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BRO1, a Novel Gene That Interacts with Components of the Pkc1p–Mitogen-Activated Protein Kinase Pathway in *Saccharomyces cerevisiae*

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Yeast cells with mutations in *BRO1* display phenotypes similar to those caused by deletion of *BCK1*, a gene encoding a MEK kinase that functions in a mitogen-activated protein kinase pathway mediating maintenance of cell integrity. *bro1* cells exhibit a temperature-sensitive growth defect that is suppressed by the addition of osmotic stabilizers or Ca^{2+} to the growth medium or by additional copies of the *BCK1* gene. At permissive temperatures, *bro1* mutants are sensitive to caffeine and respond abnormally to nutrient limitation. A null mutation in *BRO1* is synthetically lethal with null mutations in *BCK1*, *MPK1*, which encodes a mitogen-activated protein kinase that functions downstream of Bck1p, or *PKC1*, a gene encoding a protein kinase C homolog that activates Bck1p. Analysis of the isolated *BRO1* gene revealed that it encodes a novel, 97-kDa polypeptide which contains a putative SH3 domain-binding motif and is homologous to a protein of unknown function in *Caenorhabditis elegans*.

Cellular responses to a variety of environmental signals are mediated by modular protein kinase cascades whose ultimate targets are mitogen-activated protein (MAP) kinases (5). Roles for MAP kinases (also called ERKs) have been established in cells undergoing mitosis, meiosis, and differentiation (36). MAP kinase activation requires phosphorylation by a MAP kinase kinase, or MEK (for MAP kinase/ERK kinase). The MEK is, in turn, activated via phosphorylation by a member of the MEK kinase (MEKK) family, the upstream component of the MAP kinase functional unit. MAP kinase modules have been identified in a variety of eukaryotic species, ranging from mammals (46) to budding and fission yeasts (14).

Maintenance of cell integrity in the budding yeast *Saccharomyces cerevisiae* requires a distinct MAP kinase pathway, which includes a MEKK, encoded by the *BCK1*/*SLK1* gene (9, 23), two MEKs, products of the *MKK1* and *MKK2* genes (17), and a MAP kinase, encoded by the *MPK1*/*SLT2* gene (22, 30, 47; reviewed in reference 12). Null mutants lacking any one of these functional units, the MAP kinase, the two MEKs, or the MEKK, display a cell lysis defect at elevated temperatures. Addition of osmotic stabilizing agents (e.g., 1 M sorbitol) to the growth medium suppresses this lysis defect.

Genetic and biochemical data have demonstrated that signaling through the MAP kinase pathway that mediates maintenance of cell integrity is triggered by activation of yeast protein kinase C, product of the *PKC1* gene (12, 25). Disruption of *PKC1* results in a cell lysis defect at all temperatures as a result of defective cell wall construction (24, 37). Because loss of *PKC1* results in a more severe phenotype than does loss of *BCK1* or other downstream components of this cascade, one proposed model (23) suggests that the MAP kinase module constitutes one branch of a bifurcated pathway which is regulated by Pkc1p.

Here we report the isolation of a yeast mutant, *bro1*, exhibiting a temperature-dependent, osmoremedial growth defect which is suppressed by Ca^{2+} or by centromere-based plasmids containing *BCK1*. A *bro1*-null mutation produces synergistic phenotypes with null mutations in *PKC1*, *BCK1*, and *MPK1*. *BRO1* encodes a novel protein containing an SH3 domain-binding motif which has a homolog in *Caenorhabditis elegans*.

MATERIALS AND METHODS

Strains and genetic methods. Yeast strains used in this study are listed in Table 1. To create MYY550, one copy of *BRO1* was disrupted in MYY298. MYY552 is a haploid segregant of MYY550. To create MYY557, one copy of *BRO1* was disrupted in JTY2150 (previously designated FL100 [26] and provided as a gift from Elisabeth Schnieders and Jeremy Thorner) as described below. MYY560 is a haploid segregant of MYY557. MYY566 was generated by crossing MYY560 and EG123. One copy of *BCK1* was disrupted in strain MYY566 to generate MYY567 as described below. MYY596 was generated by crossing an *mpk1*-null haploid segregant of DL453 (provided as a gift from David Levin) to MYY560. Two *BRO1 bck1*::*LEU2* haploid segregants of MYY567 of opposite mating type were mated to generate MYY590, and two *bro1*::*URA3 bck1*::*LEU2* haploid segregants of MYY567 of opposite mating type were mated to generate MYY591.

MYY575 was derived by four successive backcrosses of the original *bro1-1* mutant, MYY581, to MYY290 or MYY291. Phenotypic analysis of the progeny of this backcross revealed that polymorphisms at a second genetic locus affect the expression of certain mutant phenotypes, including mitochondrial transmission, development of elongated buds, and sensitivity to caffeine. Because this second locus influences the sensitivity of cell growth to caffeine independently of mutations in *BRO1*, the locus was designated *CAF1*. *CAF1* strains grow more slowly on YPD medium containing 10 mM caffeine than do *caf1* strains. *CAF1* alleles do not affect the temperature-sensitive growth of *bro1* mutants. To determine whether *CAF1* was allelic with *BCK1*, MYY535, a *BRO1 caf1* strain carrying a copy of the *URA3* gene integrated at the *BCK1* locus (13), was crossed to MYY575, and the Caf phenotypes of Ura^- Ts^- progeny were analyzed. Some Ura^- Ts^- haploids from this cross were able to grow on YPD with 10 mM caffeine (albeit not as well as Ts^+ strains), indicating that *CAF1* and *BCK1* segregated independently and are therefore distinct loci. To create isogenic *CAF1* strains with different *BRO1* alleles, MYY575 was crossed to MYY291 and to MYY552, and strains MYY571 and MYY579 were derived as haploid spores from the respective diploid strains.

Media were prepared and standard genetic manipulations were performed as described by Rose et al. (42). Yeast cells were transformed by the lithium acetate method (18). Plasmid DNA was prepared from *Escherichia coli* DH5α and MH6.

Mutant isolation. The *bro1* mutant was isolated in a screen designed to identify genes interacting with *MDM1*. The *mdm1* strain MYY403 harboring plasmid YCp50-MDM1 (32) was mutagenized with ethyl methanesulfonate to 16% survival as described previously (50). Cells were replica plated to YPD medium at 37°C and to SD-complete medium supplemented with 1 mg of 5-fluoroorotic acid (American Biorganic) per ml at 23°C. Temperature-sensitive strains that failed to grow on fluoroorotic acid (i.e., required *MDM1* plasmid for viability) were screened for defects in mitochondrial distribution and/or mor-

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Reference
AH216 congenic		
MYY290	*MAT***a** *his3 leu2 ura3 caf1*	44
MYY291	*MATα his3 leu2 ura3 caf1*	44
MYY298	*MAT***a**/*MATα his3/his3 leu2/leu2 ura3/ura3 caf1/caf1*	32
MYY535	*MAT***a** *his3 leu2 ura3 caf1 BCK1.URA3*[a]	This study
MYY550	*MAT***a**/*MATα his3/his3 leu2/leu2 BRO1/bro1::URA3*	This study
MYY552	*MATα his3 leu2 caf1 bro1::URA3*	This study
MYY571	*MAT***a** *his3 leu2 ura3 CAF1*	This study
MYY575	*MAT***a** *his3 leu2 ura3 CAF1 bro1-1*	This study
MYY579	*MAT***a** *his3 leu2 CAF1 bro1::URA3*	This study
EG123 congenic		
EG123	*MATα his4 leu2 trp1 ura3*	26
1788	*MAT***a**/*MATα his4/his4 leu2/leu2 trp1/trp1 ura3/ura3*	26
JTY2150	*MAT***a**/*MATα his4/his4 trp1/trp1 ura3/ura3 PKC1/pkc1::LEU2*	26
DL453	*MAT***a**/*MATα his4/his4 leu2/leu2 ura3/ura3 MPK1/mpk1::TRP1*	22
MYY557	*MAT***a**/*MATα his4/his4 trp1/trp1 BRO1/bro1::URA3 PKC1/pkc1::LEU2*	This study
MYY560	*MAT***a** *his4 leu2 trp1 bro1::URA3*	This study
MYY566	*MAT***a**/*MATα his4/his4 leu2/leu2 trp1/trp1 BRO1/bro1::URA3*	This study
MYY567	*MAT***a**/*MATα his4/his4 trp1/trp1 BRO1/bro1::URA3 BCK1/bck1::LEU2*	This study
MYY590	*MAT***a**/*MATα his4/his4 trp1/trp1 ura3/ura3 bck1::LEU2/bck1::LEU2*	This study
MYY591	*MAT***a**/*MATα his4/his4 trp1/trp1 bro1::URA3/bro1::URA3 bck1::LEU2/bck1::LEU2*	This study
MYY596	*MAT***a**/*MATα his4/his4 leu2/leu2 BRO1/bro1::URA3 MPK1/mpk1::TRP1*	This study
Mixed genetic background		
MYY403	*MATα leu2 ura3 mdm1-1*	32
MYY581	*MATα leu2 ura3 mdm1-1 bro1-1* + p*CEN4/URA3/MDM1*	This study

