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The SH3-Domain Protein Bem1 Coordinates Mitogen-Activated Protein Kinase Cascade Activation with Cell Cycle Control in *Saccharomyces cerevisiae*

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The mating mitogen-activated protein kinase (MAPK) cascade has three major outputs prior to fusion: transcriptional activation of many genes, cell cycle arrest in the G_1 phase, and polarized growth. Bem1 localizes near the cortical actin cytoskeleton and is essential for polarized growth during mating. Here we show that Bem1 is required for efficient signal transduction and coordinates MAPK cascade activation with G_1 arrest and mating. *bem1*Δ null mutants are defective in G_1 arrest and transcriptional activation in response to mating pheromone. Bem1 protein stimulates Fus3 (MAPK) activity and associates with Ste5, the tethering protein essential for activation of the MAPK kinase kinase Ste11. Bem1-Ste5 complexes also contain Ste11, Ste7 (MAPK kinase), and Fus3, suggesting that Ste5 localizes the MAPK cascade to Bem1. Strikingly, Bem1 also copurifies with Far1, a Fus3 substrate required for G_1 arrest and proper polarized growth during mating. These and other results suggest that Bem1 may cross-link the Ste5-MAPK cascade complex to upstream activators and specific downstream substrates at the shmoo tip, thus enabling efficient circuitry for G_1 arrest and mating.

In the presence of mating pheromone, *Saccharomyces cerevisiae* haploid cells exit the mitotic cycle and mate (14, 48, 57). Mating is marked by transcriptional activation of mating-specific genes and changes in morphology that include cell division arrest in the G_1 phase (as shown by the absence of a bud) and polarized growth towards the highest gradient of pheromone secreted from potential partner cells (54) (as shown by the formation of a projection). Cell polarization is manifested as cell surface growth towards a mating partner (4) with actin cables accumulating along the growth axis (26) and cortical actin patches concentrating at the growth site (24, 40). This results in the formation of a uninucleate pear-shaped cell (shmoo) that can fuse to a mating partner.

The responses to pheromone are mediated by a receptor-G protein-coupled mitogen-activated protein kinase (MAPK) cascade (18, 27). The MAPKs (Fus3 and Kss1) are activated by a MAPK kinase (Ste7, termed MEK) (23) which is activated by a MAPK kinase kinase (Ste11, termed MEKK) (41). Ste5, a putative scaffolding protein, has at least two roles in this process, including complex formation with the MEKK, MEK, and MAPKs (11, 30) and activation of the MEKK (11). Once activated, the MAPKs phosphorylate many substrates, including a transcription factor (Ste12) (21) and a cyclin-dependent kinase (CDK) inhibitor (Far1) (21, 43, 60) that is also required for proper shmoo orientation during mating (61). Ste12 phosphorylation correlates with activation of mating-specific genes (56). Far1 phosphorylation correlates with G_1 arrest (43) and inhibition of Cln1 and Cln2 CDK complexes (44, 60) that promote budding (3, 16, 33). Curiously, Far1 has one region of homology to Ste5 that overlaps a metal-binding motif similar to a LIM domain found in a variety of transcription factors and cytoskeletal proteins, suggesting that the two proteins have common features of regulation (18).

It is not yet clear how the βγ subunits of the G protein transmit the signal that activates the MEKK Ste11. Ste20, a p21-activated kinase-type kinase (35), was originally placed between the G protein and Ste5 in a linear pathway (31, 47). Because Ste5 and Ste11 associate in vivo, Ste20 could regulate either Ste5 or Ste11 in response to a signal from βγ (11). Ste20 phosphorylates Ste11 in vitro and could be its MAPK kinase kinase kinase (62); however, this phosphorylation has not been shown to activate Ste11. In addition, the β subunit of the G protein can associate with Ste5 (62), suggesting that signal transmission by the G protein involves direct contact with Ste5.

The signaling pathway intersects with Cdc42, Cdc24, and Bem1, proteins that mediate polarized growth during budding and shmooing. Cdc42 is an essential Rho-type GTPase (1, 29), Cdc24 is an essential guanine-nucleotide exchange factor for Cdc42 (68), and Bem1 is a nonessential SH3-domain protein (2, 10) that binds directly to Cdc24 (45) and potentially indirectly to Cdc42 (5) in a large complex (45). Cdc42 and Bem1 localize near cortical actin patches at growth sites and regulate the actin cytoskeleton (7, 9, 34, 46). Cdc42 and Cdc24 are required for pheromone signaling; Cdc42 binds to and activates Ste20, and Cdc24 weakly interacts with Gβ in two-hybrid analysis (55, 67). Thus, Cdc42 and Cdc24 may regulate the MAPK cascade by direct links to Gβ and Ste20. Alternatively, Cdc42 and Cdc24 may indirectly affect the MAPK cascade, with Gβ and Ste20 interactions reflecting a distinct pathway that regulates morphogenesis (28). Bem1 is required for efficient shmooing and is not known to regulate pheromone signaling (10, 32).

In this report we show that in addition to regulating morphogenesis, Bem1 regulates mating at two levels: signaling and cell cycle control. Bem1 is required for efficient signaling that leads to G_1 arrest and transcriptional activation and stimulates

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the activity of the MAPK cascade via parallel inputs from Gβ and Ste20. Bem1 physically links regulators of morphogenesis to the Ste5-multikinase complex; Bem1 interacts with Ste5 in two-hybrid analysis and copurifies with Ste5 in complexes containing Ste11, Ste7, and Fus3. Overproduction of Bem1 preferentially suppresses *fus3* mutant defects in G_1 arrest and mating with little effect on transcription, suggesting that it has an additional function for these processes that is distinct from activation of the MAPK cascade. Consistent with this possibility, Bem1 also copurifies with Far1, the Fus3 substrate required for both G_1 arrest and partner selection during mating. Thus, Bem1 may connect the Ste5-MAPK cascade complex to both upstream activators and downstream MAPK substrates at sites of polarized growth, coordinating protein-protein interactions required for cell cycle control and mating. Our data suggest a direct role for cytoskeleton-associated proteins in cell cycle control in response to a growth factor.

MATERIALS AND METHODS

Microbiological techniques. See Table 1 for list of yeast strains and plasmids used in this study. Standard methods were used for microbial and molecular manipulations (25). *bem1*Δ was constructed in an *sst1*Δ *fus3*Δ strain (EY1095) (21) by using pK02 as described previously (10) to generate EY2273. All phenotypic analysis of *bem1*Δ was done on EY2273 harboring either *FUS3* or *FUS3-HA* on a *CEN* plasmid (pYEE114 or pYEE1102) (21). *ste20*Δ derivatives were made with pEL45 and pEL46-2 (31). *STE11-4* was introduced by transplacement (63) with pSL1655 (58). *far1*Δ was made by transplacement with pYBS94 (21). All disruptions were verified by Southern analysis.

Plasmids. B42 and LexA plasmids are derivatives of pJG4-5 and pJK103 and have been described previously (11).

BEM1. pYBS320 is *BEM1* 2μm *URA3* (*Xho*I-*Sac*I *BEM1* fragment in *Sal*I-*Sac*I sites of YEplac133), pYEE180 is *BEM1* 2μm *TRP1* (*Xho*I-*Sal*I *BEM1* fragment in *Sal*I-*Sac*I sites of Yeplac112), pPB583 is *BEM1* with a *Bgl*II site at the fourth codon, made by site-directed mutagenesis (reads IFHFK . . . [45]), pYEE174 is *LexA-Bem1* (*Bgl*II-*Bgl*II *BEM1* fragment of pPB583 in the *Bam*HI site of Lex202+PL [66]), pYEE186 is *B42-BEM1* (*Bgl*II-*Bgl*II *BEM1* fragment of pPB583 in the *Bam*HI site of pYBS139 [11]), pYEE183 is *GST-BEM1 URA3 LEU2* 2μm (*Bgl*II-*Kpn*I *BEM1* fragment of pPB583 cloned into pYBS305 [11]), pYEE183 is *GST-BEM1 URA3 LEU2* 2μm (*Bgl*II-*Kpn*II *BEM1* fragment of pPB583 cloned into pYBS305 [11]), pYEE185 is *GAL1p-GST-BEM1 LEU2* 2μm (pYEE183 with the *Nco*I site in *URA3* filled in).

STE5. pKC20 is STE5-myc[3] (*STE5* with three *myc* epitopes fused at the *Xho*I site Ste5M, *URA3* 2μm).

FAR1. pTP62 is *GAL1p-FAR1-Myc* (full-length *FAR1* with the *myc* epitope fused to the C terminus, *TRP1 CEN* [43]).

CDC42. pYEE188 is B42-CDC42 (*CDC42* on a *Bgl*II-*Bam*HI fragment from pPB630 with the *Bam*HI site 11 nucleotides from its ATG [gift of A. Bender, University of Indiana]).

Pheromone sensitivity assays. Halo assays were performed as described previously (20) with equal numbers of cells from an overnight culture. Plates were photographed after ~30 h at 30°C. The percentage of unbudded cells was quantitated after a 2-h exposure to 50 nM α-factor as described previously (20).

β-Galactosidase assays. LacZ assays were done as described previously (22, 30). Strain AMR70 (P. Bartel, State University of New York, Stony Brook), which has one integrated copy of *LexAop-LacZ*, was used for two-hybrid analysis.

