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Identification of the Bud Emergence Gene *BEM4* and Its Interactions with Rho-Type GTPases in *Saccharomyces cerevisiae*

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The Rho-type GTPase Cdc42p is required for cell polarization and bud emergence in *Saccharomyces cerevisiae*. To identify genes whose functions are linked to *CDC42*, we screened for (i) multicopy suppressors of a Ts$^-$ *cdc42* mutant, (ii) mutants that require multiple copies of *CDC42* for survival, and (iii) mutations that display synthetic lethality with a partial-loss-of-function allele of *CDC24*, which encodes a guanine nucleotide exchange factor for Cdc42p. In all three screens, we identified a new gene, *BEM4*. Cells from which *BEM4* was deleted were inviable at 37°C. These cells became unbudded, large, and round, consistent with a model in which Bem4p acts together with Cdc42p in polarity establishment and bud emergence. In some strains, the ability of *CDC42* to serve as a multicopy suppressor of the Ts$^-$ growth defect of Δ*bem4* cells required co-overexpression of Rho1p, which is an essential Rho-type GTPase necessary for cell wall integrity. This finding suggests that Bem4p also affects Rho1p function. Bem4p displayed two-hybrid interactions with Cdc42p, Rho1p, and two of the three other known yeast Rho-type GTPases, suggesting that Bem4p can interact with multiple Rho-type GTPases. Models for the role of Bem4p include that it serves as a chaperone or modulates the interaction of these GTPases with one or more of their targets or regulators.

During bud emergence in the yeast *Saccharomyces cerevisiae*, the actin cytoskeleton becomes polarized toward a discrete site, where the transport of secretory vesicles is directed for bud growth (16). *CDC42*, *CDC24*, and *BEM1* are implicated in the control of bud emergence: loss-of-function mutations in any of these genes lead to the loss of actin polarization and can cause cells to arrest without buds. Because secretion and entry into the nuclear division cycle are not blocked by these mutations, the mutant cells become large, round, and multinucleate (1, 3, 11, 13, 26, 47, 48).

Cdc42p is a member of the Rho family of small GTPases (26). Such GTPases act as molecular switches, through a conformational change between their GTP-bound active and GDP-bound inactive forms. Their nucleotide-bound state is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (5, 8). Cdc24p displays GEF activity toward Cdc42p (54). Mutations in *CDC24* can be suppressed by overexpression of *CDC42* and display synthetic lethality with mutations in *CDC42* (2, 55), consistent with a model in which the action of Cdc24p on Cdc42p is important for bud emergence. Cdc24p has also been linked to the SH3 domain-containing protein Bem1p. In particular, Cdc24p binds to Bem1p, and *cdc24* and *bem1* mutations are synthetically lethal (38).

In addition to Cdc42p, *S. cerevisiae* has at least four other Rho-type GTPases, Rho1p to Rho4p. Rho1p is essential for viability (33). Cells containing a Ts$^-$ *rho1* allele lyse at restrictive temperatures, suggesting that Rho1p is required for integrity of the plasma membrane and cell wall (40, 52). One of the downstream targets of Rho1p is Pkc1p, a protein kinase C homolog that is required for proper assembly of the cell wall (31, 32, 37). Rho3p is also important for normal morphogenesis (23, 35, 36). At nonpermissive temperatures, cells bearing Ts$^-$ *rho3* alleles fail to polarize actin, are defective in bud growth, and become large and round. These phenotypes suggest that Rho3p is required for the maintenance of cell polarity during bud growth (23). *RHO2* and *RHO4* are not essential, and cells that lack either one of these genes grow well at all temperatures (33, 35).

In this paper, we report the discovery of the bud emergence gene *BEM4*, whose product appears to be a novel binding protein for Rho-type GTPases.

MATERIALS AND METHODS

Microbiological techniques. Yeast transformations were performed by the method of either Ito et al. (25) or Keszenman-Pereyra and Hieda (29). Rich medium containing glucose (YPD), synthetic minimal medium (SD), and synthetic complete medium (SC) were as described previously (44). SC-Leu is SC without L-leucine. SDCA contains 0.5% Casamino Acids in SD. SDCA+Ade is SDCA with 100 μg of adenine sulfate per ml. SDCA-U is SDCA+Ade with 100 μg of tryptophan per ml. SD+Trp+Ade+Lys is SD with 20 μg of L-tryptophan, 40 μg of adenine sulfate, and 30 μg of L-lysine per ml.

DNA manipulations. Standard methods of DNA manipulation were used (44). The identical nucleotide sequence of *BEM4* was determined independently in

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TABLE 1. Strains used in this study

