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ROM7/BEM4 Encodes a Novel Protein That Interacts with the Rho1p Small GTP-Binding Protein in *Saccharomyces cerevisiae*

HISANOBU HIRANO,[1] KAZUMA TANAKA,[1] KUMI OZAKI,[1] HIROSHI IMAMURA,[1] HIDESHI KOHNO,[1] TARO HIHARA,[1] TAKAAKI KAMEYAMA,[1] KAZUHIKO HOTTA,[1] MIKIO ARISAWA,[2] TAKAHIDE WATANABE,[2] HIROSHI QADOTA,[3] YOSHIKAZU OHYA,[3] AND YOSHIMI TAKAI[1,4]*

Department of Molecular Biology and Biochemistry, Osaka University Medical School, Suita 565,[1] Department of Mycology, Nippon Roche Research Center, Kamakura 247,[2] Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113,[3] and Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki 444,[4] Japan

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The *RHO1* gene encodes a homolog of the mammalian RhoA small GTP-binding protein in the yeast *Saccharomyces cerevisiae*. Rho1p is localized at the growth site and is required for bud formation. The *RHO1*(G22S, D125N) mutation is a temperature-sensitive and dominant negative mutation of *RHO1*, and a multicopy suppressor of *RHO1*(G22S, D125N), *ROM7*, was isolated. Nucleotide sequencing of *ROM7* revealed that it is identical to the *BEM4* gene (GenBank accession number L27816), although its physiological function has not yet been reported. Disruption of *BEM4* resulted in the cold- and temperature-sensitive growth phenotypes, and cells of the Δ*bem4* mutant showed abnormal morphology, suggesting that *BEM4* is involved in the budding process. The temperature-sensitive growth phenotype was suppressed by overexpression of *RHO1*, *ROM2*, which encodes a Rho1p-specific GDP/GTP exchange factor, or *PKC1*, which encodes a target of Rho1p. Moreover, glucan synthase activity, which is activated by Rho1p, was significantly reduced in the Δ*bem4* mutant. Two-hybrid and biochemical experiments revealed that Bem4p directly interacts with the nucleotide-free form of Rho1p and, to lesser extents, with the GDP- and GTP-bound forms of Rho1p, although Bem4p showed neither GDP/GTP exchange factor, GDP dissociation inhibitor, nor GTPase-activating protein activity toward Rho1p. These results indicate that Bem4p is a novel protein directly interacting with Rho1p and is involved in the *RHO1*-mediated signaling pathway.

The Rho family belongs to the small G protein superfamily and consists of the Rho, Rac, and Cdc42 subfamilies (15, 43). Evidence is accumulating that through reorganization of the actin cytoskeleton, Rho regulates various cell functions, such as maintenance of cell morphology, formation of stress fibers and focal adhesions, cell motility, membrane ruffling, cytokinesis, cell aggregation, and smooth muscle contraction. Recently, Citron (29) and ROKα (26) have been isolated as potential targets of Rho, but their functions in reorganization of the actin cytoskeleton remain to be clarified.

Rho has two interconvertible forms, GDP-bound inactive and GTP-bound active (15, 43). The GDP-bound form is converted to the GTP-bound form by the GDP/GTP exchange reaction, which is stimulated by GDP/GTP exchange factors (GEFs) and is inhibited by GDP dissociation inhibitors (GDIs). The GTP-bound form is converted to the GDP-bound form by the GTPase reaction, which is stimulated by GTPase-activating proteins (GAPs). Two GDIs, including Rho GDI (12, 45) and Ly/D4 GDI (25, 40), and four GEFs, including Smg GDS (23, 48), Dbl (16, 47), Ost (19), and Lbc (44), have been reported to be active on Rho.

The budding yeast *Saccharomyces cerevisiae* possesses the Rho family members *RHO1* (28), *RHO2* (28), *RHO3* (31), *RHO4* (31), and *CDC42* (1, 22). Cells of this yeast grow by budding for cell division, and the actin cytoskeleton plays a pivotal role in the budding process (9). Cortical actin patches are concentrated at the site of bud emergence in unbudded cells and at the bud tip and the cytokinesis site in budded cells, whereas actin fibers are generally oriented along the long axes of the mother-bud pairs (2). *RHO1* is a homolog of the mammalian *RhoA* gene, and we have shown that cells of the *rho1* mutants stop growing with small-budded cells under restrictive conditions (49). Moreover, immunofluorescence microscopic study indicates that Rho1p is localized at the growth site where cortical actin patches are localized, including the presumptive budding site, the bud tip, and the cytokinesis site (49). These results suggest that *RHO1* is involved in the process of bud formation and that Rho1p also regulates reorganization of the actin cytoskeleton as in mammalian cells. We have recently shown that Rho1p performs its functions through at least Pkc1p, which is a homolog of mammalian protein kinase C, as a downstream target of Rho1p (34). Concerning the upstream regulators of *RHO1*, we have purified a homolog of Rho GDI in *S. cerevisiae*, cloned its gene, and named it *RDI1* (30). More recently, we have cloned two homologous genes, *ROM1* and *ROM2*, and have shown that at least Rom2p is the Rho1p-specific GEF in *S. cerevisiae* (35).

Accumulating evidence indicates that there is a small GTP-binding protein cascade operating in the budding process in *S. cerevisiae* (6). In the budding process, Rsr1p/Bud1p, a homolog of mammalian Rap1, selects the site for budding, Cdc42p initiates budding site assembly, and then Rho1p promotes bud formation. Therefore, in this cascade, it is presumed that the GTP-bound form of Rsr1p/Bud1p activates Cdc42p and then the GTP-bound form of Cdc42p activates Rho1p. In accordance with this model, it has recently been shown that the GTP-bound form of Rsr1p/Bud1p directly binds Cdc24p, which is a GEF for Cdc42p (50). However, the linkage between Cdc42p and Rho1p has not yet been established at a molecular level.

