

## Complexes between STE5 and components of the pheromone-responsive mitogen-activated protein kinase module

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**ABSTRACT** We present genetic evidence for complex formation of STE5 and the STE11, STE7, and FUS3 protein kinases, the pheromone-responsive mitogen-activated protein kinase module of *Saccharomyces cerevisiae*. Interaction between STE5 and STE11 is not dependent on STE7, and interaction between STE5 and STE7 does not require STE11. The N-terminal regulatory domain of STE11 is both necessary and sufficient for interaction with STE5. Interaction between STE7 and STE11 is bridged by STE5, suggesting the formation of a multiprotein complex. We also demonstrate biochemical interaction between STE5 and STE11 by using a combination of bacterially expressed fusion proteins and extracts prepared from yeast. Our results suggest that STE5 is a scaffolding protein that facilitates interactions between components of the pheromone-responsive mitogen-activated protein kinase module. We further propose that such scaffolding proteins serve to inhibit cross-talk between functionally unrelated mitogen-activated protein kinase modules within the same cell.

Signaling between  $\alpha$  and  $\alpha$  haploid cells of the yeast *Saccharomyces cerevisiae* is mediated by the reciprocal action of secreted peptide pheromones, termed  $\alpha$  factor and  $\alpha$  factor, respectively (1, 2). These mating pheromones bind to seven-transmembrane-domain receptors, encoded by the STE2 and STE3 genes (3–5), that signal through a heterotrimeric guanine nucleotide binding protein (G protein). The  $G_{\beta\gamma}$  subunits of the G protein, encoded by the STE4 and STE18 genes, respectively, transduce the pheromone signal to downstream components, and the  $G_{\alpha}$  subunit, encoded by the GPA1/SCG1 gene, acts as a negative control element (6–8). A group of protein kinases, which we refer to as a mitogen-activated protein kinase (MAP kinase) module (9, 10), functions downstream of the G protein (11–14). This pheromone-responsive module is composed of the STE11, STE7, and FUS3/KSS1 gene products. MAP kinase modules are highly conserved in eukaryotic organisms: FUS3 and KSS1 are structurally related to metazoan MAP/ERK kinases (15); STE7 is structurally related to MAP kinase activators, variously referred to as MAP kinase kinases and MEKs (16, 17); and STE11 is structurally related to the mammalian MEK kinase (MEKK), a MAP kinase kinase activator (18). In addition, we have shown that STE11, STE7, and FUS3/KSS1 are structurally and functionally related to the respective byr2, byr1, and spk1 protein kinases of the evolutionarily distant yeast *Schizosaccharomyces pombe* (10).

Recent genetic studies using hyperactive mutant alleles of the STE5 and STE11 genes suggest that the product encoded by the STE5 gene functions at a level between the mating pheromone receptor-coupled G protein and the MAP kinase module in *S. cerevisiae* (11, 13, 19). The predicted sequence of the STE5 gene product does not contain structural motifs that immediately suggest a catalytic function (20, 21). Therefore, as a first step toward the functional characterization of

the STE5 protein, we have utilized a genetic method, the yeast two-hybrid system (22), to detect physical interactions between STE5 and components of the *Sa. cerevisiae* pheromone response pathway. In this report, we show that STE5 forms complexes with the STE7, STE11, and FUS3 protein kinases. We also provide evidence that STE5 is required for efficient *in vivo* interaction between STE7 and STE11, two proteins that have been shown to interact *in vitro* (23). These and additional results described herein lead us to propose that STE5 may function as a molecular scaffold that is required for the proper activity of the *Sa. cerevisiae* pheromone-responsive MAP kinase module. Our results may be relevant to the function and integration of MAP kinase modules in general.