Yeast extracts and kinase assays. Strains were grown at 30°C in selective SC medium with 2% glucose to an A_{600} of 0.4 to 0.8 then induced for 1 h with 50 nM α-factor as described previously (21). Genes under control of the *GAL1* promoter (i.e., genes encoding GST-Bem1, Ste11M, Ste7M, and Far1M) were induced by growing strains in medium with 2% galactose for 5 h prior to induction with 50 nM α-factor. Whole-cell extracts were prepared as described previously (30), except that the concentration of Triton X-100 was 0.6% in extracts used for copurification. Kinase assays were done as described previously (21).

Purification of GST proteins. Glutathione *S*-transferase (GST)-Bem1 was purified with glutathione agarose from 640 to 1,000 μg of extract in 0.4 ml of modified H buffer–150 mM NaCl as described previously (30). Samples were resuspended in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min prior to electrophoresis.

Thrombin cleavage of purified GST-Bem1. Glutathione agarose beads containing GST-Bem1 were washed three times with modified H buffer containing 125 mM NaCl and once with thrombin cleavage buffer (TCB) (2.5 mM $CaCl_2$, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) and then resuspended in 20 μl of TCB. Five microliters each of thrombin (0.1 U/μl; T-6756; Sigma) and heparin (10 U/μl; H-3393; Sigma) were added, and the sample was incubated for 10 min at 30°C. Samples were then treated with SDS before resuspension in 2× loading buffer.

Western blotting (immunoblotting). Western blotting was performed as described previously (21, 30) with chemiluminescence detection kits (Amersham and Boehringer). 12CA5 and 9E10 mouse monoclonal antibody tissue culture supernatants were from the Harvard University antibody facility; anti-GST affinity-purified rabbit antiserum was a gift of R. Van Etten, Harvard Medical School.

RESULTS

Excess Bem1 suppresses G_1 arrest defect of *fus3* mutants. We isolated the *BEM1* gene as a dosage suppressor of the α-factor resistance of a *fus3-2* mutant (Fig. 1A) in a screen that identified *FAR1* and *STE5* (21, 30, 50). The isolation of *BEM1* as a suppressor of a G_1 arrest defect was surprising given that *BEM1* is thought to regulate mating by facilitating only the cell polarization that is required for projection formation (9, 10). A trivial explanation for the apparent increase in pheromone sensitivity is that overexpression of *BEM1* causes a general growth inhibition or sickness. However, three observations argue that *BEM1* overexpression specifically promotes pheromone-mediated G_1 arrest. First, *fus3* mutants harboring the *BEM1* plasmid have a higher percentage of unbudded cells after a 2-h exposure to α-factor than do cells harboring a control plasmid (Table 2), as was found for *FAR1* (21). Second, the *BEM1* plasmid restores proper G_1 arrest morphology to the arrested *fus3* cells, as shown by an increased percentage of unbudded cells that are shmoos; this contrasts with *FAR1*, which does not affect shmoo morphology (data not shown; also reference 51). Third, parallel experiments with genes that slow growth when overexpressed do not result in enhanced G_1 arrest (52).

***bem1* mutants are defective in G_1 arrest and transcription.** The effects of Bem1 overproduction on G_1 arrest might not reflect a true function of Bem1 but instead might be due to expressing high levels of Bem1 protein. We therefore examined the pheromone sensitivity of four *bem1* mutants, a *bem1*Δ null mutant (*bem1*::*LEU2*), two *sst1 bem1* double mutants (truncated *bem1* alleles) previously shown to be defective in shmoo formation (*bem1-S1* and *bem1-S2*) (10), and an *sst1*Δ *bem1*Δ double-null mutant constructed in our W303 strain background (Table 1). *SST1* (also known as *BAR1*) encodes a protease that degrades α-factor; its absence causes an ~50-fold increase in α-factor sensitivity (57). All four *bem1* mutants are less sensitive to mating pheromone than are isogenic wild-type strains (Fig. 1B), with the decrease in pheromone sensitivity correlating with the severity of the *bem1* mutation on growth and mating (e.g., that of *bem1*Δ is much greater than that of *bem1-S1*, which is greater than that of *bem1-S2*). The *bem1-S* strains exhibit a small increase in α-factor resistance that is detectable in the presence of a low concentration of α-factor (Fig. 1B, middle panel). A *bem1*Δ null mutant is significantly more α-factor-resistant than are the *bem1-S* mutants and grows at higher concentrations of α-factor in a halo assay (Fig. 1B, left and right panels [*SST1* or *sst1*Δ]). The *bem1*Δ null mutants form turbid halos with diameters not quite equal to those of isogenic *BEM1* strains (even at the earliest time point examined) (Fig. 1B, left panel), suggesting they are defective in sensing pheromone. The reduced G_1 arrest capacity of a *bem1*Δ null mutant is particularly apparent in experiments in which the signal transduction cascade is constitutively activated by overproduction of either Ste4 (Gβ) (12, 42) or by combined expression of Ste5 and Ste11 (11). Overproduction of either Gβ or Ste5-Ste11 causes significantly less growth inhibition in a *bem1*Δ null mutant than in wild-type cells, even though the *bem1* mutants ordinarily grow more poorly than wild-type cells (data not shown).

*bem1*Δ null mutants are also defective in transcriptional activation of *FUS1*, a mating-specific gene whose transcription requires the activity of the Ste12 transcription factor (36).

TABLE 1. Yeast strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
EY492	*MAT***a** *lys9* (L1543)	J. Brill
W303a	*MAT***a** *FUS3 KSS1 ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100* Gal$^+$	R. Rothstein
Isogenic derivatives of W303a		
EY700	*fus3-6*::*LEU2*	19
EY957	*sst1*Δ	21
EY940	*sst1*Δ *fus3-6*::*LEU2*	21
EY941	*sst1*Δ *fus3-7*::*HIS3*	21
EY966	*sst1*Δ *fus3-6*::*LEU2 kss1*::*HIS3*	21
EY1095	*sst1*Δ *fus3-8*::*ADE2*	21
EY1112	*sst1*Δ *fus3-8*::*LEU2 kss1*Δ::*ADE2 his3*Δ*200 lys2*::*FUS1-HIS3*	This study
EY1118	*sst1*Δ *his3*Δ*200 lys2*::*FUS1-HIS3*	This study
EY1119	*sst1*Δ *kss1*::*HIS3*	This study
EY1463	*sst1*Δ *ste20*Δ::*TRP1*	This study
EY1262	*sst1*Δ *far1*Δ *lys2*::*FUS1-HIS3 his3*Δ*200*	This study
EY1298	*sst1*Δ *STE11-4 far1*Δ *lys2*::*FUS1-HIS3 his3*Δ*200*	This study
EY1336	*sst1*Δ *STE11-4 ste5*Δ*1*::*URA3 lys2*::*FUS1-HIS3 his3*Δ*200*	This study
EY1883	*sst1*Δ *STE11-4 ste4*Δ::*LEU2 lys2*::*FUS1-HIS3 his3*Δ*200*	This study
EY1981	*sst1*Δ *STE11-4 ste20*Δ::*URA3 far1*Δ *lys2*::*FUS1-HIS3 his3*Δ*200*	This study
EY2019	*sst1*Δ *ste5*Δ::*TRP1*	12
EY1921	*sst1*Δ *ste5*Δ::*TRP1 fus3-8*::*ADE2*	12
EY1922	*sst1*Δ *ste5*Δ::*TRP1 ste7*Δ::*LEU2*	12
EY1923	*sst1*Δ *ste5*Δ::*TRP1 ste11*Δ::*URA3*	12
EY1451	*sst1*Δ *fus3-8*::*ADE2* + pYEE121 [*FUS3HA CEN URA3*]	This study
EY2307	*sst1*Δ *fus3-8*::*ADE2 bem1*Δ::*LEU2* + pYEE121	This study
EY2371	*sst1*Δ *bem1*Δ::*LEU2*	This study
BY58	*sst1*Δ *fus3-2 KSS1*	50
BY345	*sst1*Δ *fus3-205 KSS1*	50
BY369	*sst1*Δ *fus3-2 kss1*::*HIS3*	50
BY360	*sst1*Δ *fus3-205 kss1*::*HIS3*	50
Isogenic derivatives of IH1783		
IH1783	*MAT***a** *trp1 leu2 ura3 his4 can1*	10
IH2596	*MAT***a** *bem1*Δ::*LEU2 trp1 leu2 ura3 his4 can1*	10
Isogenic derivatives of JC2-1B		
JC2-1B	*MAT***a** *HML***a** *HMR***a** *bar1-1 ade2-101 ura3-52 met1*	11
JC-G11	*MAT***a** *HML***a** *HMR***a** *bar1-1 ade2-101 ura3-52 met1 bem1-S1*	11
JC-F5	*MAT***a** *HML***a** *HMR***a** *bar1-1 ade2-101 ura3-52 met1 bem1-S2*	11
Plasmids		
pSB231	*FUS1-lacZ URA3 CEN*	59
pYBS45	*FUS1-LacZ LYS2 CEN*	50
pYBS320	*BEM1 URA3* 2μm	50
pYEE180	*BEM1 TRP1* 2μm	This study
pYBS305	*GAL1p-GST URA3 LEU2* 2μm	30
pYEE161	*GAL1p-GST LEU2* 2μm	11
pYEE174	*ADH1p-LexA-BEM1 HIS3* 2μm	This study
pYEE183	*GAL1p-GST-BEM1 URA3* 2μm	This study
pYEE185	*GAL1p-GST-BEM1 LEU2* 2μm	This study
pYEE186	*GAL1p-B42-BEM1 TRP1* 2μm	This study
pYEE188	*GAL1p-B42-CDC42 TRP1* 2μm	This study
pYBS102	*FAR1 URA3* 2μm	21
pYEE74	*FUS3 URA3* 2μm	20
pYEE114	*FUS3 URA3 CEN*	21
pYEE121	*FUS3-HA URA3 CEN*	21
pYEE1102	*FUS3-HA HIS3 CEN*	21
pYEE1108	*FUS3-HA URA3 ADE2 CEN*	21
pKC20	*STE5M URA3* 2μm	This study
pKC55	*GAL1p-STE7M HIS3 CEN*	11
pNC245	*GAL1p-STE11M TRP1 CEN*	58
pTP62	*GAL1p-FAR1M TRP1 CEN*	43
pYBS146	*GAL1p-B42-STE5* (aa[a] 24–917) *TRP1* 2μm	11
pYBS216	*GAL1p-B42-STE5*Δ*1* (aa 24–336) *TRP1* 2μm	11
pYBS219	*GAL1p-B42-STE5*Δ*2* (aa 24–586) *TRP1* 2μm	11
pYBS214	*GAL1p-B42-STE5*Δ*3* (aa 336–917) *TRP1* 2μm	11
pYBS308	*GAL1p-B42-STE5*Δ*4* (aa 241–336) *TRP1* 2μm	11
pYBS323	*GAL1p-B42-STE5*Δ*5* (aa 24–917 Δ143-309) *TRP1* 2μm	11
pYBS148	*GAL1p-B42-FAR1 TRP1* 2μm	21
pYBS316	*ADH1p-LexA-STE20* (aa 71–) *HIS3* 2μm	21
LexA-Fus3	*ADH1p-LexA-FUS3 HIS3* 2μm	63
LexA-Bicoid	*ADH1p-LexA-Bicoid HIS3* 2μm	63
pSB231	*FUS1-LacZ URA3 CEN*	57
pJB207	*FUS1-LacZ LEU2* 2μm	19

