Yeast *src* Homology Region 3 Domain-binding Proteins Involved in Bud Formation

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Abstract. The yeast protein Bem1p, which bears two *src* homology region 3 (SH3) domains, is involved in cell polarization. A Rho-type GTPase, Rho3p, is involved in the maintenance of cell polarity for bud formation, and the *rho3* defect is suppressed by a high dose of *BEM1*. Mutational analysis revealed that the second SH3 domain from the NH_2 terminus (SH3-2) of Bem1p is important for the functions of Bem1p in bud formation and in the suppression of the *rho3* defect. Boi2p, which bound to SH3-2 of Bem1p, was identified using the two-hybrid system. Boi2p has a proline-rich sequence that is critical for displaying the Boi2p–Bem1p two-hybrid interaction, an SH3 domain in its NH_2-terminal half, and a pleckstrin homology domain in its COOH-terminal half. A *BOI2* homologue, *BOI1*, was identified as a gene whose overexpression inhibited cell growth. Cells overexpressing either *BOI1* or *BOI2* were arrested as large, round, and unbudded cells, indicating that the Boi proteins affect cell polarization. Genetic analysis revealed that *BOI1* and *BOI2* are functionally redundant and important for cell growth. *Δboi1 Δboi2* cells became large round cells or lysed with buds, displaying defects in bud formation and in the maintenance of cell polarity. Analysis using several truncated versions of *BOI2* revealed that the COOH-terminal half, which contains the pleckstrin homology domain, is essential for the function of Boi2p in cell growth, while the NH_2-terminal half is not, and the NH_2-terminal half might be required for modulating the function of Bem1p. Overproduction of either Rho3p or the Rho3p-related GTPase Rho4p suppressed the *boi* defect. These results demonstrate that Rho3p GTPases and Boi proteins function in the maintenance of cell polarity for bud formation.

During bud formation in the yeast *Saccharomyces cerevisiae*, cell polarity is established for the initiation of bud emergence and it is maintained during bud growth. Patches of actin filaments become concentrated at the bud site, towards which the transport of secretory vesicles is directed for the construction of the daughter cell (Tkacz and Lampen, 1972; Field and Schekman, 1980; Pringle and Hartwell, 1981; Cabib et al., 1982; Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985; Pringle et al., 1986; Drubin, 1991). The establishment and maintenance of cell polarity require the functions of Rho-type GTPases Cdc42p, Rho3p, and Rho4p, which belong to the Ras superfamily (Johnson and Pringle, 1990; Matsui and Toh-e, 1992*a,b*; Imai et al., 1996). GTPases of the Ras superfamily act as molecular switches through their conformational change between the GTP-bound active form and GDP-bound inactive form (Barbacid, 1987; Bourne et al., 1991; Boguski and McCormick, 1993). Defects in either *CDC42* or *CDC24*, which encodes a GTP–GDP exchange factor for Cdc42p, disrupt the asymmetric localization of actin filaments and cause cells to become unbudded, large, and round, an indication that Cdc42p and Cdc24p are essential for the establishment of cell polarity (Sloat and Pringle, 1978; Sloat et al., 1981; Adams et al., 1990; Johnson and Pringle, 1990; Zheng et al., 1994).

Defects in *RHO3* cause severe growth defects. Disruption of *RHO4*, which encodes a Rho3p-related GTPase, enhances the growth defect of *Δrho3* cells (Matsui and Toh-e, 1992*a*). Temperature-sensitive *rho3* mutant cells lose cell polarity at nonpermissive temperatures: the asymmetric localization of actin filaments is disrupted in the *rho3* cells, and the *rho3* cells are arrested as large, round cells, although, in contrast to *cdc42* mutant cells, not all of these cells are arrested as unbudded cells (Imai et al., 1996). Depletion of both Rho3p and Rho4p results in lysis of cells that have small buds (Matsui and Toh-e, 1992*b*). These observations strongly suggest that Rho3p is required for the maintenance of cell polarity for bud growth.

The *rho3* defect is suppressed by the overexpression of *BEM1*, an indication that Bem1p has functions that affect

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the Rho3 pathway (Matsui and Toh-e, 1992*b*). *BEM1* encodes a protein with two copies of the *src* homology region 3 (SH3)[1] domain (SH3-1 and SH3-2; see Fig. 1 *A*). Disruption of *BEM1* results in temperature-sensitive growth. At nonpermissive temperatures, *Δbem1* cells become unbudded, large, and round, with the loss of actin polarization, indicating that Bem1p is involved in cell polarization (Bender and Pringle, 1991; Chenevert et al., 1992). Bem1p can bind to Cdc24p independently of its SH3 domains (Peterson et al., 1994). Cdc24p binds to the Ras-type GTPase Rsr1p/Bud1p, which is required for the proper selection of bud sites (Chant and Herskowitz, 1991; Bender and Pringle, 1989; Zheng et al., 1995). These findings lead to the possibility that Bem1p is a component of the protein complex that is needed for bud emergence. Since SH3 domains mediate protein–protein interactions (e.g., Cicchetti et al., 1992), it is possible that Bem1p recruits another component to the complex via its SH3 domains.

To clarify the role of the SH3 domains of Bem1p, we characterized several *bem1* mutants that were defective in these domains. Using the two-hybrid system, we identified Boi2p as a protein that bound to the SH3 domain and was important for the function of Bem1p. In addition, Boi1p, which was functionally redundant with Boi2p, was identified by its inhibitory effect on bud emergence. Our genetic and morphological studies indicate that the function of Boi protein is related to the Rho3 pathway and is important for bud growth. We present a model in which Boi proteins and Rho3p are involved in the modulation of the Bem1p-containing complex for bud growth.

Materials and Methods

Microbiological Techniques

Rich medium containing glucose (YPD), synthetic minimal medium (SD), and synthetic complete medium (SC) are as described (Sherman et al., 1986). YPGal and SCGal are YPD and SC, respectively, except that 2% glucose is replaced with 5% galactose and 0.3% sucrose. YPGal0.1 is YPGal, except that the concentration of galactose is 0.1%. SC-U and SCGal-U are SC and SCGal, respectively, without uracil. SC-L and SCGal-L are SC and SCGal, respectively, without leucine. SC-UT and SCGal-UT are SC and SCGal, respectively, without uracil and tryptophan. Yeast transformations were performed by the method of Ito et al. (1983).

Strains and Plasmids

The yeast strains used are listed in Table I. Plasmid pBTM116 is a high copy number plasmid that harbors the 2-μm DNA origin *TRP1* and the sequence for the lexA DNA-binding domain. pGAD424 is a high copy number plasmid that harbors the 2-μm DNA origin *LEU2* and the sequence for the Gal4 *trans*-activation (Gal4 TA) domain (Fields and Sternglanz, 1994). Plasmid pRS316-RHO3 carries a 1.8-kb KpnI-XhoI fragment that encompasses the *RHO3* gene on pRS316, a low copy number plasmid harboring *URA3* (Sikorski and Hieter, 1989). Plasmid pKT10 is a high copy number plasmid that carries the 2-μm DNA origin *URA3*, the *TDH3* promoter before a unique EcoRI site, termination codons downstream of the EcoRI site, and the *TDH3* terminator (Tanaka et al., 1988). pOPR3 and pOPR1 are pKT10-based plasmids that carry the coding region of *RHO3* and the coding region of *RHO1*, respectively, under the control of the TDH3 promoter (Matsui and Toh-e, 1992*b*). Plasmid pOPR4 is a high copy number plasmid that carries the coding region of *RHO4* under the control of the *PYK1* promoter (Matsui and Toh-e, 1992*a*). Plasmid pKT10mycN was constructed by inserting the sequence for the initiator methionine, a myc epitope tag (EQKLISEEDL), and a multicloning site into the EcoRI site of pKT10. The KpnI-KpnI, BamHI-KpnI, HpaI-KpnI, and KpnI-DraI fragments carrying the *BEM1* sequence (see Fig. 1 *A*) were inserted in frame into the multicloning site of pKT10mycN to generate pBEM1KK, pBEM1Δ1, pBEM1Δ1Δ2, and pBEM1ΔC, respectively. The BamHI-SalI fragment carrying the sequence for SH3-2 was removed from pBEM1KK and pBEM1ΔC to create pBEM1Δ2 and pBEM1Δ2ΔC, respectively, and was religated in frame after blunting the overhangs. *BEM1* in pBEM1KK lacked the sequence for the 45 NH$_2$-terminal amino acids but was able to serve as a multicopy suppressor of *rho3* (see Fig. 1 *B*, sector *2*). The DNA sequences of the fragments, which were derived from the PCR (Saiki et al., 1988) or oligonucleotide-directed mutagenesis and used for generating plasmids, were determined to confirm precise replication during each procedure. Nucleotide sequences were determined by the method of Sanger et al. (1977).

Construction of bem1 Mutants with Mutations in the SH3 Domain

The 2.3-kb fragment, derived from pSRO1 (Matsui and Toh-e, 1992*b*), from the BamHI site in the *BEM1* coding region to the BamHI site in the 3′ noncoding region, was inserted into a derivative of pBluescriptII that had been constructed from pBluescriptII KS$^+$ (Stratagene, La Jolla, CA) by removal of the sequence between the EcoRV and XhoI sites. The 1.1-kb HindIII-HindIII fragment carrying *URA3* was inserted into the HindIII site (in the 3′ noncoding region of *BEM1*) of the resultant plasmid to generate YIpUBEM1C. The 0.6-kb fragment, derived from pSRO1, from the SmaI site in the 5′-coding region of *BEM1* to the SalI site in the sequence for SH3-2, was inserted between the SmaI site and SalI site (in the sequence for SH3-2) of YIpUBEM1C to generate YIpUBEM1. Proline 123 in SH3-1 and proline 208 in SH3-2 were replaced with leucine to generate *bem1*$^{\mathrm{Leu123}}$ and *bem1*$^{\mathrm{Leu208}}$, respectively, with an oligonucleotide-directed in vitro mutagenesis system (Amersham Corp., Arlington Heights, IL) using the primers 5′-AAAATAGGTCTTAAGGACAACACC (for *bem1*$^{\mathrm{Leu123}}$), 5′-AAACCCAACTAGTACAAGGCCG (for *bem1*$^{\mathrm{Leu208}}$), and YIpUBEM1 as the template. For the construction of *bem1-ΔSH3s*, a truncated version of *bem1* without the sequence for both SH3-1 and SH3-2, the fragment from the SmaI site in the 5′ noncoding region to the KpnI site in the coding region was inserted in frame between the SmaI and SalI sites of YIpUBEM1C after blunting the overhangs to generate YIpUBEM1ΔSH3s, which lacked the sequence between the KpnI and SalI sites of YIpUBEM1. For the construction of *bem1-ΔC*, the 1.0-kb SalI-HindIII fragment from pBEM1ΔC, which contained the *BEM1* sequence between the SalI and DraI sites, the termination codons after the DraI cleavage site, and the *TDH3* terminator, was inserted between the SalI and HpaI sites of YIpUBEM1 after blunting the overhangs of the HindIII cleavage site. For the replacement of the wild-type *BEM1* allele with a mutant allele, each derivative of YIpUBEM1, digested with SmaI and XbaI, was introduced into the cells. Replacement was confirmed by PCR.