### MATERIALS AND METHODS

**Yeast Strains, Media, and Genetic Manipulations.** *Sa. cerevisiae* YPB2 [MATa *ade2-101 his3-200 leu2-3,112 lys2-801 ura3-52 trp1-901 can<sup>r</sup> gal4-542 gal80-538 LYS2::GAL1<sub>UAS</sub>-HIS3<sub>TATA</sub>-HIS3 URA3::GAL1<sub>17mers(3x)</sub>-CYC1<sub>TATA</sub>-lacZ*] and L40 [MATa *ade2 his3 leu2 trp1 LYS2::lexA-HIS3 URA3::lexA-lacZ*] were used as hosts for two-hybrid experiments (24, 25). *ste5* $\Delta$  (L40ST5 $\Delta$ ), *ste7* $\Delta$  (L40ST7 $\Delta$ ), and *ste11* $\Delta$  (L40ST11 $\Delta$ ) derivatives of L40 were made by one-step gene replacement (26) using *ste5* $\Delta$ ::ADE2 (from pBSSTE5 $\Delta$ ::ADE2), *ste7* $\Delta$ ::ADE2 (from pBSSTE7 $\Delta$ ::ADE2), and *ste11* $\Delta$ ::ADE2 (from pBSSTE11 $\Delta$ ::ADE2), respectively. Epitope-tagged fusion proteins were expressed in *Sa. cerevisiae* YPH499 [MATa *ade2-101<sub>oc</sub> his3- $\Delta$ 200 leu2- $\Delta$ 1 lys2-801<sub>am</sub> trp1- $\Delta$ 1 ura3-52*] (27). AN43-5A [MATa *ade1 arg4 leu2-3,112 trp1 ura3-52 mfa1::FUS1::lacZ his3::FUS1::HIS3*], provided by A. Neiman and I. Herskowitz (University of California, San Francisco), was used for expression of glutathione S-transferase (GST) fusion proteins in yeast. Yeast cultures were grown in YPD [1% yeast extract/2% (wt/vol) peptone/2% (wt/vol) glucose] or in synthetic minimal (SM) medium (0.67% yeast nitrogen base/2% glucose/appropriate auxotrophic supplements). Standard yeast genetic methods were followed (28).

**Nucleic Acid Manipulation and Analysis.** The two-hybrid plasmids pGADGH [for GAL4 transcriptional activation domain (GAD) fusions], pGBT9 and pGBT10 [for GAL4 DNA binding domain (GBD) fusions], pGADHRAS<sup>R186</sup>, pGBDRAS<sup>R319</sup>, pGBDRAF, pGADbyr2, pGBDbyr2, pGADSNF1, pGBDSNF4, and pBTM116 (for expression of *lexA* fusion proteins) have been described (24, 25). pHP5 (kindly provided by H.-P. Xu, Cold Spring Harbor Laboratory) is derived from pGBT10 and contains a frame shift in the polylinker such that the *Bam*HI frame is TCG GAT CCC. pRD56 (29) and pRP259, a derivative of pGEX-3

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Abbreviations: GAD, GAL4 transcriptional activation domain; GBD, GAL4 DNA binding domain; LBD, *lexA* DNA binding domain; GST, glutathione S-transferase; G protein, guanine nucleotide binding protein; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; MEKK, MEK kinase.

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(Pharmacia), were used for expression of GST fusion proteins in yeast and bacteria, respectively. Polymerase chain reactions (PCRs) were used to generate fragments of the STE5, STE11, STE11ΔC (encoding residues 1–415), STE11ΔN (encoding residues 416–717), STE7, FUS3, byr1, spk1, MEK, and MEKK coding sequences compatible for cloning into GAD, GBD, and lexA two-hybrid expression vectors and/or into epitope-tagged or GST fusion protein expression vectors. pBSSTE5Δ::ADE2 was constructed by cloning a *Kpn* I–*Bam*HI fragment of the STE5 gene (from pSL1420, provided by B. Stevenson and G. Sprague, University of Oregon, Eugene) into the corresponding sites of pBluescript II SK– (Stratagene) and then replacing an *Eco*RI–*Xho* I fragment of the STE5 sequence with a 2.3-kb fragment of the ADE2 gene (from pASZ11; ref. 30). pBSSTE11Δ::ADE2 was constructed by cloning a *Kpn* I–*Xba* I fragment of STE11 (from pSL1507, provided by B. Stevenson and G. Sprague) into the corresponding sites of pBluescript II SK– and then replacing the *Hind*III fragment of the gene with ADE2. pBSSTE7Δ::ADE2 was constructed by cloning a *Hind*III fragment of STE7 (from pJD7, provided by Stan Fields, State University of New York, Stony Brook) into the *Bam*HI–*Sal* I sites of pUC119 and then replacing the *Msc* I–*Bgl* II fragment of the gene with ADE2. pAUD6STE5 contains a PCR fragment of STE5 cloned into the *Bam*HI site of pAUD6 (kindly provided by R. Ballester, University of California, Santa Barbara), which is derived from pRS423 (27). This plasmid provides for expression of a N-terminal c-myc epitope (31) fused to STE5 protein from the *Sa. cerevisiae ADH1* promoter. pAAUD6STE5 was constructed by cloning an ADE2 gene fragment into the *Sac* I site 3' of the STE5 insert in pAUD6STE5. pAD5STE11 was constructed by cloning a PCR fragment of the STE11 coding sequence into pAD5 (10), which allows for expression of a N-terminal hemagglutinin epitope-tagged (32) STE11 protein in yeast.

