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 SSD1-1 (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 SSD1-1 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/a 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 presum-
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, un budded cells that in most cases have also been shown to exhibit delocalized cell surface growth. CDC42 encodes a Rho-related small GTP-binding protein (Johnston 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 (pfl1) 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') 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**, **cdd55**, **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.

**Materials and Methods**

**Strains, Media, and Genetic Methods**

Yeast strains used in this study are listed in Table I. The strain CYB1829-1 was constructed by integrating, via homologous recombination, the **URA3**-plasmid pCC705 into the chromosome. 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 **SP12** locus (Chan and Botstein, 1993). The diploid strain CYB1830-30 was constructed - a one-step gene disruption procedure (Rothstein, 1983), replacing one of the **BEM2** genes in DBY1830 with the **bem2-ΔΔ3-LEU2** allele present on pCC94. This disruption was confirmed by DNA hybridization with an appropriate probe. The Escherichia coli strain DB1412 (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 × 10^9 **bem2-101** cells (CCY109-9C-1 or CCY109-ID-1) per plate, and incubating for 3-5 d at 37°C. To ensure that each revertant isolated was independent, cells from independent colonies were restreaked twice and each revertant was independently 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 **SP12** 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 low plasmid pRS316 (Sikorski and Hieter, 1989). pCC94, 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'-AGC-CAACGATCTGGGATCCAAATGCAACTACACGACATTA-3' (the mutagenic base is underlined). This generated pCC93. The sequence between the XbaI and BamHI sites of pCC93 was then replaced with the ~2-kb XbaI/BamHI fragment (containing LEU2) of pJL283 (Jones and Prakash, 1990), generating pCC94. Codons 115-2144 of **BEM2** are missing from the **bem2-ΔΔ3-LEU2** mutant allele present on this plasmid. Plasmid pCC705, used for integration of **URA3** into the genome at the **GRR6** locus, was constructed by cloning the ~3.8-kb BglII fragment (containing part of **GRR6**) of pBMY1270 (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'-CGATAAGCTTGGATCCAAATGCAACTACACGACATTA-3') and IPL2-3p (5'-TGGCCGATCTGGGATCCAAATGCAACTACACGACATTA-3'). The PCR product, which contained BamHI sites near both ends, was cleaved with BamHI and then cloned into the BamHI site of pg3 (Scheren et al., 1991). The resulting plasmid pCC408 allows expression of the COOH-terminal 247 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 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 287 residues of Bem2p under the control of the strong **TDH3** promoter.

1. **Abbreviations used in this paper:** Cs', cold-sensitive; GAP, GTPase-activating protein; Ts', temperature-resistant; Ts', temperature-sensitive.
Table 1. Yeast Strains Used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY1829</td>
<td>α lys2-801 his3Δ200 ura3-52 leu2-3,112 trpl-1</td>
</tr>
<tr>
<td>DBY1830</td>
<td>a/a ade2/+ lys2-801/+ his3Δ200/his3Δ200 ura3-52/ura3-52 leu2-3,112/trpl-1/trpl-1</td>
</tr>
<tr>
<td>CBY1829-1</td>
<td>α lys2-801 his3Δ200 ura3-52 leu2-3,112 trpl-1 URA3 (at GRR1)</td>
</tr>
<tr>
<td>CBY1830-30</td>
<td>a/a ade2/+ lys2-801/+ his3Δ200/his3Δ200 ura3-52/ura3-52 leu2-3,112/trpl-1/trpl-1</td>
</tr>
<tr>
<td>CCY-D1</td>
<td>a/a ade2/+ lys2-801/+ his3Δ200/his3Δ200 ura3-52/ura3-52 leu2-3,112/trpl-1/trpl-1</td>
</tr>
<tr>
<td>CCY-D3</td>
<td>a/a ade2/ade2 his3Δ200/his3Δ200 ura3-52/ura3-52 leu2-3,112/trpl-1/trpl-1</td>
</tr>
</tbody>
</table>

