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## Multiple Regulatory Domains on the Byr2 Protein Kinase

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**Byr2 protein kinase, a homolog of mammalian mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEKK) and *Saccharomyces cerevisiae* STE11, is required for pheromone-induced sexual differentiation in the fission yeast *Schizosaccharomyces pombe*. Byr2 functions downstream of Ste4, Ras1, and the membrane-associated receptor-coupled heterotrimeric G-protein  $\alpha$  subunit, Gpa1. Byr2 has a distinctive N-terminal kinase regulatory domain and a characteristic C-terminal kinase catalytic domain. Ste4 and Ras1 interact with the regulatory domain of Byr2 directly. Here, we define the domains of Byr2 that bind Ste4 and Ras1 and show that the Byr2 regulatory domain binds to the catalytic domain in the two-hybrid system. Using Byr2 mutants, we demonstrate that these direct physical interactions are all required for proper signaling. In particular, the physical association between Byr2 regulatory and catalytic domains appears to result in autoinhibition, the loss of which results in kinase activation. Furthermore, we provide evidence that Shk1, the *S. pombe* homolog of the STE20 protein kinase, can directly antagonize the Byr2 intramolecular interaction, possibly by phosphorylating Byr2.**

The mitogen-activated protein kinase (MAPK) module is an important and highly conserved element in eukaryotes. Composed of a series of protein kinases that can phosphorylate one another sequentially, the module is thought to transmit signals from the point of origin, typically the cell membrane, to the nucleus (2, 4, 5, 11, 15, 31). A basic MAPK module consists of three kinases: a MAPK, a MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK), and a MEK kinase (MEKK). In responding to plasma membrane-originating signals, MEKK phosphorylates and activates MEK, which in turn phosphorylates and activates MAPK/ERK (27). As a result, targets including transcription factors are phosphorylated (8, 10). This module has been found in various signaling pathways ranging from the pheromone-induced sexual differentiation in yeast to the induction of cell proliferation and differentiation in mammalian cells. In the budding yeast *Saccharomyces cerevisiae*, the MAPK module controlling pheromone-induced sexual differentiation consists of STE11, STE7, and the pair of FUS3 and KSS1 kinases. In the fission yeast *Schizosaccharomyces pombe*, the triad composed of Byr2, Byr1, and Spk1 is similarly involved in sexual differentiation (31). Mammalian cells have at least two prominent kinase cascades: MEKK, JNKK, and JNK (29, 42) and RAF, MEK, and MAPK (7, 18, 22). RAF is itself a target for Ras proteins (37, 39) and may be under very complex regulation.

Sexual differentiation in *S. pombe* is under the influence of both sex pheromones and starvation, and Byr2, like RAF, appears to be a target for regulation by Ras (38). Given the amenability of *S. pombe* for genetic analysis, Byr2 is thus an excellent candidate for the study of complex regulation. In addition to being regulated by Ras1, Byr2 also appears to be regulated by Ste4 and Gpa1. Ste4 has a leucine zipper capable of homotypic interaction and another domain capable of binding Byr2 (3). Gpa1 is the  $\alpha$  subunit of the membrane-associated pheromone receptor-coupled heterotrimeric G-protein

(32). Gpa1 is thought to be activated upon the binding of pheromones to their receptors. Ras1 and Gpa1 cooperate in regulating the sexual differentiation pathway (41), and Ste4 and Ras1 appear to activate Byr2 at least partially independently (3), but the relationship between Ste4 and Gpa1 has not been clarified.

We have been utilizing the two-hybrid system of Fields and Song to probe the mechanisms for Byr2 activation (13). Ras1 has been found to bind Byr2 in the two-hybrid system (37). Masuda et al. have demonstrated this interaction in vitro (28). Ste4, isolated as a Byr2-binding protein from a two-hybrid interaction screen, has also been shown to bind Byr2 in vitro (3). A two-hybrid bridging experiment has demonstrated that the regulatory domain of Byr2 can bind Ste4 and Ras1 simultaneously (3). We have now discovered that the Byr2 kinase catalytic domain can also bind to the regulatory domain of Byr2. We have determined the minimum binding domain for each of these interactions by characterizing the binding profile of a series of Byr2 deletion mutants, and point mutants that are defective only in the ability to bind either Ste4, Ras1, or the kinase catalytic domain have been isolated. Analysis of these mutants shows that direct interactions between the Byr2 regulatory domain and Ste4 and Ras1 are required for the proper activity of Byr2. The interaction between the Byr2 regulatory domain and the kinase catalytic domain appears to be autoinhibitory, since the loss of this interaction results in enhanced activity. With the two-hybrid system, we are able to show that Shk1, the *S. pombe* homolog of *S. cerevisiae* STE20, a kinase thought to be upstream of the sexual differentiation MAPK module in that organism (24), can antagonize the interaction of the regulatory and catalytic domains, possibly by phosphorylating Byr2, with a resultant activation of the kinase. From these data we have proposed a model for the distributive regulation of Byr2 by Ras1, Ste4, and Shk1.

### MATERIALS AND METHODS

**Yeast, 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* HF7C, L40, and YCJ4 were used as reporter strains for two-hybrid interaction experiments. HF7C is a *GAL4*-based two-hybrid reporter strain, and L40 is *lexA* based. Constructed and kindly provided by Tim Durfee and Carla Inouye at the University of California, Berkeley, YCJ4 is a dual reporter strain that has both *GAL4*-based

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TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Source or reference
<i>S. cerevisiae</i>		
HF7C	<i>MATa ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)<sub>3</sub>-CYC-lacZ</i>	H. Feilotter
L40	<i>MATa ade2 his3 leu2 trp1 LYS::lexA-HIS3 URA3::lexA-lacZ</i>	A. Vojtek
YCJ4	<i>MATa ura3-52 his3-200 leu2-3,-112 trp1-901 Can<sup>r</sup> gal4-542 gal80-538 ade2::GAL1-URA3 lys2::lexA<sub>op</sub>-lacZ</i>	T. Durfee
<i>S. pombe</i>		
SP870	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18</i> haploid	D. Beach
SP4A	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 ste4::ADE2</i> haploid	Barr et al. (3)
SP4AD	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 ste4::ADE2</i> diploid	Barr et al. (3)
SPRUD	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 ras1::ura4</i> diploid	Wang et al. (38)
SPSU	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 byr2::ura4</i> haploid	Wang et al. (38)
SPSUD	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 byr2::ura4</i> diploid	Wang et al. (38)
SPGLD	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 gpa1::LEU2</i> diploid	Neiman et al. (31)
SPBUD	<i>h<sup>90</sup> leu1-32 ade6-210 ura4-D18 byr1::ura4</i> diploid	Wang et al. (38)

and *lexA*-based two-hybrid reporter constructs (19). All *S. pombe* strains used in this study were derived from the wild-type SP870 strain, and they have been used in our previous studies.

*S. cerevisiae* cultures were grown in YPD (2% peptone, 1% yeast extract, 2% glucose) or in dropout (DO) synthetic minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) with appropriate auxotrophic supplements. *S. pombe* strains were grown in YEA (0.5% yeast extract, 0.0075% adenine, 3% glucose) or in synthetic medium PM (0.3% phallic acid, 0.018% sodium phosphate, 0.5% ammonia chloride, 2% glucose, and PM vitamins, minerals, and salts) with appropriate auxotrophic supplements. Standard lithium acetate transformation protocols were used to introduce plasmids into both *S. cerevisiae* and *S. pombe* cells (20).

**Detection of protein complex formation by the two-hybrid system.** To determine if GAD-Byr2-Reg (Byr2 regulatory domain) interacts with LBD-Byr2-Cat (Byr2 kinase catalytic domain) in the two-hybrid system, the two fusions were coexpressed in two-hybrid reporter strain L40. Cells containing these two plasmids were patched on synthetic medium lacking leucine and tryptophan (DO-Leu-Trp). The patches were then examined for histidine prototrophy as well as  $\beta$ -galactosidase synthesis, since two-hybrid interactions result in transactivation of *GAL1-HIS3* and *lexA-lacZ*. Histidine prototrophy was tested by replicating patches onto DO-Leu-Trp-His plates and was evident by growth on the His<sup>-</sup> plate.  $\beta$ -Galactosidase filter assays were conducted as previously described (37). Furthermore, in the  $\beta$ -galactosidase liquid assay, cell lysates were prepared in Z buffer, and  $\beta$ -galactosidase activities in these lysates were determined colorimetrically by using *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) (17).

**Generation of Byr2-Reg deletion mutants by PCR.** PCR (30) was performed to generate Byr2-Reg deletion mutants. The primers used in the PCR were the following: 5'-CGGAATCTATGGAATATTATACCTCG-3', the 5' oligonucleotide used in PCR to synthesize fragments starting at amino acid residue 1; 5'-GGAATCTTTAACCTTGCCTTACTG-3', the 5' oligonucleotide used to synthesize fragments starting at amino acid residue 34; 5'-GGAATCCACC GCCAAAGGCAACAA-3', the 5' oligonucleotide used for fragments starting at amino acid residue 51; 5'-GGAATTCATGTATACTGAGGTTTATT-3', the 5' oligonucleotide used for fragments starting at amino acid residue 71; 5'-GG AATTCGCCTTGTCCATCGTTTGA-3', the 5' oligonucleotide used for fragments starting at amino acid residue 151; 5'-GGAATCTAATTCATCTT CACCGAA-3', the 5' oligonucleotide used for fragments starting at amino acid residue 134; 5'-GGAATTCATTGGCTCAACCGGAGCA-3', the 5' oligonucleotide used for fragments starting at amino acid residue 167; 5'-GGAATT CCGATCCTAAAACCTCAGTATACATGG-3', the 3' oligonucleotide used in PCR to synthesize fragments ending at amino acid residue 70; 5'-GGAATT CATCGGCGGAGGTCTTCAA-3', the 3' oligonucleotide used for fragments

ending at amino acid residue 160; 5'-GGAATTC AAGGAGAAAGGGAGG ACTG-3', the 3' oligonucleotide used for fragments ending at amino acid residue 180; 5'-GGAATTCATTTTCGCATTATTTGAGCG-3', the 3' oligonucleotide used for fragments ending at amino acid residue 200; 5'-GGAATTC AAAA ATCGGAAACTCTAGA-3', the 3' oligonucleotide used for fragments ending at amino acid residue 220; 5'-GGAATTCAGTTTCTACGAAGAGAGTT-3', the 3' oligonucleotide used for fragments ending at amino acid residue 240; 5'-GGAATTC AAAATCCTCAGAGAGGAAAAG-3', the 3' oligonucleotide used for fragments ending at amino acid residue 260; 5'-GGAATTC AAAATCCTCA GAGAGGAAAAG-3', the 3' oligonucleotide used for fragments ending at amino acid residue 280; 5'-GGAATTC AAAAGTGGAGGATCAAATCC-3', the 3' oligonucleotide used for fragments ending at amino acid residue 300; and 5'-GG AATTCACATGGTTATGGATTTTG-3', the 3' oligonucleotide used for fragments ending at amino acid residue 320. PCR fragments that encode Byr2 deletion mutants were excised with *EcoRI* to expose the *EcoRI* sites at both ends and were cloned in pGAD vector to generate GAD (*GAL4* transcription activation domain) fusions. Orientation of these clones was ascertained by restriction enzyme digestion using intrinsic sites such as *XhoI*.