[a] aa, amino acid.

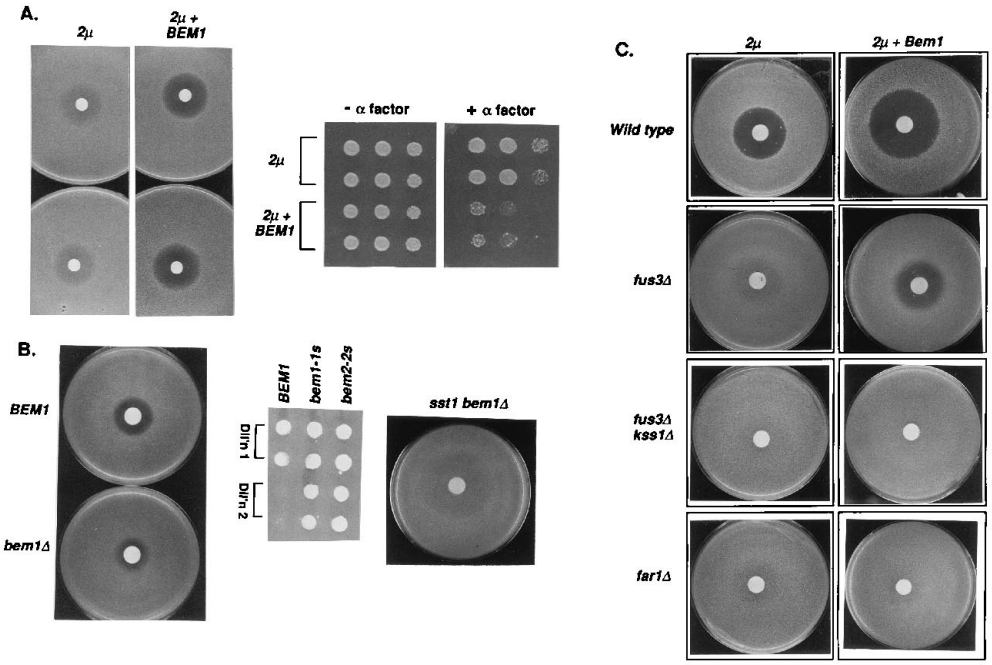

FIG. 1. *BEM1* is required for G_1 arrest. (A) Dosage suppression of a *MAT***a** *sst1*Δ *fus3-2* strain (BY58) by a *BEM1* multicopy plasmid (pYBS320). Left two panels, halo assays (5 μl of 0.1 μM α-factor on the disks) of duplicate transformants containing either YEp24 (left) or pYBS320 (right); right two panels, same strains spotted onto selective media with (+) or without (−) 5 μg of α-factor (threefold serial dilutions of 5×10^7 cells per ml). (B) Pheromone sensitivity of *bem1* mutants. Left panel, halo assays of *MAT***a** *BEM1* and *MAT***a** *bem1*Δ::*LEU2* strains (IH1783 and IH2596, respectively; 7 μl of 0.5 mM α-factor on the disk); middle panel, *MAT***a** *bar1 BEM1*, *bem1-S1*, and *bem1-S2* strains (JC-G11, JC-F5, and JC2-1B, respectively) spotted on plates containing 1.5 μg of α-factor (dilution [Dil'n] 1 = 10^8 cells per ml; dilution 2 = 4×10^6 cells per ml); right panel, halo assay of a *MAT***a** *sst1*Δ *bem1*Δ strain (EY2273 [pYEE114]) done as described for panel A except that *bem1*Δ null and *BEM1* control strains were grown at 24°C instead of 30°C. (C) *BEM1* requires either *FUS3* or *KSS1* and *FAR1* to promote G_1 arrest. Halo assays of *sst1*Δ (EY957), *sst1*Δ *fus3*Δ (EY1095), *sst1*Δ fus3Δ kss1Δ (EY966), and *sst1*Δ *far1*Δ (EY1262) strains harboring either a 2μm plasmid (Yeplac112 or pYBS305) or a 2μm-BEM1 plasmid (pYEE180 or pYEE185) done as described for panel A. EY1095 has Yeplac112 and pYEE180 on selective medium containing dextrose. All other strains have pYBS305 and pYEE185 on selective medium containing 2% galactose (pYEE185 is a *GAL1-GST-BEM1* fusion). pYEE180 has no effect in EY1262 or EY966 and a much weaker effect than pYEE185 in EY957.

Compared with wild-type strains, *bem1*Δ null mutants express approximately 30% basal levels and 20% α-factor-induced levels of a *FUS1-lacZ* reporter gene (Table 3). Thus, *BEM1* is required for efficient signal transduction that leads to both G_1 arrest and transcriptional activation. In addition, a *bem1*Δ null mutant exhibits an ~60-fold decrease in mating (Table 4). The defects in morphogenesis and mating of a *bem1*Δ mutant could be, at least in part, an indirect consequence of a defect in signal transduction.

TABLE 2. Overexpression of *BEM1* promotes G_1 arrest[a]

Strain	Plasmid description	% Unbudded cells	
		−αF	+αF
*sst1*Δ *FUS3 kss1*Δ	2μm	50	95
	2μm-*BEM1*	52	95
	2μm-*FAR1*	46	98
*sst1*Δ *fus3*Δ *KSS1*	2μm	45	52
	2μm-*BEM1*	43	79
	2μm-*FAR1*	35	81

[a] Cells were grown at 30°C in SC medium lacking uracil (to select for the plasmids) to an A_{600} of 0.3 to 0.6. Equal numbers of cells were pelleted and resuspended at an A_{600} of 0.5 in the same medium containing 50 nM α-factor (+αF) and then incubated with shaking for 2 h at 30°C (−αF, without α-factor). Cells were fixed at $t = 0$ h and $t = 2$ h as described previously (20). A total of 200 to 300 cells were counted for each sample twice after brief sonication. Similar findings were obtained for each of three transformants assayed for each plasmid. Plasmids: 2μm, YEp24; 2μm-*BEM1*, pYBS320; and 2μm-*FAR1*, pYBS102. Similar results were found for *FUS3 kss1*Δ and *FUS3 KSS1* strains. pYBS320 and pYBS102 had no effect on G_1 arrest in a *fus3*Δ *kss1*Δ double-null mutant (data not shown). The presence of Yep24-*BEM1* (pYBS320) in *FUS3 kss1*Δ (EY1119) and *fus3-2 KSS1* (BY58) strains caused a significant enhancement in the size of the projection formed in response to α-factor, with less of an effect on projection formation in the *fus3*Δ *KSS1* (EY1095) strain. Yep24-*FAR1* (pYBS102) had no effect on projection formation in any of the strains. Details of these observations are to be published elsewhere (51).

Bem1 stimulates Fus3 kinase activity. We tested the dependence of Bem1 suppression on Fus3 and its related MAPK Kss1. Overproduction of Bem1 enhances pheromone sensitivity in wild-type strains, showing that the effect is not dependent upon an enfeebled Fus3 (Fig. 1C). Bem1 also restores pheromone sensitivity to a *fus3-2 kss1*Δ strain (see Fig. 4) that is solely dependent upon the residual activity of Fus3 for signal transduction and G_1 arrest and to a *fus3*Δ *KSS1* strain dependent upon *KSS1* (Fig. 1C). By contrast, Bem1 has no effect in a *fus3*Δ *kss1*Δ double null mutant that is completely blocked for signal transduction. Thus, Bem1 requires either Fus3 or Kss1 to promoter G_1 arrest.