[a] Has *URA3* integrated next to *BCK1* at the *BCK1* locus.

phology (Mdm$^-$) at 37°C by fluorescence microscopy as described previously (31). Mdm$^-$ strains were backcrossed to strain MYY290 or MYY291, and the mitochondrial distribution and temperature-sensitive growth phenotypes of progeny were analyzed.

Isolation of the *BRO1* gene. The *BRO1* gene was isolated by complementation of the temperature-sensitive growth defect at 38°C. The *bro1-1* strain MYY575 was transformed with a yeast genomic library made in the centromere vector pSB32 (41). Six temperature-resistant clones were isolated from 33,000 Leu$^+$ transformants. Restriction maps of three of the complementing plasmids, p2-2, p2-3, and p5-1, indicated that the genomic inserts overlapped one another. These inserts also overlapped yeast DNA sequences in an independently isolated plasmid which had been identified previously by DNA hybridization and partial sequence analysis as encoding *BCK1* (13).

Two of the remaining complementing plasmids shared restriction fragments distinct from those of *BCK1*. One of these plasmids, p1-1, contained an 11-kb yeast genomic DNA insert. By subcloning portions of this insert into plasmids pRS315 or pRS316 (43) and testing these constructs for complementation of temperature-sensitive growth of *bro1* cells, a 3.0-kb fragment extending from the *Kpn*I site to the end of the genomic insert was identified as the minimum complementing region.

Chromosomal mapping. A 2.7-kb *Nco*I fragment of plasmid p1-1 was used to probe blots of DNA from phage λ and cosmid clones containing yeast genomic inserts. Blots were provided by Linda Riles (39). Hybridizing clones were identified by autoradiography.

Integrative transformation. The 2.7-kb *Nco*I fragment of p1-1 was isolated, fragment ends were filled with Klenow polymerase, and the fragment was ligated into the *Pvu*II site of plasmid YIp5 (45). The resulting plasmid, YIp1-1, was linearized with *Sac*I and transformed into yeast strain MYY291. Two Ura$^+$ transformants were crossed to MYY575, and the resulting diploids were induced to sporulate. Eleven tetrads from one clone and six tetrads from the other were dissected, and phenotypes of the progeny were analyzed. All tetrads analyzed were of the parental ditype (2 Ura$^+$ Ts$^+$:2 Ura$^-$ Ts$^-$), indicating that the two integration events had occurred within 4.5 and 8.3 centimorgans of the *BRO1* locus, respectively.

Sequence analysis of the *BRO1* gene. An 8-kb *Eco*R1 fragment from plasmid p1-1 that included the complementing region and 378 bp of vector sequence was subcloned into plasmid Bluescript KS (Stratagene, La Jolla, Calif.) to generate plasmid pBS-M11. Deletion of the 1.5-kb *Cla*I fragment from pBS-M11 yielded pBS-M11ΔC. Templates for sequencing the noncoding strand of the *BRO1* gene were generated by digesting plasmids pBS-M11 and pBS-M11ΔC with exonuclease III from the *Kpn*I side of the polylinker to create a series of nested deletion constructs. A 3-kb *Kpn*I fragment from plasmid pBS-M11 was subcloned into Bluescript KS to generate plasmid pBS-3KM11. pBS-3KM11 was digested with exonuclease III from the *Sac*I side of the polylinker to generate another series of nested deletions used as templates for sequencing the *BRO1* coding strand. The predicted amino acid sequence of Bro1p was compared with sequences in the Brookhaven Protein Data Bank, GenBank, PIR, and Swiss-Prot sequence libraries, using the FASTA and BLAST programs.

Gene disruptions. Yeast strains with null mutations in *BRO1* were generated by replacing most of the open reading frame with the *URA3* gene. Plasmid pFL1 (8) was digested with *Hin*dIII, DNA ends were filled in with Klenow polymerase, and the 1.2-kb fragment containing *URA3* was isolated. This fragment was ligated into the filled *Stu*I and *Nde*I sites of pBS-3KM11. The resulting plasmid, pM11::URA3, was cut with *Kpn*I and used to transform diploid strains MYY298 and JTY2150. Ura$^+$ transformants were selected. Disruption of one copy of *BRO1* in diploid strains was confirmed by Southern analysis.

A yeast strain with a null mutation in *BCK1* was generated by replacing the entire open reading frame with *LEU2*. Plasmid YEp13 (6) was digested with *Sal*I and *Xho*I, ends were filled with Klenow polymerase, and the 2.2-kb fragment containing *LEU2* was ligated into the *Sna*BI sites which flank the *BCK1* open reading frame in plasmid p2-3. The resulting plasmid, p2-3::LEU2b, was digested with *Sal*I and *Bgl*II and used to transform diploid strain MYY566. Leu$^+$ transformants were selected. Disruption of one copy of *BCK1* was confirmed by Southern analysis.

Determination of viable cell density of cultures. Cell viability was determined by methylene blue staining as described by Iida et al. (16). Optical densities at 600 nm (OD_{600}) were measured on samples of cell cultures. Culture samples (100 μl) diluted to an OD_{600} of 0.5 to 1.0 were added to 100 μl of a solution of 0.01% methylene blue (Sigma) in 2% sodium citrate. Mixtures were sonicated for 15 s to dissociate cell clumps, and viable (nonstained) cells were counted microscopically in a hemocytometer. At least 286 cells were counted for each time point.

Microscopy. Microscopy of yeast cells was performed as previously described (31).

Nucleotide sequence accession number. The GenBank accession number for the *BRO1* gene is U37364.

RESULTS

Isolation of the *bro1* mutant. The *bro1* mutant was isolated in a screen for strains with mutations displaying synthetic lethality with *mdm1-1*, a conditional-lethal mutation affecting mitochondrial and nuclear inheritance. After the *mdm1-1* lesion was deleted by crossing, five mutant strains that showed temperature-sensitive growth and delayed transmission of mitochondria into growing buds at 37°C were isolated. These strains were assigned to a single complementation group on the basis of temperature-sensitive growth. Phenotypic analysis of progeny from the backcross of one of these mutants demonstrated that temperature-sensitive growth was due to a single, nuclear mutation (*bro1-1*) and that the delayed mitochondrial inheritance was caused by the *bro1-1* mutation together with the dominant allele of a second unlinked locus, which we designated *CAF1* (see Materials and Methods).