**Construction of Byr2-Reg mutant library.** Byr2-Reg was randomly mutagenized by a standard PCR procedure (44) using primer pair 5'-CACCACTT TCCGGATCCTATGGAATATTA-3' (shown in boldface is the *BamHI* site, which is in frame with GAD) and 5'-TATCCATTTGGTCCGACTGATCATC AG-3' (shown in boldface is the *SalI* site, which is also in frame with GAD). pUC119-Byr2<sup>wt</sup> was used as a template for the PCR random mutagenesis. The PCR products were gel purified and ligated into pGAD vector to create in-frame fusions with GAD. The resulting pGAD-Byr2-Reg mutant library had a complexity of over 10<sup>6</sup> clones.

We also generated a Byr2 mutant library with only the N-terminal 196 amino acids, which encode Ste4 and Ras1 binding domains, mutagenized. To facilitate cloning, an *SfuI* site was silently introduced between amino acid residues 196 and 197 of the regulatory domain of Byr2. This was achieved by changing CGC TCA to CGT TCG by a PCR-based DNA manipulation technique. The N-terminal stretch of 196 amino acids was generated by PCR using primer pair 5'-CACC CACTTCCGGATCCTATGGAATATTA-3' and 5'-TTTCGCATTATTCGA ACGGACACTTCGTTT-3'. The stretch from amino acids 197 to 392 of Byr2 was generated by PCR using primer pair 5'-AAACGAAGTGTCCGTTCCGAA TAATCGCAA-3' and 5'-TATCCATTTGGTCCGACTGATCATCAG-3'. The N-terminal 196-amino-acid fragment was digested by *BamHI-SfuI*, and the other fragment encoding amino acids 197 to 392 was digested with *SfuI-SalI*. They were ligated together to form the entire Byr2 regulatory domain and cloned into pGAD and pLBD as a *BamHI-SalI* fragment. The end products were sequenced, and no undesirable alteration was found. With the creation of the *SfuI* site, the N-terminal 196 amino acids were then subjected to PCR random mutagenesis. The pool of mutants was excised with *BamHI* and *SfuI* and rejoined with the rest of Byr2-Reg to form the entire Byr2-Reg clones in pLBD. The complexity of the library was determined to be approximately 5 × 10<sup>5</sup> clones.

***S. pombe* expression constructs.** Full-length clones of Byr2 in pLBD were made by inserting *BamHI-SalI* fragments of Byr2-Reg into pLBD-Byr2-Cat, which contains a *SalI-SacI/PstI* Byr2-Cat (kinase catalytic domain) fragment. They were then excised as *BamHI-SacI* fragments and cloned immediately downstream of the *adh* promoter in *S. pombe* expression vectors pART1 and pAAU1. pART1, an *ARS LEU2*-based plasmid, was used to introduce Byr2 to SP4A, SP4AD, SPRUD, SPSU, and SPSUD. pAAU1, an *ARS ura4*-based plasmid, was used as an expression vehicle in SP4A, SP4AD, and SPGLD.

***S. pombe* sporulation assay.** *S. pombe* sporulation assays were conducted in the following fashion. Clones of Byr2 were transformed into yeast strains by the lithium acetate transformation protocol. Yeast transformants were selected in PM synthetic medium with appropriate auxotrophic supplements. After 4 or 5 days of incubation at 30°C, colonies were patched onto fresh PM plates, and after another 3 days of incubation at 30°C, the patches were examined microscopically and the number of asci in total cells was determined. At least 12 patches were tested for each plasmid and strain pair, and an average was taken.

**Generating plasmids to express Ste4 and Ras1 in two-hybrid interaction study.** pLS104, a 2 $\mu$ m *ADE2*-based plasmid, was used to express Ste4 and Ras1 in two-hybrid reporter strains. pLS104 was made and kindly provided by Siyuan Le at Cold Spring Harbor Laboratory. Ste4 and Ras1 were inserted immediately downstream of the *adh* promoter to generate pLS104-Ste4 and pLS104-Ras1. YCp405, a *LYS2*-based *S. cerevisiae* CEN plasmid (25), was used to introduce Ras1 along with Ste4. YCp405-Ras1 was made by cloning the *SphI* fragment from pLS104-Ras1 that contains the *adh* promoter-Ras1-*adh* terminator into YCp405.

**Cloning of a dominant activated Shk1 gene from an *S. pombe* cDNA library by PCR.** PCR was performed to isolate N-terminally truncated Shk1. An *S. pombe* cDNA library, kindly provided by Jim Hudson at Cold Spring Harbor Laboratory, was used as the source of template. Oligonucleotides 5'-GAGGATCC CATGGTTAAAACACTACTACCTTAATGCT-3' and 5'-AAGGATCCCTGC AGCTATTTACCAGAATGATGTATGGA-3' were used to isolate the C-terminal 385 amino acids of Shk1. To combine the amplification power of *Taq* DNA polymerase and the high fidelity of *Pfu* polymerase (Stratagene) in nucleotide incorporation, the PCR was performed in two steps. First, *Taq* DNA polymerase was used for 10 cycles of PCR. DNA was extracted from the reaction mixtures by using a Qia-quick PCR purification kit (Qiagen). The total DNA was then used

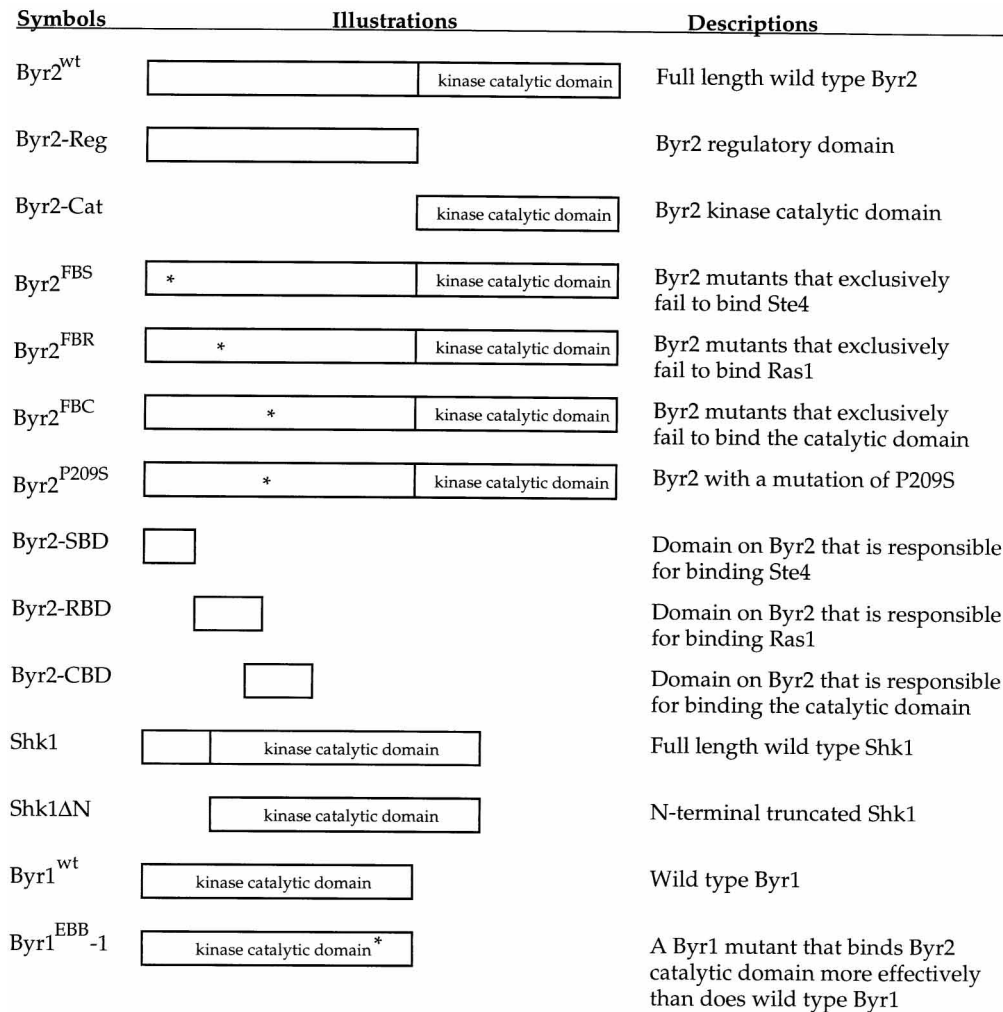


FIG. 1. Schematic illustration of Byr2, Shk1, and Byr1 wild-type proteins, domains, and mutants used in this study. Byr2-SBD, the Ste4 binding domain on Byr2, encodes amino acids 1 to 70; Byr2-RBD, the Ras1 binding domain, encodes amino acids 71 to 180; and Byr2-CBD, the catalytic binding domain, encodes amino acids 151 to 260. \*, point mutations. The Byr2<sup>FBS</sup> mutant tested has a N281 point mutation, and the Byr2<sup>FBR</sup> mutant has a K101E mutation. Shk1ΔN is the C-terminal 385 amino acids of the Shk1 protein.

as a template for 25 cycles of PCR with *Pfu* DNA polymerase. The reaction mixture was subjected to electrophoresis, and a 1.2-kb DNA fragment was excised with *Bam*HI and *Pst*I and cloned into pLS104. Forty-four clones were isolated. The first eight clones were sequenced, and all were found to encode the C-terminal 385 amino acids of *S. pombe* Shk1. The kinase-defective Shk1ΔN, Shk1<sup>K415,416R</sup>ΔN, was made with two adjacent critical lysine residues changed to arginine by PCR site-directed mutagenesis using *Pfu* DNA polymerase (16). The following four oligonucleotides were used: 5'-GAGGATCCCATGGTTAAAACTACTACCTCTAATGCT-3', 5'-AAGGATCCCTGCAGCTATTTACCAGATGATGTATGGA-3', 5'-TGGCTGTTGATTAATGTTTCATTCTCCTGATGCAACAGAAAGATTAGT-3', and 5'-ACTAATCCTTCTGTTGCCATCAGGAGAATGAACATTAATCAACAGCCA-3'. The end product was excised with *Bam*HI and *Pst*I and cloned into pLS104.

