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20. This is possible because the bandwidth of the visual signal, after transduction by the photoreceptors, is on the order of a few hertz, whereas the bandwidth of optic nerve fibers extends to 100 Hz; the difference between the bandwidths of the signal and carrier is available for multiplexing. A more detailed analysis shows that this can greatly enhance information transmission through the optic nerve as compared with a single-neuron code (M. Meister, *Proc. Natl. Acad. Sci. U.S.A.*, in press).
21. We thank R. Schneeveis for expert technical assistance. Supported by a Lucille P. Markey Scholarship and National Eye Institute (NEI) grant EY10020 to M.M., by a fellowship from the Human Frontiers in Science Project to L.L., and by NEI grants EY01543 and EY05750 to D.A.B.

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Pheromone Response in Yeast: Association of Bem1p with Proteins of the MAP Kinase Cascade and Actin

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Haploid cells of the yeast *Saccharomyces cerevisiae* respond to mating pheromones with polarized growth toward the mating partner. This morphological response requires the function of the cell polarity establishment protein Bem1p. Immunochemical and two-hybrid protein interaction assays revealed that Bem1p interacts with two components of the pheromone-responsive mitogen-activated protein (MAP) kinase cascade, Ste20p and Ste5p, as well as with actin. Mutants of Bem1p that are associated with defective pheromone-induced polarized morphogenesis interacted with Ste5p and actin but not with Ste20p. Thus, the association of Bem1p with Ste20p and Ste5p may contribute to the conveyance of spatial information that regulates polarized rearrangement of the actin cytoskeleton during yeast mating.

Haploid cells of *S. cerevisiae* secrete the peptide pheromones **a** and α factors to induce the conjugation of cells with opposite mating types. The pheromones bind to specific receptors and thereby trigger differentiation processes (*1*). The responding cells adopt a polarized cell shape with membrane projections directed toward the pheromone source (*2*). Polarized morphogenesis in the direction of an external signal is a characteristic feature of differentiated cells. Examples include chemotactic responses in neutrophils and *Dictyostelium*, polarized growth of T cells toward antigen-presenting cells, and the development of cell polarity during differentiation of epithelial and neuronal cells (*3*). The molecular mechanisms by which the positional information of an external signal is translated into the establishment of cell polarity are poorly understood.

In conjugating yeast cells, development of cell polarity requires the small Rho-like guanosine triphosphate (GTP)–binding protein Cdc42p and its guanosine diphosphate (GDP)–GTP exchange factor Cdc24p, as well as the Src homology 3 (SH3) domain–containing protein Bem1p that associates with Cdc24p (*4*–*6*). These proteins also participate in regulation of the development of cell polarity during budding; this process is under the control of an internal program that includes the function of bud-site selection proteins, which specify the site of bud formation (*7*, *8*). During mating, this internal program must be overridden by spatial information imposed by the external pheromone signal in order to specify the site of cell growth.

The binding of pheromones to their receptors activates a heterotrimeric GTP-binding protein (G protein) common to both cell types. Through the action of the Ste20p protein kinase, the G protein β and γ subunits stimulate a MAP kinase cascade whose components constitute a signaling complex by association with the scaffold protein Ste5p (*9*). Ste20p phosphorylates Ste11p, a MEK (MAP or extracellular signal–regulated kinase kinase) kinase homolog, in vitro (*10*) and is therefore likely to be a constituent of this signaling complex. The *STE20* and *BEM1* genes can function as high-dosage suppressors of a G protein β subunit mutant that is partially defective in signaling (*11*, *12*).

Specific antibodies to Ste20p, Bem1p, Ste5p, and actin (Fig. 1A) were used to investigate the relative distribution of these proteins in yeast cell fractions (*13*). About 50% of total Ste20p and >60% of total Bem1p sedimented with the particulate fraction, which also contained Ste5p and actin (55 and 65%, respectively, of total protein) (*14*). Washing the particulate fraction with increasing concentrations of NaCl solubilized increasing amounts of Ste20p, Bem1p, actin, and, to a lesser extent, Ste5p (Fig. 1B). These proteins were completely solubilized with 1% SDS but remained in the particulate fraction after treatment with NP-40 or Triton X-100 at concentrations that are known to release membrane-bound proteins. The results suggest that Ste20p, Bem1p, and Ste5p are not directly bound to membranes but rather are associated with complexes of high density. Sucrose density gradient centrifugation resolved these complexes into two fractions: a heavy fraction migrating at 46 to 48% (w/w) sucrose that contained actin, and a light fraction migrating at 38 to 40% sucrose that did not contain actin (Fig. 1C). Marker enzymes for Golgi vesicles and the plasma membrane—guanosine diphosphatase (GDPase) and adenosine triphosphatase (ATPase), respectively—did not colocalize precisely with these fractions. These observations are consistent with the view that Ste20p, Bem1p, and Ste5p are constituents of a large protein complex that is partly bound to the actin cytoskeleton and is not directly bound to the membrane.

