A Novel Suppressor of *ras1* in Fission Yeast, *byr4*, Is a Dosage-dependent Inhibitor of Cytokinesis

Kiwon Song, Kathleen E. Mach, Chih-Ying Chen, Todd Reynolds, and Charles F. Albright

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Abstract. A novel gene, designated *byr4*, was identified in *Schizosaccharomyces pombe* that affects the mitotic cell cycle and shows genetic interactions with the *ras1* signaling pathways. Null alleles of *byr4* cause cell cycle arrest in late mitosis and permit multiple rounds of septation. The multiple septa typically divide two nuclei, but the nuclei frequently do not stain equally with 4′,6-diamidino-2-phenylindole (DAPI), suggesting that *byr4* is required for proper karyokinesis. Overexpression of *byr4* inhibits cytokinesis, but cell cycle progression continues leading to multinucleate cells. When *byr4* is overexpressed, the early steps in the cytokinesis pathway, including formation of the medial F-actin ring, occur normally; however, the later steps in the pathway, including contraction of the F-actin ring, septation, and rearrangement of the medial F-actin following mitosis, rarely occur. *byr4* shows two genetic interactions with *ras1*. The inhibition of cytokinesis by *byr4* overexpression was exacerbated by null alleles of *ras1* and *scd1*, suggesting a link between pathways needed for cell polarity and cytokinesis. Overexpression of *byr4* also partially bypasses the need for *ras1* for sporulation. The electrophoretic mobility of the *byr4* protein varied in response to mutants that perturb cytokinesis and karyokinesis, suggesting interactions between *byr4* and these gene products. A more rapidly migrating *byr4* protein was found in cells with mutations in *cdc16*, which undergo repeated septation, and in *cdc15*, which fail to form a medial F-actin ring in mitosis. A slower migrating *byr4* protein was found in cells with a mutation in the β-tubulin gene, which arrests cells at the metaphase–anaphase transition.

THE ras proteins are GTPases that cycle between an active, GTP-bound form and an inactive, GDP-bound form (Boguski and McCormick, 1993). Depending on the cellular context, activated ras can stimulate the cell division cycle, alter cell shape, or cause cellular differentiation (Bourne et al., 1990). Several pathways are implicated in signaling downstream of mammalian ras proteins. The best characterized pathway activates the raf kinase. Activated ras recruits the raf kinase and activated raf, in turn, activates a mitogen–activated protein (MAP)[1] kinase cascade (Herskowitz, 1995; Marshall, 1995). A second ras effector may be phosphatidylinositol(PI)-3 kinase. Activated ras binds to PI-3 kinase and ras binding stimulates PI-3 kinase activity four-fold in vitro (Rodriguez-Vinciana et al., 1994). An activated allele of PI-3 kinase, however, activates ras and requires ras for signaling, suggesting ras is an effector of PI-3 kinase (Hu et al., 1995). A third ras effector may be ralGDS, a positive regulator of the ral GTPase (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994). Activated ral GTPase binds a protein with GTPase-activating protein (GAP) activity for the cdc42 GTPase, suggesting that this pathway may regulate the actin cytoskeleton using a GTPase cascade (Cantor et al., 1995).

We are studying ras signal transduction pathways in the fission yeast *Schizosaccharomyces pombe* because the accumulated data suggest that these pathways are similar to those in mammals and because this system is amenable to genetics (Gotoh et al., 1993; Hughes et al., 1993; Xu et al., 1994). *S. pombe* are rod-shaped yeast that usually exist as haploids (Gutz et al., 1974). *S. pombe* has a single ras gene, *ras1*, that is necessary for cellular differentiation in response to mating pheromone or nutrients and helps maintain the normal rod shape. *ras1* is not an essential gene, but null alleles of *ras1* eliminate conjugation, greatly reduce sporulation, and change cells to round shaped (Fukui et al., 1986; Nadin-Davis et al., 1986*a*,*b*). *ras1* protein signals through at least two pathways. In one pathway, *ras1* binds to and presumably activates the *byr2* kinase (van Aelst et al., 1993; Masuda et al., 1995). The *byr2* kinase is part of a MAP kinase cascade that includes *byr1* and *spk1* (Nadin-Davis and Nasim, 1988; Toda et al., 1991; Wang et al., 1991). Null alleles of *byr2*, *byr1*, and *spk1* eliminate conjugation and sporulation without affecting cell viability or cell shape. Hence, as in mammals, ras in fission yeast signals through a MAP kinase cascade. In a second path-

Address all correspondence to Charles F. Albright, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146. Tel.: (615) 343-2174; Fax: (615) 343-0704.

1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; MAP, mitogen-activated protein; MM, minimal media; YE, yeast extract.

way, *ras1* binds and presumably activates the *scd1* protein (Chang et al., 1994). *scd1* has sequence similarity to GDP dissociation stimulators for the *cdc42* GTPase. Null alleles of *scd1* eliminate conjugation and change the cell shape to round, but do not affect sporulation rates or cell viability (Chang et al., 1994; Fukui and Yamamoto, 1988). The *ras1-scd1-cdc42* pathway is very similar to the *RSR1-CDC24-CDC42* signaling pathway used to control cell polarity in *Saccharomyces cerevisiae* (Chant, 1994).

To identify new components in the *ras1* signaling pathways, we looked for genes that when overexpressed could suppress the conjugation defects of strains with a *ras1* null allele. Previous attempts to isolate multicopy suppressors of *ras1* null alleles isolated the *byr1*, *byr2*, and *byr3* genes (Nadin-Davis and Nasim, 1988; Wang et al., 1991; Xu et al., 1992). These genes partially bypass the need for *ras1* in sporulation, but do not affect cell shape and only weakly affect the conjugation rates of *ras1*$^-$ strains (Xu et al., 1992). We reasoned that *ras1* might signal through two pathways to stimulate conjugation and we might, therefore, find multicopy suppressors of the *ras1*$^-$ conjugation defect if the MAP kinase cascade was constitutively activated. One gene found in this screen was *byr4*. This study shows that *byr4* plays a critical role in the control of cytokinesis, in addition to showing genetic interactions with *ras1*.

