Interactions between the Bud Emergence Proteins Bem1p and Bem2p and Rho-type GTPases in Yeast

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Abstract. The SH3 domain-containing protein Bem1p is needed for normal bud emergence and mating projection formation, two processes that require asymmetric reorganizations of the cortical cytoskeleton in *Saccharomyces cerevisiae.* To identify proteins that functionally and/or physically interact with Bem1p, we screened for mutations that display synthetic lethality with a mutant allele of the *BEM1* gene and for genes whose products display two-hybrid interactions with the Bem1 protein. *CDC24,* which is required for bud emergence and encodes a GEF (guanine–nucleotide exchange factor) for the essential Rho-type GTPase Cdc42p, was identified during both screens. The COOH-terminal 75 amino acids of Cdc24p, outside of the GEF domain, can interact with a portion of Bem1p that lacks both SH3 domains. Bacterially expressed Cdc24p and Bem1p bind to each other in vitro, indicating that no other yeast proteins are required for this interaction. The most frequently identified gene that arose from the *bem1* synthetic-lethal screen was the bud-emergence gene *BEM2* (Bender and Pringle. 1991. *Mol. Cell Biol.* 11:1295–1395), which is allelic with *IPL2* (increase in ploidy; Chan and Botstein, 1993. *Genetics.* 135:677–691). Here we show that Bem2p contains a GAP (GTPase-activating protein) domain for Rho-type GTPases, and that this portion of Bem2p can stimulate in vitro the GTPase activity of Rho1p, a second essential yeast Rho-type GTPase. Cells deleted for *BEM2* become large and multinucleate. These and other genetic, two-hybrid, biochemical, and phenotypic data suggest that multiple Rho-type GTPases control the reorganization of the cortical cytoskeleton in yeast and that the functions of these GTPases are tightly coupled. Also, these findings raise the possibility that Bem1p may regulate or be a target of action of one or more of these GTPases.

BUD emergence in yeast requires the assembly of cytoskeletal elements at a discrete region of the plasma membrane soon after entry into the cell cycle. Early events in the formation of a bud site include the localization of a cluster of cortical actin "patches," the assembly of the neck filaments, and the capture of microtubules at the presumptive bud site (Kilmartin and Adams, 1984; Ford and Pringle, 1991; Kim et al., 1991; Snyder et al., 1991). Genes that are required for the assembly of cortical cytoskeletal structures have been identified during searches for cell-division cycle (*cdc*)[1] mutants defective in bud emergence. At restrictive temperature, cells that carry Ts⁻ mutations in *CDC24, CDC42,* or *CDC43* fail to bud and become multinucleate, large, and round, suggesting that these genes are required specifically for bud emergence but not for entry into the nuclear-division cycle or delocalized secretion (Hartwell et al., 1974; Sloat et al., 1981; Adams et al., 1990). Cdc42p is a GTPase of the Rho family (Johnson and Pringle, 1990). The functions of Cdc24p and Cdc43p are tightly linked to that of Cdc42p: Cdc24p is a GEF for Cdc42p (Zheng et al., 1994) and Cdc43p is the β subunit of a type I geranylgeranyl transferase that is required for the proper modification of the COOH terminus of Cdc42p and other GTPases (Ohya et al., 1993).

One general model for the role of Cdc42p in bud initiation is that it is directly, physically involved in the assembly of cytoskeletal structures. The observation that Cdc42p is found to be most concentrated at the presumptive bud site and remains at the tip of a bud during the early stages of bud growth (Ziman et al., 1993) is consistent with such a model. However, this pattern of localization of Cdc42p is also consistent with a second general model in which Cdc42p functions in a signal-transduction cascade. Possible support for this second model comes from recent reports that some mammalian Rho-type GTPases, including one that is 80% identical in sequence to yeast Cdc42p, can bind to the protein kinases PAK and ACK (Manser et al., 1993, 1994). The kinase domain of PAK is 70% identical in sequence to Ste20p (Manser et

1. *Abbreviations used in this paper:* cdc, cell-division cycle; DBD, DNA-binding domain; AD, activation domain.

al., 1994), a yeast protein that functions in the mating pheromone response pathway (Leberer et al., 1992; Ramer and Davis, 1993). The region of PAK that binds to human Cdc42p is similar in sequence to a portion of Ste20p (Manser et al., 1994), raising the possibility that Cdc42p might act in one or more MAP kinase pathways whose targets of phosphorylation control cytoskeletal rearrangements.

Clues to the mechanism by which Cdc42p controls the reorganization of the cortical cytoskeleton may come from the study of genes that interact with *CDC24* and *CDC42*. The *MSB1* gene was previously identified as a multicopy suppressor of the Ts$^-$ phenotype of *cdc24* and *cdc42* mutants (Bender and Pringle, 1989; D. Mack and A. Bender, unpublished data). Deletion of *MSB1* has no obvious deleterious effects on cell viability or morphology. From a screen for mutations that display synthetic lethality with a deletion allele of *MSB1*, *BEM1* and *BEM2* were identified (Bender and Pringle, 1991). *BEM2* is allelic with *IPL2*, a gene that was identified during a screen for Ts$^-$ mutants that display an increase-in-ploidy phenotype at restrictive temperature (Chan and Botstein, 1993). Cells that are defective in either *BEM1* or *BEM2* function become large and multinucleate (Bender and Pringle, 1991; Chant et al., 1991; Chenevert et al., 1992; Chan and Botstein, 1993). These phenotypes and the genetic interactions with *MSB1* suggest that *BEM1* and *BEM2* are involved in bud emergence. Support for the view that *BEM1* plays a role in bud initiation comes from the observations that Bem1p is localized to the bud site early in the cell cycle (K. Corrado and J. Pringle, personal communication) and that *bem1* mutants can display a *cdc* bud-emergence arrest phenotype when the bud-site selection gene *BUD5* is also defective (Chant et al., 1991). Consistent with the view that Bem1p is involved in the control of cytoskeletal reorganizations is the finding that *bem1* mutants are defective for mating projection formation (Chenevert et al., 1992, 1994).

Bem1p contains two copies of the SH3 domain, a protein–protein interaction motif found in a variety of proteins associated with the cortical cytoskeleton (Chenevert et al., 1992). To investigate the molecular functions of Bem1p, we have sought to identify proteins that interact with it by searching for mutations that display synthetic lethality with a mutant allele of *BEM1* and for proteins that display two-hybrid interactions with the Bem1 protein.

Materials and Methods

Plasmids, Strains, and Media

The plasmids and yeast strains used in this study are described in Tables I and II. Bacterial strain LU1 (gift of D. Hagen, University of Oregon, Eugene, OR) is a *pyrF::Tn5* derivative of SB69 (Hagen and Sprague, 1984). Bacterial strain TG1, which contains *laci^q*, is from the Amersham Corporation (Arlington Heights, IL).

Plasmid pPB443, which contains *bem2-Δ1::URA3*, was constructed in multiple steps as follows. The 1.2 kb *SmaI-NsiI* fragment of DNA that extends from ∼0.9 kb upstream of the start codon of *BEM2* to the *NsiI* site at codon 57 (Figs. 1 and 2) was inserted into the *PstI-SmaI* sites of M13mpl8 ·(which contains an *EcoRI* and an *SphI* site in the polylinker) to give phage B42. The resulting 1.2-kb *EcoRI-SphI* fragment of B42 was then inserted into the *EcoRI-SphI* sites of plasmid pBR322 to give plasmid pPB442. The 2.7-kb *BglII-NheI* fragment of *BEM2* that extends from position 5549 (within the *BEM2* open-reading frame; Figs. 1 and 2) to the *NheI* site 3' of *BEM2* (see Fig. 1) was inserted into the *BamHI-XbaI* sites of M13mpl8 to give phage B20. The portion of the *BEM2* gene 5' of that *AccI* site which is 19 bp downstream of the stop codon of *BEM2* (corresponding to position

6826 in Fig. 2) in B20 was removed, as follows, to give phage B50. First, the *SalI* site in B20 was destroyed by filling it in using the Klenow fragment of DNA polymerase. The segment of DNA between the *AccI* site in *BEM2* and the *SmaI* site in the polylinker was then deleted by blunt-ended ligation after filling in the *AccI* overhang using Klenow fragment. Phage URA3-M13 was constructed by inserting the 1.6-kb *SphI-SacI* fragment from YIpURA3 (S. Lillie, University of Michigan, Ann Arbor, Michigan) into the *SphI-SacI* sites of M13mpl8. Finally, the 1.6-kb *SphI-SacI* fragment of URA3-M13 plus the *SacI-SphI* fragment of B50 were inserted together into the *SphI* site of pPB442 to give pPB443. In this plasmid, the portion of *BEM2* between the *NsiI* site at codon 56 and the *AccI* site that is 20 bp downstream of the stop codon of *BEM2* have been replaced by *URA3* and DNA from between the *HindIII* and *SphI* sites of pBR322.

