Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11539–11543, December 1995
Cell Biology

14-3-3 proteins: Potential roles in vesicular transport and Ras signaling in *Saccharomyces cerevisiae*

DANIEL GELPERIN*, JEFFREY WEIGLE*, KAREN NELSON*, PATRICK ROSEBOOM†, KENJI IRIE‡, KUNIHIRO MATSUMOTO‡, AND SANDRA LEMMON*§

*Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106-4960; †Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, Bethesda, MD 20892; and ‡Department of Molecular Biology, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

Communicated by Anthony P. Mahowald, The University of Chicago, Chicago, IL, August 9, 1995 (received for review March 1, 1995)

ABSTRACT **Deletion of the clathrin heavy-chain gene, *CHC1*, in the budding yeast *Saccharomyces cerevisiae* results in growth, morphological, and membrane trafficking defects, and in some strains *chc1*-Δ is lethal. A previous study identified five genes which, in multicopy, rescue inviable strains of Chc⁻ yeast. Now we report that one of the suppressor loci, *BMH2/SCD3*, encodes a protein of the 14-3-3 family. The 14-3-3 proteins are abundant acidic proteins of ≈30 kDa with numerous isoforms and a diverse array of reported functions. The Bmh2 protein is >70% identical to the mammalian ε-isoform and >90% identical to a previously reported yeast 14-3-3 protein encoded by *BMH1*. Single deletions of *BMH1* or *BMH2* have no discernible phenotypes, but deletion of both *BMH1* and *BMH2* is lethal. High-copy *BMH1* also rescues inviable strains of Chc⁻ yeast, although not as well as *BMH2*. In addition, the slow growth of viable strains of Chc⁻ yeast is further impaired when combined with single *bmh* mutations, often resulting in lethality. Overexpression of *BMH* genes also partially suppresses the temperature sensitivity of the *cdc25-1* mutant, and high-copy *TPK1*, encoding a cAMP-dependent protein kinase, restores Bmh⁻ yeast to viability. High-copy *TPK1* did not rescue Chc⁻ yeast. These genetic interactions suggest that budding-yeast 14-3-3 proteins are multifunctional and may play a role in both vesicular transport and Ras signaling pathways.**

The 14-3-3 proteins comprise a family of highly related acidic proteins of ≈30 kDa (1). Originally found in abundance in mammalian brain, they have now been identified in all eukaryotes examined, including yeast, plants, insects, and humans. There are several different 14-3-3 isoforms in mammalian cells which are generally >60% identical, and homology across divergent species is similarly high. The 14-3-3 proteins are also conspicuous for the diversity of functions that have been attributed to them. They have been identified as activators of neurotransmitter synthesis enzymes (2) and implicated in a number of mitogenic signal transduction pathways, including those involving protein kinase C (PKC), Raf, and Bcr-Abl and middle-sized tumor (T) antigen (see refs. 1 and 3 and references therein). Some 14-3-3 proteins have also been isolated as factors that stimulate mitochondrial import (4), Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells (5), and ExoS, the virulence factor of *Pseudomonas aeruginosa* which ADP-ribosylates Ras and other small GTPases (6, 7). In plants 14-3-3 proteins have been found in association with DNA–protein complexes (8), and in *Schizosaccharomyces pombe* they have been shown to be important for radiation resistance and checkpoint control (9). Previously, a 14-3-3 gene, *BMH1*, was identified in *Saccharomyces cerevisiae* by its proximity to the *PDA1* gene (10). Later it was isolated by its ability to enhance the activity of Raf in a heterologous yeast reporter system (11); however, *bmh1*-Δ cells appear normal.

Our laboratory developed a screen for high-copy suppressors of inviable strains of clathrin-deficient *S. cerevisiae* (12). Clathrin is a major vesicular transport coat protein involved in receptor-mediated endocytosis and protein sorting at the late Golgi compartment. The phenotype of yeast strains carrying a disruption of the clathrin heavy-chain gene, *CHC1*, is affected by an independently segregating gene referred to as *SCD1*, suppressor of clathrin deficiency (13). With the *scd1-v* allele, Chc⁻ yeast are viable but grow poorly and display abnormal morphology, genetic instability, and defects in late Golgi protein retention and endocytosis (14). With the *scd1-i* allele Chc⁻ yeast are inviable (13). The high-copy-suppressor selection strategy identified five genes whose overexpression rescued Chc⁻ yeast of *scd1-i* genetic background to viability (12). One of these genes has been found to encode a second *S. cerevisiae* 14-3-3 protein homologue. Here, we present our isolation of this gene, *BMH2*, and initial characterization of yeast 14-3-3 mutants.¶