Isolation of BOI2 and Assay of the Two-hybrid Interaction

The construction of plasmids for the lexA DNA-binding domain fused with Bem1p (lexA-Bem1p) was performed as follows. An EcoRI site was introduced before the initiator methionine codon of *BEM1* by oligonucleotide-directed in vitro mutagenesis. The EcoRI-HpaI fragment of *BEM1* was inserted into the multicloning site of pBTM116 in frame to generate plexSHs. The SalI-SalI fragment that contained the sequence for the COOH-terminal Bem1p and the 3′ noncoding region of *BEM1* from pBEM1KK was inserted between the SalI sites of plexSHs to create plexBEM1. Yeast strain L40, with two reporter genes (*lexA-lacZ* and *lexA-HIS3*), was transformed with plexBEM1 and then with a yeast genomic library in which yeast genomic DNA was expressed as fusion proteins with the Gal4 TA domain (Chien et al., 1991). The transformants were streaked on SD+3AT plates, which are SD plates containing 100 μg/ml adenine sulfate and 40 mM 3-aminotriazole, an inhibitor of imidazoleglycerolphosphate dehydratase (His3p). Plasmids were recovered from colonies that formed on plates after incubation at 25°C for 1 wk. The recovered plasmids were reintroduced into the L40 strain harboring plexBEM1 to examine whether the plasmids could induce the expression of the reporter genes. *HIS3* expression was assessed by the formation of L40 cell colonies on SD+3AT plates after incubation at 25°C for 1 wk.

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[1] *Abbreviations used in this paper*: a.a., amino acid; GST, glutathione *S*-transferase; PH, pleckstrin homology; SC, synthetic complete medium; SD, synthetic minimal medium; SH3, *src* homology region 3; YPD, rich medium containing glucose.

Table I. Yeast Strains Used in this Study

Strain	Genotype	Reference or source
W303	*MATa/MATα ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 lys2/lys2 ade2/ade2*	Sutton et al. (1991)
W303-1A	*MATa ura3 leu2 his3 trp1 lys2 ade2*	segregant of W303
YMR505	*MATa ura3 leu2 trp1 lys2 ade2 Δrho3::LEU2 pGAL7:RHO4*	Matsui and Toh-e (1992*b*)
YMS1194	*MATa ura3 leu2 his3 trp1 lys2 ade2* $bem1^{Leu123}$	This study
YMS1199	*MATa ura3 leu2 his3 trp1 lys2 ade2* $bem1^{Leu208}$	This study
YMS1190	*MATa ura3 leu2 his3 trp1 lys2 ade2* $bem1^{Leu123,\ Leu208}$	This study
YMS1201	*MATa ura3 leu2 his3 trp1 lys2 ade2 bem1-ΔC*	This study
YMS1183	*MATa ura3 leu2 his3 trp1 lys2 ade2 bem1-ΔSH3s*	This study
YMB101	*MATa ura3 leu2 his3 trp1 lys2 ade2 Δboi1::HIS3*	This study
YMB102	*MATα ura3 leu2 his3 trp1 lys2 ade2 Δboi1::HIS3*	This study
YMB201	*MATa ura3 leu2 his3 trp1 lys2 ade2 Δboi2::URA3*	This study
YMB202	*MATa ura3 leu2 his3 trp1 lys2 ade2 Δboi2::LEU2*	YMB202 transfromed with a *ura3*-disruption plasmid
YMB1201	*MATa/MATα ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 lys2/lys2 ade2/ade2 Δboi1::HIS3/+ +/Δboi2::LEU2*	YMB102 × YMB202
YMB1207	*MATa leu2 his3 trp1 lys2 ade2 Δboi1::HIS3 Δboi2::LEU2 pGAL7:BOI2*	segregant of YMB1201 transformed with YIpUGAL7BOI2.
YMB1202	*MATa leu2 his3 trp1 lys2 ade2 Δboi1::HIS3 Δboi2::LEU2 BOI2ΔN:URA3*	segregant of YMB1201 transformed with pRS306-BOI2ΔN
DRA4	*MATa/MATα leu2/leu2 ura3/ura3 trp1/+ +/ade6 can1/+ cyh/+* GAL^+/GAL^+	derived from siblings of a cross between X2180-1B (from Yeast Genetic Stock Center) and AM1205-9B (Mitchell and Herskowitz, 1986)
KA31	*MATa ura3 leu2 his3 trp1 lys2*	Irie et al. (1991)
L40	*MATa ade2 his3 leu2 trp1 lexA-lacZ:URA3 lexA-HIS3:LYS2 gal4 gal80*	R. Sternglanz (Stony Brook)

All of the strains listed above, with the exception of YMR505, DRA4, KA31, and L40, are in the W303 background.

lacZ expression was examined by measuring the activity of β-galactosidase in L40 cells with the method described (Miller, 1972). DNA clones carrying *BOI2* were isolated from a yeast genomic library (Matsui and Toh-e, 1992*a*) in λZAPII (Stratagene) by hybridization using the *BOI2* fragment isolated by the two-hybrid method as a probe. pSKBOI2 was a plasmid derived from the yeast genomic library and carried a 6-kb fragment encompassing *BOI2* in pBluescript SK^- (Stratagene).

Construction of Plasmids for the Two-hybrid Interaction Assay

Plasmids for truncated versions of lexA-Bem1p were constructed as follows. plexΔSHs was created by removing the EcoRI-SalI (in the sequence for SH3-2) fragment of plexBEM1, with partial digestion by SalI. plexΔC was constructed by removing the PstI-PstI fragment of plexBEM1. plex-s2, plex-s1, and plex-s12 were constructed by inserting BanI-PstI fragments carrying $bem1^{Leu208}$, $bem1^{Leu123}$, and $bem1^{Leu123,\ Leu208}$, respectively, into the multicloning site of pBTM116. To create plexBEM1ΔC, the 1.0-kb SalI-HindIII fragment from pBEM1ΔC was inserted between the SalI site (in the *BEM1* coding region) and the PstI site (in the multicloning site of pBTM116) of plexSHs after blunting the overhangs of PstI and HindIII cleavage sites. Plasmids for truncated versions of Gal4 TA fused with Boi2p (Gal4TA-Boi2p) were constructed as follows. MluI-BglII, MluI-EcoRI, MluI-AatII, MluI-BanII, and MluI-XbaI fragments from pGADBOI2, a primary isolate carrying *BOI2* (see below), were inserted between the MluI site and the multicloning site of pGAD424 (Fields and Sternglanz, 1994) to create pGADBG2, pGADR1, pGADAA1, pGADBA2, and pGADXB1, respectively. BstEII-AatII and BanII-EcoRI fragments from pGADBOI2 were inserted into the multicloning site of pGAD424 in frame to create pGADE2A2 and pGADB2R1, respectively. pGADΔPRO was constructed as follows. An AatII site was introduced downstream of the 463th codon of *BOI2* in pSKBOI2 by oligonucleotide-directed in vitro mutagenesis, and the resultant plasmid was digested with AatII and religated in frame to generate pSKBOI2ΔPRO. The *BOI2* gene in pSKBOI2ΔPRO lacked the sequence between the introduced AatII site and the original AatII site for the region (a.a. 436–464) that contained the proline-rich sequence (see below). The 2.6-kb XbaI-SalI fragment carrying the *BOI2* coding region from pSKBOI2ΔPRO was inserted between the XbaI and SalI sites of pGADR1 to create pGADΔPRO. The 1.8-kb EcoRI-BamHI fragment carrying the sequence for the COOH-terminal half of Cdc24p (a.a. 167–end) was inserted between the EcoRI and BamHI sites of pGAD424 to create pGADCDC24.

In Vitro Binding Assay

The NciI-EcoRI fragment encoding Boi2p (amino acids [a.a.] 67–545) was inserted into the multicloning site downstream of the sequence for glutathione *S*-transferase (GST) in pGEXKG. Boi2p (a.a. 67–545) fused with GST (GST-Boi2p) was produced by use of this construct and affinity purified with glutathione-agarose beads (Sigma Immunochemicals, St. Louis, MO) as described (Shirayama et al., 1994). About 10^8 yeast cells (wild-type strain KA31) producing myc-tagged Bem1p were washed twice in PBS. The cell pellet was disrupted by blending with glass beads in 400 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2% [vol/vol] Triton X-100, 75 mM NaCl, 0.5 mM PMSF) and was clarified by centrifugation at 14,000 *g* for 15 min at 4°C. 30 μl of the supernatant (as the total cell lysate) was resolved by SDS-PAGE, and 160 μl of the supernatant was mixed with 50 μl GSH-agarose beads with 2 μg immobilized GST-Boi2p (or GST for control) and incubated at 4°C for 3 h. The beads were then washed extensively in washing buffer (50 mM Tris-HCl, pH 7.5, 0.1% [vol/vol] Triton X-100, 10% [vol/vol] glycerol). Bound proteins were eluted with 100 μl elution buffer (50 mM Tris-HCl, pH 9.0, 20 mM glutathione) and 20 μl of the eluate were resolved by SDS-PAGE.

Isolation of BOI1

YEp51B, which carries *LEU2*, the 2-μm DNA origin, and the *GAL10* promoter, was constructed by removing a 0.27-kb BamHI-SalI fragment adjacent to the *GAL10* promoter from YEp51 (Broach et al., 1983). Diploid strain DRA4 was transformed with a yeast genomic DNA library, based on YEp51B, in which yeast genomic DNAs were expressed under the control of the *GAL10* promoter. The resultant transformants that grew well on SC-L, but not on SCGal-L (which contained 2% galactose, instead of 5% galactose and 0.3% sucrose), were selected at 28°C, and the plasmids were recovered (details of the screening will be described elsewhere). pGA68, one of the isolates, carried *BOI1* downstream of the *GAL10* promoter. The *BOI1* gene in pGA68 was expressed from the internal methionine at position 378, as judged from a comparison of the nucleotide sequences of pGA68 and *BOI1* (Bender et al., 1996).