Cloning and analysis of *BRO1*. To determine the molecular basis for the temperature-sensitive growth defect of *bro1* cells, the wild-type *BRO1* gene was cloned by complementation with a yeast genomic library in a centromere-based plasmid. Five complementing plasmids were isolated. Three of these clones contained overlapping inserts of yeast DNA which were shown by hybridization analysis, restriction mapping, and partial nu-

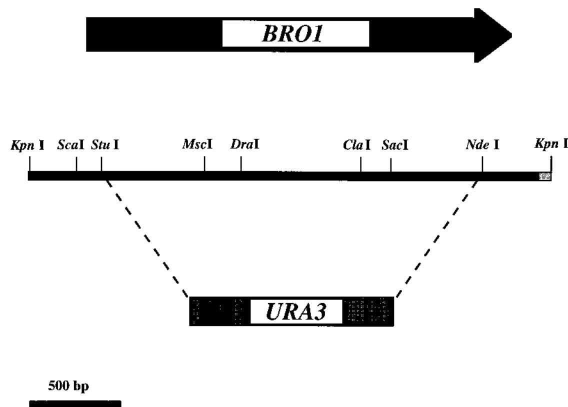

FIG. 1. Restriction map of *BRO1*. Some restriction endonuclease sites from the minimum complementing fragment are shown. Yeast genomic DNA insert (solid line) and adjacent vector sequences (stippled line) are indicated. The solid arrow indicates the location and orientation of the *BRO1* open reading frame. The region replaced by the yeast *URA3* gene for creation of the *bro1*-null mutant is indicated.

cleotide sequence characterization to contain the *BCK1* gene (35).

The other two complementing plasmids contained overlapping inserts with restriction maps distinct from those of the first set. The complementing activity of one of these plasmids, p1-1, was localized to a 3.0-kb fragment (Fig. 1) at one end of the 11-kb insert. This genomic insert was shown by integrative transformation and mapping to correspond to sequences from the locus of the temperature-sensitive mutation (see Materials and Methods). A fragment from this insert was mapped to the centromere-proximal side of *bem3* on the left arm of chromosome XVI. Because the mutant strain displays a temperature-sensitive, osmoremedial growth defect similar to that of the *bck1*-null mutant (see below) and can be rescued by additional copies of *BCK1*, we named the gene *BRO1* for its ability to confer *BCK1*-like resistance to osmotic shock.

Nucleotide sequence analysis of the 3-kb complementing fragment from plasmid p1-1 revealed a single open reading frame of 2,532 nucleotides encoding a putative 97-kDa polypeptide (Fig. 2). Comparison of the predicted amino acid sequence with sequences in the Brookhaven Protein Data Bank, GenBank, PIR, and Swiss-Prot databases showed that the *BRO1* product is homologous to the putative 98-kDa product of an open reading frame on chromosome III of *C. elegans* (GenBank accession number Z29561) (49). The amino acid sequences of the two proteins are 22% identical (Fig. 3) and 32% similar when conservative substitutions (R/K, D/E, Q/N, S/T, L/I/V/A/F) are included. The sequence similarities are shared throughout the lengths of the two proteins. Both Bro1p and its *C. elegans* homolog contain short, proline-rich regions (residues 716 to 722 of Bro1p and 754 to 762 of the *C. elegans* protein [Fig. 3]) near their carboxy termini which contain the consensus sequence (PXXP) of known SH3 domain-binding motifs (28, 33, 51).

To analyze further the cellular requirements for *BRO1*, a null mutation was created by replacing most of the *BRO1* coding sequence with the yeast *URA3* gene (Fig. 1). Like *bro1-1*, the *bro1*-null mutation conferred a temperature-sensitive growth defect in a *CAF1* genetic background, and the temperature-sensitive growth phenotype of either mutant was suppressed by a centromere-based plasmid containing *BCK1* (Fig. 4). In addition, transformation of *bro1*-null cells with a plasmid containing wild-type *BRO1* also restored normal growth at 37°C (data not shown). The same results were obtained in a background with the recessive *caf1* allele (data not shown).

Growth phenotypes of *bro1* mutants. The observation that additional copies of *BCK1* rescued the temperature-sensitive growth of *bro1* cells led us to investigate whether *bro1* mutants displayed phenotypes similar to those described for *bck1* mutants. Null mutations in *BCK1* or in other downstream components of the MAP kinase pathway that mediates maintenance of cell integrity cause a temperature-dependent cell lysis defect that is suppressed by growth on osmotically supported medium (17, 22, 23, 47). To test whether *bro1* mutants were similarly rescued at the nonpermissive temperature, *bro1-1* and *bro1*-null cells were cultured on YPD medium supplemented with 1 M sorbitol at 37°C. Both *bro1-1* and *bro1*-null cells grew under these conditions (Fig. 5). These strains also grew at 37°C on YPD medium supplemented with 0.5 M KCl (data not shown), suggesting that the rescue of growth was due to an osmotic effect rather than a specific chemical property of the solute. When *bro1* cells were first cultured at 37°C on YPD plus 1 M sorbitol and then inoculated directly onto regular YPD at 37°C, they failed to grow, indicating that the cells were sensitive specifically to osmotic shock. Microscopic inspection of *bro1* cells grown for 2 to 4 h in media lacking osmotic support at 37°C revealed many lysed or enlarged cells and morphologically normal mother cells with shriveled buds.

Growth of *bck1*-null cells is also rescued by the addition of 100 mM $CaCl_2$ or $MgCl_2$ to the growth medium (23). *bro1* mutants were tested for growth at 37°C on YPD medium supplemented with either 25 mM $CaCl_2$ or 25 mM $MgCl_2$. $CaCl_2$ rescued both *bro1-1* and *bro1*-null cells, while $MgCl_2$ rescued *bro1-1* but not *bro1*-null cells (Fig. 5). The addition of either 50 mM KCl or 75 mM sorbitol to the growth medium did not enable *bro1-1* or *bro1*-null cells to grow at 37°C but had no effect on the growth of wild-type cells at this temperature (data not shown), indicating that the rescue of *bro1* mutants by 25 mM $CaCl_2$ or $MgCl_2$ is due specifically to the cations and not to Cl^- or a general osmotic effect.

Growth of *bck1*-null and *mpk1*-null cells is inhibited by caffeine at permissive temperatures. Wild-type cells grow at 30°C on YPD containing 10 mM caffeine, but *bck1*-null and *mpk1*-null cells do not (9, 10). To test whether *bro1* mutants exhibited sensitivity to caffeine, *bro1-1* and *bro1*-null cells were cultured at 30°C on YPD agar medium containing 10 mM caffeine. Neither *bro1-1* nor *bro1*-null cells grew under these conditions (Fig. 5). These results demonstrate that *bro1* mutants display growth phenotypes similar to those reported for mutants lacking *BCK1* or other downstream components of this pathway.

In addition to its role in vegetative growth, *BCK1* is required for normal pheromone-induced morphogenesis. *MAT***a** *bck1*-null cells fail to form mating projections in regular YPD medium in response to the mating pheromone α-factor at both 30 and 38°C (9). This defect is partially rescued by the addition of 1 M sorbitol to the growth medium. To determine whether *bro1* mutants displayed a similar defect, exponentially growing cultures of *MAT***a** *BRO1*, *bro1-1*, and *bro1*-null cells were exposed to α-factor and incubated at either 30 or 37°C as described by Costigan et al. (9). The percentages of cells with mating projections were similar among the three cultures at both temperatures (data not shown), indicating that *BRO1* is not required for pheromone-induced morphogenesis.