The media used in this study were YPD (1% yeast extract, 2% peptone, and 2% glucose); SD (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose); SC (SD plus 20 µg/ml uracil, 80 µg/ml L-leucine, 20 µg/ml adenine sulfate, 20 µg/ml L-histidine, 20 µg/ml L-tryptophan, 20 µg/ml L-methionine, and 30 µg/ml L-lysine); SC-Leu (SC without L-leucine); SC-Ura (SC without uracil); SC-His (SC without L-histidine); SC-Trp (SC without L-tryptophan); SC-Trp-Leu (SC without L-tryptophan and L-leucine); and SC-Trp-Leu+X-gal (SC-Trp-Leu with 2% sucrose instead of glucose, plus 100 mM KH$_2$PO$_4$ [buffered to pH 7.0 with KOH] and 40 µg/ml X-gal [from a 40 mg/ml stock in dimethylformamide]).

Screen for Mutants That Display Synthetic Lethality with bem1

Colonies of PY429 and PY431 cells were grown on SC-His plates to select for plasmid pPB403 (*ade3* mutants are phenotypically His$^-$), resuspended and diluted in YPD, plated to give ∼2,000 cells per plate, and mutagenized to ∼10% survival using UV irradiation. Plates were incubated at 30°C for 5 d and then put at 4°C to allow the red color to develop. Thoroughly red colonies (lacking white sectors) were first streaked on YPD and incubated at 30°C to confirm that they displayed the Sect$^-$ phenotype and then streaked on YPD and incubated at 37°C to assay for Ts$^-$ growth defects. All PY429-derived Ts$^-$ Sect$^-$ mutants were crossed individually with all PY431-derived Ts$^-$ Sect$^-$ mutants by plating mixed cell suspensions on YPD medium and incubating for one day at 30°C. Mating mixes were streaked onto SD supplemented with adenine, leucine, and uracil to select for diploids. Cells from individual colonies of the resulting diploids were streaked on YPD and then checked for growth after 2 d at 37°C. Complementation tests with known mutants were done using strains Y132 and Y134 (for *cdc24*), DJTD1-14B and DJTD1-24C (for *cdc42*), JPT198BD4-4A and CJ198-2B (for *cdc43*), and Y529 and Y565 (for *bem2*), selecting when possible for diploids on an appropriate selective minimal medium.

Cloning and Localization of BEM2

The lithium-thiocyanate procedure (Keszenman-Pereyra and Hieda, 1988) was used to transform plasmids into *bem2* strain Y454. Transformants were selected on SC-Leu or SC-Ura medium at room temperature. Four individual transformants for each plasmid were streaked on YPD and assayed for growth after 2 d at 36°C.

The following colony-hybridization procedure was used to clone the remainder of the *BEM2* gene. Colonies of bacteria carrying a YCp50-based genomic library (Rose et al., 1987) were lifted onto Whatman 541 filter disks. The disks were allowed to dry at room temperature and then successively transferred onto Whatman paper wetted with 0.5 M NaOH + 1.5 M NaCl (10 min); 0.5 M Tris, pH 7.5 (5 min); 0.5 M Tris, pH 7.5 (5 min); and 2× SSC (Maniatis et al., 1982; 5 min). The disks were immersed in 95% EtOH and shaken for 10 min and then immersed in fresh 95% EtOH and shaken for an additional 10 min. Disks were allowed to dry at room temperature and then baked at 80°C for 2 h. They were then incubated in the following hybridization solution overnight at 65°C: 5× SSC, 2× Denhardt's reagent (Maniatis et al., 1982), 50 mM NaPO$_4$ (pH 6.5), 200 µg/ml sonicated salmon sperm DNA, and 0.1% SDS. [α-^{32}P]dATP was incorporated into a DNA probe by primer extension with Klenow fragment (Maniatis et al., 1982), using a segment of *BEM2* in M13 as the template. The probe was denatured by incubation at 100°C for 15 min and then incubated with the disks in hybridization solution overnight at 65°C. Disks were washed in 2× SSC + 0.1% SDS for 6 h at room temperature, with four changes of the wash solution. The positions of the colonies on the filters were determined by staining with 0.25% methyl green dye, and the colonies that contained DNA that hybridized to the probe were identified by autoradiography.