We next investigated whether Ste20p, Ste5p, and Bem1p are associated in vivo.

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Antibodies to Ste20p precipitated Bem1p and Ste5p (Fig. 2A). Ste20p and Ste5p were present in immune complexes after precipitation with antibodies to Bem1p (Fig. 2B), and Ste20p and Bem1p were precipitated with antibodies to Ste5p (Fig. 2C). The interaction between Ste20p and Ste5p was also observed in cells in which *BEM1* was deleted, and the interaction between Bem1p and Ste5p was detected in cells in which *STE20* was deleted (Fig. 2C). In cells in which *STE5* was deleted, Bem1p was co-immunoprecipitated with antibodies to Ste20p and vice versa (Fig. 2D). Antibodies to Bem1p precipitated actin, and antibodies to actin precipitated Bem1p (Fig. 3A); this association was also observed in cells that were deleted for *STE5* or *STE20* (*15*). Antibodies to actin also precipitated Ste20p, and antibodies to Ste20p precipitated actin (Fig. 3B). However, this co-immunoprecipitation of actin and Ste20p was not observed in cells in which *BEM1* was deleted (Fig. 3B) (*16*), which suggests that the association between Ste20p and actin requires Bem1p. These results indicate that Ste20p, Ste5p, and Bem1p form a complex and are associated with actin in vivo. We did not detect any differences in the various interactions between pheromone-treated and untreated cells (*15*).

We analyzed *bem1-s1* and *bem1-s2* mutations, which are associated with defective pheromone-induced polarized morphogenesis (*6*). The mutant alleles were recovered from the mutant strains by gap repair and sequenced (*17*). The two alleles contained single nucleotide exchanges that produced stop codons at codon positions 299 and 519, respectively (*18*). Thus, both mutant alleles were expected to generate truncated proteins, which was confirmed by the detection of truncated proteins of ~33 and 58 kD in *bem1-s1* and *bem1-s2* mutant cells, respectively (*15*, *19*). Antibodies to Bem1p precipitated the