Bem1 could promote G_1 arrest by stimulating MAPK activity. We assayed Fus3 kinase activity in wild-type cells that require pheromone to activate Fus3 (21) and in *STE11-4* cells with constitutive levels of active Fus3 due to a dominant activating mutation in the catalytic domain of Ste11 (58). In wild-type cells, excess *BEM1* stimulates Fus3 activity two- to threefold in the presence of α-factor (Fig. 2A). In *STE11-4* cells, Bem1 stimulates Fus3 activity both in the absence and presence of α-factor. Thus, Bem1 provides a limiting function that stimulates Fus3 kinase activity at a step in the pheromone response pathway that is upstream of Fus3 and sensitive to the level of active Ste11.

TABLE 3. Effect of *BEM1* on *FUS1* transcription as measured with a *FUS1-LacZ* fusion[a]

Expt no.	Strain	Plasmid	Mean β-galactosidase ± SD (Miller units) at αF concn (M)				
			0	5×10^{-9}	5×10^{-8}	5×10^{-7}	5×10^{-6}
1	*BEM1*		0.54 ± 0.10			24 ± 2.0	59 ± 2.0
	*bem1*Δ		0.17 ± 0.01			5 ± 1.0	11 ± 2.0
2	*FUS3 KSS1 sst1*Δ	2μm	0.36 ± 0.04	11 ± 1.8	33 ± 7.2		
		2μm-BEM1	0.44 ± 0.03	28 ± 4.3	74 ± 6.0		
3	*fus3-2 kss1*Δ *sst1*Δ	2μm	0.47 ± 0.06	0.41 ± 0.03	1.2 ± 0.5		
		2μm-*BEM1*	0.46 ± 0.02	0.60 ± 0.02	2.0 ± 0.3		
	*fus3-205 kss1*Δ *sst1*Δ	2μm	0.41 ± 0.1	3.1 ± 0.2	4.1 ± 0.4		
		2μm-*BEM1*	0.35 ± 0.1	2.9 ± 0.4	3.5 ± 0.2		

[a] Results of three separately assayed experiments are given. Cells were grown to an A_{600} of ~0.3 to 0.6 at 30°C, and then equal numbers of cells were pelleted and resuspended at an A_{600} of 0.5 in medium containing the indicated amount of α-factor and induced for 90 min. After induction, cells were pelleted, washed once in ice water and frozen at −80°C. Extracts were assayed for β-galactosidase activity as described previously (22). Values (Miller units) are given as means ± standard deviations for three or more transformants. For experiment 1, strains IH1783 and IH2596 harboring pSB231 (*FUS1-lacZ CEN URA3*) were grown at 25°C in SC medium lacking uracil. For experiment 2, strain EY1118 harboring pYBS45 (*FUS1-LacZ CEN LYS2*) and either Yep24 (2μm) or pYBS320 (2μm-*BEM1*) was grown at 30°C in SC medium lacking uracil and lysine. For experiment 3, Strains BY369 and BY360 with *FUS1-LacZ* (pSB231 *FUS1-LacZ CEN URA3*) and either Yeplac112 (2μm) or pYEE180 (2μm-*BEM1*) were grown at 30°C in SC medium lacking uracil and tryptophan. Overexpression of *BEM1* has no effect on *FUS1* expression in a *fus3*Δ *kss1*Δ strain as measured with *FUS1-LacZ* and *FUS1-HIS3* fusions. Note that pYBS320 and pYEE180 equivalently stimulate FUS3 kinase activity.

Bem1 stimulates Fus3 in the absence of Ste20. Ste20 is a potential intersection point for Bem1 in the MAPK cascade, since Bem1 interacts with the Rho-type G protein Cdc42 (e.g., Bem1 binds to Cdc24, which binds to Cdc42 [5, 45, 68], which binds to Ste20 [55]). A *ste20*Δ mutation in a W303 strain background almost completely blocks signal transduction, as shown by complete resistance to α-factor and sterility (47), absence of Fus3 activity (data not shown) and very low levels of α-factor-induced *FUS1* transcription (0.8% of wild-type levels [47]). Bem1 overproduction does not restore pheromone sensitivity to a *ste20*Δ strain; however, this absence of suppression does not prove that Bem1 acts through Ste20, because Bem1 also has no effect in other *ste* mutants tested, including *ste4*Δ (Gβ) and *ste5*Δ.

However, in the course of our experiments, we discovered that the presence of a *STE11-4* mutation allows α-factor to signal efficiently to Fus3 in the absence of Ste20. The *STE11-4* mutation restores pheromone sensitivity, fertility, and Fus3 activity to a *ste20*Δ strain (although not to wild-type levels) (Fig. 2; also see Fig. 4). By contrast, the *STE11-4* mutation does not restore pheromone sensitivity or fertility to either *ste4*Δ (Gβ) (see Fig. 4) or *ste5*Δ null mutants (data not shown), indicating that Gβ and Ste5 are both required for *STE11-4* to

TABLE 4. Quantitative mating assays

Expt no.	*MAT***a** strain	% Prototrophs from mating with plasmid[a]:			
		None	2μm	2μm-*BEM1*	2μm-*FAR1*
1	*sst1*Δ *BEM1*	2.3			
	*sst1*Δ *bem1*Δ	0.038			
2	*sst1*Δ *FUS3 KSS1*		5.6		
	*sst1*Δ *fus3*Δ *KSS1*		0.00078	0.0078	0.00056
	*sst1*Δ *fus3-2 kss1*Δ		0.011	0.62	0.013
	*sst1*Δ *fus3-205 kss1*Δ		0.016	0.49	
	*sst1*Δ *STE11-4 far1*Δ		0.60	0.60	

[a] Diploid formation was quantitated as the percentage of prototrophs formed in a 4-h mass mating between equal numbers of *MAT*α and *MAT***a** cells on solid media at 30°C as described previously (20, 22). For experiment 1, strains EY1451 and EY2307 were mated to strain EY492 (*MAT*α *lys9*) in yeast-peptone-dextrose medium. For experiment 2, strains EY957, EY940, BY369, BY360, and EY1298, each harboring a single multicopy plasmid (Yep24, pYBS320, or pYBS102), were mated to strain EY492 on SC medium lacking uracil to select for the plasmids. Numbers represent the average percentages from matings done on two transformants.

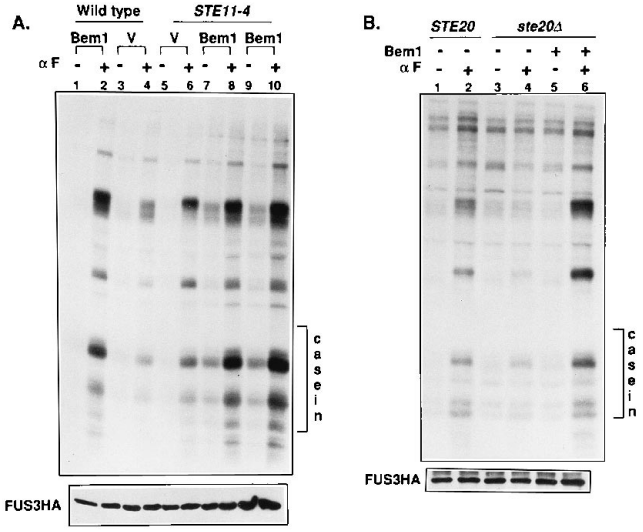

FIG. 2. Overproduction of Bem1 stimulates Fus3 kinase activity in *STE20* and *STE11-4 ste20*Δ strains. (A) Effect of Bem1 in wild-type and *STE11-4* strains. Top panel, immune complex kinase reactions of Fus3-HA in the presence of 1 μg of casein as described previously (21). Strains were induced for 1 h with α-factor where indicated (+). The autoradiogram is from an ~1-h exposure. Bottom panel, immunoblot of duplicate Fus3-HA immune complexes detected with 12CA5 antibody. V, Vector. Lanes: 1 and 2, EY1095 *fus3*Δ + Fus3-HA (pYEE1102) + Bem1 2μm (pYBS320); 3 and 4, EY1095 + Fus3-HA (pYEE1102) + 2μm (Yep24); 5 and 6, EY1298 *STE11-4 far1*Δ + Fus3-HA (pYEE1108) + 2μm (Yep13); 7 to 10, two transformants of EY1298 + Fus3-HA (pYEE1108) + *BEM1* 2μm (pYBS320). (B) Effect of Bem1 in a *STE11-4 ste20*Δ strain. Fus3-HA kinase assays were performed as described for panel A. Lanes: 1 and 2, EY1298 (*STE11-4 far1*Δ) + Fus3-HA (pYEE1108) + 2μm (YepLac112); 3 and 4, EY1981 = *STE11-4 ste20*Δ *far1* + Fus3-HA (pYEE1108) + 2μm (YepLac112); 5 and 6, EY1981 + Fus3-HA (pYEE1108) + *BEM1* 2μm (pYEE180).

suppress a *ste20*Δ block in signaling. This result is important because it (i) substantiates the existence of a parallel pathway of signaling from the G protein to Ste5 and Ste11 that can function in the absence of Ste20 (see Fig. 6), (ii) provides strong support for the view that Ste20 normally activates Ste11, and (iii) provides a strain background in which to test whether Bem1 requires Ste20 in order to stimulate the MAPK cascade.