Table I. Plasmids and Phage Used in This Study

Plasmids & phage	Characteristics
YEp24	*URA3*, origin of replication and copy number control sequences from the high copy number vector 2μm (Botstein et al., 1979).
pSL113	*LEU2*, 2μm (Bender and Sprague, 1989).
pPB321	*BEM1*, *URA3*, 2μm (Bender and Pringle, 1991); isolate from a genomic library in YEp24 (Carlson and Botstein, 1982).
pPB166	*ADE3*, *LEU2*, 2μm; made by inserting a 5-kb *Bam*HI-*Sal*I fragment bearing *ADE3* (Koshland et al., 1985) into the *Bam*HI-*Sal*I sites of pSL113.
pPB403	*BEM1*, *ADE3*, 2μm; constructed by inserting a 4-kb *Sma*I-*Xho*I fragment containing *BEM1* from pPB321 into the *Eco*RV-*Sal*I sites, and a 4-kb *Nhe*I-*Nhe*I fragment containing *ADE3* from pPB166 into the *Xba*I site, of YEp24.
pPB304	*BEM2*, *URA3*, 2μm (Bender and Pringle, 1991); isolate from a genomic library in YEp24 (Carlson and Botstein, 1982). In this plasmid, the *BEM2* open-reading frame is oriented in the direction opposite to that of the tetracycline resistance gene (*Tc*).
pPB386	*bem2*, *URA3*, 2μm; a derivative of pPB304 in which the 1.1-kb *Bgl*II-*Bgl*II fragment of DNA within *BEM2* was deleted. In this construct, the portion of *BEM2* downstream of the 3' *Bgl*II site is not in frame with the portion of *BEM2* upstream of the 5' *Bgl*II site.
pPB378	*bem2*, *LEU2*, 2μm; made by inserting a 4-kb *Spe*I (within *BEM2*)-*Sal*I (within *Tc*) fragment from pPB304 into the *Nhe*I-*Sal*I sites of pSL113.
pPB384	*bem2*, *URA3*, 2μm; a derivative of pPB304 in which the DNA between the *Sph*I site in *BEM2* and the *Sph*I site in *Tc* was deleted.
pPB318	*BEM2::LEU2*, *URA3*, 2μm; a derivative of pPB304 in which the 1.1-kb *Bgl*II-*Bgl*II fragment of *BEM2* was replaced with a 2.8-kb *Bgl*II-*Bgl*II fragment of DNA containing *LEU2*.
YEp55B	p*GAL10*, *LEU2*, 2μm (Rose and Broach, 1990).
pPB467	p*GAL10-BEM2*$_{COOH\ terminus}$, *LEU2*, 2μm; made by inserting the 1.4-kb *Bgl*II-*Hind*III fragment of *BEM2* from pPB304 into the *Bam*HI-*Hind*III sites of YEp55B.
pPB443	*bem2-Δ1::URA3* (see Materials and Methods).
pCTC52	DBD$_{lexA}$-lamin, *TRP1*, 2μm: contains the sequences encoding the DNA-binding domain (DBD) of the bacterial lexA protein fused to a cDNA that encodes human lamin C. (C. Chen and R. Sternglanz, personal communication)
pPB583	*BEM1* with a *Bgl*II site introduced at its fourth codon, *LEU2*, 2μm; constructed by ligating the linker GGAAGATCTTCC onto the ends of a 3.7-kb *Xmn*I fragment from pPB321 and then inserting the resulting 3.7-kb *Bgl*II-*Bgl*II fragment (containing *BEM1*) into the *Bgl*II-*Bgl*II sites of pSL113.
pBTM116	DBD$_{lexA}$, *TRP1*, 2μm (Bartel et al., 1993).
pPB550	DBD$_{lexA}$-BEM1$_{full-length}$, *TRP1*, 2μm; made by inserting the 2.0-kb *Bgl*II-*Nsi*I fragment of pPB583, containing *BEM1*, into the *Bam*HI-*Pst*I sites of pBTM116.
pPB594	DBD$_{lexA}$-BEM1$_{N-terminal\ half}$, *TRP1*, 2μm; made by inserting the 960-bp *Bgl*II-*Pst*I fragment of pPB583, containing codons 5–325 of *BEM1*, into the *Bam*HI-*Pst*I sites of pBTM116.
pPB623	DBD$_{lexA}$-BEM1$_{C-terminal\ half}$, *TRP1*, 2μm; made by inserting the 1.3-kb *Hpa*I-*Nsi*I fragment containing codons 235–551 of *BEM1* into the *Sma*I-*Pst*I sites of pBTM116 after the *Eco*RI site of pBTM116 had been filled in using Klenow fragment.
pPB553	AD$_{GAL4}$-CDC24$_{full-length}$, *LEU2*, 2μm; contains a >10-kb *Sau*3A fragment bearing *CDC24* fused at its third codon to the AD (activation domain) in the genomic library plasmid pGAD.2 (Chien et al., 1991). In this plasmid, a *Bam*HI site is present at the junction between the AD and *CDC24*.
JP276	*CDC24* in M13mp19; made by inserting a 2.6-kb *Bam*HI-*Spe*I fragment from pPB553, containing *CDC24*, into the *Bam*HI-*Xba*I sites of M13mp19. In this construct, a *Sal*I site is now present shortly after the stop codon of the *CDC24* open-reading frame.
pPB689	AD$_{GAL4}$-CDC24$_{COOH\ terminus}$, *LEU2*, 2μm; made by inserting the 250-bp *Bgl*II-*Sal*I fragment from JP276, containing the COOH-terminal 75 codons of *CDC24*, into the *Bam*HI-*Sal*I sites of pACTII (Harper et al., 1993).
pPB713	6xHis-BEM1; contains a 1.4-kb *Bam*HI-*Kpn*I fragment of *BEM1* (Chenevert et al., 1992), containing the COOH-terminal 410 codons of *BEM1*, inserted into the *Bam*HI-*Kpn*I sites of pQE-30 (Qiagen, Inc., Chatsworth, CA).
pPB77	TrpE-CDC24$_{COOH\ terminus}$; contains a 2.2-kb *Bgl*II-*Sal*I fragment of CDC24 from YEp103(CDC24) (Coleman et al., 1986) inserted into the *Bam*HI-*Sal*I sites of pATH1 (Koerner et al., 1991).
pPB734	GST-CDC24$_{COOH\ terminus}$; contains a 1.2-kb *Eco*RI-*Eco*RI fragment of *CDC24* from plasmid pPB77 inserted into the *Eco*RI site of pGEX-KG (Guan and Dixon, 1991). This construct contains only the COOH-terminal 75 codons of *CDC24*.

DNA Sequencing

Subclones of *BEM2* in M13 were sequenced using the dideoxy method. Both strands were completely sequenced, and all restriction sites used for subcloning were crossed. The junction sites of inserts in plasmids isolated from the GAD libraries (Chien et al., 1991) were sequenced on one strand only using the dideoxy method and the primer TACCACTACAATGGATG (corresponding to codons 855-861 in *GAL4*).

Microscopy

Before staining with DAPI, cells were first cured of mitochondrial DNA by growth in YPD containing 30 μg/ml ethidium bromide. Cells were fixed for 4 h at 24°C with 5% formaldehyde, pelleted in a microfuge, resuspended in H₂O, repelleted, and then resuspended and incubated for 1 h in 70% EtOH. Cells were then pelleted, resuspended in H₂O, pelleted, resuspended in H₂O, pelleted, and resuspended in 0.2 μg/ml DAPI. Cells were photographed with a Zeiss Axioplan fluorescence microscope using a 100× Plan-Neofluor oil immersion objective.

Two-hybrid Methodologies

Strain CTY10-5d containing plasmid pPB550 was transformed with genomic libraries constructed in the pGAD series of plasmids (Chien et al., 1991; libraries kindly provided by P. Bartel and S. Fields, State University

Table II. Yeast Strains Used in This Study

Strain	Genotype*	Source
PY429	MATa bem1-3 ura3 leu2 ade2 ade3 trp1 [BEM1 ADE3]	Segregant from PY291 (Bender and Pringle, 1991) × Y382 (Bender and Pringle, 1991) transformed with pPB403
PY431	MATα bem1-3 ura3 leu2 ade2 ade3 lys2 [BEM1 ADE3]	Same as for PY429
Y132	MATα cdc24-1 ura3 leu2 his3 met1	Segregant from SY1229 (Bender and Sprague, 1989) × PRTD-6-BD1-8C‡
Y134	MATa cdc24-1 ura3 leu2 his3 met1	Same as for Y132
DJTD1-24C	MATα cdc42-1 trp1	Johnson et al., 1987
DJTD1-14B	MATa cdc42-1 trp1	Constructed as for DJTD1-24C
JPT198BD4-4A	MATa cdc43-2	Johnson et al., 1990
CJ198-2B	MATa cdc43-2 ura3 trp1 his4	Johnson et al., 1990
Y529	MATα bem2-1 ura3 leu2 ade2 ade3	PY260 (Bender and Pringle, 1991) cured of its plasmid
Y565	MATa bem2-1 ura3 leu2 ade2 ade3 lys2	Segregant from PY308 (Bender and Pringle, 1991) × Y382 (Bender and Pringle, 1991)
Y454	MATα bem2-1 ura3 leu2 ade2 ade3 lys2	Segregant from PY260 (Bender and Pringle, 1991) × PY237 (Bender and Pringle, 1991)
Y429	MATa/MATα bem2-1::LEU2/BEM2 ura3/ura3 leu2/leu2 ade2/ade2 ade3/ade3 lys2/LYS2	Bender and Pringle, 1991
Y655	MATa/MATα bem2-Δ1::URA3/BEM2 ura3/ura3 leu2/leu2 ade2/ade2 ade3/ade3 lys2/LYS2	Gene replacement of Y429 using bem2-Δ1::URA3 from plasmid pPB443 (see text)
Y806	MATa ura3 leu2 ade2 ade3 lys2 ρ°	Segregant from Y655 made ρ° as described in Materials and Methods
Y807	MATα bem2-Δ1::URA3 ura3 leu2 ade2 ade3 lys2 ρ°	Segregant from Y655 made ρ°
CTY10-5d	MATa URA3::lexA op-lacZ ura3 leu2-3,-112 ade2 trp1-901 his3-200 gal4 gal80	Rolf Sternglanz

* Genes listed in brackets are carried on a plasmid.

‡ A MATα cdc24-1 met1 strain derived by a series of crosses from strain 5011 (Hartwell et al., 1974; D. Johnson and J. Pringle, personal communication).

of New York at Stony Brook, NY) using the lithium thiocyanate procedure, plated onto SC-Trp-Leu+X-gal medium, and incubated at 30°C. Approximately 25,000 transformants were screened for the development of blue color. 12 blue colonies were streaked onto SC-Trp-Leu+X-gal medium. Individual blue colonies from these streaks were inoculated into SC-Trp-Leu medium, and plasmids were recovered from yeast using a glass-beads procedure (Strathern and Higgins, 1991). *Escherichia coli* strain LU1, which contains a mutant allele of *leuB*, was transformed with plasmids from yeast and plated onto LB+Amp medium. Amp^r transformants were replica-plated onto selective medium lacking leucine to identify Leu$^+$ transformants.