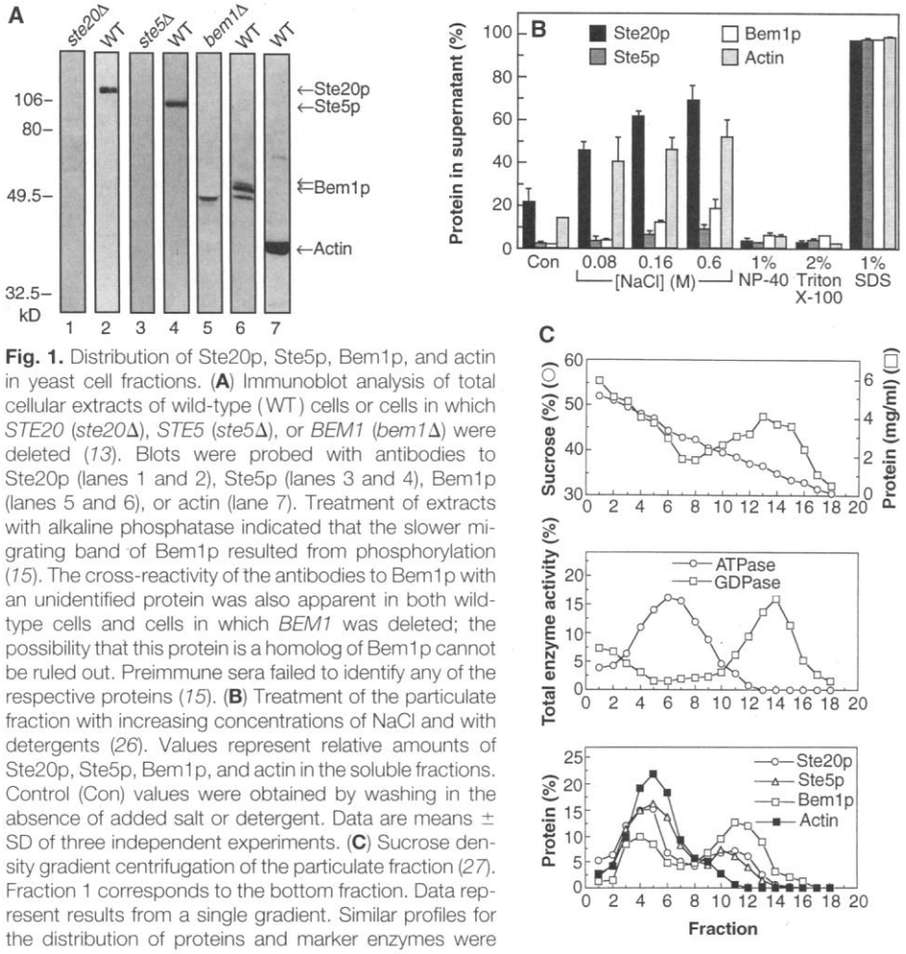

Fig. 1. Distribution of Ste20p, Ste5p, Bem1p, and actin in yeast cell fractions. (**A**) Immunoblot analysis of total cellular extracts of wild-type (WT) cells or cells in which *STE20* (*ste20Δ*), *STE5* (*ste5Δ*), or *BEM1* (*bem1Δ*) were deleted (*13*). Blots were probed with antibodies to Ste20p (lanes 1 and 2), Ste5p (lanes 3 and 4), Bem1p (lanes 5 and 6), or actin (lane 7). Treatment of extracts with alkaline phosphatase indicated that the slower migrating band of Bem1p resulted from phosphorylation (*15*). The cross-reactivity of the antibodies to Bem1p with an unidentified protein was also apparent in both wild-type cells and cells in which *BEM1* was deleted; the possibility that this protein is a homolog of Bem1p cannot be ruled out. Preimmune sera failed to identify any of the respective proteins (*15*). (**B**) Treatment of the particulate fraction with increasing concentrations of NaCl and with detergents (*26*). Values represent relative amounts of Ste20p, Ste5p, Bem1p, and actin in the soluble fractions. Control (Con) values were obtained by washing in the absence of added salt or detergent. Data are means ± SD of three independent experiments. (**C**) Sucrose density gradient centrifugation of the particulate fraction (*27*). Fraction 1 corresponds to the bottom fraction. Data represent results from a single gradient. Similar profiles for the distribution of proteins and marker enzymes were obtained in three independent experiments.

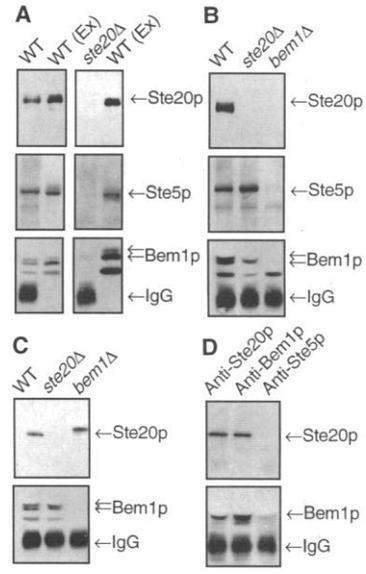

Fig. 2. Co-immunoprecipitation of Ste20p, Ste5p, and Bem1p from yeast cell extracts (*28*). (**A**) Immunoprecipitation with antibodies to Ste20p. (**B**) Immunoprecipitation with antibodies to Bem1p. (**C**) Immunoprecipitation with antibodies to Ste5p. (**D**) Immunoprecipitation with antibodies to Ste20p, Bem1p, or Ste5p in extracts of cells in which *STE5* was deleted. Immunoprecipitated proteins were subjected to immunoblot analysis (*13*) with specific antibodies to Ste20p, Ste5p, and Bem1p as indicated to the right of the panels. The specificity of the immunoprecipitations was verified by analyzing extracts of cells in which *STE20*, *STE5*, or *BEM1* were deleted (*13*). Preimmune sera failed to precipitate any of the respective proteins (*15*). Immunoprecipitation with cells in which *BEM1* was deleted also showed that the observed cross-reactivity of the antibodies to Bem1p with an unidentified protein (Fig. 1A) had no effect on the immunoprecipitation of Ste20p or Ste5p. Each precipitation was confirmed in at least three independent experiments. Ex, total cell extract (all other lanes show immunoprecipitates); IgG, immunoglobulin G.