**β-Galactosidase Filter Assay.** The filter assay for testing two-hybrid interactions was performed as described (24). We conducted lexA two-hybrid experiments with lexA DNA binding domain (LBD) and GAD pairs of fusion proteins.

**Preparation of Yeast and Bacterial Lysates, Isolation of GST and Immune Complexes, and *In Vitro* Binding Studies.** Yeast cultures expressing epitope-tagged and GST fusion proteins were grown in SM and SMGal medium [SM without glucose and containing 2% (wt/vol) galactose, 2% (vol/vol) glycerol, and 1% ethanol], respectively, at 30°C to  $2 \times 10^7$  cells per ml, and lysates were prepared as described (33). *Escherichia coli* BL21, transformed with GST, GSTSTE5, or GSTSTE11ΔC

fusion protein expression plasmids, was grown in LB with ampicillin at 100 μg/ml (LBamp) at 37°C to stationary phase, and then diluted 1:100 into fresh LBamp and grown an additional 2 hr at 37°C, at which time isopropyl β-D-thiogalactopyranoside was added to 0.1 mM. After an additional 4-hr incubation at 37°C, lysates were prepared as described (34). GST fusion proteins were purified from yeast and bacterial lysates as described (23, 34). GST fusion protein-bound glutathione-agarose beads were mixed with 500 μg of yeast cell lysate, and lysis buffer was added so that the total volume was 500 μl. The resulting slurry was incubated at 4°C for 2 hr and then washed three times with lysis buffer (33). Samples were resolved by SDS/PAGE in 10% gels and then transferred to nitrocellulose (Schleicher & Schuell). Epitope-tagged proteins were detected by immunoblot analysis with a 1:10,000 dilution of ascites fluid containing monoclonal antibody 12CA5, for hemagglutinin epitope-tagged proteins (32), or 9E10, for c-myc epitope-tagged proteins (31), followed by detection using the ECL chemiluminescent kit (Amersham).