***BRO1* is required for normal response to nutrient limitation.** *bck1*-null cells display several phenotypes indicative of a defective entry into stationary phase (9). To test whether *bro1* mutants exhibited similar phenotypes, the growth of *bro1-1* and *bro1*-null cultures was monitored during both logarithmic and

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-110  GGCTTCAGTT ACAATTCGAA GAAGTACTAA GGGGGTTGTC GTGGAAAAAT CTTGAATCAG AAAGCGGCTG ACTGGAGCAT TACTAGGTTC ATTACTCACT GGTTCTACTC

   1  ATG AAA CCT TAC TTA TTT GAC CTA AAG CTA AAA GAC ACA GAG AAG CTG GAT TGG AAA AAA GGC CTC TCC TCA TAT CTC AAG AAA TCA TAC
   1  met lys pro tyr leu phe asp leu lys leu lys asp thr glu lys leu asp trp lys lys gly leu ser ser tyr leu lys lys ser tyr

  91  GGC TCC TCA CAA TGG AGA ACA TTT TAT GAT GAG AAA GCT ACT TCA GAA CTG GAT CAC TTA AGG AAT AAT GCA AAT GGA GAA CTA GCA CCT
  31  gly ser ser gln trp arg thr phe tyr asp glu lys ala thr ser glu leu asp his leu arg asn asn ala asn gly glu leu ala pro

 181  TCT TCA TTG TCG GAA CAA AAT CTA AAA TAT TAC TCA TTT TTG GAG CAT CTT TAT TTT CGC CTG GGT AGC AAA GGA TCA AGA TTA AAA ATG
  61  ser ser leu ser glu gln asn leu lys tyr tyr ser phe leu glu his leu tyr phe arg leu gly ser lys gly ser arg leu lys met

 271  GAT TTC ACT TGG TAT GAC GCA GAA TAC TCA TCG GCC CAG AAA GGA TTG AAA TAC ACG CAA CAT ACG TTA GCA TTT GAA AAG TCT TGT ACT
  91  asp phe thr trp tyr asp ala glu tyr ser ser ala gln lys gly leu lys tyr thr gln his thr leu ala phe glu lys ser cys thr

 361  TTG TTC AAC ATT GCT GTA ATC TTT ACC CAA ATT GCG AGG GAG AAT ATC AAT GAG GAC TAC AAA AAC TCA ATC GCA AAT TTG ACA AAA GCT
 121  leu phe asn ile ala val ile phe thr gln ile ala arg glu asn ile asn glu asp tyr lys asn ser ile ala asn leu thr lys ala

 451  TTT TCC TGT TTT GAA TAT CTA TCA GAA AAT TTT TTG AAC TCA CCT TCA GTC GAT CTT CAG TCA GAA AAC ACT AGG TTT CTG GCT AAT ATT
 151  phe ser cys phe glu tyr leu ser glu asn phe leu asn ser pro ser val asp leu gln ser glu asn thr arg phe leu ala asn ile

 541  TGC CAT GCA GAA GCT CAA GAA TTG TTT GTC TTG AAA TTA TTA AAT GAT CAA ATA TCA TCC AAG CAA TAT ACA TTA ATC AGT AAA CTT TCT
 181  cys his ala glu ala gln glu leu phe val leu lys leu leu asn asp gln ile ser ser lys gln tyr thr leu ile ser lys leu ser

 631  AGA GCC ACG TGT AAC CTC TTT CAG AAA TGT CAC GAT TTT ATG AAA GAA ATA GAT GAC GAT GTG GCC ATT TAT GGT GAA CCC AAA TGG AAA
 211  arg ala thr cys asn leu phe gln lys cys his asp phe met lys glu ile asp asp asp val ala ile tyr gly glu pro lys trp lys

 721  ACG ACA GTT ACT TGC AAA CTG CAT TTC TAC AAA TCG TTA AGC GCT TAT TAT CAC GGT TTA CAC CTT GAA GAA GAA AAT AGA GTT GGC GAA
 241  thr thr val thr cys lys leu his phe tyr lys ser leu ser ala tyr tyr his gly leu his leu glu glu glu asn arg val gly glu

 811  GCA ATT GCT TTT CTC GAT TTT TCT ATG CAA CAA TTG ATT TCA TCC CTT CCA TTC AAA ACG TGG TTA GTG GAA TTT ATA GAC TTT GAT GGG
 271  ala ile ala phe leu asp phe ser met gln gln leu ile ser ser leu pro phe lys thr trp leu val glu phe ile asp phe asp gly

 901  TTT AAA GAA ACT TTA GAA AAG AAA CAA AAG GAG TTG ATT AAA GAT AAC GAT TTT ATA TAT CAT GAA AGC GTT CCA GCC GTT GTG CAG GTT
 301  phe lys glu thr leu glu lys lys gln lys glu leu ile lys asp asn asp phe ile tyr his glu ser val pro ala val val gln val

 991  GAT TCC ATT AAG GCG CTC GAT GCA ATA AAA TCT CCA ACA TGG GAG AAG ATA TTA GAA CCA TAT ATG CAA GAT GTT GCA AAT AAA TAT GAC
 331  asp ser ile lys ala leu asp ala ile lys ser pro thr trp glu lys ile leu glu pro tyr met gln asp val ala asn lys tyr asp

1081  TCT TTG TAC AGA GGA ATT ATT CCC CTA GAT GTC TAT GAA AAG GAA AGT ATT TAC TCA GAA GAA AAA GCG ACG CTG TTG AGA AAG CAA GTT
 361  ser leu tyr arg gly ile ile pro leu asp val tyr glu lys glu ser ile tyr ser glu glu lys ala thr leu leu arg lys gln val

1171  GAA GAA ACT GAG ACA GCA AAT TTG GAA TAT TCT TCC TTC ATC GAA TTT ACA AAT CTA CCC AGG CTC TTG AGT GAT TTG GAA AAA CAA TTT
 391  glu glu thr glu thr ala asn leu glu tyr ser ser phe ile glu phe thr asn leu pro arg leu leu ser asp leu glu lys gln phe

1261  AGT GAC GGA AAT ATT TTC TCG AAT ACG GAT ACA CAG GGA CAA TTG ATG AGG GAC CAA ATT CAG ACA TGG TGT AAA TTT ATC CAA ACA AAT
 421  ser asp gly asn ile phe ser asn thr asp thr gln gly gln leu met arg asp gln ile gln thr trp cys lys phe ile gln thr asn

1351  GAA TTT AGG GAT ATA GAA GAA CAG ATG AAC AAA ATT GTT TTC AAA AGG AAA CAA ATT TTA GAA ATC CTT TCT GCC TTA CCC AAT GAT CAA
 451  glu phe arg asp ile glu glu gln met asn lys ile val phe lys arg lys gln ile leu glu ile leu ser ala leu pro asn asp gln

1441  AAA GAA AAT GTT ACA AAA CTA AAA TCT TCT TTA GTA GCT GCT TCA AAC TCA GAC GAA AAA TTG TTC GCA TGC GTA AAA CCA CAT ATT GTC
 481  lys glu asn val thr lys leu lys ser ser leu val ala ala ser asn ser asp glu lys leu phe ala cys val lys pro his ile val

1531  GAG ATC AAT CTA TTG AAT GAC AAT GGA AAA ATA TGG AAG AAG TTT GAC GAA TTT AAT CGC AAT ACG CCT CCA CAA CCT AGC CTA TTG GAT
 511  glu ile asn leu leu asn asp asn gly lys ile trp lys lys phe asp glu phe asn arg asn thr pro pro gln pro ser leu leu asp

1621  ATC GAT GAT ACC AAA AAC GAC AAG ATA TTA GAG TTG TTA AAA CAA GTA AAG GGC CAT GCG GAA GAC TTA AGA ACA TTG AAA GAG GAA CGT
 541  ile asp asp thr lys asn asp lys ile leu glu leu leu lys gln val lys gly his ala glu asp leu arg thr leu lys glu glu arg

1711  TCG AGA AAT TTG TCT GAA CTA AGA GAC GAA ATC AAC AAC GAT GAT ATC ACA AAA TTA TTA ATT ATT AAT AAG GGG AAA TCC GAT GTT GAG
 571  ser arg asn leu ser glu leu arg asp glu ile asn asn asp asp ile thr lys leu leu ile ile asn lys gly lys ser asp val glu

