Associations among PH and SH3 Domain–containing Proteins and Rho-type GTPases in Yeast

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Abstract. The *src* homology region 3 (SH3) domain–bearing protein Bem1p and the Rho-type GTPase Cdc42p are important for bud emergence in *Saccharomyces cerevisiae*. Here, we present evidence that through its second SH3 domain, Bem1p binds to the structurally and functionally similar proteins Boi1p and Boi2p, each of which contain an SH3 and a pleckstrin homology (PH) domain. Deletion of *BOI1* and *BOI2* together leads to impaired morphogenesis and poor viability. A PH domain–bearing segment of Boi1p that lacks the Bem1p-binding site is necessary and sufficient for function. This segment of Boi1p displays a two-hybrid interaction with Cdc42p, suggesting that Boi1p either binds directly to or is part of a larger complex that contains Cdc42p. Consistent with these possibilities, overexpression of Boi1p inhibits bud emergence, but this inhibition is counteracted by cooverexpression of Cdc42p. Increased expression of the Rho-type GTPase Rho3p, which is implicated in bud growth, suppresses the growth defects of *boi1 boi2* mutants, suggesting that Boi1p and Boi2p may also play roles in the activation or function of Rho3p. These findings provide an example of a tight coupling in function between PH domain–bearing proteins and both Rho-type GTPases and SH3 domain–containing proteins, and they raise the possibility that Boi1p and Boi2p play a role in linking the actions of Cdc42p and Rho3p.

THE *src* homology region 3 (SH3)[1] and pleckstrin homology (PH) domains are present in many proteins that are involved in signal transduction and the organization of the cortical cytoskeleton (Pawson, 1995). The binding sites for SH3 domains generally appear to be short, proline-rich sequences (Ren et al., 1993). Less is known about the binding sites for PH domains. The findings that the PH domains in some proteins overlap with sequences that can bind to the βγ subunit of some trimeric G proteins (Touhara et al., 1994) and that some PH domains can bind PIP_2 (Harlan et al., 1994), however, raise the possibility that a general role of PH domains might be to target proteins to membranes.

Rho-type GTPases also are involved in signal transduction and the organization of the cortical cytoskeleton (Coso et al., 1995; Minden et al., 1995; Simon et al., 1995; Zhao et al., 1995; Kozma et al., 1995; Nobes and Hall, 1995). Clues to relationships between Rho-type GTPases and both SH3 and PH domains come from the study of bud formation in *Saccharomyces cervisiae*. The Rho-type GTPases Cdc42p and Rho3p are implicated in the processes of bud emergence and bud growth, respectively (Adams et al., 1990; Imai et al., 1996). The possibility that SH3 domains might have roles related to Rho-type GTPases arises from the findings that the bud emergence protein Bem1p, which contains two SH3 domains, interacts genetically with Cdc42p and Rho3p. Specifically, *CDC42* can serve as a multicopy suppressor of *bem1* (Mack, D., and A. Bender, unpublished data), and *BEM1* can serve as a multicopy suppressor of *rho3* (Matsui and Toh-e, 1992*b*). Furthermore, the discovery that the *S. pombe* Bem1p-like protein Scd2p displays a two-hybrid interaction with *S. pombe* Cdc42p (Chang et al., 1994) suggests that Bem1p might bind to Cdc42p. The possibility that some PH domains play roles related to Rho-type GTPases comes from the observations that some Cdc42p-binding proteins contain putative PH domains. Specifically, Cdc24p, which is a guanine-nucleotide exchange factor (GEF) for Cdc42p (Zheng et al., 1994), Bem3p, which is a GAP (GTPase activating protein) for Cdc42p (Zheng et al., 1994), and Cla4p, which is a protein kinase target of

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1. *Abbreviations used in this paper*: AD, transcriptional activation domain; DBD, DNA-binding domain; GEF, guanine-nucleotide exchange factor; HA, hemagglutinin; PH, pleckstrin homology; SH3, *src* homology region 3.

Table I. Plasmids and Phage Used in This Study

Plasmids and phage	Characteristics and source
D2	*BOI1* in M13mp19. Made by insertion of the 2.2-kb BamHI-BamHI fragment from PB591 (corresponding to positions 1609–3836 in Fig. 2) into the BamHI site of M13mp19.
D17	*BOI1* in M13mp18. Made by first inserting a 2.2-kb BamHI-BamHI fragment bearing *BOI1* (corresponding to positions 1609–3836 in Fig. 2) into the BamHI site of M13mp18 and then introducing an XhoI site 6 bp downstream of the stop codon of *BOI1* by site-directed mutagenesis using the oligonucleotide GACTTTGAGAACTCGAGTCATTAGTTAC.
pACTII	AD_{Gal4}, *LEU2*, 2μm ARS (Harper et al., 1993).
pCTC52	DBD_{lexA}-lamin, *TRP1*, 2μm ARS; contains the sequences encoding the DBD of the bacterial lexA protein fused to a cDNA that encodes human lamin C. (gift of C. Chien and R. Sternglanz, SUNY at Stony Brook, NY.).
pCY361	*BEM1, URA3, CEN6, ARSH4* (Chenevert et al., 1992). Made by the insertion of a 2.1-kb SmaI-KpnI fragment containing *BEM1* into the SmaI-KpnI sites of pRS316.
pGEX-KG	GST. For expression of fusion proteins in bacteria (Guan and Dixon, 1991).
pPB321	*BEM1, URA3,* 2μm ARS (Bender and Pringle, 1991). Derived from a YEp24-based genomic library (Carlson and Botstein, 1982).
pPB550	DBD_{lexA}-$BEM1_{5-551}$, *TRP1*, 2μm ARS (Peterson et al., 1994). Contains all but the first four codons of *BEM1* fused to DBD_{lexA}.
pPB561	*BEM1, URA3, CEN6, ARSH4.* Same as pCY361, except that the NotI site of the polylinker was filled in using Klenow fragment.
pPB594	DBD_{lexA}-$BEM1_{5-325}$, *TRP1*, 2μm ARS (Peterson et al., 1994). Contains codons 5–325 of *BEM1* fused to DBD_{lexA}.
pPB615	AD_{Gal4}-$BOI1_{375-980}$, *LEU2*, 2μm ARS. Contains a 12-kb Sau3AI (corresponding to position 1609 in Fig. 2)-Sau3AI fragment from the *BOI1* locus inserted into the BamHI site of the library vector pGAD (Chien et al., 1991). This is an original isolate from the Bem1p two-hybrid screen.
pPB623	DBD_{lexA}-$BEM1_{235-551}$, *TRP1*, 2μm ARS (Peterson et al., 1994). Contains codons 235–551 of *BEM1* fused to DBD_{lexA}.
pPB627	*pGAL1-CDC42, URA3, CEN4, ARS1.* Made by first inserting a BamHI site 12 bp upstream of the start codon of *CDC42* in plasmid YCp(*CDC42*) (Ziman et al., 1991) using the oligonucleotide GAAATAAACGTATTAGGGGATCCACCATATGCAAACGC and then inserting the resulting 1.1-kb BamHI-SalI fragment into the BamHI-SalI sites of 125H.
pPB644	*BEM1-215/216Bg1II, URA3, CEN6, ARSH4.* Made by introduction of a BglII restriction site at codon positions 215/216 of *BEM1* by site-directed mutagenesis of pPB561 using the oligonucleotide GTTGGGTTTGTTAGCATCAGATCTATTGCCACGGGGTAC.
pPB654	*(BEM1)-3xHA, URA3, CEN6, ARSH4.* Contains three copies of an HA epitope tag fused to the last codon of *BEM1*. Made by first introducing a NotI site at the final codon of *BEM1* by site-directed mutagenesis of pPB561 using the oligonucleotide AAAATTTCCGTTCACGATAGCGGCCGCTAGTGTTAGAAGACGAAG and then inserting into this NotI site a NotI-NotI fragment that contains the 3xHA epitope tag (Tyers et al., 1993).
pPB663	*bem1-ΔSH3#2, URA3, CEN6, ARSH4.* Similar to pPB561, except that codons 141–216, which include the second SH3 domain of *BEM1*, have been deleted. Made by deleting the 0.2-kb BamHI-Bg1II segment of *BEM1* from pPB644.
pPB699	DBD_{lexA}-$BEM1_{\Delta SH3\#2}$, *TRP1*, 2μm ARS. Similar to pPB550, but lacking codons 141–216 of *BEM1*. Made by gene conversion in yeast by cotransformation with BamHI-cut pPB550 and the 1.5-kb SmaI-DraI fragment from pPB663.
pPB713	6xHis-BEM1. Contains a 1.5-kb BamHI-KpnI fragment of *BEM1* bearing the COOH-terminal 412 codons of *BEM1* (lacking the first SH3 domain) inserted into the BamHI-KpnI sites of pQE-30.
pPB726	DBD_{lexA}-$BEM1_{140-551}$, *TRP1*, 2μm ARS. Made by inserting the 1.6-kb BamHI-NsiI fragment of pPB583 (Peterson et al., 1994), containing codons 140-551 of *BEM1*, into the BamHI-PstI sites of a version of pBTM116 (Bartel et al., 1993) in which the EcoRI site was filled in using Klenow fragment. The BamHI site of the resulting plasmid was then also filled in using Klenow fragment.
pPB734	$GST\text{-}CDC24_{COOH\ terminus}$ (Peterson et al., 1994). Contains the COOH-terminal 75 codons of *CDC24* fused to GST.
pPB735	*BOI1, URA3, CEN4, ARS1.* Contains a 9-kb *Sau*3A-*Sau*3A fragment of DNA from the *BOI1* locus inserted into the BamHI site of YCp50 (Ma et al., 1987). In this plasmid, the XhoI site shown in Fig. 2 is 1 kb away from the HindIII site in the vector. Isolated from a genomic library (Rose et al., 1987) by hybridization using a fragment of DNA from pPB591 as probe.
pPB738	AD_{Gal4}-$BOI1_{375-557}$, *LEU2*, 2 μm ARS. Contains the Pro-rich region of *BOI1*, codons 375–557, fused to AD_{Gal4}. Made by first inserting the 550-bp BamHI-NheI fragment of *BOI1* from pPB625 into the BamHI-XbaI sites of M13mp19 and then inserting the resulting 560-bp BamHI-SalI (from the polylinker) fragment from this phage into the BamHI-XhoI sites of pACTII.
pPB750	$GST\text{-}BOI1_{375-489}$. Contains the proline-rich region of BOI1 from codons 375-489 fused to GST. Made by first deleting a 1.8-kb HincII-HincII fragment from D2 and then inserting the 0.35-kb EcoRI-HindIII fragment from the resulting phage into the EcoRI-HindIII sites of pGEX-KG.
pPB768	$6xHis\text{-}BOI1_{787-980}$. Contains the COOH-terminal 194 codons of *BOI1* fused to a 6xHis tag. Made by inserting the 0.6 kb KpnI (position 2846 in Fig. 2)-XhoI fragment from D17 into the KpnI-SalI sites of pQE-30.
pPB773	*pGAL10-BOI1, LEU2,* 2μm ARS. Made by insertion of a 3.4-kb XhoI (42 bp upstream of the start codon of *BOI1*, Fig. 2)-BamHI (0.4 kb downstream of the stop codon of *BOI1*, Fig. 2) fragment from pPB735 into the SalI-BamHI sites of YEp51.
pPB775	*boi1–Δ1::LEU2* (see Materials and Methods).
pPB791	*(bem1-ΔSH3#2)-3xHA, URA3, CEN6, ARSH4.* Contains three copies of an HA epitope tag fused to the last codon of bem1-*ΔSH3#2*. Made by gene conversion in yeast by cotransformation with SalI-cut pPB654 and the 0.9-kb SmaI-PstI fragment of pPB663.
pPB799	*BOI1, ADE3, LEU2,* 2μm ARS. Made by inserting a 4.5-kb HindIII-BamHI, *BOI1*-bearing fragment from pPB735 plus and *ADE3*-bearing 5-kb BamHI-SalI fragment (Koshland et al., 1985) into the HindIII-SalI sites of pSL113 (Bender and Sprague, 1989).
pPB865	*BOI2, URA3, CEN4, ARS1.* Made by inserting a 7-kb SphI-SphI, *BOI2*-bearing fragment from cosmid 9781 (gift from F. Dietrich, Stanford University, Stanford, CA) into the SphI site of YCp50 (Ma et al., 1987).
pPB881	*boi2–Δ1::URA3* (see Materials and Methods).
pPB882	6xHis-BEM1(W192K). Same as pPB713, except that the Trp codon at position 192 of *BEM1* was changed to a Lys codon. Made by insertion of the 1.5-kb BamHI-KpnI fragment from pPB894 into the BamHI-KpnI sites of pQE-30.
pPB883	6xHis-BEM1(ΔSH3). Contains a 1.3-kb BglII-KpnI fragment from pPB644, bearing the COOH-terminal 335 codons of *BEM1* (lacking both SH3 domians) inserted into the BamHI-KpnI sites of pQE-30.