## RESULTS

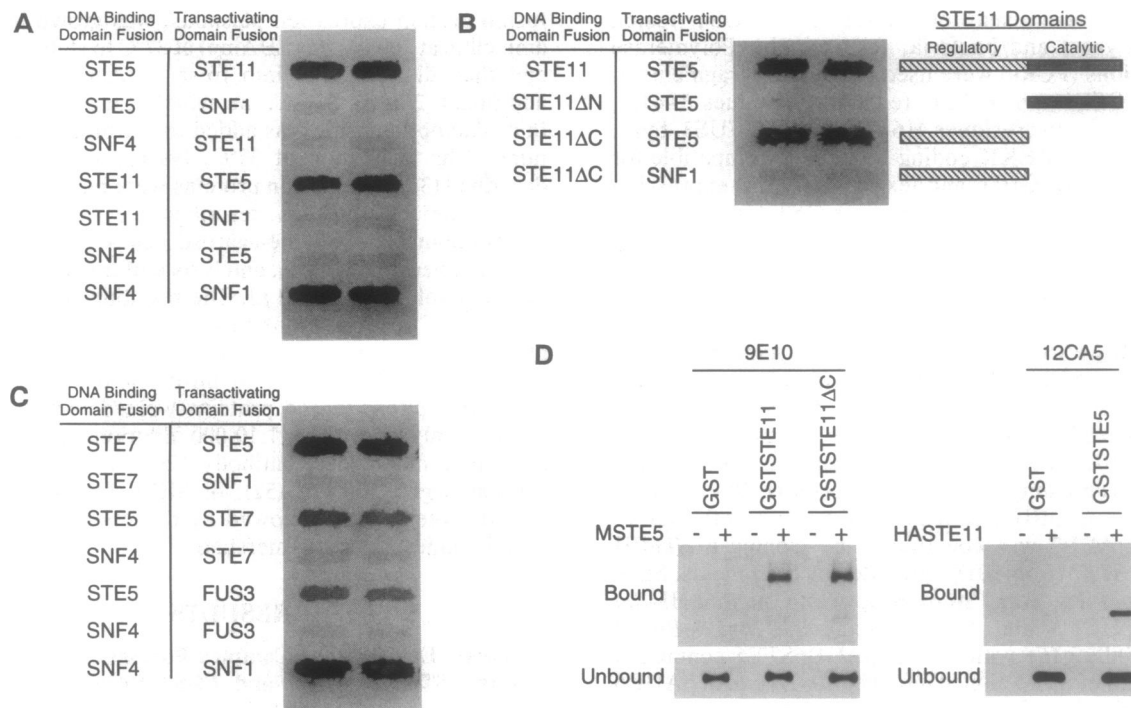
**Genetic Evidence for Complex Formation Between STE5 and the STE11, STE7, and FUS3 Protein Kinases.** We utilized the yeast two-hybrid system (22) to examine whether STE5 interacts physically with components of the pheromone-responsive MAP kinase module. Interactions between numerous proteins have been demonstrated using the two-hybrid system. We (24) and others (25) have used the system to show interactions between RAS and RAF oncoproteins, between RAS and the *Sc. pombe* byr2 protein kinase (24), and between RAF and MEK (24). A summary of the pairwise protein interactions tested in this study is presented in Table 1.

To test whether STE5 and STE11 form a complex, we constructed vectors to express STE5 or STE11 fused to GBD or to GAD in the tester strain, YPB2. As shown in Fig. 1A, interaction between STE5 and STE11 was detected, based on β-galactosidase production resulting from induction of *GAL1-lacZ* by either GADSTE5 and GBDSTE11 or by GADSTE11 and GBDSTE5. Neither STE5 nor STE11 fusion proteins interacted with GADSNF1 or GBDSNF4 fusion proteins (22, 35), which were used as controls (Fig. 1). The STE11 kinase is composed of an N-terminal regulatory domain (approximately residues 1–415) and a C-terminal catalytic domain (approximately residues 416–717) (11). As

Table 1. Pairwise combinations of GBD, LBD, and GAD fusion proteins tested in this study