Fission yeast is a good model system to study the control of the eukaryotic cell division and cytokinesis. A number of *S. pombe* cell cycle mutants were identified whose terminal phenotype suggest they participate in the control of septum formation and cytokinesis. The "early septation" genes include *cdc7*, *cdc11*, *cdc14*, and *cdc15* (Fankhauser and Simanis, 1994*b*). Cells with mutations in these genes arrest as highly elongated cells with multiple nuclei, since growth, DNA replication, and mitosis continue in the absence of cytokinesis (Nurse et al., 1976). The "late septation" genes include *cdc3*, *cdc4*, *cdc8*, and *cdc12* (Fankhauser and Simanis, 1994*b*). Cells with mutations in these genes arrest with two to four nuclei and contain disorganized septal material (Nurse et al., 1976). A third class of mutants, defined only by *cdc16* mutations, undergo repeated rounds of septation without cell cycle progression, leading to cells with two nuclei separated by multiple septa (Minet et al., 1979). Genetic studies suggest the gene products of *cdc7*, *cdc11*, *cdc14*, and *cdc16* may interact (Marks et al., 1992). Since the phenotypes resulting from mutations in these genes have similarities to the phenotypes from mutations in the *byr4* gene, *byr4* may be part of this signaling pathway.

Materials and Methods

Strains and Growth Conditions

The strains used in this study are listed in Table I. Yeast were grown in yeast extract (YE) or minimal media (MM) with required supplements at the levels of 75 mg/liter for adenine, uracil, leucine, and 0.4 mM thiamine (Moreno et al., 1991). Derivatives of MM media were used, as indicated, where ammonia was omitted (MM-N), or 10 mM glutamate was substituted for 100 mM ammonia (MM-glu).

CA5 was derived from SP870 by transforming SP870 with a DNA fragment from the HpaI site upstream of the *ras1* coding sequence to the BsmI site at the 3′ end of the *ras1* gene where the DNA sequence coding for amino acids 29 to 155 was replaced with the *ura4* gene (Nadin-Davis et al., 1986*a*). Stable ura$^+$ transformants were isolated and the authenticity of the *ras1* disruption was tested by Southern analysis (Sambrook et al., 1989). CA28 and CA29 were isolated by tetrad dissection from the diploid CA5/SPSU. CA76 was made by transforming SPSU with a linear fragment containing the entire coding sequence of the *byr2* gene, where serines 402 and 404 were changed to alanines (Wang et al., 1991). After overnight growth in 10 ml of YE media supplemented with adenine and uracil, 10^8 cells were plated on 10-cm YE plates with adenine, uracil, and 1 mg/ml 5-fluoroorotic acid (Sigma Chemical Co., St. Louis, MO) (Grimm et al., 1988). Ura$^-$ colonies were screened by Southern analysis to test the authenticity of the gene replacement (Sambrook et al., 1989; Moreno et al., 1991). CA78 and CA115 were made by tetrad dissection of the diploids CA76/CA28 and CA29/SPSCD1U, respectively. CA103 is described below.

Isolation of the byr4 Gene

Strain CA78 was transformed with a *S. pombe* genomic DNA library in pWH5 (P. Young, Queens University, Kingston, Kingston, Ontario, Canada). The transformants were plated on MM-glu plates at a density of about 5,000 colonies per 15-cm plate. The colonies were stained with iodine vapors for 3 min and those with positive staining reaction were streaked onto fresh MM-glu plates (Moreno et al., 1991). Approximately 100,000 transformants were screened. Positive clones were rescreened twice and only clones in which all of the colonies during each rescreen had a positive reaction were retained. The majority of the colonies from the primary screen arise from spontaneous diploids. Diploids containing *byr2-S402/4A* sporulated at high levels in the absence of *ras1*, giving a positive iodine staining reaction. These false positives could be largely eliminated by the repeated screening of the primary isolates. False positives (independent of the plasmid) yielded haploid colonies that were iodine-negative while true positives (dependent on the plasmid) would contain only iodine-positive colonies. As a final screen, plasmids were rescued in *E. coli*, transformed into CA78, and tested again.

Molecular Analysis of byr4

The genomic DNA insert contained in the plasmid isolated from the library, pS17, was subcloned using standard techniques (Sambrook et al., 1989). A deletion of a 2.7-kb PstI fragment yielding pS17ΔPst retained full activity and was used as a starting point for subsequent work. A vector containing a 2-kb fragment starting at the remaining BamHI site retained partial suppressor activity. This fragment and 1 kb of adjacent DNA were sequenced revealing a 1995 base open reading frame that spanned the BamHI site. Database searches were done using the BLAST program (Altschul et al., 1990).

A *S. pombe* cDNA library (J. Fikes, Massachusetts Institute of Technology, Cambridge, MA) was screened with a 2116-bp fragment (derived by PCR amplification) that encoded the entire open reading frame of the predicted *byr4* protein (Becker et al., 1991). A partial cDNA clone was isolated and the ends of this clone were sequenced to establish the exact location of the cDNA relative to the genomic sequence. A series of PCR reactions was performed using the cDNA and genomic DNA as templates. The size of these PCR products revealed no difference in size between the cDNA and genomic products, suggesting that there are no introns in this region of the *byr4* gene.

To detect the *byr4* mRNA in *S. pombe*, cells from SP870 strain were grown in MM media to a density of 5×10^6 cells/ml and harvested or shifted into MM-N for 6 h before harvesting (Watanabe and Yamamoto, 1994). 15 μg of total RNA prepared from these cells was electrophoresed in a 0.8% agarose gel containing 7.4% formaldehyde (EM Science, Gibbstown, NJ) and transferred to a nylon membrane (Hybond; Amersham Corp., Arlington Heights, IL) (Sambrook et al., 1989). This Northern blot was probed with the same fragment used to screen the cDNA library.

Assay for Bypass of ras1 in Sporulation

Plasmids were transformed into CA5/CA7, a *ras1*$^-$ diploid, and transformants were streaked on MM-glu plates (Moreno et al., 1991). After 4–5 d of growth at 29°C, single colonies were pooled in 300 μl of water and the fraction of cells that sporulated was determined by counting. At least 1,500 cells were counted for each diploid using independent transformants and samples from different days. Little variation between transformants or days was observed.