**Isolation of Byr1 mutants that bind the Byr2 kinase catalytic domain effectively.** Wild-type Byr1 binds very weakly with the Byr2 kinase catalytic domain in the two-hybrid system. To conduct a two-hybrid interaction study with Byr1, we needed a Byr1 mutant that binds the Byr2 kinase catalytic domain more effectively. Thus, we decided to screen for such a mutant with the two-hybrid system. A pool of Byr1 mutants was generated by PCR mutagenesis using our standard protocol. They were cloned into pGAD vector. The DNA of this mutant library was then cotransformed with pLBD-Byr2-Cat into strain L40. In order to select those that can bind pLBD-Byr2-Cat effectively, cells were plated onto DO-Leu-Trp-His. Colonies that grew in the absence of histidine were patched out and subjected to a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) filter assay. pGAD plasmids were recovered from the cells that were His<sup>+</sup> and LacZ<sup>+</sup>. The DNAs of these plasmids were amplified in *E. coli* and then transformed back into L40 individually with pLBD-Byr2-Cat or pLBD-Lamin to test the specificity

and reproducibility of the interaction. More than 10 independent clones were isolated in this way. One of them encoded Byr1<sup>EBB-1</sup> (EBB stands for effectively binding to Byr2) and was used in testing the kinase configuration model with the two-hybrid system.

## RESULTS

**Interaction between the regulatory and catalytic domains of Byr2 in the two-hybrid system.** There are many examples of protein kinases in which a regulatory domain binds to and inhibits the catalytic domain (35). To our knowledge, this has not yet been demonstrated for MEKKs. To test this hypothesis for Byr2, the two-hybrid system was used. The regulatory domain of Byr2, Byr2-Reg, was isolated by PCR and fused to GAD to generate GAD-Byr2-Reg (Fig. 1 illustrates the nomenclature used in this paper). Byr2-Cat, was isolated by PCR and fused to LBD (*lexA* DNA binding domain) to generate LBD-Byr2-Cat. pGAD-Byr2-Reg and pLBD-Byr2-Cat were cotransformed into the *S. cerevisiae* two-hybrid strain L40. GAD, LBD-Ras1, and LBD-Lamin were used as controls. Interactions between the GAD fusion and the LBD fusion result in histidine prototrophy and synthesis of β-galactosidase (see

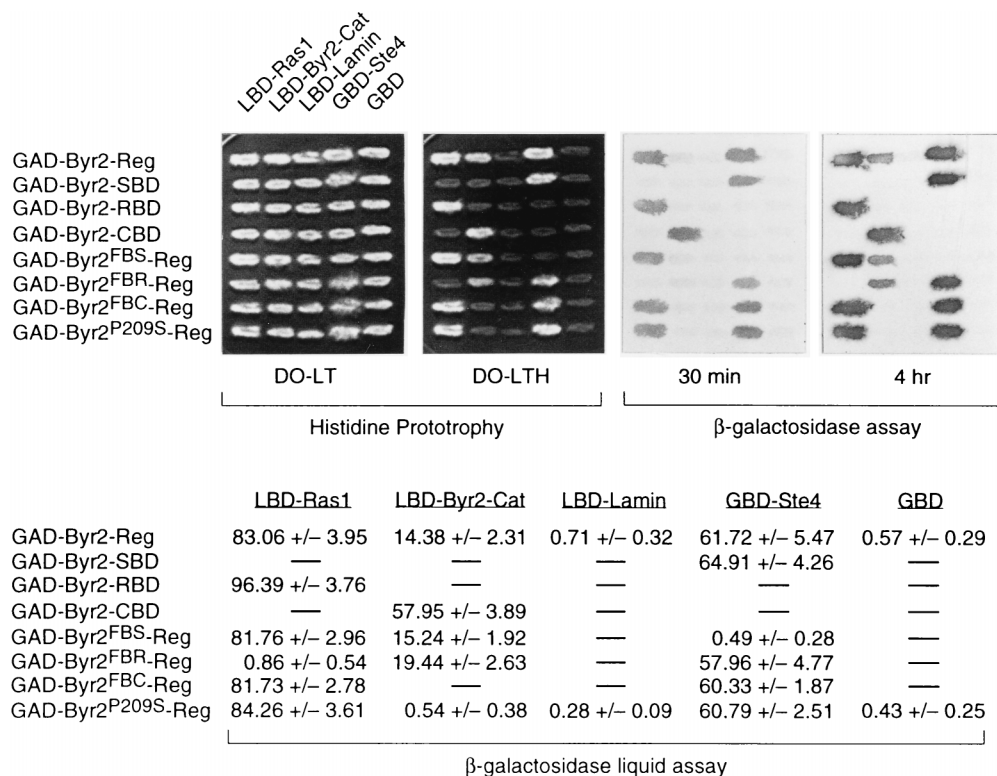


FIG. 2. Two-hybrid interaction profile of domains of Byr2 and mutants with alterations in these domains. GAD-Byr2-Reg, GAD-Byr2-SBD, GAD-Byr2-RBD, GAD-Byr2-CBD, GAD-Byr2<sup>FBS</sup>-Reg (a Byr2-Reg N281 mutant), GAD-Byr2<sup>FBR</sup>-Reg (a Byr2-Reg K101E mutant), GAD-Byr2<sup>FBC</sup>-Reg (a Byr2-Reg F221S mutant), and GAD-Byr2<sup>P209S</sup>-Reg were transformed individually with LBD-Ras1, LBD-Byr2-Cat, and LBD-Lamin in strain L40 and with GBD-Ste4 and GBD in strain HF7C. Transformants were tested for their ability to grow on media lacking histidine and assayed for  $\beta$ -galactosidase expression. Values shown are relative levels (means  $\pm$  standard deviations). DO-LT and DO-LTH are DO-Leu-Trp and DO-Leu-Trp-His, respectively.

Materials and Methods). The two-hybrid interaction results are shown in Fig. 2. Cells containing the pair pGAD-Byr2-Reg and pLBD-Ras1 or the pair pGAD-Byr2-Reg and pLBD-Byr2-Cat were able to grow on medium lacking histidine and tested positive for  $\beta$ -galactosidase activity. Cells with the pair pGAD-Byr2-Reg and pLBD-Ras1 grew better on media lacking histidine and synthesized more  $\beta$ -galactosidase, as indicated by X-Gal filter assay, than those with the pair pGAD-Byr2-Reg and pLBD-Byr2-Cat. Cells containing the pair pGAD-Byr2-Reg and pLBD-Lamin failed to grow on medium lacking histidine and tested negative for  $\beta$ -galactosidase activity; also, cells containing LBD-Ras1 or LBD-Byr2-Cat with GAD failed to grow in the absence of histidine and had no detectable  $\beta$ -galactosidase activity (data not shown in the figure). These controls demonstrated the specificity of the two-hybrid interaction between Byr2-Reg and Byr2-Cat. Thus, besides binding Ste4 and Ras1, the Byr2 regulatory domain also binds the Byr2 kinase catalytic domain in the two-hybrid system.

**Delimitation of three interaction domains.** To identify regions on the Byr2 regulatory domain that are responsible for each of the three interactions, a series of Byr2-Reg deletion mutants were generated systematically by PCR (see Materials and Methods) and were fused individually with GAD. GAD-Byr2-Reg is capable of binding LBD-Ras1 and LBD-Byr2-Cat in strain L40 and GBD-Ste4 (GBD stands for *GAL4* DNA binding domain) in another *S. cerevisiae* strain, HF7C. Therefore, the deletion mutants were tested for their ability to bind LBD-Ras1 and LBD-Byr2-Cat in L40 and GBD-Ste4 in HF7C. The shortest peptides that could bind partner proteins as effectively as could Byr2-Reg in the two-hybrid interaction assays

were considered the interaction domains. The data are summarized in Fig. 3, and a portion of the actual data is shown in Fig. 2. Clone BDM1 (Byr2 deletion mutant 1), encoding the N-terminal 70 amino acids of Byr2, was the shortest peptide that could bind Ste4 in the two-hybrid system. It failed to bind Ras1 or Byr2-Cat, and therefore it was considered to encode the Ste4 binding domain (Byr2-SBD). Clone BDM13, encoding a stretch of 110 amino acids from C71 to P180 of Byr2, was the shortest one that retained Ras1 binding ability in the two-hybrid system. This domain failed to bind Ste4 or Byr2-Cat and thus was designated the Ras binding domain (Byr2-RBD). Further truncations from either the N-terminal or the C-terminal boundary of clone BDM13 resulted in loss of Ras1 binding ability, as indicated by clones BDM12 and BDM16. Clone BDM21, encoding 110 amino acids of Byr2 ranging from P151 to L260, was the shortest one that could bind Byr2-Cat but not Ste4 or Ras1 in the two-hybrid system and thus was designated the catalytic binding domain (Byr2-CBD). Clone BDM21 in fact appears to bind the catalytic region better than the full regulatory region (Fig. 2). Although clone BDM21 may not be the exact minimum binding domain, it must be fairly close, since further trimming resulted in peptides that fail to bind Byr2-Cat, as indicated by clones BDM23 and BDM4. These results demonstrated that three distinctive binding domains on Byr2-Reg are responsible for binding Ste4, Ras1, and Byr2-Cat. The domain for Ste4 binding is completely separable from the domains responsible for binding Ras1 and Byr2-Cat, which overlap by 30 amino acids.