| GAD fusion           | <i>β</i> -Galactosidase induction |       |         |         |      |      |      |      |     |                      |      |            |      |      |     |                      |
|----------------------|-----------------------------------|-------|---------|---------|------|------|------|------|-----|----------------------|------|------------|------|------|-----|----------------------|
|                      | GBD fusion                        |       |         |         |      |      |      |      |     |                      |      | LBD fusion |      |      |     |                      |
|                      | STE5                              | STE11 | STE11ΔC | STE11ΔN | STE7 | FUS3 | byr2 | byr1 | RAF | RAS2 <sup>R319</sup> | SNF4 | STE11      | STE7 | byr2 | MEK | HRAS <sup>R186</sup> |
| STE5                 | −                                 | +     | +       | −       | +    | −    | −    | −    | −   | −                    | −    | +          | +    | −    | −   | −                    |
| STE7                 | +                                 | −     | •       | •       | −    | +    | •    | •    | •   | •                    | −    | −          | +    | •    | •   | −                    |
| STE11                | +                                 | +     | •       | •       | −    | −    | •    | •    | •   | −                    | −    | +          | +    | •    | •   | −                    |
| STE11ΔC              | +                                 | •     | •       | •       | •    | •    | •    | •    | •   | •                    | −    | •          | •    | •    | •   | •                    |
| FUS3                 | +                                 | −     | •       | •       | +    | −    | •    | •    | •   | •                    | −    | +          | +    | •    | •   | −                    |
| byr2                 | −                                 | •     | •       | •       | •    | •    | •    | •    | •   | +                    | −    | •          | •    | •    | •   | •                    |
| spk1                 | −                                 | •     | •       | •       | •    | •    | •    | +    | •   | •                    | −    | •          | •    | •    | •   | •                    |
| MEKK                 | −                                 | •     | •       | •       | •    | •    | •    | •    | •   | •                    | •    | •          | •    | •    | −   | •                    |
| MEK                  | −                                 | •     | •       | •       | •    | •    | •    | •    | +   | •                    | −    | •          | •    | •    | •   | •                    |
| HRAS <sup>R186</sup> | •                                 | •     | •       | •       | •    | •    | +    | •    | +   | •                    | −    | •          | •    | +    | •   | •                    |
| RAF                  | •                                 | •     | •       | •       | •    | •    | •    | •    | •   | •                    | •    | −          | −    | •    | +   | +                    |
| SNF1                 | −                                 | −     | −       | −       | −    | −    | −    | −    | −   | −                    | +    | •          | −    | •    | •   | •                    |

Values represent the presence of transformed colonies that expressed detectable β-galactosidase activity (+) or not (–). –, Not determined. The proteins to left of the table were fused to GAD. The proteins at the top of the table were fused to either GBD or LBD. GBD–GAD combinations were expressed in the GAL4 two-hybrid tester strain, YPB2. LBD–GAD combinations were tested in the lexA tester strain, L40. At least four transformants were tested for each determination.



**FIG. 1.** STE5 forms complexes with components of the pheromone-responsive MAP kinase module. STE5, STE11, STE11ΔC (residues 1–415), STE11ΔN (residues 416–717), STE7, and FUS3 fused to either GBD or GAD were tested for interaction by their induction of  $\beta$ -galactosidase expression (darker patches in the black and white photographs) in the host strain YPB2 (A–C). Each patch represents an independent transformant. *Sa. cerevisiae* SNF1 and SNF4 were used as positive controls. (A) STE5 forms a complex with the STE11 protein kinase. (B) N-terminal regulatory domain of STE11 is both necessary and sufficient for complex formation with STE5. (C) STE5 forms complexes with the STE7 and FUS3 protein kinases. (D) STE5 and STE11 form a complex *in vitro*. GSTSTE5 and GSTSTE11ΔC fusion proteins were purified from bacterial lysates and GSTSTE11 fusion protein was purified from yeast lysates. GST or GST fusion proteins bound to glutathione-agarose beads were mixed with lysates prepared from yeast strains expressing (+) or not (–) either c-myc epitope-tagged STE5 (MSTE5) or hemagglutinin epitope-tagged STE11 (HASTE11) proteins. After a 2-hr incubation at 4°C, the unbound fraction (one-thirtieth of the total volume) was sampled, mixed with SDS sample buffer, and boiled for 5 min. The beads containing bound fractions were washed, resuspended in a small volume of SDS sample buffer, and boiled for 5 min. Bound (one-third of the resuspended slurry) and unbound samples were electrophoresed in 10% polyacrylamide gels, and proteins were transferred to nitrocellulose. Monoclonal antibody 9E10 was used to detect MSTE5 protein (left side of panel) bound to beads, and monoclonal antibody 12CA5 was used to detect HASTE11 (right side of panel). Bacterially expressed GSTSTE11ΔC and yeast-expressed GSTSTE11 fusion proteins were each capable of binding to MSTE5 expressed in yeast. Bacterially expressed GSTSTE5 was capable of binding to HASTE11 expressed in yeast. Neither MSTE5 or HASTE11 proteins could bind to the GST moiety alone.

shown in Fig. 1B, the N terminus of STE11 was both necessary and sufficient for interaction with STE5.