* Corresponding author. Phone: 81-6-879-3410. Fax: 81-6-879-3419. Electronic mail address: ytakai@molbio.med.osaka-u.ac.jp.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
OHNY1	*MAT***a** *ura3 leu2 trp1 his3 ade2*	34
YPH499	*MAT***a** *ura3 leu2 trp1 his3 ade2 lys2*	42
YPH501	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 lys2/lys2*	42
PIO100	*MAT***a** *ura3 leu2 trp1 his3 ade2 lys2 Δbem4::HIS3*	
DPIO100B	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 lys2/lys2 BEM4/Δbem4::HIS3*	
DPIO100A	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 lys2/lys2 Δbem4::HIS3/Δbem4::HIS3*	
HNY21	*MAT***a** *ura3 leu2 trp1 his3 ade2 rho1-104*	49
DIOY20A	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 Δrom2::HIS3/Δrom2::HIS3*	35
YOC725	*MAT***a** *ura3 leu2 trp1 his3 ade2 lys2 rho1::LYS2 ade3*::[pRHO1-RhoA:HIS3]	37
DJTD2-16A	*MAT***a** *ura3 leu2 trp1 his4 cdc42-1*	22
Y190	*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 LYS2::GAL-HIS3 URA3::GAL-lacZ cyh*^{r}	

Many proteins directly interacting with Rho have been isolated as regulatory and target proteins in both yeast and mammalian cells, but the regulations and modes of actions of Rho have not yet been thoroughly elucidated. In this work, we cloned the *BEM4* gene as a multicopy suppressor of a temperature-sensitive dominant negative mutation of *RHO1*, *RHO1* (G22S, D125N). Genetic studies indicate that *BEM4* is involved in the *RHO1*-mediated signaling pathway. Moreover, two hybrid and biochemical studies indicate that Bem4p is a new type of protein directly interacting with Rho1p in *S. cerevisiae.*

MATERIALS AND METHODS

Strains, media, and yeast transformations. Yeast strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for construction and propagation of plasmids. Yeast strains were grown on YPDAU medium containing 2% glucose, 2% Bacto Peptone (Difco Laboratories, Detroit, Mich.), 1% Bacto Yeast Extract (Difco), 0.04% adenine sulfate, and 0.02% uracil. Yeast transformations were performed by the lithium acetate method (13). Transformants were selected on SD medium containing 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco), supplemented with amino acids when required. Standard yeast genetic manipulations were performed as described previously (41).

Molecular biological techniques. Standard molecular biological techniques were used for construction of plasmids, PCR, and DNA sequencing (39). Plasmids used in this study are listed in Table 2. PCRs were performed with GeneAmp PCR System 2400 (Perkin-Elmer), and DNA sequences were determined with an ALFred DNA sequencer (Pharmacia Biotech, Inc.). Point mutations were introduced by the PCR mutagenesis method (17).

Disruption of *BEM4*. pUC19-Δbem4::HIS3 was cut with *Bam*HI and *Sma*I, and the digested DNA was introduced into diploid strain YPH501. The genomic DNA was isolated from each transformant, and the proper disruption of *BEM4*

TABLE 2. Plasmids used in this study

Plasmids	Characteristics
pRS315	*LEU2*, *CEN6* (42)
pRS316	*URA3*, *CEN6* (42)
YEp352	*URA3*, 2μm (18)
pRS315-RHO1(G22S, D125N)	*RHO1*(G22S, D125N), *LEU2*, *CEN6* (35)
YEp24-RHO1	*RHO1*, *URA3*, 2μm (35)
YEp24-RHO2	*RHO2*, *URA3*, 2μm (35)
YEp24-BEM4	*BEM4*, *URA3*, 2μm; isolated from a genomic library in YEp24 (5) as a clone containing the 7-kb genomic DNA fragment of *BEM4*
YEP24-BEM4ΔNhe1	*BEM4*, *URA3*, 2μm; made by deleting the 3-kb *Nhe*I fragment from YEp24-BEM4
YEp24-BEM4ΔSalI	*URA3*, 2μm; made by deleting the 3-kb *Sal*I fragment from YEp24-BEM4
YEp352-BEM4[SalI-SalI]	*BEM4*, *URA3*, 2μm; made by inserting the 3-kb *Sal*I-*Sal*I fragment from YEp24-BEM4 into the *Sal*I site of YEp352
pRS316-P_{GAL1}-BEM4	P_{GAL1}-*BEM4*, *URA3*, *CEN6*; made by inserting the 1.9-kb *Bam*HI-*Sma*I DNA fragment containing the *BEM4* open reading frame from pACTII-HK-BEM4 into the *Bam*HI-*Sma*I site of pRS316-P_{GAL1} to place *BEM4* downstream of the *GAL1* promoter
YEp352-ROM2	*ROM2*, *URA3*, 2μm (35)
YEp24-PKC1	*PKC1*, *URA3*, 2μm (35)
pAS1-CYH2	DBD_{GAL4}, *TRP1*, 2μm (10)
pAS1-CYH2-RHO1	DBD_{GAL4}-*RHO1*, *TRP1*, 2μm; made by inserting the 0.6-kb *Nco*I-*Hpa*I PCR fragment containing the *RHO1* open reading frame into the *Nco*I-*Sma*I site of pAS1-CYH2
pAS1-CYH2-RHO1(Q68L)	DBD_{GAL4}-*RHO1*(Q68L), *TRP1*, 2μm; made as described above by use of the *RHO1*(Q68L) fragment
pAS1-CYH2-RHO1(T24N)	DBD_{GAL4}-*RHO1*(T24N), *TRP1*, 2μm; made as described above by use of the *RHO1*(T24N) fragment
pACTII-HK	AD_{GAL4}, *LEU2*, 2μm (35)
pACTII-HK-BEM4	AD_{GAL4}-*BEM4*, *LEU2*, 2μm; made by inserting the 1.9-kb *Bam*HI-*Sma*I PCR fragment containing the *BEM4* open reading frame into the *Bam*HI-*Sma*I site of pACTII-HK. The 1.9-kb *BEM4* DNA was amplified by PCR by using upstream primer 5′-CTCGGATCCATGGATTACGAAGAAATTTTA and downstream primer 5′-CTCCCCGGGCTAAAAGAACGATTTTGCAAG. The underlined sequences are portions of the *BEM4* open reading frame.
pRS316-HA-BEM4	Hemagglutinin (HA)-*BEM4*; made by inserting the 3.4-kb *Spe*I-*Sal*I fragment containing the *BEM4* gene, which is tagged at its N terminus with two HA epitopes as described previously (49) into the *Spe*I-*Sal*I site of pRS316
pYO914	GST-Rho1p; made by inserting the 435-bp *Bam*HI-*Xba*I *RHO1* fragment encoding amino acid positions 64 to 209 of Rho1p into the *Bam*HI-*Xba*I site of pGEX-KG
pGEX-2T-BEM4	GST-Bem4p; made by inserting the 1.9-kb *Bam*HI-*Sma*I *BEM4* fragment from pACTII-HK-BEM4 into the *Bam*HI-*Sma*I site of pGEX-2T
pUC19-BEM4	*BEM4*; made by inserting the 1.9-kb *Bam*HI-*Sma*I *BEM4* fragment from pACTII-HK-BEM4 into the *Bam*HI-*Sma*I site of pUC19
pUC19-Δbem4::HIS3	Derivative of pUC19-BEM4; made by replacing the 1.1-kb *Eco*RV internal fragment of *BEM4* corresponding to amino acid positions 70 to 432 of Bem4p with the 1.8-kb *HIS3* fragment