Overproduction of Bem1 causes increased Fus3 activity (Fig. 3B) and pheromone sensitivity (Fig. 4A) in a *STE11-4 ste20*Δ strain, indicating that Bem1 does not require Ste20 to stimulate Fus3. However, Ste20 is required for maximal response to pheromone in the absence and presence of excess Bem1. By contrast, Bem1 overproduction does not restore pheromone sensitivity to either *STE11-4 ste5*Δ or *STE11-4 ste4*Δ strains (Fig. 4A). Thus, Bem1 can stimulate the MAPK cascade by both Ste20-dependent and -independent mechanisms that require Gβ and Ste5, further arguing against a simple linear pathway from Gβ to Ste5 and Ste11 (see Fig. 6).

Bem1 interacts with Ste5 in two-hybrid analysis. The absence of a complete requirement for Ste20 suggested that Bem1 does not act solely through the Ste20 step in the MAPK cascade. We tested whether Bem1 interacts with other components in the mating signal transduction cascade, using a two-hybrid system (66). Bem1 was fused to the LexA DNA-binding domain and tested for interaction with signal transduction components each fused to the B42 transcriptional activation domain by using a *LexAop-LacZ* reporter gene (11). Coexpression of LexA-Bem1 with B42-Ste5 causes a 75-fold increase in β-galactosidase activity, indicating that the two proteins interact. By contrast, LexA-Bem1 did not interact with Ste20, Ste11, Ste7, or Fus3 (Table 5) (nor did B42-Bem1 interact with LexA-kinase fusions [data not shown]) and interacted only weakly (if at all) with B42-Cdc42, which interacts with Ste20 (Table 5) (55).

Results from a previous two-hybrid analysis using B42-Ste5 deletion derivatives suggests that Ste5 provides separate binding sites for Ste11, Ste7, and Fus3 (11). We attempted to map a Ste5 interaction domain for Bem1, using the same set of B42-Ste5 derivatives, and found that none interact with LexA-Bem1 (Table 5) although they each interact with either Ste11, Ste7, or Fus3 (11). While the absence of an interaction has many interpretations, one possibility is that the association between Ste5 and Bem1 requires full-length Ste5, in contrast to the protein kinases.

Ste5 copurifies with Bem1. We determined whether Ste5 copurifies with Bem1, using a GST-Bem1 fusion that complements a *bem1*Δ strain for mating and growth defects and a functional *myc* epitope-tagged derivative of Ste5 (Ste5M) (Materials and Methods). Ste5M copurifies with GST-Bem1 but not GST both in the absence and presence of α-factor (Fig. 3 lanes 5, 6, 11, and 12) (the reduction in the amount of Ste5M in lane 12 in this experiment is accounted for by the amount of Ste5M in the whole-cell extract preparation [data not shown, but see Fig. 5]). This finding is consistent with the ability of Bem1 to activate Fus3 in both the absence and presence of pheromone (Fig. 2).

Ste11, Ste7, and Fus3 copurify with GST-Bem1 in the presence of excess Ste5. Because Ste5 forms complexes with Ste11, Ste7, and Fus3, we determined whether these protein kinases also copurify with Bem1. In agreement with the two-hybrid analysis results, Ste11, Ste7, and Fus3 do not copurify with GST-Bem1 in parallel experiments using functional epitope-tagged derivatives of these proteins (Fig. 3). However, because Fus3 activity is absolutely dependent upon the activity of Ste11 and Ste7 and because Ste5 is known to bind all three protein kinases (11), we examined whether Ste11, Ste7, or Fus3 might

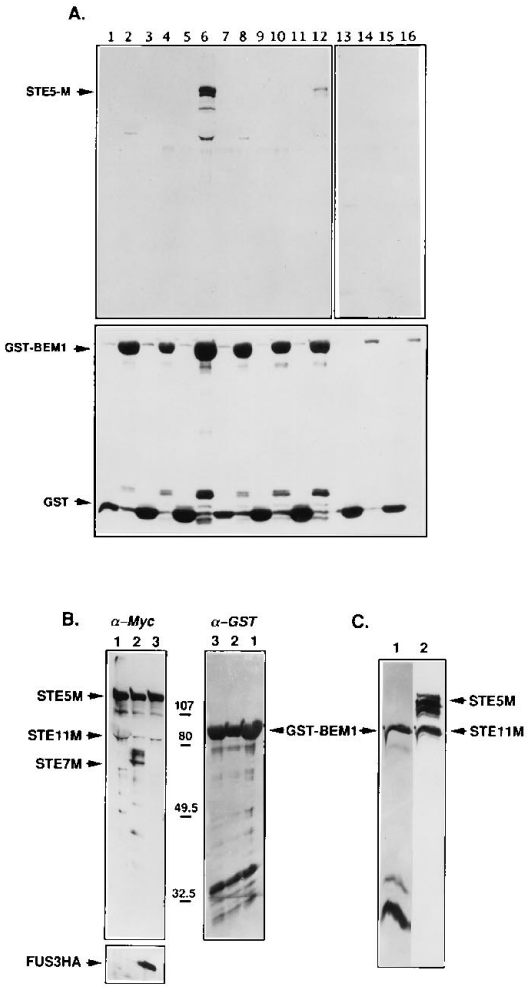

FIG. 3. Copurification of Ste5M and associated kinases with GST-Bem1. (A) Immunoblot analysis of Ste5M, Ste11M, Ste7M, and Fus3HA in purified GST-Bem1 complexes from *ste5*Δ strains. GST-Bem1 was purified from whole-cell extracts with glutathione-agarose. Duplicate samples were separated by SDS-PAGE and immunoblotted with either 9E10 (to detect Ste5M, Ste11M, and Ste7M), 12CA5 (to detect Fus3-HA), or affinity-purified rabbit antiserum against GST (α-GST) (to detect GST-Bem1). Cells were induced for 1 h with 50 nM α-factor after 5.5 h of pregrowth in galactose medium to induce expression of GST-Bem1, Ste11M, and Ste7M. Plasmids are GST (pYBS305), GST-Bem1 (pYEE185), Ste7M (pKC55), Ste11M (pNC245), Ste5M (pKC20), and Fus3-HA (pYEE1102). Lane 1, *ste5*Δ *ste7*Δ + GST + Ste7M; lane 2, *ste5*Δ *ste7*Δ + GST-Bem1 + Ste7M; Lane 3, *ste5*Δ *ste11*Δ + GST + Ste11M; lane 4, *ste5*Δ *ste11*Δ + GST-Bem1 + Ste11M; lane 5, *ste5*Δ + GST + STE5M; lane 6, *ste5*Δ + GST-Bem1 + Ste5M; lanes 7 to 12, same as lanes 1 to 6 with α-factor added; lane 13, *ste5*Δ *fus3*Δ + GST + Fus3-HA; lane 14, *ste5*Δ *fus3*Δ + GST-Bem1 + Fus3-HA; lane 15, *ste5*Δ *fus3*Δ + GST + Fus3-HA + α-factor; lane 16, *ste5*Δ *fus3*Δ + GST-Bem1 + Fus3-HA + α-factor. (B) Immunoblot analysis of Ste11M, Ste7M, and Fus3-HA in purified GST-Bem1 complexes from *ste5*Δ strains also containing Ste5M. GST-Bem1 was purified, and duplicate samples were immunoblotted with either α-Myc (left panel) or α-GST (right panel) as done for panel A. Lane 1, *ste5*Δ *ste11*Δ + GST-Bem1 (pYEE185) + Ste11M (pNC245) + STE5M (pKC20); lane 2, *ste5*Δ *ste7*Δ + GST-Bem1 + Ste7M (pKC55) + STE5M (pKC20); lane 3, *ste5*Δ *fus3*Δ + GST-Bem1 (pYEE185) + Fus3-HA (pYEE1102) + STE5M (pKC20). Ste11M, Ste7M, and Fus3-HA are not detected in parallel experiments with GST. The numbers between the panels are molecular masses (in kilodaltons). (C) Thrombin cleavage of GST-Bem1 enhances detection of Ste11M. The experiment was performed as described for panel B except that the purified GST-Bem1 complexes were treated with thrombin (Materials and Methods) prior to resuspension in 2× loading buffer. Lanes 1 and 2, *ste5*Δ *ste11*Δ + Ste5M (pKC20) + pGALSte11M (pNC245) + pGALGST-Bem1 (pYEE185). The signal indicated as Ste11M is not present in identically treated GST-Bem1 preparations from strains containing Ste5M + Ste7M or Ste5M + Fus3-HA or GST preparations from Ste5M + Ste11M strains (data not shown).