MATERIALS AND METHODS

Strains, Media, and Genetic Methods. *S. cerevisiae* strains used in this study were LP3004-1B, *MAT***a** *rad9-1 leu2-3,112* (from L. Prakash, University of Texas); SL214, *MATα GAL1:CHC1 leu2 ura3-52 trp1 his1 GAL2 scd1-i* (12); SL1320, *MATα bmh2-Δ:URA3 leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v*; SL1382, *MATα leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v*; SL1386, *MATα bmh1-Δ:LEU2 leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v*; SL1470, *MATα bmh1-Δ:LEU2 bmh2-Δ:URA3 leu2 ura3-52 trp1 his3 GAL2 scd1-v* pDG33-(*GAL1:BMH2*); *SL1558, MAT***a** *bar1-1 bmh1-Δ:LEU2 leu2 trp1* His⁻; SL1559, *MAT***a** *bar1-1 leu2 ura3-52 trp1* His⁻; SL1771, *MAT***a** *bar1-1 bmh2-Δ:URA3 leu2 ura3-52 trp1* His⁻; SL1795, *MAT***a** *bmh2-Δ:URA3 GAL1:CHC1 leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v*; SL1797, *MAT***a** *bmh1-Δ:LEU2 GAL1:CHC1 leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v*; and TT25-6, *MATα cdc25-1 leu2 ura3 trp1 can1* (15). YEP-Glc/dextrose (YEPD), YEP-Gal, and synthetic selective dropout media were prepared as described (12). Standard yeast genetic methods were followed (16).

Rescue of the *GAL1:CHC1 scd1-i* strain (SL214) depleted of clathrin heavy chains by *BMH* plasmids was determined as described (12) except that selective medium was used. All visible colonies were counted and percent rescue was calculated as (no. of colonies on glucose/no. of colonies on galac-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKC, protein kinase C; PKA, cAMP-dependent protein kinase.

§To whom reprint requests should be addressed.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U01883).

tose) × 100. Values reported are the average of four or five independent determinations.

Plasmids and Disruption Constructs. YEp*SCD3/BMH2* and pA21 were isolated from YEp24 (17) and YCp50 libraries (18), respectively. pJW18 has the 3.6-kb *Xba* I–*Sac* I fragment containing *BMH2* cloned into YEp352 (19). pJW45 contains the 5.0-kb *Bgl* II–*Bam*HI region of *BMH2* in pRS314, a *CEN, TRP1* vector (20). pDG33 contains *BMH2* under *GAL1* control in a *CEN, HIS3* plasmid. *BMH2* was PCR amplified so that the ATG initiation codon is 75 nt downstream of the *GAL1* start of transcription. pJW35 contains a 4.8-kb *Xba* I fragment including *BMH1* cloned into pBluescript SK(+) (Stratagene). pJW40 contains the 2.6-kb *Kpn* I–*Xho* I *BMH1* fragment from pJW35 cloned into pRS424, a 2μ, *TRP1* vector (21). pJW42 contains the 4.7-kb *Kpn* I–*Bam*HI *BMH1* fragment from pJW35 cloned into pUN30, a *CEN, TRP1* vector (22). YEp13-TPK1 is a 2μ, *TPK1* plasmid (15). pDG39 contains the 2.8-kb *Sph* I–*Hin*dIII *TPK1* fragment, blunted and cloned into the *Sma* I site of pRS424. YEp13-BMH1 and YEp13-BMH2 are high-copy *BMH* clones isolated from a YEp13 library (11). pAL-CDC25 contains *CDC25* in YEp13 under control of the *ADH1* promoter (23). For *bmh2*-Δ a 0.6-kb region, between *Pvu* I and *Pvu* II restriction sites in the open reading frame of *BMH2*, was replaced with the *URA3* gene (pJW28), deleting codons 68–263. For *bmh1*-Δ the region of *BMH1* between the *Bgl* II and *Hin*dIII restriction sites was replaced by the *LEU2* gene (pJW37), deleting codons 99–199.

Immunoblots. Yeast protein extracts (200 μg) prepared by glass bead homogenization (24) were subjected to SDS/PAGE and the separated proteins were transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore). Bmh proteins were detected with a rabbit antiserum (275A) made against a peptide corresponding to a conserved region of 14-3-3 proteins (FDDAIAELDTLNEDSYKDSTLIMQL-LRDNLTLWTSDQ). Antiserum 275A was made essentially as described for 274A (25) and affinity purified (26) against a bacterially expressed His_6-tagged Bmh2 protein. Immunoblots were developed by standard procedures (26).

RESULTS

***SCD3/BMH2* Encodes a Member of the 14-3-3 Protein Family.** YEpSCD3 was previously identified as a high-copy plasmid that could rescue inviable strains of clathrin heavy-chain-deficient (Chc^-) yeast to viability (12). To narrow the rescuing region of YEpSCD3, subclones were generated and transformed into a *GAL1:CHC1 scd1-i* strain (SL214) to test for growth on glucose medium. Normally, this strain grows well on galactose medium, where *CHC1* is expressed, but on glucose the *GAL1* promoter is shut off and SL214 is inviable due to the presence of the *scd1-i* allele. A single open reading frame in YEpSCD3 coding for an acidic protein (pI 4.6) of 273 aa with a predicted molecular mass of 31 kDa was found to be responsible for the rescue.