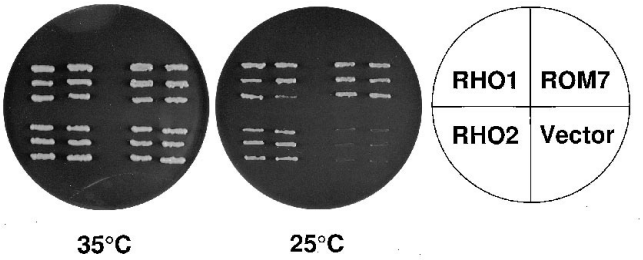

FIG. 1. Suppression of the cold-sensitive growth phenotype of the *RHO1* (G22S, D125N) mutant by overexpression of *ROM7*. OHNY1 carrying pRS315-RHO1(G22S, D125N) was transformed with YEp24-RHO1 (RHO1), YEp24-RHO2 (RHO2), YEp24-ROM7 (ROM7), or YEp24 (Vector). The resultant transformants were streaked onto SD-Ura-Leu plates and incubated at 35 or 25°C for 2 days.

was verified by PCR (data not shown). A diploid strain in which one *BEM4* allele was disrupted was named DPIO100B and was subjected to tetrad analysis. Tetrad dissection of DPIO100B resulted in a 2 His^-:2 His^+ segregation pattern; all of the His^+ clones showed cold- and temperature-sensitive growth phenotypes, whereas all of the His^- clones showed the wild-type phenotype. These Δ*bem4* mutant strains were used for further studies.

Two-hybrid method. Strain Y190 was transformed with derivatives of pAS1-CYH2 bearing the wild-type or mutant form of *RHO1* fused with the part of the *GAL4* gene encoding the Gal4p DNA-binding domain (DBD_{GAL4}). These DBD_{GAL4}-*RHO1* fusion plasmids were constructed essentially as described previously (34). In these DBD_{GAL4}-Rho1p fusions the cysteine residue of carboxyl-terminal lipid modification sites was changed to the serine residue. A resultant transformant was transformed again with vector pACTII-HK or pACTII-HK-BEM4, in which *BEM4* was fused with the *GAL4* transcriptional activation domain (AD_{GAL4}). Western blot (immunoblot) analysis using a monoclonal antibody raised against the hemagglutinin epitope confirmed that cells carrying pACTII-HK-BEM4 expressed a protein of about 85 kDa, the expected size for Bem4p fused with AD_{GAL4}. For qualitative assay, cells of each transformant were placed on a nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity as described previously (46). For quantitative assay, cells of each transformant were cultured in SD-Trp-Leu medium, and β-galactosidase activity was measured by the *o*-nitrophenyl-β-D-galactopyranoside assay method described elsewhere (14).

Assay for the sensitivity of yeast cells to echinocandin B. The sensitivity of yeast cells to echinocandin B (3) (isolated at Nippon Roche Research Center, Kamakura, Japan) was examined by halo assay. YPDAU soft agar (4 ml) warmed at 55°C was mixed with the culture of yeast cells to be tested, and the resulting mixture was poured onto a YPDAU plate. After 30 min, a dry paper disk (6 mm in diameter) was placed on the surface of the soft agar, and 3 μl of the aqueous solution of echinocandin B (20 mg/ml) was spotted onto the paper disk. The plates were subsequently incubated at 23°C for 3 days.

Cytological techniques. Actin and DNA were stained with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, Oreg.) and 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co., St. Louis, Mo.) respectively, as described previously (49). Stained cells were observed and photographed on Neopan Super Presto film (Fuji, Tokyo, Japan), using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany). Cell lysis was examined by assaying alkaline phosphatase activity, using the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma) as described previously (36).

Materials and chemicals for biochemical assays. The lipid-modified form of Rho1p was purified from the membrane fraction of *Spodoptera frugiperda* cells which were infected with a baculovirus carrying the *RHO1* gene as described previously (32, 33). The lipid-modified form of Ki-Ras was similarly purified from the membrane fraction of the insect cells. Recombinant Bem4p, yeast Rho GDI (Rdi1p) (30), and Rom2DH (35) were purified from overexpressing *E. coli* DH5α as glutathione *S*-transferase (GST) fusion proteins, using a glutathione-Sepharose 4B column (Pharmacia P-L Biochemicals Inc.) as described previously (24). The anti-Ras antibody RASK4, kindly supplied by H. Shiku (Nagasaki University School of Medicine, Nagasaki, Japan), was used. BA-85 (0.45-μm pore size) nitrocellulose filters were purchased from Schleicher & Schuell.

Production of an anti-Rho1p antibody. Purified GST-Rho1p(64-209), which is a fusion protein of GST with Rho1p from amino acid positions 64 to 209, was minced and emulsified with R-700 (RIBI ImmunoChem Research, Hamilton, Mont.), and the resulting emulsion was used to immunize four guinea pigs. After boosting was performed five times at 3-week intervals, blood was collected from the animals, and one of the immune sera was used in this study.

