The *rho*-GAP Encoded by *BEM2* Regulates Cytoskeletal Structure in Budding Yeast

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Microfilaments are required for polarized growth and morphogenesis in *Saccharomyces cerevisiae*. To accomplish this, actin cables and patches are redistributed during the cell cycle to direct secretory components to appropriate sites for cell growth. A major component of actin cables is tropomyosin I, encoded by *TPM1*, that determines or stabilizes these structures. Disruption of *TPM1* is not lethal but results in the loss of actin cables and confers a partial defect in polarized secretion. Using a synthetic lethal screen, we have identified seven mutations residing in six genes whose products are required in the absence of Tpm1p. Each mutant exhibited a morphological defect, suggesting a functional link to the actin cytoskeleton. Complementation cloning of one mutation revealed that it lies in *BEM2*, which encodes a GTPase-activating protein for the *RHO1* product. *bem2* mutations also show synthetic lethality with *rho1* and mutations in certain other cytoskeletal genes (*ACT1*, *MYO1*, *MYO2*, and *SAC6*) but not with mutations in several noncytoskeletal genes. These data therefore provide a genetic link between the GAP encoded by *BEM2* and the functional organization of microfilaments. In addition, we show that *bem2* mutations confer benomyl sensitivity and have abnormal microtubule arrays, suggesting that the *BEM2* product may also be involved directly or indirectly in regulating microtubule function.

INTRODUCTION

Microfilaments are components of all eukaryotic cells that participate in the determination of cell shape, cell movement, cytokinesis, phagocytosis, secretion, and other basic cellular functions (Stossel *et al.*, 1985, 1989; Pollard and Cooper, 1986; Bretscher, 1991; Luna and Hitt, 1992). To understand the precise contributions of microfilaments, it is necessary to both identify and characterize their components and elucidate how their organization is regulated. Budding yeast contains a single essential actin gene *ACT1* (Shortle *et al.*, 1982), making it an amenable organism for a combined genetic, biochemical, and cell biological analysis of microfilament structure, regulation, and function (Barnes *et al.*, 1990; Drubin, 1991; Huffaker and Bretscher, 1991; Solomon, 1991; Bretscher *et al.*, 1994; Welch *et al.*, 1994). Actin filaments in yeast are present as cables and cortical patches. During bud growth, the cables extend into the bud where cortical patches are concen-

trated. Thus, the distribution of actin is polarized and cell cycle regulated, suggesting an involvement of actin in directed cell growth (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Lew and Reed, 1993).

Among the several proteins known to associate with F-actin in yeast are tropomyosins. Tropomyosins are highly α -helical coiled-coil dimeric molecules that associate in a head-to-tail fashion and bind along the grooves of the actin filament. Although the function of tropomyosin in striated muscle is well understood (Ebashi *et al.*, 1969), its function and the significance of its diversity in smooth muscle and nonmuscle cells are complicated by cell-specific differential gene expression and alternate RNA splicing (reviewed in Lees-miller and Helfman, 1991). Tropomyosins in yeast are encoded by two genes, *TPM1* and *TPM2*, which provide an essential function (Liu and Bretscher, 1989a,b; Drees *et al.*, 1995). Disruption of the *TPM1* gene is not lethal, but results in a heterogeneous cell population with an overall growth rate about one-half that of isogenic wild-type cells. Moreover, Tpm1p specifically associates with actin cables in wild-type cells, struc-

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tures that are not detectable in cells lacking Tpm1p. We have therefore proposed that Tpm1p is important for the determination or stability of actin cables in yeast (Liu and Bretscher, 1989a). To extend our search for structural and regulatory components of Tpm1p-containing microfilaments, we have employed a genetic approach. Here we describe the use of a synthetic lethal screen to identify mutations that render the *TPM1* gene product essential. These mutations are likely to affect proteins that function in the same or parallel pathway as Tpm1p. Synthetic lethality screens have been successfully used for the identification of proteins important for morphogenesis and for the identification of novel components of microfilaments in yeast (e.g. Bender and Pringle, 1991; Holtzman *et al.*, 1993; Karpova *et al.*, 1993).

Recently, it has been shown that the structure of the cortical actin cytoskeleton of vertebrate cells is the target of *rho* GTPase-mediated signal transduction pathways. A number of multifunctional proteins have been identified that stimulate the intrinsic low GTPase activity of *rho* family members (for reviews, see Hall, 1994; Lamarche and Hall, 1994). Likewise in yeast, genes encoding *rho*-GTPases (*CDC42*, *RHO1*, *RHO2*, *RHO3*, and *RHO4*) have been proposed to regulate cellular morphogenesis including bud emergence and growth (Madaule *et al.*, 1987; Adams *et al.*, 1990; Johnson and Pringle, 1990; Matsui and Toh-e, 1992a,b; Yamochi *et al.*, 1994). However, biochemical or genetic data has not yet linked these signaling pathways to specific cytoskeletal effectors. Here we describe identification of mutations in a number of genes that render the Tpm1p essential. One of these mutations lies in *BEM2*, a gene that encodes a GTPase-activating protein (GAP) for the *RHO1* gene product (Kim *et al.*, 1994; Peterson *et al.*, 1994; Zheng *et al.*, 1994). Furthermore, we show that *bem2* mutations are synthetically lethal with mutations in certain other genes specifying microfilament components, thereby placing this *rho*-GAP in a signaling pathway for the regulation of specific microfilament structures. Additionally, we present evidence to suggest that Bem2p influences microtubule function in yeast.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains used in this study are listed in Table 1. Yeast media are as described by Sherman (1991). Transformation of yeast cells was performed using lithium acetate or electroporation procedures (Ito *et al.*, 1983; Meilhoc *et al.*, 1990).