Strain	Genotype[a]	Source or reference
GYM5-1	*MAT***a** *bem4Δ-1*::*URA3 ura3 leu2 ade2 his3 trp1 lys2*	Segregant from (YPH501 made *bem4Δ-1*::*URA3* by gene replacement using pGYM50-8)
GYM5-2	*MAT***a** *bem4Δ-1*::*URA3 ura3 leu2 ade2 his3 trp1 lys2*	Same as for GYM5-1
GYM5-11	*MAT***a** *bem4Δ-1*::*HIS3 ura3 leu2 ade2 his3 trp1 lys2*	GYM5-1 made *ura3*::*HIS3* (36) by gene replacement
JC24-4	*MAT***a** *cdc24-4 ura3 leu2 ade2 his3 trp1 lys2*	Strain congenic to YPH500 except for the indicated genotype (derived from a series of crosses involving YPH500 and Y147 [2])
KA31	*MAT***a**/*MAT*α *ura3*/*ura3 leu2*/*leu2 his3*/*his3 trp1*/*trp1*	24
PY83	[*CDC24*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *cdc24-4 leu2 ade3 his3*	Segregant from (Y49 × 4795-408) transformed with pPB170
PY88	[*CDC24*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *cdc24-4 ura3 leu2 ade2 ade3 met1*	Segregant from (Y108 × PY83)
PY92	[*CDC24*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *cdc24-4 ura3 leu2 ade2 ade3 met1*	Same as for PY88
PY278	[*CDC24*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *cdc24-4 bem1-4 ura3 leu2 ade2 ade3 met1*	Sect$^-$ mutant derived from PY88
PY406	[*CDC42*, *ADE3*, *LEU2*, 2μm ARS] *MAT*α *ura3 leu2 ade2 ade3 trp1*	Y382 containing pPB320
PY407	[*CDC42*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *ura3 leu2 ade2 ade3 lys2*	Y388 containing pPB320
PY457	[*BEM1*, *URA3*, 2μm ARS] *MAT***a** *bem1*::*LEU2 ura3 leu2 trp1 his4*	Segregant from (KO2-5 [13] transformed with pPB321 [3])
PY632	[*CDC42*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *bem1-4 ura3 leu2 ade2 ade3 lys2*	Sect$^-$ mutant derived from PY407
PY808	[*CDC42*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *bem4-1 ura3 leu2 ade2 ade3 lys2*	Sect$^-$ mutant derived from PY407
PY899	[*BEM1*, *URA3*, 2μm ARS] *MAT***a** *bem1*::*LEU2 ura3 leu2 ade2 ade3 lys2*	Derived from a series of crosses involving Y312, Y382, Y388, and a segregant from PY457
PY975	[*BEM1*, *URA3*, 2μm ARS] *MAT*α *bem1*::*LEU2 ura3 leu2 ade2 ade3 trp1*	Segregant from (PY899 × Y382)
Y49	*MAT*α *cdc24-4 his3 met1*	Segregant from (PTRD5-BD1-1A [2] × DC5 [9])
Y108	*MAT*α *cdc24-4 ura3 ade2 ade3 his7 met1*	Segregant from (Y49 × 4795-408)
Y253	*MAT*α *ura3 leu2 ade2 ade3 his7 trp1 can1 sap3*	Segregant from (4795-303 × 4795-408)
Y292	*MAT*α *cdc24-4 leu2 his3 GAL2*	Segregant from (Y145 [3] × DJMD4-30B [26])
Y312	*MAT*α *ura3 leu2 his3 GAL2*	Segregant from (Y124 [2] × Y292)
Y382	*MAT*α *ura3 leu2 ade2 ade3 trp1*	3
Y388	*MAT***a** *ura3 leu2 ade2 ade3 lys2*	3
Y704	*MAT***a** *bem4-2 ura3 leu2 ade2 ade3 his7* or *HIS7*	Segregant from (10.7 × Y253)
Y799	*MAT***a**/*MAT*α *ura3*/*ura3 leu2*/*leu2 ade2*/*ade2 ade3*/*ade3 trp1*/*trp1 lys2*/*lys2*	Y383 (3) × (Y383 made *MAT*α by mating-type interconversion using p*GAL-HO* [21])
Y833	*MAT*α *bem4Δ-2*::*URA3 ura3 leu2 ade2 ade3 trp1 lys2*	Segregant from (Y799 made *bem4Δ-2*::*URA3* by gene replacement using pPB1023)
Y1008	*MAT*α *bem1*::*LEU2 mfa2*::p*FUS1-lacZ ura3 leu2 ade2 ade3*	28
Y1050	*MAT***a** ura3:(*URA3*, Op_{lexA}-*lacZ*) *leu2 ade2 his3 trp1 lys2 gal80*	L40 (51) made *lys2* by growth on 5 α-aminoadipate (12)
Y1051	*MAT*α *bem4-Δ2*::*ura3 ura3 leu2 ade2 ade3 trp1 lys2*	Y833 made Ura3$^-$ on 5-fluoroorotic acid
YMR420	*MAT***a** *cdc42-1 ura3 leu2 ade2 his3 trp1 lys2*	36
YPH500	*MAT*α *ura3 leu2 ade2 his3 trp1 lys2*	45
YPH501	*MAT***a**/*MAT*α *ura3*/*ura3 leu2*/*leu2 ade2*/*ade2 his3*/*his3 trp1*/*trp1 lys2*/*lys2*	45
10.7	[*CDC24*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *cdc24-4 bem4-3 ura3 leu2 ade2 ade3 met1*	Sect$^-$ mutant derived from PY92
11B	[*CDC42*, *ADE3*, *LEU2*, 2μm ARS] *MAT***a** *bem4-2 ura3 leu2 ade2 ade3 lys2*	Sect$^-$ mutant derived from PY407
4795-303	*MAT*α *leu2 ade2 ade3 trp1 his7 can1 sap3*	L. Hartwell
4795-408	*MAT***a** ura3 leu2 ade2 ade3 his7 can1 sap3	L. Hartwell
W303	*MAT***a**/*MAT*α *ura3*/*ura3 leu2*/*leu2 ade2*/*ade2 his3*/*his3 trp1*/*trp1 lys2*/*lys2*	50

[a] Markers on plasmids are indicated in brackets.

Indiana and in Tokyo by the dideoxy method. Each strand was sequenced completely, and all sites used for subcloning were sequenced across. Deletion derivatives of plasmids for sequencing were constructed by the method of Henikoff (20), and a model 370A automatic sequencer (Applied Biosystems) was used for the sequencing done in Tokyo.

Strains and plasmids. Yeast strains used in this study are listed in Table 1. pBluescript KS$^+$ was purchased from Stratagene (La Jolla, Calif.). YEp24 contains *URA3* and the 2μm plasmid replication origin (2μm autonomously replicating sequence [ARS]) (7). The YEp24-based library (10) contains *Sau*3A fragments of yeast genomic DNA inserted into the *Bam*HI site of YEp24. pPB102 (2) and pSRO2-3 (36) are genomic clones containing *CDC42* from the YEp24-based library. pPB320, which contains *CDC42*, *ADE3*, *LEU2*, and 2μm ARS, was constructed by coinserting a 5-kb *Bam*HI-*Sal*I fragment containing *ADE3* (30) and a 4-kb *Sal*I-*Eco*RV fragment containing *CDC42* from pPB102 into the *Bam*HI-*Pvu*II sites of pSL113 (4). pPB170, which contains *CDC24*, *ADE3*, *LEU2*, and 2μm ARS, was constructed by inserting a 5-kb *Xba*I-*Bam*HI fragment containing *CDC24* from plasmid YEp103(CDC24) (14) into the *Xba*I-*Bam*HI sites of pPB166 (38). YCp50 contains *URA3*, *CEN4*, and *ARS1* (42). pPB527 is a genomic clone containing *BEM4* from a YCp50-based library (42). pGYM50, pGYM149, pGYM241, and pGYM245 are *BEM4*-bearing isolates from the YEp24-based library (see Fig. 2). pGYM50-1 was constructed by removing the *Sal*I-*Sal*I fragment from pGYM50. To construct pGYM50-2, pGYM50 was digested with *Sma*I and *Bst*XI and then religated after blunting the *Bst*XI overhang, using the Klenow fragment of DNA polymerase I. pGYM50-3 was constructed by removing the 2.7-kb *Afl*II-*Afl*II fragment of pGYM50. pGYM50-4 was constructed by inserting the 2.7-kb *Afl*II-*Afl*II fragment from pGYM50 into the *Sma*I site of the *URA3*, 2μm ARS plasmid YEplac195 (17). pGYM50-5 and pGYM50-7 were made by inserting the 4.8-kb *BEM4*-bearing *Bgl*II-*Sal*I fragment from pGYM50 into the *Bam*HI-*Sal*I sites of YEplac195 and into the *Bam*HI-*Xho*I sites of pBluescript KS$^+$, respectively. To construct pGYM50-6, pGYM50-5 was digested with *Afl*II and then religated after filling in the four-base overhang, using Klenow fragment. pSRO4 is an original isolate from the YEp24-based library (36). YEpSRO4 was made by inserting the 5.5-kb *SRO4*-bearing *Sma*I-*Sal*I fragment into the *Sma*I-*Sal*I sites of the *TRP1*, 2μm ARS plasmid YEplac112 (17). pOPR1 contains the *RHO1* coding region under the control of the *TDH3* promoter, *TRP1*, and 2μm ARS (36). Plasmids used for the two-hybrid assays are described below. Phage DM1.1.15 was created by inserting

the 0.4-kb *Spe*I-*Nru*I fragment from upstream of *BEM4* (see Fig. 2 and 3) in pPB527 into the *Xba*I-*Sma*I sites of M13mp19 (53).