Assay for glucan synthase activity. Yeast cells were grown in YPDAU containing 1 M sorbitol at 30°C to an optical density at 600 nm of 1.0. Cells were harvested by centrifugation, and glucan synthase activity in the membrane fraction was measured in the presence or absence of 0.8 μM GTPγS as described previously (21).

Assay for the binding of recombinant Bem4p with Rho1p. The GDP-bound, GTPγS-bound, or nucleotide-free form of Rho1p or Ki-Ras was prepared as described previously in the presence or absence of guanine nucleotides (48). Each form of Rho1p or Ki-Ras (10 pmol) was incubated with purified GST-Bem4p or GST (200 pmol) at 4°C for 2 h in A buffer (200 μl) containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 12 mM $MgCl_2$, 0.12% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 0.1% bovine serum albumin, and 5 μM GDP (for the GDP-bound form of Rho1p or Ki-Ras), 5 μM GTPγS (for the GTPγS-bound form of Rho1p or Ki-Ras), or no guanine nucleotides (for the nucleotide-free form of Rho1p or Ki-Ras). Each reaction mixture was loaded onto a glutathione-Sepharose 4B column (40 μl) equilibrated with A buffer. After each column was washed twice with A buffer (200 μl), GST-Bem4p or GST was eluted with B buffer (200 μl) containing 10 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 5 mM $MgCl_2$. A 30-μl aliquot of each eluate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting with the anti-Rho1p or anti-Ras antibody.

Assays for GEF and GDI activities of Bem4p. GEF and GDI activities of Bem4p were assayed under high-Mg^{2+} (5 mM) and low-Mg^{2+} (0.5 μM) conditions, respectively, by measuring the dissociation of [^{3}H]GDP from or the binding of [^{35}S]GTPγS to Rho1p (3 pmol). The dissociation of [^{3}H]GDP or the binding of [^{35}S]GTPγS was assayed at 30°C by filtration through nitrocellulose filters, measuring the radioactivity of [^{3}H]GDP or [^{35}S]GTPγS bound to each small GTP-binding protein after incubation with GST-Bem4p as described previously (48).

RESULTS

Isolation of *ROM7*, which suppresses the *RHO1*(G22S, D125N) mutation on a multicopy plasmid. We have recently isolated a temperature-sensitive dominant negative mutation of *RHO1*, *RHO1*(G22S, D125N) (35). This mutant protein, Rho1p (G22S, D125N), seems to be labile at a high temperature such as 35°C. Therefore, a wild-type strain carrying *RHO1*(G22S, D125N) shows a cold-sensitive growth phenotype. We have isolated multicopy suppressors of this mutation, including characterized genes (*RHO1*, *RHO2*, and *PKC1*) and uncharacterized genes (*ROM1*, *ROM2*, *ROM3*, *ROM4*, *ROM5*, and *ROM6* [*RHO1* multicopy suppressor]) (35). *ROM1* and *ROM2* possess the Dbl homology and pleckstrin homology domains, and at least Rom2p shows Rho1p-specific GEF activity. Further screening for multicopy suppressors of *RHO1*(G22S, D125N) identified one gene, *ROM7*, which was different from *ROM1* to *ROM6*. As shown in Fig. 1, overexpression of *ROM7* suppressed the cold-sensitive growth phenotype of OHNY1 carrying *RHO1*(G22S, D125N) to an extent similar to that of the suppression by *RHO1* or *RHO2*. This gene was characterized further in this study.

***ROM7* is identical to *BEM4*.** As shown in Fig. 2, deletion

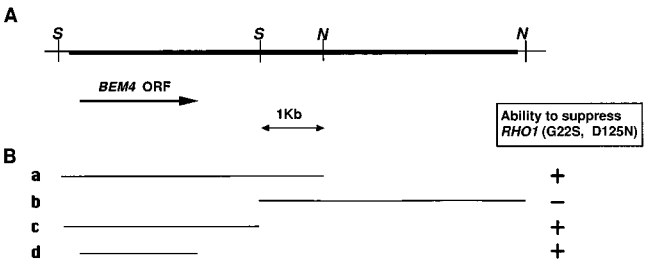

FIG. 2. Restriction enzyme map and deletion mapping of *ROM7*. (A) Restriction enzyme map of the insert DNA. The thick line represents the 7-kb original genomic DNA fragment cloned into YEp24-ROM7; thin lines represent portions of the vector, YEp24. *S*, *Sal*I; *N*, *Nhe*I. The extent and direction of the *BEM4* open reading frame (ORF) are shown by an arrow. (B) Deletion mapping of *ROM7*. Various deletion fragments of the insert DNA were cloned into YEp24 or YEp352. The constructed plasmids were transformed into OHNY1 carrying pRS315-RHO1(G22S, D125N) to examine their abilities to suppress the cold-sensitive growth phenotype. a, YEp24-BEM4ΔNheI; b, YEp24-BEM4Δ SalI; c, YEp352-BEM4[SalI-SalI]; d, pRS316-P_{GAL1}-BEM4.

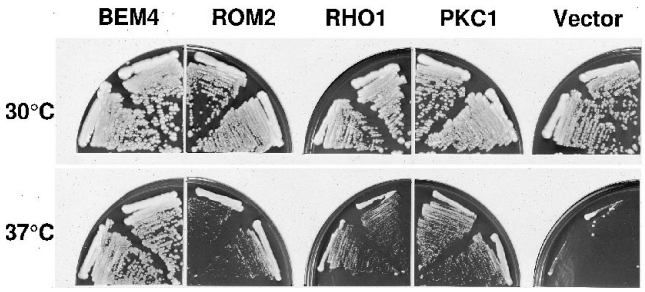

FIG. 3. Suppression of the temperature-sensitive growth phenotype of the Δ*bem4* mutant by overexpression of genes involved in the *RHO1*-mediated signaling pathway. PIO100 (Δ*bem4*::*HIS3*) was transformed with YEp24-BEM4 (BEM4), YEp352-ROM2 (ROM2), YEp24-RHO1 (RHO1), YEp24-PKC1 (PKC1), or YEp24 (Vector). Two independent transformants obtained form each transformation were streaked onto YPDAU plates, which were incubated at 30 or 37°C for 3 days.