**Isolation of Byr2-Reg mutants that fail exclusively to bind Ste4, Ras1, or Byr2-Cat.** To enable us to evaluate the physio-

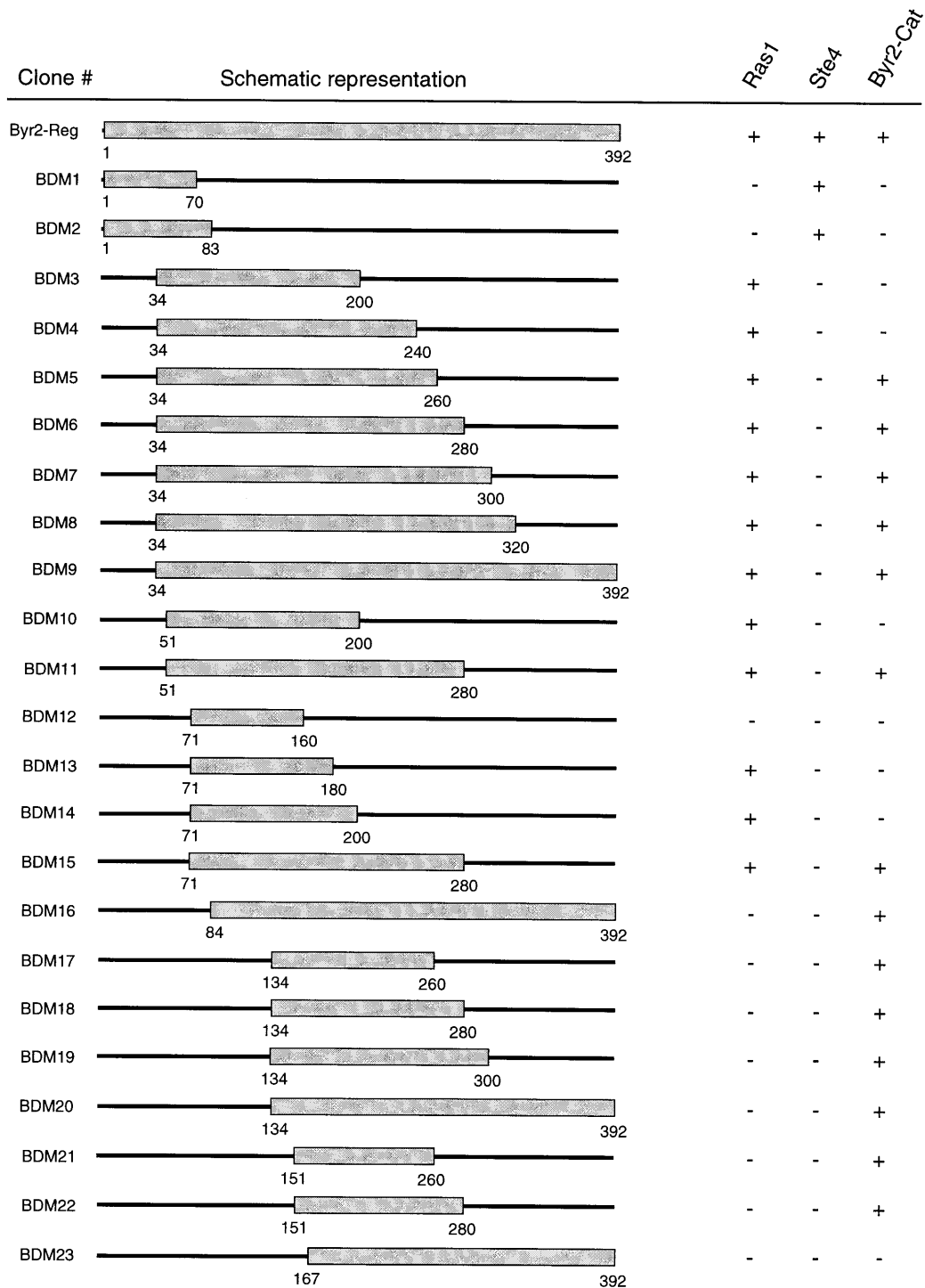


FIG. 3. Delimitation of domains on Byr2-Reg responsible for each of the three interactions. A series of Byr2 deletion mutants were made by PCR. They were assayed for two-hybrid interaction with Ste4, Ras1, and the kinase catalytic domain of Byr2. +, positive two-hybrid interaction that is at least as strong as that with Byr2-Reg; -, no detectable two-hybrid interaction. The peptide sequences expressed as GAD fusions are shown (gray rectangles), with amino acid positions indicated.

logical significance of the interaction between Ste4 and the Byr2 regulatory domain, we screened for Byr2 mutants that failed exclusively to bind Ste4 (Byr2<sup>FBS</sup>) so that we could examine them for their activities in comparison with the wild-type kinase. In this way, we could also independently confirm the location of the Ste4 binding domain. We mutagenized

Byr2-Reg by PCR random mutagenesis and generated a library of pLBD-Byr2-Reg mutants which was cotransformed with pGAD-Ste4 into *S. cerevisiae* L40. The cells were plated on DO-Leu-Trp to ensure that transformants had both GAD and LBD fusions. Transformants that failed to transactivate  $\beta$ -galactosidase were isolated. Plasmids encoding these mutants

TABLE 2. Sequence information for Byr2-Reg mutants that fail to bind Ste4 or Byr2-Cat

Mutant	Original amino acid	Residue no.	New amino acid
<b>Byr2<sup>FBS</sup> mutants</b>			
Byr2 <sup>FBS</sup> -1	L	35	P
	D	90	H
Byr2 <sup>FBS</sup> -2	G	47	R
	F	57	Y
Byr2 <sup>FBS</sup> -3	L	46	Q
	R	69	C
Byr2 <sup>FBS</sup> -4	L	35	P
Byr2 <sup>FBS</sup> -5	L	46	P
Byr2 <sup>FBS</sup> -6	L	40	S
Byr2 <sup>FBS</sup> -7	L	46	Q
Byr2 <sup>FBS</sup> -8	L	46	P
Byr2 <sup>FBS</sup> -9	N	28	S
	F	57	S
Byr2 <sup>FBS</sup> -10	N	28	I
<b>Byr2<sup>FBC</sup> mutants</b>			
Byr2 <sup>FBC</sup> -1	F	221	S
Byr2 <sup>FBC</sup> -2	I	229	P
Byr2 <sup>FBC</sup> -3	P	223	S
Byr2 <sup>FBC</sup> -4	L	213	P
Byr2 <sup>FBC</sup> -5	F	203	S
Byr2 <sup>FBC</sup> -6	F	221	S
Byr2 <sup>FBC</sup> -7	P	202	L
	I	214	T
Byr2 <sup>FBC</sup> -8	F	203	S
Byr2 <sup>FBC</sup> -9	L	213	P
Byr2 <sup>FBC</sup> -10	S	235	P
Byr2 <sup>FBC</sup> -11	I	214	M

were then cotransformed individually into L40 with pGAD-Ste4 or pGAD-Ras1. Ten Byr2-Reg mutants that were able to transactivate *lacZ* and *HIS3* reporter genes with Ras1 but not Ste4 were then recloned into pGAD vector and tested for two-hybrid interaction with pLBD-Byr2-Cat. They all could bind LBD-Byr2-Cat as effectively as could wild-type Byr2-Reg. DNA sequencing revealed that all 10 mutants have mutations between amino acids N28 and G47, which locate inside the Ste4 binding domain (Byr2-SBD). Some mutants, such as L35P (with a leucine-to-proline change at position 35), were isolated multiple times. For more information, see Table 2, and see Fig. 2 for representative data.

Similarly, to enable us to test if the physical association between Byr2-Reg and Byr2-Cat is physiologically relevant, we looked for Byr2-Reg mutants that fail to bind Byr2-Cat but are still able to bind Ste4 and Ras1 (Byr2<sup>FBC</sup> mutants). An *S. cerevisiae* two-hybrid strain with *GAL1-URA3* and *lexA-lacZ* dual reporters, YCJ4 (19), was used so that the interactions between Byr2-Reg and Ras1 and between Byr2-Reg and Byr2-Cat could be examined simultaneously in the same cells. YCJ4 was transformed with a pGAD-Byr2-Reg mutant library, pLBD-Byr2-Cat, and pHT3601-Ras1, which expresses a GBD-Ras1 fusion and an *ADE2* auxotrophic marker. Cells were plated on synthetic medium lacking leucine (for pGAD selection), tryptophan (for pLBD selection), adenine (for pHT3601 selection), and uracil (for transactivation of *GAL1-URA3* reporter construct, which results from the two-hybrid interaction between Byr2-Reg and Ras1). Transformants were tested for transactivation of the *lacZ* reporter gene, and those that tested negative were isolated. pGAD-Byr2-Reg mutants were recovered from these cells. They were then reexamined in strain L40 with pLBD-Ras1 and pLBD-Byr2-Cat and in strain HF7C with pGBD-Ste4 for Ste4 interaction. Results obtained for L40 and

HF7C were completely consistent with those for YCJ4. This demonstrated that the dual-reporter strain is functional. Eleven mutants consistently failed to bind Byr2-Cat but retained Ras1 and Ste4 binding abilities at normal levels. DNA sequencing revealed that all mutants have mutations from P202 to S235, which reside within the domain that is responsible for binding the kinase catalytic domain (Byr2-CBD). Mutations such as F221S were also encountered multiple times. Additional information and representative data are shown in Table 2 and Fig. 2.

To enable us to characterize the functions that are dependent on the physical interaction between Ras1 and Byr2, we searched for Byr2 mutants that fail to bind Ras1 specifically (Byr2<sup>FBR</sup> mutants). The DNA encoding the first 196 amino acids of Byr2, which are responsible for Ste4 and Ras1 binding, was randomly mutagenized by PCR. The mutant pool was fused to the rest of Byr2 to generate a full-length Byr2 regulatory domain in pLBD (see Materials and Methods). The DNA of the mutant library was transformed into L40 with pGAD-Ras1. More than 10,000 colonies were subjected to  $\beta$ -galactosidase filter assay, and 150 potentially interesting mutants were isolated. These pLBD-Byr2-Reg mutants were tested again with pGAD-Ras1 and pGAD-Ste4, and five mutants that failed to bind pGAD-Ras1 completely but were able to bind Ste4 as efficiently as was wild-type Byr2-Reg were isolated. These five Byr2-Reg mutants were cloned in the pGAD vector and were tested for two-hybrid binding with LBD-Byr2-Cat. Only one of these five mutants was also able to bind Byr2-Cat (Fig. 2). This result is consistent with an overlap in the Ras1 and Byr2-Cat binding domains. DNA sequencing revealed that this mutant contains a point mutation, K101E, which localizes within the Byr2 Ras1 binding domain.

These mutants confirm the results of our deletion mapping of the Byr2 interaction domains and give us reagents with which we can explore the physiological significance of these interactions.