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). Strain CY248 was obtained from Y. Matsui (University of Tokyo, Tokyo, Japan) (Matsui and Qadota, 1992a,b). Strain CCY374-2D was obtained from Y. Ohya (University of Tokyo, Tokyo, Japan) (Qadota et al., 1992). The high copy number plasmid pOPR3 and pOPR4, containing RHO1, respectively, were obtained from J. Pringle's laboratory (University of North Carolina, Chapel Hill, NC). It contains a replacement of the BglII/BamHI DNA fragment representing the 5' half of CDC53 with a DNA fragment containing URA3. The SSO-1/vl allele present in CCY475-19A was derived from CY248.

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 theipl2-1 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, un budded 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. 3). 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 purified rabbit anti-actin antibodies (gift of David Drubin) and affinity purified FITC-conjugated goat anti-rat secondary antibodies anti-α-tubulin mAb YOL1/34 (Bioproducts for Science, Indianapolis, IN) and Polarized Cell Growth

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-rabbit secondary antibodies (Organon Teknika Corp., West Chester, PA). Actin was stained with affinity purified rabbit anti-actin antibody (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).
Bern2-101 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 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.
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+ spore (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; Harihar 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., 1992b), 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., 1993; Settleman et al., 1992a, b; Barfod et al., 1993; Choi et al., 1993; 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 carboxy-terminal 287 residues of Bem2p (containing the putative GAP domain) or the carboxy-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-
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.
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 carrying pCC408 or pCC438. Since plasmids (pCC408 and pCC438) that express the GAP domains could restore normal budding pattern in bem2-101 cells, the data are consistent with this idea that the expression of the Bem2-GAP domain is sufficient for suppression of the Ts growth phenotype of bem2 mutants.
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-AIO3::LEU2 (CCY74-2D).

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.

RH01 and RH02 in High Copy Number Can Partially Suppress bem2 Mutations

The Bcr-GAP domain is active in vitro towards p21ras 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—RH01, RH02, RH03, RH04, and CDC42 (Madaule et al., 1987; Johnson and Pringle, 1990; Matsui and Toh-e, 1992a). 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, RH02, RH03, RH04, or CDC42. Our results showed that an increase in the dosage of RH01 or RH02, but not RH03, RH04, or CDC42, partially suppressed the Ts− growth phenotype of bem2-101 and bem2-AIO3::LEU2 mutants (Fig. 5, data not shown). The suppression by RH01 and RH02 is additive in that simultaneously increasing the dosage of RH01 and RH02 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 RH01 or RH02 (Table II). These results together suggest that Rholp and Rho2p may interact functionally with Bem2p in vivo.

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant features</th>
<th>Axial (%)</th>
<th>Bipolar (%)</th>
<th>Randomized (%)</th>
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<tbody>
<tr>
<td>pG-3</td>
<td>2μ, TRP1</td>
<td>40</td>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>pCC408</td>
<td>2μ, TRP1, BEM2-GAP</td>
<td>79</td>
<td>5</td>
<td>34</td>
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<tr>
<td>pCC438</td>
<td>2μ, TRP1, bcr-GAP</td>
<td>81</td>
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<tr>
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<td>32</td>
<td>5</td>
<td>63</td>
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<tr>
<td>pCC231</td>
<td>CEN, URA3, BEM2</td>
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<td>13</td>
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<tr>
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<td>CEN, URA3, SSD1-v1</td>
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<tr>
<td>YE124</td>
<td>2μ, URA3</td>
<td>35</td>
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<td>pCC743</td>
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<td>49</td>
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</table>