1801  CTC AAA GAT TTA TTC GAG GTG GAA CTG GAG AAA TTC GAG CCT TTG AGC ACA AGA ATA GAG GCG ACA ATT TAC AAA CAA TCT TCA ATG ATA
 601  leu lys asp leu phe glu val glu leu glu lys phe glu pro leu ser thr arg ile glu ala thr ile tyr lys gln ser ser met ile

1891  GAT GAC ATC AAA GCC AAG CTA GAT GAA ATT TTT CAC CTT TCA AAT TTC AAG GAT AAA TCT TCT GGG GAA GAA AAA TTT TTA GAA GAT CGT
 631  asp asp ile lys ala lys leu asp glu ile phe his leu ser asn phe lys asp lys ser ser gly glu glu lys phe leu glu asp arg

1981  AAG AAT TTT TTT GAT AAG CTG CAA GAA GCA GTG AAA TCA TTC AGT ATT TTT GCA TCC GAC TTG CCA AAA GGA ATA GAG TTC TAT GAT TCA
 661  lys asn phe phe asp lys leu gln glu ala val lys ser phe ser ile phe ala ser asp leu pro lys gly ile glu phe tyr asp ser

2071  TTA TTC AAT ATG AGT AGA GAC TTA GCA GAA AGA GTG AGA GTT GCA AAG CAG ACC GAG GAT TCA ACA GCT AAT TCT CCC GCT CCT CCC CTC
 691  leu phe asn met ser arg asp leu ala glu arg val arg val ala lys gln thr glu asp ser thr ala asn ser pro ala pro pro leu

2161  CCT CCA CTT GAT TCT AAA GCG TCT GTC GTT GGG GGT CCT CCA TTA CTG CCC CAA AAA AGT GCA GCC TTT CAG TCA TTA TCT AGA CAA GGG
 721  pro pro leu asp ser lys ala ser val val gly gly pro pro leu leu pro gln lys ser ala ala phe gln ser leu ser arg gln gly

2251  CTC AAT TTA GGG GAC CAA TTT CAA AAT CTC AAA ATA AGT GCC GGC AGT GAT TTA CCT CAA GGA CCC GGT ATT CCA CCA AGA ACT TAT GAA
 751  leu asn leu gly asp gln phe gln asn leu lys ile ser ala gly ser asp leu pro gln gly pro gly ile pro pro arg thr tyr glu

2341  GCT TCG CCA TAT GCT GCA ACG CCT ACT ATG GCA GCC CCA CCA GTA CCA CCG AAA CAA TCG CAA GAG GAT ATG TAC GAC TTG AGA AGA CGT
 781  ala ser pro tyr ala ala thr pro thr met ala ala pro pro val pro pro lys gln ser gln glu asp met tyr asp leu arg arg arg

2431  AAA GCA GTT GAA AAC GAA GAA CGT GAA CTG CAA GAG AAT CCT ACG TCC TTT TAC AAT AGA CCC TCT GTT TTT GAT GAA AAT ATG TAC TCC
 811  lys ala val glu asn glu glu arg glu leu gln glu asn pro thr ser phe tyr asn arg pro ser val phe asp glu asn met tyr ser