We next examined whether STE5 interacts with other components of the MAP kinase module. As shown in Fig. 1C, STE5 also interacted with both the STE7 and FUS3 protein kinases. Recent studies have demonstrated that FUS3 functions downstream of STE7 and STE11 (23, 36). In addition, while our manuscript was in preparation, other investigators (37) reported independently on complex formation between STE5 and FUS3. These workers also showed that STE5 is phosphorylated by FUS3. In this report, we describe experiments that focus primarily on the interactions of STE5, STE7, and STE11.

**Biochemical Evidence for Complex Formation Between STE5 and STE11.** To obtain biochemical evidence for complex formation between STE5 and STE11, we utilized GST fusion proteins purified from bacteria or yeast and extracts prepared from yeast expressing epitope-tagged proteins (32). STE5 and STE11ΔC were expressed as GST fusion proteins in bacteria, and full-length STE11 was expressed as a GST fusion protein in yeast. GST fusion proteins purified from bacterial or yeast lysates were mixed with lysates prepared from yeast strains that expressed either the c-myc epitope-tagged STE5 (MSTE5) or the hemagglutinin epitope-tagged STE11 (HASTE11) proteins. As shown in Fig. 1D, bacterially expressed GSTSTE5 was capable of binding to HASTE11 expressed in yeast. GSTSTE11 and GSTSTE11ΔC, purified

from yeast and bacteria, respectively, were each capable of binding to MSTE5 expressed in yeast. These results confirm the interaction detected between STE5 and STE11 in the two-hybrid system.

**STE5 Is Required for Strong Interaction Between STE11 and STE7.** Other investigators have shown that the STE11 protein kinase is required for pheromone-induced hyperphosphorylation of STE7 *in vivo* (14). In addition, Neiman and Herskowitz (23) have recently demonstrated that STE7 is phosphorylated and activated by STE11 *in vitro* using proteins isolated from yeast. We therefore tested whether interaction between STE7 and STE11 could be detected in the two-hybrid system. Unassisted, STE7 and STE11 failed to form a detectable complex using the GAD and GBD vectors in our host strain, YPB2 (Fig. 2A). However, because STE5 interacts with both STE7 and STE11, we examined whether the three proteins might form a complex. Indeed, when STE5 was overexpressed, interaction between STE7 and STE11 was detected. Furthermore, the N-terminal regulatory domain of STE11 was sufficient for STE5-promoted complex formation with STE7 (Fig. 2B). These results suggested that STE5, STE7, and STE11 form a multiprotein complex. To examine this possibility further, we made use of the *lexA*-based two-hybrid system, which is similar to the *GAL4*-based system but utilizes the bacterial LBD in combination with an acidic activation domain to drive transcription from a *lexA-lacZ* reporter gene (25). We have found this system in some