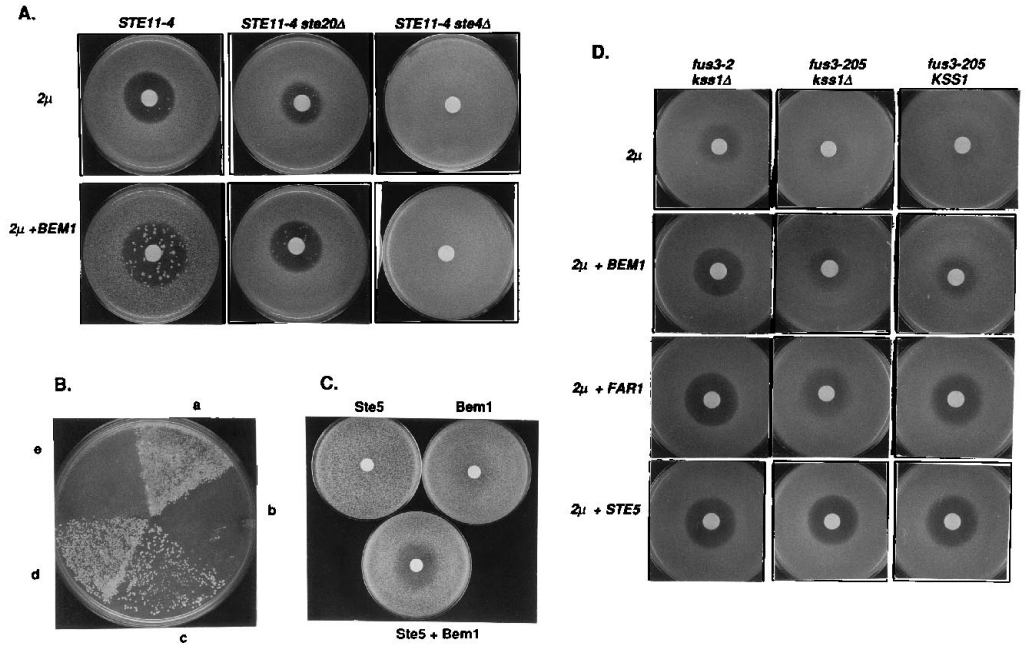

FIG. 4. Excess *BEM1* increases pheromone sensitivity of *ste20*Δ and *fus3 kss1*Δ strains. (A) Effect of Bem1 on pheromone sensitivity of *STE11-4 ste20*Δ and *ste20*Δ strains. Halo assays were performed as described in the legend to Fig. 1 except that plates were incubated at room temperature for ~30 h. (B) Mating assay of Ste$^-$ strains. Patches of *MAT***a** cells were mated to a *MAT*α *lys9* (EY492) lawn for 6 h at 30°C, and prototrophs were selected on minimal medium as described previously (20). a, *sst1*Δ (EY957); b, *sst1*Δ *ste20*Δ (EY1463); c, *sst1*Δ *STE11-4 ste20*Δ (EY1981); d, *sst1*Δ *STE11-4* (EY1298); e, *sst1*Δ *STE11-4 ste4*Δ (EY1882). (C) Suppression of *ste20*Δ G_1 arrest defect by cooverexpression of Bem1 and Ste5. Halo assays of a *MAT***a** *sst1*Δ *ste20*Δ strain (EY1463) harboring either Ste5M 2μm (pKC20), GST-Bem1 2μm (pYEE185), or both Ste5M and GST-Bem1 on selective medium with 2% galactose. (D) Suppression of the G_1 arrest defect of *fus3* point mutants by excess Bem1. Halo assays of *fus3 KSS1* and *fus3 kss1* strains harboring either *BEM1*, *STE5*, or *FAR1* on multicopy plasmids were done as described in the legend to Fig. 1. The relative amounts of *FUS1* transcription in the *fus3* strains as assessed by quantitative Northern (RNA) analysis (50, 51) are 100% for EY957 *FUS3 KSS1*, ~19% for BY369 *sst1*Δ *fus3-2 kss1*Δ, ~70% for BY345 *sst1*Δ *fus3-205 KSS1*, and ~14% for BY360 *sst1*Δ *fus3-205 kss1*Δ.

associate with Bem1 indirectly through Ste5. If this were the case, then our ability to detect a Bem1-protein kinase association would be dependent upon the levels of Ste5 in our preparations. We first tested this possibility by overexpressing Ste5 in strains harboring LexA-Bem1 and B42-kinase, because Ste5 has been shown to facilitate two hybrid interactions between Ste11 and Ste7 under the same conditions (11). However, Ste5 had no effect on the ability of LexA-Bem1 to interact with any of the kinases (data not shown).

TABLE 5. β-Galactosidase activity induced by interactions between Bem1, Ste5, and Far1 in a two-hybrid system[a]

B42 fusion with:	β-Galactosidase activity (Miller units) for LexA fusion with:			
	Bem1	Fus3	Bicoid	Ste20
Ste11	0.7			
Ste7	1.0			
Fus3	1.0			
Ste5	85	151	1.1	
Ste5Δ1	0.6			
Ste5Δ2	1.4			
Ste5Δ3	1.0			
Ste5Δ4	1.2			
Ste5Δ5	0.9			
Bem1			1.0	1.0
Far1	1,900		1.0	
Cdc42	5.0	0.4	0.9	15

[a] Assays were done in strain AMR70 containing a reporter *LexAop-LacZ* gene integrated at the *URA3* locus. Values are Miller units of β-galactosidase activity averaged from two independent plasmid-bearing transformants as described previously (11). Samples were assayed twice with the exception of B42-Ste5Δ1 to -5, which were assayed once. LexA-Ste20 does not interact with B42 (11). B42 alone with the LexA-Bem1 fusion resulted in 0.8 Miller units of β-galactosidase activity. Units have been normalized.

By contrast, Ste11M, Ste7M, and Fus3-hemagglutinin (HA) are readily detected in GST-Bem1 preparations made from strains containing increased levels of Ste5. Wild-type (Ste5$^+$) strains were constructed to contain GST-Bem1, Ste5M on a multicopy plasmid, and one of each of the three epitope-tagged protein kinases. Under the same conditions under which Ste5M copurifies with GST-Bem1, Ste11M, Ste7M, and Fus3-HA are now detected (Fig. 3B). The visualization of Ste11M by the 9E10 monoclonal antibody is somewhat obscured by GST-Bem1, which comigrates with Ste11M. Ste11M is more clearly detected when the majority of the GST-Bem1 is reduced in size by cleavage with thrombin prior to gel electrophoresis (Fig. 3C). Thus, Ste11, Ste7, and Fus3 each associate with GST-Bem1, but they most likely do so indirectly through their association with Ste5. These results strongly suggest that Bem1 activates the MAPK cascade through interactions with Ste5 in a multikinase complex(es) that contains Ste11, Ste7, and Fus3. This interaction may be sufficient to lead to activation of the MAPK cascade by Gβ, because cooverexpression of GST-Bem1 and Ste5M restores partial α-factor sensitivity to a *ste20*Δ strain (Fig. 4C).

Overproduction of Bem1 increases *FUS1-lacZ* levels and suppresses mating defects of *fus3* mutants. The results presented suggest that Bem1 mediates its stimulatory effect on G_1 arrest indirectly through activation of the MAPKs, particularly Fus3. Fus3 mediates G_1 arrest, transcription, and mating through genetically separable functions that are likely defined by Fus3 substrates involved in each process (21, 43, 51). If

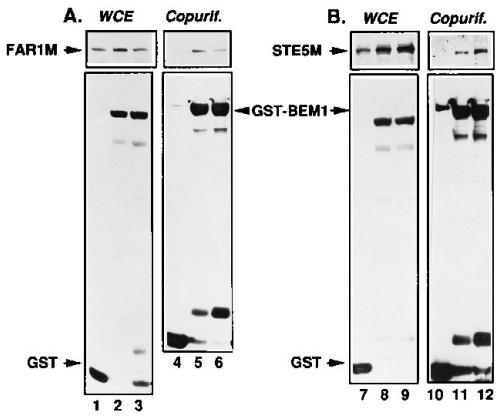

FIG. 5. Far1M copurifies with GST-Bem1. (A) Immunoblot analysis of Far1M in purified GST-Bem1 complexes. GST-Bem1 was purified from whole-cell extracts, separated by SDS-PAGE, and immunoblotted with 9E10 antibody to detect Far1M. The immunoblot was then stripped and reimmunoblotted with GST antiserum to detect GST-Bem1. Samples of whole-cell extract were analyzed in parallel. Cells were grown for 5 h in selective medium containing 2% galactose prior to a 1-h induction with α-factor. Lanes 1 and 4, EY1262 (*sst1*Δ *far1*Δ) + pGAL-GST (pYBS305) + pGAL-FAR1M (pTP62); lanes 2, 3, 5, and 6, EY1262 + pGAL-GST-BEM1 (pYEE185) + pGAL-FAR1M (pTP62). WCE, 50 μg of whole-cell extract; Copurif., glutathione agarose purified protein. Lanes 3 and 6 include α-factor. (B) Companion immunoblot analysis of Ste5M in purified GST-Bem1 complexes done as described for panel A. Lanes 7 and 10, EY2019 (*sst1*Δ *ste5*Δ) + pGAL-GST (pYBS305) + STE5M (pKC20); lanes 8, 9, 11, and 12, EY2019 + pGAL-GST-BEM1 (pYEE185) + STE5M (pKC20). Lanes 9 and 12 include α-factor.