BOI2 Expression Plasmids and Disruption of BOI1 and BOI2

YIpUGAL7 is a YIp plasmid that carries the *GAL7* promoter, *URA3*, and the BglII and SalI sites downstream of the *GAL7* promoter (Matsui and

Toh-e, 1992*b*). A BamHI site was introduced before the initiator methionine codon of *BOI2* by oligonucleotide-directed in vitro mutagenesis, and the BglII site in the 3′ noncoding region of *BOI2* was replaced with a SalI site by insertion of a SalI linker (Takara, Tokyo, Japan). The BamHI-SalI fragment carrying the complete *BOI2* coding region was inserted between the BglII and SalI sites of YIpUGAL7 to create YIpUGAL7BOI2. In this construct, designated *pGAL7:BOI2, BOI2* was expressed under the control of the *GAL7* promoter. YIpUGAL7BOI2 was digested with BglII and religated after blunting the overhangs to create YIpUGAL7BOI2ΔC. From this construct, designated *pGAL7:BOI2ΔC*, Boi2p without its COOH-terminal half (a.a. 697–end) was produced under the control of the *GAL7* promoter. Synthetic oligonucleotides containing the initiator methionine codon and the XhoI and BglII sites were inserted into the BglII site of YIpUGAL7 to create YIpUGAL7f. The 2.1-kb AatII-SalI fragment containing the sequence for the COOH-terminal half of Boi2p (a.a. 466–end) was inserted between the XhoI and SalI sites of YIpUGAL7f after blunting the overhangs of the XhoI and AatII cleavage sites to create YIpUGAL7BOI2ΔN. From this construct, designated *pGAL7:BOI2ΔN*, Boi2p without its NH_2-terminal half was produced under the control of the *GAL7* promoter. An ~6-kb SacII-SalI fragment carrying *BOI2* from pSKBOI2 was inserted into pYO324, a high copy number plasmid that carries 2-μm DNA origin and *TRP1* (Ohya et al., 1991), and into pRS314, a low copy number plasmid that carries *CEN6* and *TRP1* (Sikorski and Hieter, 1989), to create pYO324-BOI2 and pRS314-BOI2, respectively. An AatII site was introduced downstream of the 9th codon of *BOI2* in pSKBOI2 by oligonucleotide-directed in vitro mutagenesis, and the resultant plasmid was digested with AatII and religated in frame to create pSKBOI2ΔN. This construct (*BOI2ΔN*) encodes Boi2p without its NH_2-terminal half (a.a. 10–464). An ~5-kb SacII-SalI fragment carrying *BOI2ΔN* was inserted into pYO324 and pRS306, a YIp plasmid carrying *URA3* (Sikorski and Hieter, 1989), to create pYO324-BOI2ΔN and pRS306-BOI2ΔN, respectively. pYO324-BOI2 and pRS314-BOI2 were digested with BglII and religated after blunting the overhangs to create pYO324-BOI2ΔC and pRS314-BOI2ΔC, respectively. From these constructs, designated *BOI2ΔC*, Boi2p without its COOH-terminal half (a.a. 697–end) was produced. The YIpUGAL7- and pRS306-derived plasmids were digested with StuI and then introduced into cells for targeted integration at the *ura3* locus.

The *BOI2* sequence between the PmlI site (in the 5′ noncoding region) and the SpeI site of pSKBOI2 was replaced with the 1.1-kb *URA3* fragment to create pBOI2Δ. In this construct, the sequence from the position 443 bp upstream of the initiation codon to the 829th codon of *BOI2* was deleted. The plasmid for the disruption of *BOI1* was constructed as follows. The 2.8-kb SalI-HindIII fragment from the insert of pGA68 was inserted into pBluescriptII KS$^+$ to create pKSBOI1. To create pRS306-BOI1, the 2.2-kb BamHI-BamHI fragment that carried the 3′-half of the *BOI1* gene from pGA68 was inserted into the BamHI site of pRS306. To clone the 5′-half of *BOI1*, the yeast genomic DNA in which pRS306-BOI1 had been integrated at the *BOI1* locus was digested with EcoRV, religated, and introduced into *Escherichia coli*. With the recovered plasmid as a template, a 840-bp fragment carrying the 5′ noncoding region of *BOI1* and the sequence for the NH_2-terminal portion (a.a. 1–87) of Boi1p was amplified by PCR. In the PCR, the primer was designed to replace the 87th codon of *BOI1* in the amplified fragment with a stop codon, TAG. The amplified fragment was inserted into the BamHI site of pKSBOI1 to create pKSBOI1-2, which carried a 3.3-kb insert encompassing a version of *BOI1* in which the sequences for a.a. 91–374 were deleted. A 1.8-kb BamHI fragment carrying *HIS3* was inserted into the BglII site in the *BOI1* coding region of pKSBOI1-2 to create pBOI1Δ. In this construct, the *BOI1* gene was disrupted with the termination codon at the 87th codon, with the deletion of the sequence for a.a. 91–374 and with the insertion of *HIS3*. pBOI1Δ and pBOI2Δ were digested with PvuII and introduced into the cells by replacement transformation to disrupt *BOI1* and *BOI2*, respectively. These replacements did not disrupt any open reading frames other than *BOI1* and *BOI2*, as judging from nucleotide sequences. The disruptions were confirmed by Southern analysis and PCR.

Cell Lysis Assay

Cell lysis was assayed by monitoring the leakage of alkaline phosphatase, a yeast intracellular protein, into the culture medium by the method described (Paravicini et al., 1992). Cells were streaked on YPD plates. After incubation, the plates were overlaid with a solution of BCIP (10 mM 5-bromo-4-chloro-3-indolyl phosphate [Sigma], 0.1 M Tris-HCl, pH 9.5, and 1% agar) and incubated for 1 h at 37°C.

Detection of myc Epitope–tagged Protein

Proteins containing the myc epitope tag were detected by Western blotting analysis with anti-myc antibodies as described (Yoko-o et al., 1995).

Morphological Observations

Cells were stained with rhodamine-phalloidin to reveal actin filaments as described (Pringle et al., 1989). Cells were fixed with 5% formaldehyde for 10 min, washed with PBS, stained with 1:50 diluted rhodamine-phalloidin solution (Molecular Probes, Inc., Eugene, OR) for 2 h, and washed five times with PBS. The sample was then mounted in *p*-phenylenediamine (1 mg/ml in 90% glycerol) and observed under an epifluorophotomicroscope (BH-2; Olympus, Tokyo, Japan). Dead cells were stained with methylene blue (Rose, 1975). A solution of methylene blue (0.02% methylene blue, 2% sodium citrate) was mixed with an equal volume of cell culture, and the cells were observed immediately with Nomarski optics.

Results

Bem1p Domains Required for Suppression of the Δrho3 Defect

Overexpression of *BEM1* suppresses the growth defect caused by the disruption of *RHO3* (Matsui and Toh-e, 1992*b*; Fig. 1 *B*, sector *2*). To examine whether the SH3 domains of Bem1p are necessary for the suppression or not, we constructed truncated *BEM1* sequences (shown diagrammatically in Fig. 1 *A*) and analyzed the suppression activities of the various constructs. *Δrho3* cells that overexpressed *BEM1* without the sequence for SH3-1 grew as well as *Δrho3* cells that overexpressed *BEM1* (Fig. 1 *B*, *right*, sectors *2* and *3*), indicating that SH3-1 is not required for the ability of *BEM1* to serve as a multicopy suppressor of *rho3*. However, *Δrho3* cells that overexpressed *BEM1* without the sequence for SH3-2 (Fig. 1 *B*, *right*, sectors *4*, *5*, and *7*) or the COOH terminus of 35 a.a. (Fig. 1 *B*, *right*, sectors *6* and *7*) formed colonies as tiny as those of *Δrho3* cells that harbored a control plasmid (Fig. 1 *B*; *right*, *sector 8*), indicating that these truncated versions of *BEM1* did not suppress the *Δrho3* defect. The deletion of SH3-2 or of the COOH-terminal sequence did not reduce the amount of the myc-tagged Bem1p, as judged by Western analysis (data not shown). These results indicate that SH3-2 and the COOH-terminal sequence are critical if *BEM1* is to function as a multicopy suppressor of *rho3*.

The Effects of Substitutions in the SH3 Domains and of Deletion of the COOH-terminal Region of Bem1p on Cell Growth

The replacement of the conserved proline residue by leucine destroys the function of an SH3 domain in the sem5 protein of *Caenorhabditis elegans* (Rozakis-Adcock et al., 1992). We introduced the corresponding mutation into SH3-1 ($bem1^{Leu123}$) and SH3-2 ($bem1^{Leu208}$). The $bem1^{Leu208}$ cells and $bem1^{Leu123,Leu208}$ cells displayed temperature-sensitive growth (Fig. 1 *C*, sectors *3* and *4*). At 37°C, the cells were arrested as large, round, unbudded cells, and the asymmetric organization of actin filaments was disrupted in these cells (data not shown). These phenotypes are similar to those of the *Δbem1* cells. By contrast, $bem1^{Leu123}$ cells grew as well as wild-type cells, even at elevated temperatures (Fig. 1 *C*, sector *2*). These results indicate that SH3-2 is important for the function of Bem1p in bud emergence.