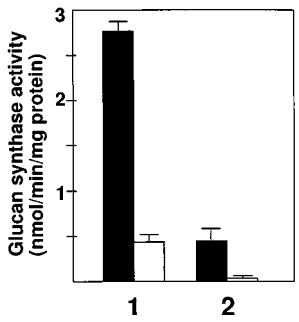

FIG. 5. Deficiency of glucan synthase activity in the Δ*bem4* mutant. Membrane fractions of the wild-type strain (YPH501) and the Δ*bem4* mutant (DPIO100A) cultured at 30°C were prepared, and glucan synthase activity was measured in the presence (closed bars) or absence (open bars) of 0.8 μM GTPγS. 1, YPH501; 2, DPIO100A. The values are averages ± standard errors for four experiments.

mapping of the cloned DNA segment indicated that the suppressor gene was located on the 3-kb *Sal*I-*Sal*I fragment. This DNA fragment was partially sequenced, and a search with the obtained nucleotide sequence against the GenBank data library revealed that the 3-kb fragment contains the *BEM4* gene (accession number L27816), but its characterization has not yet been reported. Bem4p is a protein of 634 amino acids, and its amino acid sequence is not related to that of any known protein. To confirm that *BEM4* is the suppressor gene itself, the 1.9-kb open reading frame amplified by PCR was cloned into pRS316-P_{GAL1} to express *BEM4* under the control of the *GAL1* promoter. As shown in Fig. 2B, expression of *BEM4* under the control of the *GAL1* promoter suppressed the cold-sensitive growth phenotype of OHNY1 carrying *RHO1*(G22S, D125N). Therefore, we concluded that the *BEM4* gene is the suppressor gene itself.

***BEM4* is functionally relevant to *RHO1*.** To analyze the functions of *BEM4* genetically, *BEM4* was disrupted as described in Materials and Methods. In this disruption, the region of *BEM4* corresponding to amino acid positions 70 to 432 was replaced with the *HIS3* gene. Cell growth of the Δ*bem4* mutant was examined at various temperatures, and it was found that the Δ*bem4* mutant shows cold- and temperature-sensitive growth phenotypes: the Δ*bem4* mutant grew slowly at 30°C but not at 14 or at 37°C (data not shown). By use of the Δ*bem4* mutant, genetic interactions between *BEM4* and the *RHO1*-mediated signaling pathway were further investigated. A multicopy plasmid carrying *BEM4*, *ROM2*, *RHO1*, or *PKC1* or a control vector was transformed into the Δ*bem4* mutant cells. As shown in Fig. 3, overexpression of *ROM2*, *RHO1*, or *PKC1* partially suppressed the temperature-sensitive growth phenotype of the Δ*bem4* mutant. This result indicates that *BEM4* is functionally relevant to *RHO1*, as the results suggested that the overexpression of *BEM4* suppressed the *RHO1* (G22S, D125N) mutation. Interestingly, the cold-sensitive growth phenotype of the Δ*bem4* mutant was not suppressed by overexpression of these genes but was suppressed by overexpression of *RHO3*, which did not suppress the temperature-sensitive growth phenotype of the Δ*bem4* mutant (data not shown). These results indicate that at least at the higher temperature, Bem4p is involved in the *RHO1*-mediated signaling pathway. We have recently shown that *rho1* and Δ*rom1* Δ*rom2* mutants show the cell lysis phenotype and that the temperature-sensitive growth phenotype of the *rho1* or Δ*rom2* mutant is suppressed by an osmotic stabilizer such as 1 M sorbitol (35, 49). We examined whether the Δ*bem4* mutant shows similar phenotypes. As shown in Fig. 4, the Δ*bem4* mutant exhibited the cell lysis phenotype at 37°C that was found for the *rho1* and Δ*rom2* mutants. Moreover, the temperature-sensitive growth phenotype of the Δ*bem4* mutant was partially suppressed by 1 M sorbitol (data not shown). These results also indicate that Bem4p is involved in the *RHO1*-mediated signaling pathway.

Deficiency of glucan synthase activity in the Δ*bem4* mutant. We have recently shown that β(1→3)glucan synthase (glucan synthase) is another target of Rho1p in *S. cerevisiae* (8, 38). The activity of glucan synthase from wild-type cells is stimulated by GTPγS and is deficient in the *rho1* mutants. Moreover, glucan synthase is activated by the GTPγS-bound form, but not by the GDP-bound form, of recombinant Rho1p. To confirm the genetic result that *BEM4* is involved in the *RHO1*-mediated signaling pathway, glucan synthase activity was measured in the Δ*bem4* mutant. As shown in Fig. 5, glucan synthase activity in the membrane fraction assayed in the presence or absence of GTPγS was significantly reduced in the Δ*bem4*

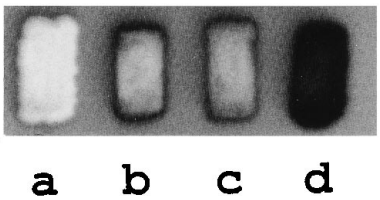

FIG. 4. Cell lysis phenotype of the Δ*bem4* mutant. Cells of strains to be tested were streaked onto a YPDAU plate. The plate was incubated at 37°C for 7 h, and the patches were subsequently stained with a chromogenic substrate, BCIP, of alkaline phosphatase at 37°C overnight. a, YPH501 (wild type); b, DIOY20A (Δ*rom2*); c, DPIO100A (Δ*bem4*); d, HNY21 (*rho1*). HNY21 was strongly stained because of its severe temperature sensitivity for growth.