**Two-hybrid interaction profile of an activated Byr2 mutant, Byr2<sup>P209S</sup>.** An activated Byr2 mutant, Byr2<sup>P209S</sup>, was investigated to see if its two-hybrid interaction profile is different from that of the wild-type kinase. Byr2<sup>P209S</sup>, which has a point mutation resulting in a serine substitution for proline on residue 209 within Byr2-CBD, was made by site-directed mutagenesis in a previous study (31). The P209S mutation is structurally equivalent to an activating P279S mutation of STE11 of *S. cerevisiae* (36), because the six amino acids flanking the proline are shared by Byr2 and STE11 even though the rest of the kinase regulatory domains of these two genes show little homology. Neiman et al. have shown that overexpression of Byr2<sup>P209S</sup> could rescue the sporulation defect of *gpa1<sup>null</sup>* cells more effectively than could the overexpression of wild-type Byr2 (31).

The regulatory domain of Byr2<sup>P209S</sup>, Byr2<sup>P209S</sup>-Reg, was isolated by PCR and was cloned into pGAD and pLBD vectors. pGAD-Byr2<sup>P209S</sup>-Reg was cotransformed into L40 with pLBD-Ras1, pLBD-Byr2-Cat, and pLBD-Lamin individually. Transformants were then tested for the transactivation of *lacZ* and *HIS3* reporter genes. pGAD-Byr2-Reg was tested in parallel for comparison. Byr2<sup>P209S</sup>-Reg was able to bind Ras1 as efficiently as was wild-type Byr2-Reg (Fig. 2). However, it completely failed to bind Byr2-Cat. Byr2<sup>P209S</sup>-Reg was also tested in *S. cerevisiae* HF7C with Ste4 and was found to be able to bind Ste4 as well as did wild-type Byr2-Reg. Thus, the P209S mutation appears to abolish specifically the interaction between Byr2-Reg and Byr2-Cat. This finding suggests a link between Byr2 intramolecular interaction and kinase autoregulation.

TABLE 3. Suppression of mating and/or sporulation defects in *ste4<sup>null</sup>*, *ras1<sup>null</sup>*, *byr2<sup>null</sup>*, and *gpa1<sup>null</sup>* haploid or diploid cells by overexpression of Byr2 mutants<sup>a</sup>

Mutant	% Asci in total cells					
	<i>ste4<sup>null</sup></i>		<i>ras1<sup>null</sup></i> diploid	<i>byr2<sup>null</sup></i>		<i>gpa1<sup>null</sup></i> diploid
	Haploid	Diploid		Haploid	Diploid	
None (vector alone)	<0.01	<0.01	1.5 ± 0.3	<0.01	<0.01	<0.01
Byr2 <sup>wt</sup>	0.5 ± 0.3	4.8 ± 0.8	33.8 ± 2.5	54.2 ± 7.2	75.0 ± 3.5	0.4 ± 0.2
Byr2 <sup>P209S</sup>	14.3 ± 4.3	22.3 ± 6.1	35.0 ± 3.5	61.7 ± 2.9	78.3 ± 2.9	21.4 ± 4.8
Byr2 <sup>FBC-1</sup>	13.0 ± 4.2	30.0 ± 3.5	ND <sup>b</sup>	ND	ND	23.5 ± 4.8
Byr2 <sup>FBC-2</sup>	ND	ND	ND	ND	ND	24.1 ± 3.3
Byr2 <sup>FBC-3</sup>	ND	ND	ND	ND	ND	24.1 ± 4.3
Byr2 <sup>FBC-4</sup>	ND	ND	ND	ND	ND	23.1 ± 2.9
Byr2 <sup>FBS</sup>	0.3 ± 0.1	2.4 ± 1.6	3.2 ± 0.6	1.6 ± 0.9	1.3 ± 0.6	ND
Byr2 <sup>FBR</sup>	<0.01	0.4 ± 0.3	33.7 ± 2.9	1.1 ± 0.5	1.6 ± 0.6	ND
Byr2 <sup>FBS-FBR</sup>	<0.01	0.3 ± 0.2	ND	<0.01	<0.01	ND
Byr2 <sup>FBS-P209S</sup>	14.8 ± 0.4	27.2 ± 2.2	ND	64.0 ± 4.2	82.0 ± 5.7	ND

<sup>a</sup> *ste4<sup>null</sup>*, *ras1<sup>null</sup>*, and *byr2<sup>null</sup>* haploid and/or diploid cells were transformed with pART1-Byr2<sup>wt</sup>, pART1-Byr2<sup>P209S</sup>, pART1-Byr2<sup>FBC-1</sup>, pART1-Byr2<sup>FBS</sup>, pART1-Byr2<sup>FBR</sup>, pART1-Byr2<sup>FBS-FBR</sup>, pART1-Byr2<sup>FBS-P209S</sup>, or pART1 vector alone. *gpa1<sup>null</sup>* diploid cells were transformed with pAAU1-Byr2<sup>wt</sup>, pAAU1-Byr2<sup>P209S</sup>, four different pAAU1-Byr2<sup>FBC</sup> mutants, or pAAU1 vector alone. Transformants were selected in synthetic medium with appropriate auxotrophic supplements on plates. After 4 or 5 days of incubation at 30°C, colonies were patched onto fresh plates. After another 3 days of incubation at 30°C, when the cells start to undergo nitrogen starvation, the patches were examined microscopically and the percentages of asci in the total cell populations were determined. At least 10<sup>4</sup> cells were examined for each entry in the table. The percentages shown in the table are averages from at least 10 colonial patches.

<sup>b</sup> ND, not determined.

**Genetic analysis of domain mutants.** Upon nitrogen starvation, *S. pombe* haploid cells conjugate with cells of the opposite mating type and form diploid cells. If nitrogen starvation persists, diploid cells typically undergo meiosis followed immediately by sporulation, in which four spores (haploid cells) are produced, forming a zygotic ascus. We monitor the production of zygotic spores to assay the ability of haploid cells to conjugate.

If nitrogen sources are introduced immediately after conjugation, newly formed diploid cells can be propagated asexually without entering meiosis. When starved for nitrogen, these diploid cells are induced to enter meiosis and sporulate, a process that requires the same components of the sexual differentiation pathway as conjugation (reviewed in reference 9). In this case, azygotic spores form. We can thus assay the functionality of the sexual differentiation signaling pathway by depriving diploid cells of nitrogen sources and monitoring azygotic sporulation.

In both cases, whether sexual functions are monitored in haploid or diploid cells, the ability of the cells to sporulate is measured by determining the percentage of sporulated cells. Sporulation of diploid cells is a less stringent test for the integrity of the pathway than conjugation and subsequent sporulation.

*S. pombe* cells that lose the function of Byr2, Ste4, Ras1, or Gpa1 are defective in mating and sporulation. Wild-type Byr2 acts, to various extents, as a high-copy-number suppressor of these defects. By comparing Byr2 mutants with wild-type Byr2 as high-copy-number suppressors, we can begin to analyze the physiological significance of each interaction involving Byr2. As a starting point, haploid cells deficient in Byr2, Ste4, Ras1, or Gpa1 fail to mate (less than 0.01%; see Materials and Methods for a description of the sporulation assay). Introduction of a high-copy-number plasmid expressing the wild-type Byr2 gene yields 60% zygotic spores (percentage of total cells) in *byr2<sup>null</sup>* cells and 0.5% in *ste4<sup>null</sup>* cells but none in *ras1<sup>null</sup>* or *gpa1<sup>null</sup>* cells (Table 3). Diploid cells deficient in Byr2, Ste4, or Gpa1 are unable to sporulate, and those deficient in Ras1 can sporulate only at a level of about 1.5%. Overexpression of the wild-type Byr2 gene dramatically increases sporulation effi-

ciency in these cells: 80% in *byr2<sup>null</sup>* diploid cells, 5% in *ste4<sup>null</sup>* diploid cells, 35% in *ras1<sup>null</sup>* diploid cells, and 0.5% in *gpa1<sup>null</sup>* diploid cells.

One Byr2<sup>FBS</sup> (fails to bind Ste4) mutant, which contains a N28I point mutation, was tested and compared with wild-type Byr2 for its ability to act as a high-copy-number suppressor for sporulation defects. It was transformed into *byr2<sup>null</sup>* haploid cells and diploid cells, *ste4<sup>null</sup>* haploid cells and diploid cells, and *ras1<sup>null</sup>* diploid cells. The cells were plated on synthetic medium with appropriate auxotrophic supplements. After 4 or 5 days of incubation at 30°C, transformants were patched onto fresh plates. After an additional 3 days of incubation, the patches were examined microscopically for the percentage of asci. The averages from more than 10 patches are presented in Table 3. In *byr2<sup>null</sup>* haploid cells and diploid cells, Byr2<sup>FBS</sup> yielded about 1 to 2% asci, which is 50-fold less than the value for wild-type Byr2. In *ste4<sup>null</sup>* haploid cells and diploid cells, Byr2<sup>FBS</sup> was comparable to wild-type Byr2. It yielded about 0.2% zygotic asci in *ste4<sup>null</sup>* haploid cells and about 2% azygotic asci in *ste4<sup>null</sup>* diploid cells. In *ras1<sup>null</sup>* diploid cells, however, it yielded only about 3.5% azygotic asci, about 10-fold less than the value for wild-type Byr2. The diminished ability of Byr2<sup>FBS</sup> to complement these mutants indicates that the interaction between Byr2 and Ste4 is required for normal function. This interaction is irrelevant when Ste4 itself is missing, indicating that the Byr2<sup>FBS</sup> mutant has no other defect besides the failure to bind Ste4.

Byr2<sup>FBR</sup>, which contains a K101E point mutation and fails to bind Ras1, was tested similarly. It was expressed in *byr2<sup>null</sup>* haploid cells and diploid cells, *ste4<sup>null</sup>* haploid cells and diploid cells, and *ras1<sup>null</sup>* diploid cells. Like Byr2<sup>FBS</sup>, Byr2<sup>FBR</sup> yielded only 1 to 2% asci in *byr2<sup>null</sup>* cells. However, it failed to yield any spores in *ste4<sup>null</sup>* cells, whereas it behaved similarly to wild-type Byr2 in *ras1<sup>null</sup>* cells, yielding about 35% asci. These results suggest that the interaction between Byr2 and Ras1 is necessary for the proper function of Byr2 and that Byr2<sup>FBR</sup> is defective only in this interaction.