Table 1. Interactions of Bem1p with Ste20p. Interactions were detected with the two-hybrid system (*30*). Numbers in parentheses indicate the expressed amino acids. Data (Miller units) are means ± SD of three independent experiments. Growth on −His medium was evaluated as follows: (−) no growth, (+) slow growth, and (++) normal growth. The SH3 domains of Bem1p are located from amino acids 79 to 127 and 162 to 212 (*6*).

Gal4p-AD fusions	LexA-DBD fusions	β-Galactosidase activity	Growth on −His medium
Bem1p(1–551)	Ste20p(1–939)	0.21 ± 0.02	−
Bem1p(1–551)	Ste20p(1–497)	2.76 ± 0.20	+
Bem1p(1–551)	Ste20p(498–939)	0.22 ± 0.01	−
Bem1p(1–325)	Ste20p(1–497)	0.34 ± 0.07	−
Bem1p(157–551)	Ste20p(1–497)	8.92 ± 1.57	++
Bem1p(298–551)	Ste20p(1–497)	0.31 ± 0.04	−

truncated forms (Fig. 3C). Although actin and Ste5p coprecipitated with the products of the mutant alleles, Ste20p was not detected in the immune complexes (Fig. 3, C and D). Antibodies to actin coprecipitated the truncated Bem1p proteins but antibodies to Ste20p did not (Fig. 3, C and D). In *bem1-s* mutant cells, antibodies to actin did not precipitate Ste20p and vice versa (*15*). Thus, the Bem1p mutant proteins are defective in association with Ste20p but proficient in association with Ste5p and actin.

We used the yeast two-hybrid system (*20*) to define interacting regions within Bem1p and Ste20p (Table 1). Full-length Bem1p, fused to the transcriptional activation domain of Gal4p, interacted with fusions of the DNA-binding domain of LexA to the NH_2-terminal half of Ste20p, but not with fusions to the catalytic, COOH-terminal domain of Ste20p

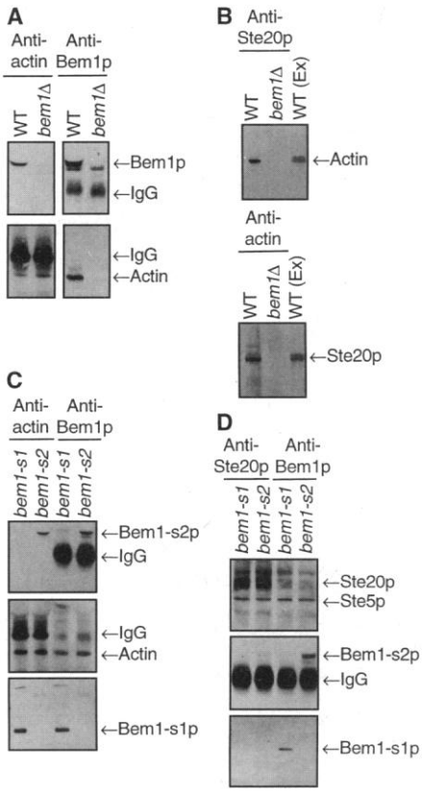

Fig. 3. Immunoprecipitation with antibodies to Bem1p, Ste20p, and actin (*28*). (**A**) Association of Bem1p with actin. Immunoprecipitates from wild-type cells (WT) and cells in which *BEM1* was deleted (*13*) were analyzed with antibodies to either Bem1p or actin. (**B**) Association of actin and Ste20p. Immunoprecipitates were analyzed with antibodies to Ste20p or to actin. (**C**) Association of proteins encoded by *bem1-s* mutant alleles with actin (*29*). Immunoprecipitates were analyzed with antibodies to Bem1p or to actin. (**D**) The *bem1-s* mutant proteins were defective in the association with Ste20p but associated with Ste5p. Immunoprecipitates were analyzed with antibodies to Bem1p, Ste20p, or Ste5p. Each result was confirmed in at least three independent experiments.