Bem1 functions as an upstream activator of Fus3, one might expect overproduction of Bem1 to enhance the transcription of mating specific genes and to suppress additional defects associated with *fus3* mutants (such as mating) (19, 20), as has been found for *STE5* (30). The effect of Bem1 on transcriptional activation was measured for the *FUS1* gene, whose expression is tightly linked to the activity of the MAPK cascade (19). Overproduction of Bem1 causes an ~2.5-fold increase in the level of *FUS1* expression in the presence of α-factor (Table 3), as measured with a *FUS1-lacZ* fusion (59), but has no effect on *FUS1* expression in a *fus3*Δ *kss1*Δ strain (see Table 3 footnote). Furthermore, overproduction of Bem1 efficiently suppresses the mating defect of *fus3 kss1*Δ double mutants that are dependent upon residual Fus3 activity for signal transduction (Table 4). These results, taken together with the effects of Bem1 on Fus3 kinase activity and the association between Bem1 and Ste5-multikinase complexes, strongly support the notion that Bem1 functions as an upstream activator of the MAPK cascade.

Bem1 preferentially suppresses G_1 arrest and mating defects of a *fus3* mutant. Although Bem1 functions as an upstream activator of Fus3, overproduction of Bem1 more readily suppresses mating and G_1 arrest defects of certain *fus3* mutants, than it does their transcription defects. For example, although Bem1 overproduction suppresses the G_1 arrest and mating defects of *fus3-2 kss1*Δ and *fus3-205 kss1*Δ mutants (Fig. 4D; Table 4), it has little or no effect on the expression of *FUS1* in these strains (Table 3), which have greatly reduced levels of *FUS1* transcription compared with those of the wild type both in the absence or presence of excess Bem1 (Fig. 4 legend; Table 3). That the effects of Bem1 on G_1 arrest may be distinguishable from transcriptional activation is further suggested by the observation that the ability of Bem1 to restore pheromone sensitivity to a *fus3-205* mutant does not improve when the strain is *KSS1*$^+$ and has higher levels of Ste12-dependent transcription (Fig. 4D and legend). This pattern of suppression contrasts that of Far1, where the strength of suppression increases when the strain is *KSS1* (Fig. 4D) and has higher levels of *FAR1* transcription (51) and that of Ste5, which stimulates *FUS1* expression in both *fus3-2 kss1*Δ and *fus3-205 kss1*Δ strains (30). Thus, Bem1 may regulate G_1 arrest and mating in a manner that is distinct from transcriptional activation by the MAPK cascade.

Bem1 requires Far1 to promote G_1 arrest and mating. Far1 is a key regulator of both G_1 arrest and mating with no known role in Ste12-dependent transcription (6) or Fus3 activation (21). Fus3 is thought to positively regulate Far1 in two ways, by phosphorylating Ste12 and activating *FAR1* transcription (a function shared by Kss1 [6, 19, 21]), and by phosphorylating Far1 and facilitating its inhibition of a subset of G_1 cyclin-Cdc28 complexes (43). It is possible that Fus3 also regulates the mating function of Far1, since Far1 phosphorylation by Fus3 has not been shown to be solely required for G_1 arrest (43, 44, 60), and overproduction of Far1 does not suppress the mating defect of *fus3* mutants (Table 4). Overproduction of Bem1 does not restore pheromone sensitivity or mating to a *far1* deletion mutant (Fig. 1C; Table 4). Thus, Bem1 requires Far1 in addition to Fus3 and Kss1 to promote G_1 arrest and mating.

Bem1 associates with Far1. One explanation for why Bem1 might preferentially suppress G_1 arrest and mating defects of certain *fus3* mutants is that Bem1 affects the regulation of Far1. This idea is attractive because Far1 and Ste5 have similar LIM domains that could mediate a common protein-protein interaction (18) as well as functional homology (Ste5 overexpression restores pheromone sensitivity to a *far1*Δ mutant [50]). We therefore tested the possibility that Bem1 might also associate with Far1. As shown in Table 3, Bem1 and Far1 associate in the two-hybrid system, yielding levels of β-galactosidase >10-fold higher than that quantitated for LexA-Bem1 and B42-Ste5. Furthermore, a functional epitope-tagged form of Far1 (Far1M) (43) copurifies with GST-Bem1 both in the absence and presence of pheromone, as found for Ste5 (Fig. 5). Relatively similar amounts of Far1M and Ste5M appear to be present in whole-cell extracts and to copurify with GST-Bem1 (Fig. 5), suggesting the possibility that the proteins associate with Bem1 with similar affinities.

DISCUSSION

Novel role for a morphogenesis protein in cross-linking a MAPK cascade to a cell cycle control substrate. Bem1 is known to be required for the polarized growth that leads to shmoo formation during mating (9). Here we show that Bem1 positively regulates both signal transduction and G_1 arrest during mating and associates with two key regulators of G_1 arrest, Ste5 and Far1. Functional evidence demonstrates that Bem1 is required for efficient G_1 arrest. A *bem1*Δ null mutant is defective in G_1 arrest (Fig. 1; Table 2), even when signaling is enhanced either by an *sst1*Δ mutation or by overexpression of signal transduction components (e.g., Gβ or Ste5-Ste11). The *BEM1* gene is a potent dosage suppressor of *fus3* mutants, functioning nearly as well as *FAR1* (Table 1; Fig. 1 and 4) (21, 50, 52). *BEM1* positively regulates the MAPK cascade that effects G_1 arrest; *bem1*Δ null mutants are defective in both basal and induced transcription of *FUS1* (Table 3), a gene whose expression is strictly dependent upon MAPK activity (19, 21), and Bem1 overproduction stimulates Fus3 activity (Fig. 2) and transcription of the *FUS1* gene (Table 3). Thus, the defect in G_1 arrest in *bem1*Δ mutants is likely to involve inefficient signal transduction, in contrast to *far1*Δ mutants, which have normal signaling (6, 21).

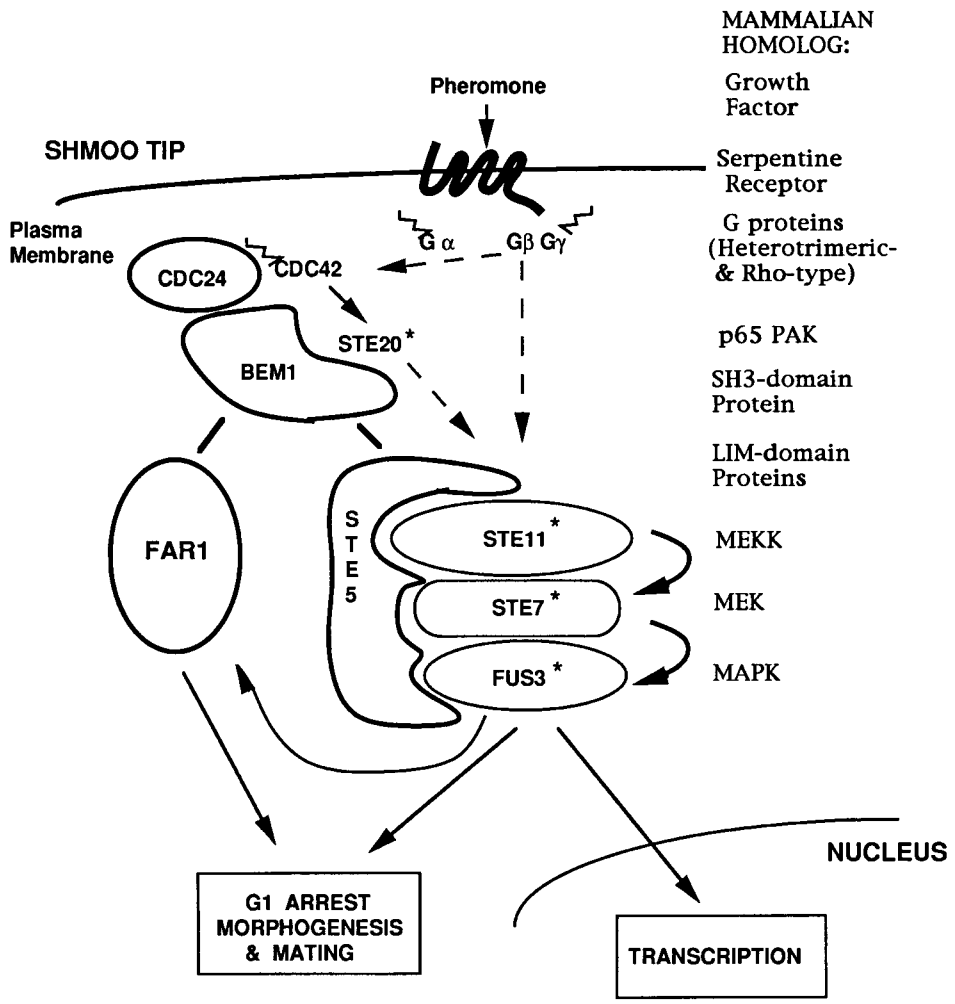

FIG. 6. Summary of genetic and biochemical interactions between Bem1 and components of the pheromone response pathway. Signal transmission from the receptor involves parallel inputs from Ste20 and Gβ (Ste4) to a Ste5-multikinase complex (11, 18), resulting in activation of Ste11, Ste7, and Fus3. Gβ associates with Ste5 (61), and Ste20 may phosphorylate Ste11 (61) in a manner that leads to increased Ste11 activity (as suggested by results shown in Fig. 2 and 4). Cdc42 is shown activating Ste20 at a step downstream of the G protein as speculated (55, 67). Bem1 is likely to associate with Ste20 (32), Cdc24, and Cdc42 (5, 45, 68) at or near the cortical actin cytoskeleton of the shmoo tip (9, 32, 46). Bem1 associates with Ste5 in a complex with Ste11, Ste7, and Fus3 and facilitates activation of Ste11 by either Ste20 or Gβ (Fig. 2 to 4; Table 5). Bem1 also associates with Far1 (Fig. 5; Table 5), which is required for both G_1 arrest and shmoo orientation (6, 17, 59) and is a Fus3 substrate (21, 43). The MAPK cascade may regulate morphogenesis by direct phosphorylation of proteins required for polarized growth such as Bem1, Cdc24, Cdc42, Ste20, and the actin cytoskeleton. These hypothesized events could occur either simultaneously or sequentially during the response to pheromone. Kss1 is not shown for simplicity, although it functions redundantly with Fus3 for some aspects of signal transduction such as transcriptional activation (19). Asterisks indicate protein kinases, and zigzags indicate membrane anchors.