A search of GenBank revealed that the Scd3 protein (Scd3p) is highly homologous to the 14-3-3 family of proteins and to a previously identified *S. cerevisiae* protein encoded by the *BMH1* gene (10). Alignment of Scd3p and Bmh1p shows that the two *S. cerevisiae* proteins are 91% identical overall and >97% identical over their first 256 residues (Fig. 1). Due to this high degree of homology we now refer to *SCD3* as *BMH2*. The Bmh proteins are most similar to the animal ε isoforms, with Bmh2p/Scd3p showing >70% identity to mouse ε (Fig. 1); however, homology to other animal isoforms is still extensive (60–65% identity). Both Bmh proteins are ≈75% identical to the *Sch. pombe* 14-3-3 homologues (rad24p and rad25p), which are themselves only ≈75% identical (9). However, phylogenetic analysis (27) places the fission-yeast proteins in a class of their own, distinct from Bmh1p, Bmh2p/Scd3p, and mammalian ε.

The carboxyl terminus is the most divergent region of the 14-3-3 proteins. Most of the mammalian isoforms are truncated relative to the yeast forms, and Bmh2p contains an unusual stretch of 17 glutamines in this region. It is of interest that a *Pvu* II deletion of *BMH2*, which results in a truncation of 10 carboxyl-terminal amino acids and addition of 6 vector-derived residues, is essentially nonfunctional in the *GAL1:CHC1 scd1-i* rescue assay. Therefore, the carboxyl terminus of Bmh2p may be important for function and the divergent carboxyl termini of the 14-3-3 isoforms could contribute to the different activities of these proteins.

Disruption of *BMH1* and *BMH2*. To determine the phenotype of cells lacking Bmh proteins, *BMH1* and *BMH2* deletions were generated by using the *LEU2* and *URA3* genes as disruption markers, respectively. Dissection of *bmh1*-Δ:*LEU2/BMH1 bmh2*-Δ:*URA3/BMH2* double heterozygotes yielded tetrads with four, three, or two viable spores (1:4:1 ratio). Approximately equal numbers of *BMH1 BMH2, bmh1*-Δ, and *bmh2*-Δ segregants were recovered and all had wild-type growth rates. The inviable class (the missing *bmh1*-Δ *bmh2*-Δ spore progeny) germinated and formed microcolonies of 8–32 cells. This indicates that disruption of both *BMH* genes is lethal and that at least one 14-3-3 gene is required for vegetative growth. Identical results were obtained with *scd1-i* and *scd1-v* strains.

Although the *bmh1*-Δ and *bmh2*-Δ single mutants grew as well as wild-type strains under normal conditions (YEPD, 30°C), we put the mutants through a number of tests in an attempt to identify any phenotypes. Cells were grown on a variety of nonfermentable carbon sources (e.g., acetate, glycerol, and galactose), YEPD containing 0.9 M NaCl (high-osmolarity medium), 100 mM $CaCl_2$ or 10 mM EGTA, the microtubule inhibitor benomyl at 1–50 μg/ml, and pH 5.5 medium. Single *bmh* null mutants were also tested for heat and cold sensitivity (growth at 37°C and 17°C, and heat shock at 55°C for 30 min). In all cases no differences from wild-type were observed. Cells had normal growth rates, morphology, mating, sporulation as homozygous diploids, and mating pheromone response in all conditions tested.

Mutation of both *Sch. pombe* 14-3-3 genes, *rad24* and *rad25*, is lethal, but *rad24* or *rad25* single mutants are viable (9), similar to *S. cerevisiae bmh* null mutants. However, *rad24*

```
MSQT-REDSVYLAKLAEQAERYEEMVENMKAVASSGQELSVEERNLLSVAYKNVIGARRASWRIVSSIEQKEESKEKSEHQVELIRSYRSKIETELTKISD  Bmh2p
..TS-.....T.....C.....  Bmh1p
..NS-..N.....K..C.NDK.....I.....I.....RGNTR-.AA..KE..K...D..SD.CH  rad25p
..T.S...A.....G.....S...TD...T.....GNTA-.....KE..Q...Q..DT.CQ  rad24p
.-DD-...L..Q.....D....S..K..GMDV..T.....I.....N.GG-.DKLKM..E..QMV....KL.CC  ε-14-3-3

DILSVLDSHLIPSATTGESKVFYYKMKGDYHRYLAEFSSGDARERKATNSSLEAYKTASEIATTELPPTHPIRLGLALNFSVFYYEIQNSPDKACHLAKQA
.....A.....
.V....EK....A.....Y.....TV.EVCKE.AD.....A..D..VA.....D.M.....LD..ES.....V
...T..EK....N.ASA.....Y.....AV.EK.QHSADQ...G..A.....A..A.....L....R..Y.....
...D...K....A.N.....AT.ND.KE.AEN..V...A..D..M.....L....R..R...A.

FDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDISESGQ-EDQQQQQQQQQQQQQQQQQAPAEQTQGEPTK  273
.....M.....A.....H...------P..AAE-..AP.  267
..E..S...S.....-A.YN.SAKEEAPAAAAASENEHPEPKESTTDTVKA  270
..E..S...S.....-A.YSAAAAGGNTEGA.ENAPSNAPEGER.PKATHR  271
.....MQGD.--.E.NKEAL.DVEDEN.  255
```

FIG. 1. Alignment of Bmh1p and Bmh2p/Scd3p amino acid sequences with those of *Sch. pombe* rad25p and rad24p and the mouse ε-14-3-3 isoform. Identities are indicated with periods. Dashes indicate gaps. The overlined sequence is the region used for generation of anti-peptide antiserum.

mutants, and to a much lesser degree *rad25* mutants, enter mitosis prematurely and *rad24* mutants are hypersensitive to DNA-damaging agents (9). This suggested that fission-yeast 14-3-3 genes are important for checkpoint control. Therefore, we tested whether the *S. cerevisiae* single *bmh* mutants were hypersensitive to DNA damage (Fig. 2). We found the viability of a known budding-yeast radiation-sensitive/checkpoint control mutant, *rad9* (28), declined rapidly with increasing UV irradiation. In contrast, survival curves of the *bmh1*-Δ and *bmh2*-Δ mutants were identical to that of the wild-type strain. Similar results were observed with γ irradiation (data not shown).