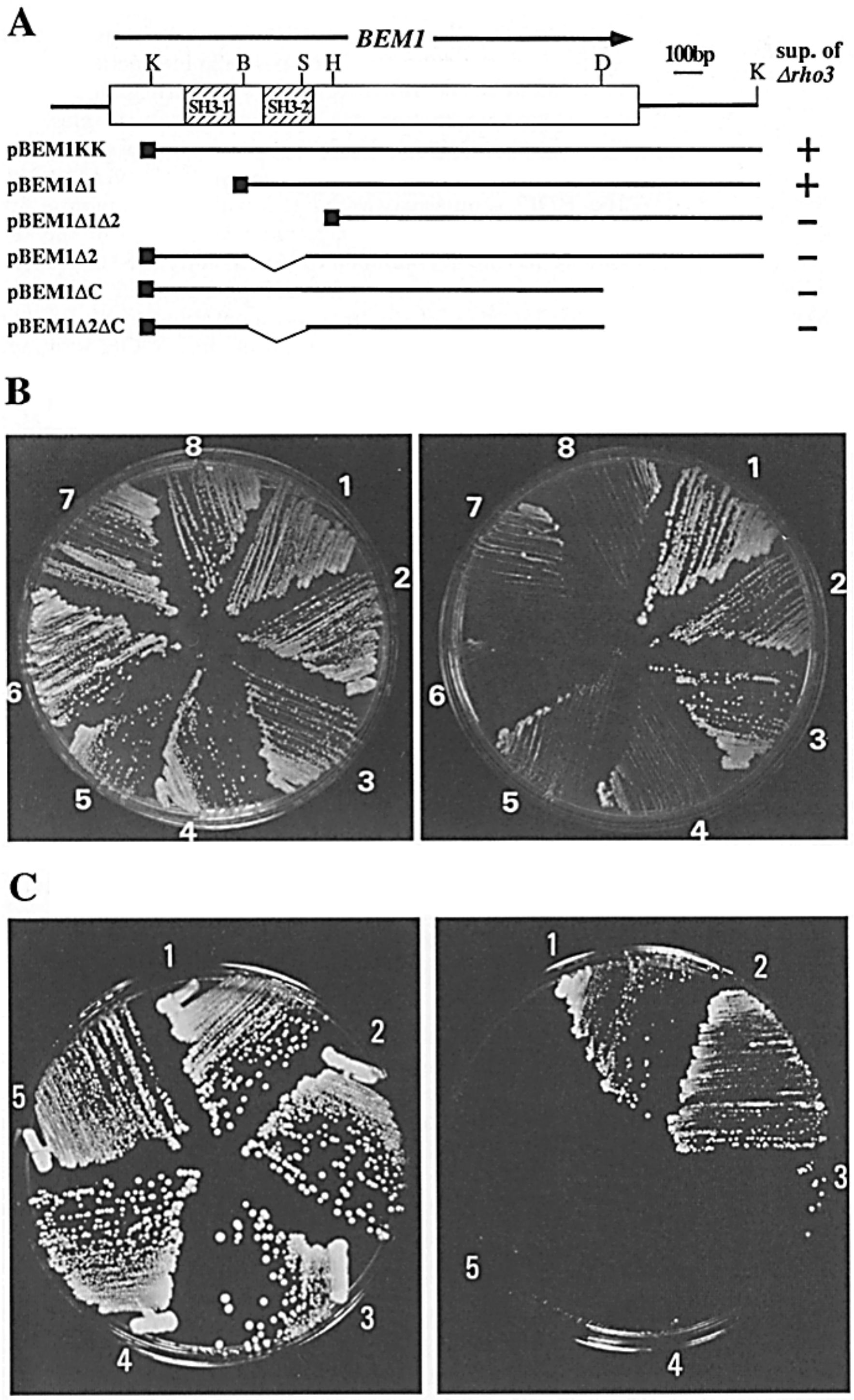


Figure 1. The importance of SH3-2 in the functions of Bem1p. (*A*) The constructs used for overexpression of the various versions of *BEM1* are shown schematically below the map of *BEM1*. *Open box*, the coding region of *BEM1*; *hatched boxes*, the regions encoding the SH3 domains (*SH3-1* and *SH3-2*); *black boxes*, the sequence that encodes the myc epitope tag; *lines*, the *BEM1* sequences that are under the control of the *TDH3* promoter (narrow lines indicate deleted regions). The ability of each plasmid to suppress the growth defect of *Δrho3* cells (shown in *B*) is indicated. *K*, KpnI; *B*, BamHI; *S*, SalI; *H*, HpaI; and *D*, DraI. (*B*) Cells of the *Δrho3 pGAL7:RHO4* strain YMR505 carrying the indicated plasmids were incubated on YPD (*right*) and SCGal-U (*left*) plates at 30°C for 3 d. The *Δrho3* strain YMR505, which carries *pGAL7:RHO4*, *RHO4* under the control of the *GAL7* promoter, grows very poorly on glucose-containing medium but grows well on galactose-containing medium because *RHO4* can serve as a multicopy suppressor of *rho3* (Matsui and Toh-e, 1992*a*,*b*). Cells harbored pRS316-RHO3, a centromeric plasmid carrying *RHO3* (sector *1*), pBEM1KK (sector *2*), pBEM1Δ1 (sector *3*), pBEM1Δ2 (sector *4*), pBEM1Δ1Δ2 (sector *5*), pBEM1ΔC (sector *6*), pBEM1Δ2ΔC (sector *7*), and a dummy plasmid, pKT10mycN (sector *8*) as a negative control. (*C*) Wild-type cells (strain W303-1A, sector *1*), *bem1*$^{\mathrm{Leu123}}$ cells (strain YMS1194, sector *2*), *bem1*$^{\mathrm{Leu208}}$ cells (strain YMS1199, sector *3*), *bem1*$^{\mathrm{Leu123,Leu208}}$ cells (strain YMS1190, sector *4*), and *bem1-ΔC* (strain YMS1201, sector *5*) were streaked and incubated on YPD plates for 3 d at 25°C (*left*) and at 37°C (*right*).

The COOH-terminal 35 a.a. of Bem1p is essential for the suppression of the *Δrho3* defect (Fig. 1 *B*). We examined whether the COOH-terminal 35 a.a. is also important for the function of Bem1p in cell growth or not. We replaced the wild-type *BEM1* allele with a truncated allele that lacked the sequence for the COOH-terminal 35 a.a. (*bem1-ΔC*). The *bem1-ΔC* cells displayed temperature-sensitive growth (Fig. 1 *C*, sector *5*) and were arrested as large, round, unbudded cells at 37°C (data not shown). These results indicate that the COOH-terminal 35 a.a. is important for the function of Bem1p in bud emergence.

It has been reported that the COOH-terminal half of Bem1p interacts with the COOH-terminal half of Cdc24p (Peterson et al., 1994). We examined whether the COOH-terminal 35 a.a. of Bem1p is required for the Cdc24p-Bem1p interaction using two-hybrid system (Fields and Sternglanz, 1994). The lexA–Bem1p fusion (from plexBEM1) increased the level of expression of *lacZ* in L40 cells (*lexA-lacZ* strain), with the increase depending on the presence of the Gal4TA–Cdc24p fusion from pGADCDC24. The lexA-fused Bem1p without the COOH terminus of 35 a.a. (from plexBEM1ΔC), however, did not (Table II). These results indicate that the

Table II. Assay of Two-hybrid Interaction between Cdc24p and Bem1p

	pGAD424	pGADCDC24
pBTM116	0.4	0.7
plexBEM1	7	54
plexBEMΔC	4	0.5

β-Galactosidase activities (units) of L40 cells that harbored plasmids in the indicated combinations are shown.

COOH-terminal 35 a.a. of Bem1p is required for the two-hybrid interaction between Cdc24p and Bem1p.

Isolation of BOI2

A screening was made for genes that encode the Bem1p-binding protein using the two-hybrid system. Plasmid plexBEM1, carrying the sequence encoding the lexA-Bem1p fusion and the yeast *S. cerevisiae* genomic library expressed as fusion proteins with the Gal4 TA domain (Chien et al., 1991), were introduced into L40 cells that carried *lexA-lacZ* and *lexA-HIS3.* The plasmids were then recovered from the colonies that formed on selective plates (SD+3AT). Among the plasmids recovered from the His^+ transformants, only plasmid pGADBOI2 increased the level of expression of both *HIS3* and *lacZ* in L40 cells in a plexBEM1-dependent manner. DNA clones were isolated from the yeast genomic library (Matsui and Toh-e, 1992*a*) with a fragment from pGADBOI2 as a probe. The nucleotide sequence of the clones revealed a gene that encoded a 1,040-a.a. protein with an SH3 domain, a proline-rich sequence, and a pleckstrin homology (PH) domain (Musacchio et al., 1993; Fig. 2). A homology search of the Genbank database using the FASTA program revealed a homologue with 38% identity. This homologous gene was identified on the basis of its two-hybrid interaction with Bem1p (Bender et al., 1996). Both groups designated these genes *BOI1* and *BOI2* (as bem one interacting), and the gene on pGADBOI2 was designated *BOI2.* Four domains of Boi2p, namely, domain I (residues 39–113) including an SH3 domain (residues 50–102), domain II (residues 266–331), domain III (residues 436–462) including a proline-rich sequence (residues 438–458), and domain IV (residues 731–943) including a PH domain (residues 767–891), were highly homologous to those of Boi1p (Fig. 2 *B*), with the extent of identity being 71, 65, 78, and 69%, respectively.

Domains That Are Required for the Two-hybrid Interaction between Bem1p and Boi2p

To identify the sequence responsible for the two-hybrid interaction between Bem1p and Boi2p, several constructs that encoded lexA-Bem1p fused proteins with truncation and/or mutations (shown diagrammatically in Fig. 3 *A*) were introduced into L40 cells that harbored pGADBOI2, and the two-hybrid interaction was examined. The *BEM1* sequence for the NH_2-terminal half (a.a. 1–325), which contained both SH3 domains, was sufficient for the interaction (Fig. 3 *A*, *plexΔC*). Introduction of the $bem1^{Leu208}$ mutation into this *BEM1* sequence, however, abolished the increase in the expression level of the reporter genes (Fig. 3 *A*, *plex-s2* and *plex-s12*). By contrast, the plasmid carrying only the $bem1^{Leu123}$ mutation increased the expression (Fig. 3 *A*, *plex-s1*). These results indicate that SH3-2 is essential for the Bem1p–Boi2p interaction.

To identify the Boi2p domain that participates in the two-hybrid interaction, we constructed several plasmids that encoded Gal4TA–Boi2p fused proteins, as shown diagramatically in Fig. 3 *B*. In the original isolate, pGADBOI2, the *BOI2* sequence was ligated with the sequence for Gal4TA at the Sau3AI site that was located in the sequence for the SH3 domain of Boi2p, and the SH3 domain in the fusion was disrupted (see Figs. 2 *A* and 3 *B*). Thus, the SH3 domain of Boi2p was dispensable for the Boi2p–Bem1p interaction. The deletion of the COOH-terminal sequence of Boi2p (threonine 465–end) did not reduce the interaction (Fig. 3 *B*, *pGADAA1*), an indication that the NH_2-terminal half of Boi2p is sufficient for the interaction. More extensive deletion of the COOH-terminal sequence (alanine 442–end including the proline-rich sequence), however, abolished the interaction (Fig. 3 *B*, *pGADBA2*). The deletion of the sequence for a.a. 436–464, including the proline-rich sequence, also abolished the interaction (Fig. 3 *B*, *pGADΔPRO*). Proline-rich sequences are reported to be the motif of SH3 domain–binding sites (e.g., Cicchetti et al., 1992). These results suggest that the interaction between Boi2p and Bem1p is mediated by the interaction between the proline-rich sequence of Boi2p and SH3-2 of Bem1p. Deletion of the NH_2-terminal sequence (a.a. 1–266), however, also abolished the interaction (Fig. 3 *B*, *pGADE2A2*). It is likely that the NH_2-terminal sequence affects the conformation of Boi2p, allowing the proline-rich sequence to bind efficiently or, alternatively, the NH_2-terminal sequence might stabilize the Gal4–Boi2p fusion protein.

The Interaction of Bem1p and Boi2p In Vitro

The interaction of Boi2p with Bem1p was examined in vitro with purified GST-Boi2p. Yeast cells were transformed with the plasmids for myc-tagged Bem1p, shown diagrammatically in Fig. 1 *A*. The myc-tagged Bem1p in the lysates was detected with anti-myc antibodies (Fig. 3 *C*, *left*). The lysates containing myc-tagged Bem1p were incubated with GST-Boi2p that had been bound to GSH beads. The versions of Bem1p that possessed SH3-2 were coprecipitated with GST-Boi2p (Fig. 3 *C*, *right*, lanes *1*, *2*, and *5*), whereas the versions of Bem1p that lacked SH3-2 only coprecipitated at very low levels, if at all, with GST-Boi2p (Fig. 3 *C*, *right*, lanes *3*, *4*, and *6*). Both the two-hybrid experiments and the in vitro binding assays indicate that SH3-2 is critical for the Boi2p–Bem1p interaction and that SH3-1 is not essential for this interaction.

Phenotypes of Cells that Overexpressed BOI1 and BOI2

BOI1 was identified during a screening for genes whose expression, under the control of the *GAL10* promoter, was lethal to yeast cells. The isolated *BOI1* was fused to the *GAL10* promoter at the Sau3AI site located in the sequence for codons 374–375 of *BOI1*. In this fused gene, the 378th methionine codon is expected to be the initiation codon. Cells that carried the multicopy plasmid that harbored the *BOI1* sequence under the control of the *GAL10* promoter did not grow on SCGal-L, a galactose-containing selective medium, and they were arrested as large,