bern2-101 trpl 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 grrl-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 grrl-102 (CCY487-21D), and bem2-101 grrl-102 (CCY488-16A). Similar results were obtained with the grrl-101 and grrl-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 (grrl) 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-A103::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 grrl (Flick and Johnston, 1991; Vallier and Carlson, 1991; Conklin et al., 1993), cdc55 (Healy et al., 1991), ipd3 (van Zyl et al., 1992), cdc3, cdc10, cdc1l, 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 grrl::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 grrl::LEU2 null mutation can suppress the Ts phenotype of bem2-101 mutants at 37°C. A similar grrl::URA3 null mutation (Flick and Johnston, 1991) also can suppress the Ts phenotype of bem2-103::LEU2 mutants 33°C. Thus, these suppressor mutations are named grrl-101, grrl-102, and grrl-103. Suppression of bem2-101 Ts phenotype by grrl::LEU2 and grrl-101 is recessive, whereas that by grrl-102 and grrl-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 grrl mutations suppress the Ts growth phenotype of bem2-101 mutants, which are typically grown on glucose-containing
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.

**grrl Mutants Are Defective in Chitin Localization and Cell Separation**

To better understand the nature of the defect seen in grrl cells, we examined cytologically the grrl-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 diffusible 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 grrl-102 cells became slightly more elongated and many elongated cells became somewhat swollen at one end. About 90% of grrl 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 zymolase (Fig. 7, g and h), thus suggesting that grrl 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 grrl 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).

**grrl cdc55 Double Mutants Are Inviable**

CDC55 encodes a protein homologous to the regulatory B subunit of mammalian protein phosphatase 2A. Like grrl 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 grrl::LEU2 and cdc55::URA3 (CCY469-3A × CCY469-4A) revealed that grrl::LEU2 cdc55::URA3 double mutants are inviable at 26°C on YEPA medium. Among 36 tetrad analyzed, 4 tetrad produced viable spores, all of which were Ura+ or Leu+, but not Ura+ Leu+. 22 tetrad produced 3 viable spores and 10 tetrad produced 2 viable spores. None of these viable spores were Ura+ Leu+. Among the 42 inviable spores, 38 had the inferred genotype of grrl::LEU2 cdc55::URA3. These results clearly show that the grrl::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).

**SSDI-vl 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 SSDI-vl (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), INSI, PDE2, BCY1 (Wilson et al., 1991), SLK1/BCK1/SPP31 (Costigan et al., 1992), SLT2/MPK1 (Mazzoni et al., 1993), CLNI, CLN2 (Cvrcková and Nasmyth, 1993), and RPC33 (Chiannilkulchai et al., 1992). The SSDI-vl 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 SSDI-vl(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 SSDI-vl mutants are Ts+ for growth at 37°C, bem2-101 ssd1-d and bem2-101 ssd1-d Δ2::URA3 mutants are Ts+, and bem2-101 ssd1-d mutants carrying SSDI-vl 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 SSDI-vl 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 SSDI-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 SSDI-vl 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 SSDI-v suppressor allele. Tetrad analysis of a diploid strain (CY248 × CCY475-19A) homozygous for SSDI-vl, leu2 and his3, and heterozygous for bem2-Δ103::LEU2 and Δsit4::HIS3 revealed that bem2-Δ103::LEU2 Δsit4::HIS3 SSDI-vl mutants are inviable at 26°C on YEPD medium. Among 46 tetrad analyzed, 10 tetrad pro-
duced 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 \( \text{bem2-} \Delta 03:: \text{LEU2 and } \Delta 4:: \text{HIS3 SSD1-vI} \). These results clearly show that the \( \text{bem2-} \Delta 03:: \text{LEU2 and } \Delta 4:: \text{HIS3 SSD1-vI} \) mutations together produce a synthetic lethal phenotype, which cannot be suppressed by the SSD1-vI allele.