Byr2<sup>P209S</sup> and four Byr2<sup>FBC</sup> mutants, containing F221S, I229P, P223S, and P213S mutations and failing to bind the catalytic domain, were tested in similar fashion. They were



transformed into *byr2*<sup>null</sup> haploid cells and diploid cells, *ste4*<sup>null</sup> haploid cells and diploid cells, *ras1*<sup>null</sup> diploid cells, and *gpa1*<sup>null</sup> diploid cells. *Byr2*<sup>P209S</sup> behaved indistinguishably from the *Byr2*<sup>FBC</sup> mutants, and they all were superior to wild-type *Byr2* as high-copy-number suppressors in *ste4*<sup>null</sup> and *gpa1*<sup>null</sup> cells. They yielded about 15% zygotic asci and about 20% azygotic asci in *ste4*<sup>null</sup> haploid cells and diploid cells, respectively, which is 4- to 30-fold higher than the values for wild-type *Byr2*. They yielded about 20% azygotic asci in *gpa1*<sup>null</sup> diploid cells, 40-fold higher than the value for wild-type *Byr2*. *Byr2*<sup>FBC</sup> mutants behaved similarly to wild-type *Byr2* in complementing the sexual defects of *byr2*<sup>null</sup> and *ras1*<sup>null</sup> cells. These data suggest that *Byr2*<sup>P209S</sup> is indeed in the same group as *Byr2*<sup>FBC</sup> and that the abrogation of the interaction between the *Byr2* regulatory and kinase domains appears to result in *Byr2* activation.

Two double mutants, *Byr2*<sup>FBS-FBR</sup>, which contains both N28I and K101E mutations, and *Byr2*<sup>FBS-P209S</sup>, which contains both N28I and P209S mutations, were tested similarly in the sporulation assay. They were made by joining together *Byr2* fragments containing each mutation. They were expressed in *byr2*<sup>null</sup> haploid cells and diploid cells, *ste4*<sup>null</sup> haploid cells and diploid cells, and *ras1*<sup>null</sup> diploid cells. The *Byr2*<sup>FBS-FBR</sup> mutant was a very poor suppressor for all strains tested. It failed to effectively rescue the sexual defects of *byr2*<sup>null</sup>, *ste4*<sup>null</sup>, or *ras1*<sup>null</sup> cells. This result confirms the notion that both Ste4 binding and Ras1 binding are required for proper *Byr2* function. The *Byr2*<sup>FBS-P209S</sup> mutant, however, behaved like the *Byr2*<sup>P209S</sup> single mutant in the sporulation assay. It yielded about 60 to 80% asci in *byr2*<sup>null</sup> cells, 15 to 25% asci in *ste4*<sup>null</sup> cells, and 35% asci in *ras1*<sup>null</sup> cells. This result confirms that *Byr2*<sup>P209S</sup> is more active than wild-type *Byr2*. It also further confirms that the release of *Byr2* autoinhibition bypasses the requirement for Ste4 interaction under these conditions.

To ensure that the increased activity of *Byr2*<sup>P209S</sup> which we observed is due to its effects on the MAPK pathway, we tested whether *Byr2*<sup>P209S</sup> could bypass *Byr1*, which functions downstream of *Byr2*. Overexpression of wild-type *Byr2* cannot rescue the sporulation defect of *byr1*<sup>null</sup> cells (38). pART1CM, pART1CM-*Byr2*, pART1CM-*Byr2*<sup>P209S</sup>, and pART1CM-*Byr1* were transformed into SPBUD, an *S. pombe* *byr1*<sup>null</sup> diploid strain. Transformants were tested in the sporulation assay. As expected, cells expressing *Byr1* yielded about 50% spores, while those expressing vector alone, wild-type *Byr2*, or *Byr2*<sup>P209S</sup> had no spores. Moreover, to ensure that the increased activity of *Byr2*<sup>P209S</sup> was the result of its altered regulation and not the result of a change in protein stability, we checked the protein expression level of *Byr2*<sup>P209S</sup> in comparison with that of wild-type *Byr2*. 9E10 epitope-tagged *Byr2*, *Byr2*<sup>P209S</sup>, and *Byr1* were expressed in SPBUD. Transformants were grown in PM medium, and cells were harvested when the culture became turbid. Equal amounts of cells were taken and were extracted in 0.5% sodium dodecyl sulfate (SDS) by vortexing with glass beads. The cell extracts were boiled with SDS sample buffer, cleared, and subjected to SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining and Western blot analysis with 9E10 monoclonal antibodies (data not shown). Bands at 80 kDa were found specifically in the lanes from cells expressing 9E10-*Byr2* or 9E10-*Byr2*<sup>P209S</sup>. The intensities of the bands were indistinguishable. This experiment rules out the possibility that the P209S mutation activates *Byr2* by enhancing the stability of the protein.

**Two-hybrid assay for an open configuration of *Byr2*.** From our studies of the isolated *Byr2* regulatory and kinase domains, we concluded that they interact in the two-hybrid system and that point mutations in the regulatory domain, such as P209S, disrupt this interaction. Thus, we suspected that wild-type *Byr2*

might exist in a closed configuration, whereas the *Byr2*<sup>P209S</sup> and *Byr2*<sup>FBC</sup> mutants might be in an open configuration, with the kinase catalytic domain released. Since the *Byr2* kinase domain can be recognized by both the *Byr2* regulatory domain and *Byr2*-CBD in the two-hybrid system, it is possible to test this prediction. GAD-Ste4, GAD-Ras1, GAD-*Byr2*-Reg, GAD-*Byr2*-CBD, or GAD was expressed in L40 with LBD-*Byr2*, LBD-*Byr2*<sup>P209S</sup>, LBD-*Byr2*<sup>FBC</sup>-1, or LBD-Lamin. Two-hybrid interactions again were determined by the transactivation of *HIS3* and *lacZ* reporter genes. Results are shown in Fig. 4. LBD-*Byr2*, LBD-*Byr2*<sup>P209S</sup>, and LBD-*Byr2*<sup>FBC</sup>-1 were able to bind GAD-Ste4 and GAD-Ras1 equally well. *Byr2*-Reg was able to bind *Byr2*<sup>P209S</sup> and *Byr2*<sup>FBC</sup>-1 but not wild-type *Byr2*. *Byr2*-CBD was able to interact very weakly with *Byr2* but much more strongly with *Byr2*<sup>P209S</sup> and *Byr2*<sup>FBC</sup>-1. This suggests that the catalytic domain of wild-type *Byr2* was restrained, whereas that of the point mutants was released. It also shows that both *Byr2*-Reg and *Byr2*-CBD could be used to detect the release of *Byr2*-Cat. It further indicates that the interaction between *Byr2*-Reg and *Byr2*-Cat does not significantly interfere with Ras1 or Ste4 binding to *Byr2*-Reg.

To further test our hypothesis, namely, that the *Byr2* regulatory domain binds to and inhibits access to the kinase catalytic domain and that the P209S mutation releases this intramolecular interaction, we decided to perform the same two-hybrid experiments using GAD-*Byr1*. *Byr1* is the presumed MEK substrate for *Byr2*. We have observed a very weak interaction between GAD-*Byr1* and LBD-*Byr2*-Cat in the two-hybrid system. To study this complex formation more clearly, a *Byr1* mutant that binds the *Byr2* kinase catalytic domain more effectively was sought, and found, by screening a GAD-*Byr1* mutant library for clones that can establish a strong two-hybrid interaction with LBD-*Byr2*-Cat (see Materials and Methods). Fifteen mutants were isolated, and one of them, *Byr1*<sup>EBB-1</sup>, was used in the two-hybrid experiments.

We first tested the two-hybrid binding between GAD-*Byr1*<sup>EBB-1</sup> and LBD-*Byr2*-Cat. GAD-*Byr1*<sup>wt</sup> and LBD-Lamin served as negative controls. The data are presented in Fig. 5. GAD-*Byr1*<sup>EBB-1</sup> was able to bind very well with LBD-*Byr2*-Cat, whereas GAD-*Byr1*<sup>wt</sup> binds LBD-*Byr2*-Cat very poorly. GAD-*Byr1*<sup>EBB-1</sup> failed to bind LBD-Lamin, establishing the specificity of the interaction between GAD-*Byr1*<sup>EBB-1</sup> and LBD-*Byr2*-Cat. We then tested the interaction between GAD-*Byr1*<sup>EBB-1</sup> and LBD-*Byr2*<sup>wt</sup> and LBD-*Byr2*<sup>P209S</sup>. GAD-*Byr1*<sup>EBB-1</sup> was able to bind much more effectively to LBD-*Byr2*<sup>P209S</sup> than to LBD-*Byr2*<sup>wt</sup>, once again supporting the notion that the kinase catalytic domain of wild-type *Byr2* is restrained whereas that of *Byr2*<sup>P209S</sup> is released.

**Disruption of the closed configuration of *Byr2* by *Shk1*.** Our success in monitoring the kinase configuration in the two-hybrid system raised the possibility that we might be able to test or even screen for signaling components that are responsible for the opening of the closed configuration of wild-type *Byr2*. For this purpose, we used two-hybrid testing strains containing GAD-*Byr2*-CBD and LBD-*Byr2*<sup>wt</sup>. The switch from a closed form to an open form in *Byr2* would result in the binding of GAD-*Byr2*-CBD to LBD-*Byr2*<sup>wt</sup>, detected in the usual manner. The components we tested for opening of *Byr2* were expressed from either an *ADE2* based or a *LYS2* based vector or both vectors. We tested Ras1, Ste4, and *Shk1*. We suspected *S. pombe* *Shk1* to be an activator of *Byr2* because it appears to be involved in sexual differentiation in *S. pombe* (33) and is a close homolog of *S. cerevisiae* STE20/PAK (26). STE20 has been determined to function in the sexual differentiation of *S. cerevisiae*, upstream of STE11 (24), which is a close homolog of *Byr2* (31). Moreover, Wu et al. have dem-

