Control of Cellular Morphogenesis by the Ipl2/Bem2 GTPase-activating Protein: Possible Role of Protein Phosphorylation

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Abstract. The *IPL2* gene is known to be required for normal polarized cell growth in the budding yeast *Saccharomyces cerevisiae.* We now show that *IPL2* is identical to the previously identified *BEM2* gene. *bem2* mutants are defective in bud site selection at 26°C and localized cell surface growth and organization of the actin cytoskeleton at 37°C. *BEM2* encodes a protein with a COOH-terminal domain homologous to sequences found in several GTPase-activating proteins, including human Bcr. The GTPase-activating protein-domain from the Bem2 protein (Bem2p) or human Bcr can functionally substitute for Bem2p. The Rho1 and Rho2 GTPases are the likely in vivo targets of Bem2p because *bem2* mutant phenotypes can be partially suppressed by increasing the gene dosage of *RHO1* or *RHO2*.

CDC55 encodes the putative regulatory B subunit of protein phosphatase 2A, and mutations in *BEM2* have previously been identified as suppressors of the *cdc55-1* mutation. We show here that mutations in the previously identified *GRR1* gene can suppress *bem2* mutations. *grr1* and *cdc55* mutants are both elongated in shape and cold-sensitive for growth, and cells lacking both *GRR1* and *CDC55* exhibit a synthetic lethal phenotype. *bem2* mutant phenotypes also can be suppressed by the *SSD1-v1* (also known as *SRK1*) mutation, which was shown previously to suppress mutations in the protein phosphatase-encoding *SIT4* gene. Cells lacking both *BEM2* and *SIT4* exhibit a synthetic lethal phenotype even in the presence of the *SSD1-v1* suppressor. These genetic interactions together suggest that protein phosphorylation and dephosphorylation play an important role in the *BEM2*-mediated process of polarized cell growth.

The generation of differentiated cellular subdomains is critical for the functioning of many eukaryotic cells. A wide variety of cellular constituents, including plasma membrane proteins, organelles, and cytoskeletal filaments, must be organized asymmetrically. The resulting polarization of cell structures is important, for example, for the transmission of a nerve impulse, the transport of molecules across an epithelial cell, and the crawling of a fibroblast. The molecular mechanisms by which cell polarity is established are poorly understood, and very little is known about how sites of directional cell growth are selected.

The development of cell polarity has been studied extensively in the budding yeast *Saccharomyces cerevisiae* (reviewed in Chant and Pringle, 1991; Drubin, 1991; Madden et al., 1992). Initiation of normal cell growth involves the selection of a proper bud site on the surface of the elliptical cell. The choice of this bud site is nonrandom (Friefelder, 1960; Chant and Herskowitz, 1991); wild-type haploid cells bud from only one pole in an axial fashion (i.e., from sites near the site of the previous cell division), whereas wild-type **a**/α diploid cells bud in a bipolar fashion (i.e., from either pole). After the selection of a bud site, subsequent growth is localized mostly to this selected site, eventually giving rise to a bud. Thus, growth of yeast cells is highly asymmetrical, and this is accomplished by localized vesicle fusion and cell wall synthesis.

A number of genes have been identified as being important for bud site selection. They include *RSR1/BUD1*, *BUD2*, *BUD3*, *BUD4*, *BUD5*, *CDC3*, *CDC10*, *CDC11*, *CDC12*, *CDC24*, *SPA2*, and *RVS167* (Sloat et al., 1981; Bender and Pringle, 1989; Snyder, 1989; Chant et al., 1991; Chant and Herskowitz, 1991; Bauer et al., 1993; Flescher et al., 1993). Mutations in these genes result in the selection of inappropriate bud sites for the initiation of growth but, with the exception of temperature-sensitive *cdc24* mutations at elevated temperatures, do not prevent the subsequent localization of growth to these selected sites. *RSR1/BUD1* encodes a Ras-related small GTP-binding protein that is regulated by the Bud2 GTPase-activating protein and the Bud5 GDP/GTP exchange factor (Chant et al., 1991; Powers et al., 1991; Bender, 1993; Park et al., 1993). *CDC3, CDC10, CDC11, CDC12*, and *SPA2* encode proteins that appear at presump-

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tive bud sites early in the cell cycle (Haarer and Pringle, 1987; Snyder, 1989; Ford and Pringle, 1991; Kim et al., 1991; Snyder et al., 1991). Cdc3p, Cdc10p, Cdc11p, and Cdc12p are putative components of the 10-nm neck filaments which are required for cytokinesis.

Once a bud site has been selected, several other genes are known to be required for the subsequent localization of growth to this site. They include *CDC24*, *CDC42*, *CDC43*, *BEM1*, and *BEM2* (Sloat et al., 1981; Adams et al., 1990; Bender and Pringle, 1991; Chant et al., 1991; Chenevert et al., 1992). Mutations in these genes result in large, multinucleate, unbudded cells that in most cases have also been shown to exhibit delocalized cell surface growth. *CDC42* encodes a Rho-related small GTP-binding protein (Johnson and Pringle, 1990) that is regulated by the Cdc24 GDP/GTP exchange factor (Zheng et al., 1994), the Bem3 GTPase-activating protein (Zheng et al., 1994), and the Cdc43/Ram2 geranylgeranyltransferase I (Ohya et al., 1993; Trueblood et al., 1993). It is concentrated on the plasma membrane at the site of bud emergence and also over the surface of the growing bud (Ziman et al., 1993). Bem1p and Rvs167p both contain SH3-domains (Chenevert et al., 1992; Bauer et al., 1993) similar to those found in signal transducing proteins that function at the membrane/cytoskeleton interface (Pawson and Schlessinger, 1993).

Indeed, putative components of the 10-nm neck filaments (see above) and the actin cytoskeleton are known to be important for the spatial control of cell growth in yeast. For example, actin (*act1*) and profilin (*pfy1*) mutants are defective in localized cell surface growth and bud site selection (Novick and Botstein, 1985; Haarer et al., 1990; Drubin et al., 1993), and conditional myosin (*myo2*) mutants become arrested predominantly as large, unbudded cells and also exhibit delocalized cell surface growth at their restrictive temperatures (Johnston et al., 1991). Thus, normal polarized cell growth requires the coordinated function of a large number of signal transducing proteins and components of different cytoskeletal systems.

BEM2 was first identified through its genetic interaction with *MSB1*, which can function as a dosage-dependent suppressor of the temperature-sensitive (Ts$^-$)[1] growth phenotype of *cdc24* and *cdc42* bud emergence mutants (Bender and Pringle, 1989). Yeast cells lacking *MSB1* have no detectable phenotype but become inviable when *BEM1* or *BEM2* is also mutated in these cells (Bender and Pringle, 1991). We have previously isolated the Ts$^-$ *ipl2-1* mutant as a conditional mutant that gains entire sets of chromosomes at the restrictive growth temperature of 37°C (Chan and Botstein, 1993). Cytological studies of this mutant revealed that the observed change in chromosome number is associated with a failure in bud growth but not DNA replication and nuclear division. *ipl2-1* mutants become arrested as large, multinucleate, unbudded cells at 37°C. This mutant phenotype is similar to that exhibited by a number of previously identified mutants (see above), including *bem2*. Here we show that *IPL2* is identical to *BEM2*. For this reason, the *ipl2-1* mutation will be referred to as *bem2-101* in this report. Bem2p is related in sequence and function to a number of GTPase-activating proteins, and it probably functions in vivo with Rho1p and Rho2p, two Ras-related small GTPases (Madaule et al., 1987), to control polarized cell growth. Results from the genetic analysis of *bem2*, *grr1*, *cdc55*, *SSD1*, and *sit4* mutants further suggest that protein phosphorylation and dephosphorylation may play an important role in the *BEM2*-mediated process of polarized cell growth.

1. *Abbreviations used in this paper*: Cs$^-$, cold-sensitive; GAP, GTPase-activating protein; Ts$^+$, temperature-resistant; Ts$^-$, temperature-sensitive.

Materials and Methods

Strains, Media, and Genetic Methods

Yeast strains used in this study are listed in Table I. The strain CBY1829-1 was constructed by integrating, via homologous recombination, the *URA3*-bearing plasmid pCC705 into the genome at the *GRR1* locus of DBY1829. The strains CCY432-1D and CCY432-15C, used for identifying extragenic suppressor mutations of *bem2-101*, were derived from a strain carrying the *URA3* gene integrated next to the *SPT2* locus (Chan and Botstein, 1993). The diploid strain CBY1830-30 was constructed by a one-step gene disruption procedure (Rothstein, 1983), replacing one of the two *BEM2* genes in DBY1830 with the *bem2-Δ103::LEU2* allele present on pCC394. This disruption was confirmed by DNA hybridization with an appropriate probe. The *Escherichia coli* strain DB1142 (*leu pro thr hsdR hsdM recA*) was routinely used as a host for plasmids.

Rich medium YEPD, synthetic minimal medium SD, and SD medium with necessary supplements were prepared as described previously (Sherman et al., 1974). These different media contained glucose as carbon source. Cells were routinely grown at 26°C unless otherwise specified. Yeast genetic manipulations were performed as described by Sherman et al. (1974).

Isolation of Extragenic Suppressors of bem2-101

Spontaneous, temperature-resistant (Ts$^+$) revertants were isolated by seeding YEPD plates with about 2×10^7 *bem2-101* cells (CCY109-9C-1 or CCY109-1D-1) per plate, and incubating for 3–5 d at 37°C. To ensure that each revertant isolated was independent, cells from independent colonies grown at 26°C were used to seed each plate, and only one revertant was studied per plate. Extragenic suppressors were identified by tetrad analysis after mating revertants to *bem2-101* strains (CCY432-1D or CCY432-15C) that carry a *URA3* marker next to the *SPT2* locus, which is tightly linked to *bem2-101* (Chan and Botstein, 1993). Initially, suppressors were not chosen on the basis of a cold-sensitive (Cs$^-$) growth phenotype. However, several of them (see Results) turned out to have a Cs$^-$ growth phenotype.

DNA Manipulation

Functional localization of the cloned *BEM2* gene was done by subcloning DNA fragments into the low copy number *URA3*-CEN-plasmid pRS316 (Sikorski and Hieter, 1989). pCC394, used for disruption of *BEM2*, was constructed in two steps. First, a BamHI site was created in pCC231 (see Fig. 2) near the 3′ end of *BEM2* (at codons 2144–2145) by site-directed mutagenesis (Kunkel et al., 1987), using the primer IPL2.1p: 5′-AGCCAACAGCATGGGATCCAGATTA-3′ (the mutagenic base is underlined). This generated pCC393. The sequence between the XbaI and BamHI sites of pCC393 was then replaced with the ∼2-kb XbaI/BamHI fragment (containing *LEU2*) of pJJ283 (Jones and Prakash, 1990), generating pCC-394. Codons 115–2144 of *BEM2* are missing from the *bem2-Δ103::LEU2* mutant allele present on this plasmid. Plasmid pCC705, used for integration of *URA3* into the genome at the *GRR1* locus, was constructed by cloning the ∼3.8-kb BglII fragment (containing part of *GRR1*) of pBM1720 (Flick and Johnston, 1991) into the BamHI site of the *URA3*-plasmid YIp5 (Scherer and Davis, 1979).