2521  AAA TAC AGC AGT
 841  lys tyr ser ser

2581  TAGGTGCATT TCTTATTTCT TAATTTTATT GTAGCTTTTT TTTTTCAACT AATATGATAA TTATATATAT ACCGGATTTG TTGAAAGCCA GTTTTGCAAG GATAAAGTAA
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FIG. 2. Sequences of the *BRO1* gene and its predicted protein product.

```
Bro1p    1 MKPY.LFDLKLKDTEKLDWKKGLSSYLKKSYGSSQWRTFYDEKATSELDH 49
Ce98     1 MATFGFLSAPLKSTNEVDLVKPLTSYIDNVYNTSDNNRSDVAEAVQELNK 50

Bro1p   50 LRNNANGELAPSSLSEQNL..KYYSFLEHLYFRLGSKGSRLKMDFTWYDA 97
Ce98    51 LRSKACCQPLDKHQSALDVLTRYYDQLVAIENKIIISATQNPVVFKWKDA 100

Bro1p   98 ....EYSSAQKGLKYTQHTLAFEKSCTLFNIAVIFTQIARE...NINEDY 140
Ce98   101 FDKGSLFSSRASLSLSDG..SFERAAVLFNIGSLMSQIGAAQQFHTDDEI 148

Bro1p  141 KNSIANLTKAFSCFEYLSENFL....NSPSVDLQSENTRFLANICHAEAQ 186
Ce98   149 KVSAKLFQQSAGVFARLRDVVLGMVQQEPTPDLMPDTLAALSALMTAQAQ 198

Bro1p  187 ELFVLKLLNDQISSKQYTLISKLSRATCNLFQKCHDFMKEIDDDVAIYGE 236
Ce98   199 EAIYIKGHKDKMKA...TSMVKISAQVAEFYSEAQKMMS..KDIVRGLWD 243

Bro1p  237 PKWKTTVTCKLHFYKSLSAYYHGLHLEEENRVGEAIAFLDFSMQQLISSL 286
Ce98   244 KDWSAIVSGKNLAYQALAQYHQSEVCGEARQIGEQLSRLAESLKLFDTAQ 293

Bro1p  287 PFKTWLVEFIDFDGFKETLEKKQKELIKDNDFIYHESVPAVVQVDSIKAL 336
Ce98   294 KYLPR..DITGIWDIYPSVSKAHAAAKKDNDFIYHEKVSDFRTLPTLPKA 341

Bro1p  337 DAIKSPTWEKILEPYMQDVANKYDSLYRGIIPLDVYEKESIYSEEKATLL 386
Ce98   342 VLAKPTPMQTPMTPSFRD......MFAVLVPVQVHNAMQSYDARKAELV 384

Bro1p  387 RKQVEETETANLEYSSFIEFTNLPRLLSDL.......EKQFSDGNIFSNT 429
Ce98   385 NMETVRMREATQLMNGVLASLNLPAALDDVTSTETLPESLKLKSAKLKQN 434

Bro1p  430 DTQGQLMR..DQIQTWCKF...IQTNEFRDIEEQMNKIVFKRKQILEILS 474
Ce98   435 GGSSEIMRLFSELPTLYQRNEDILTETSRILNEEKESDDTMRKQLGTKWT 484

Bro1p  475 ALPNDQ.....KENVTKLKSSLVAASNSDEKLFACVKPHIVEINLLNDNG 519
Ce98   485 RMSSEQLTGPLVTEIGKYRGILHTASNADKMVKEKFESHRQGIELLSKNE 534

Bro1p  520 KIWKKFDEFNRNTPPQPSLLDIDDTKNDKILELLKQVKGHAEDLRTLKEE 569
Ce98   535 ......SELRSSIPGQTA....HATGETDTVRQLRQFMSQWNEVTTDREL 574

Bro1p  570 RSRNLSELRDEINNDDITKLLIINKGKSDVELKDLFEVELEK....FEPL 615
Ce98   575 LEKELKNTNCDIAND.......FLKAMAENQLINEEHISKEKIAQIFGDL 617

Bro1p  616 STRIEATIYKQSSMIDDIKAKLDEIFHLSNFKDKSSGEEKFLEDRKNFFD 665
Ce98   618 KRRVQSSLDTQETLMNQIQAANNT...FTGEKTGSSTG....AERERILK 660

Bro1p  666 KLQEAVKSFSIFASDLPKGIEFYDSLFNMSRDLAERVR...VAKQTE... 709
Ce98   661 MLAQASDAYVELKANLEEGTKFYNDLTPILVRLQQKVSDFAFARQTEKED 710

Bro1p  710 ..................................DSTANSPAPPLPP 722
Ce98   711 LMRQLQLSIVSGQAAKAVVDGVNSLVSSYLTGGTNAAQSPANAPPRPPPP 760

Bro1p  723 LDSKASVVGGPP.........LLPQKSAAFQSLSRQGLNLGDQFQNLKIS 763
Ce98   761 RPAAPSVESPIPPPRTQQSMQATPGAPPQYNPYQQQQQPQMQQFQQHPGY 810

Bro1p  764 AGSDLPQGPGIP..PRTYEASPYAATPTMAAPPVP.....PKQSQEDMYD 806
Ce98   811 YQQPMPYGQPQPMFQPQYQPTFAAPYPTFPGAFPSYQQQWPQQQQQGGFP 860

Bro1p  807 LRRRKAVENEERELQENPTSFYNRPSVFDENMYSKYSS 844
Ce98   861 PNPQFGQQNQQQGGGGGANPFQ................ 882
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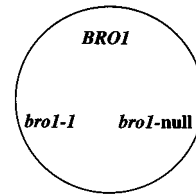
FIG. 3. Sequence comparison of Bro1p and a putative 98-kDa polypeptide from *C. elegans*. Amino acid sequences derived from nucleotide sequences were aligned by the method of Needleman and Wunsch (34). Identical residues are indicated with vertical lines. Putative SH3 domain-binding motifs are underlined.



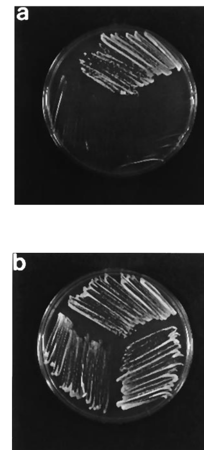


FIG. 4. Complementation of temperature-sensitive growth of *bro1* mutants by additional copies of *BCK1*. Yeast strains MYY571 (*BRO1*), MYY575 (*bro1-1*), and MYY579 (*bro1*-null) were cultured in different sectors as shown at the top for 2 days at 37°C on YPD agar medium. (a) Untransformed cells; (b) cells transformed with the *BCK1*-containing plasmid p2-2.

postlogarithmic phases. The growth rates of cultures of *bro1* mutant cells at 30°C during logarithmic phase were indistinguishable from that of an isogenic *BRO1* strain (data not shown). At higher culture densities, however, *bro1*-null mutants displayed aberrant growth patterns (Fig. 6). In wild-type cultures, the number of viable cells rapidly increased at OD_{600} values below 10 and showed only modest changes at higher optical densities. This pattern reflects an initial stage of rapid cell division followed by a period of slower cell division and increased nutrient storage. In *bro1*-null cultures, the number of viable cells continued to increase rapidly at OD_{600} values above 10, nearly doubling between 12 and 24 h, and then decreased to less than half its peak value by 48 h. A similar but less severe effect was observed in a culture of *bro1-1* cells (data not shown). These results suggest that a defective regulation of cell proliferation in response to nutrient limitation leads to decreased viability of *bro1* cells.

Microscopic analysis of *bro1* cells in postlogarithmic phase cultures revealed morphological abnormalities among budded cells. Cells with elongated buds were observed in both *bro1-1* and *bro1*-null cultures but not in wild-type cultures grown at 30°C for 4 or 9 days in YPD liquid medium (Fig. 7). Twenty-two percent ($n = 386$) of *bro1-1* and 9% ($n = 436$) of *bro1*-null cells in the 4-day experiment had buds with lengths longer than the longest diameter of the mother cell, whereas the ratio of bud length to diameter of the mother portion of the cell never exceeded 1 in wild-type cells ($n = 415$). Most *bro1* cells with elongated buds were nonrefractile by phase-contrast microscopy (Fig. 7), suggesting a failure to enter stationary phase (48). *bro1* cells with elongated buds were observed within 4 days of growth on either solid or liquid YPD medium, in the presence or absence of 1 M sorbitol, and at 23, 30, or 37°C. Similar phenotypes have been reported for *pkc1*-null cells and

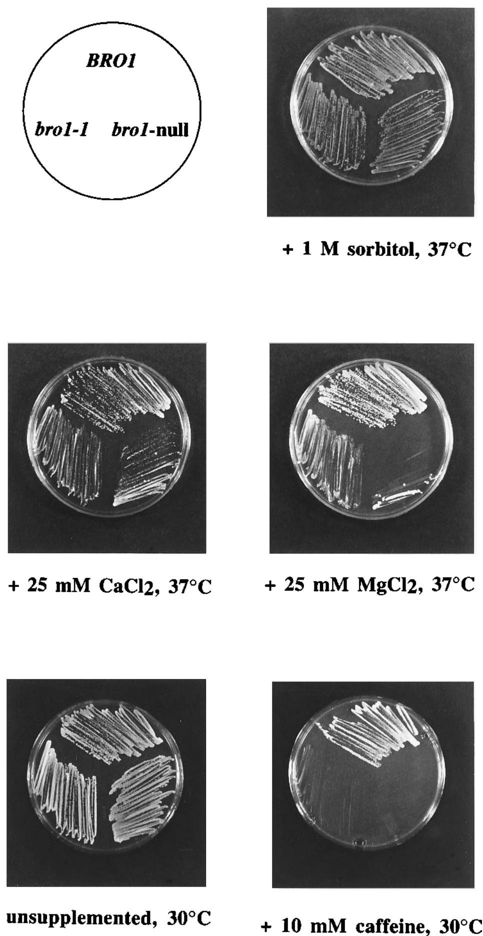


FIG. 5. Growth properties of *bro1* mutants. Yeast strains MYY571 (*BRO1*), MYY575 (*bro1-1*), and MYY579 (*bro1*-null) were cultured in different sectors as shown at the top left on YPD-agar medium with various supplements at either 37 or 30°C. Cells cultured at 37°C were photographed after 2 days, and cells cultured at 30°C were photographed after 3 days.