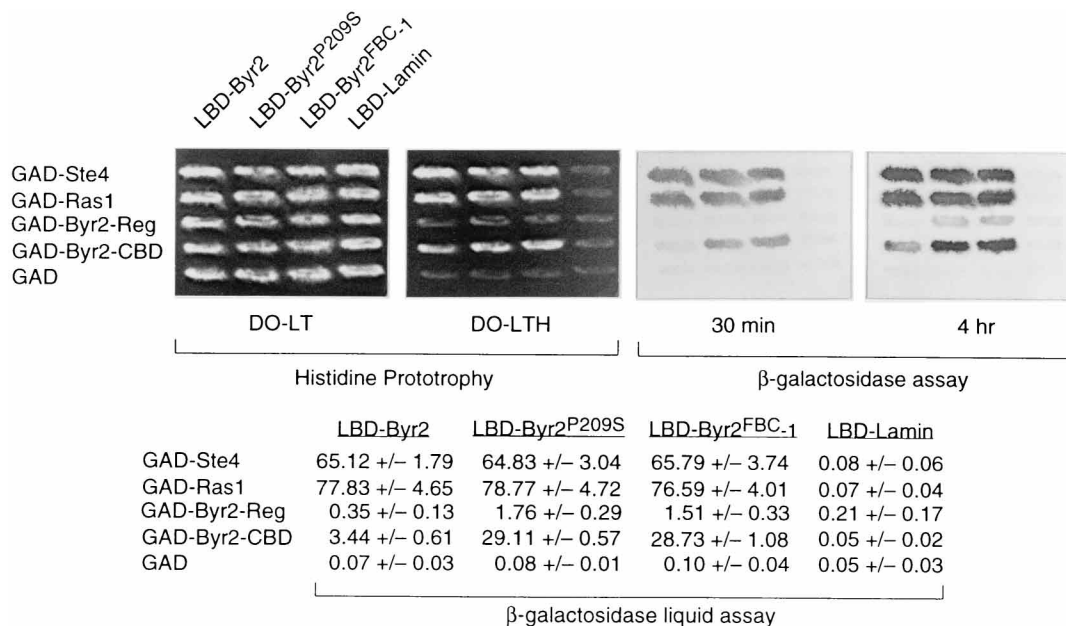


FIG. 4. Comparison of two-hybrid binding characters of Byr2<sup>wt</sup>, Byr2<sup>P209S</sup>, and Byr2<sup>FBC-1</sup>. L40 was transformed with either pGAD-Ste4, pGAD-Ras1, pGAD-Byr2-Reg, pGAD-Byr2-CBD, or pGAD and either pLBD-Byr2<sup>wt</sup>, pLBD-Byr2<sup>P209S</sup>, pLBD-Byr2<sup>FBC-1</sup>, or pLBD-Lamin. Transformants were tested for their ability to grow on the media lacking histidine and assayed for  $\beta$ -galactosidase expression. Values shown are relative levels (means  $\pm$  standard deviations). DO-LT and DO-LTH are DO-Leu-Trp and DO-Leu-Trp-His, respectively.

onstrated that STE20 can directly phosphorylate STE11 in vitro (40).

We first tested if Ste4 or Ras1 or the two together could induce the opened configuration of Byr2. Ste4 and Ras1 are candidates for this task because they both bind to the regulatory domain of Byr2, and Byr2-RBD even overlaps with Byr2-CBD. LBD-Byr2 or LBD-Byr2<sup>P209S</sup> was expressed in L40 and YCJ4 cells containing GAD-Byr2-CBD. A third plasmid, an *ADE2*-based pLS104, was used to express Ste4 alone. A fourth plasmid, a *LYS2*-based YCp405, was used to express Ras1. Two-hybrid interaction data are presented in Fig. 6. Again, GAD-Byr2-CBD was able to bind strongly with LBD-Byr2<sup>P209S</sup> but only very weakly with LBD-Byr2. Expression of Ste4 or Ras1 or the pair did not induce interaction between LBD-Byr2 and GAD-Byr2-CBD, nor did it visibly affect the interaction between LBD-Byr2<sup>P209S</sup> and GAD-Byr2-CBD. In this experi-

ment, we failed to demonstrate that wild-type Byr2 can be opened by Ste4 or Ras1, singly or jointly.

In contrast to the above results, Shk1, the *S. pombe* homolog of *S. cerevisiae* STE20/PAK, can open Byr2. A dominant activated form of Shk1, Shk1 $\Delta$ N, was isolated from an *S. pombe* cDNA library by PCR (see Materials and Methods). It was cloned into pLS104. Forty-four independent clones were obtained, and the first eight were tested for their ability to induce the interaction between GAD-Byr2-CBD or GAD-Byr1<sup>EBB-1</sup> and the kinase catalytic domain of LBD-Byr2. A kinase-defective Shk1, Shk1<sup>K415,416R</sup> $\Delta$ N, was made with two adjacent critical lysine residues changed to arginine by PCR site-directed mutagenesis (see Materials and Methods). pLS104-Shk1 $\Delta$ N clones were transformed into L40 with pGAD-Byr2-CBD or pGAD-Byr1<sup>EBB-1</sup> and pLBD-Byr2. pLS104, pLS104-Shk1<sup>K415,416R</sup> $\Delta$ N, pGAD, and pLBD-Lamin were tested as

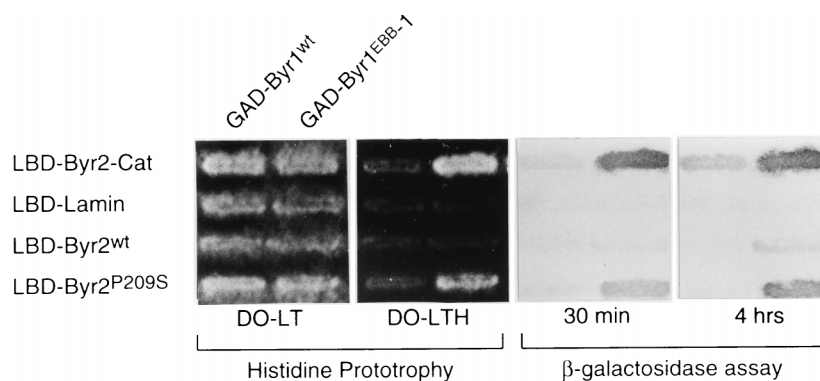


FIG. 5. Two-hybrid interaction with Byr1<sup>EBB-1</sup>. L40 was transformed with either pGAD-Byr1 or pGAD-Byr1<sup>EBB-1</sup> and either pLBD-Byr2-Cat, pLBD-Lamin, pLBD-Byr2<sup>wt</sup>, or pLBD-Byr2<sup>P209S</sup>. Transformants were tested for their ability to grow on the media lacking histidine and assayed for  $\beta$ -galactosidase expression. DO-LT and DO-LTH are DO-Leu-Trp and DO-Leu-Trp-His, respectively.

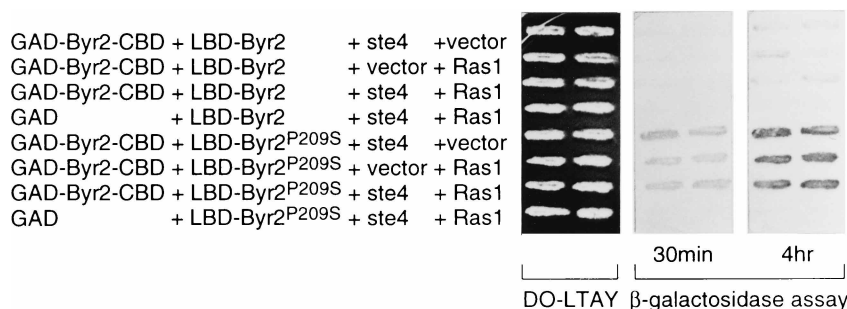


FIG. 6. Effect of overexpression of Ste4 or Ras1 or the two together on the two-hybrid interaction between GAD-Byr2-CBD and LBD-Byr2<sup>wt</sup>. YCJ4 was transformed with either pGAD-Byr2-CBD or pGAD, either pLBD-Byr2<sup>wt</sup> or pLBD-Byr2<sup>P209S</sup>, either pLS104-Ste4 or pLS104, and either pYcP405-Ras1 or pYcP405. Transformants were tested for their ability to grow on the media lacking histidine and assayed for  $\beta$ -galactosidase expression. DO-LTAY is DO-Leu-Trp-Ade-Lys.

negative controls. Cells were plated on DO-Leu-Trp-Ade, and transformants were tested for transactivation of *HIS3* and *lacZ* reporter genes. As shown in Fig. 7, Shk1 $\Delta$ N was able to specifically induce the interaction between GAD-Byr2-CBD or GAD-Byr1<sup>EBB-1</sup> and LBD-Byr2, while the kinase-defective Shk1<sup>K415,416R</sup> $\Delta$ N was unable to do so. Only one Shk1 $\Delta$ N clone failed to induce the interaction, and this clone was shown by DNA sequencing to have a premature stop codon. Thus, we determined that Shk1 $\Delta$ N can induce the two-hybrid interaction between Byr2-CBD or GAD-Byr1<sup>EBB-1</sup> and the kinase catalytic domain of wild-type Byr2. Shk1 $\Delta$ N presumably achieves this by abolishing Byr2 intramolecular interaction, thus releasing the Byr2 kinase catalytic domain.

To test if Shk1 $\Delta$ N can enhance the interaction between Byr1<sup>wt</sup> and Byr2-Cat, we overexpressed Shk1 $\Delta$ N in L40 cells that contain GAD-Byr1<sup>wt</sup> and LBD-Byr2-Cat. We used the pair GAD-Byr1<sup>wt</sup> and LBD-Byr2-Cat since they have a weak two-hybrid interaction and any enhancement would be readily observed. The overexpression of Shk1 $\Delta$ N was unable to en-

hance that weak two-hybrid interaction. Thus, it appears that Shk1 $\Delta$ N affects specifically Byr2 intramolecular interaction, between Byr2-Reg and Byr2-Cat.