Construction of disruption alleles of *BEM4*. pGYM50-8, which contains *bem4Δ-1*::*URA3*, was constructed by replacing the 1.1-kb *Eco*RV fragment (see Fig. 2 and 3) of pGYM50-7 with the *URA3*-bearing 1.1-kb *Sma*I-*Sma*I fragment from pJJ242 (27). In *bem4Δ-1*::*URA3*, the sequence between codons 70 and 433 of *BEM4* was removed. pPB1023, which contains *bem4Δ-2*::*URA3*, was constructed by inserting fragments into pBR322 (6) as follows: (i) the 0.4-kb *Hin*cII-*Eco*RI fragment from DM1.1.15 (in which the *Hin*cII and *Eco*RI sites are from the polylinker) was inserted into the *Ssp*I-*Eco*RI sites, (ii) a 0.7-kb *Hpa*I-*Sau*3A fragment from the 3′ end of *BEM4* (see Fig. 2 and 3) from pPB527 was inserted into the *Eco*RV-*Bam*HI sites, and (iii) a 1.2-kb *Hin*dIII-*Hin*dIII fragment containing *URA3* from YIpURA3 (S. Lillie, University of Michigan, Ann Arbor) was inserted into the *Hin*dIII site. In *bem4Δ-2*::*URA3*, the DNA between 101 bp upstream of the start codon and 57 codons upstream of the stop codon of *BEM4* was removed. For gene replacements, a 2.6-kb *Bst*XI-*Pvu*II fragment from pGYM50-8 and a 3-kb *Pvu*I (from within pBR322 sequences)-*Bam*HI (corresponding to the *Sau*3A site in Fig. 2 and 3) fragment from pPB1023 were used. In each case, the success of the transplacement into the yeast genome was confirmed by Southern analysis.

Isolation of multicopy suppressors of the *cdc42* defect. Ts$^-$ *cdc42-1* strain YMR420 containing YEpSRO4 was transformed with the YEp24-based genomic library and plated on SDCA+Ade plates. After incubation at 25°C for 1 day followed by incubation at 37°C for 3 days, cells that could form visible colonies were isolated and cultured in SDCA+Ade. To confirm the ability of the plasmids to suppress the *cdc42* Ts$^-$ growth defect, each plasmid was reintroduced at 25°C into YMR420 containing YEpSRO4. Transformants were streaked on SDCA+Ade plates and incubated at 37°C for 2 days. Plasmids that carried *CDC42* were identified by amplification of a 740-bp DNA fragment, using PCR (43) with the two convergent primers 5′-AAAAAAAGATCTTCCACAAAATGCAAA and 5′-GGGGGGGTCGACGGATGATCCTGGCGC (underlined sequences indicate extensions that were introduced to allow for more efficient restriction of the amplified fragment).

Colony-sectoring assays. A previously described colony-sectoring assay (3) was used to identify mutants that required multiple copies of *CDC42* for survival. This assay is based on the facts that *ade2* mutants are red, *ade2 ade3* mutants are white, and 2μm ARS-based plasmids typically are segregated imperfectly between mother cell and daughter cell. Thus, when grown on YPD (nonselective) medium, *ade2 ade3* cells that contain the *CDC42*- and *ADE3*-bearing plasmid pPB320 form colonies that contain red and white sectors (Sect$^+$ phenotype), derived from cells that had retained and those that had lost the plasmid, respectively. However, in mutants that need multiple copies of *CDC42* for survival, only those cells that inherit the plasmid can proliferate, resulting in uniformly red colonies (Sect$^-$ phenotype). To search for mutants, strains PY406 and PY407 were plated on YPD and mutagenized with UV irradiation to approximately 10% survival, and Sect$^-$ colonies were isolated. To test for dominance of the Sect$^-$ phenotype, mutants were crossed to Y382 or Y388. To test whether mutants required *CDC42* itself (as opposed to some other gene on the plasmid, such as *ADE3*), representative members of each complementation group were transformed separately with the *CDC42*-bearing plasmid pPB102 and with the control plasmid YEp24 (selecting for Ura$^+$ transformants). Those mutants that became Sect$^+$ when transformed with pPB102 and remained Sect$^-$ when transformed with YEp24 were judged to be *CDC42* requiring. Representative members of each group were then mated to known Ts$^-$ *cdc42* and *cdc24* mutants. The resulting diploids were cured of the plasmid at room temperature and then tested for the ability to grow at 37°C.

To identify mutations that display synthetic lethality with *cdc24*, we used essentially the procedure just described, except that the *cdc24-4* strains PY88 and PY92 (containing the *CDC24*- and *ADE3*-bearing plasmid pPB170) were used. To test whether the Sect$^-$ mutants required *CDC24* itself from plasmid pPB170 for survival, we assayed the sectoring abilities of mutants after transformation with YEp103(CDC24) or with YEp24.

Isolation of multicopy suppressors of the Δ*bem4* defect. *bem4Δ-1*::*HIS3* strain GYM5-11 was transformed with the YEp24-based library and plated on SDCA-U plates. After incubation at 37°C for 4 to 6 days, cells from visible colonies were isolated and cultured in SDCA-U. Plasmids were recovered and reintroduced into GYM5-11 to confirm the ability to suppress the *bem4* Ts$^-$ phenotype. Suppressing plasmids were classified by restriction analysis. Plasmids containing *MSB1* (3) were identified by PCR using the following two sets of convergent primers, which were designed to amplify two distinct regions in *MSB1*: (i) 5′-AGTATGGATGACGAAGAG and 5′-TTTTAAGCTTTTCGGATGTTTG and (ii) 5′-GAATGTATTGAGGTTAA and 5′-GTATTTGTACTAAGCTTC AATGA (underlined residues indicate substitutions that were introduced to create sites for restriction). Plasmids pMSB1 and pMSB4, which were isolated from this screen, carried *MSB1*. pMSB4Δ, bearing a disrupted *MSB1* coding region, was made by removing a *Bgl*II-*Bgl*II fragment from pMSB4.