For β-galactosidase activity assays in liquid culture, overnight cultures of yeast were diluted 10- to 40-fold in the appropriate synthetic liquid medium (SC-Leu, SC-Trp, or SC-Trp-Leu) and then grown for 8-9 h with shaking at 30°C. Cultures were harvested at an OD$_{600}$ of 0.1 to 0.5. β-galactosidase assays were performed as previously described (Reynolds and Lundblad, 1989).

Biochemical Analyses

The purification of GST-Rho1p (using the baculovirus expression system in Sf9 cells) and GST-Bem2p (from bacteria) and the GTPase activity assay were performed as described previously (Zheng et al., 1994).

E. coli strain LU1 containing plasmid pGEX-KG (Guan and Dixon, 1991) or pPB734 (for expression of GST or GST-Cdc24p, respectively) and strain TG1 containing plasmid pPB713 (for expression of 6xHis-Bem1p) were grown at 30°C in LB + 100 μM ampicillin to an OD$_{600}$ of 0.7-0.9. (The 6xHis-Bem1p fusion was used simply as a source of Bem1p; the 6xHis tag itself was not used for these experiments.) Expression of fusion proteins was induced with IPTG (0.2-1 mM), and growth at 30°C was continued for 3-5 h. Cells were harvested and resuspended in a volume of ice-cold sonication buffer (50 mM Tris-HCl [pH 8], 300 mM NaCl, 5 mM β-mercaptoethanol, 100 mM PMSF, 400 ng/ml bestatin, 500 ng/ml leupeptin, 350 ng/ml pepstatin A) that was 10% of the volume of the original culture. Lysozyme was added to 1 mg/ml, and the mixtures were incubated on ice for 30 min. After sonication, the lysate was centrifuged at 17,000 g for 20 min at 4°C. The supernatants, to which was added Triton X-100 (1% final concentration), were used for the binding studies (see below).

Glutathione-agarose resin (Sigma Chem. Co., St. Louis, MO) was prepared according to the manufacturer's instructions. Aliquots of the resin were equilibrated in binding buffer (sonication buffer containing 1% Triton X-100) and then incubated with rocking for 30 min at 4°C with extracts containing either GST or GST-Cdc24p. The resin was washed extensively with binding buffer and then incubated with the 6xHis-Bem1p-containing extract for 2 h at 4°C. After extensive washing with binding buffer, the samples were boiled for 5 min in SDS sample buffer and then subjected to SDS-PAGE analysis (Laemmli, 1970).

Results

Screen for Mutations That Display Synthetic-Lethal Interactions with bem1

Cells that lack Bem1p function appear to have an incompletely penetrant bud emergence defect at 30°C; some of the cells display normal morphologies while others become large and multinucleate or lyse (Bender and Pringle, 1991; Chenevert et al., 1992). Although a fair fraction of individual *bem1* cells display morphological defects and have poor viability even at such "permissive" temperatures, cultures of *bem1* cells can be propagated at temperatures up to ~38°C. To identify genes that interact with *BEM1*, we screened for mutants that require a wild-type copy of *BEM1* for survival at 30°C. The general strategy used to find such mutants was to screen through clones of *bem1* cells for mutants that were unable to survive the loss of a plasmid containing wild-type *BEM1*. To identify such mutants, we used an *ADE3/ade2 ade3*-based colony-sectoring assay (Bender and Pringle, 1991). In this scheme, the *BEM1* plasmid contains the *ADE3* color marker and the *bem1* strain carries mutant alleles of *ADE2* and *ADE3*. Cells that contain the plasmid are red;

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cells that have lost it are white. On rich medium, the starting strain forms red colonies that contain white sectors (Sect⁺ phenotype). However, mutants that become dependent upon Bemlp function for survival are predicted to form thoroughly red colonies that lack white sectors (Sect⁻ phenotype).

To facilitate complementation analyses, *beml* synthetic-lethal mutants were sought from both a and α strains (PY429 and PY431). 365 Sect⁻ mutants were identified from PY429 and 315 Sect⁻ mutants were identified from PY431. All of the first 164 diploids analyzed from crosses involving 34 PY429-derived Sect⁻ mutants and 13 PY431-derived Sect⁻ mutants were Sect⁻, suggesting that it may not be possible to organize the mutants into complementation groups using the Sect phenotype. However, we found that those Sect⁻ mutants that were also Ts⁻ for viability could be organized into complementation groups based upon their Ts⁻ phenotype. We therefore restricted our analysis to those Sect⁻ mutants that either failed to grow or grew only poorly at 37°C. 34 Ts⁻ Sect⁻ mutants, at least 14 of which were independently derived, were isolated from strain PY429, and 29 Ts⁻ Sect⁻ mutants, at least 14 of which were independently derived, were isolated from strain PY431. Because substantial mutagenesis (to 10% survival) was used in the generation of these mutants, we expected that the Ts⁻ growth defects in many of them may be functionally unrelated to the mutations responsible for the Sect⁻ phenotype (i.e., they may be fortuitous Ts⁻ mutations). We therefore chose to analyze only those Ts⁻ Sect⁻ mutants that arose more than once from the screen. Each of the PY429-derived mutants was therefore crossed to each of the PY431-derived mutants, and the resulting diploids were tested for Ts⁻ growth defects. 15 of the diploids failed to grow at 37°C. These mutants fell into four complementation groups (Table III).

To determine whether any of these complementation groups contained mutations in genes known to be involved in bud emergence, every member of each group was crossed to Ts⁻ *cdc24*, *cdc42*, *cdc43*, and *bem2* mutant strains, and the resulting diploids were assayed for growth at 37°C (see Materials and Methods). Although all of the mutants complemented both the *cdc42* and *cdc43* mutants, the two members of group I failed to complement the Ts⁻ growth defect caused by *cdc24*, and all eight members of group II failed to complement the Ts⁻ phenotype of *bem2* mutants. When transformed with a low copy number plasmid containing *CDC24*, both of the group I mutants could grow at 37°C, consistent with the view that these strains indeed are defective in *CDC24* function. In addition, these transformants now displayed a Sect⁺ phenotype at 30°C, confirming that *cdc24* is indeed synthetically lethal with *beml*. Analogous

Table III. Ts⁻ Sect⁻ Mutants Identified from a beml Synthetic-Lethal Screen

Complementation group	No. of independent isolates	Gene
I	2	CDC24
II	7–8*	BEM2
III	3	?
IV	2	?

* The range indicates the uncertainty as to how many isolates were actually independently derived.

results with group II mutants transformed with a low copy number plasmid carrying *BEM2* confirmed that these mutants are defective in *BEM2* function and that *bem2* and *beml* display a synthetic-lethal interaction. The analyses of the mutants in groups III and IV will be presented elsewhere.

Subcloning of BEM2

A plasmid containing a fragment of DNA from the *BEM2* locus was previously isolated by complementation of the Ts⁻ phenotype of a genomic *bem2* mutation (Bender and Pringle, 1991). To localize *BEM2* within this clone, various segments of the insert were tested for the ability to complement the Ts⁻ phenotype of a *bem2* mutant (Fig. 1). The results of the subcloning analysis suggested that *BEM2* extends across the left-hand *Bgl*II site, but that in appropriate contexts (e.g., plasmids pPB318 and pPB467), sequences to the left of that *Bgl*II site are sufficient for suppression (Fig. 1). By sequence analysis, a single large open-reading frame was identified that crossed that *Bgl*II site and extended beyond the right end of the original insert (Fig. 1). This open-reading frame was found to be in frame with an ATG in the tetracycline-resistance gene of the vector, starting 64 codons upstream of the *Bam*HI site into which the insert was originally cloned (data not shown). Colony hybridization was used to isolate a plasmid containing the remainder of *BEM2* (see Materials and Methods). The sequence of *BEM2* (Fig. 2) is identical to that of *IPL2* (Kim et al., 1994). The *BEM2* open-reading frame is predicted to encode a protein of 2,167 amino acids (245 kD).