Table I. S. pombe Strains Used in This Study

Strain	Genotype	Source
SP870	*h^{90} leu1-32 ura4-d18 ade6-210*	M. Wigler
SPSU	*h^{90} leu1-32 ura4-d18 ade6-210 byr2::ura4*	M. Wigler
SPSCD1U	*h^{90} leu1-32 ura4-d18 ade6-210 scd1::ura4*	M. Wigler
CA5	*h^{90} leu1-32 ura4-d18 ade6-216 ras1::ura4*	This study
CA7	*h^{90} leu1-32 ura4-d18 ade6-210 ras1::ura4*	This study
CA21	*h^{90} leu1-32 ura4-d18 ade6-216*	This study
CA28	*h^{90} leu1-32 ura4-d18 ade6-216 byr2::ura4 ras1::ura4*	This study
CA29	*h^{90} leu1-32 ura4-d18 ade6-216 byr2::ura4*	This study
CA76	*h^{90} leu1-32 ura4-d18 ade6-210 byr2-S402/4A*	This study
CA78	*h^{90} leu1-32 ura4-d18 ade6-216 byr2-S402/4A ras1::ura4*	This study
CA103	*h^{90} leu1-32 ura4-d18 ade6-210/ h^{90} leu1-32 ura4-d18 ade6-216 byr4::ura4*	This study
KGY444	*h^{+} leu1-32 ura4-d18 ade6-210 cdc25-22*	K. Gould
CA91	*h^{-} ura4-d18 ade6-216 nda3-KM311*	This study
CA117	*h^{-} leu1-32 ade6-210 cdc16-116*	This study
CA110	*h^{90} leu1-32 ura4-d18 ade6-216 scd1::ura4*	This study
KGY247	*h^{+} leu1-32 ura4-d18 ade6-210*	K. Gould
CA115	*h^{90} leu1-32 ura4-d18 ade6-216 byr2::ura4 scd1::ura4*	This study
KGY433	*h^{-} cdc3-124 leu1-32*	K. Gould
KGY439	*h^{-} cdc4-8 ura4-d18 leu1-32*	K. Gould
MBY105	*h^{+} cdc7-24 ade6-210 ura4-d18*	M. Balasubramanian
MBY106	*h^{+} cdc11-123 ade6-210*	M. Balasubramanian
MBY97	*h^{+} cdc14-118 ade6-210 leu1-32 ura4-d18*	M. Balasubramanian
MBY107	*h^{+} cdc15-136 ade6-210 leu1-32*	M. Balasubramanian
KGY657	*h^{+} cdc8-110 leu1-32 ura4-d18*	K. Gould
MBY81	*cdc12-112 mei1-102 lys1-131*	M. Balasubramanian

Construction of byr4 Null Allele

To construct a null allele of *byr4*, a BclI site was added at the stop codon of pS17ΔPst by PCR mutagenesis. The coding sequence and 426 bp of 5′ untranslated region were then removed by deleting the 2.4-kb BclI fragment. The resulting 2.4-kb HindIII fragment from this vector was cloned into pBSK^{+} (Stratagene, La Jolla, CA) and the *ura4* gene containing BamHI ends was inserted into the BclI site (Grimm et al., 1988; Lees-Miller et al., 1992). The 4.1-kb HindIII to SmaI fragment from this vector, where the entire coding region of *byr4* was replaced with the *ura4* gene, was transformed into the diploid strain CA21/SP870. Stable ura^{+} colonies were isolated and screened by Southern blot analysis to identify those strains in which one copy of the *byr4* gene was disrupted. One such transformant was designated as CA103. Tetrads from CA103 were dissected to determine the effect of deletion of *byr4*.

For germination of spores in liquid culture, CA103 was grown to mid-exponential phase in YE and then transferred to MM sporulation media (MM-glu with 1% glucose) (Fankhauser et al., 1993; Hagan and Hyams, 1988). After growth for 48 h in MM sporulation media, vegetative cells were lysed by treatment with 0.5% (vol/vol) glusulase (DuPont, Wilmington, DE) for 24 h at 29°C. The isolated spores were incubated at 3×10^6 cells/ml in MM supplemented with adenine and leucine but without uracil, so that only those spores which carry the *byr4::ura4* allele could germinate. After incubation for 24 h at 29°C, cells were fixed and observed microscopically as described below.

To isolate haploids containing the *byr4::ura4* allele, the CA103 diploid strain was transformed with pbyr4/REP41 or pbyr4-409/REP41 and transformants were selected and maintained in MM media with thiamine (Basi et al., 1993). The resulting strain was allowed to sporulate and purified spores were prepared as described before (Moreno et al., 1991). The resulting spore preparation was germinated on plates containing adenine and thiamine but without uracil and leucine. Under these conditions, only haploids containing both the *byr4::ura4* allele and pbyr4/REP41 plasmid can germinate and grow. To determine the effect of the *byr4* null allele, this strain was grown in MM media with leucine, adenine, and thiamine for 16 h. The growth with leucine supplementation allows cells to lose the complementing plasmid and dilute the endogenous *byr4* protein, resulting in some fraction of the cells exhibiting the null phenotype.

FACS Analysis

For flow cytometry, samples were prepared as previously described (Paulovich and Hartwell, 1995). Briefly, cells were fixed in 70% ethanol for 12 to 24 h at 4°C and washed with 50 mM sodium citrate (pH 7.5). 4×10^6 cells were suspended in 0.5 ml of 50 mM sodium citrate, incubated with 250 μg/ml RNase A for 1 h at 50°C, and then incubated with 1 mg/ml proteinase K for 1 h at 50°C. After adding 0.5 ml of 50 mM sodium citrate containing 8 μg/ml propidium iodide, samples were incubated in the dark for 12 to 24 h at 4°C and analyzed with a Becton Dickinson fluorescence-activated cell analyzer. For each sample, 20,000 cells were analyzed.

Overexpression of byr4 Protein in E. coli and Western Analysis

To construct an *E. coli* expression vector for the *byr4* protein, a three-way ligation was performed. The vector pET14b (Novagen, Madison, WI) was digested with NdeI and BamHI. The 5′ end of the *byr4* gene was isolated from a PCR reaction that amplified the region of the *byr4* gene from the start codon to the SpeI site (+90). An NdeI site was added at the start codon by modifying the oligonucleotide used for the PCR reaction. The 3′ end of the gene was isolated as an SpeI to BclI fragment. The BclI site was created by attaching oligonucleotide linkers at the SmaI site in pS17ΔPst. The resulting vector, pbyr4/ET14b, contained the entire open reading frame of *byr4* with an NH_2 terminal His tag.

For production of *byr4* antibodies, *E. coli* strain BL21(DE3)pLysS (Novagen) containing pbyr4/ET14b was grown at 37°C to a density of 0.6, induced with 0.5 mM Isopropylthio-β-D-galactoside for 3 h, and harvested. Inclusion bodies were purified and separated by SDS polyacrylamide gel electrophoresis. The *byr4* protein was visualized by incubating the gel in 0.25 M KCl, 1 mM DTT and excised. The gel fragment was emulsified in Freund's adjuvant, and rabbits were injected at monthly intervals with ~100 μg protein per injection (Harlow and Lane, 1988).

Antisera were purified by affinity chromatography. *byr4* protein from the soluble fraction of the bacterial lysate were purified by incubation with Ni-agarose (Qiagen, Chatsworth, CA) for 30 min on ice. The beads were washed three times with 60 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl (pH 7.9) and five times with crosslinking buffer (20 mM Hepes, 5% glycerol, 0.03% Brij-35, 100 mM NaCl [pH 7.4]), and eluted three times with 1 ml of crosslinking buffer with 100 mM EDTA. The material purified from a 500 ml bacterial culture was coupled to 1 ml of 6-aminohexanoic acid *N*-hydroxysuccinimide ester Sepharose 4B (Sigma Chemical Co., St. Louis, MO) at 4°C for 16 h. The coupled beads were washed and used to affinity purify an equal volume of serum using standard techniques (Harlow and Lane, 1988).