continued

Table I. (continued)

Plasmids and phage	Characteristics and source
pPB884	*boi2-Δ1::LEU2* (see Materials and Methods).
pPB894	*BEM1-W192K, URA3, CEN6, ARSH4*. pPB561 in which the Trp codon at position 192 (in the second SH3 domain of *BEM1*) was changed to Lys by site-directed mutagenesis using the oligonucleotide CCATAACTGTGAAAAGTTCATTGCTAAGC.
pPB898	GST-BOI2$_{424\text{-}488}$. Contains the Pro-rich segment of *BOI2* from codons 424–488 (Fig. 3 *a*) fused to GST. The primers GCGCGGATCCAGGTGGTAGAGGAAATGG and CGCGCGTCGACAAGTTCTTTTAGGCCC were used to PCR amplify this segment of *BOI2* from pPB865. The resulting 0.25 kb EcoRI-SalI fragment was then inserted into the EcoRI-XhoI sites of pGEX-KG.
pPB900	*pGAL10-[boi1-(735-980)], LEU2*, 2 μm ARS. Contains the COOH-terminal 246 codons of *BOI1* under the control of the *GAL10* promoter. Made by insertion of a 1.1-kb BglII (corresponding to position 2687 in Fig. 2)-HindIII fragment from phage D2 into the BamHI-HindIII sites of YEp55C.
pPB939	*BOI1, URA3, CEN6, ARSH4*. Made by inserting a 4.5-kb HindIII-BamHI fragment from pPB735 into the HindIII-BamHI sites of pRS316.
pPB945	DBD$_{lexA}$-BEM1$_{W192K}$, *TRP1*, 2μm ARS. Same as pPB550, except that the Trp codon at position 192 has been changed to a Lys codon. Made by replacing the 0.6-kb BamHI-PstI fragment of pPB550 with the 0.6-kb BamHI-PstI fragment from pPB894.
pPB952	*boi1-W53K, URA3, CEN6, ARSH4*. Same as pPB939, except that the Trp codon at position 53 in the SH3 domain of *BOI1* was changed to Lys. Made by site-directed mutagenesis of pPB939 using the oligonucleotide CGCCCATAATACTTGCCGTCATTG.
pPB954	*boi1-P7A7, URA3, CEN6, ARSH4*. Same as pPB939, except that each of the Pro codons at positions 398, 400, 401, 404, 406, 409, and 410 in *BOI1* were changed to Ala codons by site-directed mutagenesis of pPB939 using the oligonucleotide GGGCGATTGTGCAGCTTGAACTGCACTTGCATATGATGCTGCTTTTGCAGCCCTTCCCG.
pPB967	*boi1-(K795A,R797A), URA3, CEN6, ARSH4*. Same as pPB939, except that the Lys codon at position 795 and the Arg codon at position 797 were changed to Ala codons by site-directed mutagenesis of pPB939 using the oligonucleotide CCATGAAGTGTGAAAAACGCTTGTGCCCAAGTCCCC.
pPB1004	*boi1-Δ(5-733), URA3, CEN6, ARSH4. BOI1* in which codons 5–733 were deleted. Created by first introducing a BglII site at the fifth codon of BOI1 in pPB939 using the oligonucleotide GCCTAGGGTAGATCTTTCGAGACTC and then deleting the resulting 2.2-kb BglII-BglII segment from *BOI1*.
pPB1013	*RHO3, URA3, CEN6, ARSH4*. Made by insertion of a 1.8-kb KpnI-XhoI fragment bearing *RHO3* (Matsui and Toh-e, 1992*a*) into the KpnI-XhoI sites of pRS316.
pPB1142	*BOI1, TRP1*, 2μm ARS. Made by inserting the 4.5-kb BamHI-SalI (from the polylinker) fragment of DNA from pPB939 into the BamHI-SalI sites of pYO324.
pPB1144	*boi1-P7A7, TRP1*, 2μm ARS. Made by inserting the 4.5-kb BamHI-SalI (from the polylinker) fragment of DNA from pPB954 into the BamHI-SalI sites of pYO324.
pPB1148	*(bem1-W192K)-3xHA, URA3, CEN6, ARSH4*. Contains three copies of an HA epitope tag fused to the last codon of *bem1-W192K*. Made by ligating the 2.8-kb PstI-PstI fragment of DNA from pPB894 with the 4.2-kb PstI-PstI fragment of DNA from pPB654.
pPB1152	AD$_{Gal4}$-BOI1$_{731\text{-}980}$, *LEU2*, 2μm ARS. Contains the PH domain–bearing segment of *BOI1*, from codons 731–980 fused to the AD$_{Gal4}$. Made by first filling in the NcoI site of pACTII using Klenow, inserting a 760-bp EcoRI-XhoI fragment of *BOI1* from D17 into the EcoRI-XhoI sites of the NcoI-filled pACTII, and then filling in the BamHI site of resulting plasmid using Klenow.
pQE-30	6xHis. For expression of histidine-tagged fusion proteins in bacteria (Qiagen, Inc., Chatsworth, CA).
pRS316	*URA3, CEN6, ARSH4* (Sikorski and Hieter, 1989).
pY0324	*TRP1*, 2μm *ARS* (Y. Ohya). Contains the 2.2-kb EcoRI-EcoRI fragment from YEp24 (Botstein et al., 1979) inserted into the AatII site of pRS304 (Sikorski and Hieter, 1989).
YEp51	*pGAL10, LEU2*, 2μm ARS (Rose and Broach, 1990).
YEp55B,C	*pGAL10, LEU2*, 2μm ARS (Rose and Broach, 1990).
YIpGAL7-RHO3	*pGAL7-RHO3, URA3* (Matsui and Toh-e, 1992*a*).
125H	*pGAL1, URA3, CEN4, ARS1*.

Cdc42p (Cvrcková et al., 1995), all contain putative PH domains (Zheng et al., 1994; Musacchio et al., 1993; Mayer et al., 1993; Crvcková et al., 1995).

From a small-scale screen for proteins that displayed two-hybrid interactions with Bem1p, we previously isolated Cdc24p. Using bacterially expressed proteins, we found that Bem1p can bind directly to Cdc24p, and that neither the SH3 domain-bearing portion of Bem1p nor the putative PH domain of Cdc24p was required for this interaction (Peterson et al., 1994). In the current study, we have conducted a more exhaustive screen for proteins that display two-hybrid interactions with Bem1p, focusing on those proteins that interact with an SH3 domain. We report the isolation and initial characterization of the Bem1p-interacting proteins Boi1p and Boi2p, the analysis of which supports the possibility that the roles of some PH domains, SH3 domains, and Rho-type GTPases may be interconnected.

Materials and Methods

Plasmids, Strains, and Media

The plasmids and yeast strains used in this study are described in Tables I and II. pPB775, which contains *boi1-Δ1::LEU2*, was made by first deleting the DNA from between the XhoI site in *BOI1* (corresponding to position 446 in Fig. 2) to the XhoI site in the YCp50 vector portion of pPB735, inserting into the remaining XhoI site a 2.2-kb SalI-XhoI, *LEU2*–bearing fragment from YEp13 (Broach et al., 1979), and then inserting into the resulting plasmid a 0.4-kb XhoI-BamHI fragment from D17 (Table I). In *boi1-Δ1::LEU2*, *LEU2* replaces the sequences from 37 bp upstream of the start codon to 6 bp downstream of the stop codon of *BOI1*. For gene replacements, a 3.8-kb HindIII-PstI fragment from pPB775 was used. (The HindIII and PstI sites are from within YCp50 vector sequences.)

pPB881, which contains *boi2-Δ1::URA3*, and pPB884, which contains *boi2-Δ1::LEU2*, were constructed as follows. The primers GCGCAAGCTTGCTTGTCAGGACCCG plus CGCGGATCCTTTGCGGAGAAGTTGG were used to PCR amplify a 0.8-kb HindIII-BamHI fragment containing sequences from upstream of *BOI2*, and the primers CGCG-

Table II. Yeast Strains Used in This Study

Strains	Genotype*	Source
AMR70	*MATα ura 3: (URA3, Op_{lexA}-lacZ) leu2 ade2 his3 trp l lys2 gal80*	R. Sternglanz
BJ5405	*MATα ura3 leu2 his3 trp1 lys2 Gal$^+$*	E. Jones
BJ5407	*MATa ura3 leu2 his3 trp1 lys2 Gal$^+$*	E. Jones
CTY10-5d	*MATa ura3: (URA3, Op_{lexA}-lacZ) leu2 ade2 his3 trp1 gal4 gal80*	R. Sternglanz
KO2-5c	*MATa bem1::LEU2 ura3 leu2 his4 trp1*	Chenevert et al. (1992)
PY788	*[BEM1 URA3] MATα bem1::LEU2 ura3 leu2 his3 trp1*	This study‡
PY864	*[BOI1 ADE3 LEU2] MATα boi1-Δ1::LEU2 boi2-Δ2::LEU2 ura3 leu2 ade2 ade3 trp1*	This study§
PY867	*[BOI1 ADE3 LEU2] MATa/MATα boi1-Δ1::LEU2/boi1-Δ1::LEU2 boi2-Δ1::LEU2/boi2-Δ::LEU2 ura3/ura3 leu2/leu2 ade2/ade2 ade3/ade3 trp1/TRP1 lys2/LYS2*	This study§
PY899	*[BEM1 URA3] MATa bem1::LEU2 ura3 leu2 ade2 ade3 lys2*	Segregant from [Y382 × segregant from (PY788 × Y 388)]
PY970	*[BOI1 ADE3 LEU2] MATα boi1-Δ1::LEU2 boi2-Δ2::LEU2 bem1::LEU2 ura3 leu2 ade2 ade3*	Segregant from (PY864 × PY899)
Y246	*MATa/MATα ura3/ura3 leu2/leu2 his3/HIS3*	Bender and Pringle (1989)
Y312	*MATα ura3 leu2 his3 Gal$^+$*	This study‖
Y314	*MATa ura3 leu2 his3 Gal$^+$*	Same as for Y312.
Y382	*MATα ura3 leu2 ade2 ade3 trp1*	Bender and Pringle (1991)
Y388	*MATa ura3 leu2 ade2 ade3 lys2*	Bender and Pringle (1991)
Y890, Y892	*MATa/MATα boi1-Δ1::LEU2/BOI1 boi2-Δ1::URA3/BOI2 ura3/ura3 leu2/leu2 his3/HIS3*	Gene replacement of Y246 with *boi1-Δ1::LEU2* using pPB775 and with *boi2-Δ1::URA3* using pPB881
Y893	*MATa/MATα boi2-Δ2::LEU2/BOI2 ura3/ura3 leu2/leu2 his3/HIS3*	Gene replacement of Y246 with *boi2-Δ1::LEU2* using pPB884
Y926	*MATα boi1-Δ1::LEU2 ura3 leu2 his3 trp1 lys2 Gal$^+$*	Segregant from [(BJ5405 × BJ5407) made *boi1-Δ1::LEU2* by gene replacement using pPB775]
Y995	*MATa/MATα ura3/ura3 leu2/leu2 his3/his3 Gal$^+$*	Y312 × Y314
Y1050	*MATa ura3:(URA3, Op_{lexA}-lacZ) leu2 ade2 his3 trp1 lys2 gal80*	L40 (Vojtek et al., 1993) made *lys2* by growth on 5 α-aminoadipate (Chattoo et al., 1979).

*Genes listed in brackets are carried on a plasmid.
‡Derived from a series of crosses involving Y124 (Bender and Pringle, 1989), Y145 (Bender and Pringle, 1991), DJMD4-30B (Johnson and Pringle, 1990), and pPB321-bearing K02-5 (Chenevert et al., 1994).
§pPB799 in a strain derived from a series of crosses involving Y382, Y388, and segregants from Y890 and Y893.
‖Derived from a series of crosses involving Y124 (Bender and Pringle, 1989), Y145 (Bender and Pringle, 1991), and DJMD4-30B (Johnson and Pringle, 1990).