or full-length Ste20p. Removal of the NH_2-terminal SH3 domain of Bem1p improved the interaction with the NH_2-terminus of Ste20p, whereas removal of both SH3 domains abrogated the interaction. The interaction was also abolished by removal of the COOH-terminal region after amino acid 325 of Bem1p. Thus, the second SH3 domain and COOH-terminal sequences of Bem1p—including the segment from amino acids 316 to 399, which shows weak similarity to SH3 domains and homology to a region of human p47 (*7*)—are required for the interaction with the NH_2-terminal region of Ste20p. Removal of this region of Ste20p that interacts with Bem1p results in a lethal phenotype (*21*).

Ste5p is thought to act as a scaffolding protein of the pheromone-response MAP kinase cascade, interacting with Ste11p, Ste7p, Fus3p, and Kss1p (9), and it is directly associated with the βγ subunits of the G protein (*22*) (Fig. 4). Because the products of the *bem1-s1* and *bem1-s2* mutant alleles fail to interact with Ste20p and are associated with defects in polarized growth in response to pheromone but not during budding, we propose that Ste20p may serve as a link between components of the pheromone signaling pathway and regulators of cell polarity such as Bem1p, Cdc42p, and Cdc24p (Fig. 4). Our results suggest that the interaction between Ste20p and Bem1p is important in polarized growth in response to the external pheromone signal.

The activated form of Cdc42p binds and thereby activates Ste20p and $p65^{PAK}$, a mammalian homolog of Ste20p, in vitro (*23*). Therefore, Cdc42p may function in the G protein–dependent activation of Ste20p (Fig. 4). The similarity of Ste20p to mammalian $p65^{PAK}$ (*23*) and of Cdc42p to the mammalian Rho-like guanosine triphosphatases Rac1, Cdc42Hs, and RhoA that are known to participate in the activation of the JNK-SAPK (c-Jun NH_2-terminal kinases or stress-activated protein kinases) signaling cascade (*24*) and in the regulation of actin reorganization (*25*) in response to extracellular signals indicates that our results may be relevant to the understanding of similar signaling mechanisms in mammalian cells.

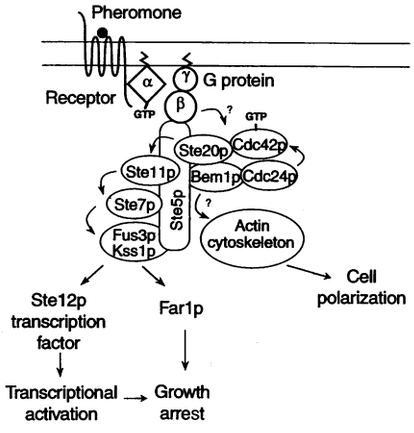

Fig. 4. Model for the role of Ste20p and Bem1p as mediators of spatial information for the rearrangement of the actin cytoskeleton.

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13. Yeast media and culture conditions were as described [M. D. Rose, F. Winston, P. Hieter, *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990)]. The *ste20-*, *bem1-*, and *ste5*-deleted cells were derivatives of strain W303-1A (*MAT***a** *ade2 his3 leu2 ura3 can1*) (*11, 12*) [E. Leberer *et al.*, *Mol. Gen. Genet.* **241**, 241 (1993)]. Cells were washed with 1.2 M sorbitol, resuspended in 1.2 ml of homogenization buffer [20 mM Hepes (pH 7.4), 0.5 M sorbitol, 10 mM NaCl, 2 mM EDTA, and 1 mM Na_3VO_4] containing protease inhibitor cocktail (PIC) [1 mM phenylmethylsulfonyl fluoride, 1 mM pefabloc, and pepstatin, leupeptin, and aprotinin each at 2 μg/ml], and disrupted in a Mini Beadbeater (Biospec) at 4°C. The homogenate was centrifuged at 6000*g* for 5 min and the protein concentration of the supernatant was determined by the Bio-Rad DC method. Samples were separated by SDS–polyacrylamide gel electrophoresis [U. K. Laemmli, *Nature* **227**, 680 (1970)] and transferred (MultiPhor chamber, Pharmacia) to nitrocellulose filters, which were then incubated sequentially with primary (rabbit or mouse) and secondary [horseradish peroxidase-conjugated goat antibodies to either rabbit or mouse immunoglobulin (Bio-Rad)] antibodies in tris-buffered saline containing 1% bovine serum albumin. Immune complexes were detected with the Amersham ECL kit. Standard protocols [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)] were used to generate rabbit antibodies to glutathione-S-transferase (GST) fusion proteins of Ste5p and Bem1p expressed in *Escherichia coli*. Antibodies were af-