Plasmids used for the expression of GAP domains were constructed in the following way. DNA sequence spanning the putative Bem2-GAP domain was amplified from the *BEM2*-plasmid pCC231 by PCR, using the primers IPL2.2p (5′-CGATAAGCTTGGATCCAAATGCAACTACACGACTTA-3′) and IPL2.3p (5′-TCGCGGATCCAAGCTTCAAGGAGAAGAG-3′). The PCR product, which contained BamHI sites near both ends, was cleaved with BamHI and then cloned into the BamHI site of pG-3 (Schena et al., 1991). The resulting plasmid pCC408 allows expression of the COOH-terminal 287 residues of Bem2p under the control of the strong *TDH3* promoter. In a similar fashion, the plasmid pCC438, which allows expression of the COOH-terminal 304 residues of Bcr, was constructed, using the *bcr*-

Table I. Yeast Strains Used

Strain		Genotype
DBY1829	α	*lys2-801 his3-Δ200 ura3-52 leu2-3,112 trp1-1*
DBY1830	a/α	*ade2/+ lys2-801/+ his3-Δ200/his3-Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-1/trp1-1*
CBY1829-1	α	*lys2-801 his3-Δ200 ura3-52 leu2-3,112 trp1-1 URA3 (at GRR1)*
CBY1830-30	a/α	*ade2/+ lys2-801/+ his3-Δ200/his3-Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-1/trp1-1 bem2-Δ103::LEU2/+*
CCY-D1	a/α	*ade2/+ lys2-801/+ his3-Δ200/his3-Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-1/trp1-1 bem2-101/bem2-101*
CCY-D3	a/α	*ade2/ade2 his3-Δ200/his3-Δ200 ura3-52/ura3-52 lys2-Δ101::HIS3::lys2-Δ102/lys2-Δ101::HIS3::lys2-Δ102 leu2-Δ101::URA3::leu2-Δ102/+ grr1-102/grr1-102*
CCY109-1D-1	a	*ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 bem2-101*
CCY109-9C-1	α	*ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 bem2-101*
CCY354-1C	α	*ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 grr1-102*
CCY355-4C	α	*ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 grr1-103*
CCY362-7B	α	*ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 grr1-101*
CCY363-1C	a	*ade2 ura3-52 his4-619 grr1-102*
CCY363-1D	α	*ura3-52 his4-619 grr1-102*
CCY374-2D	α	*lys2-801 his3-Δ200 ura3-52 leu2-3,112 trp1-1 bem2-Δ103::LEU2*
CCY416-12D	α	*lys2-801 his3-Δ200 ura3-52 leu2-3,112 trp1-1 bem2-101*
CCY432-1D	α	*leu2-3,112 his3-Δ200 ura3-52 lys2-801 bem2-101 URA3 (at SPT2)*
CCY432-15C	a	*leu2-3,112 his3-Δ200 ura3-52 lys2-801 bem2-101 URA3 (at SPT2)*
CCY450-8A	a	*lys2-801 his3-Δ200 ura3-52 leu2-3,112 grr1::LEU2*
CCY469-38A	a	*lys2-801 his3-Δ200 ura3-52 leu2-3,112 grr1::LEU2*
CCY469-41A	α	*ade2 his3-Δ200 ura3-52 leu2-3,112 cdc55::URA3*
CCY471-13C	a	*ade2 his3-Δ200 ura3-52 leu2-3,112 bem2-101*
CCY475-19A	α	*his3-Δ200 ura3-52 leu2-3,112 SSD1-v1 bem2-Δ103::LEU2*
CCY487-21D	a	*ade2 his3-Δ200 ura3-52 leu2-3,112 grr1-102*
CCY488-16A	a	*ade2 his3-Δ200 ura3-52 leu2-3,112 grr1-102 bem2-101*
CCY802-11C	a	*ade2 his3-Δ200 ura3-52 leu2-3,112*
CY248	a	*Δsit4::HIS3 SSD1-v1 his3 leu2-3 ura3*

Most of the strains were constructed specifically for this study, the exceptions being DBY1829 and DBY1830, which are from D. Botstein's laboratory collection (Stanford University, Stanford, CA), and CY248, which is from K. Arndt's laboratory collection (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The *cdc55::URA3* allele was constructed in J. Pringles's laboratory (University of North Carolina, Chapel Hill, NC). It contains a replacement of the BglII/BamHI DNA fragment representing the 5′ half of *CDC55* with a DNA fragment containing *URA3*. The *SSD1-v1* allele present in CCY475-19A was derived from CY248.

plasmid bcr-3 (Hariharan and Adams, 1987) as template and the primers BCR.1p (5′-CGCGGATCCAAATGGGCTCCCAGACCCTGAGG-3′) and BCR.2p (5′-TCGCGGATCCGGAGATGGACTGGGACCT-3′).

The high copy number *URA3*-2μ-plasmids YEpU-RHO1, YEpU-RHO2, and YEpU-CDC42, containing *RHO1*, *RHO2*, and *CDC42*, respectively, were obtained from Y. Ohya (University of Tokyo, Tokyo, Japan) (Qadota et al., 1992). pCC743, containing both *RHO1* and *RHO2*, was constructed by inserting the ∼2.7-kb SalI/EagI fragment (containing *RHO2*) of YEpU-RHO2 into the SalI/EagI sites of YEpU-RHO1. The high copy number plasmids pOPR3 and pOPR4, containing *RHO3* and *RHO4*, respectively, were obtained from Y. Matsui (University of Tokyo, Tokyo, Japan) (Matsui and Toh-e, 1992*a*,*b*).

Cytological Techniques

Some of the cytological experiments were carried out using diploid cells because their larger size makes it easier to visualize the actin and microtubule cytoskeletons. Immunofluorescence staining of cells was carried out as described (Pringle et al., 1989). Microtubules were stained with the rat anti-α-tubulin mAb YOL1/34 (Bioproducts for Science, Indianapolis, IN) and affinity purified FITC-conjugated goat anti-rat secondary antibodies (Organon Teknika Corp., West Chester, PA). Actin was stained with affinity purified rabbit anti-actin antibodies (gift of David Drubin) and affinity purified FITC-conjugated goat anti-rabbit secondary antibodies (Organon Teknika Corp., West Chester, PA). DNA was stained with DAPI (1 μg/ml; Accurate Chemical Co., Westbury, NY), and chitin was stained with Calcofluor (0.2 mg/ml; Sigma Chemical Co., St. Louis, MO). Stained cells were viewed with a Zeiss Axioskop fluorescence microscope and photographed with Kodak Type 2415 Technical Pan hypersensitized film (Lumicon, Livermore, CA).

Results

We have previously cloned the *IPL2* gene (Chan and Botstein, 1993). Molecular analysis of *IPL2* described below revealed that *IPL2* is identical to *BEM2* (Bender and Pringle, 1991; Zheng et al., 1993, 1994), which has been independently cloned in Alan Bender's laboratory (Peterson et al., 1994). For this reason, *IPL2* will be referred to as *BEM2*, and the *ipl2-1* mutation will be referred to as *bem2-101* in this and future reports.

bem2-101 Mutants Are Defective in Bud Site Selection and Polarized Cell Growth

We have previously shown that *bem2-101* mutant cells become arrested as large, unbudded cells when incubated at 37°C (Chan and Botstein, 1993). To determine whether this arrest is associated with defects in cell surface growth, we examined the deposition of cell wall chitin in diploid wild-type and *bem2-101* mutant cells by Calcofluor staining (Hayashibe and Katohda, 1973). At 26°C, chitin-staining in these cells was restricted mostly to bud scars, which define previous bud sites. For ∼95% of wild-type cells, these bud scars were found exclusively near the two poles, indicative of the expected bipolar budding pattern of diploid cells (Fig.

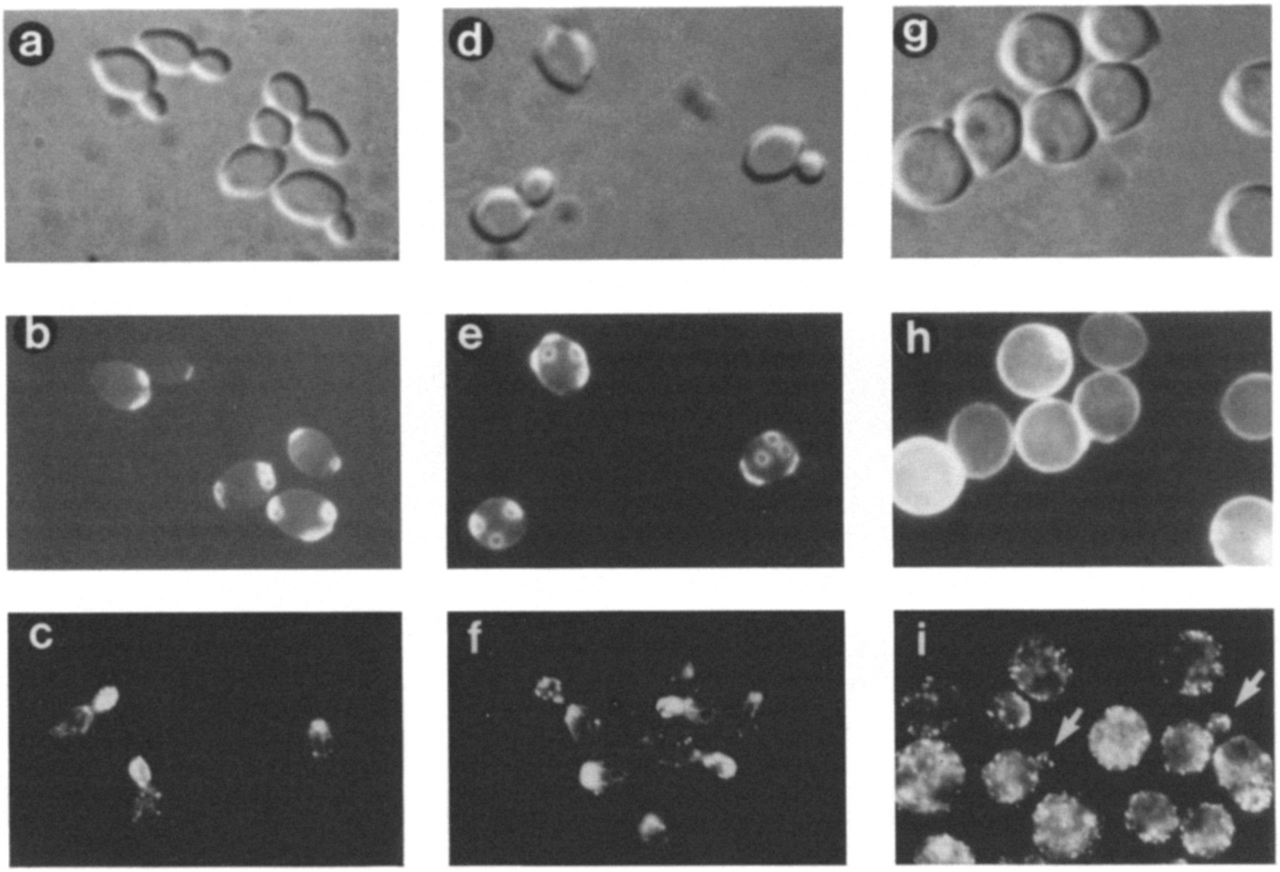

Figure 1. Cytological examination of the *bem2-101* mutant. Wild-type (DBY1830) (*a–c*) and *bem2-101* (CCY-D1) (*d–i*) diploid cells grown at 26°C (*a–f*) or for 2 h at 37°C (*g–i*) were stained with Calcofluor (*b*, *e*, and *h*) or anti-actin antibodies (*c*, *f*, and *i*). The DIC images (*a*, *d*, and *g*) and Calcofluor-staining images were obtained from the same cells. The arrows in *i* highlight small-budded cells that have uniform distributions of actin patches. All cells are shown at the same magnification.