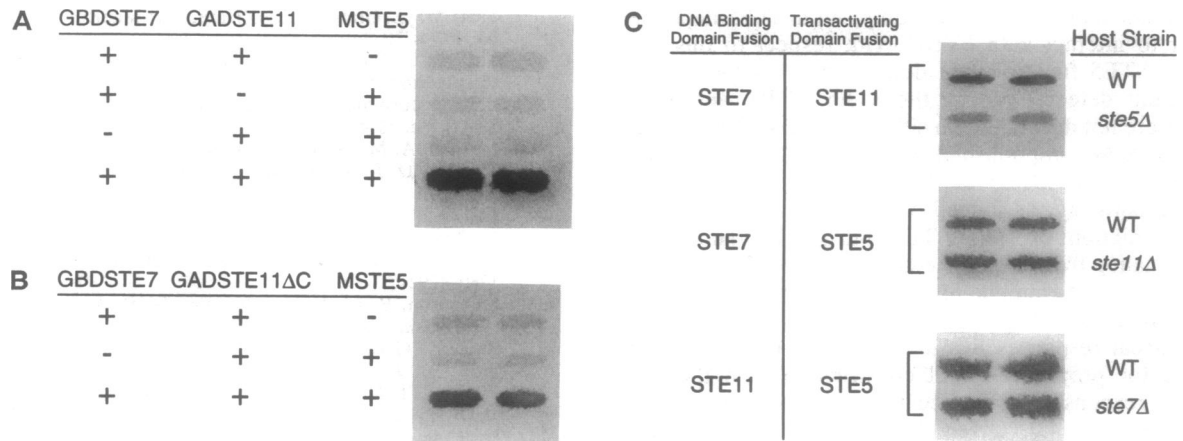


FIG. 2. Evidence that STE5, STE7, and STE11 form a multiprotein complex. The GAL4 two-hybrid tester strain, YPB2, was transformed (+) or not (-) with plasmids expressing STE7 fused to GBD, STE11 or STE11ΔC fused to GAD, and/or MSTE5 and assayed for  $\beta$ -galactosidase expression (shown to the right of A and B). Complex formation between STE11 and STE7 in the GAL4-based two-hybrid system requires overexpression of STE5 (A). The N-terminal regulatory domain of STE11 is sufficient for multiprotein complex formation with STE5 and STE7 (B). Complex formation among STE5, STE7, and STE11 in the LexA-based two-hybrid system is shown in C. STE5, STE11, and STE7 fused to LBD or GAD were tested for interaction by their induction of  $\beta$ -galactosidase expression in the LexA two-hybrid tester strain L40 or in strains derived from L40 carrying deletions of *STE5*, *STE7*, or *STE11* genes, as indicated. In the more-sensitive LexA-based system, interaction between STE11 and STE7 does not require overexpression of STE5. However, the interaction between these kinases is significantly reduced in a *ste5Δ* mutant. Interaction between STE5 and STE7 in the LexA system is not dependent on STE11, and interaction between STE5 and STE11 does not require STE7.

cases, when using the tester strains described above, to be more sensitive than the GAL4-based system. Using the LexA two-hybrid system, we were able to detect interaction between STE7 and STE11 fusion proteins (Table 1). We also detected interactions between STE5 and STE11 and between STE5 and STE7 as was observed in the GAL4-based system.

We next examined pairwise interactions of STE5, STE7, and STE11 in various *Sa. cerevisiae* deletion mutants derived from the LexA two-hybrid tester strain, L40. Deletion of the *STE7* gene did not markedly affect the interaction between STE5 and STE11, and interaction between STE5 and STE7 was not affected by deletion of the *STE11* gene (Fig. 2B). However, compared to the interaction detected in a wild-type (*STE5*<sup>+</sup>) strain, the interaction between STE7 and STE11 was significantly reduced in a *ste5* null mutant. In addition, we observed a stronger interaction between STE7 and STE11 fusion proteins when STE5 was overexpressed from a third plasmid than was observed in cells expressing wild-type levels of STE5 (data not shown). These results suggest a role for STE5 in facilitating interaction between STE7 and STE11.

**Additional Interactions Between MAP Kinase Module Components.** We used the two-hybrid system to detect additional interactions between components of the pheromone-responsive MAP kinase module. Interaction between LEXSTE7 and GADSTE7 was detected, as was interaction between LEXSTE11 and GADSTE11 (Table 1), consistent with the observations of other investigators that STE7 and STE11 protein kinases autophosphorylate *in vitro* (23, 36). Formation of STE7 or STE11 complexes was unaffected by deletion of the *STE5* gene (data not shown). Errede *et al.* (36) have provided evidence that STE7 protein kinase purified from yeast extracts phosphorylates and activates FUS3 kinase purified from a bacterial expression system. Consistent with their observations, we detected interaction between STE7 and FUS3 (Table 1). These interactions were maintained even in strains carrying deletions of the *STE5* or *STE11* genes (data not shown). We also detected interaction between STE11 and FUS3 in the two-hybrid system (Table 1). Interaction between these two kinases was maintained in strains carrying deletions of *STE5* or *STE7* (data not shown).