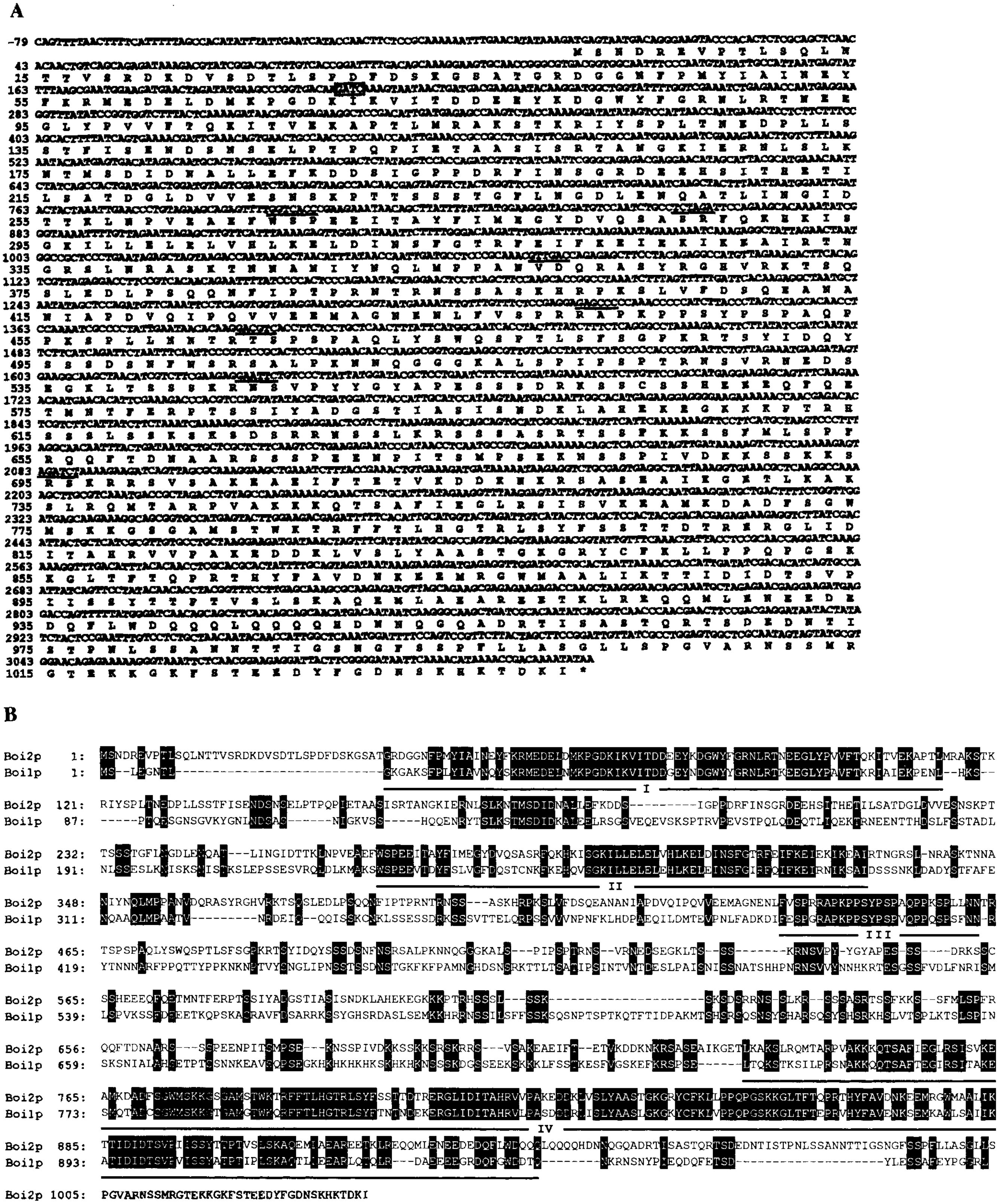


Figure 2. Amino acid sequence of Boi2p. (*A*) The nucleotide and deduced amino acid sequences of *BOI2*. The nucleotide sequence of *BOI2* has been deposited in DDBJ/EMBL/Genbank under the accession No. D38310. The Sau3AI restriction site, namely, the site of the junction with the sequence for Gal4 TA in pGADBOI2, is indicated by a box. The restriction sites for BstEII, XbaI, HincII, BanII, AatII, EcoRI, and BglII, which were used for the construction of truncated versions of *BOI2* (shown schematically in Fig. 3 *B*) are underlined. (*B*) Comparison of the amino acid sequences of Boi2p and Boi1p. Identical residues are boxed in black. Domains I, II, III, and IV are underlined.

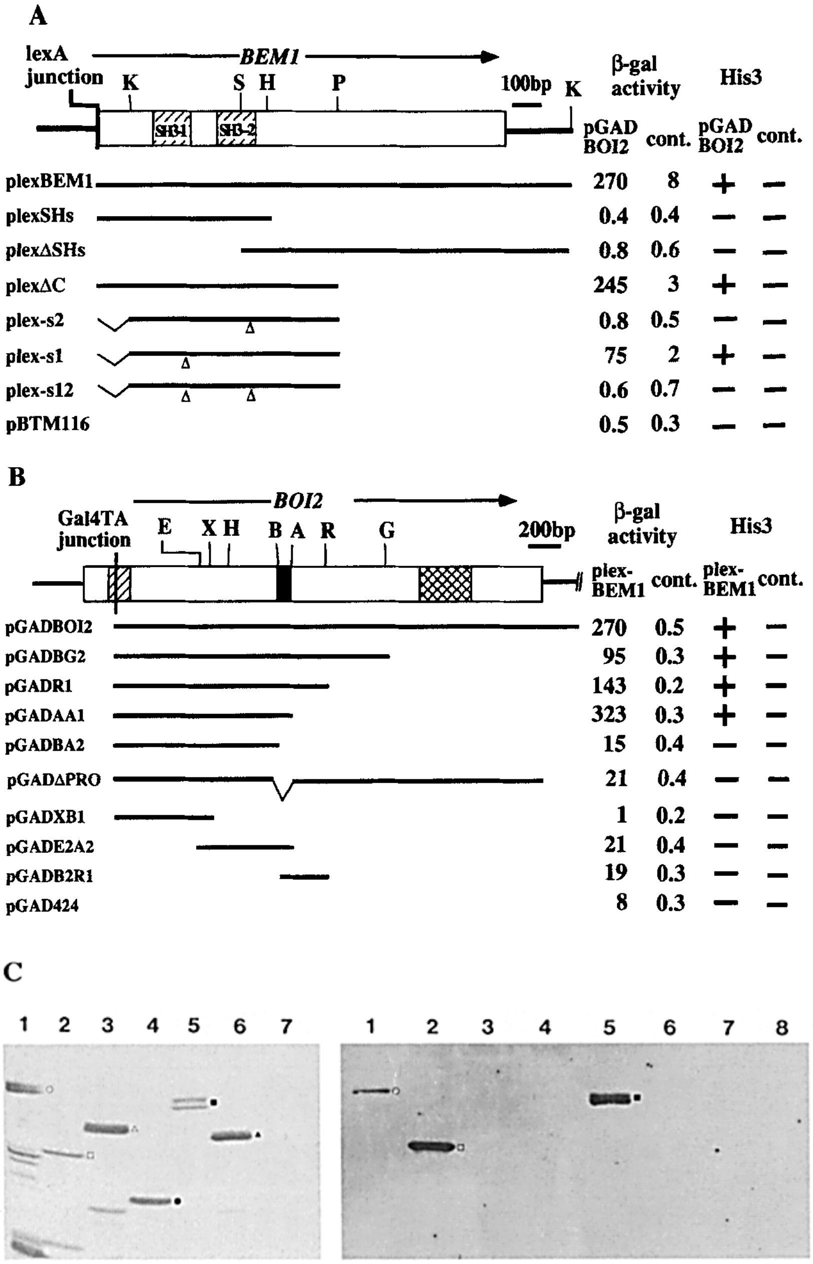
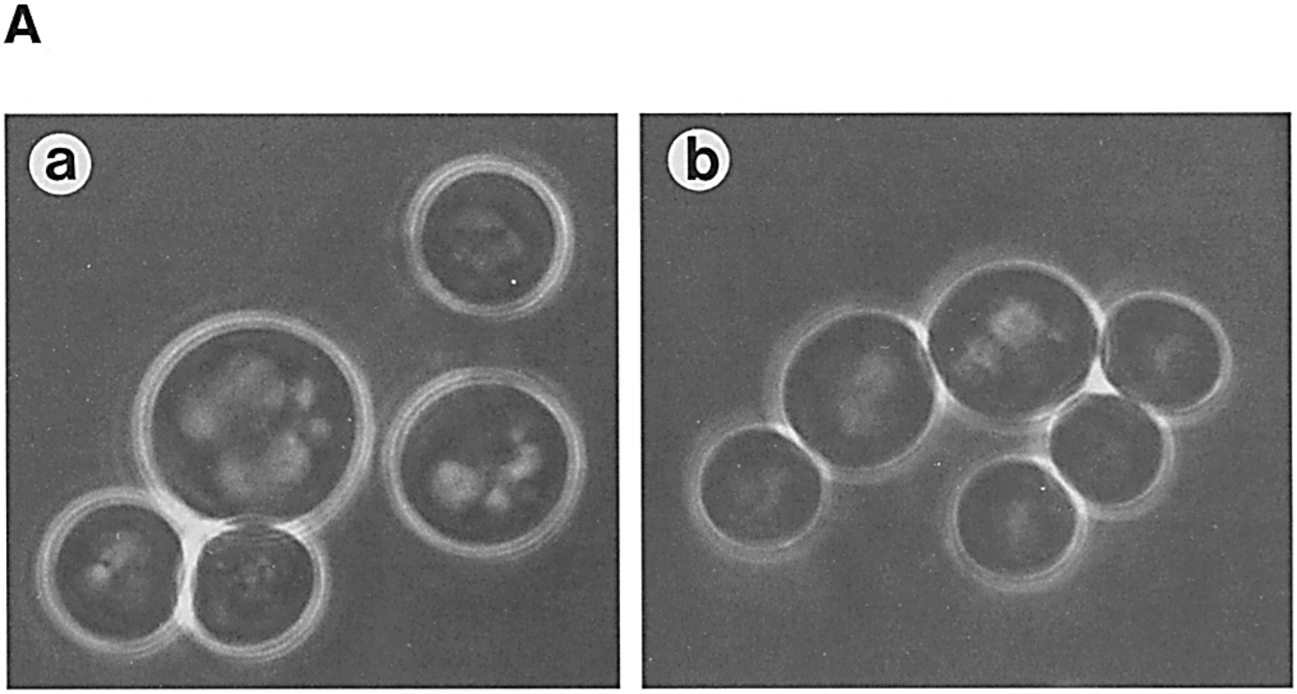

A
lexA junction
BEM1
K S H P
100bp K
SH1 SH2
β-gal activity
His3
pGAD BOI2 cont. pGAD BOI2 cont.
plexBEM1 270 8 + −
plexSHs 0.4 0.4 − −
plexΔSHs 0.8 0.6 − −
plexΔC 245 3 + −
plex-s2 0.8 0.5 − −
plex-s1 75 2 + −
plex-s12 0.6 0.7 − −
pBTM116 0.5 0.3 − −
B
Gal4TA junction
BOI2
E X H B A R G
200bp
β-gal activity
His3
plex-BEM1 cont. plex-BEM1 cont.
pGADBOI2 270 0.5 + −
pGADBG2 95 0.3 + −
pGADR1 143 0.2 + −
pGADAA1 323 0.3 + −
pGADBA2 15 0.4 − −
pGADΔPRO 21 0.4 − −
pGADXB1 1 0.2 − −
pGADE2A2 21 0.4 − −
pGADB2R1 19 0.3 − −
pGAD424 8 0.3 − −
C
1 2 3 4 5 6 7
1 2 3 4 5 6 7 8