Two-hybrid analysis. The DBD (DNA-binding domain of LexA) fusions were constructed in pEG202 (15, 18). (These DBD fusion plasmids were kindly provided by C. De Virgilio, The University of North Carolina.) In the DBD fusion proteins, the Cys of the CAAX box (plus the adjacent Cys in Rho2p) of each GTPase was mutated to Ser to prevent prenylation (15, 56). pACTII contains the AD (transcriptional activation domain) of *GAL4*, *LEU2*, and 2μm ARS (19).

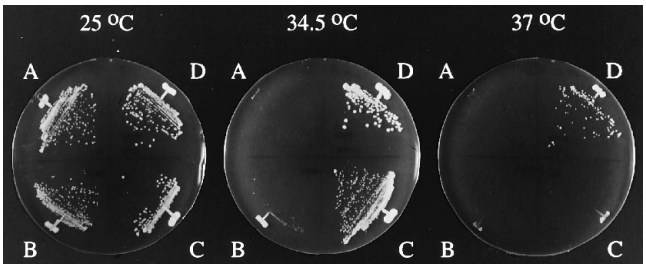

FIG. 1. Suppression of *cdc42*. Cells of *cdc42-1* strain YMR420 containing the vectors YEp24 plus YEplac112 (A), YEp24 plus YEpSRO4 (a high-copy-number plasmid carrying *SRO4*) (B), pGYM50 (a high-copy-number plasmid carrying *BEM4*) plus YEplac112 (C), and pGYM50 plus YEpSRO4 (D) were streaked on SDCA+Ade plates and incubated at the indicated temperatures for 2 days.

pPB725 contains the AD of *GAL4* fused to the third codon of *BEM4* in pACTII. pPB725 was constructed by first introducing a *Bam*HI site at the third codon of *BEM4* by site-directed mutagenesis using the oligonucleotide TTTCTTCGG GATCCATTTAG and then inserting a 2.5-kb *Bam*HI-*Sau*3AI fragment containing *BEM4* into the *Bam*HI site of a version of pACTII in which the *Nco*I site had been filled in with Klenow fragment. pACTII and pPB725 were introduced separately into Y1050, selecting for transformants on SC-Leu. The plasmids that encode the DBD fusions were introduced into the resulting pACTII- and pPB725-bearing strains, selecting for transformants on SD+Trp+Ade+Lys. Overnight liquid cultures were diluted 40-fold in SC+Trp+Ade+Lys and grown for 8 h with shaking at 30°C to an optical density at 600 nm of 0.1 to 0.5. β-Galactosidase assays were performed as described previously (41). Four independent transformants were tested for each combination of plasmids.

Morphological observations. Cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI) to reveal DNA and with rhodamine-phalloidin to reveal actin filaments as described previously (39). Cells were fixed with 5% formaldehyde for 10 min, washed with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.3]), and then stained with a 1:50-diluted solution of rhodamine-phalloidin (Molecular Probes, Eugene, Oreg.) for 2 h, and DAPI was added to a final concentration of 125 ng/ml. The cells were washed five times with PBS. These samples were mounted in 10 mg of *n*-propyl-gallate per ml in 90% glycerol and observed with an epifluorophotomicroscope (Olympus BH-2).

Nucleotide sequence accession number. The sequence shown in Fig. 3 is available from EMBL/GenBank/DDBJ under accession number L27816.

RESULTS

Isolation of multicopy suppressors of *cdc42*. *SRO4*, which was identified originally as a multicopy suppressor of *rho3* (36), can serve as a weak multicopy suppressor of the growth defect of a *cdc42-1* mutant at 34.5°C but fails to do so at 37°C (Fig. 1). To identify other genes whose functions are linked to Cdc42p, we screened for plasmids from a YEp24-based yeast genomic library that, in combination with a high-copy-number *SRO4*-bearing plasmid, would allow suppression of a *cdc42-1* mutant at 37°C. Seventy-eight plasmids were isolated in this screen. PCR analysis revealed that 74 of these contained *CDC42*. Restriction analysis indicated that the remaining plasmids, pGYM50, pGYM149, pGYM241, and pGYM245, harbored inserts derived from a single other genomic locus (Fig. 2). Although the ability of these plasmids to suppress *cdc42* effectively at 37°C required co-overexpression of *SRO4*, each plasmid was sufficient for suppression at 34.5°C (Fig. 1).

The suppressing activity was mapped to a 4.8-kb *Bgl*II-*Sal*I segment (Fig. 2). The insertion of 4 bp (by using Klenow fragment) at the unique *Afl*II site within this segment (in pGYM50-6) destroyed the suppressing activity. Sequence analysis of a 3.2-kb fragment of DNA from this region revealed that the *Afl*II site is located within a large open reading frame for a new gene, which we call *BEM4* (Fig. 3). The sequence of the inferred Bem4 protein is 633 amino acids long (Fig. 3). It is not similar to the sequence of any other known protein and contains no obvious motifs.