Bem2p Contains a Rho-GAP Domain

Bem2p contains a single copy of a Rho-GAP homology domain (Boguski and McCormick, 1993) near its COOH terminus, within a region of the protein found to be sufficient
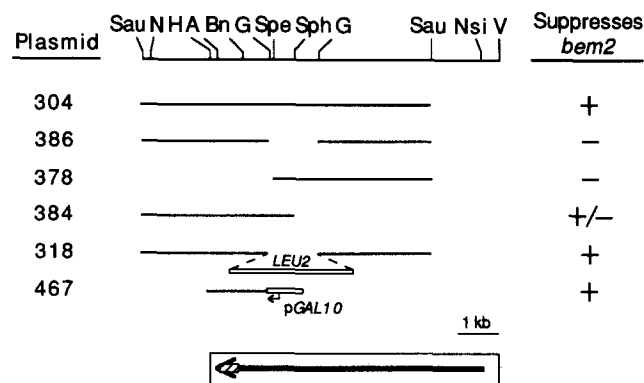

Figure 1. Localization of *BEM2*. Line segments indicate fragments of DNA obtained from the *BEM2* locus. The ability of these fragments, when present on high copy number vectors, to suppress the Ts⁻ phenotype of *bem2-1* strain Y454 (see Materials and Methods) is indicated. All sites for *Nhe*I (*N*), *Sph*I (*Sph*), and *Eco*RV (*V*) are shown. Relevant *Sau*3A (*Sau*), *Hind*III (*H*), *Acc*I (*A*), *Ban*I (*Bn*), *Bgl*II (*G*), *Spe*I (*Spe*), and *Nsi*I (*Nsi*) sites are also shown. The open box for plasmid pPB318 indicates a fragment of DNA containing *LEU2*. The open box for plasmid pPB467 represents a fragment of DNA containing the *GAL10* promoter. The open box at the bottom of the figure indicates the segment of DNA that was sequenced. The arrow within that box represents the *BEM2* open-reading frame. The hatched box marks the Rho-GAP domain.

Figure 2. Nucleotide and predicted amino acid sequences of *BEM2*. The Rho-GAP domain is boxed. The following restriction sites (see Fig. 1) are overlined: *Eco*RV (1), *Nsi*I (472), *Sau*3A (1684), *Bgl*II (4347), *Sph*I (4953), *Spe*I (5481), *Bgl*II (5549), *Ban*I (6201), *Acc*I (6826), and *Hin*dIII (6994). These sequence data are available from EMBL/GenBank/DDBJ under accession number L33832.

for the suppression of the Ts⁻ phenotype of *bem2* mutants (Figs. 1, 2, and 3). Because *BEM2* appears to be involved in bud emergence (Bender and Pringle, 1991; Chan and Botstein, 1993), and *BEM2* was identified genetically through its interaction with *MSB1*, itself a multicopy suppressor of Ts⁻ mutations in *CDC42*, it initially seemed likely that Bem2p would prove to be a GAP for Cdc42p. To investigate this possibility, a Rho-GAP domain-containing portion of Bem2p was previously expressed and purified as a GST fusion protein from bacteria and assayed for GAP activity upon a GST-Cdc42p fusion purified from a baculovirus expression system. Although the Rho-GAP domain-containing protein Bem3p can stimulate the GTPase activity of Cdc42p in this assay, the GST-Bem2p fusion was not able to serve as a GAP for Cdc42p (Zheng et al., 1993, 1994).

Rho1p is the only other known essential Rho-type GTPase in yeast. Strong overexpression of *RHO1* causes cells to become large and multinucleate (Madaule et al., 1994), implicating Rho1p as a potential target of Bem2p. To test this possibility, the GST-Bem2p fusion (Fig. 4 *a*) was assayed for GAP activity toward a GST-Rho1p fusion (Fig. 4 *a*) from Sf9 cells. As shown in Fig. 4 *b*, Bem2p can stimulate the rate of release of ³²P from [γ-³²P]GTP, but not from [α-³²P]GTP, bound to Rho1p, indicating that Bem2p can indeed stimulate the hydrolysis of GTP on Rho1p in vitro.

Morphological Defects of bem2 Mutants

BEM2 was deleted from the genome by gene replacement using a 4-kb *SspI-SalI* fragment of *bem2-Δ1::URA3*-containing DNA from plasmid pPB443 (Materials and Methods). (The *SspI* site in pPB443 is ~0.6 kb upstream of the *NsiI* site shown in Fig. 1; the *SalI* site is within the tetracycline resistance gene.) The *bem2-Δ1::URA3* deletion was constructed in a/α *bem2-1::LEU2/BEM2* diploid strain Y429. A Ura⁺
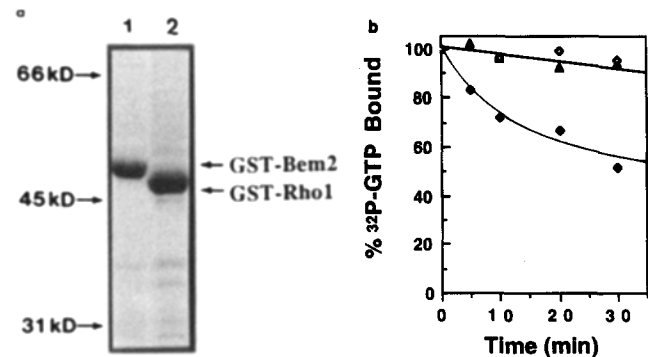

Figure 4. Stimulation of the GTPase activity of Rho1p by GST-Bem2p. (*A*) Coomassie blue-stained SDS–polyacrylamide gel of GST-Bem2p and GST-Rho1p fusions. The sizes of molecular mass markers are indicated. (*B*) Effect of GST-Bem2p on the rate of GTP hydrolysis by Rho1p. 1 μg of GST-Rho1p was preloaded with [γ-³²P]GTP (♦, ▲) or [α-³²P]GTP (◊) and then incubated with 1 μg of GST-Bem2p (♦, ◊) or 5 μg of GST (▲) at 22°C.

Leu⁻ transformant (Y655) was sporulated and subjected to tetrad analysis at 24°C. Of 12 tetrads dissected, 10 gave 2 Ura⁺: 2 Ura⁻ viable segregants, one gave 1 Ura⁺: 2 Ura⁻ viable segregants, and one gave 2 Ura⁺: 1 Ura⁻ viable segregants, indicating that *BEM2* is not essential at this temperature. The success of the construction of the *bem2-Δ1::URA3* deletion was confirmed by Southern analysis on chromosomal DNA from the starting diploid strain Y429 and both a Ura⁺ and a Ura⁻ segregant derived from Y655 (data not shown).

Whereas the Ura⁻ segregants from Y655 were healthy and grew well at temperatures up to 36°C, all of the Ura⁺