GATCCGAGGATTACTTCGGG plus TAGCCATTGTACGACG were used to amplify a 1-kb BamHI-SphI fragment containing sequences from downstream of *BOI2*. For these amplifications, pPB865 was used as the template. The resulting HindIII-BamHI plus BamHI-SphI fragments were then ligated together into the HindIII-SphI sites of pBR322. Into the resulting plasmid was then inserted either a 3.8-kb BamHI-Bg1II, *URA3*-bearing fragment from pNKY51 (Alani et al., 1987) to make pPB881, or a 2.9-kb Bg1II-Bg1II, *LEU2*-bearing fragment from YEp13 to make pPB884. In both pPB881 and pPB884, DNA from between 18 bp upstream of the start codon to 15 codons upstream of the stop codon of *BOI2* were removed. For gene replacements, a 5.6-kb EcoRI-SphI fragment from pPB881 and a 4.7-kb HindIII-SphI fragment from pPB884 were used.

The media used in this study were YP (1% yeast extract, 2% peptone); YPD (YP plus 2% glucose); SD (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose); SD + Trp + Ade + Lys (SD plus 20 μg/ml L-tryptophan, 40 μg/ml adenine sulfate, and 30 μg/ml L-lysine); SC (SD plus 20 μg/ml uracil, 80 μg/ml L-leucine, 20 μg/ml adenine sulfate, 20 μg/ml L-histidine, 20 μg/ml L-tryptophan, 20 μg/ml L-methionine, and 30 μg/ml L-lysine); SC-Ura (SC without uracil); SC-Leu (SC without L-leucine); SC-His (SC without L-histidine); SC-Ura-Leu (SC without uracil and L-leucine), SC-Trp-Leu (SC without L-tryptophan and L-leucine); and SC-Trp-Leu+X-gal (SC-Trp-Leu with 2% sucrose instead of glucose, plus 100 mM KH_2PO_4 [buffered to pH 7.0 with KOH] and 40 μg/ml X-gal [from a 40 mg/ml stock in dimethylformamide]).

Two-hybrid Screening

The two-hybrid screen was performed as described previously (Peterson et al., 1994), except that in this study, a cDNA library was used in addition to genomic libraries (libraries kindly provided by S. Elledge, Baylor College of Medicine, Houston, TX) and by P. Bartel and S. Fields (State University of New York, Stony Brook, NY). Strain CTY10-5d containing plasmid pPB550 (encoding DBD_{lexA}-Bem1) was transformed with library plasmids using the lithium thiocyanate procedure (Keszenman-Pereyra and Hieda, 1988) and plated on SC-Trp-Leu+X-gal. Blue transformants were restreaked on SC-Trp-Leu+X gal. Plasmids from blue colonies were recovered in *Eschericia coli* and introduced separately into strain AMR70 containing pPB550 and into AMR70 containing pCTC52 (encoding DBD_{lexA}-lamin), selecting for transformants on SC-Trp-Leu. Colonies of transformed cells were imprinted onto Whatman 50 filter paper (Whatman Chemical Co., Clifton, NJ), frozen on dry ice, and incubated in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0) containing 0.3 mg/ml X-gal. Colonies were scored for the development of blue color 6 h after incubation at 30°C. Only those clones that caused expression of β-galactosidase in combination with the DNA-binding domain (DBD)–Bem1p fusion, but not in combination with the DBD–lamin fusion, were studied further. Library plasmids were then introduced into AMR70 containing the plasmids that encode the DBD–Bem1p fusions shown in Fig. 1, plated on SC-Trp-Leu, and tested for β-galactosidase activity using the filter-lift technique described above. In each case, multiple independent transformants were tested for activity, and all experiments were repeated at least once using transformants that had been isolated on separate days. There was some uncertainty in scoring the results of the test with pPB594 (Fig. 1, *Test 6*) because this plasmid by itself caused cells to turn light blue in X-gal. Therefore, for this test, only those transcriptional activation domain of Gal4p (AD) fusions that caused cells to turn dark blue were retained. For each library plasmid that passed all six tests shown in Fig. 1, a portion of the library insert adjacent to the AD was sequenced by the dideoxynucleotide chain termination technique using either primer TACCACTACAATGGATG or ATACCCCACCAAACCC.

Quantitative β-Galactosidase Activity Assays

For the two-hybrid experiments in Table IV, all of the DNA-binding domain (DBD) fusions were constructed in pEG202 (Golemis et al., 1994; Cvrcková et al., 1995). (All of these DBD fusion-encoding plasmids were kindly provided by C. De Virgilio and J. Pringle, The University of North Carolina, Chapel Hill, NC.) In the DBD fusions to Rho3 and to the different versions of Cdc42, the Cys codon of the CAAX box was mutated to

Ser to prevent attachment to membrane (Cvrcková et al., 1995; Ziman et al., 1991). The plasmids that encode the DBD fusions were introduced into strain Y1050, selecting for transformants on SC-His. The plasmids that encode the AD fusions were then introduced into the resulting DBD-containing strains, selecting for transformants on SD+Trp+Ade+Lys. Liquid overnight cultures were diluted 10–40-fold in SD+Trp+Ade+Lys and grown for 8–12 h with shaking at 30°C. Cultures were harvested at an OD_{600} of 0.1–0.6. β-galactosidase assays were performed as described (Reynolds and Lundblad, 1989).

Cloning and Sequencing of BOI1

pPB735 bearing full-length *BOI1* was identified using the previously described protocol for colony hybridization (Peterson et al., 1994). The 3.8-kb SphI-BamHI segment of DNA from pPB735 was sequenced by the dideoxynucleotide chain-termination method. Both strands were sequenced completely, and all sites used for subcloning were sequenced across.

Biochemical and Immunological Techniques

Binding experiments using bacterially expressed proteins were performed essentially as described previously (for details, see Peterson et al., 1994). The expression of fusion proteins was induced with IPTG, and cells were opened by sonication in sonication buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 400 ng/ml bestatin, 500 ng/ml leupeptin, 350 ng/ml pepstatin A). After the addition of Triton X-100 to 0.1%, lysates were centrifuged at 17,000 *g* for 20 min. Aliquots of extract supernatants were incubated with glutathione-agarose resin (Sigma Chemical Co., St. Louis, MO) for 1 h, and the resin was then washed three times with binding buffer (sonication buffer plus 0.1% Triton X-100). The supernatant of the appropriate second bacterial extract was incubated with the resin for an additional hour, and the resin was washed three times with binding buffer. The samples were then boiled in 2× sample buffer (125 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.2% bromophenol blue dye) and subjected to SDS-PAGE analysis.

The histidine-tagged Boi1 ($6\times$His-$Bio1_{787\text{-}980}$) fusion protein that was used as a source of antigen was purified from pPB768-bearing bacterial strain TG1 (Amersham Life Sciences Inc., Arlington Heights, IL) according to the manufacturer's procedure for isolation of denatured proteins (Qiagen, Inc., Chatsworth, CA). Elution of the fusion protein from the Ni-NTA resin (Invitrogen Corp., San Diego, CA) was accomplished using column wash buffer at pH 4.5. The eluate was dialyzed against PBS (0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) to remove the urea, and the resulting precipitated protein was used for immunization of a NZW rabbit. For the primary injection, a suspension of 1 mg of $6\times$His-$Boi1_{787\text{-}980}$ in PBS was emulsified with complete Freund's adjuvant and injected intradermally at multiple sites. Boosts with 0.5 mg of the fusion protein in an emulsion with incomplete Freund's adjuvant were given subcutaneously 6 and 12 wk later. Serum obtained 21 d after the second boost was used without further purification. A histidine-tagged Bem1 fusion protein was produced from TG1 cells bearing pPB713 and was injected into a rabbit as described above. Serum obtained 13 d after the second boost was used without further purification.

The yeast extracts used in lanes *1* and *2* of Fig. 5 *A* were prepared by diluting 5 ml of overnight cultures form SC-Leu into 100 ml of YP plus 2% raffinose and then incubating the cultures at 30°C until they reached an OD_{600} of 0.2. Galactose was then added to a final concentration of 1%, and cells were incubated an additional 6 h. Cells were pelleted and washed with IP buffer (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.4 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A) and then opened by vigorous vortexing with glass beads. Cell debris that pelleted after centrifugation at 3,000 *g* for 5 min was discarded. Concentrations of total protein were determined using the Bradford assay (Bradford, 1976). 10 μg of protein was boiled in 2× sample buffer before SDS-PAGE.

The yeast extracts used for immunoprecipitation studies were prepared from cultures grown in SC-Ura (Fig. 5 *A*) or SC-Ura-Trp (Fig. 5, *B* and *C*) to an OD_{600} of 0.8. Cells were pelleted and washed with IP buffer and then opened using a French press (Fig. 5 *A*) or by vortexing with glass beads (Fig. 5, *B* and *C*). Cell debris was discarded. After incubation on ice for 20 min, DTT was added to a final concentration of 1 mM. 3 mg (Fig. 5 *A*) or 6 mg (Fig. 5, *B* and *C*) of each extract was precleared by incubation with 50 μl (Fig. 5 *A*) or 30 μl (Fig. 5, *B* and *C*) of a slurry of protein G–agarose beads (Boehringer Mannheim Co., Indianapolis, IN) for 1 h at 4°C. 50 μl of protein G–agarose was washed once with nondenaturing binding buffer (20 mM Tris-Cl, pH 7.5, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 0.1 mM PMSF, 0.4 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A) without added NaCl (Fig. 5 *A*) or with 200 mM NaCl (Fig. 5, *B* and *C*) and then mixed with 10 μl of 12CA5 (anti-HA epitope) ascites fluid (Berkeley Antibody Co., Berkeley, CA) plus 190 μl of nondenaturing binding buffer and incubated with gentle shaking at 4°C for 2 h. The beads were washed three times with nondenaturing binding buffer and incubated with the precleared yeast extracts at 4°C for 3 h. The beads were then washed four times with nondenaturing binding buffer. 2× sample buffer was added to each immunoprecipitate, and each sample was boiled for 10 min and then loaded onto an 8% SDS-polyacrylamide gel. After electrophoresis, gels were soaked for 30 min in gel transfer buffer (25 mM Tris-Cl, pH 8.3, 0.2 M glycine, 15% methanol). Proteins were electroblotted to a nitrocellulose membrane (Optitran BA-S85; Schleicher & Schuell, Inc., Keene, NH). Membranes were soaked in PBS-T (PBS + 0.1% Tween 20) plus 0.1% India ink for 30 min, washed twice briefly with PBS-T, and incubated with Blotto (PBS-T + 5% nonfat dry milk) for 1 h. Membranes were then incubated overnight in fresh Blotto containing anti-Boi1p serum at a dilution of 1:5,000, washed three times with PBS-T, and then incubated for 1 h in PBS-T containing HRP-conjugated goat anti-rabbit IgG antibodies (Sigma Chemical Co.) at a dilution of 1:25,000. Membranes were washed three times with PBS-T, and the HRP was detected by chemiluminescence according to the protocol of the manufacturer (Amersham Life Sciences, Inc.). The primary and secondary antibodies were washed off by incubation with stripping buffer (62.5 mM Tris-Cl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) at 42°C for 30 min. Membranes were reprobed with anti-Bem1p serum at a dilution of 1:50,000 and then with secondary antibody as described above.

Microscopy

Cells were fixed in 70% EtOH and stained with DAPI (4′,6-diamidino-2-phenylindole) in 1 mg/ml *p*-phenylenediamine essentially as described previously (Pringle et al., 1989). Cells were mounted under a coverslip and photographed with an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) using a 100× Plan-Neofluor oil immersion objective.