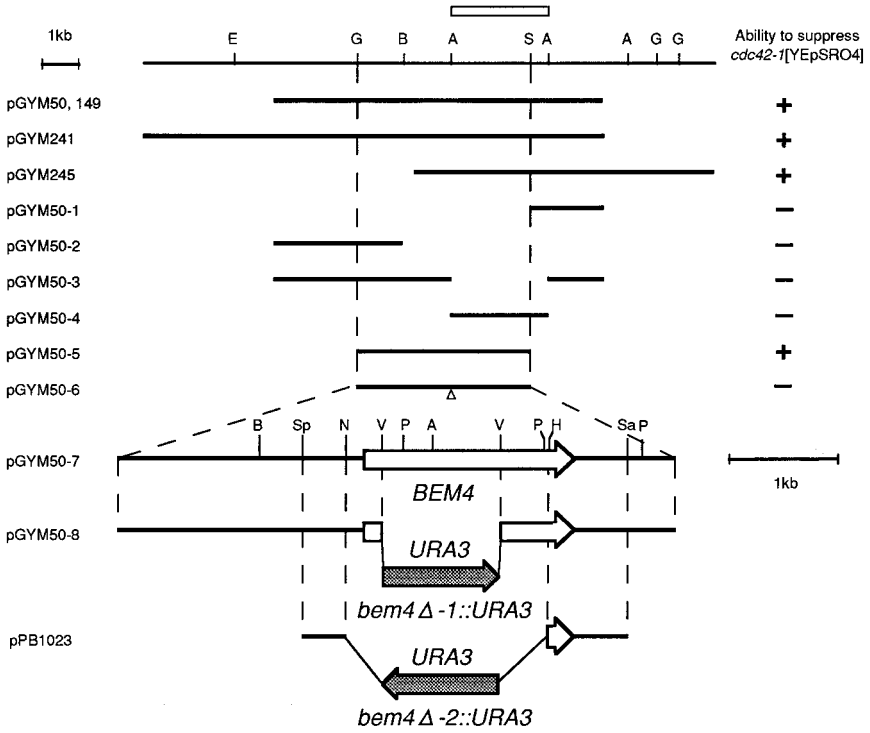

FIG. 2. Subcloning of *BEM4*. The inserts of the isolated *BEM4* clones and subclones, and the Δ*bem4* disruption alleles, are diagrammed below the restriction map of the *BEM4* region. + and − indicate growth and lack of growth of transformants (in YEpSRO4-bearing strain YMR420) after incubation for 3 days at 37°C. The coding region of *BEM4* is represented by an open arrow. Shadowed arrows represent the coding region of *URA3*. The open box indicates the fragment used as a probe for physical mapping. The small arrowhead represents the *Afl*II site at which 4 bp were inserted into pGYM50-6. All A (*Afl*II), B (*Bst*XI), E (*Eco*RI), G (*Bgl*II), P (*Pvu*II), S (*Sal*I), and V (*Eco*RV) and some H (*Hpa*I), N (*Nru*I), Sa (*Sau*3AI), and Sp (*Spe*I) sites are shown.

Disruption of *BEM4*. To investigate the function of *BEM4*, we deleted it from the *MAT***a**/*MAT*α diploid strain YPH501 (see Materials and Methods). Segregants deleted for *BEM4* were viable at 30°C but inviable at 37°C. When an exponentially growing culture of Δ*bem4* strain GYM5-1 was shifted from 30 to 37°C, cells began to stop dividing after approximately 5 h (data not shown). Eight hours after the shift, approximately 80% of the cells were unbudded, round, and large (Fig. 4). Approximately 20% of all cells contained two nuclei. These phenotypes are similar to those caused by loss of Cdc42p function (1). Although the distribution of actin appeared normal at 30°C (Fig. 4A), actin patches were distributed throughout the cell cortex at 37°C (Fig. 4B).

Identification of *CDC42* multicopy-suppressee mutants. Mutants that require multiple copies of *CDC42* for survival were sought from strains PY406 and PY407, which contain the *CDC42*- and *ADE3*-bearing plasmid pPB320, using a colony-sectoring assay (see Materials and Methods). In this assay, a mutant that requires multiple copies of *CDC42* for survival forms uniformly red colonies that lack white sectors (Sect$^-$ phenotype). We imagined that different mutations might be suppressed by *CDC42* at different temperatures, and so we searched for *CDC42*-requiring mutants at 30, 34, and 37°C. Prior experience (3) suggested that the Sect$^-$ phenotype of most or all of the dominant Sect$^-$ mutants would be due to integration of pPB320 into the genome. Therefore, we did not pursue any dominant Sect$^-$ mutants.

Among the remaining mutants, we identified four complementation groups that required specifically *CDC42*, as opposed to *ADE3* or *LEU2*, from pPB320 for survival (see Materials and Methods) (Table 2). By a combination of complementation and plasmid-based suppression tests, the group I and group II mutants were deduced to be defective in *CDC42* and *CDC24*, respectively. The isolation of mutations in *CDC42* and *CDC24* was expected because *CDC42* is essential for viability (26) and *CDC42* was previously isolated as a multicopy suppressor of *cdc24* (2). The following results suggest that strain PY632, the sole member of group III, is defective in *BEM1*. First, the diploid formed by crossing PY632 to *bem1*::*LEU2* strain PY975, when cured of plasmid at 23°C, was unable to grow at 37°C. Second, when transformed with a low-copy-number plasmid bearing *BEM1*, PY632 became Sect$^+$. The growth defects of the *bem1*::*LEU2* strain Y1008 at 35°C could be partially suppressed by overexpression of *CDC42* (from plasmid pPB102), demonstrating that *CDC42* can indeed serve as a multicopy suppressor of *bem1*.

From the results of complementation and plasmid-based suppression tests, we concluded that the group IV mutants were not defective in any of the known bud emergence genes *CDC24*, *CDC42*, *CDC43*, *BEM1*, or *BEM2* (data not shown). Members of this group, although Sect$^-$ at 30°C or higher temperatures, were Sect$^+$ at 23°C. From the YCp50-based genomic library, we isolated a plasmid that could suppress both the Sect$^-$ phenotype of group IV mutant PY808 at 30°C and the growth phenotype of the same strain at 37°C (after loss of pPB320 at 23°C). A 2.6-kb *Nru*I-*Sau*3A segment of the insert from this plasmid (Fig. 2 and 3) was sufficient for the suppression of both the Ts$^-$ growth defect and the Sect$^-$ phenotype. A 3.2-kb segment of DNA that encompasses this suppressing region was sequenced and found to contain *BEM4* (Fig. 2 and 3). Δ*bem4* strain Y1051, which grows only poorly at 30°C and is inviable at 33°C, could grow well at temperatures up to 37°C when transformed with the high-copy-number, *CDC42*-bearing plasmid pPB320 (data not shown), indicating that the Ts$^-$