For Western analysis, approximately 2.5×10^8 cells from mid-log growth were harvested by centrifugation, washed twice with PBS, 50 mM NaF, 1 mM NaN_3, and used immediately or frozen at −80°C. Cells were resuspended in 125 μl of HB buffer (25 mM Hepes, 60 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EGTA, 0.1 mM Na vanadate, 1 mM pefabloc (Boehringer Mannheim Corp., Indianapolis, IN), 20 μg/ml leupeptin, 40 μg/ml aprotinin, 1 mM DTT (pH 7.4) supplemented with 0.2% Triton X-100 and a protease inhibitor cocktail (0.1 μg/ml chymostatin, 1 μg/ml pepstatin A, 1.1 μg/ml phosphoramidon, 7.2 μg/ml E-64, 2.5 μg/ml antipain, 100 μM benzamidine, and 100 μM sodium metabisulfite). 1 ml of cold glass beads (Sigma Chemical Co.) were added, and the cells were lysed by mixing twice for 20 s medium speed with a beadbeater (Biospec Products, Inc., Bartlesville, OK). 0.4 ml of HB/Triton buffer was added to the beads, the buffer was removed, and the resulting extract was clarified by centrifugation for 15 min at 350,000 *g* to yield a high-speed supernatant and high-speed pellet. Protein concentrations of the high-speed supernatant were measured by the dye-binding assay (Pierce, Rockford, IL) and these samples were adjusted to contain equivalent protein concentrations. The high-speed pellet fractions were adjusted by the same dilution factor. Approximately 100 μg protein of high-speed supernatant or an equivalent cellular amount of high-speed pellet fraction were separated by SDS polyacrylamide electrophoresis and transferred to nitrocellulose membranes. *byr4* protein was detected by Western blotting using purified serum at a dilution of 1:2000, relative to the starting serum. Bound antibodies were detected with a 1:10,000 dilution of anti-rabbit IgG-HRP (Jackson Immunochemicals, West Grove, PA) and enhanced chemiluminescence reagents.

Overexpression of byr4 in S. pombe

A *S. pombe* expression vector, pbyr4/REP41, was constructed by ligating the NdeI to EcoRV fragment from pbyr4/ET14b into pREP41 that was digested with NdeI and SmaI (Basi et al., 1993; Maundrell, 1993). pbyr4/REP41 was introduced into various strains to overexpress the *byr4* protein. An NH_2-terminal truncation of the *byr4* protein was generated by site-directed mutagenesis. Using pbyr4R/REP41, the sequence coding the NH_2-terminal 199 amino acids was removed, leaving the methionine at codon 200 as the new initiator methionine. The resulting plasmid is designated pbyr4-409/REP41. After pbyr4/REP41 transformation, strains were maintained on MM-glu plates with thiamine to repress *byr4* protein expression. MM-glu plates were used since these cells accumulate red pigments when ammonium chloride is used as a nitrogen source.

To characterize the phenotype resulting from *byr4* overexpression in asynchronous cultures, these transformants were first grown to mid-log phase in MM with thiamine, washed twice in thiamine-free media, and incubated for 16–24 h in thiamine-free media at 29°C. Aliquots of the culture were taken for cytological analysis.

To examine the effect of *byr4* overexpression in cell-cycle synchronized cultures, pbyr4/REP41 or pREP41, as a control, were transformed into KGY444, a strain containing *cdc25-22* temperature-sensitive allele (Moreno et al., 1989). The resulting strains were grown to mid-log phase in MM with thiamine at 18°C, washed twice in thiamine-free media, and grown for 24–33 h in thiamine-free media at a permissive temperature, 18°C. The culture was shifted to the restrictive temperature, 35°C, for 4 h to impose the *cdc25-22* block and then shifted back to the permissive temperature, 23°C, to allow synchronized resumption of the cell cycle. This protocol was empirically determined to result in the *byr4* overexpression phenotype appearing during the next mitotic division after release from the *cdc25-22* block. Aliquots of cells were taken every 30 min for 6 h and processed for cytological and protein analyses as described below. Cell concentrations were kept below 5×10^6 cells/ml. The time for the initial mitosis to occur varied from experiment to experiment and was significantly longer than reported by previous investigators. This variation resulted from the use of minimal media instead of rich media (data not shown).

Microscopic Techniques

For 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.), calcofluor (fluorescent brightner No. 28, Sigma Chemical Co.), and phalloidin staining, cells were fixed by adding 1/4 volume of a freshly prepared 17% (wt/vol) paraformaldehyde to an exponentially growing culture (Moreno et al., 1991). To visualize DNA, cells were stained with 1 μg/ml DAPI (Moreno et al., 1991). To simultaneously visualize DNA and septa, cells were stained with 20 μg/ml DAPI and 2 mg/ml calcofluor (Toda et al., 1981). To visualize F-actin, cells were permeabilized by washing with 1% NP40 in PBS and stained with 5 μg/ml rhodamine-phalloidin (Sigma Chemical Co.). For immunostaining, cells were fixed by adding 17% paraformaldehyde and 70% glutaradehyde in PBS to final concentrations 3.4% (wt/vol) and 0.25%, respectively. Fixed cells were permeabilized by treatment with lysing enzyme (L-2265, Sigma Chemical Co.) and Zymolyase 20T (ICN Biomedicals, Inc., Costa Mesa, CA) followed by washing with 1% NP40 in PBS. Microtubules were stained by anti-α-tubulin rat monoclonal antibody YOL1/34 (Accurate Chemical & Science Corp., Westbury, NY) followed by Texas-red conjugated anti-rat goat IgG (Molecular Probes, Inc., Eugene, OR). Fluorescence microscopy was performed using Zeiss Axioskop and Axiovert with 100× objective. Photographs were taken using Tmax 400 (Kodak, Rochester, NY) and TriX-pan 400 (Kodak).