Plasmid Constructions

Plasmid pDS1-TPM1 contains the *TPM1*, *URA3*, and *ADE3* genes. The original plasmid pDK221 (Koshland *et al.*, 1985) has a mutated *CEN3/ARS1*, which allows the vector to be lost at high frequency. pDS1 was constructed by replacing the *LEU2* of pDK221 with *amp^R* and *URA3* genes. Specifically, an *EcoRI-Sall* fragment containing

ADE3, *CEN3*, and the mutated *ARS1* was isolated from pDK221 and ligated to the *EcoRI-Sall* sites of YIp5 (Struhl *et al.*, 1979). The *tet^R* gene of YIp5 was also disrupted (Don Sullivan, personal communication). To construct the pDS1-TPM1, primers containing the *Sall* linker that hybridize to -233 bp to -212 bp and 727 bp to 743 bp regions of the *TPM1* gene (Liu and Bretscher, 1989a) were used for PCR amplification from genomic DNA; this introduces *Sall* sites at -239 bp and 244 bp, respectively. The *Sall* fragment that flanks the *TPM1* gene was then inserted into pDS1. The expression of Tpm1p protein was confirmed by immunoblotting and complementation of defects associated with *tpm1Δ*.

Plasmid pSL001 was isolated from a genomic library in a centromeric plasmid (Rose *et al.*, 1987) by complementation of the defects of *tsl1-1* (*bem2-10*). The pSL002, pSL003, pSL008, pSL009, and pSL010 represent different and overlapping regions of the genomic fragment in pSL001 and were subcloned into pRS316 (Sikorski and Hieter, 1989). pTW003 is the *BEM2* disruption plasmid and was constructed as follows: the 0.9-kb *XbaI-BamHI* fragment of *BEM2* was subcloned into the *XbaI-BamHI* site of YIp352, an integrating plasmid that contains the *LEU2* marker (Hill *et al.*, 1986). The 1.0-kb *HindIII-XbaI* fragment of *BEM2* was then inserted into the *XbaI-HindIII* site.

Tetrad Analyses of *Tsl⁻* Mutants

To determine whether the 65 *Tsl⁻* mutants contained single mutations, the *Tsl⁻ tpm1Δ/pDS1-TPM1* haploid cells were crossed to parental *tpm1Δ* cells (ABY204 or ABY205). Sporulation and tetrad analyses were done using standard methods (Sherman, 1991), and at least 12 tetrads were analyzed for each cross. If the mutant carries a single gene mutation, two spores should inherit the *Tsl⁻* mutation and therefore be either dead or give rise to a red colony (if carrying pDS1-TPM1) and two should be *Tsl⁺* and give rise to white or sector (if carrying pDS1-TPM1) colonies. Mutations in two or more genes would give rise to more white and *Sect⁺* spores. The *Sect⁻* spores (*Tsl⁻ tpm1Δ/pDS1-TPM1*) with potential single gene mutation derived from the above crosses were backcrossed again to ABY204 or ABY205, and their growth phenotypes were followed subsequently. In 10 to 14 tetrads analyzed, the abnormal morphology and corresponding growth phenotypes (such as *Ts⁻*, *Cs⁻*, and osmotic sensitivity) cosegregated with the *tsl* mutation.

Isolation of *tsl* Mutations

Each single *tsl* mutation (except for *tsl2-1*) was isolated by backcrossing the *tsl tpm1Δ::LEU2/pDS1-TPM1* mutant to a wild-type *TSL TPM1* haploid strain (ABY165 or ABY164 depending upon the mating type). Sporulation of the resulting diploid and analyses of the meiotic products yielded the desired *tsl TPM1* strain by following the *Leu⁻ Ura⁻* spores, which are either *TSL TPM1* or *tsl TPM1*. These two types of spores were easily distinguished by the growth phenotype as well as the abnormal morphology conferred by *tsl* mutations. Each *tsl TPM1* strain was confirmed to carry *TPM1* by immunoblotting.

Complementation Analyses between *tsl* Mutants and *tpm2Δ*, *myo2-66*, *anc4-1*, *act1-4*, *myo1::LEU2*, and *sac6::URA3*

To test whether any of the *tsl* mutants were alleles of *TPM2*, *MYO2*, or *ANC4*, each mutant was crossed to *tpm2Δ::HIS3* (ABY439), *myo2-66* (*Ts⁻*, ABY117), or *anc4-1* (*Ts⁻*, ABY244) haploid cells. *tsl/+ myo2-66/+* and *tsl/+ anc4-1/+* are *Ts⁺*; *tsl/+ tpm2Δ/+* gave normal cell morphology, suggesting that these mutations do not reside in *TPM2*, *MYO2*, or *ANC4*. To further test whether *tsl* mutations reside in *ACT1*, *SAC6*, or *MYO1*, combinations of diploids were made by crossing each *tsl* mutant to *act1-4* (ABY104), *sac6::URA3* (ABY111), or *myo1::LEU2* (ABY187). Because the resulting diploids exhibit normal cell morphology whereas the parental