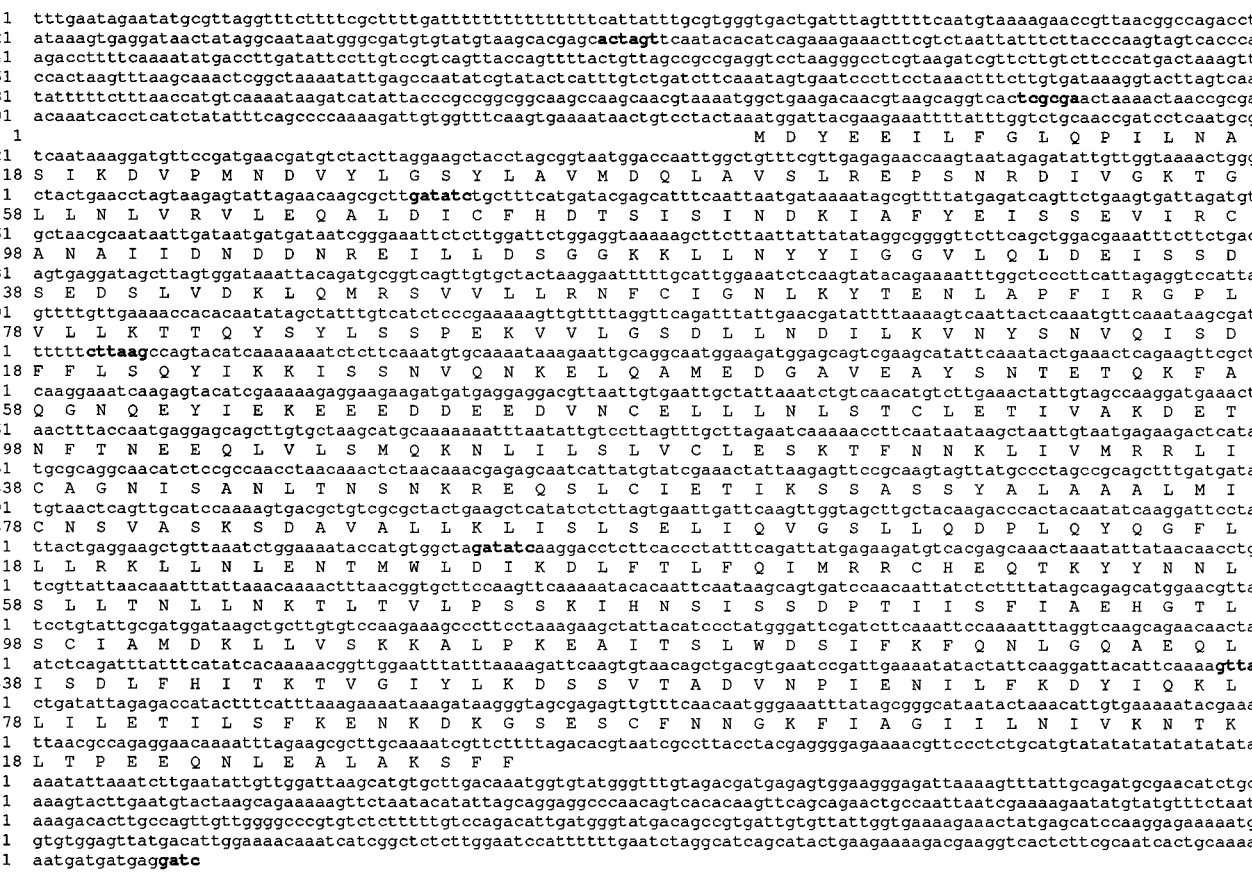

FIG. 3. Nucleotide and deduced amino acid sequences of *BEM4*. The following sites are indicated with boldface letters: *Spe*I (175), *Nru*I (575), *Eco*RV (877), *Afl*II (1326), *Eco*RV (1963), *Hpa*I (2394), and *Sau*3A (3133).

growth defect of *bem4* mutants can indeed be suppressed by overexpression of *CDC42*. However, for reasons that are not yet clear, high-copy-number *CDC42* was not sufficient for suppression of the Δ*bem4* growth defect in the unrelated strain GYM5-11 (see Fig. 5C; discussed below).

Although mutations in *CDC42* and *CDC24* were isolated from screens using both PY406 and PY407, all 32 of the *bem4* mutants were derived from PY407 (and none were derived from PY406), suggesting that PY407 may contain one or more other mutations that are required for the Sect$^-$ phenotype of *bem4* cells. In crosses between each of two group IV (*bem4* Sect$^-$ Ts$^-$) mutants and Y382 (*BEM4* Sect$^+$ Ts$^+$), the Ts$^-$:Ts$^+$ phenotype segregated 2:2, confirming that loss of *BEM4* function is sufficient for the Ts$^-$ phenotype at 37°C. However, only approximately half of the Ts$^-$ segregants, and none of the Ts$^+$ segregants, were Sect$^-$ at 30°C (Table 3), suggesting that a single other mutation is required for the Sect$^-$ phenotype of *bem4* mutants. We have not yet identified this second mutation.

Identification of mutations that display synthetic lethality with *cdc24*. Cells carrying the *cdc24-4* mutation, although unable to grow at 37°C, can grow well at 30°C. To search for mutations that displayed synthetic lethality with *cdc24*, we used a colony-sectoring assay to screen for mutations that caused *cdc24-4* strains PY88 and PY92 to require *CDC24* (on the *ADE3*-bearing plasmid pPB170) for survival at 30°C. From this screen, 161 recessive Sect$^-$ mutants were isolated. From the results of complementation and linkage analyses, we determined that 159 of these mutants were likely to be defective in *CDC24*. These mutants were not studied further. One of the two remaining Sect$^-$ mutants, PY278, was judged to be defective in *BEM1* because (i) a *BEM1*-containing, low-copy-number plasmid was able to suppress its Sect$^-$ phenotype and (ii) the diploid created by crossing it to *bem1*::*LEU2* strain PY899, when cured of plasmid at 23°C, was unable to grow at 37°C.

The single remaining Sect$^-$ mutant that was identified from this screen is strain 10.7. From the results of tetrad analysis, we judged that the Sect$^-$ phenotype of 10.7 was due to a synthetic-lethal interaction between *cdc24-4* and a second mutation that, by itself, also gave a Ts$^-$ growth phenotype (data not shown). A single plasmid that could suppress the Ts$^-$ phenotype was isolated from the YCp50-based genomic library. Restriction analysis indicated that this plasmid contained *BEM4*. Moreover, the diploid strain formed by crossing Y704 (a derivative of 10.7, cured of plasmid) to *bem4*Δ*-2*::*URA3* strain Y833 was unable to grow at 37°C, strongly suggesting that Y704 does indeed contain a mutation in *BEM4*. In a cross between *bem4*Δ*-1*::*URA3* strain GYM5-2 and *cdc24-4* strain JC24-4, although both the *cdc24-4 BEM4* and *CDC24 bem4*Δ*-1*::*URA3* segregants grew well at 32°C, the *bem4*Δ*-1*::*URA3 cdc24-4* segregants were inviable at this temperature (data not shown), confirming that *cdc24-4* is synthetically lethal with *bem4*.

Two-hybrid interactions involving Bem4p and Rho-type GTPases. Cdc42p displays a two-hybrid interaction with Bem4p (Table 4). Combined with the multicopy suppressor and multicopy suppressee relationships between *BEM4* and *CDC42*, this two-hybrid interaction suggests that Cdc42p binds to Bem4p during the normal functions of these proteins. Cdc42p^{G12V} and