Figure 3. Domains required for the Bem1p–Boi2p interaction. (*A*) The *BEM1* sequences, expressed as fusion proteins with lexA, are represented by lines below the map of *BEM1*. The site of the mutations in *bem1*$^{\mathrm{Leu123}}$ and *bem1*$^{\mathrm{Leu208}}$ are indicated by triangles. Plasmids indicated on the left were introduced into L40 cells that harbored either pGADBOI2 or pGAD424 (as a control), and the activities of β-galactosidase (units) and the His3 phenotypes were assayed. +, growth; −, no growth. *K*, KpnI and BanI; *S*, SalI; *H*, HpaI; and *P*, PstI. (*B*) The *BOI2* sequences expressed as fusion proteins with Gal4TA are represented by lines below the map of *BOI2*. The *BOI2*-coding region is indicated by an open box. The SH3 domain (*hatched*), the proline-rich sequence (*black*), and the PH domain (*cross-hatched*) are indicated. Plasmids indicated on the left were introduced into L40 cells that harbored either plexBEM1 or pBTM116 (*cont.*), and the expression of the reporter genes was examined. *E*, BstEII; *X*, XbaI; *H*, HincII; *B*, BanII; *A*, AatII; *R*, EcoRI; *G*, BglII. (*C*) Cell lysates were prepared from the wild-type strain KA31 that harbored plasmids pBEM1KK (lanes *1* and *8*), pBEM1Δ1 (lane *2*), pBEM1Δ2 (lane *3*), pBEM1Δ1Δ2 (lane *4*), pBEM1ΔC (lane *5*), pBEM1Δ2ΔC (lane *6*), or a dummy plasmid pKT10mycN (lane *7*). The plasmids are shown schematically in Fig. 1 *A*. Total cell lysates were incubated with GST-Boi2p beads (*right panel*, lanes *1–7*) or GST beads (*right panel*, lane *8*) as a control. Total cell lysates (*left panel*), proteins coprecipitated with GST-Boi2p (*right panel*, lanes *1–7*), and proteins coprecipitated with GST (*right panel*, lane *8*) were resolved by SDS-PAGE, and myc-tagged Bem1p was detected by Western blotting analysis. The positions of the various forms of myc-tagged Bem1p, produced from pBEM1KK (*open circle*), pBEM1Δ1 (*open square*), pBEM1Δ2 (*open triangle*), pBEM1Δ1Δ2 (*closed circle*), and pBEM1ΔC (*closed square*), pBEM1Δ2ΔC (*closed triangle*) are indicated.

round, and unbudded cells (Fig. 4 *Aa*). Overexpression of *BOI2* from the *GAL7* promoter (*pGAL7:BOI2*) also inhibited cell growth (Fig. 4 *B*, sector *4*), and cells overexpressing *BOI2* were arrested as large, round, unbudded cells (Fig. 4 *Ab*). The asymmetric organization of actin filaments was disrupted in cells that overexpressed *BOI1* and in cells that overexpressed *BOI2* (data not shown). These results indicate that overexpression of *BOI1* and *BOI2* inhibits bud emergence, and that the Boi proteins affect cell polarization.

To identify the domain that is important for the inhibitory effect, we constructed two truncated versions of *BOI2*, namely *pGAL7:BOI2ΔN* and *pGAL7:BOI2ΔC*. The overproduction of Boi2p without its NH_2-terminal half from *pGAL7:BOI2ΔN* inhibited cell growth (Fig. 4 *B*, sector *3*) and cells expressing *pGAL7:BOI2ΔN* were arrested as large, round, unbudded cells (data not shown). The overproduction of Boi2p without its COOH-terminal half from *pGAL7:BOI2ΔC*, however, did not have any inhibitory effect on cell growth (Fig. 4 *B*, sector *2*). These results indicate that the COOH-terminal half that contains domain IV is important for inhibition of bud emergence, while the NH_2-terminal half that contains domains I, II, and III is not essential for the inhibition.

The finding that the NH_2-terminal half is not essential for the inhibition suggests that the Bem1p–Boi2p interaction is not critical for the inhibitory effect. To clarify this, we introduced both constructs for the overexpression of *BOI* genes (*pGA68 and pGAL7:BOI2*) into *bem1* mutant cells in which *bem1* lacked the sequence for both SH3 domains (*bem1-ΔSH3s*). The overexpression of either *BOI1* or *BOI2* inhibited the growth of the *bem1-ΔSH3s* cells (data not shown). These results indicate that the protein–protein interaction between Bem1p and the Boi proteins is not critical for the inhibitory activity of the overexpressed *BOI* gene.



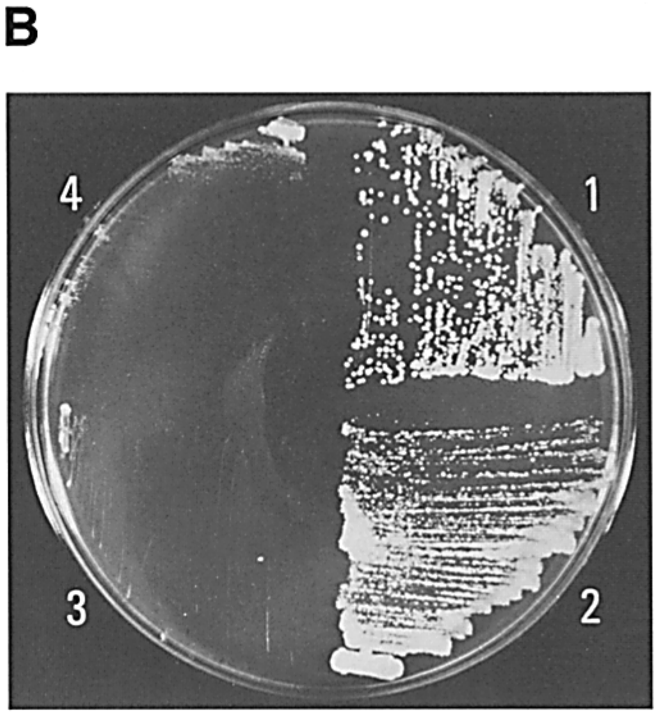


Figure 4. Overexpression of *BOI*. (*A*) The morphology of *BOI*-overexpressing cells (*a* and *b*). (*a*) Cells (strain W303-1A) carrying pGA68 and cultured in SC-L were shifted to SCGal-L, and (*b*) cells (strain W303-1A) carrying *pGAL7:BOI2* and cultured in YPD were shifted to YPGal. Cells were harvested 8 h after the shift. (*B*) Cells (strain W303-1A) carrying a dummy plasmid YIpUGAL7 (sector *1*), *pGAL7:BOI2ΔC* (sector *2*), *pGAL7:BOI2ΔN* (sector *3*), or *pGAL7:BOI2* (sector *4*) were streaked on an SCGal-U plate and incubated at 30°C for 2 d.

Phenotypes of the boi-disrupted Cells

Disruption of neither *BOI1* nor *BOI2* alone resulted in a cell growth defect (Fig. 5 *A*, sectors *2* and *3*). However, all but one of ~200 *Δboi1 Δboi2* spores isolated from *Δboi1/+ Δboi2/+* heterozygous diploid cells failed to form colonies. The single viable *Δboi1 Δboi2* spore formed a tiny colony that grew very poorly. *Δboi1 Δboi2* cells with *BOI2* under the control of the *GAL7* promoter (*pGAL7:BOI2*) grew on medium that contained 0.1% galactose, which induced the expression of *pGAL7:BOI2* with low efficiency. *Δboi1 Δboi2 pGAL7:BOI2* cells dramatically reduced the growth rate 24 h after a shift to glucose-containing medium, which repressed the expression of *pGAL7:BOI2* and then grew very poorly (Fig. 5 *A*, sector *4*). *Δboi1 Δboi2 pGAL7:BOI2* cells grew very poorly on glucose-containing medium at all temperatures tested (20, 25, 30, and 37°C). These results indicate that *BOI1* and *BOI2* are functionally redundant, and that the Boi proteins are important for cell growth.

About 24 h after the shift to glucose-containing medium, *Δboi1 Δboi2 pGAL7:BOI2* cells began to stop growing and lysed, as judged from the leakage of alkaline phosphatase into the medium (Fig. 5 *B*). From the staining of dead cells with methylene blue (Rose, 1975), it appeared that ∼30% of all the cells in the culture 24 h after the shift were dead, and ∼95% of these dead cells were budded (Fig. 6 *a*). Within 48 h after the shift, the fraction of dead budded cells increased to ∼60% of the total cells. The remaining cells were large and round (Fig. 6 *a*), and in these large round cells, the asymmetric organization of actin filament (as observed in wild-type cells; Fig. 6 *b*) was disrupted (Fig. 6 *d*). A majority of *Δboi1 Δboi2* cells lysed with buds, and the death of these *Δboi1 Δboi2* cells was not suppressed by the addition of an osmotic stabilizer into the medium (data not shown). In the presence of an osmotic stabilizer that partially prevented cell lysis, however, most of the *Δboi1 Δboi2* cells were uniformly observed as large and round cells in the glucose-containing medium rather than as dead budded cells. Furthermore, the asymmetric organization of actin filaments was disrupted in these large round cells (Fig. 6, *e* and *f*), indicating that these *Δboi1 Δboi2* cells have lost cell polarity. These morphological observations strongly suggest that cells without the Boi function failed to maintain cell polarity, and that the loss of cell polarity caused a defect in bud growth and subsequent cell lysis.

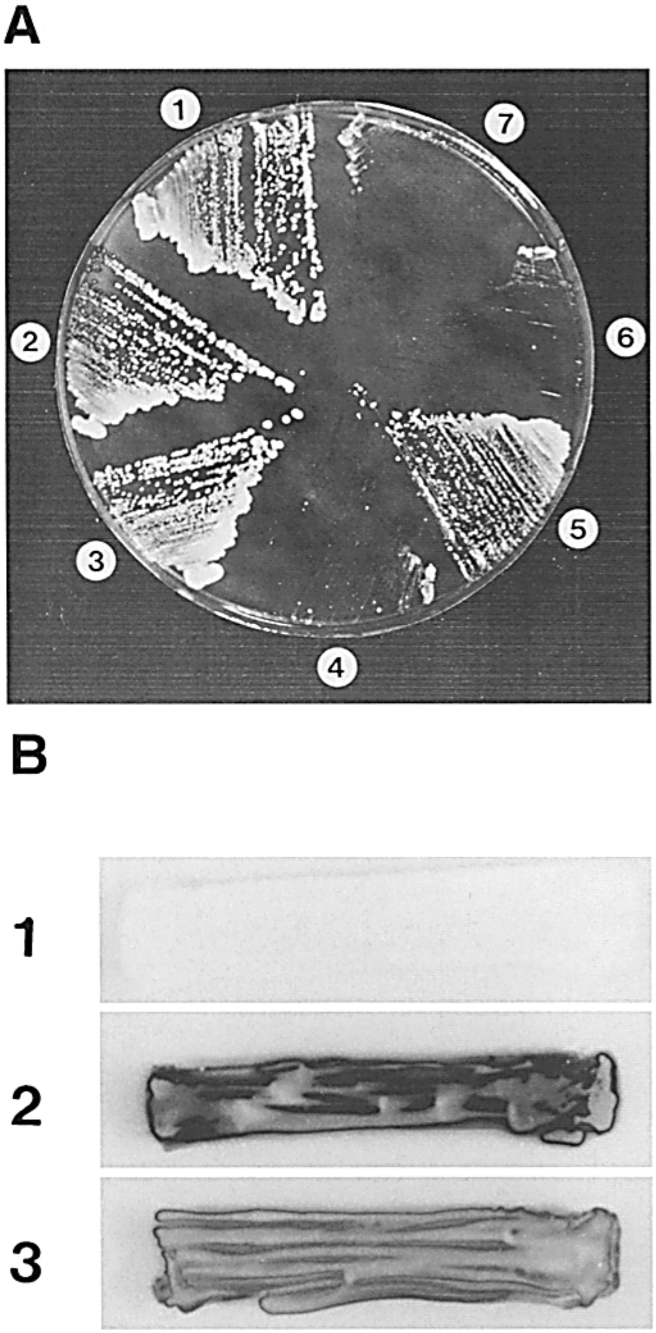


Figure 5. Disruption of *BOI*. (*A*) Strains to be tested were streaked on a YPD plate and incubated at 30°C for 2 d as follows. Wild-type strain W303-1A (sector *1*); *Δboi1* strain YMB101 (sector *2*); *Δboi2* strain YMB202 (sector *3*); *Δboi1 Δboi2 pGAL7:BOI2* strain YMB1207 (sector *4*); *Δboi1 Δboi2 BOI2ΔN* strain YMB1202 (sector *5*); *Δboi1 Δboi2 pGAL7:BOI2* strain YMB1207 carrying pRS314-BOI2ΔC (sector *6*); and *Δboi1 Δboi2 pGAL7:BOI2* strain YMB1207 carrying pYO324-BOI2ΔC (sector *7*). (*B*) Wild-type strain W303-1A (*1*), *Δboi1 Δboi2 pGAL7:BOI2* strain YMB1207 (*2*), and *Δrho3 pGAL7:RHO4* strain YMR505 (*3*) were streaked on a YPD plate, incubated for 40 h at 30°C, and overlaid with a solution of BCIP. Black regions in the photograph were blue and indicated cell lysis.