Bmh1p Is More Abundant Than Bmh2p. Bmh1p and Bmh2p were identified in immunoblots of yeast extracts by an antiserum raised against a synthetic peptide highly conserved among 14-3-3 proteins (Fig. 3). In wild-type extracts Bmh1p was consistently detected as a 34-kDa peptide, which was absent in a *bmh1*-Δ strain and increased 3- to 5-fold when expressed from a *CEN* plasmid or ≈10-fold when expressed from a high-copy 2μ plasmid. Bmh2p was difficult to detect in immunoblots of wild-type yeast proteins, but an additional band of 36 kDa was observed when *BMH2* gene dosage was supplemented on a *CEN* or 2μ vector. In wild-type strains carrying no plasmids, levels of Bmh1p appeared at least 5-fold greater than those of Bmh2p. This is consistent with a recent two-dimensional gel electrophoresis study which showed both proteins to be fairly abundant in budding yeast, but the amount of Bmh1p to be 3- to 4-fold greater than that of Bmh2p (29).

Clathrin-Deficient Yeast Are Sensitive to the Dosage of *BMH1* and *BMH2*. In searching for *SCD* genes, we identified two plasmids from a YCp50 low-copy plasmid library corresponding to *SCD3/BMH2*. To examine the rescue in low copy in more detail and to determine whether *BMH1* could also rescue Chc⁻ yeast, strains of SL214 carrying various doses of *BMH1* and *BMH2* were generated and tested for growth on glucose. Both *BMH1* and *BMH2* rescued the *GAL1:CHC1 scd1-i* strain on glucose when present on low- or high-copy plasmids. However, the percentage of rescue indicated there was a strong dosage effect of the *BMH* genes. One extra copy generated by integrating *BMH1* or *BMH2* on YIp vectors into the genome did not permit growth of the *GAL1:CHC1 scd1-i* strain on glucose, whereas low-copy expression from a *CEN* plasmid gave a low, but significant, percent (10–11%) recovery of viable Chc⁻ yeast. In high copy, rescue was increased to 51.1 ± 6% for *BMH1* and 69.0 ± 4.6% for *BMH2*. In addition, colony size was significantly smaller for SL214 carrying the *BMH, CEN* plasmids as compared to *BMH*, 2μ vectors (data not shown).

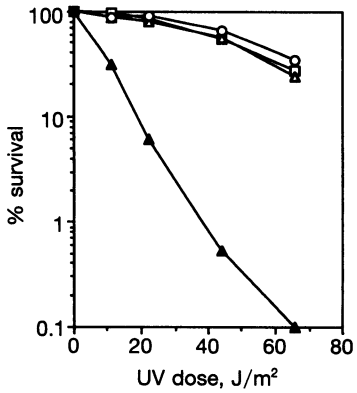

FIG. 2. *bmh1*-Δ and *bmh2*-Δ strains are not supersensitive to UV irradiation. Cells (10^7) were synchronized in G_1 with α-factor and then grown in fresh medium for 1 hr to allow entry into S phase. Cells were plated in duplicate on YEPD and irradiated for various times with a source delivering 2.2 $\mu J \cdot mm^{-2} \cdot sec^{-1}$ to yield the indicated UV exposures. Colonies were counted after 3 days at 30°C. Percent viability is expressed relative to unirradiated plates. Note that higher doses of UV radiation did not uncover any further differences in sensitivity between *bmh* and wild-type strains. Strains were: SL1559, *BMH1 BMH2* (□); SL1558, *bmh1*-Δ (△); SL1771, *bmh2*-Δ (○); and LP3004-1B, *rad9-1* (▲).

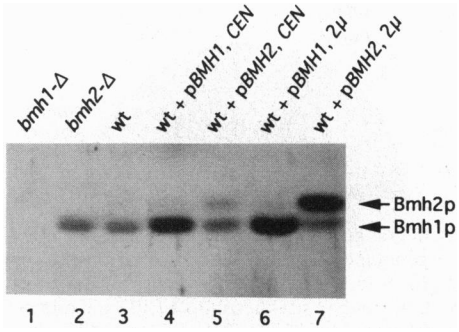

FIG. 3. Immunoblot analysis of Bmh1p and Bmh2p. Strains were grown in selective medium where appropriate and cell extracts were analyzed by immunoblotting, with anti-14-3-3 peptide antibodies. Strains were SL1386, *bmh1*-Δ (lane 1); SL1320, *bmh2*-Δ (lane 2); SL1382, *BMH1 BMH2* (wild type, wt) (lane 3); and SL1382 transformed with pJW42 (p*BMH1, CEN*) (lane 4), pA21 (p*BMH2, CEN*) (lane 5), pJW40 (p*BMH1*, 2μ) (lane 6), or pJW18 (p*BMH2*, 2μ) (lane 7).