Results

Screen for Proteins that Display Two-hybrid Interactions with Bem1p

To identify proteins that bind to either SH3 domain of

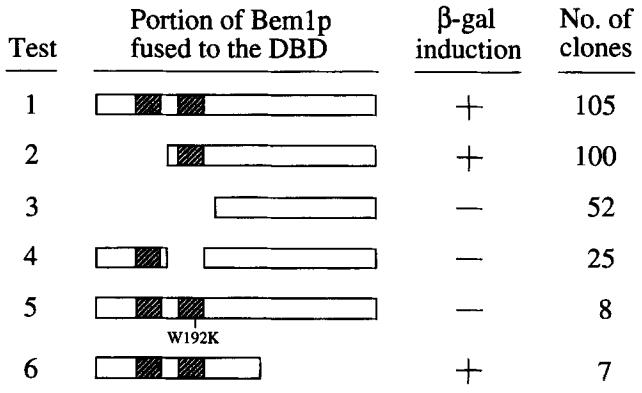


Figure 1. Tests for two-hybrid interactions between DBD (DNA-binding domain)–Bem1p fusions and the AD fusions encoded by the library clones (Materials and Methods). The segments of Bem1p that were fused to the DBD are indicated by boxes. Striped segments represent the SH3 domains. The number of AD fusions that affected β-galactosidase expression in the manner indicated is shown. For each test, only those fusions that also gave the indicated results in the previous tests were included. The DBD-Bem1p–encoding plasmids used for these two-hybrid tests were pPB550 (*1*), pPB726 (*2*), pPB623 (*3*), pPB699 (*4*), pPB945 (*5*), and pPB594 (*6*).

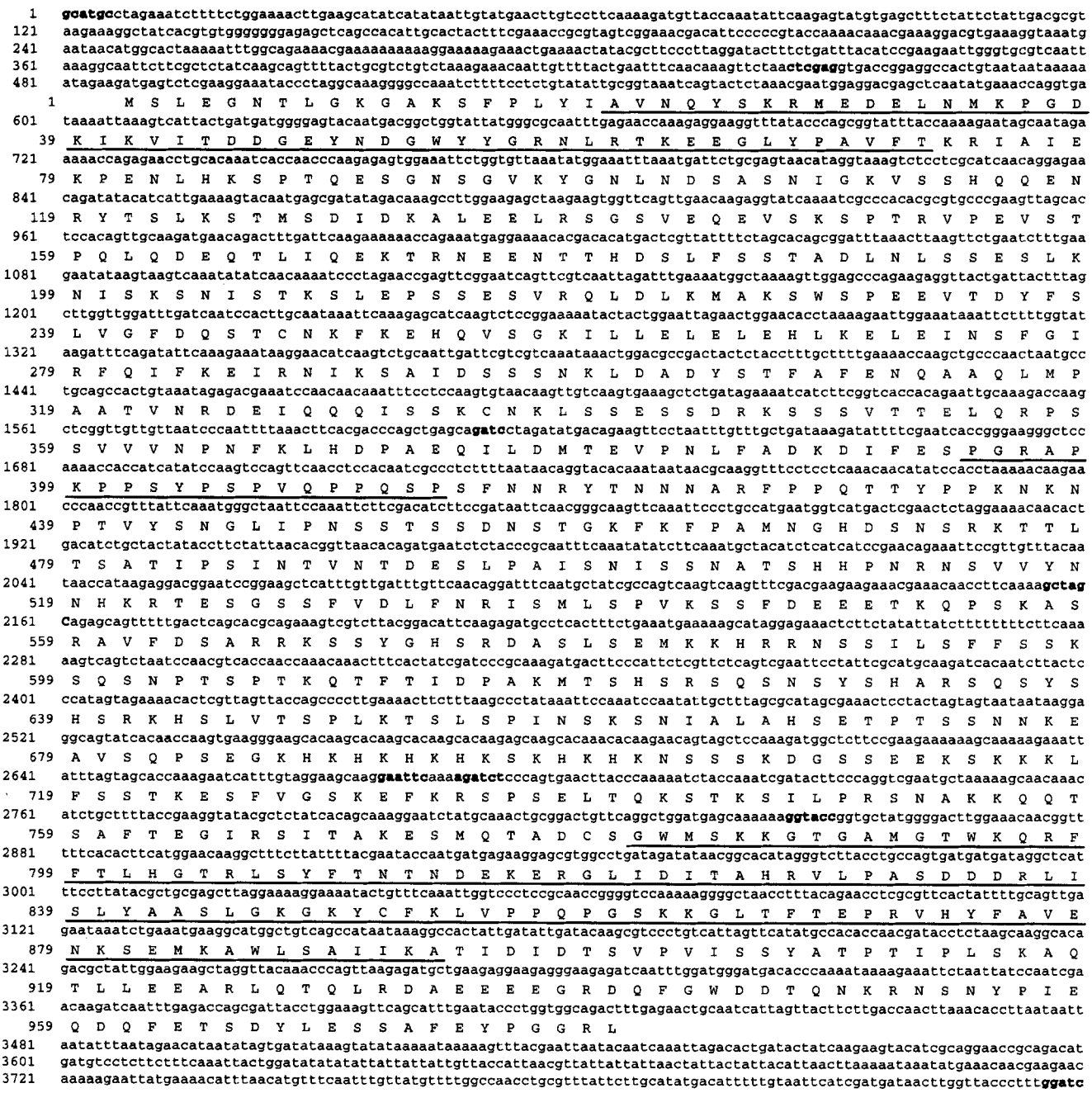


Figure 2. Sequence of the *BOI1* gene and the inferred Boi1 protein. The SH3 domain, Pro-rich region, and PH domain are underlined. All XhoI (446), NheI (2156), BglII (2687), KpnI (2846), BamHI (3836), and relevant SphI (1), Sau3AI (1609), and EcoRI (2678) sites are indicated in bold type. These sequence data are available from GenBank/EMBL/DDBJ under accession No. L31406.

Bem1p, we first screened for proteins that displayed two-hybrid interactions with full-length Bem1p and then tested these for the ability to interact with mutant versions of Bem1p that lacked one or both SH3 domains. 105 clones were identified from this screen. A segment of Bem1p that contains the second SH3 domain was required for the interaction with 25 of these clones (Fig. 1, *Tests 1–4*). 8 of these 25 clones failed to interact with a mutant version of Bem1p in which a conserved Trp residue (position 192) in the second SH3 domain was changed to Lys (Fig. 1, *Test 5*). 7 of these 8 clones could interact with a portion of Bem1p that lacks a fair amount of the non-SH3 domain sequence (Fig. 1, *Test 6*), including sequences required for the two-hybrid interaction with Cdc24p (Peterson et al., 1994).

Sequence analysis revealed that each of these seven clones was derived from one of the four following genes: (*a*) the new gene *BOI1* (Bem1p-interacting; one clone); (*b*) the related gene *BOI2* (two clones), which was also identified during the yeast genome sequencing project (as *YER114C*) and during an independent screen for genes whose products displayed two-hybrid interactions with Bem1p (Matsui et al., 1996); (*c*) *YER124C* (three clones), a gene of unknown function that was identified during the

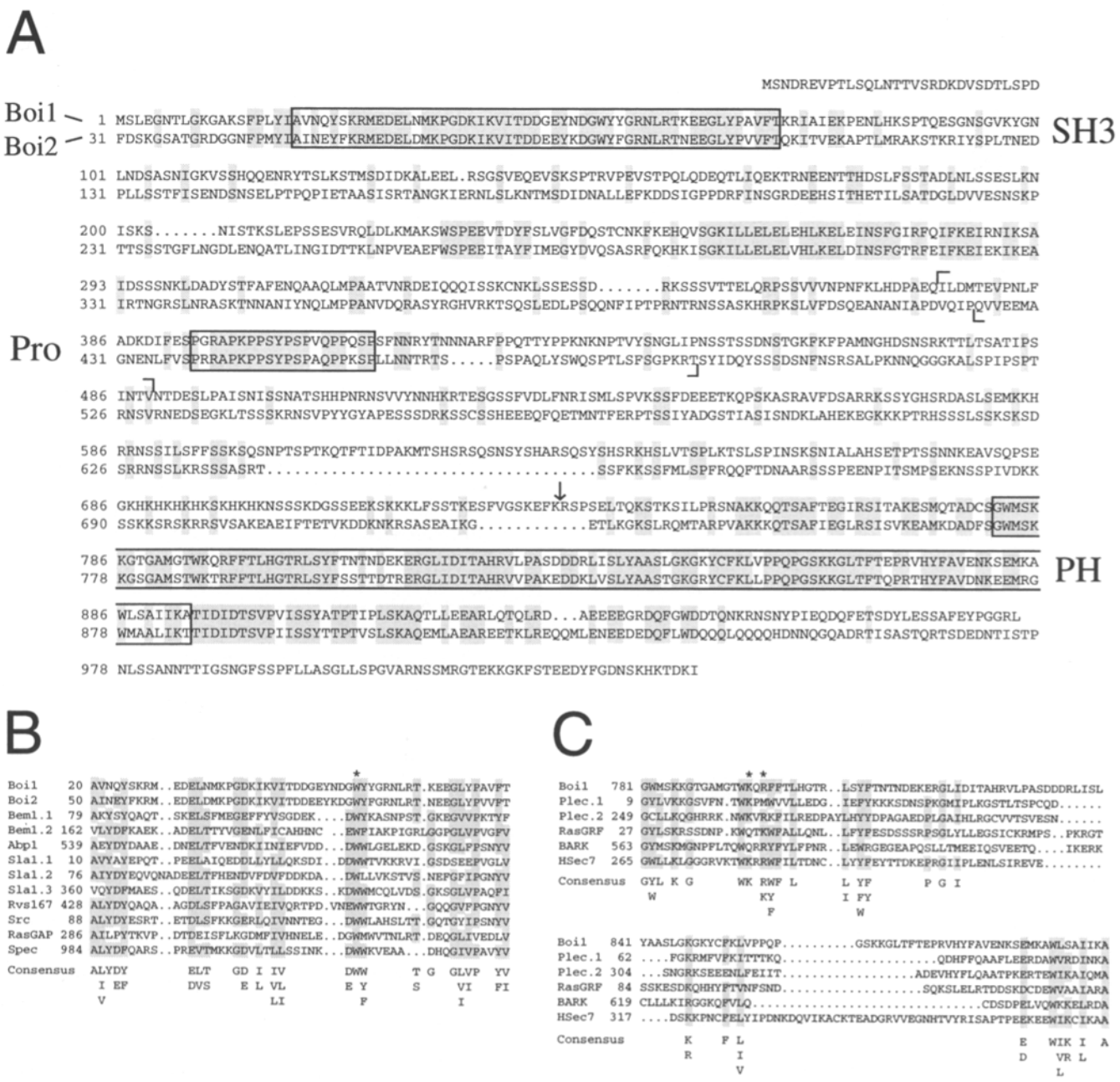


Figure 3. Sequence comparisons involving Boi1p and Boi2p. (*A*) Alignment of Boi1p and Boi2p. Identical amino acids are shaded. The SH3 domains, proline-rich regions, and PH domains are boxed. Brackets indicate the segments of Boi1p and Boi2p used in the binding studies shown in Fig. 4. The arrow marks the site in Boi1p that was used for the construction of *boi1-Δ(5-733)* and *pGAL10-[boi1-(735-980)]*. (*B*) Alignment of the SH3 domains of Boi1p and Boi2p with those from the yeast proteins Bem1p (Chenevert et al., 1992), Abp1p (Drubin et al., 1990), Sla1p (Holtzman et al., 1993), and Rvs167p (Bauer et al., 1993) and with chicken Src (Takeya and Hanafusa, 1983), human Ras-GAP (Trahey et al., 1988), and human erythroid α-spectrin (*Spec*; Sahr et al., 1990). The asterisk marks the Trp residue that was mutated to create *boi1-W53K*. (*C*) Alignment of the PH domain of Boi1p with those from pleckstrin (*Plec*; Tyers et al., 1988), Ras-GRF (Shou et al., 1992), β-adrenergic receptor kinase (BARK; Benovic et al., 1989), and HSec7 (Liu and Pohajdak, 1992). The asterisks mark the positions that were changed to create *boi1-(K795A, R797A)*. The consensus sequences shown in *B* and *C* were derived from the sequences shown here. Matches to the consensus are shaded.

yeast genome sequencing project; and (*d*) *MPS1/RPK1* (one clone), which encodes an essential, dual-specificity protein kinase required for spindle pole body duplication and cell cycle control (Lauze et al., 1995; Poch et al., 1994). The segments of Boi1p and Boi2p present in the AD fusion clones contained proline-rich regions (nine prolines over a length of 20 amino acids), consistent with the possibility that Boi1p and Boi2p may be ligands for the second SH3 domain of Bem1p. Further analyses of *BOI1* and *BOI2* are presented below. Yer124p does not contain either of the canonical SH3 domain-binding motifs R/KxxPxxP or PxxPxR/K, raising the possibility that the interaction between Yer124p and Bem1p might be indirect (e.g., via a bridging protein). Mps1p/Rpk1p contains the potential