finity-purified with nitrocellulose strips containing immobilized GST-Ste5p or GST-Bem1p as described (*10*). Antibodies to Ste20p were as described (*10*). Detection of actin was performed with C4 monoclonal antibody to actin (Boehringer Mannheim).
14. Extracts of yeast W303-1A cells were prepared as described (*13*). The supernatant was further fractionated by centrifugations at 10,000*g* for 20 min and 100,000*g* for 90 min. Pellets were combined to yield the particulate fraction. Equal amounts of proteins of the soluble and particulate fractions were subjected to immunoblot analysis (*13*). After densitometric evaluation of immunoblots, relative percentages of Ste20p, Ste5p, Bem1p, and actin were corrected for the total protein content of the two fractions.
15. T. Leeuw, A. Fourest-Lieuvin, C. Wu, J. Chenevert, K. Clark, M. Whiteway, D. Y. Thomas, E. Leberer, unpublished results.
16. Preimmune sera or antibodies to the calnexin homolog Cne1p [F. Parlati, M. Dominguez, J. J. M. Bergeron, D. Y. Thomas, *J. Biol. Chem.* **270**, 244 (1995)] failed to precipitate actin, and antibodies to actin failed to precipitate Cnep1. These results demonstrate the specificity of the immune reactions.
17. The centromere plasmid pBEM324 containing *BEM1* was constructed by converting the Pst I site in *BEM1* in plasmid pCENBEM (*12*) into an Eco RI site. Plasmid pBEM324 was then digested with Hind III and Eco RI within the coding region of *BEM1*, and transformed into *bem1-s1* strain JC-G11 and *bem1-s2* strain JC-F5 (*6*). The repaired plasmids were isolated from transformants that were defective in mating with a *far1* tester strain and sequenced by the dideoxy chain termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
18. The *bem1-s1* allele contains a G → A mutation at nucleotide position 896, and the *bem1-s2* allele contains a C → T mutation at nucleotide position 1555 of the coding sequence of *BEM1*.
19. J. Chenevert and I. Herskowitz, unpublished results.
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26. The particulate fraction of yeast W303-1A cells was washed by pipetting for 3 min at 4°C with homogenization buffer (*13*) containing the indicated concentrations of NaCl or detergent. After centrifugation at 100,000*g* for 60 min, proteins in the resulting supernatant were precipitated with 7% (w/v) trichloroacetic acid and both fractions were resuspended in equal volumes of lysis buffer [10% glycerol, 5% β-mercaptoethanol, 2% SDS, and 60 mM tris (pH 6.8)]. Equal volumes were subjected to immunoblot analysis, and the relative amounts of Ste20p, Bem1p, Ste5p, and actin were determined densitometrically.
27. The particulate fraction of W303-1A cells was obtained as described (*14*), except that the homogenization buffer did not contain Na_3VO_4. The resulting pellet was resuspended in 2 ml of separation buffer [54% (w/w) sucrose in 20 mM Hepes (pH 7.4)] containing PIC (*13*) and homogenized by eight strokes in a 2-ml Wheaton tissue grinder (Wheaton Scientific). The homogenate was placed at the bottom of a tube (Beckman Instruments) and overlaid with 1.15 ml each of 50, 47.5, 45, 42.5, 40, 37.5, 35, and 32% (w/w) sucrose, all in 20 mM Hepes (pH 7.4). Gradients were centrifuged at 170,000*g* for 16 hours. Eighteen fractions were collected from the bottom. Portions (200 μl) of each fraction were stored for determination of protein amounts (*13*) and sucrose concentrations [with a refractometer (Fisher)]. Assays for plasma membrane ATPase and Golgi GDPase activities were performed as described [B. J. Bowman and C. W. Slayman, *J. Biol. Chem.* **254**, 2928 (1979); C. Abeijon, P. Orlean, P. W. Robbins, C. B. Hirschberg, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6935 (1989)]. Fractions were precipitated with trichloroacetic acid and resuspended in lysis buffer (*26*), and equal volumes were subjected to immunoblot analysis (*13*).
28. Equal volumes of cell cultures were harvested at an optical density at 598 nm of ~1.3, and cells were washed in 1.2 M sorbitol, resuspended in precipitation buffer [50 mM tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, and 0.07% Triton X-100] containing PIC (*13*) and 1% (w/v) bovine serum albumin, and disrupted in a Mini Beadbeater. After two sequential centrifugations at 5000 and 10,000*g* (each for 4 min), the supernatant was incubated with antibodies for 1 hour. Antibody-antigen complexes were incubated with protein A or protein A–protein G Sepharose beads (1:1) (Pharmacia) for polyclonal and monoclonal antibodies, respectively. Beads were sedimented at 1000*g*, washed three times with precipitation buffer, and resuspended in equal volumes of lysis buffer (*26*). Equal volumes of the various precipitates were subjected to immunoblot analysis (*13*).
29. The yeast strains used were the *bem1-s1* and *bem1-s2* mutant derivatives of strain JC2-1B (*6*).
30. The two-hybrid protein interaction assays were performed as described (*20*) in a *far1-1* derivative strain of L40. Fusions of Bem1p with the transcriptional activation domain (AD) of Gal4p were constructed by cloning polymerase chain reaction (PCR) fragments of *BEM1* into plasmid pGAD424. Ste20p fusions with the LexA DNA-binding domain (DBD) were constructed by cloning PCR fragments of *STE20* into plasmid pBTM116. The fusion of the AD of Gal4p with the *BEM1* fragment encoding amino acids 157 to 551 was isolated from a genomic DNA library fused to the AD of Gal4p (*20*) in a two-hybrid screen in which the NH_2-terminus of Ste20p (amino acids 1 to 497) fused to the LexA DBD was used as a bait. From 189,000 transformants, eight were specifically positive and identified as *BEM1*. β-Galactosidase activity assays were performed as described (*20*).
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Requirement of *Saccharomyces cerevisiae* Ras for Completion of Mitosis