Although percent rescue by *BMH1* was similar to rescue by *BMH2* at each gene dosage, colony sizes formed revealed a major difference between the genes. *GAL1:CHC1 scd1-i* cells rescued by *BMH1* on a 2μ vector grew significantly more slowly than cells rescued by *BMH2* on a 2μ vector (Fig. 4). Comparable differences were found for *CEN* vectors. Considering that Bmh1p is more abundant than Bmh2p, this suggests that Bmh2p is more potent than Bmh1p in its ability to rescue Chc⁻ yeast.

Since overexpression of Bmh1p or Bmh2p could rescue inviable strains of Chc⁻ yeast and this was dose dependent, we tested whether viable strains of clathrin-depleted cells (*scd1-v* genotype) would show any synthetic growth phenotypes in the presence of *bmh1*-Δ or *bmh2*-Δ. *GAL1:CHC1 scd1-v* strains carrying *bmh1*-Δ or *bmh2*-Δ were generated and analyzed for growth on glucose (Fig. 5). The *bmh1*-Δ mutants were inviable on glucose, whereas *bmh2*-Δ mutants grew very slowly (Fig. 5) or were inviable (data not shown). This synthetic growth defect could be reversed by transformation with the *BMH* gene on a plasmid, so that growth was similar to that of *chc1*-Δ *scd1-v BMH1 BMH2* strains. This further demonstrates that clathrin-deficient yeast are sensitive to the cellular levels of Bmh proteins.

The low-copy rescue by *BMH1* and *BMH2* suggested that either of these might, in fact, be allelic to *SCD1*, since single- or low-copy *scd1-v* can rescue clathrin-deficient yeast carrying the *scd1-i* allele (12). However, segregation analysis indicated

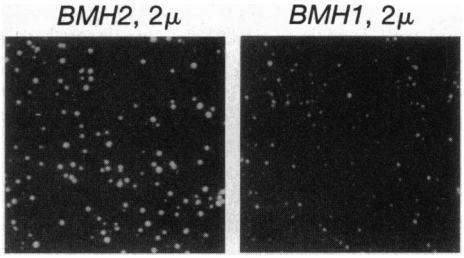

FIG. 4. Differential rescue of growth of a *GAL1:CHC1 scd1* strain by overexpression of *BMH1* and *BMH2*. SL214 transformed with pJW18 (*BMH2*, 2μ) or pJW40 (*BMH1*, 2μ) was inoculated into selective glucose medium and grown for 15 hr to repress *CHC1* expression. Cells were plated on selective glucose medium and incubated at 30°C for 7 days.

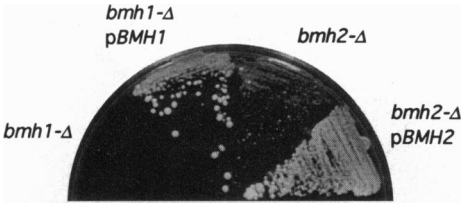

FIG. 5. Synthetic growth defect of *bmh1*-Δ and *bmh2*-Δ in Chc$^-$ yeast of *scd1-v* genotype. Strains were inoculated into selective glucose medium and grown for 15 hr at 30°C to repress clathrin heavy-chain expression. Cells were then streaked onto YEPD and grown for 6 days. Strains from left to right: SL1797, *GAL1:CHC1 scd1-v bmh1*-Δ, plus no vector (bmh1-Δ) or pJW42 (p*BMH1*); SL1795, *GAL1:CHC1 scd1-v bmh2*-Δ, plus pRS314 (bmh2-Δ) or pJW45 (p*BMH2*).

that *BMH1* and *BMH2* are not allelic to *SCD1* (data not shown).

Evidence for a Role for 14-3-3 Proteins in the Ras Signaling Pathway. The 14-3-3 proteins have been implicated in several kinase signaling pathways, including those involving PKC, Bcr, and Raf-1, a downstream target of the Ras signaling cascade (see refs. 1 and 3 and references therein). In *S. cerevisiae* three independent kinase signaling pathways are known to be required for cell viability. These include the *RAS*/cAMP-dependent protein kinase (PKA, encoded by *TPK* genes) pathway (30), the *SLN1/HOG1* osmotic sensor pathway (31, 32), and the PKC (*PKC1*) pathway (33). To determine whether the inviability of the Bmh$^-$ strains resulted from perturbation of any one of these pathways, we tested whether conditions that allow bypass rescue of lethal mutations in these kinase cascades could rescue *bmh1*-Δ *bmh2*-Δ mutants.

In *S. cerevisiae*, cells with mutations in any of the genes in the *PKC1* pathway (*pkc1, bck1, mkk1/mkk2*, or *mpk1*) acquire a lethal cell lysis defect but can be rescued by plating on medium containing 1 M sorbitol or 100 mM $CaCl_2$ (33). However, *bmh1*-Δ *bmh2*-Δ spore segregants were not rescued by these osmoremedial agents, indicating that the lethality of Bmh$^-$ yeast is not due solely to a defect in PKC signaling.