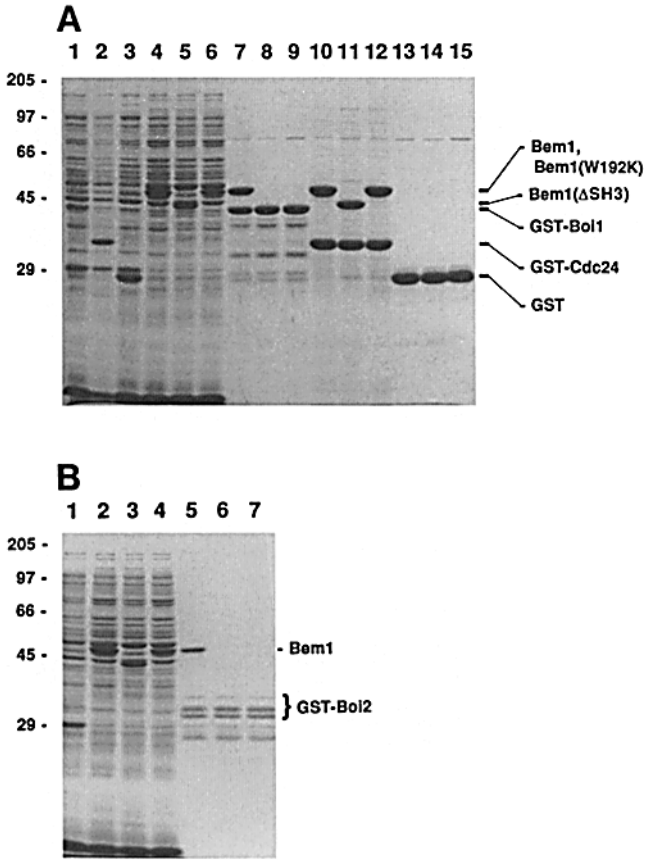


Figure 4. Binding of wild-type and mutant versions of Bem1p to Boi1p, Boi2p, and Cdc24p. Proteins were separated electrophoretically and then stained with Coomassie brilliant blue. (*A*) Proteins from extracts of bacteria expressing GST-Boi1 (lane *1*), GST-Cdc24 (lane *2*), GST (lane *3*), Bem1 (lane *4*), Bem1(ΔSH3) (lane *5*), and Bem1(W192K) (lane *6*). Proteins bound to glutathione *S*-agarose resin from extracts containing GST-Boi1 (lanes *7–9*), GST-Cdc24 (lanes *10–12*), and GST (lanes *13–15*) mixed with extracts containing Bem1 (lanes *7*, *10*, and *13*), Bem1(ΔSH3) (lanes *8*, *11*, and *14*), and Bem1(W192K) (lanes *9*, *12*, and *15*). (*B*) Proteins from extracts of bacteria expressing GST-Boi2 (lane *1*), Bem1 (lane *2*), Bem1(ΔSH3) (lane *3*), and Bem1(W192K) (lane *4*). Proteins bound to glutathione *S*-agarose resin from extracts containing GST-Boi2 mixed with extracts containing Bem1 (lane *5*), Bem1(ΔSH3) (lane *6*), and Bem1(W192K) (lane *7*). The positions of molecular mass standards are indicated on the left, and the positions of the fusion proteins are shown on the right. The following plasmids were used for expression of the relevant proteins: pGEX-KG (GST), pPB750 (GST-Boi1), pPB898 (GST-Boi2), pPB734 (GST-Cdc24), pPB713 (Bem1), pPB883 [Bem1(ΔSH3)], and pPB882 [Bem1(W192K)].

SH3 domain–binding sequence RPAPKPP near its kinase domain. We have not analyzed Yer124p or Mps1p/Rpk1p any further.

A segment of DNA containing full-length *BOI1* was cloned from a low copy number library by colony hybridization and was sequenced (see Materials and Methods). The sequences of *BOI1* and the inferred Boi1 protein are shown in Fig. 2. In addition to containing a proline-rich region, Boi1p and Boi2p both have an SH3 and a PH domain (Figs. 2 and 3). In a multiple sequence alignment analysis, the PH domain of Boi1p (whose sequence we originally deposited into data bases under the name "Bob1p") was found to have one of the best alignment scores of any PH domain identified to date (Gibson et al., 1994).

Binding of Bem1p to Boi1p and Boi2p

Bacterially expressed Bem1p could bind to a bacterially expressed GST fusion that contains a 115–amino acid long, proline-rich segment of Boi1p (Figs. 3 *A* and 4 *A*, lane *7*) and to a GST fusion containing a 65–amino acid long, proline-rich segment of Boi2p (Figs. 3 *A* and 4 *B*, lane *5*), but it could not bind to GST alone (Fig. 4 *A*, lane *13*). A mutant version of Bem1p that completely lacks the second SH3 domain and one containing a mutation at a conserved Trp residue in the second SH3 domain failed to bind to GST-Boi1p and GST-Boi2p (Fig. 4, *A*, lanes *8* and *9*, and *B*, lanes *6* and *7*). These mutant versions of Bem1p were expressed well and could bind Cdc24p (Fig. 4, *A*, lanes *4–6* and *10–12*, and *B*, lanes *2–4*), indicating that the failure to bind Boi1p and Boi2p was not caused by instability or gross misfolding of the mutant Bem1 proteins. Given that the only region of similarity between these segments of Boi1p and Boi2p is the proline-rich stretch (Fig. 3 *A*), these data strongly suggest that the second SH3 domain of Bem1p binds directly to the proline-rich region of Boi1p and Boi2p, and they demonstrate that no other yeast proteins are required for this interaction.

To investigate whether Boi1p associates with Bem1p in yeast, we asked whether Boi1p could be coimmunoprecipitated from yeast extracts with a hemagglutinin (HA) epitope–tagged version of Bem1p that was expressed from a low copy number plasmid under the control of its own promoter. HA-tagged Bem1p, but not untagged Bem1p, could be precipitated using anti-HA antibodies (Fig. 5 *A*, *bottom panel*, lanes *3–5*). A protein that is recognized by polyclonal antibodies raised against Boi1p and that is the same size as Boi1p is present in the anti-HA immunoprecipitate (Fig. 5 *A*, *top panel*, lanes *1–3*). This protein is not present in immunoprecipitates from isogenic strains that lack either Boi1p (Fig. 5 *A*, lane *4*) or the HA tag on Bem1p (Fig. 5 *A*, lane *5*).

HA-tagged versions of Bem1p that contain a point mutation in or a deletion of the second SH3 domain could be immunoprecipitated with anti-HA antibodies (Fig. 5 *B*, *bottom panel*, lanes *3* and *4*). However, even when overexpressed from a high copy number plasmid, Boi1p failed to coimmunoprecipitate with these mutant versions of Bem1p (Fig. 5 *B*, *top panel*, lanes *3* and *4*), indicating that the second SH3 domain of Bem1p is important for the association with Boi1p. Similarly, even when overexpressed from a high copy number plasmid at levels comparable to that at which wild-type Boi1p is overexpressed in this experiment (Fig. 5 *C*, *middle band in the bottom panel*), a mutant version of Boi1p that lacks seven of the nine prolines in the proline-rich region failed to coimmunoprecipitate with HA-tagged Bem1p (Fig. 5 *C*, *top panel*, lane *5*). These results strongly suggest that Boi1p is present in a complex with Bem1p in yeast, and that the ability of these two proteins to be in the same complex requires the second SH3 domain of Bem1p and the proline-rich region of Boi1p.

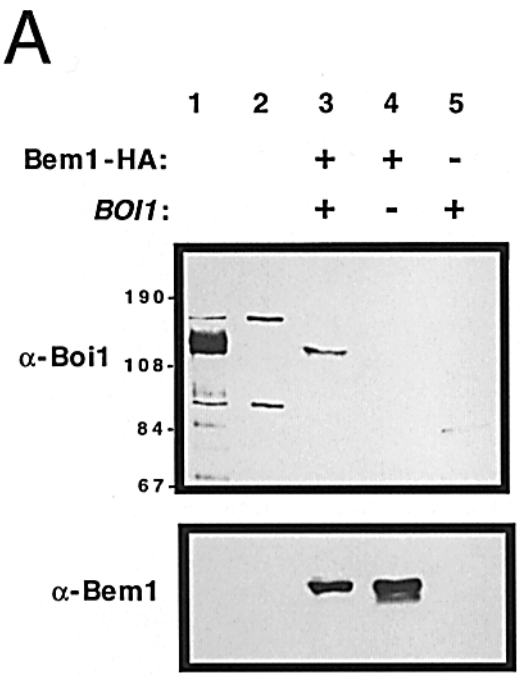
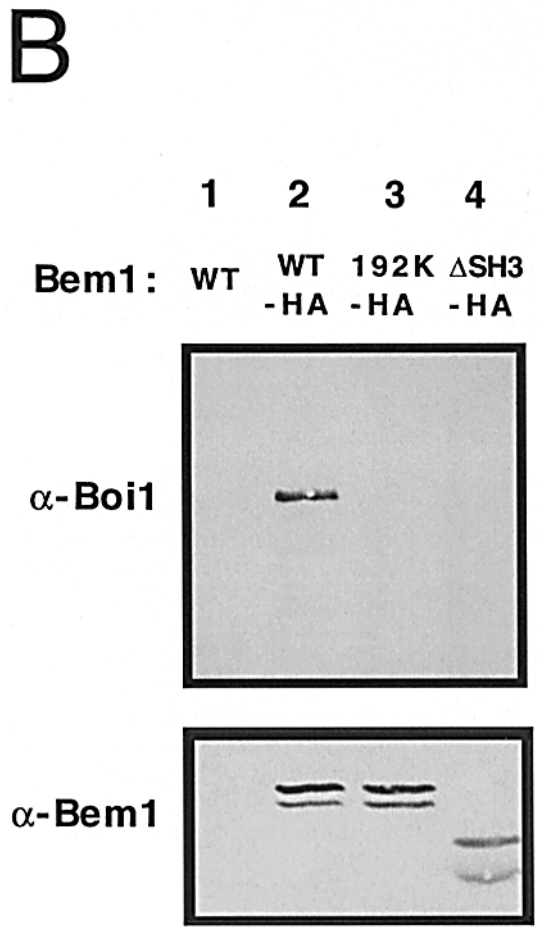
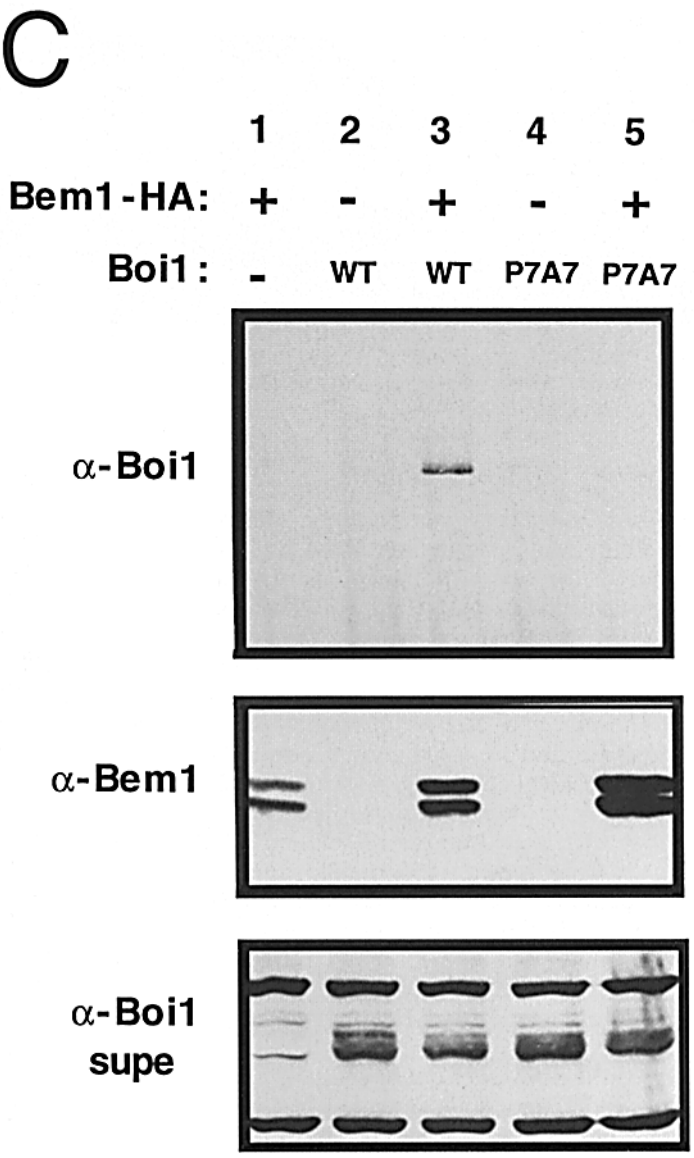