1, *a* and *b*). In contrast, the localization of bud scars was randomized in >75% of *bem2-101* cells (Fig. 1, *d* and *e*). After a 2-h incubation at 37°C, the chitin-staining pattern of wild-type cells remained unchanged (data not shown), whereas that of *bem2-101* cells was greatly altered (Fig. 1, *g* and *h*). Chitin-staining was no longer restricted to bud scars; instead, many *bem2-101* cells became brightly and uniformly stained, indicating delocalized chitin deposition and loss of cell polarity. This delocalized growth resulted in large, round cells that were mostly unbudded. Thus, *bem2-101* cells are defective in bud site selection at the permissive growth temperature and are defective in polarized cell surface growth at the restrictive growth temperature. This dual defect is also exhibited by some *cdc24* mutants (Sloat et al., 1981).

bem2-101 Mutants Are Defective in Organization of the Actin Cytoskeleton

Since the actin cytoskeleton plays an important role in polarized cell surface growth and chitin localization (Novick and Botstein, 1985; Drubin et al., 1988, 1993; Haarer et al., 1990; Johnston et al., 1991), we examined this structure in wild-type and *bem2-101* cells by anti-actin immunofluorescence microscopy. At 26°C, wild-type and *bem2-101* cells had similar actin-staining patterns, characterized by actin cables that run along the mother-bud axis and cortical actin patches that are concentrated in areas of active cell growth (i.e., buds and presumptive bud sites) (Fig. 1, *c* and *f*). Thus, even though *bem2-101* cells are defective in bud site selection at 26°C, they are not noticeably defective in the organization of the actin cytoskeleton at this temperature. After a 2-h incubation at 37°C, the actin-staining pattern of wild-type cells remained unchanged (data not shown), whereas that of *bem2-101* cells was greatly altered (Fig. 1 *i*). Actin cables were no longer detectable and cortical actin patches became uniformly distributed throughout the *bem2-101* cells, which were predominantly enlarged and unbudded. For the small number of *bem2-101* cells that remained budded, actin patches were often not concentrated in the buds. Thus, loss of cell polarity and delocalization of chitin deposition are associated with a failure to organize an asymmetric, polarized actin cytoskeleton in *bem2-101* cells at 37°C.

BEM2 Encodes a Protein that Is Required for Growth at Elevated Temperatures

To elucidate the cause of the defects described above, we carried out a molecular analysis of the previously cloned *BEM2* gene (Chan and Botstein, 1993). *BEM2* was localized to a region that spans over 4.2 kb (Fig. 2). Interestingly, the plasmid pCC42, which does not contain the entire predicted *BEM2*-encoding sequence, can complement the Ts^- phenotype of a *bem2-101* mutant. This apparent discrepancy will

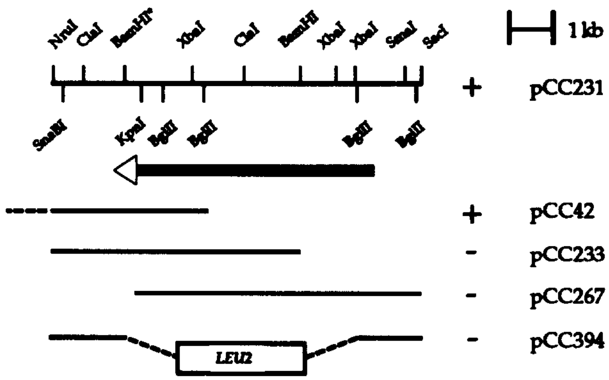

Figure 2. Functional localization of the cloned *BEM2* gene. The ability (+) or inability (−) of the different *URA3*-CEN plasmids (containing the DNA fragments shown) to complement the temperature-sensitive growth phenotype of a *bem2-101* mutant at 37°C is listed. The plasmid pCC42 contains additional yeast sequence present to the left of the NruI site. The asterisk denotes the BamHI site that is not normally present but was created in pCC393 for the purpose of constructing pCC394, which has the sequence between the BamHI and XbaI sites replaced by the *LEU2* gene. The location and orientation of the predicted *BEM2* open reading frame is represented by the arrow.

be discussed below. Sequencing of the *BEM2* region revealed a long open reading frame (Fig. 3) that potentially encodes a protein of 2167 amino acids, with a pI of 8.4 and a predicted molecular mass of 246 kD, which is consistent with the apparent molecular mass (>200 kD) of Bem2p as determined by immunoblotting (data not shown). A search of the GenBank database revealed no protein with primary sequence identical to that of the predicted Bem2p.

To determine the *bem2*-null mutant phenotype, a diploid yeast strain with one of its two *BEM2* genes replaced by the *LEU2* gene was constructed (see Fig. 2 and Materials and Methods). In this construction, codons 115–2144, representing 94% of the *BEM2* coding sequence, were removed. Sporulation and tetrad analysis of this heterozygous (*BEM2/bem2-Δ103::LEU2 leu2/leu2*) diploid strain (CBY1830-30) showed that all four spores per tetrad were viable at 26°C on rich YEPD medium, indicating that *BEM2* is not essential for cell viability at this temperature. However, a Leu^+ spores (carrying the *bem2-Δ103::LEU2* mutation) gave rise to smaller colonies, indicating that deletion of *BEM2* results in a slower growth rate at 26°C. *bem2-Δ103::LEU2* cells are also temperature-sensitive for growth at 33°C on YEPD medium, and this temperature sensitivity can be partially suppressed by the presence of 1 M sorbitol (Fig. 4), suggesting that *bem2-Δ103::LEU2* cells may be osmotically fragile and prone to lyse at this elevated temperature. This is consistent with our previous observation that *bem2-101* mutant cells look abnormal at 37°C when examined by phase contrast microscopy, some appearing to have especially enlarged vacuoles (Chan and Botstein, 1993). Indeed, the Ts^- growth phenotype of *bem2-101* mutants also can be suppressed by the presence of 1 M sorbitol (Fig. 4). Overall, the mutant phenotypes caused by *bem2-Δ103::LEU2* are similar to, but more severe than, those caused by *bem2-101*, suggesting that the latter mutation does not result in a total loss of *BEM2* function (at temperatures below 35°C, the restrictive temperature for *bem2-101* mutants). The fact that yeast cells lacking *BEM2* are viable at 26°C, but not at 37°C, suggests that localization of cell growth to selected bud sites does not absolutely require the function provided by Bem2p at 26°C. Alternatively, this function may be (partially) provided by other gene products at this temperature (but not at 37°C). Indeed, at least four other genes (*BEM3*, *DBM1*, *LRG1* and *YBR1728*) encoding proteins related in sequence to Ipl2p are present in yeast (Doignon et al., 1993; Zheng et al., 1993, 1994; Müller et al., 1994; our unpublished results).

Bem2p Has Sequence Homology with GTPase-activating Proteins

Analysis of the predicted Bem2p sequence revealed two interesting features. First, the amino-terminal 310 residues of Bem2p is very rich (36%) in serine and threonine. The functional significance of this is unknown, but it is interesting to note that human Bcr described below also contains a region rich in these two amino acids. Second, the carboxyl-terminal 203 residues of Bem2p is homologous to sequences found in a large family of proteins (Boguski and McCormick, 1993), including human Bcr (Heisterkamp et al., 1985; Hariharan and Adams, 1987; Lifshitz et al., 1988), chimaerin (Hall et al., 1990, 1993), CDC42GAP (Barfod et al., 1993), rho-GAP (Lancaster et al., 1994), the rat RasGAP-associated protein p190 (Settleman et al., 1992*b*), and yeast Bem3p (Zheng et al., 1993, 1994), Lrg1p (Müller et al., 1994), and Ybr1728p (Doignon et al., 1993). The sequence homology with human Bcr is highest (35% identity) and that with yeast Bem3p, Lrg1p, and Ybr1728p is lower (26%, 25%, and 29% identity, respectively). The Bem2p-related domains from five of these eight proteins have been shown to function in vitro as GTPase-activating proteins that are specific for members of the Rho-subfamily of Ras-related small GTP-binding proteins (Diekmann et al., 1991; Settleman et al., 1992*a*; Barfod et al., 1993; Chen et al., 1993*a*, *b*; Hall et al., 1993; Ridley et al., 1993; Zheng et al., 1993, 1994; Lancaster et al., 1994). Thus, Bem2p may also serve as a GTPase-activating protein (GAP) in vivo.

bem2 Mutant Phenotypes Can Be Suppressed by Expression of the GAP Domain from Bem2p or Human Bcr

To determine the biological significance of the sequence homology found between Bem2p and the different (putative) GAPs, we tested whether *bem2* mutant phenotypes can be suppressed by expression of the putative GAP domain from wild-type Bem2p or human Bcr. For this purpose, high copy number plasmids that allowed expression of the carboxyl-terminal 287 residues of Bem2p (containing the putative GAP domain) or the carboxyl-terminal 304 residues of human Bcr (containing the previously demonstrated GAP domain [Diekmann et al., 1991; Ridley et al., 1993]) under the control of the *TDH3* promoter were introduced into *bem2* cells. Our results showed that *bem2-101* and *bem2-Δ103::LEU2* cells containing either plasmid could grow at 37°C (Fig. 5; data not shown), indicating that expression of the putative GAP domain from Bem2p or the previously demonstrated GAP domain from Bcr can suppress the Ts^- growth phenotype of *bem2* mutants. Furthermore, in our ini-

1 TCCTGAAACATATTCCCCTCGGTGAAACACGCTCTTGAAAATGACCTGAGAACACAAGATATCAGACGGCTCTTGTTGCACAAAAAACAAAAACAAAAGAAGCAAGCTACGTTGCAGCCA

121 CTGGTACTGTCTGCTAACTCAAACATTCTTTTTCATTTTTAATATACAGATTTCGTATTCTCCCGTTCCCGCTATACGCAATTTTCTTTCTTTTTTTTCCCTCTTTGGTTAGGAGACTGC

241 AGATCAGTCCCATTTTTTCCAACAGAACAAACAAGCAAACGTTTCAGTTTAAAAGAACACACGGTCGTCGTGCCTTTTCTGGATAGACACAAAAAAAACAAATAACGAAGCAGGAGTCTA
*

361 ATGAAAGGTCTTCTCTGGTCTAAGAACAGGAAATCTTCAACGGCCAGTGCTAGTTCAAGCTCAACTTCCACGTCTCATAAGACAACTACTGCGTCTACAGCATCGTCGTCGTCGCCGTCA
1 M K G L L W S K N R K S S T A S A S S S S T S T S H K T T T A S T A S S S S P S

481 TCATCCTCTCAAACTATTCGAAACTCGACAAGCGGTGCTTCTCCATACATGCATTCCCACCATCACCATGGCCAGGGACACAGTCACCACAGAGGGGAAGACAACAACAGAGATAAGAGA
41 S S S Q T I R N S T S G A S P Y M H S H H H H G Q G H S H H R G E D N N R D K R