The *SLN1/HOG1* pathway is induced in response to osmotic stress (31). In the absence of Sln1p, the putative osmosensor at the plasma membrane, this kinase cascade is constitutively active. This results in lethality in nonhyperosmotic medium (32). The lethality of *sln1* mutants on normal media can be suppressed by inactivation of *HOG1*, which encodes the downstream mitogen-activated protein kinase in this pathway. Although Hog1 kinase is crucial during osmotic stress, loss of *HOG1* alone has no detrimental effect on cell growth in nonhyperosmotic conditions. To determine whether *bmh1*-Δ *bmh2*-Δ mutants are inviable due to constitutive activation of the *SLN1/HOG1* pathway, we tested whether deletion of *HOG1* could suppress the lethal phenotype; however, *hog1*-Δ (31) also could not rescue Bmh$^-$ cells.

The Ras/cAMP pathway in *S. cerevisiae* is a nutrient-sensing pathway (30). In nutrient-rich conditions, Ras proteins (coded for by *RAS1* and *RAS2*) bind GTP, stimulated by the GDP/GTP exchange protein Cdc25p. This leads to increased activity of adenylate cyclase and synthesis of cAMP. cAMP activates the PKAs, redundantly encoded by *TPK1, TPK2*, and *TPK3*, thereby promoting cell cycle initiation. Cells lacking functional *RAS* or *CDC25* genes arrest growth in the G_1 stage of the cell cycle, but this can be suppressed by overexpression of *TPK1* (15). To examine whether Bmh$^-$ cells are inviable due to a block in Ras/cAMP signaling, we overexpressed *TPK1* in a strain (SL1470) in which the only source of Bmh protein was provided by *BMH2* under control of the *GAL1* promoter. On galactose medium SL1470 grew as well as wild-type cells, but upon shift to glucose Bmh protein was depleted and after ≈20 hr cells stopped growing, although they showed no obvious uniform cell cycle-arrest phenotype. Interestingly, high-copy

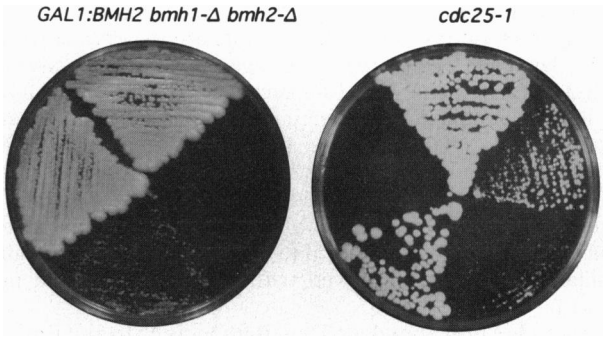

FIG. 6. Genetic interactions of *BMH1* and *BMH2* with the Ras signaling pathway. (*Left*) Overexpression of *TPK1* partially suppresses the lethality of the Bmh$^-$ phenotype in yeast. Cells were grown overnight in glucose medium to repress Bmh protein expression, streaked onto glucose plates, and grown for 6 days. Strains clockwise from the top: SL1470, *bmh1*-Δ *bmh2*-Δ *GAL1:BMH2*, transformed with pJW45 (*BMH2, CEN*), no plasmid, pDG39 (*TPK1*, 2μ), or pJW42 (*BMH1, CEN*). (*Right*) Overexpression of *BMH1* and *BMH2* partially suppresses the temperature sensitivity of a *cdc25-1* mutant. Cells were streaked onto YEPD and grown at 37° for 5 days. Strains clockwise from the top: TT25-6, *cdc25-1*, transformed with pAD-CDC25, YEp13-BMH2 (*BMH2*, 2μ), YEp13-BMH1 (*BMH1*, 2μ), YEp13-*TPK1* (*TPK1*, 2μ), or no plasmid.

TPK1 was able to suppress the lethality of Bmh protein depletion (Fig. 6 *Left*). We also found that overexpression of *BMH1* or *BMH2* was able to partially suppress the temperature sensitivity of the *cdc25-1* mutation (Fig. 6 *Right*). Similar to the Chc$^-$ rescue, overexpression of *BMH2* appeared to suppress *cdc25-1* better than *BMH1*, but neither *BMH* gene suppressed as well as *TPK1*. High-copy *BMH* genes could not suppress the dominant/negative temperature-sensitive $RAS2^{Val\text{-}19\ Ala\text{-}22}$ mutation (34) (data not shown). This suggests that Bmh proteins function in the cAMP-dependent signaling pathway, possibly at the level of Ras. However, since *TPK1* overexpression only partially suppressed Bmh$^-$ cells, Bmh proteins are likely to have additional functions in yeast.

It was possible that the clathrin defect itself perturbs Ras signaling at the plasma membrane and that high-copy *BMH* genes rescue clathrin-deficient yeast via suppression of a Ras signaling defect. If this were the case then overexpression of *TPK1* should also rescue Chc$^-$ yeast. However, YEp13-TPK1 could not restore viability to the *GAL1:CHC1 scd1-i* strain SL214 grown on glucose (data not shown).