Table 1. Yeast strains used in this study

Strain	Genotype	Source
ABY173	MAT α <i>ade2-101/ + his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ ura3-52 lys2-801/ + trp1-am/+</i>	T. Huffaker (Cornell University, Ithaca, NY)
ABY164	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys2-801</i>	T. Huffaker
ABY165	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys2-801 ade2-101</i>	T. Huffaker
ABY205	MAT α <i>ura3-52 leu2-3,112 ade2-101 ade3 tpm1Δ::LEU2</i>	This study
ABY204	MAT α <i>ura3-52 leu2-3,112 ade2-101 ade3 tpm1Δ::LEU2</i>	This study
ABY248	MAT α <i>tsl1-1 ura3-52 leu2-3,112 1 ade2-101 ade3</i>	This study
ABY249	MAT α <i>tsl1-1 ura3-52 leu2-3,112 lys2-801 ade2-101 ade3</i>	This study
ABY250	MAT α <i>tsl2-2 ura3-52 leu2-3,112 lys2-801 ade2-101 ade3</i>	This study
ABY251	MAT α <i>tsl3-1 ura3-52 leu2-3,112 lys2-801 ade2-101 ade3</i>	This study
ABY601	MAT α <i>tsl4-1 ura3-52 leu2-3,112 lys2-801 ade2-101 ade3</i>	This study
ABY602	MAT α <i>tsl5-1 ura3-52 leu2-3,112 lys2-801 ade2-101 ade3</i>	This study
ABY603	MAT α <i>tsl6-1 ura3-52 leu2-3,112 lys2-801 ade2-101</i>	This study
ABY604	MAT α <i>tsl2-1 ura3-52 leu2-3,112 ade2-101 ade3 tpm1Δ::LEU2 [pDS1-TPM1]</i>	This study
ABY611	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys2-801 ade2-101 bem2Δ::LEU2</i>	This study
ABY612	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 ade2-101 trp1-1(am) bem2Δ::LEU2</i>	This study
ABY168	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys2-801 tpm1Δ::LEU2</i>	Liu and Bretscher, 1992
ABY167	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 ade2-101 tpm1Δ::LEU2</i>	Liu and Bretscher, 1992
ABY117	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 ade2-101 trp1 myo2-66</i>	Johnston <i>et al.</i> , 1991
ABY111	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys2-801 trp1 sac6::URA3</i>	Adams <i>et al.</i> , 1991
ABY106	MAT α <i>ura3-52 leu2-3,112 abp1::URA3</i>	Drubin <i>et al.</i> , 1988
ABY187	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 ade2-101 trp1 myo1::LEU2</i>	Watts <i>et al.</i> , 1987
ABY244	MAT α <i>his4-619 ura3-52 anc4-1</i>	Vinh <i>et al.</i> , 1993
ABY104	MAT α <i>ura3-52 ade2-101 act1-4</i>	A. Adams' lab
ABY651	MAT α <i>ura3 leu2 trp1 his3 ade2 rho1-104</i>	Yomachi <i>et al.</i> , 1994
ABY430	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys-801 trp1 myo4::URA3</i>	Haarer <i>et al.</i> , 1994
ABY439	MAT α <i>ura3-52 leu2-3,112 his3Δ-200 lys-801 tpm2Δ::LEU2</i>	Drees <i>et al.</i> , 1995
ABY268	MAT α <i>tub2-401 ura3-52 ade2-101</i>	Huffaker <i>et al.</i> , 1988
ABY263	MAT α <i>tub2-406 ura3-52 his 4</i>	Pasqualone and Huffaker, 1994
ABY262	MAT α <i>tub2-104 ura3-52 his 4</i>	Huffaker <i>et al.</i> , 1988
FW1711	MAT α <i>spt2-150 SPT15::pFW218[URA3] leu2Δ1 ura3 pet140</i>	F. Wiston (Harvard Medical School, Boston, MA)

act1-4, *sac6::URA3*, *myo1::LEU2*, and *tsl* haploids all have morphological defects, the *tsl* mutations do not appear to be alleles of *ACT1*, *SAC6*, or *MYO1*.

Disruption of BEM2 and Synthetic Lethality Analyses Using *bem2 Δ* Cells

The *BEM2* gene was disrupted by the one-step gene disruption method (Rothstein, 1983). To disrupt the *BEM2* gene, the pTW003 plasmid was digested with *Xba*I and transformed into diploid cells ABY173. The cells were sporulated and all the spores were viable; Leu⁺ and temperature-sensitivity segregated 2:2. *BEM2* disruption was confirmed by Southern analysis. The derived *bem2 Δ* haploid cells were used for synthetic lethality analyses. For each cross between *bem2 Δ* and other mutations, at least 10 tetrads were analyzed. In cases where synthetic lethality was implicated (haploids containing both mutations are inviable), the cross was repeated with a strain carrying *BEM2* on a *URA3*-containing plasmid and showing that the double mutant was only viable in cells retaining the plasmid.

Generation and Affinity Purification of Antibodies to Yeast Actin

Yeast actin was prepared by a former graduate student Ruby Ye as described (Water *et al.*, 1980) and standard procedures were used to prepare yeast actin polyclonal antibodies in rabbits. To affinity purify the serum, actin was prepared using the same procedure and the monomeric actin was electroeluted according to the method of Hunkapiller *et al.* (1983). Two hundred micro-

grams of actin was conjugated to 1 ml of CNBr-activated Sepharose beads (Sigma Chemical, St. Louis, MO) to make an affinity column. One milliliter of serum was diluted twofold with Tris-buffered saline (pH 7.5) and loaded on the column. After extensive washing with Tris-buffered saline, the antibodies were eluted with 0.1 M glycine (pH 2.8) and neutralized quickly to pH 7.5 with Tris base.

Microscopic and Fluorescence Analyses of Cells

Immunofluorescence microscopy of tubulin and actin was performed as described (Pringle *et al.*, 1989). Rabbit antibodies to yeast actin were used at a 1:50 dilution. Rabbit anti- β -tubulin polyclonal antibodies 206 (Bond *et al.*, 1986) were a gift from F. Solomon (Massachusetts Institute of Technology, Cambridge, MA) and were used at 1:2,000. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgGs (ICN, Costa Mesa, CA) were used at 1:50. DNA was stained with 4', 6'-diamidino-2-phenylindole dihydrochloride at a concentration of 1 μ g/ml. All samples were examined by Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