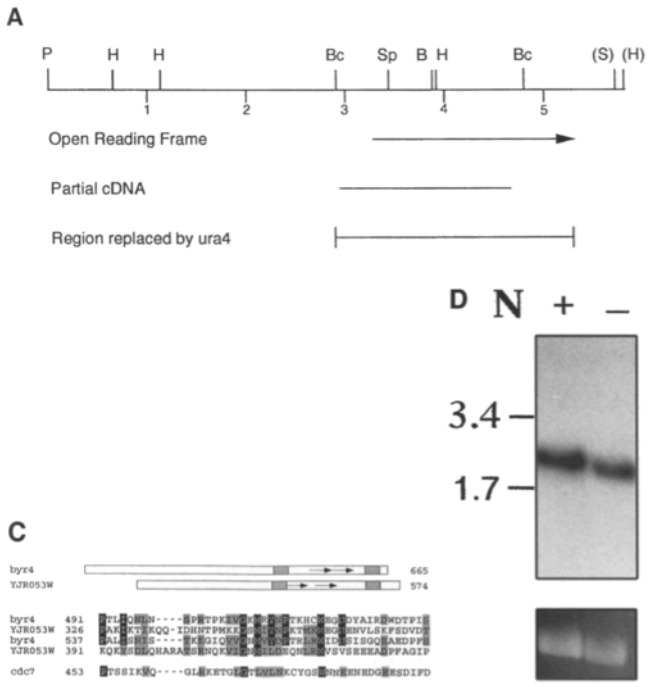
Results

byr4 Encodes a Novel Protein that Partially Bypasses ras1⁻ Sporulation Defects

To isolate new components of *ras1* signaling pathways, we screened for multicopy suppressors of the *ras1⁻* conjugation defect in a strain, CA78, that contains an activated allele of *byr2*. (The isolation and characterization of this *byr2* allele will be described elsewhere.) We screened yeast transformed with a genomic DNA library for colonies that stained black with iodine vapors, a characteristic of conjugating or sporulating strains. A single novel gene was isolated on a plasmid designated pS17. Microscopic examination revealed that the iodine staining was due to the sporulation of diploids. We found that diploids accumulated whenever the pS17 plasmid was introduced into CA78 haploid cells. These diploids probably arise from the inhibition of cytokinesis and not from conjugation since they accumulate even in media with plentiful nitrogen and since *byr4* overexpression inhibits cytokinesis (see later results). Since strain CA78 contains an activated allele of *byr2*, these diploids sporulated at a high rate to yield a black iodine staining reaction.

Several deletion mutations in the genomic insert of pS17 were made and tested for their ability to induce sporulation in strain CA78. These experiments suggested that the suppressor activity mapped near the end of the genomic insert. This region was sequenced and found to contain an open reading frame of 1995 bases, yielding a predicted protein of 665 amino acids with a predicted mass of 75.7 kD (Fig. 1 *B*). This gene is designated *byr4*, for bypass of yeast ras. Sequence from the other end of the genomic insert was identical to the *atp2* gene that was mapped to chromosome I, showing that *byr4* resides on chromsome I (Vassoroti et al., 1984).

A comparison of the protein sequence to itself revealed an imperfect direct repeat of 43 amino acids (Fig. 1 *B*). A search of the protein sequence database revealed only one potentially meaningful similarity to a novel gene from *S. cerevisiae*, designated *YJR053W*, that was identified as a result of the genome sequencing effort. The region of similarity between *byr4* and *YJR053W* was confined to the previously identified repeats in *byr4* and sequences surrounding these repeats (Fig. 1 *C*). An alignment of these repeats revealed a core region of 12 amino acids that was most similar in these repeats. When this core region of 12 amino acids was used to search the database, no exact matches were found. Among these sequences that most closely resembled this consensus sequence was a region of



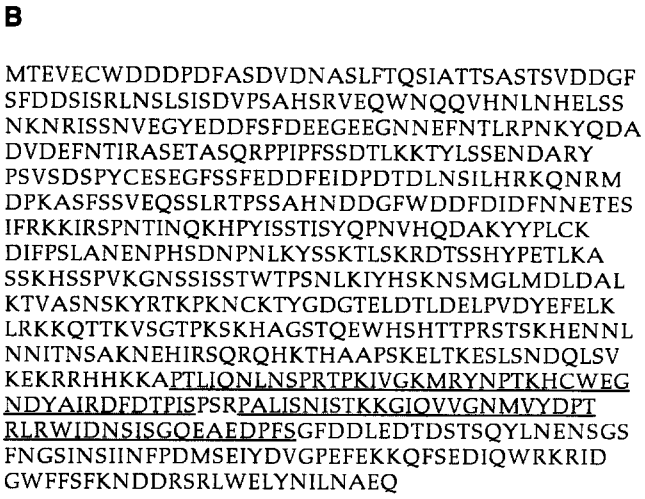


Figure 1. Molecular analysis of the *byr4* gene. (*A*) Restriction site map of *byr4* region. The upper line shows the *S. pombe* genomic region in pS17ΔPst. The numbers below this line correspond to the length in kb. The letters designate restriction sites, including PstI (*P*), HindIII (*H*), BclI (*Bc*), SpeI (*Sp*), BamHI (*B*), and SmaI (*S*). The SmaI and HindIII sites on the right end are part of the pWH5 vector. The location of the open reading frame, partial cDNA, and region deleted to construct the *byr4* null allele are indicated below the restriction map. (*B*) Predicted protein sequence. The two imperfect direct repeats are underlined. These repeats were found and judged statistically significant using the MACAW program (Schuler et al., 1991). These sequence data are available from Genbank/EMBL/DDBJ under accession number U59224. (*C*) Alignment of *byr4* and *YJR053W* sequences. A cartoon depicting the regions of similarity, as identified by the BLAST algorithm, is shown above. The arrows represent the direct repeats and the shaded areas represent other regions of significant sequence similarity. The protein sequence of the direct repeats, as aligned by the CLUSTAL algorithm, are shown below. Identical amino acids found in at least three sequences are shown in black and similar amino acids found in at least three repeats are shaded. Note that several positions are more conserved between the first repeat of *byr4* and *YJR053W* or the second repeat of *byr4* and *YJR053W* than between the two repeats of *byr4* or *YJR053W*. The similar region of *cdc7*, identified by the BLOCKS algorithm, is shown on the last line [Henikoff et al., 1995]. (*D*) Northern blot analysis of *byr4*. Total RNA from actively growing (+N) or nitrogen starved (−N) cells were separated and probed with a fragment of the *byr4* gene. A single mRNA of 2.5 kb was detected in both samples. Approximately equal amounts of RNA samples were loaded in each lane as illustrated by ethidium bromide staining of 23S rRNA (lower). A duplicate filter was probed with a fragment of the *mei2* gene to confirm that the nitrogen-starved culture had accumulated mRNAs that were known to be expressed in response to nitrogen starvation (data not shown).

the *cdc7* kinase of *S. pombe* (Fig. 1 *C*). The potential significance of this sequence similarity is under investigation.

Northern analysis showed a single mRNA of approximately 2.5 kb that was present at the same levels in actively growing and in nitrogen-starved cells (Fig. 1 *D*). A partial cDNA was isolated that begins 362 bases upstream of the presumed initiator methionine. A comparison of this cDNA with genomic sequences revealed no introns in the region encoding the NH_2-terminal 447 amino acids. The location of the partial cDNA, the size of the mRNA, and the lack of apparent splicing signals are consistent with the predicted gene structure (Fig. 1 *A*).