DISCUSSION

Two *S. cerevisiae* genes that encode 14-3-3 proteins have now been identified. Similar to results in *Sch. pombe*, deletion of both *BMH1* and *BMH2* is lethal, indicating that 14-3-3 proteins have crucial functions in eukaryotic cell physiology. However, the Bmh proteins, unlike fission-yeast 14-3-3 proteins, have not been implicated in checkpoint control, although further studies will be required to rule out this possibility completely.

The basis for our initial isolation of *BMH2* was its ability, when overexpressed, to rescue inviable strains of clathrin-deficient yeast (12). Our studies indicate that overexpression of *BMH1* can also restore viability to these strains and that deletion of *BMH1* or *BMH2* further impairs the slow growth phenotype of viable strains lacking clathrin. These results provide genetic evidence that 14-3-3 proteins may be involved in vesicular transport. Thus far we have not seen remediation of clathrin sorting defects upon overexpression of *BMH* genes (unpublished observations); however, this does not rule out a role for these 14-3-3 proteins in membrane transport in yeast. At least two other *SCD* genes identified in the same multicopy suppressor screen, *SCD4* and *SCD5*, encode proteins that have

clear roles in vesicular trafficking, yet they also do not rescue Chc$^-$ sorting defects (unpublished observations).

Studies in mammalian cells also support a role for 14-3-3 proteins in membrane transport. Members of this protein family have been isolated as cofactors that stimulate Ca^{2+}-dependent regulated exocytosis in a permeabilized adrenal chromaffin cell system (5). Some 14-3-3 proteins have been shown to be associated with synaptosomes (35), and some isoforms localize to the Golgi compartment in cultured cells (36). A 14-3-3 protein has also been isolated as an activator of ExoS, the *P. aeruginosa* virulence factor which ADP-ribosylates small GTP-binding proteins, including Rab3, Rab4, and Ras (6, 7). It has been proposed that ADP-ribosylation of Rab proteins by ExoS may disrupt vesicle trafficking during infection, inhibiting the normal antimicrobial functions of cells (7).

The recent connections of 14-3-3 proteins to protein kinase signaling pathways led us to test whether the lethality of Bmh$^-$ yeast could be due to a perturbation of any one of the three known essential kinase cascades in *S. cerevisiae.* Although we cannot completely rule out that 14-3-3 proteins act downstream of the steps assayed in our studies, our data indicate that more than one signal transduction pathway or other essential cellular processes are affected. The partial suppression of *cdc25-1* by overexpression of *BMH1* or *BMH2* suggests a potential interaction with the Ras signaling pathway. In addition, two other lines of genetic evidence link Bmhp function to Ras signaling. First, we found that overexpression of *TPK1* restored viability to Bmh$^-$ yeast. Second, the *tpk1-wimp1* allele, which encodes a weakened PKA, shows temperature-sensitive synthetic lethality with *bmh1*-Δ (S. Garrett, personal communication). Normal, *BMH1 BMH2* cells carrying *tpk1-wimp* as their sole source of PKA grow quite well.

Nonetheless, that *TPK1* overexpression only partially rescued the *bmh1*-Δ *bmh2*-Δ lethality suggests that not all 14-3-3 functions occur through the Ras/PKA pathway and supports a multifunctional role for these proteins. The genetic interactions with clathrin mutants are also consistent with this. Since overexpression of *TPK1* could not rescue inviable strains of Chc$^-$ yeast, it is unlikely that the suppression of clathrin deficiency by high-copy *BMH* genes is mediated through suppression of the Ras pathway. Still, the exact biochemical functions of the 14-3-3 proteins are not understood. We find the potential connection of 14-3-3 proteins to small GTPases such as Ras intriguing, given the importance of Rab and Arf proteins in vesicular transport (37). The data presented here could also be accounted for if 14-3-3 proteins are more generalized factors or stress-response proteins that stabilize polypeptides or protein–protein interactions such as those perturbed in Chc$^-$ and Ras signaling mutants.

A key question concerns whether Bmh1p and Bmh2p are functionally interchangeable. That single *BMH* mutations show no obvious phenotypes but deletion of both genes is lethal indicates that Bmh1p and Bmh2p must be able to overlap significantly in their functions. The high degree of sequence identity (>90%) between Bmh1p and Bmh2p is also consistent with this view. However, the ability of *BMH1* and *BMH2* to rescue inviable strains of Chc$^-$ yeast and to rescue the temperature sensitivity of *cdc25-1* distinguishes the two genes, because *BMH2* was consistently more potent than *BMH1.* Differential expression does not appear to account for this, since Bmh1p is more abundant than Bmh2p. It is possible that the functional differences reside in the divergent carboxyl termini of the proteins, since we have evidence that a short carboxyl-terminal stretch of Bmh2p may be functionally important. This region of all 14-3-3 proteins is the most variable and thus could be crucial for differences in isoform function and cellular localization.

We thank David Levin and Mike Gustin for strains, plasmids, and helpful discussions; Takashi Toda for strains; and Steve Garrett and Michael Wigler for sharing unpublished results. This work was supported by grants from the National Science Foundation and American Cancer Society (S.L.) and by Public Health Service Training Grants HD07104 (D.G.) and HL07415 (K.N.).