601 AAAAGCTCCGTATTTCCACCCTCCAAGCAGTACACATCCACTAGTTCCAGTCAGGTGAACTTGGGGATGTATCATTCGGATACTAACACCAGATCTTCTAGAAGCATAGCCTCTACGCTA
81 K S S V F P P S K Q Y T S T S S S Q V N L G M Y H S D T N T R S S R S I A S T L

721 AAAGACGATTCTCCGTCTGTCTGCTCCGAAGATGAAATAAGCAATTCTTCTAGCCAGAAGTCCAATGCCCAGGATGAGACTCCCATTGCGTACAAGAAGAGTGCCCATTCGAAAGACTCA
121 K D D S P S V C S E D E I S N S S S Q K S N A Q D E T P I A Y K K S A H S K D S

841 TTGTTGCCTTCACGTTCCTCGTCTTTATCACCTCCTCAGTCTCGATGTAGCACTGGAACTACGCTAGAGAAATCGTTGAACACTTCAGGTATCAGTAACAGTAGCGGCACTAATAATAAC
161 L L P S R S S S L S P P Q S R C S T G T T L E K S L N T S G I S N S S G T N N N

961 AATAGCAATAATAACAACGATAACGAGCAAAAGCAAAGAAATGTTATCCACTTAAACTCTGAAAACTATGATACAACTGTTTTTAAGACTGGATGGGTTAATAAGTCCCATGGCCAGACT
201 N S N N N N D N E Q K Q R N V I H L N S E N Y D T T V F K T G W V N K S H G Q T

1081 GTTGCTACAAACTATAATAGCTCAATGACAGCCCCTTCCTCTTCCTCTTCGTCCTCCTCTCAGAATCTCCGTAATGATGCGTATTCTAGAAACAGGGAAAGCAGGTTTTATGGGAACGAC
241 V A T N Y N S S M T A P S S S S S S S S Q N L R N D A Y S R N R E S R F Y G N D

1201 GGTTCGTCTTTAAAAAATGATGACAGCAGTAGTACTACGGCCACTAACAGTGGCAATGATGTGGCCTCAGCAAGAAGCAGCATGGCTATAGATCCTCAAATGCTAGTACCTGATTACCGT
281 G S S L K N D D S S S T T A T N S G N D V A S A R S S M A I D P Q M L V P D Y R

1321 CTTTATAGGGCTCAATTGAAAGGTTGCGTTCTCAACCTTTACAAAAGTGGTTTGAATAGTAACATTAAGTTTTTTGATCCAACTTTACCAGCTTCAAATAGTAGTATAGCAAATGAAAAC
321 L Y R A Q L K G C V L N L Y K S G L N S N I K F F D P T L P A S N S S I A N E N

1441 CATCAACAGAAAAAACAACAAACAAACAACCAAGCACAAGCGGAAGCTCTGCACCAAAAGCAATCATTTGGCCAAATGGGTGAACCTATTACACTGGACCTGAAGTATTTATCTGAAGTT
361 H Q Q K K Q Q T N N Q A Q A E A L H Q K Q S F G Q M G E P I T L D L K Y L S E V

1561 TACCCGCACCCAGATTTAAGACAAGACAGTGATGGTAAAATTATATCAGGCACAATAGAAAGTTTATGTCACACAGTCTTATTTTACCCCGGTCCAAAGCAATCGGACGTACCTAATGAG
401 Y P H P D L R Q D S D G K I I S G T I E S L C H T V L F Y P G P K Q S D V P N E

1681 AAATCATTATCTAAGACACATAGAGCTGTTATTAATCTGTTATTGATGTTTCCATTACTAGATCATTTCATTAAGTTTCTAAAGGTATTCAACCAATTCGGTCTTTCTTTTACCAAGAAT
441 K S L S K T H R A V I N L L L M F P L L D H F I K F L K V F N Q F G L S F T K N

1801 AAATCAAGATTGACAAATAATTCCACACAATTCTACAACATATCCCCTGCTGTCGATGACTCCATGACTCAAAGATTAGCTTTAACTGCTAAAACAATCCTTGATGTATTTCCGGGATTT
481 K S R L T N N S T Q F Y N I S P A V D D S M T Q R L A L T A K T I L D V F P G F

1921 TTGCTGGATGAACCAATGCTGAAGACTATAATTTCCTTATTAGATACTATATCTTTGCATAACGATGAAATTTCCAACAATTTGAAAATTAAAATCGCCAATAAACATAACGAATTAATG
521 L L D E P M L K T I I S L L D T I S L H N D E I S N N L K I K I A N K H N E L M

2041 AAACTTACCGCATTCACTCGATCTCTTCCAATGGCAACATCATCTACACATGAATTAGAGATTATATTGGATCCAAGTCATTTCCTGTCTTTAGACATTACAACATTGGCTGATGAAGTT
561 K L T A F T R S L P M A T S S T H E L E I I L D P S H F L S L D I T T L A D E V

2161 CATCATATCAATTTAAAGTTTGATAAAGTATGGGCACCGAAATTTGACTATTCTCTGCTGTATGATTCGAAGTTTATTAATCGAAGAATTGTCTCGTTAAATCCATTGGTATTCAACAAC
601 H H I N L K F D K V W A P K F D Y S L L Y D S K F I N R R I V S L N P L V F N N

2281 GACCAAAATATCCATTTTCTTGGAAGGCTACTAATATCACATTTATTTCCAACAAATCCTGAGTTTAGTAAAAAAGTGACTCCAAAAGTGAGAGCTGAGTTATTAGATAAATGGGTCCAA
641 D Q N I H F L G R L L I S H L F P T N P E F S K K V T P K V R A E L L D K W V Q

2401 ATTGGCTGTAGATTTGAACATTTAGGAGACATGGTATCATGGCTAGCTGTCGCCACTATCATTTGTTCAATTCCTGTACTAAGGTCATCTTCATGGAAATATGTTCCTGATCAGTCATTG
681 I G C R F E H L G D M V S W L A V A T I I C S I P V L R S S S W K Y V P D Q S L

2521 AAAACTATTTTTAAAGATTGGGTTCCTACCATTATTCAGTTAGAGAGGCGACAAAGGACATCTAAATCTACAAGTAGTGTTTTCATACTCGCTCCTCCAAATCTGGATGACGACTTTACG
721 K T I F K D W V P T I I Q L E R R Q R T S K S T S S V F I L A P P N L D D D F T

2641 AGAGCTAACGTGATTTCTTATTTTGGCGATTTATTAATTCATGCTGATGATTTACCATCTGATACGAAATTCAAGTACTTGGAAAAGAAAATTAACAGAACAAAAAACGCTTTTCATAAA
761 R A N V I S Y F G D L L I H A D D L P S D T K F K Y L E K K I N R T K N A F H K

2761 TGGCAACAAAGATTACAAGCAATCGACTCTACGAGACATAAAACTAATTCGACAGAAAACGTCAGGGATAATGATTCTCCTAATAACGTTGTTTATCAGCTCTGGAAATTTCATTTATCA
801 W Q Q R L Q A I D S T R H K T N S T E N V R D N D S P N N V V Y Q L W K F H L S

2881 CAACCTCCGTTGAACATTGAAGGAATAATGAAATTAAGTGTCCAACACGAACCTCCAATAATCGACCAGAAAGCGTATTCTACTATTGGTTCTCAAAGGAGTGCTTTAGTAACTGGAAGT
841 Q P P L N I E G I M K L S V Q H E P P I I D Q K A Y S T I G S Q R S A L V T G S

3001 TATTTACCCATTTTATTCAATGAACTATTCCCAAATTATTCACTATTTCCTAAGAATACATTAGTGGGTGCTGCCAGTGATGCGAAGTTGCCGCCTCCAAGATCATCCGCGCGCCTCTCA
881 Y L P I L F N E L F P N Y S L F P K N T L V G A A S D A K L P P P R S S A R L S

3121 AAATCACTTTCAATCAGTGAACCAATTCCAATAGCTTCAAACAGTCACACTATGGGCTCATTAACAGATGATGCTATGTCCTCAAAAAATGATAATAATAAAGTTACAGGTGTTGGAAAA
921 K S L S I S E P I P I A S N S H T M G S L T D D A M S S K N D N N K V T G V G K

3241 ATTGACGGACCTGTGATCAAAGAGATGTCGTCAAAACAATCAAATAAACAGAGACTATTAAAATCTGTGAGAGATGTTTTTAATATTGACATGGATGTGTTTCATATATCAGATGAGTTA
961 I D G P V I K E M S S K Q S N K Q R L L K S V R D V F N I D M D V F H I S D E L

3361 GTATTTAAATCGGTATACGATAATGATGGTAAATCCCGTCCTGCTAGTATGGTCATAGAAACACCAAAGAGATTCTCACAACACTCTTCCATGCTTATTAATAATCCAGCTACTCCAAAC
1001 V F K S V Y D N D G K S R P A S M V I E T P K R F S Q H S S M L I N N P A T P N

3481 CAAAAGATGAGAGATTCCTTGGATACCACTGGAAGACTAAGTAAGACATTAGAAAATATGGACTTTTTCAACAATATTGGTCAAGTATCAGATTCACTAAAAGAATCGATTATTCGTGTC
1041 Q K M R D S L D T T G R L S K T L E N M D F F N N I G Q V S D S L K E S I I R V

3601 GTATTGAAATCGTCGTCGCTAGAAAAGATTTTTGATTTACTGGTATTAACTTCAAATATCTTTTCAAAATTGGTAGACACTAAAGACCTTGAGAATTATTACTATCATCAAAGACAAAGG
1081 V L K S S S L E K I F D L L V L T S N I F S K L V D T K D L E N Y Y Y H Q R Q R

3721 GGTCATTCTACCAGAGGGCTTTCCGATGATAACATAGGACTACTAGATTATGCATTCGTTAAACTAACTATGGACAATGATATTTTCACAGAAACATTCTTCAACACTTATAAAAGCTTT
1121 G H S T R G L S D D N I G L L D Y A F V K L T M D N D I F T E T F F N T Y K S F

Figure 3. Nucleotide sequence of the *BEM2* region and predicted sequence of the Bem2 protein. The upstream and downstream in-frame stop codons are shown by asterisks. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z35159.