Identification of Mutations that Are Synthetically Lethal with *tpm1 Δ*

A synthetic lethal screen using the color sectoring assay (Koshland *et al.*, 1985) was set up using hap-

loid *tpm1Δ* yeast of both mating types, ABY204 and ABY205. The cells were transformed with pDS1-TPM1 containing the *URA3*, *ADE3*, and *TPM1* genes in a *CEN3/ARS1* vector that is lost at high frequency. The presence of *ADE3* on the plasmid confers a red color to the *ade2 ade3 tpm1Δ* strain. As *tpm1Δ* cells are viable, they are able to lose the pDS1-TPM1 plasmid spontaneously, resulting in red colonies with white sectors. Mutations showing synthetic lethality with *tpm1Δ* render the plasmid essential and therefore give rise to nonsectoring red colonies. A total of 70,000 colonies from EMS mutagenized cells (about 20% viability) from six independent cultures were screened for the nonsectoring red phenotype at 25°C. Sixty-five nonsectoring potential *Tsl⁻* (*TPM1* synthetic lethal) mutants were recovered. Because their nonsectoring phenotype was stable on nonselective YPD media and they were inviable under conditions selecting for plasmid loss (on 5-FOA; Boeke *et al.*, 1984), the nonsectoring phenotype was not caused by increased plasmid stability or reversion of the chromosomal *ade3* gene. Diploids formed between each mutant and parental *tpm1Δ* cells gave rise to sectoring colonies that could grow on 5-FOA, indicating that all the mutations were recessive and that the nonsectoring phenotype was not a result of plasmid integration. The diploids were sporulated and tetrads for all 65 *tsl* mutants were analyzed. Fifty-five of the mutants were discarded as they did not segregate as a single gene or gave rise to a leaky, unworkable, *Tsl⁻* phenotype. One diploid would not sporulate, one did not yield viable spores, and one probably had a mutation in the uracil biosynthetic pathway. The seven remaining mutants contained single mutations conferring a clear *Tsl⁻* phenotype. To determine the number of the complementation groups, these mutants were mated to each other and the resulting diploids were examined for their viability to sector on YPD plates and their viability on 5-FOA-containing plates. They fell into six complementation groups that were designated *tsl1-1*, *tsl2-1*, *tsl2-2*, *tsl3-1*, *tsl4-1*, *tsl5-1*, and *tsl6-1*.

Phenotypic and Morphological Effects Conferred by *tsl* Mutations

The growth characteristics of strains carrying *tsl* mutations were assessed in a *TPM1* background at a variety of temperatures. Most mutants, like the wild-type cells, grew normally over a wide temperature range and were resistant to high osmotic stress (Figure 1). However, *tsl1-1* and *tsl2-2* confer temperature sensitivity, whereas *tsl3-1* and *tsl5-1* confer cold sensitivity. Strains harboring *tsl2-2* are also osmotically sensitive. Importantly, all of the mutants have morphological defects, showing abnormally enlarged cells

Figure 1. Growth properties of *tsl* mutants. Haploid *tsl1-1* (ABY249), *tsl2-1* (ABY604), *tsl2-2* (ABY250), *tsl3-1* (ABY251), *tsl4-1* (ABY601), *tsl5-1* (ABY602), *tsl6-1* (ABY603), and wild-type (ABY165) cells were incubated at 12°C (12 days), 25°C (4 days), 30°C (3 days), and 37°C (2 days) on YPD plates, and at 25°C on a YPD plate containing 0.9 M NaCl (6 days). *tsl2-1* grew at 25°C and 30°C, although with a glassy appearance that is reflected in the photograph.

in a population that is often quite heterogeneous (Figure 2). In addition, two cold-sensitive mutants, *tsl3-1* and *tsl5-1*, grew as chains of cells (arrows in Figure 2), perhaps suggesting a defect in cell separation. Overall, the morphology of *tsl* cells is reminiscent of strains having defects in genes specifying actin binding proteins (Welch *et al.*, 1993; Bretscher *et al.*, 1994), suggesting an effect on the actin cytoskeleton. To test whether any of these mutations were alleles of *TPM2*, *MYO2*, or *ANC4* (gene mutations that are known to show synthetic lethality with *tpm1Δ* [Liu and Bretscher, 1992; Vinh *et al.*, 1993; Drees *et al.*, 1995]), each mutant was crossed to *tpm2Δ::HIS3*, *myo2-66* (*Ts⁻*), or *anc4-1* (*Ts⁻*) haploids. Complementation analyses (for details, see MATERIALS AND METHODS) indicated that none of the mutations resides in these genes. Further crosses indicated that *tsl* mutations are not alleles of *ACT1* (actin), *SAC6* (fimbrin), and *MYO1* (myosin II), genes in which mutations confer a clear morphological phenotype. Therefore, we have identified a new set of genes that show synthetic lethality with *tpm1Δ* and have clear morphological defects. In the rest of this report, we characterize the *TSL1* gene; other *tsl* mutants are yet to be explored.

Figure 2. Morphology of *tsl* mutants. Haploid *tsl1-1* (ABY249), *tsl2-1* (ABY604), *tsl2-2* (ABY250), *tsl3-1* (ABY251), *tsl4-1* (ABY601), *tsl5-1* (ABY602), *tsl6-1* (ABY603), and wild-type (ABY165) cells were grown at 25°C to early log phase, and visualized under differential interference contrast. All pictures were photographed and printed at the same magnification. Bar, 2 μ m.

The *tsl1-1* Mutant Has a Defect in the Actin Cytoskeleton

The morphology of the *tsl1-1* mutant suggests that it might have a defect in its actin cytoskeleton (Figure 2), so the localization of actin was examined. At the permissive temperature (25°C), the actin cytoskeleton is disorganized, particularly in abnormally enlarged cells that frequently contain more than one nucleus (Figure 3, A and B), which is similar to the phenotype of *tpm1Δ* cells (arrows in Figure 3). Moreover, the actin cortical patches that seem to be concentrated in the growing buds at the permissive temperature are more delocalized upon shifting to the restrictive temperature (37°C) for 2 h (Figure 3, C and D). However, a few actin cables are evident in *tsl1* cells at 25°C (arrowheads in Figure 3A), suggesting an intact but perhaps incorrectly assembled cytoskeleton, whereas cables are not detectable in *tpm1Δ* cells (Figure 3, E and F). Given the fact that Tpm1p colocalizes with actin cables (Liu and Bretscher, 1989a), the morphological results argue that *TSL1* is important for the correct localization of microfilaments and the genetic results suggest that *TSL1* may function in the same pathway as *TPM1*. For example, *TSL1* may be important for the correct distribution of actin cables that are stabilized by Tpm1p.