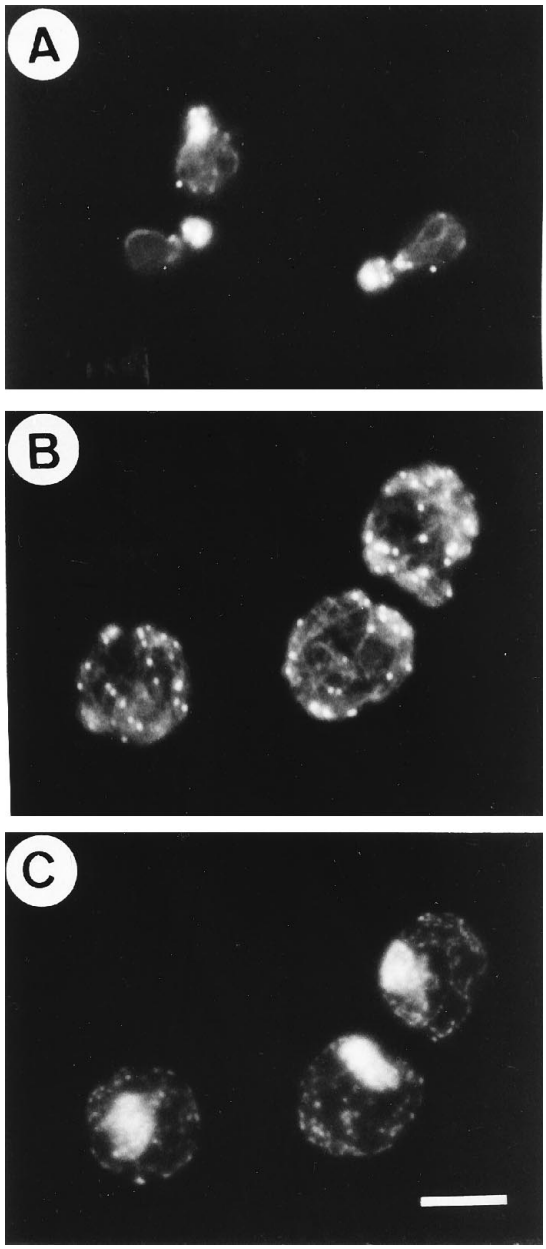

FIG. 4. Phenotypes of Δ*bem4* mutants. A culture of *bem4*Δ*-1*::*URA3* strain GYM5-1 that was growing exponentially at 30°C was split in two; one culture was kept at 30°C, and the other was grown at 37°C. Cultures were harvested after 8 h at 30°C (A) or 37°C (B and C) and stained with rhodamine-phalloidin to reveal actin (A and B) and with DAPI to reveal DNA (C). The bar represents 5 μm.

$Cdc42p^{Q61L}$ are mutant versions of Cdc42p that are predicted to exist predominantly in the GTP-bound state. $Cdc42p^{D118A}$ is a mutant version of Cdc42p that gives a dominant negative phenotype and is predicted to have a conformation similar to that of the GDP-bound, inactive form (55, 56). All three of these mutant versions of Cdc42p displayed comparable two-hybrid interactions with Bem4p, suggesting that Bem4p can interact with both the active and inactive forms of Cdc42p.

Bem4p also displays two-hybrid interactions with Rho1p, Rho2p, and Rho4p (Table 4), suggesting that Bem4p binds to multiple Rho-type GTPases. However, we failed to detect two-hybrid interactions between Bem4p and either Rho3p (Table 4) or the Ras-type GTPase Ras2p (15a).

TABLE 2. *CDC42*-requiring mutants isolated at different temperatures[a]

Temp (°C)	No. of mutants in complementation group (gene):			
	I (*CDC42*)	II (*CDC24*)	III (*BEM1*)	IV (*BEM4*)
30	38	9	1	12
34	83	3	0	17
37	14	0	0	3

[a] Sect$^-$ mutants were isolated from strains PY406 and PY407 and analyzed as described in Materials and Methods.

Genetic interactions between *BEM4* and *RHO1*. The two-hybrid interaction between Bem4p and Rho1p suggests that these two proteins may interact functionally with each other. To explore this possibility, we examined whether *RHO1* could serve as a multicopy suppressor of the Ts$^-$ growth defect caused by deletion of *BEM4*. To do so, we exploited the finding that overexpression of *CDC42* was not sufficient for suppression of the growth defect of Δ*bem4* strain GYM5-11 at 37°C (Fig. 5C). Overexpression of *RHO1* alone was also insufficient for suppression at 37°C (Fig. 5B). However, co-overexpression of *RHO1* and *CDC42* effected strong suppression (Fig. 5D), suggesting that Bem4p does indeed play a role in the function of Rho1p in addition to its putative role in the function of Cdc42p.

Identification of multicopy suppressors of *bem4*. To investigate further the function of Bem4p, we screened for multicopy suppressors of the Ts$^-$ growth defect of a Δ*bem4* mutant. From a high-copy-number yeast genomic library, we identified 11 plasmids that enabled GYM5-11 cells to grow well at 37°C (see Materials and Methods). The results of restriction analysis indicated that two of these plasmids carry *BEM4* itself and the other nine contain DNA from four other loci (data not shown). From restriction and PCR analysis (see Materials and Methods), we determined that one of these loci contained *MSB1* (data not shown), a gene that was shown previously to be a multicopy suppressor of both *cdc24* and *cdc42* in sorbitol-containing medium (2) and to display synthetic-lethal interactions with *bem1* and *bem2* (3). Deletion of a 0.5-kb *Bgl*II-*Bgl*II fragment from within the *MSB1* open reading frame of the isolated plasmid eliminated its ability to suppress the Ts$^-$ growth defect of GYM5-11, indicating that the relevant gene in this insert is indeed *MSB1* (data not shown). The characterization of the three remaining multicopy suppressors of *bem4*, which we call *SBE1-3*, will be presented elsewhere.

DISCUSSION

The goal of this study was to identify genes whose functions are linked to that of Cdc42p. We discovered *BEM4* from screens for (i) multicopy suppressors of *cdc42*, (ii) mutations

TABLE 3. Segregation patterns of the Sect$^-$ and Ts$^-$ phenotypes in crosses involving *bem4*

Cross (relevant genotype)	No. of segregants			
	Ts^{+a}		Ts$^-$	
	Sect^{+b}	Sect$^-$	Sect$^+$	Sect$^-$
PY808 (*bem4-1*) × Y382 (*BEM4*)	24	0	14	10
11B (*bem4-2*) × Y382 (*BEM4*)	22	0	11	6

[a] Growth at 37°C was assayed after cells were cured of plasmid at 23°C.
[b] The sectoring phenotype was assayed at 30°C.

TABLE 4. Two-hybrid interactions between Bem4p and Rho-type GTPases

DBD fused to[a]:	β-Galactosidase activity[b] (U)	
	AD−[c]	AD-Bem4[d]
—	0.09 ± 0.08	0.17 ± 0.08
$Cdc42^{C188S}$	2.2 ± 0.6	280 ± 20
$Cdc42^{G12V,C188S}$	9.5 ± 1.4	270 ± 30
$Cdc42^{Q61L,C188S}$	10 ± 3	250 ± 60
$Cdc42^{D118A,C188S}$	0.13 ± 0.03	380 ± 60
$Rho1^{C206S}$	0.08 ± 0.03	99 ± 34
$Rho2^{C188S/C189S}$	0.15 ± 0.01	66 ± 18
$Rho3^{C228S}$	0.08 ± 0.03	0.14 ± 0.07
$Rho4^{C188S}$	0.12 ± 0.02	330 ± 70

[a] The DBD fusions were all expressed from derivatives of pEG202 (see Materials and Methods). —, unfused DBD.
[b] Mean ± the standard deviation derived from four independent transformants.
[c] Unfused AD of Gal4p, encoded by pACTII.
[d] Encoded by pPB725.

that required multiple copies of *CDC42* for survival, and (iii) mutations that displayed synthetic lethality with a mutant allele of *CDC24*. The only other genes that were isolated from these screens were *CDC24* (from the second screen) and *BEM1* (from the second and third screens), suggesting that these screens were indeed selective for genes whose functions are tightly linked to that of Cdc42p. At high temperatures, deletion of *BEM4* can cause cells to become unbudded, round, large, and multinucleate, consistent with the view that Bem4p functions with Cdc42p in bud emergence.