The Boi2p Domain That Is Required for Cell Growth

To identify the domain that is critical for the Boi function, we constructed two truncated versions of *BOI2*: *BOI2ΔN*, which lacked the sequence for domains I, II, and III; and *BOI2ΔC*, which lacked the sequence for domain IV. *Δboi1 Δboi2* cells carrying *BOI2ΔN* grew as well as wild-type cells (Fig. 5 *A*, sector *5*), but *Δboi1 Δboi2* cells carrying *BOI2ΔC* on either a low copy number plasmid or a high copy number plasmid grew as poorly as *Δboi1 Δboi2* cells (Fig. 5 *A*, sectors *6* and *7*). These results indicate that the COOH-terminal half is essential and sufficient for cell growth while the NH_2-terminal half is dispensable.

The Effect of BOI2 on Δrho3 Cells

Boi2p interacted with SH3-2, which was required for suppression by Bem1p of the *Δrho3* defect. This result may suggest the involvement of Boi2p in the suppression. We examined whether or not multiple copies of *BOI2* could suppress the *Δrho3* defect. Although the overexpression of *BOI2* from *pGAL7:BOI2* inhibited cell growth, the introduction of pYO324-BOI2, a high copy number plasmid that harbored *BOI2*, into cells did not inhibit the growth of wild-type cells or *Δrho3* cells that had been rescued by overexpression of *RHO4* (data not shown; Fig. 7, *left*). The growth of *Δrho3* cells that carried pYO324-BOI2 was as poor as that of *Δrho3* cells without pYO324-BOI2 (Fig. 7, *right*, sectors *1* and *2*), indicating that *BOI2* can not serve as a multicopy suppressor of the *Δrho3* defect. Conversely, multiple copies of *BOI2* inhibited the growth of *Δrho3* cells that overexpressed *BEM1*. The growth defect of *Δrho3* cells was rescued by overexpression of *BEM1* (Fig. 7, *right*, sector *3*), but in the presence of pYO324-BOI2, the growth of the *Δrho3* cells that overexpressed *BEM1* was as poor as that of *Δrho3* cells without the *BEM1* overexpression plasmid (Fig. 7, *right*, sector *4*). However, neither *BOI2ΔN* nor *BOI2ΔC* on a high copy number plasmid did not show the inhibitory effect (Fig. 7, *right*, sectors

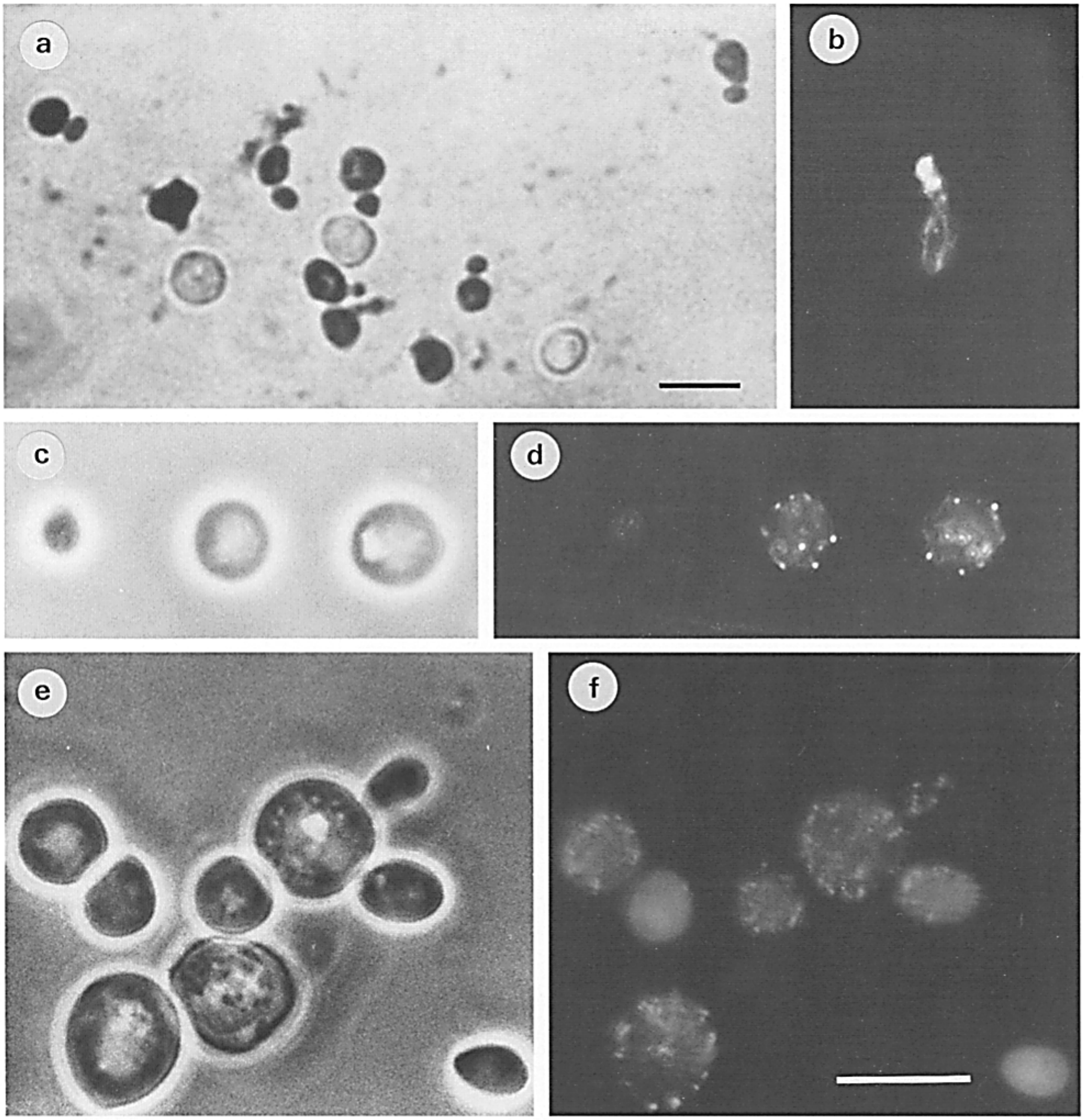


Figure 6. Morphology of *Δboi1 Δboi2* cells. (*a*) *Δboi1 Δboi2 pGAL7:BOI2* cells (strain YMR1207) were grown in YPGal0.1, shifted to YPD, harvested 24 h after the shift, and stained with methylene blue. Black cells in the photograph were blue, an indication of cell death. Bar, 10 μm. (*b*) Rhodamine-phalloidin staining of a wild-type cell. (*c-f*) *Δboi1 Δboi2 pGAL7:BOI2* cells (strain YMR1207) were grown in YPGal0.1, shifted to YPD (*c* and *d*) or to YPD with 1 M sorbitol (*e* and *f*), harvested 24 h after the shift, and stained with rhodamine-phalloidin (*d* and *f*). Yeast cells were cultured at 28°C. (*c* and *e*) Phase-contrast images. (*b*, *d*, and *f*) Fluorescence microscopy. Bar in *f* (valid for *b-f*),10 μm.

5 and 6). These results indicate that the NH_2-terminal half and the COOH-terminal half of Boi2p is critical for the inhibitory activity. It might be possible that the *BEM1*-overexpressing *Δrho3* cells are hypersensitive to the inhibitory effect of *BOI2* on bud emergence, which was observed in case of *pGAL7:BOI2*. Such was not the case, however, since in addition to the difference in required domains, the *BEM1*-overexpressing *Δrho3* cells that harbored pYO324-BOI2 showed morphology similar to that of *Δrho3* cells without the *BEM1* overexpression plasmid (showing an increase of the proportion of lysed cells with buds) instead of becoming large, round, unbudded cells (data not shown). These results suggest that Boi2p inhibits the function of Bem1p in the suppression of the *Δrho3* defect.

Genetic Interactions among the BOI Genes, RHO3, and RHO4

Since (*a*) Boi2p interacts with Bem1p, (*b*) *BEM1* interacts genetically with *RHO3*, and (*c*) the morphological pheno-

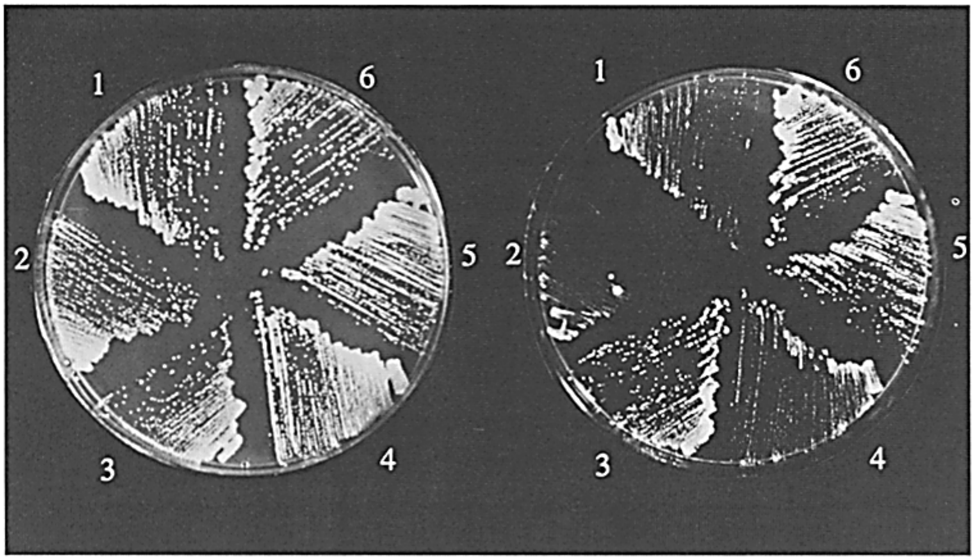


Figure 7. Effect of multiple copies of *BOI2* on the growth of *Δrho3* cells. *Δrho3 pGAL7:RHO4* strain YMR505, carrying the indicated plasmids, was incubated on SCGal-UT (*left*) and SC-UT (*right*) plates at 30°C for 4 d. The *Δrho3* strain YMR505 grew very poorly on glucose-containing medium but grew well on galactose-containing medium as a result of the overexpression of *RHO4* from *pGAL7:RHO4* (Matsui and Toh-e, 1992*b*). Plasmids were as follows: pYO324 and pKT10, dummy plasmids (sector *1*); pYO324-BOI2, a high copy number plasmid that harbored *BOI2*, and pKT10 (sector *2*); pBEM1KK, a *BEM1*-overexpressing plasmid, and pYO324 (sector *3*), pBEM1KK and pYO324-BOI2 (sector *4*); pBEM1KK and pYO324-BOI2ΔN, a high copy number plasmid that harbored *BOI2ΔN* (sector *5*); pBEM1KK and pYO324-BOI2ΔC, a high copy number plasmid that harbored *BOI2ΔC* (sector *6*).