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In the yeast *Saccharomyces cerevisiae*, Ras regulates adenylate cyclase, which is essential for progression through the G_1 phase of the cell cycle. However, even when the adenosine 3′,5′-monophosphate (cAMP) pathway was bypassed, the double disruption of *RAS1* and *RAS2* resulted in defects in growth at both low and high temperatures. Furthermore, the simultaneous disruption of *RAS1*, *RAS2*, and the *RAS*-related gene *RSR1* was lethal at any temperature. The triple-disrupted cells were arrested late in the mitotic (M) phase, which was accompanied by an accumulation of cells with divided chromosomes and sustained histone H1 kinase activity. The lethality of the triple disruption was suppressed by the multicopies of *CDC5*, *CDC15*, *DBF2*, *SPO12*, and *TEM1*, all of which function in the completion of the M phase. Mammalian *ras* also suppressed the lethality, which suggests that a similar signaling pathway exists in higher eukaryotes. These results demonstrate that *S. cerevisiae* Ras functions in the completion of the M phase in a manner independent of the Ras-cAMP pathway.

Ras functions in the regulation of cell proliferation and differentiation in various eukaryotes (*1*, *2*). The yeast *S. cerevisiae* has two *RAS* genes, *RAS1* and *RAS2* (*3*), whose products stimulate adenylate cyclase encoded by *CYR1* (*4*). Ras thereby regulates the cAMP pathway, which is essential for progression through the G_1 phase of the cell cycle (*3*, *5*). However, Ras does not appear to regulate adenylate cyclase in other eukaryotes (*6*, *7*). Given the diversity of Ras effectors (*8*), *S. cerevisiae* Ras may have a function other than the regulation of adenylate cyclase. Wigler and colleagues described genetic evidence that predicts the alternative function of Ras (*5*). Here, we show that *S. cerevisiae* Ras and Ras-related protein Rsr1 are functionally redundant and that they jointly regulate the completion of the M phase.

Cells with a single disruption of *CYR1* (*cyr1*Δ) or a triple disruption of *RAS1*,

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