Consistent with the functional evidence for a role in signal transduction, Bem1 interacts with Ste5 in two-hybrid analysis (Table 5) and copurifies with Ste5 in complexes that also contain the MAPK cascade enzymes Ste11, Ste7, and Fus3 (Fig. 3 and 5). Taken together, our data strongly argue that Bem1 potentiates the MAPK cascade through interactions with the Ste5-MAPK cascade complex that lead to enhanced activation of Ste11 (Fig. 6). These findings extend the recent observations of Zao et al. (65) and Leeuw et al. (32), in that they provide functional evidence of a role for Bem1 in pheromone-mediated signal transduction as well as evidence for a physical connection between Bem1 and the MAPK cascade enzymes. Moreover, the demonstration that the MAPK cascade enzymes associate with Bem1 in a Ste5-dependent manner (Fig. 5) provides further evidence for the existence of a Ste5-multikinase complex that may be regulated by other proteins (11, 18, 30).

Bem1 promotes G_1 arrest through Far1 (Fig. 1), a Fus3 substrate (21, 43) that inhibits two G_1-specific cyclin-dependent kinases (CDKs), Cln1-Cdc28 and Cln2-Cdc28 (43, 44, 60). Bem1 could regulate Far1 indirectly through activation of Fus3, which phosphorylates both Ste12 (and activates transcription of *FAR1* [20, 21]) and Far1 (and facilitates Far1 binding to CDKs [43]). However, Bem1 physically associates with Far1 (Table 3; Fig. 5), suggesting that Bem1 plays a more direct role in the regulation of Far1. Two observations suggest that the requirement for Bem1 in G_1 arrest is not strictly linked to transcription of *FAR1*. First, in *fus3-2 kss1*Δ and *fus3-205 kss1*Δ strains, overproduction of Bem1 promotes G_1 arrest yet has no obvious effect on transcriptional activation of the *FUS1* gene, which is tightly regulated by Ste12 (6, 36). Second, the ability of Bem1 to restore G_1 arrest to a *fus3-205* mutant is not enhanced by *KSS1*, which increases the transcriptional capacity of the strain (Fig. 4). Bem1 could directly regulate Far1 at many levels that include accessibility to Fus3 for phosphoryla-

tion, cell cycle stability (37), and accessibility to CDK targets (44). This last possibility is intriguing, given that Bem1 localizes at growth sites (9, 46) potentially near Cln1-Cdc28 and Cln2-Cdc28 complexes that may promote budding (3, 15, 16, 33) and be inhibited during G_1 arrest and projection formation (43, 44, 57, 60). Thus, by associating with both Ste5 and Far1, Bem1 may coordinate multiple events required for cell cycle control that lead to the formation of a shmoo (Fig. 6).

Far1 may regulate shmoo orientation through Bem1 interactions. *FAR1* was originally shown to be required for mating in addition to G_1 arrest (6). Recent work suggests that *FAR1* regulates mating by ensuring proper shmoo orientation in response to a pheromone gradient, a process essential for efficient partner selection (17, 59). That Far1 has two distinct roles in mating is suggested by the existence of *far1* point mutations that abolish oriented growth but not G_1 arrest (59). It is possible that Far1 regulates shmoo orientation through interactions with morphogenesis proteins. The association between Bem1 and Far1 (Table 5; Fig. 5) is highly consistent with the proposed functions for Far1 in shmoo orientation (17, 59), and suggests that Bem1 may also play a role in this process, perhaps by localizing Far1 to the growth site near other proteins that regulate polarized growth. Furthermore, it is conceivable that phosphorylation of Far1 by Fus3 is also required for shmoo orientation in addition to G_1 arrest. Thus, Bem1 may coordinate signal transduction with both G_1 arrest and partner selection.

Bem1 stimulates the MAPK cascade by both Ste20-dependent and -independent mechanisms. We find that efficient pheromone-dependent signaling from Gβ to Ste11 can occur in the absence of Ste20 in a W303 strain background but that it requires Ste5 and a hyperactive form of Ste11 (*STE11-4* [Fig. 2 and 4]). The ability of a hyperactive Ste11 to bypass the *ste20*Δ block in pheromone signaling suggests that Ste20 normally functions to activate Ste11. Our findings also support recent work indicating that Gβ interacts with Ste5 (34a, 60) and suggests that full activation of Ste11 involves a complex set of interactions between Gβ, Ste20, and Ste5 (Fig. 6).

Bem1 stimulates the MAPK cascade both in the absence and presence of Ste20, although Ste20 is needed for maximal effects (Fig. 2 and 4). One possibility is that Bem1 helps to localize Ste5 and associated Ste11 to both Gβ and Ste20 (Fig. 6). This possibility is consistent with two observations. First, Bem1 associates with Ste20 (32), possibly indirectly through direct linkages between Bem1 and Cdc24 (45), Cdc24 and Cdc42 (68), and Cdc42 and Ste20 (55). Second, Bem1 and Cdc42 localize at sites of cortical actin near the pheromone receptor at the shmoo tip (9, 28, 32, 69). In the absence of Ste20, Bem1 may stimulate the MAPK cascade through a Gβ-dependent mechanism that could involve either a direct interaction between Gβ and Ste5 (64) or an indirect one, in which Cdc42 activates another PAK-type kinase that has functional redundancy with Ste20 (15).

Potential for regulation of signal transduction by LIM domains. Ste5 and Far1 have in common a putative LIM-domain structural motif that is known to mediate protein-protein interactions that include dimerization between LIM domains and dimerization with non-LIM-domain proteins (18, 49, 53). The existence of this shared motif in Ste5 and Far1 raises the possibility that it is involved in the Bem1 interactions. The LIM domain appears to be important for both Ste5 and Far1 function. A *ste5* deletion mutant that is missing the LIM domain is nonfunctional, although it still binds Ste11 and Ste7 (11), and a LIM-domain point mutant inhibits Ste5 function (29a). A point mutation in the LIM domain of Far1 inhibits its function in shmoo orientation, although it is not known whether it affects G_1 arrest (61). Thus, regulation of the LIM domain in both Ste5 and Far1 has the potential to affect their function. Finally, if Ste5 and Far1 were to associate with Bem1 through a common motif, they might compete for binding to Bem1, providing another level of coordinate regulation.

Bem1 may organize specific signaling circuits in response to a growth factor. It is noteworthy that overproduction of Bem1 has a minor effect on transcription of the *FUS1* gene in a wild-type strain and little or no effect in *fus3-2 kss1*Δ and *fus3-205 kss1*Δ mutants, despite readily detectable effects on G_1 arrest and mating. This is striking because transcriptional activation of *FUS1* is thought to be the most sensitive assay for signal transduction. One interpretation of these findings is that when Bem1 is overexpressed, it colocalizes the MAPK cascade (through Ste5) to specific cellular sites that match its normal location in a cell. Thus, while Bem1 might have little effect on the population of MAPK molecules that phosphorylate the transcription factor Ste12 (possibly in the nucleus), it might have a large effect on those that phosphorylate Far1. This possibility is consistent with immunolocalization studies that indicate that Fus3 is in the cytoplasm as well as the nucleus (18) whereas Kss1 is primarily in the nucleus (34b). Our observations may reveal the existence of signaling circuits in which Bem1 physically couples pheromone signaling to cell cycle control and mating responses at the shmoo tip. Proper spatial organization of the signaling cascade may be important for many aspects of mating. For example, the Bem1-Ste5 interaction could enhance receptor-mediated signal transduction needed for shmoo formation, which requires 100-fold more pheromone than does G_1 arrest or agglutination (39), or this interaction could target Fus3 to substrates involved in shmoo formation or fusion. Several observations suggest that Bem1 has a function required for shmoo formation that involves Ste20 (10, 32) yet may be distinct from activation of the MAPK cascade; a *bem1-S* protein that does not associate with Ste20 still associates with Ste5 yet remains defective in shmoo formation (32), and overexpression of Bem1 does not restore shmoo formation to a *STE11-4 ste20*Δ strain (data not shown), despite stimulatory effects on the MAPK cascade (Fig. 2). Thus, Bem1 may localize the MAPK cascade to specific substrates involved in shmoo formation (and fusion) that localize at the shmoo tip (9, 22).

The concept of a spatial organizer is likely to be of general significance to other signal transduction cascades that involve complex sets of biological responses. The existence of mammalian homologs of morphogenesis proteins such as Ste20 and Cdc42 (8) and the recent observation that activated forms of Rho- and Rac-type GTPases stimulate specific MAPK cascades (e.g., c-Jun NH_2-terminal kinases or stress-activated protein kinases) (13, 38) suggest that signaling in higher systems involves related circuitry.

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