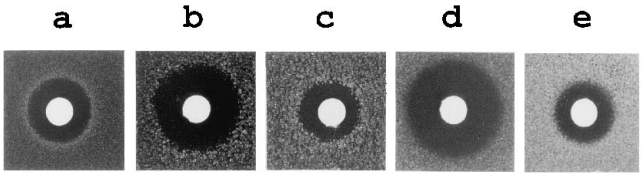

FIG. 6. Sensitivity of the Δ*bem4* mutant to echinocandin B. a, YPH501 (wild type); b, DPIO100A (Δ*bem4*); c, DPIO100A (Δ*bem4*) transformed with pRS316-HA-BEM4; d, YOC725 (*RhoA*); e, DJTD2-16A (*cdc42*). The diameters (in centimeters; averages ± standard errors) of halos measured in two independent experiments were as follows: a, 1.45 ± 0.05; b, 2.1 ± 0.1; c, 1.4 ± 0.05; d, 2.25 ± 0.05; and e, 1.45 ± 0.05.

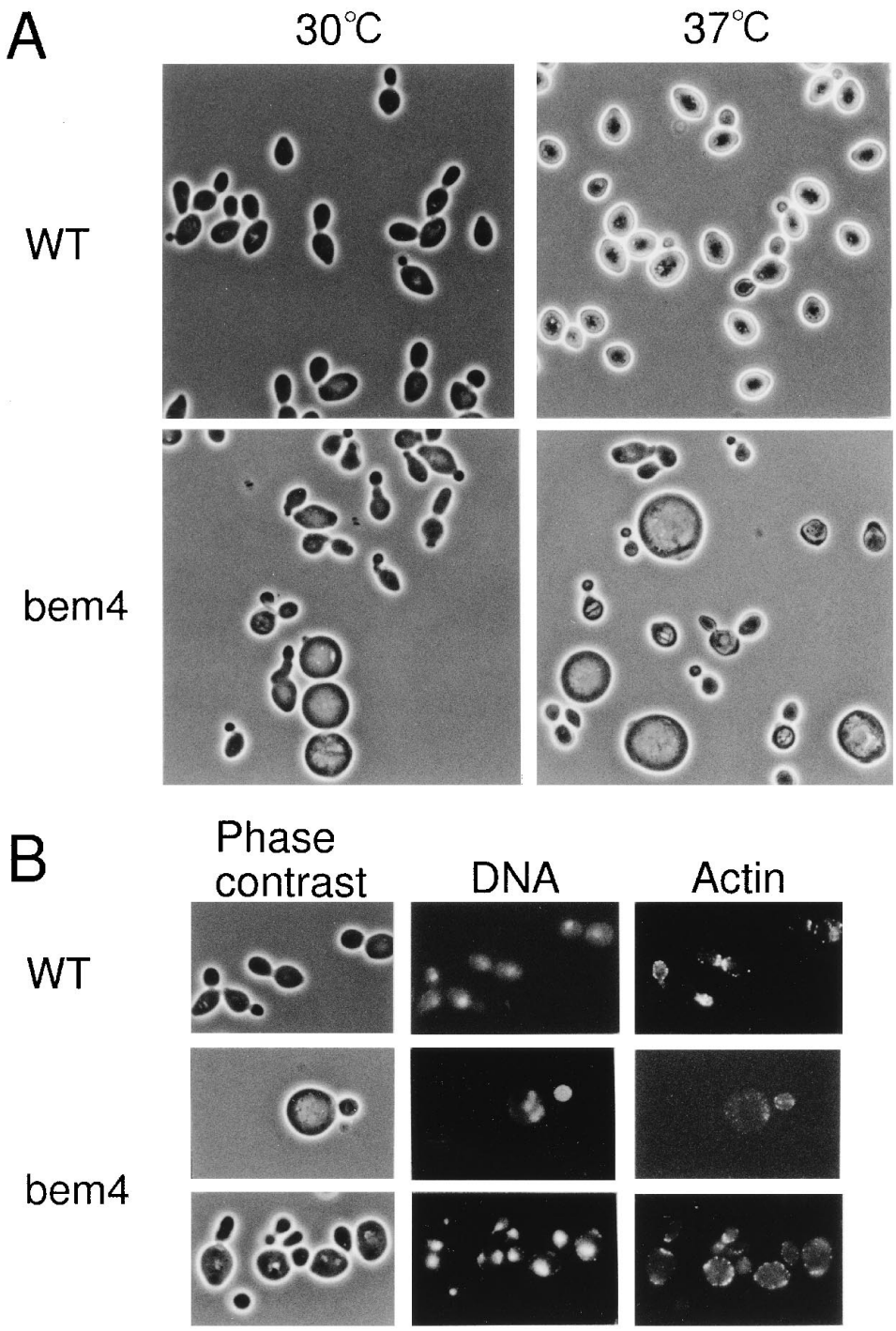

FIG. 7. Morphology of Δ*bem4* mutant cells. (A) Cells of diploid strains YPH501 (wild type [WT]) and DPIO100A (Δ*bem4* [bem4]) incubated at 30 or 37°C for 2 h were subjected to phase-contrast microscopy. (B) Cells of YPH501 (WT) and DPIO100A (Δ*bem4*) incubated at 37°C for 2 h were fixed, double-stained with DAPI and rhodamine-phalloidin for DNA and actin, respectively, and subjected to microscopy. All fields were photographed at the same magnification.

mutant. Moreover, the Δ*bem4* mutant was hypersensitive to echinocandin B, an antibiotic which specifically inhibits glucan synthase activity (20), as was the *RhoA* mutant, which carries the mammalian *RhoA* gene in place of the *RHO1* gene (38) (Fig. 6). It was also found that the cell growth of the Δ*bem4* mutant, but not of the wild-type strain, was clearly inhibited in medium containing 3 μg of echinocandin B per ml (data not shown). From these results, we concluded that Bem4p functions in the *RHO1*-mediated signaling pathway in *S. cerevisiae.*

***BEM4* is not simply involved in the *RHO1*-mediated signaling pathway.** We have recently shown that cells of the *rho1* or Δ*rom1* Δ*rom2* mutant are arrested with a small bud under restrictive conditions (35, 49). The morphological phenotype of the Δ*bem4* mutant was investigated in the next experiment