tsl1-1* Is a New Allele of *BEM2*, *bem2-10

The *TSL1* gene was cloned from a CEN-based genomic library (Rose *et al.*, 1987) by complementation of *tsl1-1* temperature sensitivity. The plasmid was designated as pSL001 (Figure 4) and recovered 11 times in several separate experiments. Because a single copy of pSL001 rescued both the temperature sensitivity and morphological defects of *tsl1-1* (Figure 3, G and H), pSL001 is likely to contain the *TSL1* gene. This was subsequently confirmed by complementation and linkage analyses (see below). Further subcloning indicated that a 9-kb fragment (pSL009) was required for full complementation (Figure 4), suggesting that *TSL1* is large. This was confirmed by Northern analyses using different probes from pSL009 (black bars in Figure 4); all hybridized to a 7- to 7.5-kb transcript (unpublished observation). Restriction mapping and partial sequence analysis identified the cloned gene as *BEM2* (for bud emergence 2), which encodes a *rho*-type GTPase-activating protein (*rho*-GAP) that has a coding sequence encompassing 6.5 kb (Bender and Pringle, 1991; Chan and Botstein, 1993; Kim *et al.*, 1994; Peterson *et al.*, 1994). *tsl1-1* was redesignated *bem2-10*. The GAP homology domain is about 150 residues long (1981–2135) and is indicated by the box in Figure 4. The fact that pSL010 (lacking the GAP domain) fails to complement *tsl1-1* is consistent with the results showing that the GAP homology domain is essential and sufficient for the function of *BEM2* (Kim *et al.*, 1994; Peterson *et al.*,

1994). To confirm the identity of *BEM2* and *TSL1*, a *bem2Δ::LEU2* haploid (see MATERIALS AND METHODS and Figure 4) was found to have a conditional lethal phenotype and morphological defects similar to *tsl1-1*, including a delocalized actin cytoskeleton (Figure 3, I and J; Kim *et al.*, 1994). The *bem2Δ::LEU2* haploid was mated to a *tsl1-1* haploid carrying the complementing pSL009 plasmid. The resulting diploid had a wild-type morphology and was not temperature sensitive, but surprisingly could not grow after plasmid loss on 5-FOA. Moreover, *bem2Δ/tsl1-1* or *tsl1-1/tsl1-1* diploids are inviable whereas *bem2Δ/bem2Δ* is viable. This indicates that *BEM2* and *TSL1* are either the same gene or they show unlinked noncomplementation. Linkage between *tsl1-1* and *bem2Δ* could not be determined because *bem2Δ/tsl1-1* diploids did not sporulate even in the presence of pSL009 plasmid. Because *BEM2* is closely linked to *SPT15* (Bender and Pringle, 1991), we determined the linkage of *tsl1-1* to *SPT15* by crossing to strain FW1711 (*SPT15::URA3*). Analyses of 19 tetrads revealed close linkage (~8 cM) of *TSL1* to *SPT15*. *TSL1* is therefore *BEM2*.

Regulation of Microfilaments by the *BEM2* Signaling Pathway

If Bem2p is important for the functional organization of microfilaments, the combination of *bem2* with mutations affecting other cytoskeletal proteins might also be lethal. Because mutations in some genes encoding cytoskeletal proteins (*TPM1* and *MYO2*) appear to have defects in a vesicular trafficking pathway (Jonston *et al.*, 1991; Liu and Bretscher, 1992), the *bem2Δ* and *tpm1Δ* haploids were crossed with strains containing mutations affecting cytoskeletal proteins or components necessary for the secretory pathway. The meiotic products of the resulting diploids were then examined. In this way haploids could be recovered containing both mutations, and their viability analyzed (Table 2). If inviability was indicated, this was confirmed by repeating the cross with a strain carrying *BEM2* on a plasmid, and showing that the double mutant was only viable in cells retaining the plasmid. The *bem2* mutation shows synthetic lethality with mutations in *TPM1* (tropomyosin I), *MYO1* (myosin II), *MYO2* (myosin V), *SAC6* (fimbrin), and a specific allele of *ACT1* (actin), but not with mutations in various *SEC* genes or other alleles of *ACT1*.

Of particular interest is the finding of synthetic lethality between *bem2Δ* and *myo2-66*. Previous studies have implicated Myo2p and Tpm1p in vesicle delivery because mutations in *MYO2* or *TPM1* result in the accumulation of vesicles and defects in proper targeting of secretory products (Jonston *et al.*, 1991; Liu and Bretscher, 1992). Electron microscopy and biochemical analyses of the *bem2-10* mutant grown at either the

Figure 3. Actin localization (A, C, E, G, and I) and morphology (B, D, F, H, and J) of *tsl1-1*, *tpm1* Δ , and *bem2* Δ cells. Haploid *tsl1-1* (ABY249) cells (A and B, grown at 25°C; C and D, grown for 2 h at 37°C), haploid *tpm1* Δ ::*LEU2* (ABY167) cells (E and F), haploid *tsl1-1* (ABY249) cells carrying the plasmid pSL009 that complements the *tsl1-1* mutation (G and H), and haploid *bem2* Δ (ABY611) cells. Some cells with the *tsl1-1*, *tpm1* Δ , or *bem2* Δ mutations are abnormally large and the actin cytoskeleton is delocalized (arrows). The arrowheads in A and I point to *tsl1-1* and *bem2* Δ cells with actin cables. Cells were visualized after decoration with anti-actin antibody. Bar, 5 μ m.