type of the *Δboi1 Δboi2* cells is similar to that of *Δrho3* cells (Matsui and Toh-e, 1992*b*), we examined whether *BEM1*, *RHO3*, or *RHO3*-related genes could serve as multicopy suppressors of the *boi* defect. Plasmids were introduced into *Δboi1*/+ *Δboi2*/+ heterozygous diploid cells, and the transformants were sporulated and dissected. After incubation at 25°C for 3 d, *Δboi1 Δboi2* segregants carrying plasmids that overexpressed either *RHO3* or *RHO4* (i.e., plasmids pOPR3 or pOPR4) formed visible colonies. They grew as well as wild-type cells and much better than *Δboi1 Δboi2* cells without pOPR3 and pOPR4 (Fig. 8). These results indicate that both *RHO3* and *RHO4* can serve as a multicopy suppressor of *boi*. Plasmids overexpressing either *BEM1* or *RHO1*, encoding a Rho-type GTPase, (i.e., pBEM1KK or pOPR1) did not rescue the growth defect of *Δboi1 Δboi2* segregants (data not shown).

Discussion

Functions of the Boi Proteins

The phenotypes of *Δboi1 Δboi2* cells resemble those of cells depleted of both Rho3p and Rho4p. *Δrho3 Δrho4* cells carrying *pGAL7:RHO4, RHO4* under the control of the *GAL7* promoter cease to grow in a glucose-containing medium, and the Rho3p- and Rho4p-depleted cells lyse with buds (Matsui and Toh-e, 1992*b*; Fig. 5 *B*). In the presence of an osmotic stabilizer, Rho3p- and Rho4p-depleted cells become large and round, and the asymmetric organization of actin filaments is disrupted (Matsui and Toh-e, 1992*b*). The phenotypes of the Boi protein-depleted cells (Figs. 5 *B* and 6) were quite similar to those of Rho3p- and Rho4p-depleted cells, and they strongly suggest that these cells are defective in the maintenance of cell polarity for bud growth. Moreover, both *RHO3* and *RHO4* can serve as a multicopy suppressor of the *boi* defect. Both the strong genetic interactions and the phenotypes of mutants strongly suggest that the Boi proteins, Rho3p, and Rho4p are all involved in the same process that maintains cell polarity for bud growth.

Domains of Boi Proteins

Boi2p possesses four domains that are highly conserved in Boi1p (Fig. 2 *B*), and the high degree of conservation suggests that the domains play an important role in the function of the Boi proteins. Domain III contains a proline-

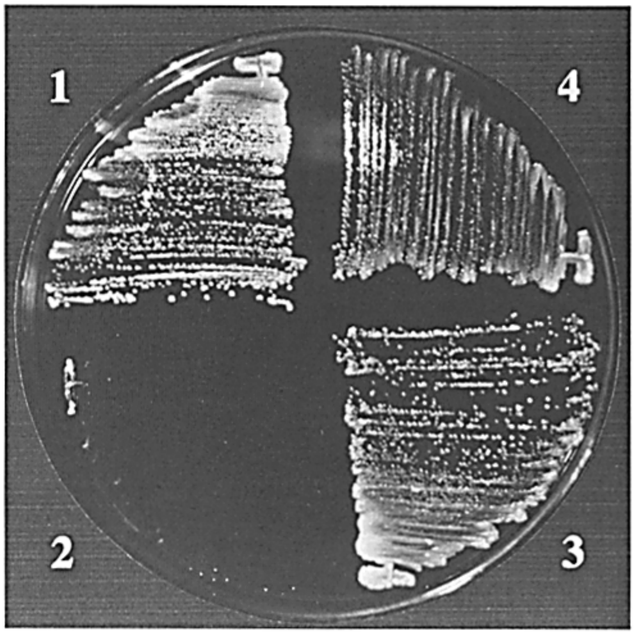


Figure 8. Suppression of the *boi* defect. Wild-type cells (strain W303-1A, sector *1*), *Δboi1 Δboi2 pGAL7:BOI2* cells (strain YMR1207, sector *2*), a *Δboi1 Δboi2* segregant carrying pOPR3 (sector *3*), and a *Δboi1 Δboi2* segregant carrying pOPR4 (sector *4*) were streaked on a YPD plate and incubated for 2 d at 30°C.

rich sequence that is required for displaying the two-hybrid interaction with SH3-2 of Bem1p (Fig. 3). Domain I contains an SH3 domain that can interact with an SH3 domain–binding protein. Domain IV contains a PH domain. We analyzed the role of the domains using truncated versions of Boi2p, and the results are summarized in Fig. 9. *BOI2ΔN*, which lacked the sequence for domains I-III, complemented *Δboi1 Δboi2*, while *BOI2ΔC*, which lacked domain IV, did not, an indication that the COOH-terminal half, including domain IV, of Boi2p is essential and sufficient for the function of Boi2p in cell growth, while the NH$_2$-terminal half is dispensable. The role of a PH domain is still obscure, but it has been reported that PH domains participate in interactions with lipid moiety and proteins (Musacchio et al., 1993; Harlan et al., 1994). It is possible that Boi proteins interact with proteins other than Bem1p via the PH domain. In this context, it is of interest to recall that the overexpression of *BOI* genes under the control of the galactose-dependent promoter inhibited bud emergence (Fig. 4). As in the case of the function of Boi2p in cell growth, the COOH-terminal half of Boi2p (from *pGAL7:BOI2ΔN*) is essential and sufficient for the inhibitory effect, and the interaction of Bem1p and the Boi proteins is not required. These findings suggest that the COOH-terminal half of Boi2p interacts with a factor(s) involved in bud emergence. It might be possible that the interaction between the COOH-terminal half and the component(s) for bud emergence can replace, in part, the role of the Bem1p–Boi2p interaction and can allow cells to grow well without the NH$_2$-terminal half of the Boi proteins.

The Role of the Bem1p–Boi2p Interaction

Although the NH$_2$-terminal half of Boi2p is not essential for either cell growth or inhibition of bud emergence, the NH$_2$-terminal half of Boi2p might be required to inhibit the suppression of the growth defect of *Δrho3* cells by Bem1p. These findings strongly suggest that the Boi proteins possess the potential to modulate the function of Bem1p and that the NH$_2$- and COOH-terminal portions of the Boi proteins play a critical role in modulating the function of Bem1p. The NH$_2$-terminal portion of Boi2p interacts with SH3-2 of Bem1p, and SH3-2 is critical for suppression of the *Δrho3* defect. Thus, it is likely that the Bem1p–Boi2p interaction plays a role in the modulating activity.

The role of the Bem1p–Boi2 interaction in the suppression of the *Δrho3* defect is suggested to be negative; multiple copies of *BOI2* did not suppress the *Δrho3* defect, but they inhibited the growth of *Δrho3* cells that had been rescued by the overproduction of Bem1p (Fig. 7). From these results, we cannot exclude the possibility that the intrinsic amount of Boi2p might play a positive role in the suppression of the *Δrho3* defect. It is possible, however, that another protein that interacts with SH3-2 might play an important role in suppressing the *Δrho3* defect, acting in concert with or independently of Boi2p, and that Boi2p might modulate the function of Bem1p by interacting competitively with SH3-2. It was reported very recently that Bem1p interacts with Ste20p and Ste5p, two components of the pheromone-responsive mitogen-activated protein kinase cascade, as well as with actin. The Ste20p protein kinase requires both SH3-2 and the COOH-terminal half of Bem1p for the Ste20p–Bem1p interaction (Leeuw et al., 1995). Ste20p is therefore a candidate for a protein that is critical for the suppression of the *Δrho3* defect. At this time, however, we have no experimental evidence to suggest the involvement of Ste20p.

Protein Complex for Bud Formation

The COOH-terminal 35 a.a. of Bem1p is required for bud emergence at elevated temperatures and for the suppression of the *rho3* defect (Fig. 1). Thus, it appears possible that protein–protein interaction at the COOH terminus of Bem1p is critical for the functions of Bem1p. One of the candidates for an interacting protein is Cdc24p (Peterson et al., 1994). Indeed, the COOH-terminal 35.a.a. was required for the two-hybrid interaction between Bem1p and Cdc24p (Table II). Cdc24p also interacts with Cdc42p as a GTP–GDP exchange factor and with Rsr1p/Bud1p, which is needed for determination of the bud site (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Zheng et al., 1994, 1995). The finding that Bem1p required the Cdc24p-interacting COOH terminus for suppression of the *Δrho3* defect suggests the possibility that a protein complex containing Bem1p and Cdc24p might play a role in the process that involves Rho3p. It is plausible that the protein complex for bud-site selection is developed by the association of Cdc42p, Cdc24p, and Bem1p for the polarization of cells. After the initiation of bud emergence, the protein complex for cell polarization should be developed and/or rearranged to terminate the process of initiation of bud emergence and for the continuation of bud growth. In this context, it is of great interest that factors involved in bud growth exhibit genetic and protein–protein interactions with factors that are involved in bud emergence and, moreover, that Boi2p might be able to modulate the function of Bem1p and to interact with a factor(s) other than Bem1p that is involved in cell polarization. Our present working hypothesis is that for the regulation of cell mor-

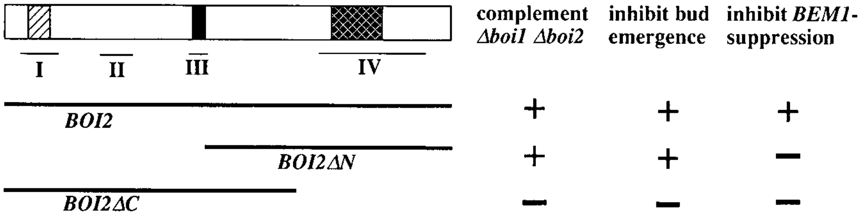


Figure 9. Summary of the domain analysis of *BOI2*. The *BOI2* sequence of each allele is indicated by a thick line below the coding region of *BOI2* (*open box*). The SH3 domain (*hatched*), the proline-rich sequence (*black*), and the PH domain (*cross-hatched*) are indicated. The regions of domains I, II, III, and IV are indicated by narrow lines. The abilities of each allele to complement *Δboi1 Δboi2*, to inhibit bud emergence of wild-type cells when overexpressed under the control of the *GAL7* promoter, and to inhibit the growth of the *BEM1*-overexpressing *Δrho3* cells are indicated.

phogenesis, Rho3p and Boi proteins control the development of the protein complex for bud growth, and the Boi proteins mediate the connections between the protein complex for cell polarization and the machinery for bud growth via its affinities for Bem1p and for the factor(s) involved in cell polarization.

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