TABLE 3. Two-hybrid interactions between Bem4p and Rho1p

DBD_{GAL4} fused to:	AD_{GAL4} fused to:	β-Galactosidase activity[a] (U)
RHO1	*BEM4*	82 ± 10
RHO1	Vector	9 ± 3
RHO1(T24N)	*BEM4*	161 ± 20
RHO1(T24N)	Vector	10 ± 5
RHO1(Q68L)	*BEM4*	65 ± 7
RHO1(Q68L)	Vector	8 ± 3
SNF1	*BEM4*	7 ± 3
SNF1	Vector	9 ± 6

[a] Average ± standard error for three transformants.

and found to be distinct from that of the *rho1* or Δ*rom1* Δ*rom2* mutant, as described below. Diploid cells of the Δ*bem4*/Δ*bem4* homozygous mutant growing slowly at 30°C or in growth arrest at 37°C were observed by microscopy. As shown in Fig. 7A, abnormally enlarged, round cells (about 8% at 30°C and about 15% at 37°C) were observed in the culture of the Δ*bem4* mutant. Moreover, at 30°C, smaller cells of the Δ*bem4* mutant often had an elongated or crooked bud. Staining of actin and DNA revealed that in the Δ*bem4* mutant cells, cortical actin patches which are normally seen at the growing sites, including a bud tip or a cytokinesis site, were delocalized and that there were often more than two nuclei (Fig. 7B). These results indicate that the Δ*bem4* mutant is deficient in the budding process, although the deficiency is not simply caused by the impairment of the *RHO1*-mediated signaling pathway.

Bem4p interacts with Rho1p in the two-hybrid assay. The isolation of *ROM1* and *ROM2*, which encode Rho1p-specific GEFs, as multicopy suppressors of the *RHO1*(G22S, D125N) mutation (35) prompted us to examine whether Bem4p interacts with Rho1p in the two-hybrid assay (11). A DNA fragment encoding the entire open reading frame of *BEM4* was fused with AD_{GAL4}. Yeast strains carrying plasmids encoding various DBD_{GAL4}-*RHO1* fusions, including DBD_{GAL4}-*RHO1*, DBD_{GAL4}-*RHO1*(T24N), and DBD_{GAL4}-*RHO1*(Q68L), were transformed with AD_{GAL4}-*BEM4*. As shown in Table 3, Bem4p interacted with Rho1p, Rho1p(T24N), and Rho1p(Q68L) but not with Snf1p, which was used as a negative control (11). It is noteworthy that Bem4p bound Rho1p(T24N) most efficiently. Since Rho1p(T24N), Rho1p, and Rho1p(Q68L) seem to be in the nucleotide-free or GDP-bound (7), GDP-bound, and GTP-bound (4) forms in vivo, respectively, these results suggest that Bem4p binds the nucleotide-free form of Rho1p and, to lesser extents, the GDP- and GTP-bound forms of Rho1p.

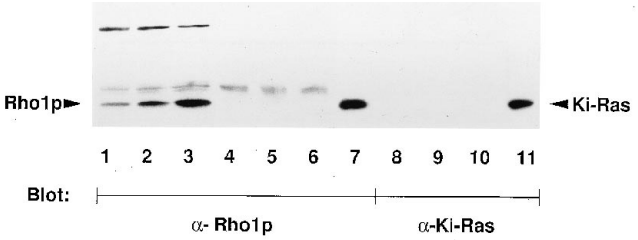

FIG. 8. Binding of GST-Bem4p to Rho1p. The GDP-bound, GTPγS-bound, or nucleotide-free form of Rho1p or Ki-Ras was incubated with GST-Bem4p or GST at a Rho1p or Ki-Ras concentration of 50 nM, and each reaction mixture was loaded onto glutathione-Sepharose 4B column to recover GST-Bem4p or GST. The recovered sample was subjected to SDS-PAGE followed by Western blotting with the anti-Rho1p or anti-Ki-Ras antibody. Lanes 1 to 3 and 8 to 10, GST-Bem4p; lanes 4 to 6, GST. Lanes 1, 4, and 8, GDP-bound form; lanes 2, 5, and 9, GTPγS-bound form; lanes 3, 6, and 10, nucleotide-free form. Lanes 7 and 11, 0.5 pmol of Rho1p and Ki-Ras, respectively, as controls for Western blotting. The bands of Rho1p and Ki-Ras are indicated with arrowheads. One hundred percent binding of input of Rho1p or Ki-Ras to GST-Bem4p or GST corresponds to 1.5 pmol.

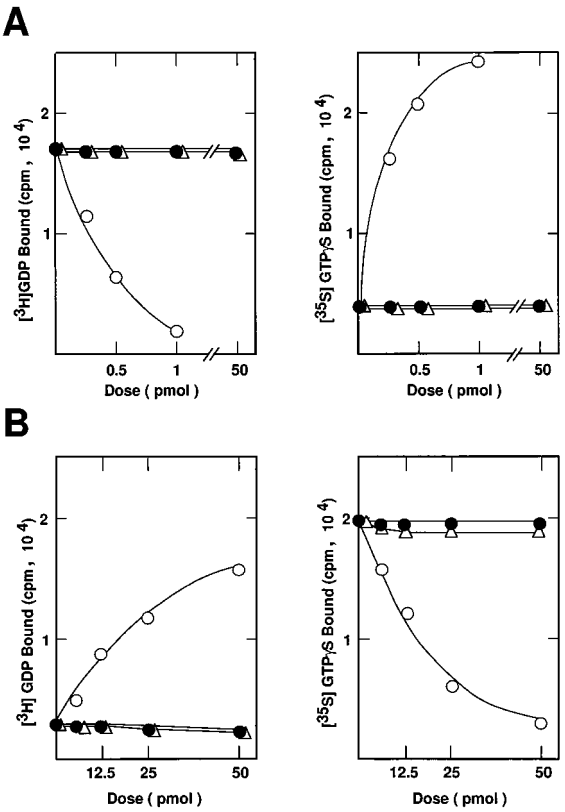

FIG. 9. Absence of GEF and GDI activities in Bem4p. (A) Absence of a GEF activity in Bem4p. The GEF activity to stimulate the dissociation of [^{3}H]GDP from or the binding of [^{35}S]GTPγS to Rho1p was assayed for 10 min in the presence of various amounts of GST-Bem4p (●), GST-Rom2DH (○), or GST (△). (B) Absence of GDI activity in Bem4p. The GDI activity to inhibit the dissociation of [^{3}H]GDP from or the binding of [^{35}S]GTPγS to Rho1p was assayed for 10 min in the presence of various amounts of GST-Bem4p (●), GST-Rdi1p, (○), or GST (△).