Figure 3. Sequence alignment of Rho-GAP domains. The Rho-GAP domains of Bem2p, Bcr (Heisterkamp et al., 1985), Chimaerin (Hall et al., 1990), p85 (Skolnik et al., 1991), and p190 (Settleman et al., 1992) are shown. Amino acids that are identical between Bem2p and any of the other proteins are indicated by shading. In the consensus sequence, each position in which all five proteins contain the same amino acid is indicated by a capital letter, each position in which all five proteins contain a hydrophobic amino acid is indicated by ∅, each position in which four of the proteins contain the same amino acid is indicated by a lowercase letter, and each position in which at least four of the proteins contain a positively or a negatively charged amino acid is indicated by + or −, respectively.
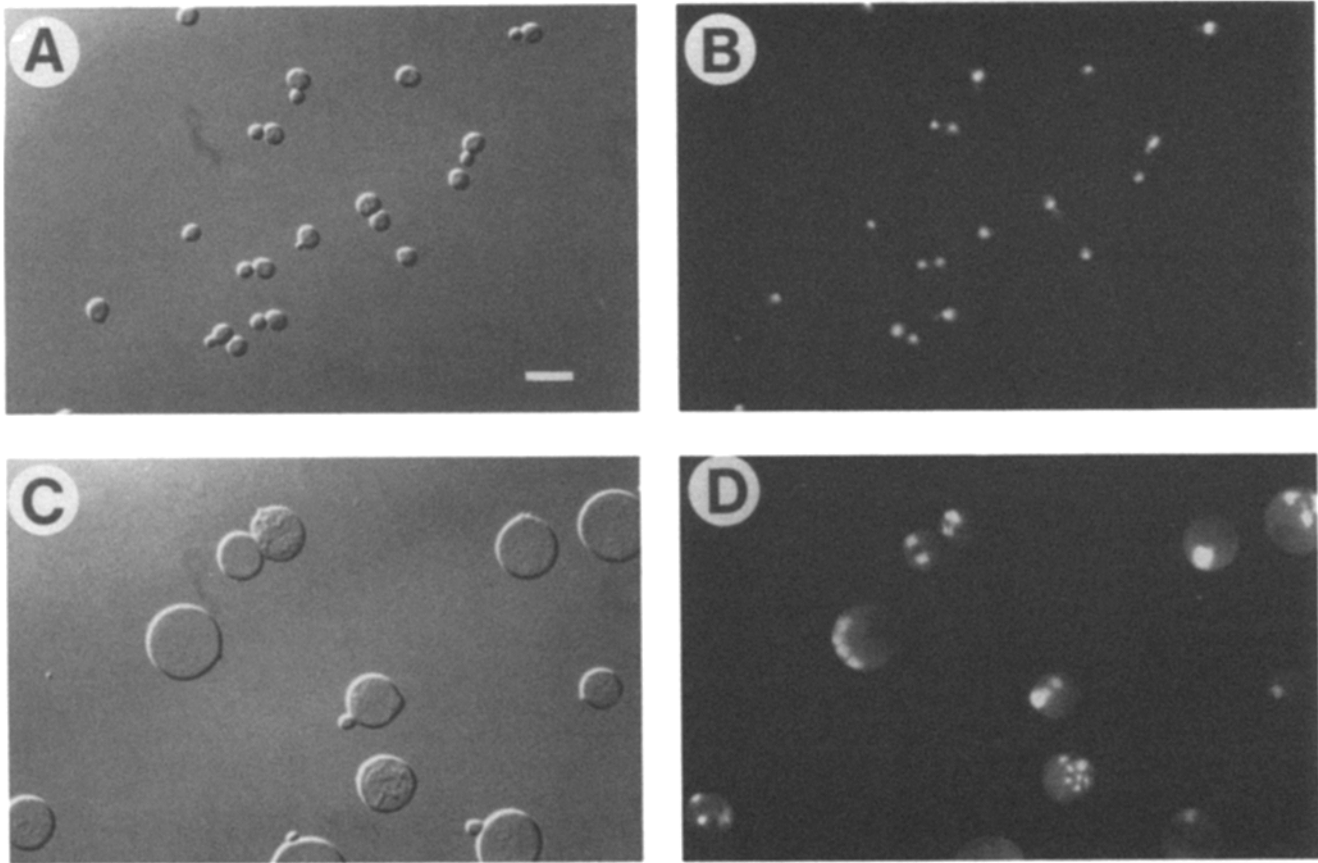
```
Bem2   1981   PTIVVKLLEEIELRGLDEVGLYRIPGSIGSINALKNAFDEEGATDNSFTLED
Bcr    1068   PYIVRQCVEEIERRGMERVGIYRVSGVATDIQALKAAFD..VNNKDVSVMMS
Chim    157   PMVVDMCIREIESRGLNSEGLYRVSGFSDLIEDVKAFDRDGEKADISVNMY
p85     129   PPLLIKLVEAIEKKGLECSTLYRTQS.SSNLAEIRQLLDCDTPSVDLEMI..
p190   1242   PIFIERCIEYIEATGLSTEGIYRVSGNKSEMESLQRQFD...QDHNLDLAEK

Consensus     P.∅∅...∅e.IE.+GL...g.YR..g.......l+...fD............


Bem2   2053   DRWPEVNAIAGCFKMYLRELPDSLFSHAMVNDFTDLAIKYKAHAMVNEEYKRM
Bcr    1118   E..MDVNAIAGTLKLYFRELPEPLFTDEFYPNFAEGIALSDPVAKESC.....
Chim    209   E...DINIITGALKLYFRDLPIPLITYDAYPKFIESAKIMDPDEQLET.....
p85     178   ....DVHVLADAFKRYLLDLPNPVIPAAVYSEMISLA....PEVQSSEEYIQL
p190   1291   D..FTVNTVAGAMRSFFSELPDPLVPYSMQIDLVEAHKINDREQKLHA.....

Consensus     -...-vn.∅ag..K.y∅.-LP.pl∅.........-................


Bem2   2126   MNELLQK..LPTCYYQTLKRIVFHLNKVHQHVVNNKMDASNLAIVFSMSFIN
Bcr    1164   MLNLLLS..LPEANLLTFLFLLLDHLKRVAEKEAVNKMSLHNLATVFGPTLLR
Chim    254   LHEALKL..LPPPAHCETLRYLMAHLKRVTLHEKENLMNAENLGIVFGPTLMR
p85     223   LKKLIRSPSIPHQYWLTLQYLLKHFFKLSQTSSKNLLNARVLSEIFSPMLFR
p190   1337   LKEVLKK..FPKENHEVFKYVISHLNKVSHNNKVNLMTSENLSICFWPTLMR

Consensus     ....l....∅P.....t∅..∅..Hl.+∅......N.m...nL...F.p.l.r
```

Figure 5. Phenotypes of *bem2* mutants. DIC images (*a* and *c*) and fluorescence images of DAPI-stained (*b, d*) wild-type Y806 (*a* and *b*) and *bem2* deletion mutant Y807 (*c* and *d*) cells. Dilute cultures of exponentially growing cells at 24°C were shifted to 36°C and incubated for 6 h before fixation (see Materials and Methods). Bar, 10 μm.

segregants grew poorly at 24°C and were completely inviable at temperatures above 32°C. Even at temperatures permissive for colony formation, many *bem2-Δ1::URA3* cells became large and multinucleate (data not shown), as was seen previously for the original, presumed point-mutant alleles of *bem2* (Bender and Pringle, 1991). When grown at 36°C, the majority of *bem2-Δ1::URA3* cells display these phenotypes (Fig. 5). These results are consistent with the view that *BEM2* plays some role in bud-site assembly and/or bud growth.

Physical Interaction between Bem1p and Cdc24p

To search for proteins that display two-hybrid interactions with Bem1p, plasmid pPB550, which encodes a fusion between the DBD (DNA-binding domain) of lexA and nearly full-length Bem1p (see Materials and Methods), was transformed into yeast strain CTY10-5d, which contains the *lacZ* reporter gene under the control of the lexA DNA-binding site. This DBD-Bem1p-bearing strain was then transformed with a plasmid library that contains random *Sau*3A fragments of genomic DNA fused to the coding sequence for the AD (transcriptional activation domain) of Gal4p (Chien et al., 1991).

Of ~50,000 transformants obtained, 12 expressed β-galactosidase activity as detected by X-gal plate assays. The library plasmids from these 12 transformants were isolated and individually retransformed into CTY10-5d containing either no other plasmid or pPB550. In all cases, β-galactosidase expression was dependent upon the presence of both the library plasmid and the DBD-Bem1p fusion. To investigate whether Bem1p itself was required for these inductions of β-galactosidase expression, each library plasmid was introduced into a strain that contains pCTC52, a plasmid that encodes a fusion between the DBD of lexA and human nuclear lamin C, a protein unrelated to Bem1p. All of these transformants were white on X-gal medium, suggesting that the products of these library clones might interact specifically with DBD-Bem1p.

Based upon restriction analysis, at least seven of the clones from the library were judged to contain DNA from a single genomic region (data not shown). DNA sequence analysis indicated that at least six of these clones had non-overlapping, short (2 to 38 codons) open-reading frames in frame with the AD. This result indicates that the gene responsible for the induction of β-gal expression in this set of clones need not be fused to the AD. These clones were not analyzed further.