1. Aitken, A., Collinge, D. B., van Heusden, B. P. H., Isobe, T., Roseboom, P. H., Rosenfeld, G. & Soll, J. (1992) *Trends Biochem. Sci.* **17,** 498–501.
2. Ichimura, T., Isobe, T., Okuyama, T., Yamauchi, Y. & Fujisawa, H. (1987) *FEBS Lett.* **219,** 79–82.
3. Morrison, D. (1994) *Science* **266,** 56–57.
4. Alam, R., Hachiya, N., Sakaguchi, M., Kawabata, S., Iwanaga, S., Kitajima, M., Mihara, K. & Omura, T. (1994) *J. Biochem. (Tokyo)* **116,** 416–425.
5. Morgan, A. & Burgoyne, R. D. (1992) *Nature (London)* **355,** 833–836.
6. Fu, H., Coburn, J. & Collier, R. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2320–2324.
7. Coburn, J. & Gill, D. M. (1991) *Infect. Immun.* **59,** 4259–4262.
8. Lu, G., DeLisle, A. J., de Vetten, N. C. & Ferl, R. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11490–11494.
9. Ford, J. C., Al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J. F. & Carr, A. M. (1994) *Science* **265,** 533–535.
10. Van Heusden, G. P. H., Wenzel, T. J., Lagendijk, E. L., de Steensma, H. Y. & Van den Berg, J. A. (1992) *FEBS Lett.* **302,** 145–150.
11. Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E. & Matsumoto, K. (1994) *Science* **265,** 1716–1719.
12. Nelson, K. K. & Lemmon, S. K. (1993) *Mol. Cell. Biol.* **13,** 521–532.
13. Lemmon, S. K. & Jones, E. W. (1987) *Science* **238,** 504–509.
14. Payne, G. & Lemmon, S. K. (1994) in *Guidebook to the Secretory Pathway,* eds. Rothblatt, J., Novick, P. & Stevens, T. H. (Oxford Univ. Press, New York), pp. 267–269.
15. Toda, T., Cameron, S., Sass, P., Zoller, M. & Wigler, M. (1987) *Cell* **50,** 277–287.
16. Guthrie, C. & Fink, G. R. (1991) *Guide to Yeast Genetics and Molecular Biology* (Academic Press, San Diego).
17. Carlson, M. & Botstein, D. (1982) *Cell* **28,** 145–154.
18. Rose, M. D., Novick, P., Thomas, J. H., Botstein, D. & Fink, G. R. (1987) *Gene* **60,** 237–243.
19. Hill, J. E., Meyers, A. M., Koerner, T. J. & Tzagoloff, A. (1986) *Yeast* **2,** 163–167.
20. Sikorski, R. S. & Hieter, P. (1989) *Genetics* **122,** 19–27.
21. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. (1992) *Gene* **110,** 119–122.
22. Elledge, S. J. & Davis, R. W. (1988) *Gene* **70,** 303–312.
23. Kim, J.-H. & Powers, S. (1991) *Mol. Cell. Biol.* **11,** 3894–3904.
24. Stepp, J. D., Pellicena-Palle, A., Hamilton, S., Kirchhausen, T. & Lemmon, S. K. (1995) *Mol. Biol. Cell.* **6,** 41–58.
25. Roseboom, P. H., Weller, J. L., Babila, T., Aitken, A., Sellers, L. A., Moffett, J. R., Namboodiri, M. A. A. & Klein, D. C. (1994) *DNA Cell Biol.* **13,** 629–640.
26. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
27. Higgins, D. G. & Sharp, P. M. (1988) *Gene* **73,** 237–244.
28. Weinert, T. A. & Hartwell, L. H. (1988) *Science* **241,** 317–322.
29. Garrels, J. I., Futcher, B., Kobayashi, R., Latter, G. I., Schwender, B., Volpe, T., Warner, J. R. & Mclaughlin, C. S. (1994) *Electrophoresis* **15,** 1466–1486.
30. Broach, J. R. (1991) *Trends Genet.* **7,** 28–33.
31. Brewster, J. L., Devaloir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. (1993) *Science* **259,** 1760–1763.
32. Maeda, T., Wurgler-Murphy, S. M. & Saito, H. (1994) *Nature (London)* **369,** 242–245.
33. Levin, D. E., Bowers, B., Chen, C. Y., Kamada, Y. & Watanabe, M. (1994) *Cell. Mol. Biol. Res.* **40,** 229–239.
34. Powers, S., O'Neill, K. & Wigler, M. (1989) *Mol. Cell. Biol.* **9,** 390–395.
35. Martin, H., Rostas, J., Patel, Y. & Aitken, A. (1994) *J. Neurochem.* **63,** 2259–2265.
36. Leffers, H., Madsen, P., Rasmussen, H. H., Honore, B., Andersen, A. H., Walbum, E., Vandekerckhove, J. & Celis, J. E. (1993) *J. Mol. Biol.* **231,** 982–998.
37. Ferro-Novick, S. & Novick, P. (1993) *Annu. Rev. Cell Biol.* **9,** 575–599.