**STE5 Does Not Interact with the *Sc. pombe* byr1 or byr2 Protein Kinases or with the Mammalian MEK, MEKK, or**

**RAF Kinases.** As shown in Table 1, we did not detect interactions between STE5 and the *Sc. pombe* homologs of STE7 and STE11, byr1 or byr2, respectively, suggesting that determinants required for interaction with STE5 have not been conserved in the *Sc. pombe* kinases. STE5 also failed to interact with the mammalian MEK and MEKK, which are structurally related to STE7 and STE11, respectively. Interestingly, interactions between byr1 and byr2 (M.B., unpublished results) or between MEK and MEKK (Table 1) were not detected even when STE5 was overexpressed. We also failed to detect complex formation between STE5 and the mammalian RAF protein kinase (Table 1), a MAP kinase kinase activator that is not related structurally to either MEKK or STE11 (38–40).

## DISCUSSION

Previous studies suggested that STE5 functions at a level between the mating pheromone receptor-coupled G protein and the pheromone-responsive MAP kinase module in *Sa. cerevisiae*. We have used the yeast two-hybrid system to show that STE5 forms complexes with the STE11, STE7, and FUS3 protein kinases that compose the pheromone-responsive MAP kinase module. *Sa. cerevisiae* strains carrying *ste7* or *ste11* null mutations were used to show that the interaction detected between STE5 and STE11 was not bridged by STE7 and that the interaction between STE5 and STE7 was not bridged by STE11. However, interaction between STE7 and STE11 in the two-hybrid system was bridged by STE5. In addition to genetic experiments, we used a combination of epitope-tagged and GST fusion proteins to obtain biochemical evidence for complex formation between STE5 and STE11. In an independent study, Choi *et al.* (41) have also demonstrated complex formation between STE5 and the pheromone-responsive MAP kinase module.

Our results suggest possible functions for STE5. One function of STE5 may be to directly modulate STE11 activity, since it is the N-terminal regulatory domain of STE11 with which STE5 interacts. Another is that STE5 functions as a molecular scaffold that promotes and/or modulates interaction between STE7 and STE11 by interacting directly with both proteins. Alternatively, the dependence on STE5 for interaction between STE7 and STE11 may result from a

requirement that STE11 be modified in a STE5-dependent manner to interact with STE7. With respect to this latter possible STE5 function, we determined that the pairwise interactions detected among the STE5, STE7, and STE11 proteins are not dependent on either the *STE4* or *STE20* gene products (S.M., unpublished observations), each of which acts upstream of STE5 in the *Sa. cerevisiae* pheromone signaling pathway, as judged by genetic analyses (19, 42). Thus, interactions of STE5, STE7, and STE11 do not require basal signaling from either the G protein or STE20.

Besides the pheromone-responsive MAP kinase module, two additional MAP kinase modules are involved in distinct physiological responses in *Sa. cerevisiae*. One of these is required for proper cell wall construction, and the other mediates responses to extracellular salt concentration (for review, see ref. 43). Given the diversity of MAP kinase modules exemplified in yeast, it seems inevitable that an even greater diversity will emerge from metazoans. Indeed, to date at least four distinct MAP kinase homologs (15), three MAP kinase kinase homologs (44, 45), and two MAP kinase kinase kinases, RAF and MEKK (18, 38–40), have been identified in mammals. Furthermore, recent studies suggest that MAP kinases can be activated by multiple pathways (18, 46–48). The existence of multiple MAP kinase modules in a single cell presents an obvious problem—that of potential cross-talk between functionally similar, but distinct, modules. Indeed, we have found that the *STE11* gene, when overexpressed, can suppress the loss of *BCK1* (M.B., S.M., and L. Van Aelst, unpublished results), which encodes the protein kinase structurally related to STE11 that is required for proper cell wall construction in *Sa. cerevisiae* (49). The existence of proteins like STE5, required for interaction of the proper MAP kinase module components, allows the evolution of multiple MAP kinase modules in the same cell that can process signals without interference. Our failure to detect interactions between *Sc. pombe* *byr1* and *byr2* or between mammalian MEK and MEKK, may reflect a requirement for proteins similar to STE5 that facilitate interactions between these pairs of protein kinases. The two-hybrid system we have used to detect interactions between STE5 and members of the pheromone-responsive MAP kinase module of *Sa. cerevisiae* may be used to search for functional homologs of STE5 in *Sc. pombe* and metazoan cDNA–GAD fusion libraries.

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