regulatory (STE11ΔC, encoding amino acids 1 to 415), but not catalytic (STE11ΔN, encoding residues 416 to 717) domain of STE11 (Table 5). And, like Ste4, STE50 is capable of homotypic interaction. It is remarkable that the region of Ste4 that complexes with itself and Byr2 shows no significant homology to STE50. Similarly, the regulatory domains of STE11 and Byr2 are structurally diverged. Nonetheless, STE50 possesses analogous binding abilities: it is able to undergo homotypic interaction and to form a complex with the regulatory domain of STE11. These results suggest that STE50 could be a functional homolog of Ste4 and could regulate STE11 but does not share the precise molecular mechanisms of Ste4. The existence of homologous domains of Ste4 and STE50 might point to the binding of these to an as yet unidentified component of mating pheromone-MAPK pathways.

DISCUSSION

Signal transduction is the first step in the process by which living organisms respond to their environment. The great variety of signal transduction pathways in eukaryotes are created by combinations of a smaller set of evolutionarily conserved components. One conserved motif found throughout evolution is the MAPK module, exemplified in pheromone signaling in yeasts and hormone signaling in metazoans (2, 4, 5, 9, 13, 22). We have chosen to study the MAPK module of *S. pombe* because it mediates sexual differentiation and is induced by the combination of starvation and pheromones. In particular, it has been our expectation that at least one molecule receives multiple molecular signals that represent these physiological conditions.

S. pombe Byr2 is a good candidate to be such a molecule. It is the most upstream element of its MAPK module (22), and we have now shown that it interacts directly with two potential regulatory molecules, first, Ras1, and second, Ste4. Genetics indicate that both Ras1 and Ste4 act upstream and through Byr2 and that they bind at separable sites to the Byr2 regulatory region. Moreover, Ste4 and Ras1 each appear to be able to activate Byr2 in partial independence of the other.

Both Ras1 and Ste4 are candidates for mediating starvation. In particular, the synthesis of Ste4 is known to be nutritionally regulated, and it is expressed only under conditions of starvation (24). Ras1 expression is constitutive, but Ste6, a Ras1 exchange factor, like Ste4, is expressed only upon starvation and is required for conjugation. However, either or both Ste4 and Ras1 could mediate some other aspects of regulation, and in fact the mechanism of action of the pheromone receptor and its coupled G protein upon Byr2 is still unknown. A third influence upon Byr2 could be feedback regulation, by which the cell avoids hypersensitivity to pheromones. Both Ras1 and Ste4 are also candidates for these regulatory roles.

Ste4 shows weak homology to *S. cerevisiae* STE50. STE50, like Ste4, binds to the most upstream element of the pheromone-responsive MAPK module, and again like Ste4, is capable of homotypic interaction. Paradoxically, the homologous region between Ste4 and STE50 does not include the domains of Ste4 needed for physical interaction with Byr2 or homotypic binding. This suggests that some yet-unidentified component common to pheromone signaling in budding and fission yeasts interacts with this homologous domain.

Our studies do not test any biochemical mechanisms by which Ras1 and Ste4 can activate Byr2. Many possibilities are readily envisioned. For example, Ras1 might induce the proper localization of Byr2 while Ste4, through its homotypic interactions, might induce the dimerization and catalytic *trans*-autoactivation of Byr2. Indeed, preliminary data suggest that dimerization of Byr2 increases its kinase activity (29a). However, a domain of Ste4 capable of dimerization and binding Byr2, but lacking the STE50 homology domain, does not have Ste4 function (Table 3). Nor have we been able to demonstrate using the two hybrid system that Ste4 can induce the dimerization of Byr2. Whatever the mechanism of Ste4 activation of Byr2, the existence of STE50 and its interactions with STE11 suggest the existence of a conserved mechanism for the activation of MAPK modules.

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