To test if *byr4* could bypass the need for *ras1* in sporulation, we introduced a *byr4* containing plasmid, pS17ΔPst, into a *ras1*$^-$ diploid and measured the sporulation rate. pS17ΔPst increased the sporulation rate of these cells from 0.7% to 7.7%, representing an 11-fold stimulation. A strain containing the *ras1* gene sporulated at about 40% in this assay. The predicted open reading frame was placed downstream of the attenuated *nmt1* promoter in pREP41 to create pbyr4/REP41. This plasmid caused a threefold stimulation of the sporulation rate of *ras1*$^-$ diploids. This effect was observed with thiamine in the media, which inhibits expression of the *nmt1* promoter. Removing thiamine from the media to generate higher levels of expression led to growth defects that interfered with the sporulation assay. Therefore, both genomic and expression plasmids containing *byr4* partially rescue the sporulation defects of diploid cells without *ras1*.

byr4 Null Alleles Cause Cell Cycle Arrest with Multiple Septa and Abnormal Nuclei

A null allele of *byr4* was constructed by replacing one allele of *byr4* with the *ura4* gene in a diploid strain (Fig. 1 *A*). When tetrads from this heterozygous diploid, CA103, were dissected, only two colonies per tetrad were viable. The viable colonies were ura$^-$ suggesting *byr4* encodes an essential gene. To ensure that the lethal phenotype was due to loss of the *byr4* protein, we transformed CA103 with pbyr4/REP41 and analyzed random spores. It was now possible to isolate ura$^+$ haploids since the *byr4::ura4* allele was complemented by *byr4* on the plasmid. The null allele was also complemented by a mutant of *byr4* that contained only the COOH-terminal 466 amino acids (data not shown).

To better characterize the phenotype of the *byr4* null allele, spores containing the *byr4* null allele were germinated and examined. The cells resulting from the germinated spores arrested with multiple septa (Fig. 2, *A–C*). The septa were visible by phase-contrast microscopy or following staining with calcofluor (Fig. 2 *B*). These cells contained either one nucleus, two nuclei of unequal size, or two nuclei of equal size as visualized by DAPI staining (Fig. 2 *A* and Table II). In cells with a single nucleus, the nucleus was either in one end of the cell with multiple septa or was divided by a septum. In cells with two nuclei, nuclei were usually found at the ends of the cell and not in the intermediate compartments defined by the multiple septa (Fig. 2 *A* and Table II). When the nuclei were of unequal DAPI staining, the brightest nucleus was typically larger than a nucleus from a normal cell or a cell with equal DAPI staining. This suggests DNA replication occurred in cells with unequal DAPI staining and that the unequal DAPI staining was not due to the separation of single chromatids. As a further test, the DNA content of these cells was determined by FACS analysis (Fig. 3). This analysis revealed the cells contained a 2N amount of DNA, suggesting that the abnormal nuclei in the arrested cells were not due to anaphase occurring with single chromatids. An explanation of the unequal DAPI staining will require additional experiments.

The location of F-actin in these cells was demonstrated by staining with rhodamine-conjugated phalloidin (Fig. 2 *C*). In normal cells, F-actin is found as dots at the ends of the cells during most of interphase, where new growth occurs. During mitosis, these F-actin dots disappear and a F-actin ring forms around the middle of the cell where the septum will form. This ring forms part of the structure that contracts to divide the cytoplasm during cytokinesis (Marks and Hyams, 1985). In cells with a null allele of *byr4*, F-actin is concentrated around the multiple septa (Fig. 2 *C*). Some F-actin dots were most intense at the septum closest to the cell end, which is presumably the septum most recently formed (Fig. 2 *C*). At this septum, typical F-actin structures were observed, including medial rings and contractile rings (Fig. 2 *C* and data not shown). A medial F-actin ring without a septum was also observed in some cells that contained one or more septa, suggesting that this medial F-actin ring precedes the formation of the septum as in a normal cell cycle (Fig. 2 *C*). Part of the cell was frequently dark-field and did not contain any F-actin, suggesting this region of the divided cell was dead (Fig. 2 *C* and data not shown). The multiple septa appeared fully formed because digestion of these cells with small amounts of lysing enzyme liberated the individual compartments defined by the septa (data not shown). These results suggest *byr4* function is needed to limit the cell to a single septum per cell cycle.

The phenotype of the *byr4* null allele was also examined in actively growing cells with a plasmid loss experiment. Haploid cells with the *byr4::ura4* allele are propagated by complementing the null allele with the *byr4* gene on a plasmid. When these cells are grown in media that are not selective for the plasmid, some cells lose the plasmid through random segregation, deplete their supply of *byr4* protein, and express the null phenotype. Using this procedure, approximately 1/8 of the culture arrested with multiple septa (Fig. 2 *D*). When these cells were stained with DAPI to reveal the DNA, we again observed that the nuclei were at the ends of the cell and the relative DNA staining of these nuclei was frequently unequal (Table II). Cells with a single nucleus, as observed by DAPI staining, were seen in a fraction of the cells with multiple septa, and in some of these cells, the nucleus was divided by a septum (Fig. 2 *D* and Table II).