Figure 5. Coimmunoprecipitation of Boi1p with Bem1p-HA from yeast extracts. (*A*) anti-HA immunoprecipitates from the following yeast extracts were analyzed after electrophoresis by immunoblotting using polyclonal antibodies against Boi1p. pPB654 [*BEM1*-(3x*HA*)]–bearing strain BJ5405 (*BOI1*), lane *3*; pPB654-bearing strain Y926 (*boi1Δ*), lane *4*; and pCY361 (*BEM1* without an HA tag)-bearing strain BJ5405, lane *5*. The blot was stripped and reprobed with polyclonal antibodies against Bem1p (*bottom panel*). Lanes *1* and *2* are control lanes to determine the position of Boi1p. Extracts were from BJ5405 bearing either pPB773 (*pGAL-BOI1*) (lane *1*) or YEp51 (*pGAL*), grown in galactose-containing medium. Positions of molecular mass standards are indicated on the left. (*B*) Anti-HA immunoprecipitates from *bem1Δ* strain KO2-5c containing pPB1142 (*BOI1*, 2 μm ARS) plus either pPB561 (*BEM1* without an HA tag), lane *1*; pPB654 [(*BEM1*)-3x*HA*], lane *2*; pPB1148 [(*bem1-W192K*)-3x*HA*], lane *3*; or pPB791 [(*bem1-ΔSH3#2*)-3x*HA*], lane *4*. The anti-HA immunoprecipitates were probed with antibodies against Boi1p (*top panel*) and Bem1p (*bottom panel*). (*C*) Anti-HA immunoprecipitates from *boi1Δ* strain Y926 containing the following pairs of plasmids: pPB654 and pYO324 (empty vector), lane *1*; pPB561 and pPB1142 (*BOI1*, 2μm ARS), lane *2*; pPB654 and pPB1142, lane *3*; pPB561 and pPB1144 (*boi1-P7A7*, 2μm ARS), lane *4*; and pPB654 and pPB1144, lane *5*. The anti-HA immunoprecipitates were probed with antibodies against Boi1p (*top panel*) and Bem1p (*middle panel*). The supernatant fractions (after removal of the immunoprecipitates) were probed with antibodies against Boi1p (*bottom panel*). The middle band in the bottom panel is Boi1p.





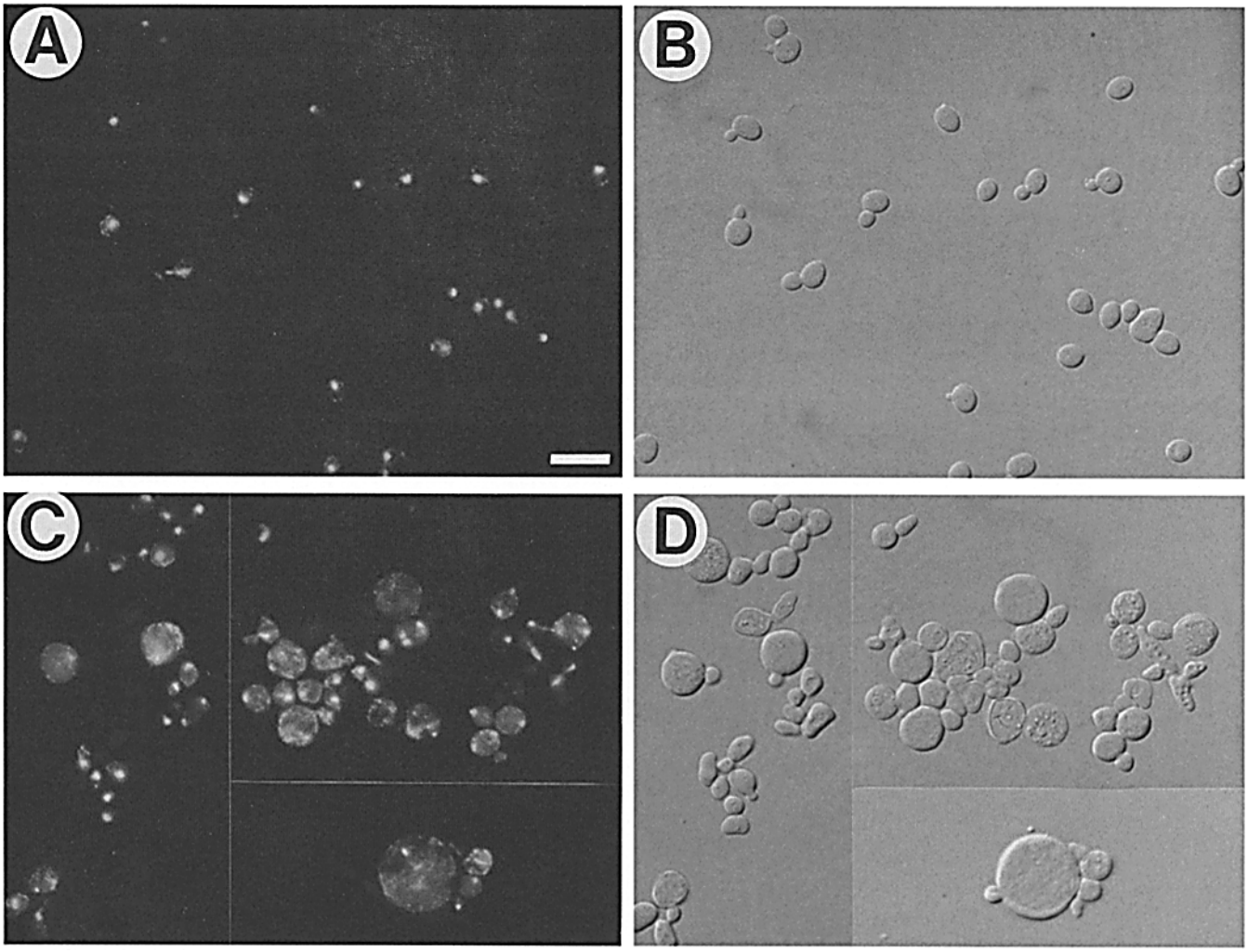


Figure 6. Phenotypes of *boi1 boi2* mutants. Fluorescence (*A* and *C*) and DIC images (*B* and *D*) of DAPI-stained cells from a wild-type (*A* and *B*) and a *boi1 boi2* mutant (*C* and *D*) segregant from Y892. Cells were grown at 20°C in YPD. Bar, 10 μm.

Phenotypes of boi1 boi2 Mutants

BOI1 and *BOI2* were deleted from the genome of an a/α diploid strain (Materials and Methods). Southern analysis confirmed the success of the deletions (data not shown). In tetrad analyses of *boi1-Δ1::LEU2/BOI1 boi2-Δ1::URA3/BOI2* strains Y890 (five tetrads) and Y892 (seven tetrads) at 30°C, 8 viable segregants were Ura^+ Leu^- and 10 were Ura^- Leu^+, indicating that neither *BOI1* nor *BOI2* is essential. Indeed, cells deleted for either *BOI1* or *BOI2* alone grew well and displayed normal morphologies. None of the viable segregants were Ura^+ Leu^+, and 15 of the 17 inviable segregants were inferred to be Ura^+ Leu^+, indicating that cells deleted for both *BOI1* and *BOI2* were inviable at 30°C. However, viable double-mutant segregants could be obtained at 24 or 20°C. Those obtained at 24°C grew very poorly, but those obtained at 20°C grew only somewhat more slowly than wild-type cells (data not shown). *boi1 boi2* double-mutant segregants obtained at 20°C failed to grow or grew very poorly when streaked at 30° or 37°C.

Many of the *boi1 boi2* double-mutant cells were misshapen or round and large, and a fair fraction of the cells appeared to be lysed (Fig. 6). Even at the "permissive" temperature of 20°C, ~30% of the cells were dead, as judged by the inability to stain with methylene blue dye. In those cells that had normal morphologies, the distribution of actin appeared normal. In large, round cells, cortical actin patches were delocalized, as expected of cells that were growing isotropically (data not shown). In many cases, dividing nuclei failed to orient properly with respect to the bud, and ~5% of the cells contained two or more nuclei (Fig. 6). These phenotypes are consistent with a model in which Boi1p and Boi2p are required for the proper assembly or function of structures at the bud site. However, further analyses will be required to elucidate the primary defect of these mutants.

Mutational Analysis of Boi1p

To investigate whether the proline-rich region, the SH3 domain, or the PH domain of Boi1p is important for function, we tested the ability of the following mutant versions of *BOI1* to substitute for the wild-type gene: (*a*) *boi1-P7A7*, in which seven of the nine proline codons in the proline-rich region were changed to Ala codons (encoding

Table III. Ability of Mutant Versions of BOI1 to Substitute for Wild-type BOI1 and BOI2 in the Presence or Absence of BEM1

Plasmid*	Sectoring phenotype‡	
	+BEM1§	*−BEM1*‖
pPB939 (*BOI1*)	+	+
pPB954 (*boi1-P7A7*)	+	+
pPB952 (*boi1-W53K*)	+	−
pPB967 [*boi1-(K795A,R797A)*]	−	−
pPB1004 [*boi1-Δ(5-733)*]	+	−

*The allele of *BOI1* present on each plasmid is shown in parentheses or brackets.
‡The ability of colonies of cells containing the indicated plasmids to form white sectors (indicating the ability to grow in the absence of the wild-type *BOI1*-bearing plasmid pPB799) was assayed on SC-Ura transformation plates. Each experiment was performed at least three times using transformants isolated on at least three separate days. Tens to hundreds of colonies were analyzed in each experiment.
§Sectoring phenotype at 30°C of PY867 (*boi1/boi1 boi2/boi2 BEM1/BEM1*) transformants.
‖Sectoring phenotype at 24°C of PY970 (*boi1 boi2 bem1*) transformants.

a mutant version of Boi1p that failed to coimmunoprecipitate with Bem1p from yeast extracts; Fig. 5 *C*); (*b*) *boi1-W53K*, in which a conserved Trp codon in the SH3 domain was changed to a Lys codon; and (*c*) *boi1-(K795A,R797A)*, which contains a mutation of Arg 797 that is analogous to a mutation that impairs the function of the PH domain of Burton's tyrosine kinase (in immunodeficient XID mice) (Thomas et al., 1993; Rawlings et al., 1993), plus a second mutation in a nearby basic residue (Lys 795). (The latter mutation was introduced in case Lys 795 can participate in binding interactions that normally involve Arg 797.)

The ability of these mutant alleles of *BOI1* to substitute for *BOI1* and *BOI2* was determined using a red/white (*ADE3/ade2 ade3*) colony-sectoring assay. The basis for this assay is that strain PY867, which contains homozygous mutant alleles of *boi1*, *boi2*, *ade2*, and *ade3* in the genome, and which carries wild-type *BOI1* and *ADE3* on plasmid pPB799, forms uniformly red colonies (Sect$^-$ phenotype) on nonselective medium caused by the requirement for *BOI1* and the red color conferred by *ADE3*. When transformed with a second plasmid containing an allele of *BOI1* that is sufficient for growth, however, PY867 cells now formed colonies containing many white sectors (Sect$^+$ phenotype) because pPB799 is no longer needed for viability. When transformed with low copy number plasmids bearing *boi1-P7A7* or *boi1-W53K*, PY867 became Sect$^+$ (Table III), indicating that neither the proline-rich sequence (needed for binding to Bem1p) nor the SH3 domain of Boi1p are required for growth. In contrast, when transformed with a plasmid bearing *boi1-(K795A,R797A)*, PY867 remained Sect$^-$, suggesting that the PH domain is important for function. *boi1-Δ(5-733)*, which lacks sequences from codon positions 5–733 (and so lacks the SH3 domain and the Pro-rich sequence), but which retains the PH domain (Fig. 3 *A*), gave a Sect$^+$ phenotype (Table III) and conferred good growth to *boi1 boi2* mutant cells (Fig. 7). Thus, the COOH-terminal, PH domain–bearing segment appears to be both necessary and sufficient for Boi1p/Boi2p function.