3841 ACTACTACAACTACGGTATTAGAAAATATGGCAAAACGGTATGTTGGTGCAAAGAGTTGCAGCGTGTCAATTTCCAAGATTTTAGATCGCAGTGATGATTCTAAAATGAAGATAAATGAG
1161 T T T T T V L E N M A K R Y V G A K S C S V S I S K I L D R S D D S K M K I N E

3961 GATACAAATTTAGTGTCCTCTTCATTATACGACCAAAATTTTCCTGTTTGGGATATGAAAGTAACTGACGATGAAAACATAAACCTTATTTATATGGCAAAAATCCAAATCGGCGCCGCG
1201 D T N L V S S S L Y D Q N F P V W D M K V T D D E N I N L I Y M A K I Q I G A A

4081 GAAGCTATACTACATTTAGTGAAAAATCATTACTCTGACTTTACTGACGATTTATGTAACAACTCTACTCTATTGGATATTATAAAAATAATGGAACAAGAAGTATCCACTGAATGGCCT
1241 E A I L H L V K N H Y S D F T D D L C N N S T L L D I I K I M E Q E V S T E W P

4201 ACGCGAATTGCAAACTCAAAGTTACAAAAAAGCCTACCAGAAAATTTTGTGATTGAAACAGAAAATTTGTTGACTACGTTAACTGATCTATTTCATGGTATTAAATCAGCATACCAAAAG
1281 T R I A N S K L Q K S L P E N F V I E T E N L L T T L T D L F H G I K S A Y Q K

4321 CAACTATACAGACCTATTGGTGTCAATAGAACTCAGAAGAGAATAACTGACATTTTGAATTCCTTTAACACATTCTCTTTTACAGATCTTAACAACATCATTGATGATCCTTCCTTTAGC
1321 Q L Y R P I G V N R T Q K R I T D I L N S F N T F S F T D L N N I I D D P S F S

4441 GATGACATGATTAGATCTTTTCAAAAGTTACACTCAACCAATTACGAAGATATACTAGAATGGATCTACCAGTTAGATAACTTTATTTCTAAGAAGTTCAATTTAGTTTCTAAGAAAGAT
1361 D D M I R S F Q K L H S T N Y E D I L E W I Y Q L D N F I S K K F N L V S K K D

4561 TGGATAGTTCTTTTCCAGGAGTTGGAATTGCTTTCAAAGGAATCTCTGGTCTCTTTTTTCAATTATCCGCTACATTTTAAGTCAAGCAAATTGATAAACCCCGGATATTTGCAATTGCAC
1401 W I V L F Q E L E L L S K E S L V S F F N Y P L H F K S S K L I N P G Y L Q L H

4681 GAGTTTGAAATTTCCAATCTTTTTACTTGGATTTCTACATTGATTCTTAAGGATGATAATGGTACGGAGAGTCTTTTTTTTGAAAAACTACCACAATCTATAAAATTGTTAATAAAGTTA
1441 E F E I S N L F T W I S T L I L K D D N G T E S L F F E K L P Q S I K L L I K L

4801 CACACGAGTCTAACTACGTTTTTCGTGATGGAAATTTCAAACGTCAACAAAAGCAGTAGTGAAAGGTTAACTACTTGTAAAGTTATATTACAAATTTTGAACTACATCCGCTGGAAAAAC
1481 H T S L T T F F V M E I S N V N K S S S E R L T T C K V I L Q I L N Y I R W K N

4921 GGAAGTTTGGATTTATTTGATTCAGAGGAGGATGAAAGTCCTCATGCTATTTGTCCACACATTCCAGCTTTTATTGAAACTGCTATTGCGCATGCCATAATATCACCAGAGTCTAGAAAC
1521 G S L D L F D S E E D E S P H A I C P H I P A F I E T A I A H A I I S P E S R N

5041 TATGAACTTTCGTGGATCAAAGCTTCTGAAAAACTTTCGGACCCTACTAAAGGAACCCAAAATCTAAGATCGATTAGTAACGTTTTAGAGAAGATAGATGACATACATATAAAGAGATTC
1561 Y E L S W I K A S E K L S D P T K G T Q N L R S I S N V L E K I D D I H I K R F

5161 ATTGAGATTGATGATGTATTCAGCAAAAACTGCAAGAATTTATGCCCCTGCCCTGGTTGGTTCATTTCAAGGTTACTAGAAATATCTCAGTTTGTCCCTAACATGAGTATCACTAATTCG
1601 I E I D D V F S K N C K N L C P C P G W F I S R L L E I S Q F V P N M S I T N S

5281 AAACTTATTAATTTTGACAAAAGGAGATTTGTTAACAACATAATATCTAATGTCTTGGATTTAATTCCGAACGAAAGGGAGTTTCCACTTGATATTGAAATGAGTGACGAAAATCCTTCG
1641 K L I N F D K R R F V N N I I S N V L D L I P N E R E F P L D I E M S D E N P S

5401 AAAAGAACTACATTTGGCCGCATTCTGTTCAATAATTTTGAAGATGTTAATAAAGTATACAGAAAGAAGACTAAAAAAGTATCAGAATCAGAAGCCATATCGGAGAGATTTCAAGAGCAG
1681 K R T T F G R I L F N N F E D V N K V Y R K K T K K V S E S E A I S E R F Q E Q

5521 GGAGTATTTAATGAAATACTAGTCAATGAAATTGAAAAAATAAAGAGGGAAGCCAGAAAATTAGAGGTTTTACTCGATCAAGAAAAGATCTTGAAGAACTCTGCTGCTTTACATCAGGCC
1721 G V F N E I L V N E I E K I K R E A R K L E V L L D Q E K I L K N S A A L H Q A

5641 GTTCCGAAAAAGAATAGGAAGTCTGTTATTATCTCAGGTACTCATAGTGACAACGATCATAGCTATAATATCAATAAGAACACCGGTCAAACGCCTTCTCTAGGTAGTGTTATGGAAAGC
1761 V P K K N R K S V I I S G T H S D N D H S Y N I N K N T G Q T P S L G S V M E S

5761 AATAATTCAGCAAGGAATAGAAGAGATTCAAGGGCCTCCTTTTCCACAAATAGATCATCAGTAGTGTCAAATTCCAGCCATAATGGTGTTAGTAAAAAAATAGGTGGATTTTTCAGAAGA
1801 N N S A R N R R D S R A S F S T N R S S V V S N S S H N G V S K K I G G F F R R

5881 CCATTTTCAATCGGTGGATTCAACACATCCAGTTCTAATTATTCACTCAACAGTATATTATCGCAAGAAGTTTCAAGTAACAAATCCATTCTACCAAGTATACTTCCAGAGGTTGATTCC
1841 P F S I G G F N T S S S N Y S L N S I L S Q E V S S N K S I L P S I L P E V D S

6001 ATGCAACTACACGACTTAAAACCTAGTTATAGTTTGAAGACCTTTGAAATCAAGTCAATAATGGAAATAATCAATCACAGAAACATTCCGGCTTACTACTATGCGTTCAAGATAGTTATG
1881 M Q L H D L K P S Y S L K T F E I K S I M E I I N H R N I P A Y Y Y A F K I V M

6121 CAAAATGGCCATGAATATCTGATTCAAACGGCTAGTTCTAGTGACCTCACAGAATGGATAAAAATGATCAAGGCTTCCAAGAGATTTTCATTCCATTCGAAAAAATATAAAGGTAAAACT
1921 Q N G H E Y L I Q T A S S S D L T E W I K M I K A S K R F S F H S K K Y K G K T

6241 CATAATAAGATATTTGGGGTACCATTAGAAGACGTTTGTGAAAGAGAGAATACCTTGATACCTACAATTGTTGTGAAATTGCTGGAAGAGATTGAGCTGCGTGGGCTAGATGAAGTGGGA
1961 H N K I F G V P L E D V C E R E N T L I P T I V V K L L E E I E L R G L D E V G

6361 TTGTACAGAATTCCTGGTTCCATCGGCAGTATAAATGCACTAAAGAACGCATTTGACGAAGAAGGTGCAACAGATAATTCCTTTACATTAGAGGACGATAGATGGTTTGAAGTGAATGCG
2001 L Y R I P G S I G S I N A L K N A F D E E G A T D N S F T L E D D R W F E V N A

6481 ATCGCAGGCTGTTTTAAGATGTATTTAAGAGAGTTACCAGATTCTTTATTTTCTCATGCTATGGTGAACGACTTTACCGATTTGGCGATTAAGTATAAAGCACATGCAATGGTAAATGAA
2041 I A G C F K M Y L R E L P D S L F S H A M V N D F T D L A I K Y K A H A M V N E

6601 GAATACAAGAGAATGATGAATGAGTTACTCCAAAAGTTACCTACATGCTATTACCAAACTTTGAAAAGAATTGTATTTCATTTGAATAAAGTACATCAGCATGTTGTAAATAATAAAATG
2081 E Y K R M M N E L L Q K L P T C Y Y Q T L K R I V F H L N K V H Q H V V N N K M

6721 GACGCATCAAACTTGGCTATTGTATTTTCCATGAGTTTTATAAACCAAGAAGACTTAGCCAACAGCATGGGGTCCAGATTAGGTGCAGTTCAGACAATCCTGCAAGATTTTATTAAGAAT
2121 D A S N L A I V F S M S F I N Q E D L A N S M G S R L G A V Q T I L Q D F I K N

6841 CCAAATGATTATTTCAAGCAATAAACAATTTTCGTGGTAAATGTATACAATCCACTGCTGAGAGAGAAAAAAATTTGGTCCCGCCTATCGAGGGTTTGAGTTTATATTTTTATATTTACA
2161 P N D Y F K Q *

6961 GAGAGATATAGAGCCTTTATTCATTTTTTGTTATTAATGAAGTTTGATTTGTGAACAATTTTGATTTTGGAATTCTGCCTCTTCTCCTTG

tial cloning of the *BEM2* gene, we isolated the plasmid pCC42, which is a low copy number plasmid that contains a truncated version of *BEM2* (Fig. 2), missing sequences 5′ of the Sau3A site present at codons 1818–1819 (Fig. 3). Transcription of this truncated *BEM2* gene, perhaps from a cryptic promoter fortuitously located near this codon, would be expected to allow production of a truncated Bem2p containing at most the carboxyl-terminal 349 residues (i.e., similar to the putative Bem2–GAP domain described above). *bem2-101* cells containing pCC42 grow fairly well at 37°C, again suggesting that expression of the Bem2-GAP domain is sufficient for suppression of the Ts⁻ growth phenotype of *bem2* mutants.

Since *bem2* mutants are also defective in bud site selection at the permissive growth temperature of 26°C, we examined whether plasmids (pCC408 and pCC438) that express the GAP domains could restore normal budding pattern in *bem2-101* cells. As shown in Table II, *bem2-101* haploid cells exhibited a randomized budding pattern, whereas the same cells carrying pCC408 or pCC438 budded predominantly in an axial pattern, which is characteristic of wild-type haploid cells. The ∼20% of *bem2-101* cells that did not bud axially might have lost the plasmids during the growth of the cell cultures and thus did not express the GAP domains. Consistent with this idea is the observation that *bem2-101* cells carrying the plasmid pCC231, which contains the entire *BEM2*

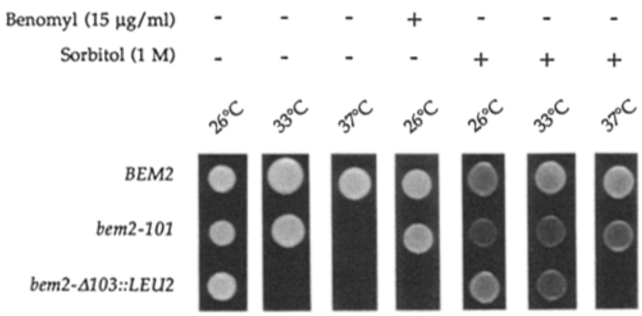

Figure 4. Growth phenotype of *bem2* mutants. Suspensions of the following yeast strains were spotted on YEPD plates that did (+) or did not (−) contain sorbitol or benomyl and allowed to grow at the indicated temperatures for two days: *BEM2* (DBY1829), *bem2-101* (CCY416-12D), and *bem2-Δ103::LEU2* (CCY374-2D).

gene, also exhibited this residual level of nonaxial budding. Thus, expression of the GAP domain from Bem2p or Bcr fully suppresses the randomized budding phenotype of *bem2-101* mutant cells.