Based upon DNA sequence and restriction analyses, it was determined that one of the remaining library clones contains *CDC24* fused in-frame at its third codon to the AD (data not shown). Quantitative β-gal activity assays confirmed that the induction of β-gal expression required the presence of both the DBD-Bem1p- and the AD-Cdc24p-encoding plasmids (Fig. 6, combinations 1–3). A segment of Bem1p that lacks

Combination	DBD-Bem1 fusions	AD-Cdc24 fusions	β-gal activity
1	▭▪▪▭	—	2×10^{-2}
2	—	▭▨▨░▭	2×10^{-2}
3	▭▪▪▭	▭▨▨░▭	1 0
4	▭▪▪▭	—	4×10^{-2}
5	▭▪▪▭	▭▨▨░▭	8×10^{-2}
6	▭▭	—	1×10^{-2}
7	▭▭	▭▨▨░▭	2.3
8	—	▭	2×10^{-2}
9	▭▭	▭	140

■ SH3 domain   ▨ GEF domain   ▨ PH domain (potential)

Figure 6. Two-hybrid interaction between Bem1p and Cdc24p. The portions of Bem1p fused to the DBD (DNA-binding domain) of LexA and the portions of Cdc24p fused to the AD (activation domain) of Gal4p are represented by boxes. β-galactosidase activities are given for the average values from three independent transformants for each combination of plasmids used. The range of activities for each set of measurements was always less than 60% of the average. The following combinations of plasmids were used: pPB550 (1), pPB553 (2), pPB550 + pPB553 (3), pPB594 (4), pPB594 + pPB553 (5), pPB623 (6), pPB623 + pPB553 (7), pPB689 (8), and pPB623 + pPB689 (9).

both SH3 domains can display a two-hybrid interaction with Cdc24p (Fig. 6, combinations 6 and 7), indicating that the SH3 domains are not required for this interaction. Conversely, a segment of Bem1p that contains both SH3 domains but lacks its COOH terminus fails to interact with Cdc24p (Fig. 6, combinations 4 and 5). A segment of the COOH terminus of Cdc24p that lacks both the GEF domain and a putative PH (Pleckstrin homology) domain, a motif found in various proteins implicated in signal transduction (Musacchio et al., 1993), can display a two-hybrid interaction with Bem1p (Fig. 6, combinations 8 and 9). This is the same portion of Cdc24p that has recently been shown to be lethal
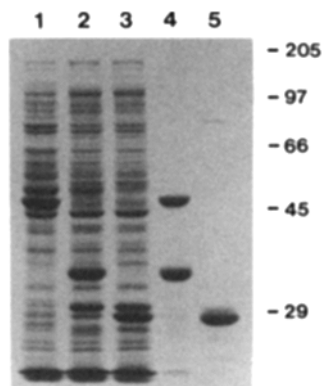
1 2 3 4 5
- 205
- 97
- 66
- 45
- 29

Figure 7. In vitro binding studies using bacterially expressed Bem1p and Cdc24p fusion proteins. Proteins were separated electrophoretically through a 12% SDS–polyacrylamide gel and then stained with Coomassie brilliant blue. Lanes *1–3*: extracts of cells expressing 6xHis-Bem1p (lane *1*), GST-Cdc24p (lane *2*), or GST (lane *3*). Lanes *4* and *5*: proteins bound to glutathione-agarose resin from mixed extracts containing GST-Cdc24p plus 6His-Bem1p (lane *4*) or GST plus 6His-Bem1p (lane *5*). The predicted sizes of 6xHis-Bem1p, GST-Cdc24p, and GST are 47, 36, and 28 kD, respectively. Positions of molecular mass markers are shown on the right.

when overexpressed in yeast (S. Sakaguchi, Y. Ohya, and Y. Anraku, personal communication).

To investigate whether Bem1p binds directly to Cdc24p, the ability of bacterially expressed Bem1p to bind to a bacterially expressed GST-Cdc24p fusion was tested (Materials and Methods). As shown in Fig. 7, Bem1p can indeed bind to GST-Cdc24p, but not to GST alone, indicating that the interaction between Bem1p and Cdc24p is direct and that no other yeast proteins are required for this interaction.

The analyses of the remaining clones that were identified from the two-hybrid screen will be presented elsewhere.

Discussion

Interaction between Bem1p and Cdc24p

Given that Bem1p and Cdc24p are both involved in bud emergence, and that *bem1* displays synthetic lethality with *cdc24*, the physical interaction between Cdc24p and Bem1p identified in this study almost certainly reflects a biologically relevant association. One model for the role of this interaction is that Bem1p acts to physically link Cdc24p to some "landmark" protein that marks the site at which a bud or a mating projection is supposed to form. In this scenario, Bem1p might serve simply as an adaptor between the putative landmark protein and Cdc24p, or Bem1p might both link Cdc24p to the landmark protein and stimulate its GEF (or other) activity there. A second model is that Bem1p is a component of a complex of proteins that becomes assembled at the bud or mating projection site in response to the action of Cdc24p and/or Cdc42p. For example, Cdc24p may play a role in positioning Bem1p at the bud site, and Bem1p might in turn help to nucleate the assembly of cytoskeletal structures there. A third model is that the primary role of Bem1p is to regulate the GTPase cycle of Cdc42p and perhaps other Rho-type GTPases (see below). We are currently investigating whether Bem1p affects the ability of Cdc24p to stimulate GTP-GDP exchange on Cdc42p in vitro.

Although there are insufficient data to distinguish among specific models for the role of Bem1p, it seems likely, based upon the following immunolocalization and genetic data, that Bem1p acts at the bud site and plays an early role in bud emergence. In particular, Bem1p has recently been shown to be localized to the bud site early during the process of bud site assembly (Kathy Corrado and John Pringle, personal communication), and *BEM1* has been found to display a synthetic-lethal interaction with mutant alleles of the bud site selection gene *BUD5* (Chant et al., 1991; Fig. 8). Mutations in *BUD5* can both lower the restrictive temperature (from 39 to 37°C) and confer a *cdc* arrest phenotype to *bem1* mutants (Chant et al., 1991). These genetic interactions between *bud5* and *bem1* might reflect a direct physical association between the two gene products and so support the idea that Bem1p serves as a linker between the bud site selection machinery and Cdc24p. However, for the following reasons, these genetic interactions between *bud5* and *bem1* are also consistent with the possibility that Cdc24p links the bud-site selection machinery to Bem1p, a situation in which Bud5p need not physically interact with Bem1p. Bud5p has recently been shown to behave genetically as a GEF for Rsr1p (Bender, 1993; Fig. 8), a Ras-type GTPase that is required for proper bud-site selection (Bender and Pringle, 1989; Chant and
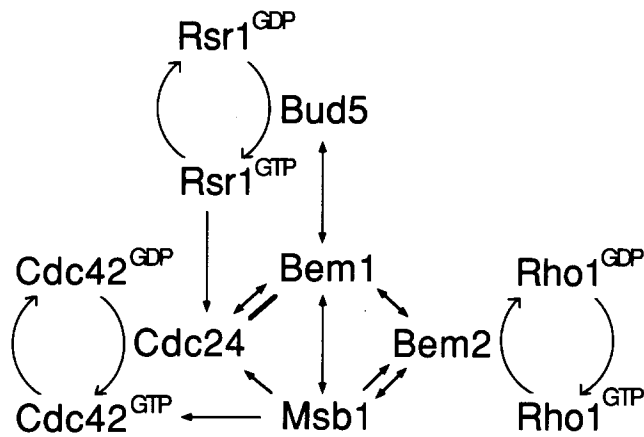
Figure 8. Genetic and two-hybrid interactions among proteins implicated in cell polarity development. Double-headed arrows indicate synthetic-lethal interactions; straight, single-headed arrows represent multicopy suppression; and the straight, thick line indicates a physical interaction.

Herskowitz, 1991). *RSR1* was itself identified as a multicopy suppressor of Ts⁻ mutations in *CDC24* (Bender and Pringle, 1989; Fig. 8), raising the possibility that Rsr1p binds to Cdc24p. Thus, if the activated form of Rsr1p binds to Cdc24p (Ruggieri et al., 1992), then mutations in *BUD5* that prevent the activation of Rsr1p may interfere with the normal functioning of Cdc24p, a situation that could be lethal in the absence of functional Bem1p (given that *cdc24* and *bem1* display synthetic-lethal interactions; Fig. 8). Consistent with this possibility is the observation that *BUD5* is required for wild-type *RSR1*, but not for mutationally activated *rsr1ᵛᵃˡ¹²*, to serve as a multicopy suppressor of *cdc24* (Bender, 1993).