in cells lacking both *NHP6A* and *NHP6B*, a pair of genes encoding functionally redundant HMG1-like proteins which act downstream of Mpk1p (10, 20, 37). The development of these highly elongated buds may indicate a failure to cease polarized cell growth or may be a response to nutrient scarcity prior to cell death. Taken together, these results demonstrate that *BRO1* is required for normal cellular response to nutrient limitation.

Genetic interactions of *BRO1* with *PKC1*, *BCK1*, and *MPK1*. To investigate further genetic interactions between *BRO1* and components of the Pkc1p-MAP kinase cascade, the phenotypes of strains lacking *BRO1* and either *PKC1*, *BCK1*, or *MPK1* were examined. As strains with null mutations in several genes in this pathway have been constructed in strain 1788 (derived from strain EG123), *BRO1* was disrupted in this strain background to generate isogenic mutant strains. The *bro1*-null mutation does not result in temperature-sensitive lethality in this strain background but does confer sensitivity to caffeine at 30°C. Diploid strains which were heterozygous for null mutations in *BRO1* and either *PKC1*, *BCK1*, or *MPK1* were created and induced to sporulate. Spores with the *pkc1*-null allele are inviable on regular YPD medium at all temperatures but grow on osmotically supported medium (24, 26). Twenty tetrads from the *BRO1*/*bro1*-null *PKC1*/*pkc1*-null strain were dissected onto YPD medium containing 1 M sorbitol, and the spores were cultured at 30°C. None of the 18 *bro1*-null *pkc1*- null spores were viable (Table 2). *bck1*-null and *mpk1*-null spores are viable on regular YPD medium at 30°C but grow more slowly than do wild-type spores (9, 22, 23). Thirty-seven tetrads from the *BRO1*/*bro1*-null *BCK1*/*bck1*-null strain and 25 from the *BRO1*/*bro1*-null *MPK1*/*mpk1*-null strain were dissected onto regular YPD, and the spores were cultured at 30°C. Thirty-four of 36 *bro1*-null *bck1*-null spores and all 26 *bro1*-null *mpk1*-null spores were inviable (Table 2). Both *bro1*-null *bck1*-null and *bro1*-null *mpk1*-null spores were viable when germinated at 30°C on YPD medium containing 1 M sorbitol, and cells grown from these spores were capable of growth on this medium at 37°C. These results indicate that synthetic lethality results from the combination of *bro1*-null with either *pkc1*-null, *bck1*-null, or *mpk1*-null mutation.

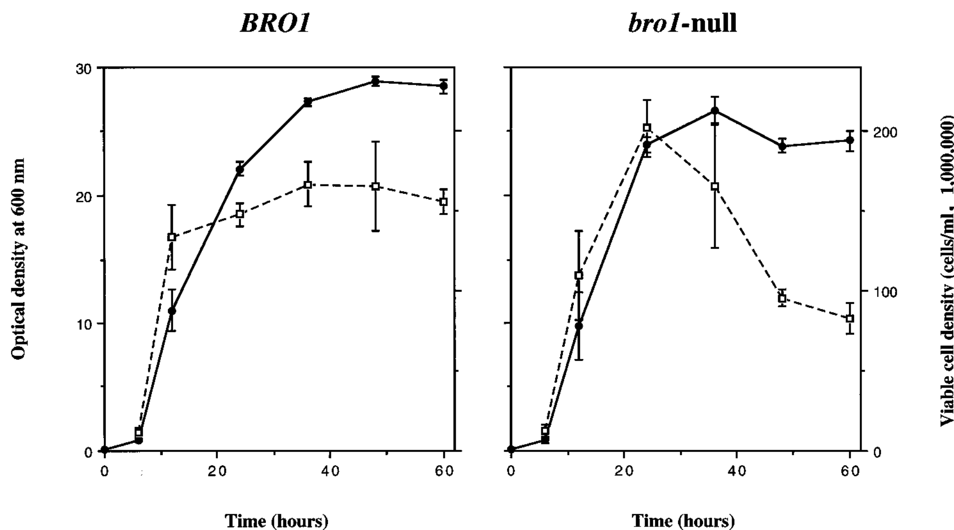


FIG. 6. Decreased viability of *bro1*-null cells at high culture density. Yeast strains MYY571 (*BRO1*) and MYY579 (*bro1*-null) were cultured at 30°C in YPD liquid medium with vigorous shaking. Samples of each culture were taken at indicated time points and diluted appropriately to determine OD_{600} (circles) and viable cell densities (squares). Cell viability was determined by methylene blue staining as described in Materials and Methods. Each datum point represents the mean ± standard deviation for values obtained from three separate cultures.

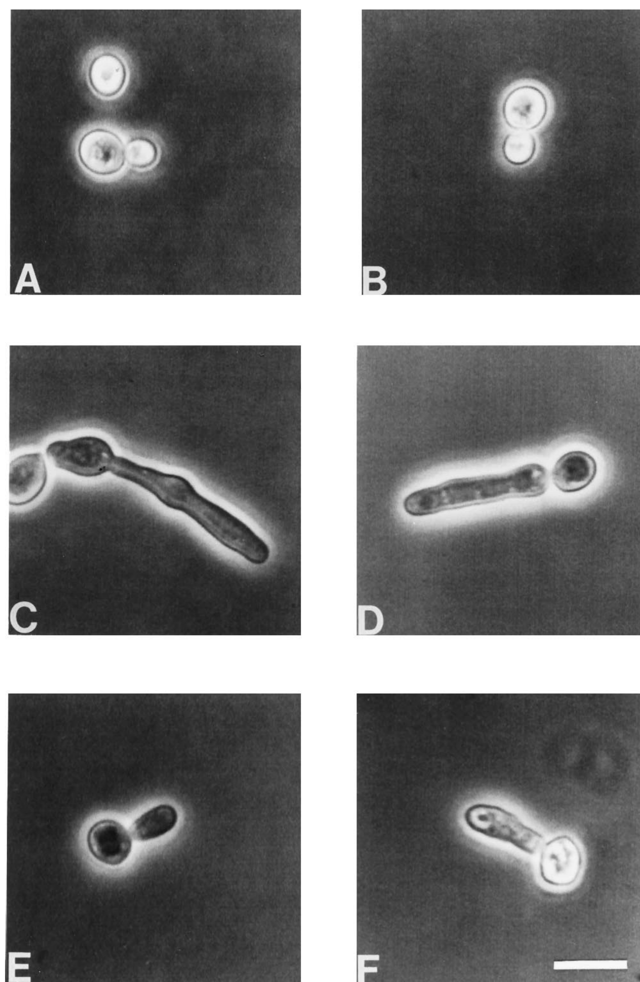


FIG. 7. Elongated bud morphology of *bro1* cells in postlogarithmic phase cultures. Yeast strains MYY571 (*BRO1*; A and B), MYY575 (*bro1-1*; C and D), and MYY579 (*bro1*-null; E and F) were cultured at 30°C in YPD liquid medium for 4 days. Cells were viewed by phase-contrast microscopy. *BRO1* cells shown are representative of total population; *bro1-1* and *bro1*-null cells are representative of a subpopulation of cells with elongated buds (see text). The bar represents 10 μm.

TABLE 2. Synthetic lethality of the *bro1*-null mutation

Parental genotype	Spore phenotype	No. of spores	
		Viable	Total
BRO1/bro1::*URA3 PKC1/pkc1*::*LEU2*[a]	Ura^- Leu^-	18	18
	Ura^+ Leu^-	21	22
	Ura^- Leu^+	15	22
	Ura^+ Leu^+	0	18
BRO1/bro1::*URA3 BCK1/bck1*::*LEU2*[b]	Ura^- Leu^-	36	36
	Ura^+ Leu^-	36	38
	Ura^- Leu^+	29	38
	Ura^+ Leu^+	2	36
BRO1/bro1::*URA3 MPK1/mpk1*::*TRP1*[c]	Ura^- Trp^-	26	26
	Ura^+ Trp^-	24	24
	Ura^- Trp^+	19	24
	Ura^+ Trp^+	0	26

[a] Strain MYY557.
[b] Strain MYY567.
[c] Strain MYY596.

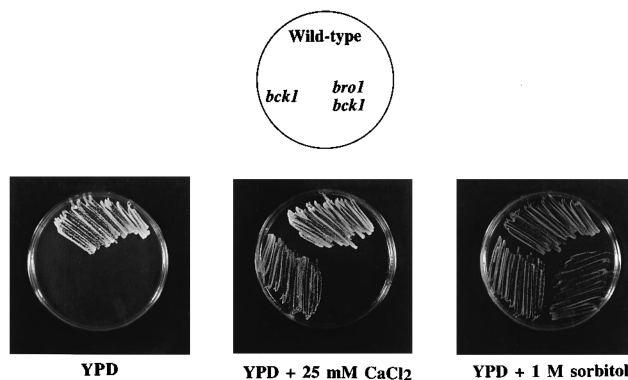


FIG. 8. Calcium-mediated rescue of temperature-sensitive growth of the *bck1*-null mutant is *BRO1* dependent. Isogenic yeast strains 1788 (wild type), MYY590 (*bck1*), and MYY591 (*bro1 bck1*) were cultured in different sectors as shown at the top at 37°C for 2 days on the media indicated. Homozygous diploid strains were used because haploid *bck1*-null mutants accumulate recessive extragenic suppressor mutations at a high frequency (23).

Null mutations in several other genes confer synthetic phenotypes when combined with mutations in the Pkc1p-MAP kinase pathway. These include mutations in a pair of genes, *PPZ1* and *PPZ2*, that encode structurally similar protein phosphatases (21, 38) and a mutation in *BCK2*, a gene encoding a serine/threonine-rich protein of unknown function (21). Double-mutant *bro1 ppz1*, *bro1 ppz2*, and *bro1 bck2* strains were tested for growth on YPD medium at both 30 and 37°C, and the triple-mutant *bro1 ppz1 ppz2* strain was tested for growth on YPD at 30°C. No synthetic phenotypes were observed for any of the mutation combinations (data not shown).

The temperature-sensitive growth defects of *bro1* mutants in the AH216 strain background are complemented by additional copies of *BCK1* (see above). Multicopy plasmids containing *PPZ2*, *BCK2*, or *KRE6*, all of which have been shown to suppress phenotypes caused by mutations in the Pkc1p-MAP kinase pathway (21, 40), were tested for the ability to complement the temperature-sensitive growth of *bro1* mutants. None of these plasmids were able to rescue *bro1* cells. In addition, a multicopy plasmid encoding *PKC1* also failed to rescue growth of *bro1* cells.

Rescue of *bck1*-null cells by Ca^{2+} depends on *BRO1*. The temperature-sensitive growth of the *bck1*-null mutant can be rescued by the addition of 100 mM $CaCl_2$ to the growth medium (as described above). At this salt concentration, it is unclear whether the rescue of *bck1*-null cells is due to an osmotic effect or a specific effect of one of the ions. The addition of 25 mM $CaCl_2$ to YPD medium supported growth of *bck1*-null cells at 37°C (Fig. 8). The *bck1*-null cells failed to grow at this temperature on YPD supplemented with either 50 mM KCl or 75 mM sorbitol (data not shown), indicating that the rescue of *bck1*-null cells at 37°C is due specifically to Ca^{2+}.