RHO1 and RHO2 in High Copy Number Can Partially Suppress bem2 Mutations

The Bcr-GAP domain is active in vitro towards p21^{rac} and the human homolog of Cdc42p (Diekmann et al., 1991; Ridley et al., 1993), both of which belong to the Rho subfamily of Ras-related small GTP-binding proteins. Five genes encoding Rho-related small GTP-binding proteins have been identified in *S. cerevisiae*–*RHO1*, *RHO2*, *RHO3*, *RHO4*, and *CDC42* (Madaule et al., 1987; Johnson and Pringle, 1990; Matsui and Toh-e, 1992*a*). To find out whether these small GTP-binding proteins interact functionally with Bem2p in vivo, we examined the phenotype of *bem2-101* cells bearing high copy number plasmids that contain *RHO1*, *RHO2*, *RHO3*, *RHO4*, or *CDC42*. Our results showed that an increase in the dosage of *RHO1* or *RHO2*, but not *RHO3*, *RHO4*, or *CDC42*, partially suppressed the Ts⁻ growth phenotype of *bem2-101* and *bem2-Δ103::LEU2* mutants (Fig. 5, data not shown). The suppression by *RHO1* and *RHO2* is

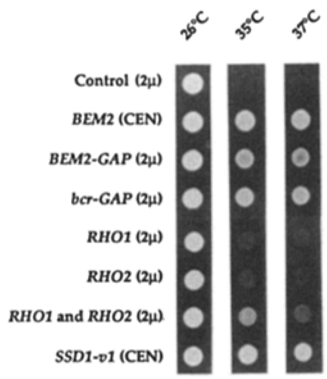

Figure 5. Growth of a *bem2-101* mutant (CCY416-12D) carrying different plasmids. Suspensions of cells carrying high copy number control 2μ-plasmid pSM217, *BEM2*-containing low copy number CEN-plasmid pCC231, *BEM2-GAP*-containing high copy number 2μ-plasmid pCC408, *bcr-GAP*-containing high copy number 2μ-plasmid pCC438, *RHO1*-containing high copy number 2μ-plasmid YEpU-RHO1, *RHO2*-containing high copy number 2μ-plasmid YEpU-RHO2, *RHO1-RHO2*-containing high copy number 2μ-plasmid pCC743, or *SSD1-v1*-containing low copy number CEN-plasmid pCC75 were spotted on YEPD plates and allowed to grow at the indicated temperatures for 2 d.

additive in that simultaneously increasing the dosage of *RHO1* and *RHO2* led to improved suppression. Furthermore, the randomized budding defect of *bem2-101* cells at 26°C was also weakly suppressed by an increase in the dosage of *RHO1* or *RHO2* (Table II). These results together suggest that Rho1p and Rho2p may interact functionally with Bem2p in vivo.

Mutations in GRR1 Can Suppress bem2 Mutations

To identify other gene products that function with Ipl2p in the regulation of cellular morphogenesis, we isolated and characterized seven extragenic suppressors of the *bem2-101* mutation. Three of these suppressor mutations confer a Cs⁻ growth phenotype at 13°C (Fig. 6). All three were found to be alleles of *GRR1* (see below), which is known to be required for: (*a*) high-affinity glucose transport (Erickson and Johnston, 1994; Vallier et al., 1994); (*b*) the repression of many yeast genes caused by the presence of glucose in the growth medium (Bailey and Woodword, 1984; Flick and Johnston, 1991; Vallier and Carlson, 1991); and (*c*) glucose-dependent divalent cation transport (Conklin et al., 1993). These three mutations suppress the Ts⁻ growth phenotype,

Table II. Budding Pattern of bem2-101 Haploid Cells Carrying Different Plasmids

		Budding pattern (%)		
Plasmid	Relevant features	Axial	Bipolar	Randomized
pG-3	2μ, *TRP1*	40	5	55
pCC408	2μ, *TRP1*, *BEM2-GAP*	79	5	16
pCC438	2μ, *TRP1*, *bcr-GAP*	81	6	13
pRS316	CEN, *URA3*	32	5	63
pCC231	CEN, *URA3*, *BEM2*	80	7	13
pCC75	CEN, *URA3*, *SSD1-v1*	70	6	24
YEp24	2μ, *URA3*	35	3	62
YEpU-RHO1	2μ, *URA3*, *RHO1*	55	4	41
YEpU-RHO2	2μ, *URA3*, *RHO2*	57	8	35
pCC743	2μ, *URA3*, *RHO1*, *RHO2*	47	4	49

bem2-101 trp1 ura3 (CCY416-12D) cells carrying the different plasmids were grown at 26°C in supplemented SD medium (with selection for *URA3* or *TRP1* present on the different plasmids) to a density of ~2×10^6 cells/ml, fixed and then stained with Calcofluor. For each sample, 200 cells with at least two bud scars were examined. In scoring the bud scar pattern, each mother cell body was divided into three equal sectors along its length. Cells with an axial budding pattern had bud scars located exclusively in one terminal sector; cells with a bipolar budding pattern had bud scars located in both terminal, but not the middle, sectors; cells with a randomized budding pattern had bud scars in the middle sector.

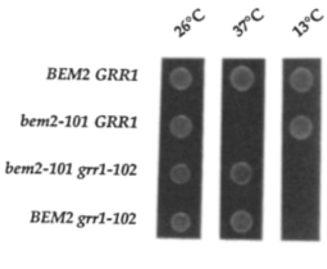

Figure 6. Suppression of the phenotype of *bem2-101* mutants by the *grr1-102* mutation. Suspensions of the following yeast strains were spotted on YEPD plates and allowed to grow at 26 or 37°C for 2 d, or at 13°C for 6 d: *BEM2 GRR1* (CCY802-11C), *bem2-101 GRR1* (CCY471-13C), *BEM2 grr1-102* (CCY487-21D), and *bem2-101 grr1-102* (CCY488-16A). Similar results were obtained with the *grr1-101* and *grr1-103* mutations.

but not the morphological defects, of *bem2-101* mutants at 37°C (Fig. 6; data not shown). At this temperature, the budding pattern of cell carrying *bem2-101* and any one of these suppressor mutations is still randomized, and these cells are often irregular in shape. However, at 26°C, bud site selection is normal in ~90% of these cells (data not shown). At this temperature, cells carrying these suppressor (*grr1*) mutations are slow-growing, elongated in shape (Fig. 7 *a*), and mildly supersensitive to the microtubule destabilizing drug benomyl (being unable to grow in the presence of 15 μg/ml of benomyl on YEPD medium). This latter phenotype is also shared by *bem2-Δ103::LEU2* mutants (Fig. 4), and it suggests that cellular morphogenesis may play a role in determining microtubule stability. The suppressor mutant phenotypes described above are recessive and are not greatly affected by the presence or absence of the *bem2-101* mutation.

A number of mutations that confer an elongated cell phenotype similar to that of the suppressor mutants have been described, including *grr1* (Flick and Johnston, 1991; Vallier and Carlson, 1991; Conklin et al., 1993), *cdc55* (Healy et al., 1991), *tpd3* (van Zyl et al., 1992), *cdc3*, *cdc10*, *cdc11*, and *cdc12* (Hartwell, 1971). Several lines of evidence indicate that the three extragenic suppressor mutations described above reside within the *GRR1* gene. First, mating of a known Cs^- *grr1::LEU2* mutant (CCY450-8A) (Flick and Johnston, 1991) with the Cs^- suppressor mutants (CCY354-1C, CCY355-4C, and CCY362-7B) generated diploids that were Cs^- for growth at 13°C (i.e., noncomplementation). Second, the Cs^- growth phenotype of one suppressor mutant tested (CCY363-1D) was complemented by a low copy number plasmid containing the *GRR1* gene. Third, tetrad analysis of a diploid strain heterozygous for the suppressor mutation (CCY363-1C × CBY1829-1) showed that the suppressor mutation is very tightly linked to *GRR1* (76 parental ditypes, 0 nonparental ditypes, 0 tetratypes). Fourth, the *grr1::LEU2* null mutation can suppress the Ts^- phenotype of *bem2-101* mutants at 37°C. A similar *grr1::URA3* null mutation (Flick and Johnston, 1991) also can suppress the Ts^- phenotype of *bem2-Δ103::LEU2* mutants 33°C. Thus, these suppressor mutations are named *grr1-101*, *grr1-102*, and *grr1-103*. Suppression of *bem2-101* Ts^- phenotype by *grr1::LEU2* and *grr1-101* is recessive, whereas that by *grr1-102* and *grr1-103* is weakly semi-dominant.

Since *GRR1* is required for the repression of many yeast genes caused by the presence of glucose in the growth medium, the loss of repression could be the reason why *grr1* mutations suppress the Ts^- growth phenotype of *bem2-101* mutants, which are typically grown on glucose-containing

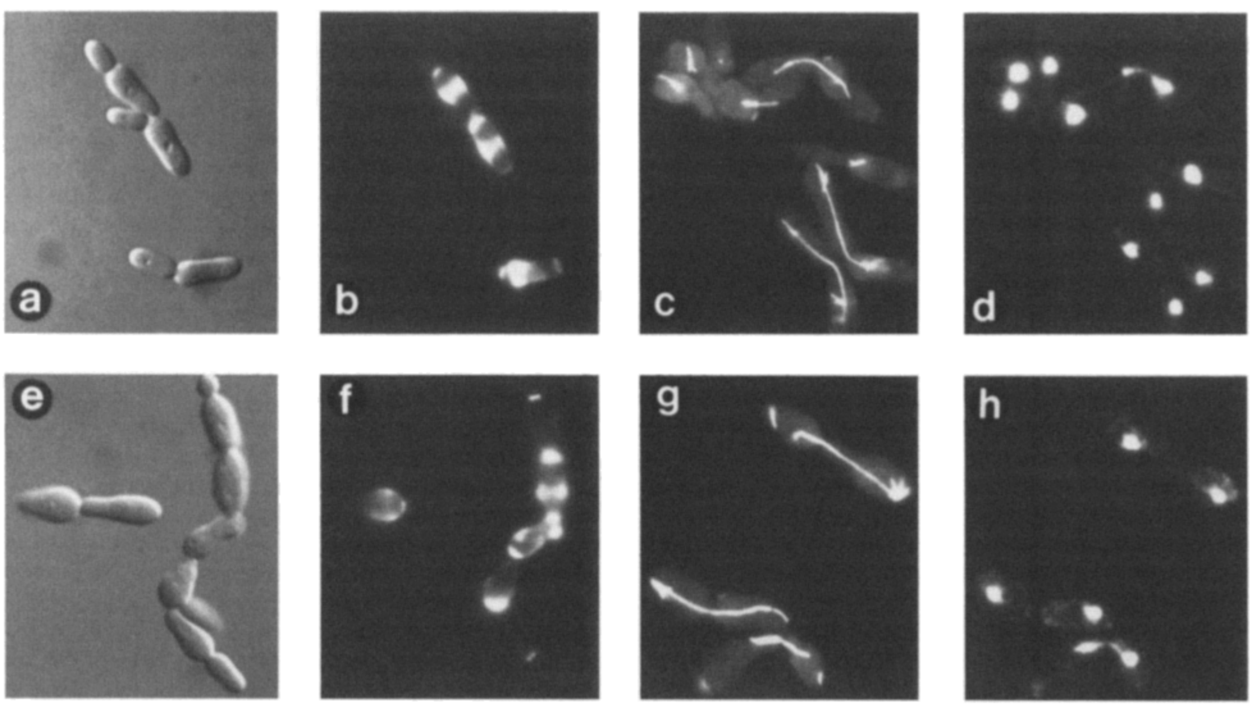

Figure 7. Cytological examination of *grr1-102* diploid mutant cells (CCY-D3). Cells grown at 26°C (*a–d*) or for 24 h at 13°C (*e–h*) were stained with Calcofluor (*b* and *f*), anti-tubulin antibodies (*c* and *g*), or DAPI (*d* and *h*). The DIC (*a* and *e*) and Calcofluor-staining images were obtained from the same cells; the anti-tubulin–staining and DAPI-staining images were from the same cells. All cells are shown at the same magnification.