Figure 4. The *tsl1-1* mutation lies in the *BEM2* gene. The 13-kb fragment in pSL001 that was isolated from a genomic library that complements *tsl1-1* is shown with selected restriction enzyme sites. The ability of subfragments to complement *tsl1-1* is also shown; the smallest complementing fragment is present in plasmid pSL009. Partial sequence analysis of pSL009 revealed identity to *BEM2*, which contains an open reading frame of 2167 residues.

dues (Peterson *et al.*, 1994; Kim *et al.*, 1994), indicated by stippling. The box indicates the *rho*-GAP homologous domain. Black bars show the extent of the probes used in Northern blots; all detected a message of about 7 kb. The construct used to disrupt *BEM2* is shown above pSL001.

restrictive or permissive temperature failed to show cytoplasmic accumulation of vesicles or of the secretory protein invertase (unpublished observation). Moreover, *bem2Δ* does not show synthetic lethality with a number of conditional mutations affecting the secretory pathway (Table 2). Therefore, Bem2p does not appear to regulate the secretory pathway directly but appears to be important for the organization of the machinery that delivers components for cell wall growth; inappropriate targeting in *bem2* mutants results in morphologically altered cells.

Genetic Interaction between *BEM2* and *RHO1*

The above results suggest that Bem2p is important for microfilament organization. Previous studies suggested that *BEM2* is involved in bud emergence, a process regulated by the *rho* protein Cdc42p (Adams *et al.*, 1990; Johnson and Pringle, 1990). However, Bem2p does not have GAP activity on Cdc42p, whereas it can activate the GTPase of Rho1p (Zheng *et al.*, 1993, 1994), a *rho*-GTPase involved in the subsequent assembly and growth of buds (Madaule *et al.*, 1987; Yamochi *et al.*, 1994). A likely scenario places Bem2p as the GAP that regulates Rho1p. The phenotype of a conditional *rho1-104* (*Ts*⁻) mutant and the localization of Rho1p to sites of cell growth (Yamochi *et al.*, 1994) are consistent with Rho1p regulating microfilament structure. The small bud arrest phenotype of the *rho1-104* conditional mutation is different from the large cell phenotype of the conditional *bem2-10* mutant (Figures 2 and 3). This is to be expected, as in the former case Rho1p is presumably inactivated (Yamochi *et al.*, 1994), whereas in the latter case it is hyperactivated. This is also consistent with the finding that overexpression of *RHO1* causes cells to have a similar phenotype to

bem2 alleles (Madaule *et al.*, 1994). To directly test whether *BEM2* interacts genetically with *RHO1*, the *rho1-104* haploid (ABY651) was crossed with the *bem2Δ::LEU2* haploid (ABY611). Although the resulting diploid grew well, it failed to sporulate. Therefore the pSL009 plasmid was introduced into the diploid that was now able to sporulate. Ten tetrads were analyzed for *Leu*⁺ and *Ts*⁻ phenotypes; the ratio of PD:NPD:TT was 2:2:6. In NPD (nonparental ditype) and TT (tetratype) tetrads, spores inferred to be *bem2Δ::LEU2 rho1-104* were either inviable, or carried the pSL009 plasmid and required it for viability; *bem2Δ::LEU2* and *rho1-104* therefore display synthetic lethality at 25°C. A similar experiment was also performed for *tpm1Δ* and *rho1-104* cells, but the analysis was severely hampered by inefficient sporulation and poor viability, a phenomenon previously encountered with *tpm1Δ* alleles, so we were not able to determine whether these two mutations also show synthetic lethality. Nevertheless, the synthetic lethality between *tpm1Δ* and *bem2Δ* shows that the cytoskeletal defect imposed by loss of Tpm1p cannot be tolerated in combination with a defect in the *BEM2* signaling pathway that involves *RHO1*. Thus *RHO1* and *BEM2* are important for regulating Tpm1p-containing microfilaments.

Effect of *bem2Δ* on Microtubule Function

The functions of microfilaments and microtubules are generally thought to be distinct (Huffaker *et al.*, 1988). However, evidence has emerged recently suggesting that there might be a link between these two elements (Lillie and Brown, 1992; Palmer *et al.*, 1992). To investigate whether microtubule function is compromised in *tpm1Δ*, *rho1*, or *bem2Δ* cells, their sensitivity to the

Table 2. Synthetic lethal relationships between *bem2* and *tpm1* and mutations in cytoskeletal and secretory genes

	<i>bem2</i> Δ	<i>tpm1</i> Δ
<i>act1-4</i>	+	+
<i>act1-101::HIS3^a</i>	+	+
<i>act1-113::HIS3^a</i>	+	+
<i>act1-117::HIS3^a</i>	+	+
<i>act1-119::HIS3^a</i>	+	+
<i>act1-123::HIS3^a</i>	+	+
<i>act1-125::HIS3^a</i>	+	+
<i>act1-133::HIS3^a</i>	+	+
<i>act1-135::HIS3^a</i>	+	+
<i>act1-105::HIS3^a</i>	SL	+
<i>tpm1</i> Δ::LEU2	SL	
<i>tpm2</i> Δ::HIS3	+	SL ^d
<i>myo1</i> Δ::LEU2	SL ^c	nd
<i>myo2-66</i>	SL	SL ^e
<i>myo4</i> Δ::URA3	+	+
<i>abp1::URA3</i>	+	+
<i>sac6::URA3</i>	SL	+
<i>sec1-1^b</i>	+	+ ^e
<i>sec4-8^b</i>	+	+ ^e
<i>sec6-4^b</i>	+	+ ^e
<i>sec13-1^b</i>	+	+ ^e
<i>sec14-3^b</i>	+	+ ^e
<i>sec18-1^b</i>	+	+ ^e

Isogenic strains, except as noted, were kindly provided by ^aD. Drubin and ^bP. Novick.

^c *bem2-10* used instead of the disruption allele.

^d Drees *et al.*, 1995.

^e Liu and Bretscher, 1992.