Recombinant Bem4p binds Rho1p in vitro. To examine whether Bem4p binds Rho1p in vitro, the entire open reading frame of Bem4p was fused to GST. GST-Bem4p was expressed in *E. coli* and purified. Purified GST-Bem4p or GST was incubated with the GDP-bound, GTP-bound, or nucleotide-free form of lipid-modified Rho1p or Ki-Ras and we examined whether these forms of Rho1p or Ki-Ras were bound to GST-Bem4p as described in Materials and Methods. As shown in Fig. 8, GST-Bem4p, but not GST, bound the nucleotide-free form of Rho1p and, to lesser extents, the GDP- and GTPγS-bound forms of Rho1p. Since GST-Bem4p did not bind Ki-Ras, the binding of Bem4p to Rho1p was specific. These results are consistent with the results of the two-hybrid assay, and we concluded that Bem4p directly binds Rho1p.

Bem4p does not stimulate or inhibit the GDP/GTP exchange reaction of Rho1p. The finding that Bem4p directly interacts with Rho1p raised the possibility that Bem4p possesses a GEF, GDI, or GAP activity toward Rho1p. We have already shown that Rom2p and Rdi1p are a GEF (35) and a GDI (30) for Rho1p, respectively. However, as shown in Fig. 9A, GST-Bem4p did not stimulate the dissociation of GDP from or the binding of GTPγS to Rho1p, indicating that Bem4p does not possess a GEF activity toward Rho1p. Moreover, as shown in

Fig. 9B, GST-Bem4p did not inhibit the dissociation of GDP from or the binding of GTPγS to Rho1p, indicating that Bem4p does not possess a GDI activity toward Rho1p. We also examined whether GST-Bem4p stimulated or inhibited the GEF activity of Rom2p or the GDI activity of Rdi1p, but GST-Bem4p did not show any effect on these activities (data not shown). Moreover, GST-Bem4p did not possess a GAP activity toward Rho1p (data not shown). These experiments were repeated with full-length Bem4p which was not fused with GST, and essentially the same results were obtained (data not shown). Therefore, we concluded that Bem4p does not show a GEF, GDI, or GAP activity to Rho1p by itself, at least under the conditions used in this study.

DISCUSSION

In this present study, we cloned *BEM4* as a multicopy suppressor of the temperature-sensitive dominant negative mutation of *RHO1*, *RHO1*(G22S, D125N). This result and further genetic analyses indicate that *BEM4* is involved in the *RHO1*-mediated signaling pathway. Overexpression of *RHO1*, *ROM2*, or *PKC1* suppressed the temperature-sensitive growth phenotype of the Δ*bem4* mutant. Furthermore, we have shown that the activity of glucan synthase, which is a target of Rho1p (8, 38), is significantly reduced in the Δ*bem4* mutant. Interestingly, it has been found that *BEM4* is also involved in the *CDC42*-mediated signaling pathway: the *BEM4* gene has independently been identified as a multicopy suppressor of the *cdc42* mutation, as a gene whose mutation is suppressed by overexpression of *CDC42*, and as a gene whose mutation results in synthetic lethality with the *cdc24* mutation (27). Moreover, two-hybrid analysis indicates that Bem4p interacts not only with Rho1p and Cdc42p but also with Rho2p and Rho4p (27). We have also found that *CDC42* is a multicopy suppressor of the Δ*bem4* mutant and that Cdc42p binds Bem4p in the two-hybrid assay (data not shown). Therefore, *BEM4* seems to be involved in multiple signaling pathways regulated by the Rho family members in *S. cerevisiae*. Consistent with this inference, the Δ*bem4* mutant is deficient in the budding process, which is regulated by the Rho family members.

Both the two-hybrid and biochemical studies consistently indicate that Bem4p binds the nucleotide-free form of Rho1p and, to lesser extents, the GDP- and GTP-bound forms of Rho1p. In addition, Bem4p showed neither GEF, GDI, nor GAP activity toward Rho1p. Since this type of protein has not yet been reported for other families of small GTP-binding proteins, and since its amino acid sequence is not related to that of any known protein, Bem4p should play a novel role, which has not yet been demonstrated, in the signaling pathways mediated by the Rho family members.

It is not known whether Bem4p is involved in the regulation of activities, downstream signaling, or localization of the Rho family members. One possible function of Bem4p is in the folding or stability of the Rho family members as a kind of chaperone specific to them. This idea seemed to be consistent with the fact that *BEM4* is required for growth at 14 or 37°C. However, we have discerned no difference in the soluble or particulate amount of Rho1p between the wild-type and Δ*bem4* mutant strains (data not shown). This result also suggests that Bem4p is not required for localization of Rho1p at the membranes, excluding the possibility that Bem4p is involved in the prenylation step of Rho1p. The finding that glucan synthase activity was reduced in the Δ*bem4* mutant suggests that Bem4p may be involved in the activation step of Rho1p or in the coupling of Rho1p with its target. Although our current data do not clarify these points, they should be examined in future experiments.

Since Bem4p interacts with the Rho family members, Bem4p should be involved in a process common to the Rho family members, not in a process specific to each member. In this respect, Bem4p is similar to Rho GDI, which interacts with the Rho family members, although Rho GDI preferentially interacts with the GDP-bound form of the Rho family members (45). Although no biochemical correlation between Bem4p and Rdi1p has been detected, we have found that the temperature-sensitive growth phenotype of the Δ*bem4* mutant is somewhat strengthened by the Δ*rdi1* mutation, which does not cause any phenotype by itself (reference 30 and data not shown). Further studies should be undertaken to clarify the functional relationship between *BEM4* and *RDI1*.

In conclusion, Bem4p is a novel protein directly interacting with the Rho family members, and its functional importance in the signaling pathways mediated by them is apparent. Further studies on Bem4p will reveal a new aspect of the functions and modes of actions of the Rho family small GTP-binding proteins.

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