YEPD medium. If this were true, we would expect conditions that lead to the derepression of glucose-repressed genes in *bem2-101 GRR1* mutants also to result in suppression of the Ts⁻ growth phenotype. We found this not to be true because *bem2-101 GRR1* mutants are still Ts⁻ on YEP medium containing glycerol, galactose, or raffinose, instead of glucose (data not shown), thus arguing against the loss of glucose repression as being the basis for suppression of *bem2-101*.

grr1 Mutants Are Defective in Chitin Localization and Cell Separation

To better understand the nature of the defect seen in *grr1* cells, we examined cytologically the *grr1-102* mutant in greater detail. At 26°C, the permissive growth temperature, budding pattern (Fig. 7, *a* and *b*, data not shown) as well as organization of the actin cytoskeleton (data not shown) and microtubules were normal (Fig. 7 *c*), but chitin localization was not. Chitin staining was not restricted to bud scars; instead, additional chitin staining was often seen, typically as a diffuse, broad band that goes around the circumference of a portion of the elongated cell (Fig. 7 *b*). After a 24-h incubation at 13°C, some *grr1-102* cells became slightly more elongated and many elongated cells became somewhat swollen at one end. About 90% of *grr1* cells appeared interconnected and could not be separated by sonication (Fig. 7 *e*). However, these cells were readily separable after the removal of cell wall material by zymolyase (Fig. 7, *g* and *h*), thus suggesting that *grr1* mutants are defective in cell separation but not cytokinesis at 13°C. The chitin delocalization defect seen at 26°C was exaggerated at 13°C, and the diffuse chitin staining appeared more patchy in some cells. This patchiness may not be apparent in Fig. 7 *f*. While the organization of the actin cytoskeleton remained normal (data not shown), the organization of microtubules was altered. Staining of microtubules by anti-tubulin antibodies became more intense; a significant fraction (∼23%) of *grr1* cells also contained microtubules that did not appear to be connected to the spindle pole body or the nucleus (Fig. 7, *g* and *h*). In spite of the observed microtubule defect, nuclear migration and division remained normal (Fig. 7 *h*).

grr1 cdc55 Double Mutants Are Inviable

CDC55 encodes a protein homologous to the regulatory B subunit of mammalian protein phosphatase 2A. Like *grr1* mutants, *cdc55* mutants are elongated at 14°C, and mutations in *BEM2* have been identified previously as extragenic suppressors of *cdc55-1* (Healy et al., 1991). Thus, we were interested in studying the functional relationship between *GRR1* and *CDC55*. To this end, we examined the consequence of simultaneous inactivation of both genes. Tetrad analysis of a diploid strain heterozygous for *grr1::LEU2* and *cdc55::URA3* (CCY469-38A × CCY469-41A) revealed that *grr1::LEU2 cdc55::URA3* double mutants are inviable at 26°C on YEPD medium. Among 36 tetrads analyzed, 4 tetrads produced 4 viable spores, all of which were Ura⁺ or Leu⁺, but not Ura⁺ Leu⁺. 22 tetrads produced 3 viable spores and 10 tetrads produced 2 viable spores. None of these viable spores were Ura⁺ Leu⁺. Among the 42 inviable spores, 38 had the inferred genotype of *grr1::LEU2 cdc55::URA3*. These results clearly show that the *grr1::LEU2* and *cdc55::URA3* mutations together produce a synthetic lethal phenotype, thus suggesting that *CDC55* and *GRR1* may be involved in the regulation of a common process (possibly one that involves protein phosphatase [2A]). We also determined whether the *bem2-101* mutation can be suppressed by other perturbations of protein phosphatase 2A activity. Our results showed that neither increased dosage of *PPH3*, *PPH21*, or *PPH22*, which encode catalytic subunits of protein phosphatase 2A (Sneddon et al., 1990; Ronne et al., 1991; Sutton et al., 1991), nor deletion of the *PPH22* gene can suppress the Ts⁻ growth phenotype of *bem2-101* mutants (data not shown).

SSD1-v1 Can Suppress *bem2* Mutations

In the initial attempt to clone the *BEM2* gene, two classes of low copy number plasmids that contain yeast genomic DNA sequences capable of complementing the Ts⁻ for growth and random budding phenotypes of *bem2-101* mutants were isolated (Fig. 5 and Table II). One class contains the bona fide *BEM2* gene; the other contains a different gene that is unrelated to *BEM2* (Chan and Botstein, 1993). Sequence analysis of this latter gene revealed that it is identical to *SSD1-v1* (also known as *SRK1*), which was identified previously as a gene that can suppress the mutant phenotypes caused by mutations in *SIT4* (Sutton et al., 1991), *INS1*, *PDE2*, *BCY1* (Wilson et al., 1991), *SLK1/BCK1/SSP31* (Costigan et al., 1992), *SLT2/MPK1* (Mazzoni et al., 1993), *CLN1*, *CLN2* (Cvrcková and Nasmyth, 1993), and *RPC53* (Chiannilkulchai et al., 1992). The *SSD1-v1* gene also can suppress the Ts⁻ growth phenotype of *bem2-Δ103::LEU2* mutants at 35 but not 37°C (data not shown). As first reported by Sutton et al. (1991), we found different laboratory yeast strains to have *SSD1-v(1)* or *ssd1-d* alleles on their chromosomes (data not shown). The molecular basis of the difference between these alleles is not known. *bem2-101 SSD1-v(1)* mutants are Ts⁺ for growth at 37°C, *bem2-101 ssd1-d* and *bem2-101 ssd1-Δ2::URA3* mutants are Ts⁻, and *bem2-101 ssd1-d* mutants carrying *SSD1-v1* on a low copy number plasmid are Ts⁺. All the *bem2* strains used in this study are presumed to carry *ssd1-d* alleles unless otherwise stated.

bem2-Δ103::LEU2 Δsit4::HIS3 SSD1-v1 Mutants Are Inviable

The *SIT4* gene encodes a protein closely related to, but not identical to, the catalytic subunit of protein phosphatase 2A (Arndt et al., 1989). Yeast cells that are simultaneously deleted for *SIT4* and *TPD3*, which encodes the regulatory A subunit of protein phosphatase 2A, are inviable even in the presence of the *SSD1-v* allele (van Zyl et al., 1992). Like *BEM2*, *SIT4* is required for bud emergence and/or growth (Fernandez-Sarabia et al., 1992). Since the *SSD1-v1* gene suppresses the Ts⁻ growth phenotype of *bem2* mutants and the inviability of *sit4*-deletion mutants (Sutton et al., 1991), we were interested in studying the functional relationship between *BEM2* and *SIT4*. Thus, we examined the consequence of simultaneously deleting *BEM2* and *SIT4* in a cell that contained an *SSD1-v1* suppressor allele. Tetrad analysis of a diploid strain (CY248 × CCY475-19A) homozygous for *SSD1-v1*, *leu2* and *his3*, and heterozygous for *bem2-Δ103::LEU2* and *Δsit4::HIS3* revealed that *bem2-Δ103::LEU2 Δsit4::HIS3 SSD1-v1* mutants are inviable at 26°C on YEPD medium. Among 46 tetrads analyzed, 10 tetrads produced 4 viable spores, all of which were His$^+$ or Leu$^+$, but not His$^+$ Leu$^+$. 25 tetrads produced 3 viable spores and 11 tetrads produced 2 viable spores. None of these viable spores were His$^+$ Leu$^+$. Among the 47 inviable spores, 45 had the inferred genotype of *bem2-Δ103::LEU2 Δsit4::HIS3 SSD1-v1*. These results clearly show that the *bem2-Δ103::LEU2* and *Δsit4::HIS3* mutations together produce a synthetic lethal phenotype, which cannot be suppressed by the *SSD1-v1* allele.

Discussion

Previous studies of *S. cerevisiae* mutants defective in cellular morphogenesis have identified two Ras-related small GTP-binding proteins, Rsr1p/Bud1p and Cdc42p, and their regulatory proteins as important components that control polarized cell growth in yeast. Here we show that this control also involves the Bem2 GTPase-activating protein, which may regulate the Rho1p and Rho2p Ras-related GTP-binding proteins in vivo.

The *BEM2* gene is required for bud site selection at 26°C and localization of cell growth to selected bud sites at 37°C. Conditional *bem2* mutants incubated at 37°C exhibit uniform cell surface growth, disorganization of the actin cytoskeleton, and they become arrested as large, round, multinucleate, unbudded cells that are osmotically fragile. The carboxyl-terminal 203 residues of the predicted Bem2 protein is homologous to sequences found in a large family of eukaryotic proteins, some of which have been shown to function in vitro as GAPs for members of the Rho-subfamily of Ras-related small GTP-binding proteins (Diekmann et al., 1991; Settleman et al., 1992*a*; Barfod et al., 1993; Hall et al., 1993; Ridley et al., 1993; Zheng et al., 1993). Bem2p most likely also functions as a GAP in vivo because *BEM2* function required for polarized cell growth (at 26°C and 37°C) can be fulfilled by simply expressing the GAP-domain of Bem2p or that of human Bcr, which is the protein most homologous to Bem2p identified so far.

In animal cells, Rho-related small GTP-binding proteins are involved in controlling the organization of the actin cytoskeleton (Hall, 1992). In *S. cerevisiae*, five genes (*CDC42*, *RHO1*, *RHO2*, *RHO3*, and *RHO4*) encoding Rho-related GTP-binding proteins have been identified (Madaule et al., 1987; Johnson and Pringle, 1990; Matsui and Toh-e, 1992*a*). The gene product of *CDC42* shares the highest degree of sequence homology with human Rac1 and Cdc42Hs, while the gene product of *RHO1* is most homologous to RhoA. The Bcr-GAP domain can function in vitro as a GAP for Rac1 and Cdc42Hs, but not RhoA (Diekmann et al., 1991; Ridley et al., 1993). Microinjection experiments also suggest that the Bcr-GAP domain can inhibit Rac1-mediated, but not RhoA-mediated, processes in fibroblasts (Ridley et al., 1993). Since Bem2p can be functionally substituted by the Bcr-GAP domain, we might expect Bem2p also to function in yeast as a GAP for Cdc42p, but perhaps not for Rho1p, Rho2p, Rho3p, or Rho4p. However, this may not be true because increased dosage of *RHO1* or *RHO2*, but not *RHO3*, *RHO4*, or *CDC42*, can partially suppress the *bem2-101* and *bem2-Δ103::LEU2* mutations. This observation can be interpreted in several ways. First, since overproduction of Rho1p or Rho2p may result in activation of the Cdc43/Ram2 geranylgeranyltransferase I (Qadota et al., 1992), this activation may somehow be responsible for the suppression of the *bem2* mutations. Second, unusually high levels of Rho1p or Rho2p may partially provide the function normally performed by another Rho-related protein whose activity is affected in *bem2* mutants. Third, Bem2p may function in vivo as a GAP for Rho1p and Rho2p. We favor this last possibility because we have preliminary results which suggest that *SSD1-v1* can suppress the Ts$^-$ growth phenotype of not only *bem2*, but also *rho1^{ts}*, mutants (our unpublished results). This interpretation is also consistent with the recent finding that the Bem2-GAP domain functions in vitro as a GAP for yeast Rho1p, but not yeast Cdc42p or human Cdc42Hs (Zheng et al., 1993, 1994; Peterson et al., 1994).