Synthetic Lethality between boi1 boi2 and bem1

The finding that mutant versions of Boi1p that lack the proline-rich region are sufficient for good growth of *boi1 boi2* mutants suggests that the ability to bind Bem1p is not essential for Boi1p/Boi2p function. To further explore the relationship between Boi1p and Bem1p, we used the colony-sectoring assay to determine whether those sequences in Boi1p that were dispensable in an otherwise wild-type strain were still unnecessary in the absence of Bem1p. *boi1-Δ(5-733)* could not substitute for wild-type *BOI1* and *BOI2* in cells lacking *BEM1* (Table III), indicating that sequences outside of the PH domain–bearing segment of Boi1p are important in a process for which Bem1p normally is sufficient. *boi1-P7A7* could substitute for *BOI1* and *BOI2* in a *bem1* mutant, indicating that the proline-rich region of Boi1p is not needed, even in the absence of Bem1p (Table III). In contrast, *boi1-W53K* could not substitute for *BOI1* and *BOI2* in a *bem1* mutant, indicating that the SH3 domain of Boi1p is important for Boi1p/Boi2p function in the absence of Bem1p (Table III).

Interactions between Boi1p and Rho3p

Given that Bem1p displays genetic interactions with Cdc42p, Rho3p, and Bem2p, which is a potential GAP for Rho1p, we were curious to know whether Boi1p and Boi2p also had functions linked to any Rho-type GTPases (Mack, D., and A. Bender, unpublished data; Matsui and Toh-e, 1992*b*; Peterson et al., 1994). To address this possibility, we used a colony-sectoring assay to investigate whether any of the known yeast Rho-type GTPases could serve as multicopy bypass suppressors of *boi1 boi2*. *boi1/boi1 boi2/boi2 ade2/ade2 ade3/ade3* strains bearing pPB799 (*BOI1*, *ADE3*, 2 μm ARS) remained Sect$^-$ after transformation with high copy number plasmids containing *CDC42*, *RHO1*, or *RHO2*, indicating that these transformants still required wild-type *BOI1* for survival. In contrast, these strains became Sect$^+$ when transformed with a high copy number plasmid bearing *RHO3*. The suppression of *boi1 boi2* by *RHO3* was strong enough that even

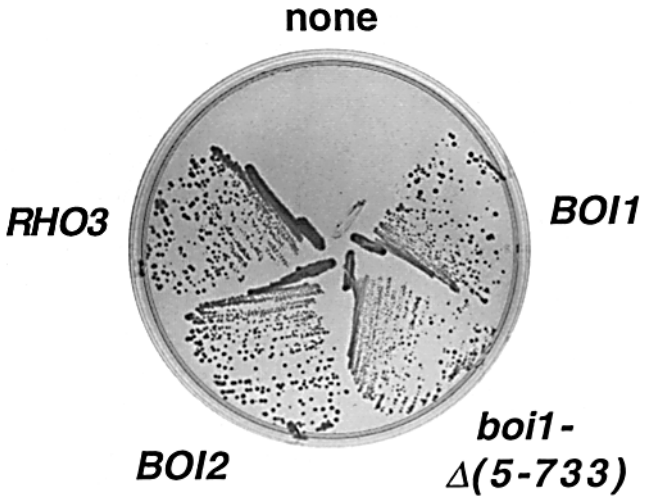


Figure 7. Effects of *boi1-Δ(5-733)* and *RHO3* on the growth of cells deleted for *BOI1* and *BOI2*. Growth of *boi1/boi1 boi2/boi2* strain PY867 cured of plasmid (none) or containing (in place of pPB799) the low copy number plasmids pPB939 (*BOI1*), pPB1004 (*boi1-Δ*[5-733]), pPB865 (*BOI2*), or pPB1013 (*RHO3*). Cells grown on YPD at 20°C were streaked on YPD and grown at 30°C for 48 h.

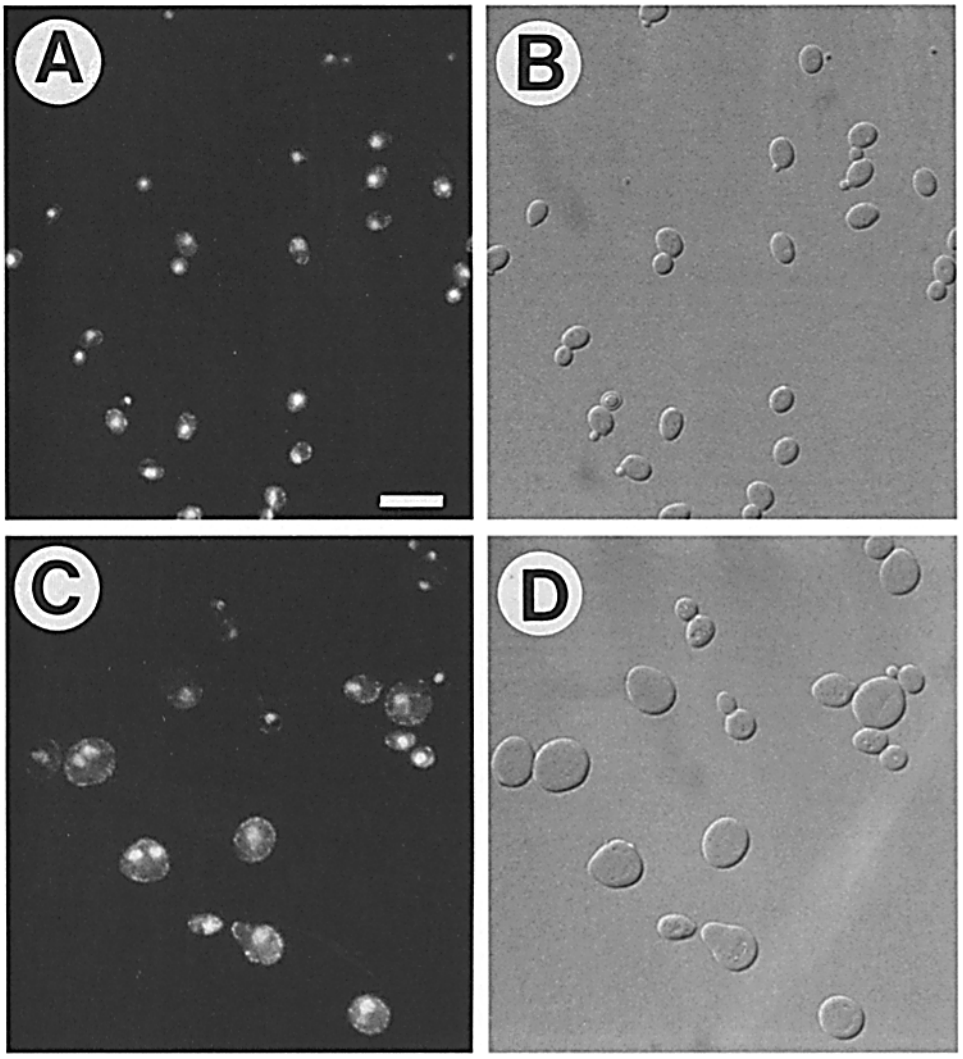


Figure 8. Phenotypes for overexpression of the PH domain–bearing region of Boi1p. Fluorescence images (*A* and *C*) and differential interference contrast images (*B* and *D*) of DAPI-stained wild-type cells (strain Y314) containing either the empty vector YEp55B (*pGAL*) (*A*, *B*) or pPB900 [*pGAL-boi1(735-980)*] (*C* and *D*). Cultures were grown to an OD_{600} of 0.1 in SC-Leu containing 2% raffinose instead of glucose, pelleted and resuspended in the original volume of YP + 2% raffinose, and grown at 30°C to an OD_{600} of 0.1–0.2. Galactose was then added to 3% final concentration (from a 30% stock), and the cells were grown for an additional 8 h to an OD_{600} of 0.5–0.7 before fixation. Bar, 10 μm.

when present on a low copy number plasmid, *RHO3* was able to allow frequent sectoring of PY867 (data not shown), as well as good growth of cells lacking *BOI1* and *BOI2* (Fig. 7). *RHO4*, a multicopy suppressor of *rho3* (Matsui and Toh-e, 1992*a*), about which little is known, could also serve as a multicopy suppressor of *boi1 boi2* (data not shown).

The ability of *RHO3* to serve as an efficient multicopy suppressor of *boi1 boi2* raised the possibility that Boi1p and Boi2p play a role in the activation or function of Rho3p. Given that overexpresson of *RHO3* can inhibit the growth of cells that have a partial loss of function mutation in *CDC42* (Matsui and Toh-e, 1992*b*), we were curious to know whether overexpresson of Boi1p could also impair bud emergence. Overexpression of either full-length *BOI1* (data not shown) or of the COOH-terminal, PH domain–bearing region of *BOI1* (amino acids 735–980) from *GAL* promoter constructs greatly impaired growth, causing cells to become unbudded, large, and multinucleate (Fig. 8). Although overexpression of full-length *BOI1* strongly inhibited growth in both haploid (data not shown) and diploid strains (Fig. 9 *B*), the overexpression of *boi1-(735-980)* strongly impaired growth only in a haploid (Fig. 9 *C*), but not in a diploid (Fig. 9 *A*), perhaps because the ratio of Boi1p to other relevant proteins may be lower in a diploid than in a haploid. Overexpression of *boi1-(735-980)* and *RHO3* together (but not of *RHO3* alone), however, greatly impaired cell growth in a diploid (Fig. 9 *A*). This synergistic inhibition of growth caused by cooverexpression of Rho3p and the PH domain–bearing region of Boi1p is consistent with the possibilities that Boi1p either promotes the activation of Rho3p or in some other way facilitates some process that is controlled by Rho3p. It is also possible, however, that Boi1p (when overexpressed) interferes with bud emergence in a manner that is distinct from its potential role in Rho3p function (e.g., by inhibiting Cdc42p; see below).

Interactions between Boi1p and Cdc42p

The finding that overexpression of the COOH-terminal, PH domain–bearing region of Boi1p inhibits bud emergence raises the possibility that this portion of Boi1p binds to some factor that is required for bud emergence. Given that Cdc42p is required for bud emergence and that some