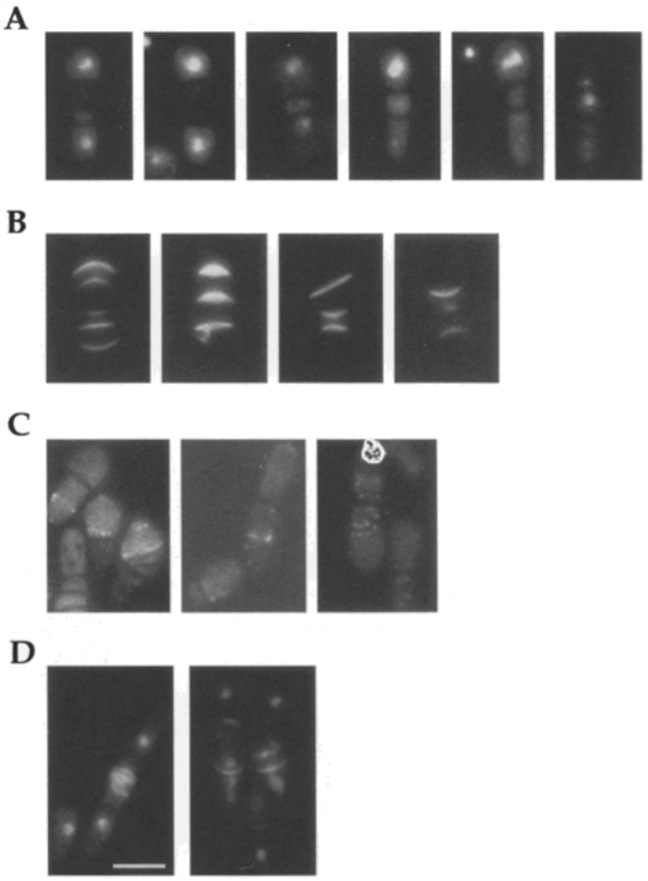


Figure 2. Phenotype of cells with the *byr4* null allele. (*A–C*) Phenotypes of germinated spores with *byr4* null allele. Spores containing the *byr4::ura4* allele were germinated, fixed, and stained with DAPI (*A*), calcofluor (*B*), or rhodamine-phalloidin (*C*). In *A*, representative cells containing two nuclei of equal DAPI staining (left two), two nuclei of unequal DAPI staining (middle two), one nucleus (second from right), or one nucleus divided by a septum (right) are shown. In *B*, representative cells containing multiple septa are shown. In *C*, F-actin is seen adjacent to septa or at the approximate medial location of the remaining cell. (*D*) Phenotype of vegetative cells with *byr4* null allele created by plasmid loss. Actively growing cells, in which the *byr4::ura4* allele was complemented by pbyr4/REP41, were allowed to lose the plasmid resulting in expression of the null phenotype. The resulting mixed cell population was fixed and stained with both DAPI and calcofluor. The left panel shows a cell arrested with multiple septa between two nuclei of equal DAPI staining and the right panel shows cells with multiple septa where one or more divide the nucleus. Bar, 5 μm.

The size of the cells without *byr4* were typical of cells in mitosis and the cell size did not increase with prolonged incubation, suggesting that these cells were not entering the next cell cycle. The FACS analysis was consistent with

Table II. Number and Relative DNA Staining of Nuclei in Cells without byr4

Nuclei/cell	Relative DNA staining of nuclei	Fraction of cells*	Fraction of cells‡
		%	%
One	–	15	10
Two	Unequal	52	34
Two	Equal	24	41
Divided§	–	9	15

*Spores from strain CA103, a strain heterozygous for the *byr4* null allele, were germinated for 16 h at 29°C under conditions where only cells containing the null allele will germinate. During this period, 52% of the 1035 spores examined had germinated. The resulting cells were stained with DAPI and the number and relative size of the nuclei were determined by microscopy.

‡Haploid cells, containing the *byr4* null allele complemented by pbyr4/REP41, were grown in medium that was not selective for the plasmid. Multiseptate cells, that presumably lost the plasmid through random segregation, were identified and their relative DNA staining was determined following DAPI staining. More than 500 multiseptate cells were scored.

§Cells were designated divided if the DAPI staining material was divided by a septum.

this hypothesis since few cells with 4N DNA content were observed (Fig. 3). As an additional test, we examined the conjugation rates of these cells during the plasmid loss experiment since cells arrested in G1 before start should be capable of conjugation and sporulation (Nurse and Bissett, 1981). The abnormal cells, as identified by those with multiple septa, were never observed to form zygotes or spores (data not shown). These observations together suggest that the cells without *byr4* were arrested in late mitosis and did not enter the G1 phase of the next cell cycle.

byr4 Overexpression Induces Multinucleate and Branched Cells

To test the consequence of *byr4* overexpression, we introduced pbyr4/REP41 into wild-type cells and induced *byr4* expression by removing thiamine from the media. Overexpression of *byr4* led to decreased colony size and elongated cells. Some of the elongated cells became branched or developed protrusions that we will refer to as bumps (Fig. 4). The elongated cells contained multiple nuclei, typically in close proximity to each other, but did not contain septa that were visible by phase-contrast microscopy. In some cases, weak calcofluor staining existed where septa would normally be found (data not shown). Elongated cells with two or four nuclei appeared within 16 h of thiamine removal. As the time of induction increased, the cells became further elongated, developed bumps and branches, and eventually lysed.

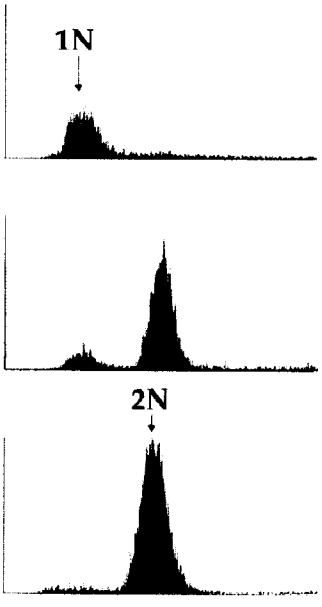


Figure 3. Analysis of DNA content in cells from germinated spores with a *byr4* null allele. Spores and germinated cells were washed, fixed with ethanol, washed several times with buffer, stained with propidium iodide, and analyzed by FACS. The upper panel shows the DNA content of the spore population before germination. Approximately 74% of this spore mixture has 1N DNA content. The middle panel shows the FACS analysis of cells after germination. Approximately 77% of the cells have 2N DNA content. When these samples were examined using phase-contrast microscopy, 79% of the cells were germinated spores, a value in reasonable agreement with the fraction of cells in the 2N peak. The ungerminated spores are less than 50% of the cells in this experiment because they were preferentially lost during the washing steps. The lower panel shows a control sample with 2N DNA content.

The abnormal morphology prompted us to analyze the distribution of microtubules and F-actin in cells overexpressing *byr4*. The location of microtubules was observed by indirect immunofluorescence using an anti-α-tubulin antibody. In normal cells, microtubules span the length of the cell during interphase. These cytoplasmic microtubules are dismantled during mitosis and are replaced by the mitotic spindle (Hagan and Hyams, 1988). When we visualized the microtubules in cells overexpressing *byr4*, some cells contained interphase arrays of microtubules and some cells contained mitotic arrays of microtubules (Fig. 4 *A*). When cells contained more than one mitotic spindle, we observed that a single spindle was connected to two nuclei. In these cells, each nucleus was only connected to a single spindle. These observations suggest *byr4* overexpression does not alter the normal distribution of microtubules or their dependence on the cell cycle. In addition, these phenotypes also suggest that growth, DNA replication, and mitosis continue in the absence of cytokinesis.