## DISCUSSION

Previous studies showed that Byr2 acts downstream of both Ste4 and Ras1 in the sexual differentiation pathway in *S. pombe* (3, 38), and two-hybrid data indicated that all three can form a complex (3). In this study, we used deletion analysis to define the nonoverlapping Ras1 binding domain of Byr2 and the Ste4 binding domain. These conclusions were confirmed by point mutation analysis. Moreover, we used the *byr2* gene with mutations in its Ste4 and Ras1 regulatory domains in high-copy-number genetic suppression studies to show that Byr2 activity is dependent upon each of these interactions. Thus, a Byr2 mutant that fails to bind Ste4 (Byr2<sup>FBS</sup>) is a poor suppressor in cells lacking Ras1, but such a mutant is comparable to Byr2<sup>wt</sup> as a suppressor in cells lacking Ste4. Similarly, a Byr2 mutant

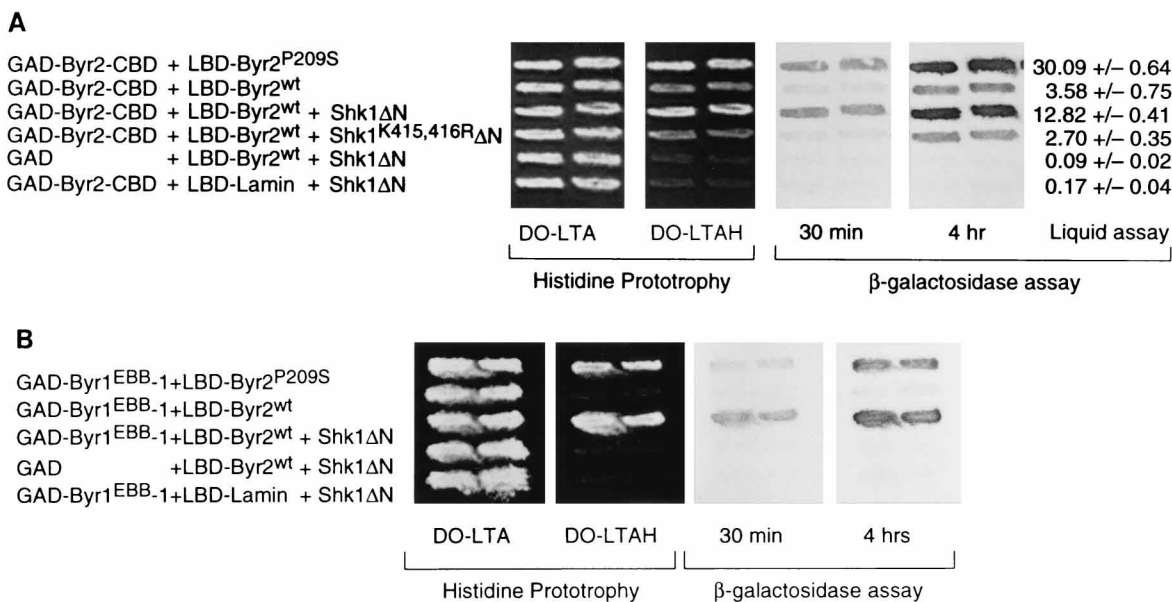


FIG. 7. Effect of overexpression of activated Shk1 on the two-hybrid interaction between GAD-Byr2-CBD or GAD-Byr1<sup>EBB-1</sup> and LBD-Byr2<sup>wt</sup>. (A) L40 was transformed with either pGAD-Byr2-CBD or pGAD, either pLBD-Byr2, pLBD-Byr2<sup>P209S</sup>, or pLBD-Lamin, and either pLS104-Shk1 $\Delta$ N or pLS104-Shk1<sup>K415,416R</sup> $\Delta$ N or just with the expression vector without an insert. Transformants were tested for their ability to grow on the media lacking histidine and assayed for  $\beta$ -galactosidase expression. (B) L40 was transformed with either pGAD-Byr1<sup>EBB-1</sup> or pGAD, either pLBD-Byr2<sup>wt</sup>, pLBD-Byr2<sup>P209S</sup>, or pLBD-Lamin, and pLS104-Shk1 $\Delta$ N or just the expression vector. Transformants were tested for their ability to grow on the media lacking histidine and assayed for  $\beta$ -galactosidase expression. DO-LTA and DO-LTAH are DO-Leu-Trp-Ade and DO-Leu-Trp-Ade-His, respectively.

that fails to bind Ras1 (Byr2<sup>FBR</sup>) is a poor suppressor in cells lacking Ste4 but is comparable to Byr2<sup>wt</sup> in cells lacking Ras1. Thus, not only does Byr2 act downstream of Ste4 and Ras1 and not only does it also interact with these proteins, but it is also dependent upon them. Furthermore, Byr2 can respond to either Ste4 or Ras1 in the absence of the other.

An inhibitory role for the MEKK regulatory domain has long been suspected since truncation or alteration of the N terminus often yields constitutively active kinases (14, 36). Physical interaction between the regulatory domains and kinase catalytic domains, however, has not been reported for RAF, MEKK, or STE11. Fortuitously, we observed a weak two-hybrid interaction between the separated regulatory and catalytic domains of Byr2. We mapped the catalytic binding domain of Byr2 by both deletion and point mutation analyses to a region which minimally overlapped with the Ras1 binding domain. We have tried but failed to demonstrate this interaction using purified protein from *E. coli* expression systems. This failure to confirm interaction by biochemical studies might be due to the weakness of the interaction. The two-hybrid system is recognized as being a more sensitive indicator of interaction than simple coprecipitation or affinity chromatography. A weak interaction between the regulatory and catalytic domains of Byr2 might be predicted because strong intramolecular interactions would interfere with regulation. Alternatively, bacterially produced proteins might not fold properly or might lack a critical posttranslational modification. Nevertheless, further genetic and two-hybrid studies strongly suggest the physiological relevance of this interaction.

Point mutations that abolish interaction between the regulatory and catalytic domains activate Byr2. Byr2<sup>P209S</sup>, which was previously shown to be an activated mutant of Byr2 (31), is mutated in the regulatory region which we have defined as the catalytic binding domain. While this in itself might have been fortuitous, all four Byr2<sup>FBC</sup> mutants that we examined were activated and behaved indistinguishably from Byr2<sup>P209S</sup> in our genetic tests. These results provide confirmation of the two-hybrid studies and strongly suggest that intramolecular binding between the regulatory and catalytic domains is autoinhibitory.

If the hypothesis that intramolecular binding of regulatory and catalytic domains occurs is correct, then we should be able to observe the breaking of this interaction. Using two-hybrid analysis, we compared Byr2<sup>wt</sup> and Byr2<sup>P209S</sup> for the ability to interact with proteins that bind the catalytic domain. We used three such proteins: the separate regulatory domain itself (Byr2-Reg), the separate catalytic binding domain (Byr2-CBD), and a mutant Byr1 that was selected for tighter binding to the Byr2 catalytic domain. All three proteins had much stronger interaction with Byr2<sup>P209S</sup> than with Byr2<sup>wt</sup>. We conclude that mutants altered in the catalytic binding domain open up the conformation of Byr2 by breaking intramolecular binding.

Because mutants with alterations in the Byr2 regulatory domain can apparently activate the kinase and alter its conformation, we suspected that other regulatory components of the signaling pathway might perform this function physiologically. Using the two-hybrid assay for the open configuration of Byr2, we were able to test components of the system for their ability to induce this conformational change. We failed to observe opening of Byr2 by Ras1 or Ste4 or both. Failure to observe opening with Ras1 or Ste4 may indicate that those proteins have other functions (see below) or may merely reflect a technical obstacle. On the other hand, an activated form of Shk1 was extremely effective at opening Byr2.

Shk1 is one of the *S. pombe* homologs of *S. cerevisiae* STE20. Like STE20, Shk1 is also implicated in the sexual differentiation of *S. pombe* (33). A direct demonstration of the involve-

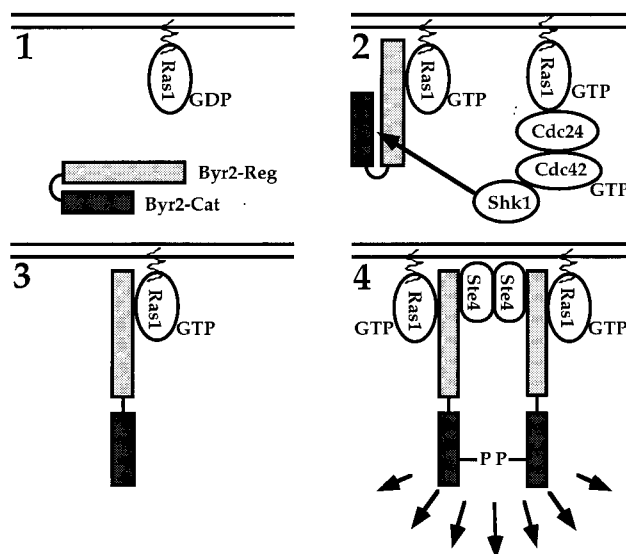


FIG. 8. Model for Byr2 activation. See Discussion.

ment of the *shk1* gene in the sexual differentiation of *S. pombe* has not been straightforward, since disruption of Shk1 is lethal (26, 33). Shk1 is associated with Cdc42, a member of the Rho family of guanine nucleotide-binding proteins, which is implicated in its activation and localization and is required for both morphological and sexual differentiation (6, 26, 33, 34). Cdc42, in turn, is associated with Ras1 through their mutual and cooperative interaction with Cdc24 (6), a guanine nucleotide exchange protein (43).

We can assemble a consistent model for the activation of Byr2 that incorporates our findings (Fig. 8). This model is neither complete nor necessarily correct. In the depicted model, Byr2 recognizes GTP-bound Ras1, associated with the membrane. In this cellular region and through its association with Ras1, Byr2 may encounter other components necessary for its activation, including Shk1. We have depicted a further association between Ras1 and Shk1, as discussed above. Upon encountering an activated form of Shk1, Byr2 assumes an open configuration. In the presence of Ste4, which is a leucine zipper protein capable of homodimerization, the open configuration of Byr2 might dimerize, leading to autophosphorylation and further activation of Byr2.

This model does not depict the influence of the sex pheromone pathway upstream of Byr2. In principle, the pheromone pathway could interact with any of the depicted components or with yet another component. Shk1 is a leading candidate for the site of this influence, because mutants that open the Byr2 configuration are best able to suppress the loss of Gpa1, the  $\alpha$  subunit of the heterotrimer thought to report sex pheromone receptor occupancy. Moreover, STE20, the *S. cerevisiae* homolog of Shk1, has been reported to function closely downstream of the  $\beta\gamma$  subunit of the heterotrimer, which mediates receptor occupancy in that organism (1, 24). Furthermore, several mammalian PAK homologs have been shown to function downstream of heterotrimeric G-proteins (21).

Many aspects of this model can and should be challenged. First, we have not demonstrated that Shk1 phosphorylates Byr2, nor have we demonstrated autophosphorylation or the subsequent activation of the kinase. It is not clear that Ras1 and/or Ste4 do not directly participate in opening the configuration of Byr2. We may have merely failed to observe such an involvement. The role of Ste4 is very unclear. Dimerization

and autocatalytic activation by transphosphorylation are attractive possibilities, with precedent in the tyrosine kinase family. Furthermore, the protein kinase RAF can be activated by dimerization (12). In support of this idea, we have generated dimerizable Byr2 fusion proteins by attaching the leucine zipper domain of Ste4 and c-Jun (23) to Byr2 and have found that they behave much like the activated Byr2<sup>FBC</sup> mutants (unpublished data). However, we have never observed Ste4 induction of the dimerization of Byr2 in the two-hybrid system, nor does the dimerization model explain the need for the domain of Ste4 that is homologous to *S. cerevisiae* STE50. An alternative hypothesis is that the STE50-like domain of Ste4 brings another protein to the Byr2 complex. Finally, the temporal sequence of events, if important, is still unclear. Further research is clearly needed to confirm, extend, or correct this model.

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