+ denotes a viable combination; SL denotes an inviable combination; nd denotes not determined.

microtubule-destabilizing drug benomyl was assessed and the localization of microtubules was determined. *tpm1*Δ and *rho1* showed normal sensitivity to benomyl whereas *bem2*Δ and *bem2-10* mutations conferred increased sensitivity to benomyl compared with an isogenic wild-type strain (Figure 5). After growth at 25°C the *tpm1*Δ and *bem2*Δ mutants both had a slightly increased proportion of cells with aberrant microtubule structures (Figure 6 and Table 3). These phenotypes were greatly exaggerated after 2 h of growth at 37°C, where a high percentage of cells had misoriented spindles and longer dangling cytoplasmic microtubules that frequently appeared to split into two separate misoriented bundles (Figure 6). Quantitation revealed that misoriented spindles were more prevalent in *bem2*Δ cells, which is also reflected in the much higher percentage of cells with two or more nuclei (Table 3). Thus *bem2*Δ appears to confer a more severe defect in microtubule function than is seen in *tpm1*Δ cells, either due to a direct effect on microtubule organization, or indirectly through its affect on microfilament organization. Analysis of microtubule distribution in the *rho1-104* mutant was complicated by the finding that many of the cells are sick, even at the

Figure 5. *bem2* mutations confer sensitivity to benomyl at 30°C. Wild-type (ABY165), *tpm1*Δ (ABY167), *bem2-10* or *tsl1-1* (ABY249), *bem2*Δ (ABY611), *rho1-104* (ABY651), and *rho1-104* carrying *RHO1* plasmid (pWT; Yomachi *et al.*, 1994) cells were suspended uniformly in a multi-well plate and spotted on YPD plates containing 0, 10, 15, 20, and 30 μg/ml benomyl, respectively, by a multi-pronged transferring device. Cells were incubated at 30°C for 3 days.

permissive temperature, and cell death occurs dramatically upon shifting to 37°C. To further examine the possibility that *Bem2p* might be involved in microtubule function, the viability of combinations of *bem2*Δ with seven different alleles of *TUB2* (encoding β-tubulin) was examined (Table 4). The *bem2*Δ shows synthetic lethality with one *tub2* allele (*tub2-429*), which has a defect in nuclear microtubules (Reijo *et al.*, 1994).

DISCUSSION

Mutations that Show Synthetic Lethality with *tpm1*Δ

We have used a synthetic lethal screen to search for genes whose products become essential in the absence of tropomyosin I in yeast. Previous studies employing this strategy have been highly successful in identifying genes whose products either interact or function in the same pathway. Here we describe the isolation of seven mutations, which fall into six complementation groups, that show synthetic lethality with *tpm1*Δ. We have clearly not saturated the screen because only one gene was recovered twice and none of the mutations were in *TPM2*, *MYO2*, or *ANC4*, which are known to show synthetic lethality with *tpm1*Δ (Liu and Bretscher, 1992; Vinh *et al.*, 1993; Drees *et al.*, 1995).

Despite the large number of different genes recovered, three arguments suggest that the screen is quite specific. First, combinations of *bem2-10* or *bem2*Δ with a variety of other conditional mutations, such as *sec* genes and some *cdc* genes, do not show synthetic lethality. Second, one of ten alleles of *act1* tested shows

Figure 6. Microtubule (A, D, G, J, and M) and nuclear (B, E, H, K, and N) staining and their Nomarski images (C, F, I, L, and O). Wild-type (ABY165), *tpm1* Δ (ABY167), and *bem2* Δ (ABY611) were either grown at 25°C (D, E, F, J, K, and L) or shifted to 37°C for 2 h (A, B, C, G, H, I, M, N, and O) before processing for immunofluorescence analysis using rabbit anti- β -tubulin polyclonal antibody 206. The insert in M shows abnormal arrays of cytoplasmic microtubules in *bem2* Δ cells after shifting to 37°C for 2 h. Bars, 5 μ m.

synthetic lethality with *bem2* Δ . Although the reason for allele specificity in this case is not clear, the fact that it occurs with the *act1-105* allele and not with an

allele (*act1-125*) having a more severe phenotype (Wertman *et al.*, 1992), indicates that the lethality is not simply the combination of two highly deleterious mu-

Table 3. Spindle orientation and number of nuclei in wild-type, *tpm1Δ*, and *bem2Δ* strains

		Microtubule					DAPI

wild-type (ABY165) ^a	37°C	52%	24%	24%	0%	0%	0%	100%
<i>tpm1Δ</i> (ABY167)	25°C	57%	17%	13%	11%	2%	2%	98%
<i>tpm1Δ</i> (ABY167)	37°C	44%	0%	2%	40%	14%	25%	75%
<i>bem2Δ</i> (ABY611)	25°C	44%	7%	12%	23%	14%	19%	81%
<i>bem2Δ</i> (ABY611)	37°C	39%	0%	3%	23%	35%	50%	50%

^a Cells were grown in YPD at 25°C with or without shift to 37°C for 2 h and then stained with tubulin antibody and DAPI. 200–300 cells of each type were scored for microtubule structure and number of nuclei.

tations but rather a specific allele of *ACT1* that is particularly sensitive to a defect in signaling. Third, and most importantly, each *tsl* mutation confers a morphological defect. The abnormally enlarged cell phenotypes are similar to *tpm1Δ* cells (Figures 2 and 3). The results suggest that these mutants have a defect in or associated with the actin cytoskeleton because the polarity of cell growth is compromised. For example, some might be impaired in actin-based vesicular transport because they show synthetic lethality with *tpm1Δ* and Tpm1p has been implicated in vesicular transport (Liu and Bretscher, 1992). Interestingly, the two cold-sensitive mutants, *tsl3-1* and *tsl5-1*, also appear to have defects in cytokinesis, resulting in chains of cells (Figure 2). This defect is typically seen in mutations in *CDC3*, *CDC10*, *CDC11*, and *CDC12*, which encode protein components of the 10-nm neck-filaments (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim *et al.*, 1991). It is not surprising that genes required for cytokinesis are also important for the

establishment of cell polarity as both are microfilament-based processes (Snyder, 1989; Flescher *et al.*, 1993). Future work will be needed to determine whether the *tsl3* and *tsl5* mutations reside in genes specifying 10-nm neck-filament proteins.