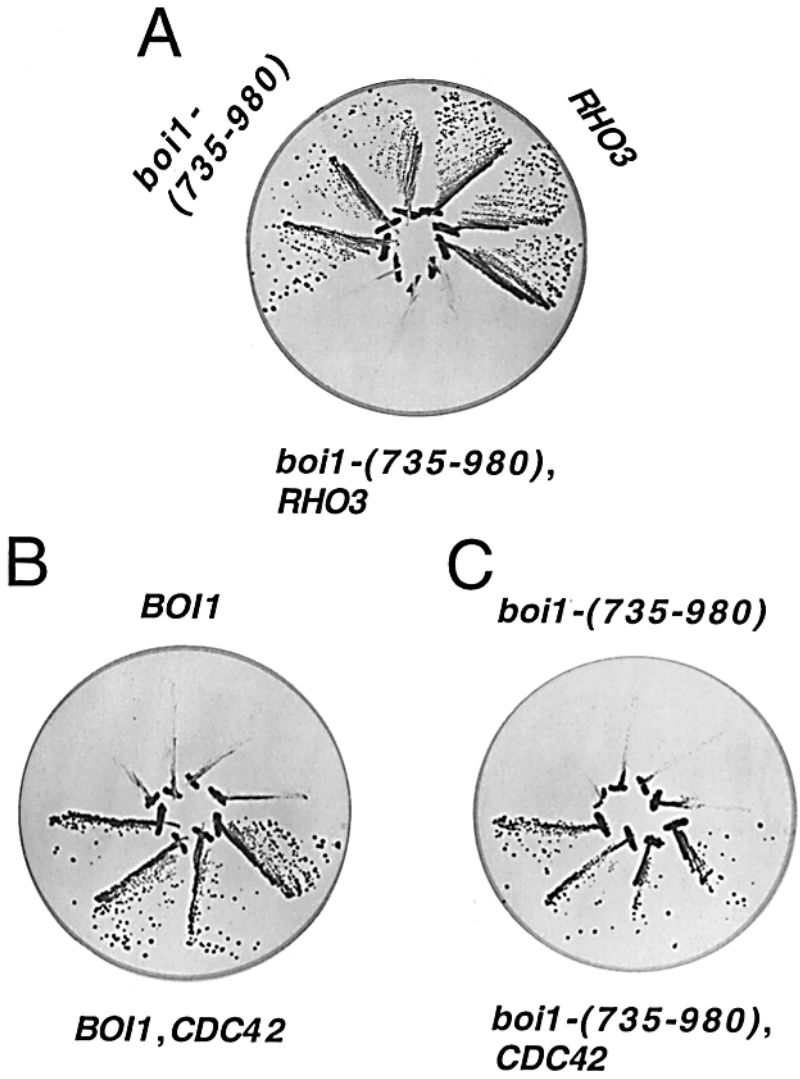


Figure 9. Growth of cells that overexpress either the PH domain–bearing portion of *BOI1* [*boi1-(735-980)*] or full-length *BOI1*, alone or in combination with *CDC42* or *RHO3*. (*A*) Diploid strain Y995 bearing the following pairs of plasmids: pPB900 [*pGAL-boi1(735-980)*, *LEU2*] plus the empty vector 125H (*pGAL*, *URA3*); the integrated plasmid YIpGAL7-RHO3 (*pGAL-RHO3*, *URA3*) plus the empty vector YEp55C (*pGAL*, *LEU2*); and pPB900 plus YIpGAL7-RHO3. (*B*) Y995 bearing pPB773 (*pGAL-BOI1*) plus either 125H or pPB627 (*pGAL-CDC42*, *URA3*). (*C*) Haploid strain Y314 bearing plasmid pPB900 plus either 125H or pPB627. Cells were streaked from SC-U-L to SC-U-L containing 3% galactose and 2% raffinose instead of glucose, and the plates were incubated at 30°C for 3 d (*A*), 30°C for 6 d (*B*), or 34°C for 6 d (*C*).

Cdc42p-binding proteins contain putative PH domains, we considered the possibility that Boi1p, when overexpressed, titrates out either Cdc42p or some factor that is required for Cdc42p function. Consistent with either possibility, we found that overexpression of *CDC42* could restore growth to cells that overexpressed either full-length Boi1p (Fig. 9 *B*) or the COOH-terminal, PH domain–bearing segment of Boi1p (Fig. 9 *C*).

As shown in Table IV, Boi1p can display a two-hybrid interaction with Cdc42p, suggesting that Boi1p either binds directly to Cdc42p or is present in a larger complex with Cdc42p. Whereas Bem1p interacts with a portion of Boi1p that contains the proline-rich region (amino acids 375–557), Cdc42p interacts with the PH domain–bearing region of Boi1p (amino acids 731–980; Table IV). $Cdc42p^{Q61L}$, which is a mutant version of Cdc42p that is predicted to be predominantly in the GTP-bound state (Ziman et al., 1991), displays a two-hybrid interaction with Boi1p (Table IV). In contrast, $Cdc42p^{D118A}$, which is a mutant version of Cdc42p that gives a dominant-negative phenotype and is predicted to exist predominantly in the GDP-bound or nucleotide-depleted state (Ziman et al., 1991, 1994), fails to interact with Boi1p (Table IV). These data suggest that Boi1p might interact preferentially with the GTP-bound form of Cdc42p. We failed to detect a two-hybrid interaction between Boi1p and either Rho3p (Table IV), Rho1p, Rho2p, or Rho4p (data not shown), suggesting that Boi1p

Table IV. Two-hybrid Interactions Involving Boi1p

	β-Galactosidase activity*			
	Segments of *BOI1* fused to the AD§			
DBD fusion‡	None	375–980	375–557	731–980
–	0.06	0.03	0.06	0.07
Bem1	0.03	28	1.4×10^2	0.15
$Cdc42^{C188S}$	2.6	5.5×10^2	4.8	3.8×10^2
$Cdc42^{Q61L/C188S}$	14	4.0×10^2	15	2.3×10^2
$Cdc42^{D118A/C188S}$	<0.02	<0.02	<0.02	<0.02
$Rho3^{C228S}$	0.12	0.08	0.13	0.11

*Activities are given as the average value from at least three independent transformants. Each measured value was within 1/3 of the average.

‡The DBD_{lexA} fusions are all expressed from derivatives of pEG202 (Materials and Methods).

The dash represents unfused DBD_{lexA}.

§The segments of *BOI1* (in codons) fused to the AD are indicated. The plasmids encoding the AD_{Gal4} fusions are pACTII (none), pPB615 (codons 375–980), pPB738 (codons 375–557), and pPB1152 (codons 731–980).

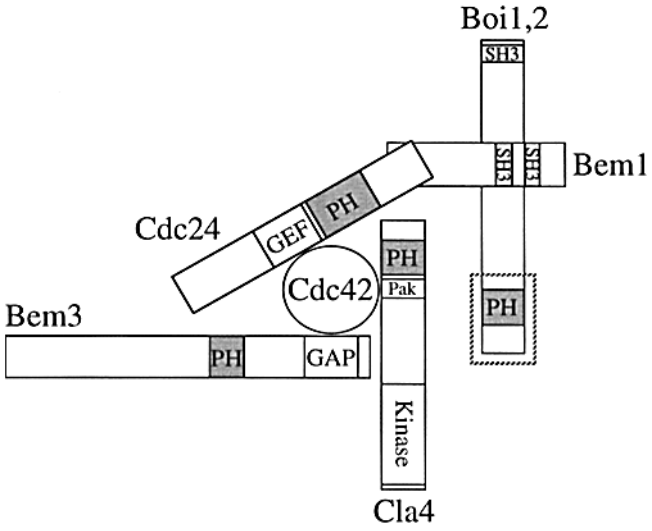


Figure 10. Interactions among some PH domain-containing proteins, SH3 domain-bearing proteins, and Cdc42p. Positions of PH, SH3, GEF, GAP, and Pak domains are indicated. The portion of Boi1p that displays a two-hybrid interaction with Cdc42p and is sufficient for good cell growth is indicated with a hatched box.

may interact specifically with Cdc42p, not with other Rho-type GTPases.

Discussion

PH and SH3 Domains

Boi1p and Boi2p are structurally similar to each other and appear to have redundant or overlapping functions. Both proteins contain an SH3 domain, a PH domain, and a proline-rich sequence. Cells deleted for *BOI1* and *BOI2* together (but of neither gene alone) display morphological defects and poor viability. The large-cell, multinucleate, and cell-lysis phenotypes of *boi1 boi2* mutants resemble those caused by mutations in *BEM1* and in other genes involved in bud emergence (Bender and Pringle, 1991; Chant et al., 1991; Chenevert et al., 1992; Adams et al., 1990).

The following results suggest that Boi1p and Boi2p bind Bem1p, and that this binding is mediated by the proline-rich region of Boi1p/Boi2p and the second SH3 domain of Bem1p. First, Boi1p and Boi2p display two-hybrid interactions with Bem1p, and mutations in the second SH3 domain of Bem1p destroy these interactions. Second, bacterially expressed short portions of Boi1p and Boi2p that contain the proline-rich region can bind to bacterially expressed Bem1p, and mutations in the second SH3 domain of Bem1p destroy this binding. Third, Boi1p can be coimmunoprecipitated with Bem1p from yeast, and this coimmunoprecipitation requires the second SH3 domain of Bem1p and the proline-rich sequence of Boi1p.

The observations that deletion of the Pro-rich region of *BOI1* has no obvious deleterious effect (even in a strain that lacks *BOI2*), but that mutations in the second SH3 domain of Bem1p make cells Ts^- for growth (Bender, L., and A. Bender, unpublished data), suggest that the second SH3 domain of Bem1p may bind to other proteins in addition to Boi1p and Boi2p. Other proteins that were isolated from the Bem1p two-hybrid screen that are candidates for binding to the second SH3 domain of Bem1p are Yer124p, about which nothing except its sequence is known, and Mps1p/Rpk1p, which is a protein kinase that is required for spindle pole body duplication and cell cycle control (Lauze et al., 1995; Poch et al., 1994). However, we have no further evidence that either of these proteins normally binds to Bem1p in yeast.

We have not yet identified proteins that bind to the SH3 domain of Boi1p/Boi2p. However, the finding that this SH3 domain of Boi1p/Boi2p is not necessary in otherwise wild-type cells, but that it becomes essential in cells that lack Bem1p, raises the possibility that it binds to some protein whose function overlaps with or is tightly linked to Bem1p.

We previously demonstrated that Cdc24p, which contains a putative PH domain, also binds to Bem1p. The presence of putative PH domains in both Cdc24p and Boi1p/Boi2p, and of SH3 domains in both Bem1p and Boi1p/Boi2p, hints at a potential relatedness in function between at least some SH3 and PH domains in yeast (Fig. 10). One general possibility is that some SH3 and PH domains share in common roles in the assembly of protein complexes that are regulated by or that regulate one or more Rho-type GTPases.

PH Domain–bearing Proteins and Cdc42p

Overexpression of the COOH-terminal, PH domain–bearing segment of Boi1p (or of full-length Boi1p) inhibits bud emergence, suggesting that the COOH-terminal segment of Boi1p binds to some protein that is important for budding. Overexpression of Cdc42p suppresses the effects of overexpression of Boi1p, suggesting that Boi1p binds to either Cdc42p itself or to some protein involved in a process that is regulated by Cdc42p. Boi1p displays a two-hybrid interaction with Cdc42p, consistent with the possibility that Boi1p either binds to or is in a larger complex with Cdc42p. The PH domain–bearing region of Boi1p, which is sufficient for the two-hybrid interaction with Cdc42p, is distinct from the region of Boi1p that binds to Bem1p, arguing against the possibility that the two-hybrid interaction between Boi1p and Cdc42p is caused by an indirect bridging interaction involving Bem1p.

The presence of putative PH domains in the Cdc42p-binding proteins Cdc24p, Bem3p, and Cla4p suggests that at least some PH domains in yeast may play roles in the function of Cdc42p. All of these proteins contain other distinct Cdc42p-binding domains (i.e., a GEF domain in Cdc24p, a GAP domain in Bem3p, and a Pak domain in Cla4p, Fig. 10), and in the two cases in which the issue has been addressed (for Bem3p and Cla4p), the PH domains are not needed for binding to Cdc42p (Zheng et al., 1994; Crvcková et al., 1995). Therefore, rather than being required for binding to Cdc42p, the main role of the PH domains in these proteins might instead be to either regulate binding interactions involving Cdc42p or to serve as targeting sequences to bring these proteins to sites where Cdc42p is localized.

Unlike Cdc24p, Bem3p, and Cla4p, each of which contain a separate, other domain with catalytic activity crucial for function, the most important portion of Boi1p/Boi2p is the

COOH-terminal region that contains no recognizable motifs other that the PH domain. Further work is required to determine whether Boi1p binds directly to Cdc42p, and if it does, whether the Cdc42p-binding site is distinct from, overlaps with, or is coincident with the PH domain.

Boi1p/Boi2p as a Potential Link between Cdc42p and Rho3p

Little is known about the roles of Rho3p or the relationship of Rho3p to Cdc42p. If Cdc42p functions mainly in bud emergence and Rho3p functions mainly in bud growth, then one general possibility is that Rho3p functions subsequently to Cdc42p with, for example, Rho3p acting at sites containing protein complexes (e.g., cytoskeletal structures) whose assembly or localization is regulated by Cdc42p.

RHO3 can serve as an efficient multicopy suppressor of *boi1 boi2*, and, when overexpressed, Rho3p exacerbates the bud emergence defects caused by overexpression of Boi1p. Models to account for these interactions between Boi1p/Boi2p and both Rho3p and Cdc42p include that Boi1p/Boi2p either helps to position an activator of Rho3p at a site where Cdc42p had acted previously, or that Boi1p/Boi2p helps to displace Cdc42p or a regulator of Cdc42p from a site where Rho3p is to act subsequently. Future efforts will be directed toward elucidating the physical and functional relationships between Boi1p/Boi2p and both Cdc42p and Rho3p.

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