Suppression of the Ts^- growth defect of Δ*bem4* strain GYM5-1 required co-overexpression of *RHO1* and *CDC42*, suggesting that Bem4p also plays a role in Rho1p function. *MSB1*, which can serve as a multicopy suppressor of the Ts^- growth defect of Δ*bem4* cells, can also serve as a multicopy suppressor of the Ts^- growth phenotypes of both *cdc42* (3) and *rho1* (40a) mutants, consistent with a model in which Bem4p promotes the actions of both Cdc42p and Rho1p. Strong support for the idea that Bem4p promotes the action of Rho1p comes from the recent isolation of *BEM4* as a multicopy suppressor of the growth defect caused by a dominant negative allele of *rho1* (22). Additional support for this view is the finding that *SBE1*, which we isolated as a multicopy suppressor of the Ts^- growth defect of the Δ*bem4* mutant GYM5-1, maps to the same locus (adjacent to *PHO85* on chromosome XVI [36a]) that was identified recently as a multicopy suppressor of *rho1* (40a).

The phenotypes caused by deletion of *BEM4* varied with genetic background. For example, the restrictive temperatures for growth caused by deletion of *BEM4* were different for different strains. Also, although deletion of *BEM4* from some strains (e.g., YPH501 [Fig. 4], W303, and KA31 [data not shown]) caused cells to become unbudded, large, round, and multinucleate at 37°C, its deletion from other strains (e.g., Y799) did not cause such obvious bud emergence defects. Instead, Δ*bem4* cells derived from Y799 became misshapen and lysed at 37°C (32a). We have observed similar effects with *BEM1* and *BEM2*, whose products are also linked to the functions of Rho-type GTPases; in some strains, the deletion of either gene leads to a bud emergence defect, whereas in other strains, these deletions result in misshapen cells that lyse at high temperatures. In addition, the restrictive temperature for deletion of either *BEM1* or *BEM2* can vary from one strain to another (1a). We have not yet identified the background mutations that affect the phenotypes of strains deleted for the *BEM* genes.

Because genetic and two-hybrid interactions suggest that Bem4p can affect the functions of both Cdc42p and Rho1p, it is possible that the phenotypic variation of different Δ*bem4* strains is due to one or more background mutations that affect the functions of one or the other or both of these GTPases. The Ts^- growth defect of Δ*bem4* strain Y1051 can be efficiently suppressed by overexpression of *CDC42* alone, suggesting that this strain may be relatively insensitive to the Δ*bem4* effects on Rho1p function. In contrast, the finding that co-overexpression of *CDC42* and *RHO1* is required for suppression of the Ts^- growth defect of Δ*bem4* strain GYM5-1 suggests that this strain may have one or more mutations that make it particularly sensitive to impaired Rho1p function.

Bem4p displays two-hybrid interactions with Cdc42p, Rho1p, Rho2p, and Rho4p. These GTPases are not so similar in sequence to one another that any protein that displays a two-hybrid interaction with one will inevitably interact with the others; various other proteins that display two-hybrid interactions with Cdc42p (e.g., Cla4p, Ste20p, Bem3p, and Rga1p) failed to display two-hybrid interactions with Rho1p-Rho4p (15, 46, 49). Thus, these GTPases can be discriminated from one another in two-hybrid assays. The genetic interactions between Bem4p and both Cdc42p and Rho1p support the view that Bem4p binds to Cdc42p and Rho1p during normal function in vivo. In this context, it is likely that the two-hybrid interactions between Bem4p and both Rho2p and Rho4p also reflect interactions that occur in vivo.

The ability of Bem4p to display two-hybrid interactions with wild-type Cdc42p, $Cdc42p^{G12V}$, $Cdc42p^{Q61L}$, and $Cdc42p^{D118A}$ (Table 4) suggests that Bem4p can bind to both the active and inactive forms of Cdc42p. Of the proteins known to bind to Rho-type GTPases (e.g., GDIs, GEFs, GAPs, and effectors), only Rho GDIs can bind to both the GDP- and GTP-bound states of multiple Rho-type GTPases, raising the possibility that Bem4p itself may serve one or more roles associated with Rho GDI. Some support for this possibility is the observation that although *RDI1*, which encodes a yeast Rho GDI, normally can be deleted without obvious effect on cell growth (34), *RDI1* becomes essential, or nearly so, in cells that lack *BEM4* (34a). However, Rdi1p appears to account for all of the Rho GDI activity in yeast strains (34). Furthermore, Bem4p is not similar in sequence to known Rho GDIs, and *RDI1* cannot serve as a

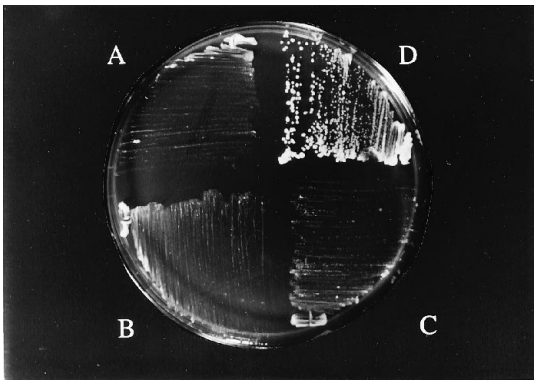

FIG. 5. Suppression of Δ*bem4*. Cells of *bem4Δ-1*::*URA3* strain GYM5-11 containing the vectors YEp24 plus YEplac112 (A), YEp24 plus pOPR1 (a high-copy-number plasmid carrying *RHO1* under the control of the *TDH3* promoter) (B), pSRO2-3 (a high-copy-number plasmid carrying *CDC42*) plus YEplac112 (C), and pSRO2-3 plus pOPR1 (D) were streaked on YPD plates and incubated at 37°C for 2 days.

multicopy suppressor of Δ*bem4* (34a). Thus, although Bem4p may have a function related to that of Rho GDI, we think that Bem4p itself is unlikely to be a Rho GDI. Other potential roles for Bem4p include promoting the folding, stability, or localization of multiple Rho-type GTPases or regulating the binding of these GTPases to their targets or regulatory proteins (e.g., GEFs, GAPs, GDIs, processing proteins, or degradation machinery).

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