**Discussion**

Previous studies of *S. cerevisiae* mutants defective in cellular morphogenesis have identified two Ras-related small GTP-binding proteins, Raslp/Budlp 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 GTP-activating protein, which may regulate the Rholp 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, un budded cells that are osmotically fragile. The carboxy-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 hydrophobic subfamily of Ras-related small GTP-binding proteins (Diekmann et al., 1991; Settleman et al., 1992a; 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, Ras-related small GTP-binding proteins are involved in controlling the organization of the actin cytoskeleton (Hall, 1992). In *S. cerevisiae*, five genes (*CDC42, RH01, RH02, RH03, and RH04*) encoding Ras-related GTP-binding proteins have been identified (Madalena et al., 1987; Johnson and Pringle, 1990; Matsui and Toh-e, 1992a). The gene product of *CDC42* shares the highest degree of sequence homology with human Ral and Cdc42Hs, while the gene product of *RH01* is most homologous to RhoA. The Bcr-GAP domain can function in vitro as a GAP for Ral1 and Cdc42Hs, but not RhoA (Diekmann et al., 1991; Ridley et al., 1993). Microinjection experiments also suggest that the Bcr-GAP domain can inhibit RalC-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 Rholp, Rho2p, Rho3p, or Rho4p. However, this may not be true because increased dosage of *RH01* or *RH02*, but not *RH03*, *RH04*, or *CDC42*, can partially suppress the *bem2-101* and *bem2-}\( \Delta 03:: \text{LEU2} \) mutations. This observation can be interpreted in several ways. First, since overproduction of Rholp or Rho2p may result in activation of the Cdc43/Ram2 geranylgeranytransferase I (Qadota et al., 1992), this activation may somehow be responsible for the suppression of the *bem2* mutations. Second, unusually high levels of Rholp or Rho2p may partially provide the function normally performed by another Ras-related protein whose activity is affected in *bem2* mutants. Third, Bem2p may function in vivo as a GAP for Rholp and Rho2p. We favor this last possibility because we have preliminary results which suggest that SSD1-vI can suppress the Ts- growth phenotype of not only *bem2*, but also *rho1*, 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 Rhol, but not yeast Cdc42p or human Cdc42Hs (Zheng et al., 1993, 1994; Peterson et al., 1994).

The identification of Rholp as a potential in vivo target of Bem2p is interesting because Rholp 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* mutants, like that of *bem2* 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, un budded cells that are enlarged. We do not know the basis for this difference, but it may be explained, at 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, Rholp, Rho2p, and their human counterparts, even though yeast Cdc42p and Rholp 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 Rholp and Rho2p are regulated by the Bem2 GAP in vivo, mutations that reduce *BEM2* function should result in Rholp 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 *RH01* and *RH02* function because GTP-bound Rholp and Rho2p would be in the activated state, and increasing the dosage of *RH01* or *RH02* 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 Rholp and Rho2p. In addition, controlled cycling between the GTP- and GDP-bound states may be important, as proposed for the Sec4 and Sarl GTP-binding proteins (Walworth et al., 1989, 1992; Oka and Nakano, 1994). In this model, increasing the dosage of *RH01* or *RH02* in *bem2* mutants would result in an increased amount of Rholp 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
SARI 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 Sarlp (Yoshihisa et al., 1993). In principle, Bem2p may also function as an effector of Rholp and Rho2p. However, since overproduction of Rholp 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 Racl have been identified (Manser et al., 1993, 1994). They may function as in vivo targets of Cdc42Hs and Racl. 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, Rholp, or Rho2p have not been identified, the genetic interactions summarized in Fig. 8 suggest 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 Tdp3p 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 PPPH22, 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. 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 SSDL-vl 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 SSDL-vl suppressor. The SSDL 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 pep1- mutants, which are defective in cell shape control due to a defective Slt4-related protein phosphatase (Shimizu et al., 1993). These observations together suggest that the SSDL (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 SSDL-vl can suppress mutations in many genes (SIT4, PDE2, BCY1, SIKI/BCK1/SSP31, SLT2/MPK1, CLN1, and CLN2) that encode proteins involved in (the control of) protein kinase or phosphatase function.

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 Mummy and Walter, 1993) that can be compensated by appropriate changes in protein kinase activities. Since both SSDL-vl 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/Slt1, Bck1/Slk1/Ssp31, Mkk1, Mkk2, and Mpk1/Slt2 protein kinase cascade results in Ts-growth defects that can be suppressed by osmotic stabilizing conditions.

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.

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agents and, at least in some cases, by the SSD1-v 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.

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