To test whether this Ca^{2+}-mediated restoration of growth required *BRO1*, *bck1*-null and *bro1*-null *bck1*-null cells were cultured at 37°C on YPD supplemented with 25 mM $CaCl_2$. *bck1*-null cells grew into visible colonies under these conditions, whereas *bro1*-null *bck1*-null cells did not (Fig. 8). Both *bck1*-null and *bro1*-null *bck1*-null cells grew at 37°C on YPD supplemented with 1 M sorbitol (Fig. 8). These results demonstrate that the Ca^{2+}-mediated rescue of *bck1*-null cells at 37°C on regular (i.e., lacking osmotic support) medium requires functional *BRO1*.

DISCUSSION

We have described the isolation and analysis of a novel gene, *BRO1*, and presented three lines of evidence for a functional relationship between *BRO1* and components of the Pkc1p-MAP kinase pathway which mediates maintenance of cell integrity in *S. cerevisiae*. First, *bro1* mutants display phenotypes similar to those resulting from mutations directly affecting the Pkc1p-MAP kinase cascade. Similar to null mutations in *BCK1*, mutations in *BRO1* result in a temperature-sensitive, osmoremedial growth defect that can be rescued by Ca^{2+}. Like *bck1*-null and *mpk1*-null mutants, *bro1* mutants are sensitive to caffeine. As seen in populations of *pkc1*-null and *nhp6*-null cells, a significant fraction of *bro1* cells develop highly elongated buds. Finally, both *bck1*-null and *bro1*-null mutants display aberrant responses to nutrient limitation.

Further support for an interaction between *BRO1* and the Pkc1p-MAP kinase pathway is the finding that additional copies of wild-type *BCK1* restore growth of *bro1* cells on regular medium at the nonpermissive temperature. The presumed effect on cells having additional copies of *BCK1* is an amplification of the signal transmitted through this MAP kinase pathway. This explanation is consistent with the observation that Ca^{2+} also restores growth of *bro1* cells at the nonpermissive temperature, as previous studies have suggested that the addition of Ca^{2+} to the growth medium causes an increase in Pkc1p activity (24) and, presumably, in the downstream components of the MAP kinase pathway.

A third line of evidence for a genetic interaction between *BRO1* and the Pkc1p-MAP kinase pathway is the synergistic effect of null mutations in *BRO1* and either *PKC1*, *BCK1*, or *MPK1*. Deletion of *BRO1* renders these genes essential for viability under growth conditions in which they would otherwise be dispensable. Moreover, *bro1* and *bck1* mutants are viable at 37°C on YPD with 25 mM $CaCl_2$, whereas the *bro1 bck1* mutant is not. Synthetic lethality between null mutations may indicate that the two gene products or the pathways in which they act perform related functions. For example, proper assembly of the actin cytoskeleton in yeast requires Cap2p, β subunit of the capping protein (3), and Sac6p, the yeast fimbrin homolog (2). Cells with null mutations in either *SAC6* or *CAP2* are viable and display similar alterations in actin distribution, but the *sac6 cap2* mutant is inviable (1, 19).

Two models may explain the functional relationship between Bro1p and the Pkc1p-MAP kinase cascade. One possibility is that Bro1p and the Pkc1p-MAP kinase pathway produce signals which converge to activate one or more common targets (Fig. 9A). A similar convergent model which relates the activity of Ppz1p and Ppz2p to the Pkc1p-MAP kinase pathway has been proposed by Lee et al. (21). An alternative model is that Bro1p and the Pkc1p-MAP kinase pathway generate parallel signals to distinct targets which have redundant or overlapping functions (Fig. 9B). An analogous model has been proposed to relate the roles of Kre6p and Pkc1p in cell wall biosynthesis (40). Null mutations in either *KRE6* or *PKC1* cause a substantial decrease in the level of the cell wall polymer (1→6) β-glucan, and together the two mutations confer lethality. Overexpression of *KRE6* rescues the osmotic sensitivity of the *pkc1*-null mutant but has no effect on the level of (1→6) β-glucan in these cells, suggesting that Kre6p and Pkc1p have distinct but partially redundant functions in cell wall assembly. The essential feature of both models is that Bro1p is able to function independently of the Pkc1p-MAP kinase pathway. This does not exclude, however, the possibility that direct signalling between Bro1p and the kinase cascade also occurs. For example, the activity of Bro1p may regulate or be regulated by one of the kinase components.

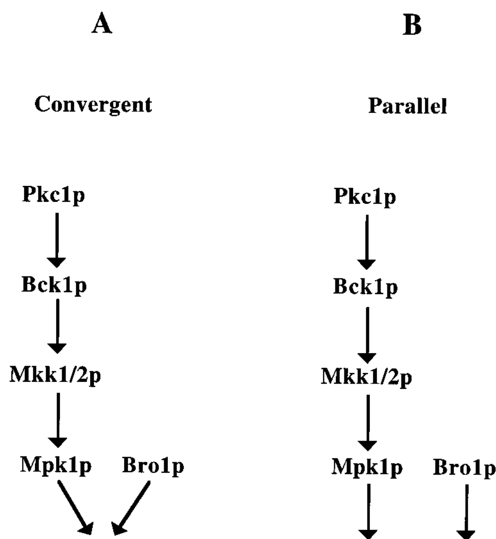


FIG. 9. Models for interaction of Bro1p with the Pkc1p-MAP kinase pathway.

Does Bro1p function in a common pathway with Ppz1p, Ppz2p, and/or Bck2p? Phenotypic analysis of various null mutants generated in yeast strain EG123 provides two pieces of evidence consistent with this possibility. First, similar to *ppz1* and *bck2* mutations, a *bro1* mutation exacerbates the osmotic sensitivity of both *pkc1* and *mpk1* mutants. Second, a *bro1* mutation does not alter the osmotic phenotypes of *ppz1*, *ppz2*, *ppz1 ppz2*, or *bck2* mutants. Alternatively, Bro1p may function in a third cell integrity pathway, distinct from both the kinase and phosphatase pathways. Comprehensive epistasis and combinatorial-mutant experiments should resolve this question at the genetic level.

How does Bro1p function at the molecular level? The presence of a proline-rich motif near the carboxy terminus of Bro1p suggests that it may interact directly with SH3 domain-containing proteins. Five *S. cerevisiae* genes encoding proteins with SH3 domains have been identified to date: *ABP1*, *BEM1*, *RVS167*, *SLA1*, and *SHO1* (4, 7, 11, 15, 29). It is interesting that the products of several of these genes are involved in processes which are defective in *bro1* cells. For example, Bem1p and Sla1p are required for polarized bud growth (7, 27), Sho1p is involved in osmosensory signaling (29), and Rvs167p is required for proper response to nutrient deprivation (4). A more precise understanding of the role of Bro1p in these processes will emerge from future studies of the interaction of this protein with other cellular components.

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