The identification of Rho1p as a potential in vivo target of Bem2p is interesting because Rho1p is believed to be concentrated to the periphery of yeast cells where cortical actin patches are clustered, and because the Ts$^-$ growth defect of *rho1-104^{ts}* mutants, like that of *bem2^{ts}* mutants, can be suppressed by the presence of 1 M sorbitol (Yamochi et al., 1994). However, the mutant phenotypes of *rho1-104* and *bem2* mutant cells are not identical. At 37°C, *rho1-104* mutants become arrested as uninucleate, tiny- or small-budded cells that are normal in size, whereas *bem2* mutants become arrested as multinucleate, unbudded cells that are enlarged. We do not know the basis for this difference, but it may be explained, as least partly, by our finding that Bem2p may also function as a GAP for Rho2p in vivo.

The apparent discrepancy between the proposed function of the Bcr-GAP domain in fibroblasts and in yeast cells may reflect functional differences that may exist between yeast Cdc42p, Rho1p, Rho2p, and their human counterparts, even though yeast Cdc42p and Rho1p can be substituted in vivo (at least partially) by human Cdc42Hs and RhoA, respectively (Munemitsu et al., 1990; Shinjo et al., 1990; Yamochi et al., 1994; Qadota et al., 1994). Alternatively, the Bcr-GAP domain, which was expressed in yeast under the control of the strong *TDH3* promoter without the amino-terminal 80% of the intact Bcr protein, might have lost its substrate specificity. This potential problem also applies to most in vitro (and in vivo) studies of GAPs, which typically utilize truncated recombinant proteins. In this context, it is interesting to note that the Bem2-GAP domain constitutes <10% of the full-length Bem2p.

If Rho1p and Rho2p are regulated by the Bem2 GAP in vivo, mutations that reduce *BEM2* function should result in Rho1p and Rho2p that are more frequently associated with GTP. According to the model commonly used to explain the functioning of Ras-related small GTP-binding proteins (Bourne et al., 1991), *bem2* mutants may have excessive *RHO1* and *RHO2* function because GTP-bound Rho1p and Rho2p would be in the activated state, and increasing the dosage of *RHO1* or *RHO2* in *bem2* mutants should result in an exacerbation of *bem2* mutant phenotypes. This prediction is precisely opposite to what we observed. Thus, association with GTP may be insufficient for the functioning of Rho1p and Rho2p. In addition, controlled cycling between the GTP- and GDP-bound states may be important, as proposed for the Sec4 and Sar1 GTP-binding proteins (Walworth et al., 1989, 1992; Oka and Nakano, 1994). In this model, increasing the dosage of *RHO1* or *RHO2* in *bem2* mutants would result in an increased amount of Rho1p or Rho2p that is GTP bound, which would then lead to increased cycling between the GTP- and GDP-bound states due to the intrinsic GTPase activity of these proteins. In fact, an increase in the dosage of

SAR1 is known to result in a partial suppression of the Ts⁻ growth defect of *sec23-1* mutants (Oka and Nakano, 1994), which carry a defective GAP for Sar1p (Yoshihisa et al., 1993). In principle, Bem2p may also function as an effector of Rho1p and Rho2p. However, since overproduction of Rho1p or Rho2p can partially suppress the Ts⁻ phenotype caused by a deletion of *BEM2*, Bem2p cannot be the only effector of these proteins.

The mechanisms by which most Ras-related small GTP-binding proteins transduce signals to downstream components are not known. In fibroblasts, Ras-mediated mitogenic signaling in response to various growth factors appears to involve upstream phosphorylation events that lead to the complexing of activated Ras with the Raf protein kinase, which in turn activates the MEK and MAP protein kinases (for review, see Crews and Erikson, 1993). The latter protein kinase can be dephosphorylated and inactivated by the MKP-1/PAC1 protein phosphatase, resulting in termination of mitogenic signaling (Sun et al., 1993; Zheng and Guan, 1993; Ward et al., 1994). Recently, two protein kinases that can bind to GTP-bound human Cdc42Hs or Rac1 have been identified (Manser et al., 1993, 1994). They may function as in vivo targets of Cdc42Hs and Rac1. The Bcr protein is also known to have protein kinase activity in vitro (Maru and Witte, 1991). While proteins that clearly function upstream or downstream of Bem2p, Rho1p, or Rho2p have not been identified, the genetic interactions summarized in Fig. 8 suggests that protein phosphorylation or dephosphorylation may also play an important role in the *BEM2*-mediated process.

TPD3 and *CDC55* encode the regulatory A and B subunit of yeast protein phosphatase 2A, respectively. Cells lacking *TPD3* or *CDC55* are cold-sensitive for growth; these cells are elongated in shape and defective in cell separation at reduced temperatures (Healy et al., 1991; van Zyl et al., 1992). In addition, *cdc55* mutants are known to exhibit delocalized cell surface chitin deposition at the restrictive temperature (Healy et al., 1991), and they are especially proficient in undergoing pseudohyphal differentiation in response to nitrogen starvation (Blacketer et al., 1993). The growth phenotype of *cdc55* mutants can be suppressed by mutations in *BEM2* (Healy et al., 1991). Here we show that the Ts⁻ growth phenotype of *bem2-101* mutants can be suppressed by mutations in *GRR1*. *grr1* mutants have Cs⁻ and morphological phenotypes similar to those of *tpd3* and *cdc55* mutants. Yeast cells lacking both *GRR1* and *CDC55* exhibit a synthetic lethal phenotype. Both Grr1p and Tpd3p contain tandem repeats that are similar in being leucine and isoleucine rich (Flick and Johnston, 1991; van Zyl et al., 1992). This combination of genetic interactions, mutant phenotypes, and sequence similarities observed among *BEM2*, *CDC55*, *GRR1*, and *TPD3* suggest that *GRR1* may also be involved (directly or indirectly) in the regulation of protein phosphatase (2A) activity. In this context, it is interesting to note that yeast cells overexpressing *PPH22*, which encodes a catalytic subunit of protein phosphatase 2A, are elongated in shape (Ronne et al., 1991).

SIT4 encodes a protein closely related, but not identical, to the catalytic subunit of protein phosphatase 2A (Arndt et al., 1989). It is required for bud emergence and/or growth and *SWI4*-mediated accumulation of G_1 cyclin RNAs (Fernandez-Sarabia et al., 1992). Yeast cells lacking both *SIT4* and *TPD3* exhibit a synthetic lethal phenotype (van Zyl et al., 1992). *BEM2* and *SIT4* are related genetically in two ways. First, the *SSD1-v1* gene suppresses the Ts⁻ growth phenotype of *bem2* mutants and the inviability of *sit4*-deletion mutants (Sutton et al., 1991). Second, yeast cells lacking both *BEM2* and *SIT4* exhibit a synthetic lethal phenotype even in the presence of the *SSD1-v1* suppressor. The *SSD1* gene product is homologous in sequence to the Dis3 protein of *Schizosaccharomyces pombe*. Cells mutated simultaneously in *dis3⁺* and *dis2⁺*, which encodes a catalytic subunit of protein phosphatase 1, exhibit a synthetic lethal phenotype (Kinoshita et al., 1991). The *dis3⁺* gene can also function as a dosage-dependent suppressor of *S. pombe ppe1⁻* mutants, which are defective in cell shape control due to a defective Sit4-related protein phosphatase (Shimanuki et al., 1993). These observations together suggest that the *SSD1* (and *dis3⁺*) gene product may be involved in the regulation of protein phosphatase activity. This is an idea that has been proposed previously (Sutton et al., 1991; Wilson et al., 1991) and is consistent with the observation that *SSD1-v1* can suppress mutations in many genes (*SIT4*, *PDE2*, *BCY1*, *SLK1/BCK1/SSP31*, *SLT2/MPK1*, *CLN1*, and *CLN2*) that encode proteins involved in (the control of) protein kinase or phosphatase function.

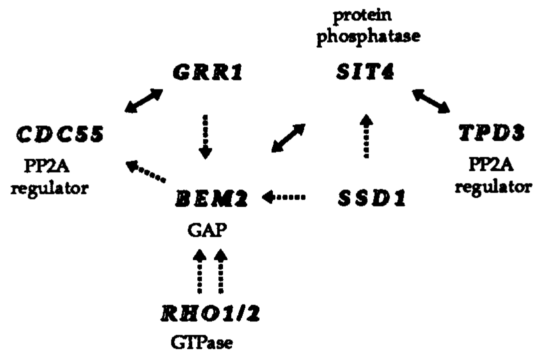

Figure 8. Summary of observed genetic interactions. Synthetic lethal relationship revealed by simultaneous deletion of two genes is depicted by a solid line. Suppression of mutation in one gene (near *arrowhead*) by mutation in a second gene or increased dosage of a second gene is depicted by a single broken line or two broken lines, respectively.

While we do not know the molecular mechanisms underlying the genetic interactions outlined in Fig. 8, we believe that these interactions all point towards a likely role for protein phosphorylation or dephosphorylation in the *BEM2*-mediated pathway or one that functionally overlaps with this pathway. Mutations that inactivate regulatory subunits of protein phosphatases may result in increases or decreases in phosphatase activities towards different substrates (reviewed in Mumby and Walter, 1993) that can be compensated by appropriate changes in protein kinase activities. Since both *SSD1-v1* and *grr1* can suppress the *bem2*-null mutation, the postulated phosphorylation or dephosphorylation event probably does not occur upstream of Bem2p. Instead, it probably occurs downstream or in a parallel pathway with overlapping functions. In one simple model, Bem2p may directly or indirectly control the activity of a protein phosphatase (or kinase) or a protein whose function requires appropriate phosphorylation or dephosphorylation. In this context, it is interesting to note that mutational inactivation of components of the Pkc1/Stt1, Bck1/Slk1/Ssp31, Mkk1, Mkk2, and Mpk1/Slt2 protein kinase cascade results in Ts⁻ growth defects that can be suppressed by osmotic stabilizing agents and, at least in some cases, by the *SSD1-v1* allele (reviewed in Errede and Levin, 1993). These properties are very similar to those of *bem2* mutants. Further identification of other proteins that function in the *BEM2*-mediated process should help to elucidate the relationship between these signaling proteins.

We would like to thank Louis Lim for bringing our attention to the identity between Ipl2p and Bem2p; Alan Bender, and Yoshi Ohya for communication of results before publication; David Drubin, Annette Healy, Yoshi Ohya, Mark Johnston, Jerry Adams, Yasushi Matsui, and Kim Arndt for supply of antibodies, strains, and plasmids; Brian Haarer, David Drubin, and Alison Adams for comments on the manuscript.

This work was supported by a National Institutes of Health Grant (GM45185) and an Advanced Research Program grant (003658-510) from The Texas Higher Education Coordinating Board.

Received for publication 15 June 1994 and in revised form 8 September 1994.

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