Importance of a Rho-GAP Encoded by *BEM2* for Regulating Microfilaments

BEM2 was first identified in a synthetic lethal screen with *MSB1*, which was originally recovered as a multicopy suppressor of *CDC24* (Bender and Pringle, 1989, 1991). Defects in *BEM2* cause cells to become large and multinucleated. Sequence analyses indicates that *BEM2* encodes a protein containing a *rho*-GAP domain (Peterson *et al.*, 1994; Kim *et al.*, 1994). The finding that *BEM2* shows synthetic lethality with *tpm1Δ* provides the first genetic evidence for a link between the actin cytoskeleton and a specific *rho* signaling pathway. Because *rho* proteins have very low GTPase activity (Anderson and Lacal, 1987; Zheng *et al.*, 1993, 1994; Peterson *et al.*, 1994), the specificity of the *rho*-GAP is very important. Although yeast contains at least five members of the *rho* family of GTPases (*CDC42*: Johnson and Pringle, 1990; *RHO1,2*: Madaule *et al.*, 1987; Yomachi *et al.*, 1994; *RHO3,4*: Matsui and Toh-e, 1992a,b), several lines of evidence suggest that the target of Bem2p is likely to be the product of the *RHO1* gene. First, a conditional mutation in *RHO1* arrests cells with small buds at the restrictive temperature (Yamochi *et al.*, 1994), indicating that Rho1p is important for microfilament-mediated polarized cell growth. Second, Bem2p has GAP activity on Rho1p, but not on Cdc42p (Zheng *et al.*, 1994). Third, the direct genetic interaction between *RHO1* and *BEM2* demonstrated

Table 4. Synthetic lethal analyses between *bem2Δ* and *tub2* mutations^a

	<i>bem2Δ::LEU2</i>
<i>tub2-104</i>	+
<i>tub2-406</i>	+
<i>tub2-401</i>	+
<i>tub2-429::URA3</i>	SL
<i>tub2-428::URA3</i>	+
<i>tub2-423::URA3</i>	+
<i>tub2-418::URA3</i>	+

^a Isogenic strains, except at the *TUB2* locus, kindly provided by T. Huffaker.

+ denotes viable; SL denotes synthetic lethal.

here places them in the same signaling pathway that regulates microfilaments.

The fact that *BEM2* shows synthetic lethality with *TPM1*, *SAC6*, *MYO2*, and *MYO1*, but not with *ABP1*, *MYO4*, and *TPM2*, indicates that mutations causing clear defects in microfilaments are more sensitive to the *BEM2* signaling pathway. This is not surprising as *ABP1*, *MYO4*, and *TPM2* all have functions largely overlapping with the products of other cytoskeletal genes (Holtzman *et al.*, 1993; Haarer *et al.*, 1994; Drees *et al.*, 1995). Among the ten *ACT1* alleles tested, only *act1-105*, which has mutations at E311A and R312A, is inviable in combination with *bem2Δ*. Although these two residues are not among the known crucial ones, such as for ATP binding (Wertman and Drubin, 1992), the *act1-105* itself is conditionally lethal, indicating that E311 and R312 are important for the function of actin. Interestingly, none of the actin mutations show synthetic lethality with *TPM1*. This is not unexpected as *TPM2* shares some overlapping functions with *TPM1* (Drees *et al.*, 1995).

Possible Function for *BEM2* in the Regulation of Microtubules

Whether the functions of microtubules and microfilaments are inter-related remains controversial. Earlier studies suggested that microtubules might have a function in polarized cell growth as the cytoplasmic microtubules point to the growing bud (Byers and Goetsch, 1975; Byers, 1981). Huffaker *et al.* (1988) then demonstrated that loss of cellular microtubules in *tub2* conditional alleles has no effects on bud formation, secretion, and bud growth, processes that involve microfilament participation. However, recent results have raised the possibility that microfilaments and microtubules might still have overlapping functions. For example, *SMY1*, a multicopy suppressor of *myo2-66*, appears to be a kinesin-related microtubule motor protein (Lillie and Brown, 1992). However, because both Myo2p and Smy1p are localized to sites of bud growth, a primary function with microfilaments and not microtubules may be indicated. In another example, a conditional actin mutant was found to be defective in microtubule orientation and nuclear migration at the restrictive temperature (Palmer *et al.*, 1992). However, normal microtubule arrays have been observed in strains with defects in microfilament-associated proteins such as *myo2-66* (Johnston *et al.*, 1991). Strikingly, we have observed both microfilament and microtubule defects in *tpm1Δ* and *bem2Δ* cells (Figures 3 and 6), suggesting that microfilaments and microtubules may be regulated through the same signaling pathway, or that defects in microfilaments may in turn affect the microtubule orientation and function. For example, a component necessary for microtubule organization might normally be positioned on the cell

Figure 7. Diagram of the known genetic interactions involving *TPM1* and *BEM2*. ↔ indicates synthetic lethality between alleles in these genes. The synthetic lethal interactions between *TPM1* and *MYO2*, and *TPM2* are described elsewhere (Liu and Bretscher, 1992; Drees *et al.*, 1995).

surface by the polarized microfilament system. However, the *BEM2* gene was independently isolated in a screen for mutants defective in chromosome segregation (Chan and Botstein, 1993), which is a microtubule-based process. These authors also noted that *bem2* mutations confer sensitivity to benomyl (Kim *et al.*, 1994).

Conclusion

The results presented here provide a genetic link between a specific G-protein signaling pathway, involving the *RHO1* product with the GAP encoded by *BEM2* and several components of the actin cytoskeleton (Figure 7). These interactions reveal an involvement of Bem2p with actin, fimbrin, and tropomyosin I, the three known components of the polarized actin cables in yeast, and a myosin V that appears to be involved in polarized vesicular transport. Additionally, our data suggest that Bem2p participates in functions involving myosin II, which includes cytokinesis (Watts *et al.*, 1987). Finally, our results also implicate a direct or indirect involvement of Bem2p in the regulation of microtubules. Now that these links have been established, genetic and biochemical approaches can be used to identify additional regulatory components that participate in these aspects of yeast morphogenesis.

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