Interaction between BEM1 and BEM2

From preliminary studies using sucrose velocity gradient sedimentation and gel filtration, it appears that Bem1p may be part of a complex that is ∼80 S in size (L. Bender and A. Bender, unpublished data). It is possible that a number of genes whose products are either constituents of this complex or are required for the proper functioning of those constituents would display genetic interactions with *BEM1*. Because of difficulty in organizing potential *bem1* synthetic-lethal mutants into complementation groups using the Sect phenotype, we limited our analyses to those *bem1* synthetic-lethal mutants that were also Ts⁻ for viability. *BEM2* was one of four such genes that were identified at least twice each during this screen. The synthetic-lethal interaction between *bem1* and *bem2* suggests that the functions of Bem1p and Bem2p may be closely linked, an idea that is supported by the following genetic and phenotypic data.

In addition to displaying synthetic lethality with each other, *bem1* and *bem2* both display synthetic lethality with *msb1* (Bender and Pringle, 1991: Fig. 8). These interactions with *msb1* seem particularly significant given that deletion of *MSB1* in an otherwise wild-type strain does not have any obvious deleterious effect, and *bem1* and *bem2* are the only mutants known to display synthetic lethality with *msb1* at temperatures lower than 37°C (Bender and Pringle, 1991; D. Fouts and A. Bender, unpublished data). Msb1p is not similar in sequence to other known proteins. The most distin-

guishing feature of the Msb1p sequence is that it is rich in prolines; over a stretch of 300 amino acids near its COOH terminus, 16% of the amino acids are prolines. Given that proline-rich sequences in some proteins have been found to bind to SH3 domains, it is possible that Msb1p binds to one or both of the SH3 domains of Bem1p.

Unlike *CDC24* and *CDC42* (Coleman et al., 1986; Johnson and Pringle, 1990), *BEM1* (Chenevert et al., 1992) and *BEM2* are not essential for viability. *bem1* and *bem2* mutants also have in common a lack of penetrance of their mutant phenotypes: some of the cells in a culture of *bem1* or *bem2* mutants grown even at permissive temperatures can be large and multinucleate, whereas the rest appear normal (Bender and Pringle, 1991). The following evidence is consistent with the view that both *bem1* and *bem2* mutants also have a tendency to become aneuploid and/or polyploid. First, *BEM2* (as *IPL2*) has recently been identified during a screen for mutations that cause cells to become polyploid (Kim et al., 1994). Second, colonies of *MATα bem1* or *bem2* mutant cells often produce abnormally low amounts of α-factor pheromone and in some cases instead produce a-factor, a situation that could result from instability of the *MATα* locus-containing chromosome (A. Bender, unpublished data). Third, crosses involving either *bem1* or *bem2* mutants frequently give only inviable segregants (A. Bender, unpublished data), consistent with the possibility that triploids or aneuploids are being formed in these crosses.

Multiple Rho-type GTPases and Bud Emergence

Its inferred amino acid sequence suggested that Bem2p would prove to be a GAP for one or more Rho-type GTPases. For the following reasons, Rho1p was considered to be a possible target of the presumed GAP activity of Bem2p. Strong overexpression of *RHO1* can cause cells to become large and multinucleate (Madaule et al., 1994), suggesting that the presence of too much Rho1p can interfere with bud emergence. Expression of a constitutively activated allele of *RHO1* also causes cells to become large, round, and unbudded (Madaule et al., 1994). Since the phenotypes caused by an activating mutation in *RHO1* would be expected to resemble those caused by a loss of function of the GAP for Rho1p, and mutations in *BEM2* apparently cause defects in bud emergence, we considered the possibility that Bem2p might act as a GAP for Rho1p. We found that the Rho-GAP domain of Bem2p can indeed stimulate the GTPase activity of Rho1p in vitro.

Because the intrinsic GTPase activity of Rho1p is very low, we were not able to accurately calculate the amount of stimulation by Bem2p of the Rho1p GTPase activity. However, given that the apparent rate of Bem2p-stimulated GTPase activity of Rho1p appears to be low (e.g., it is approximately fourfold lower than the measured rate of the Bem3p-stimulated GTPase activity of Cdc42p [Zheng et al., 1994]), it appears that Bem2p has only weak GAP activity toward Rho1p in vitro. It is possible that the GST-Bem2p fusion used in these assays lacks sequences and/or modifications that are necessary for full activity and that the GAP activity of Bem2p toward Rho1p in vivo is therefore higher than the GAP activity measured in vitro. However, it is also possible that the observed in vitro GAP activity of Bem2p approximates its in vivo activity and that this level of GAP activity

is sufficient for Bem2p to serve as an effective down-regulator of Rholp.

Given its role in bud emergence, Cdc42p was considered to be another potential target of Bem2p. However, we have been unable to detect any GAP activity of Bem2p toward Cdc42p (Zheng et al., 1994). The ability of the Rho-GAP domain of Bem3p to serve as a GAP for GST-Cdc42p indicates that the GST-Cdc42p fusion used in those experiments was functional. The finding that the GST-Bem2p fusion is active as a GAP toward Rholp demonstrates that the GST-Bem2p fusion used in those assays also was functional. Combined, these results argue strongly that Bem2p does not act as a GAP toward Cdc42p. Furthermore, the finding that GST-Bem2p will not competively interfere with the ability of Bem3p to serve as a GAP toward Cdc42p suggests that Bem2p cannot even bind to Cdc42p (Zheng et al., 1993, 1994).

The other known Rho-type GTPases in *S. cerevisiae* are Rho2p, Rho3p, and Rho4p (Madaule et al., 1987; Matsui and Toh-e, 1992a). Depletion experiments suggest that Rho3p and Rho4p may be involved in bud growth (Matsui and Toh-e, 1992b), raising the possibility that one or both of these GTPases may be targets of Bem2p. Efforts to test whether Bem2p can act as a GAP for Rho3p in vitro have been frustrated by our inability to purify a functional GST-Rho3p fusion (Y. Matsui, Y. Zheng, and R. Cerione, unpublished data). In principle, genetic data could help identify the relevant in vivo targets of Bem2p. However, if the functions of multiple Rho-type GTPases are interconnected, such data may be difficult to interpret.

Interactions between Bem1p and Multiple Rho-type GTPases

BEM1 is able to serve as a multicopy suppressor of the lethality caused by depletion of both Rho3p and Rho4p (Matsui and Toh-e, 1992b). When combined with the genetic data linking Bem1p with Bem2p and the two-hybrid, biochemical, and genetic data linking Bem1p with Cdc24p, the finding that *BEM1* is a multicopy suppressor of *rho3 rho4* double mutants raises the possibility that Bem1p is a common target of action for, or regulator of, multiple Rho-type GTPases. Alternatively, it is possible that the main role of Bem1p involves its interaction with Cdc24p and that the genetic interactions between *BEM1* and other Rho-type GTPases reflect a tight coupling of function between Cdc42p (a target of Cdc24p) and other Rho-type GTPases. Consistent with the latter view is the finding that *CDC42* itself can also serve as a multicopy suppressor of *rho3 rho4* mutants (Matsui and Toh-E, 1992b).

Loss of Rholp function, either by depletion (Madaule et al., 1994) or by using a Ts⁻ allele (Yamochi et al., 1994), causes cells to arrest with small buds, suggesting that Rholp plays a role in bud development. Rholp appears to be concentrated at sites of cortical actin patches, supporting the view that Rholp may play a role in the control of actin organization (Yamochi et al., 1994). The view that multiple Rho-type GTPases control cytoskeletal reorganizations in preparation for bud emergence in yeast parallels findings that multiple Rho-type GTPases control the reorganization of the cortical cytoskeleton in fibroblasts in response to growth factors (Ridley and Hall, 1992; Ridley et al., 1992). In vertebrates as in yeast, there appear to be functional interac-

tions between Ras- and Rho-type GTPases and between Rho-type GTPases and SH3 domain-containing proteins (Settleman et al., 1992; Birge and Hanafusa, 1993; Musacchio et al., 1993). Future studies in yeast and animals may reveal whether there exist evolutionarily conserved functional relationships among these and other sets of proteins and protein motifs involved in cellular morphogenesis and signal transduction.

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