When F-actin was visualized in cells that overexpressed *byr4*, two F-actin staining patterns were observed (Fig. 4 *B*). Some cells contained F-actin at the ends and some cells contained F-actin at both the ends and the middle of the cell. Cells with F-actin dots at both the ends and a medial F-actin ring are not observed in a normal population of cells and result from *byr4* overexpression. We also found F-actin concentrated in the bumps of these cells.

byr4 Overexpression Inhibits Cytokinesis

Examining the phenotypes resulting from *byr4* overexpression in asynchronous cultures revealed several unusual features. To more accurately determine which stage of the cell cycle is blocked by *byr4* overexpression, *byr4* was overexpressed in cells that were synchronized with respect to the cell cycle using the *cdc25-22* allele that reversibly blocks cells at the G2/M border at restrictive temperature (Booher et al., 1989; Moreno et al., 1989). We introduced the pbyr4/REP41 into KGY444, a strain containing *cdc25-22*, induced *byr4* expression in thiamine-free media at permissive temperature, shifted the cells to restrictive temperature to impose the cell cycle block, and returned the cells to permissive temperature for sample collection of cycling cells. These conditions were empirically determined to result in the *byr4* overexpression phenotype appearing during the next cell cycle after release from the *cdc25-22* block.

At time zero, cells from both control and *byr4* overexpressing cultures contained a single nucleus, and F-actin

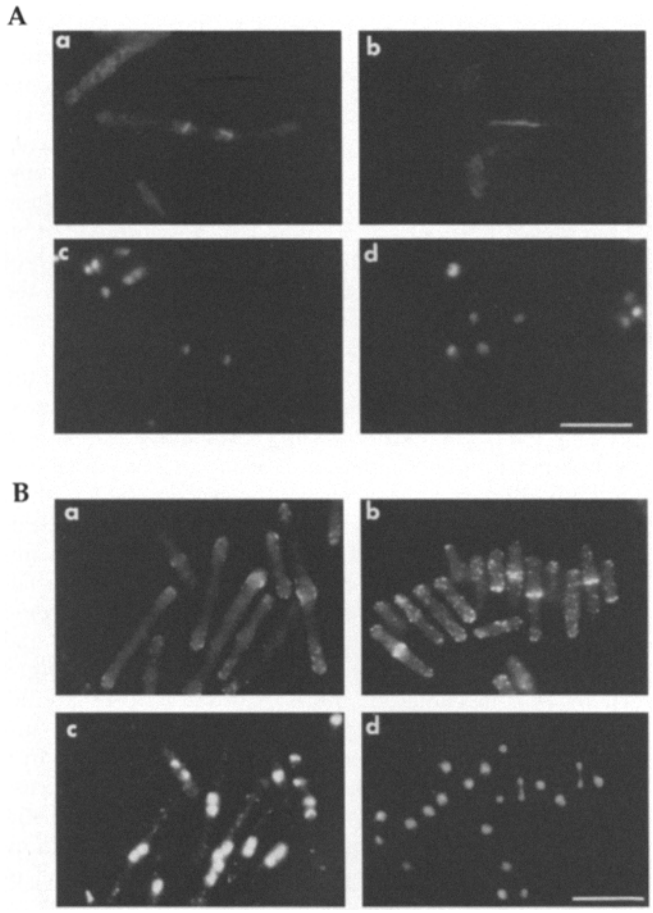


Figure 4. Localization of microtubules and F-actin in cells overexpressing *byr4*. (*A*) Localization of microtubules. Wild-type strain SP870 containing pbyr4/REP41 or pREP41 was incubated in thiamine-free media for 16 h at 29°C. Cells were then fixed and stained with DAPI (*c* and *d*) and anti-α-tubulin antibody (*a* and *b*). The left panels (*a* and *c*) show cells overexpressing *byr4* from pbyr4/REP41 and the right panels show cells with only pREP41 (*b* and *d*). The *byr4* overexpressing cells become elongated and contain two nuclei in close proximity to each other (*c*). Both nuclei contain intra-nuclear microtubules suggesting this cell is in metaphase (*a*). The control cells have a typical cell length and have either one or two nuclei per cell (*d*). When two nuclei are present, they are almost always connected by a mitotic spindle suggesting they are in mitosis (*b*). Bar, 10 μm. (*B*) Localization of F-actin. Cells, prepared as above, were stained with rhodamine-conjugated phalloidin (*a* and *b*) and DAPI (*c* and *d*). Cells expressing *byr4* were elongated and multinucleate (*c*) and frequently developed bumps or branches. F-actin was concentrated in the bumped regions (*a*) as well as the ends of the cell. In control cells, F-actin was found at the ends of the cell during interphase or as a ring around the middle of the cell during mitosis (*b*). Bar, 10 μm.

was localized to the cell ends, consistent with arrest at the G2/M border (Fig. 5). By 150 min, cells from both cultures had two nuclei and medial F-actin, which is consistent with entry into anaphase. The peak of septation occurred at 240 min in the control culture, indicating these cells were completing cytokinesis and entering the next cell cycle. Few septa or divided cells were found in the cells that overexpressed *byr4*, even when these cells were incubated for another cell generation time. Consistent with this observation, F-actin in a contractile ring configuration was rarely observed in these cells, though medial F-actin was present. Instead, at 300 min cells overexpressing *byr4* frequently formed bumps (80%) and lysed (40%). At this point, the medial F-actin ring was replaced with medial F-actin dots and F-actin dots at the cell ends. The location of the bumps coincided with the location of the medial F-actin. By 360 min, the nuclei in the *byr4* overexpressing cells were closer together than at 300 min, suggesting these nuclei migrated back together after anaphase B. The control cells and cells overexpressing *byr4* start a second mitosis at 360 min. In separate experiments, control cells completed a second mitosis while cells overexpressing *byr4* typically accumulated four nuclei per cell but rarely formed septa or underwent cell division (data not shown). These results suggest that *byr4* overexpression inhibits cytokinesis, leading to the formation of multinucleate cells. F-actin localizes to the presumptive site of septation during mitosis, but this F-actin rarely forms a contractile ring or relocalizes following mitosis.

The Electrophoretic Mobility and Localization of the byr4 Protein Vary Due to Mutations that Affect Cytokinesis

To better understand the mechanism of *byr4* action, polyclonal antibodies to the *byr4* protein were generated and used to characterize the *byr4* protein. Rabbits were immunized with recombinant *byr4* protein produced in *E. coli*. The resulting serum was purified by affinity chromatography and used in Western analysis with lysates of *S. pombe* (Fig. 6 *A*). Serum from the immunized rabbit recognized a protein of 97 kD apparent molecular weight in lysates from wild-type yeast (Fig. 6 *A*, lane *2*). This apparent size is significantly larger than the calculated mass of 75.7 kD. This protein was absent from Western blots probed with preimmune serum from this rabbit (Fig. 6 *A*, lane *6*). A protein of the same apparent molecular weight was found in a strain where a null allele of *byr4* was complemented by pbyr4/REP41 (Fig. 6 *A*, lane *3*) but was absent when the *byr4* null allele was complemented by a truncated version of the *byr4* gene (Fig. 6 *A*, lane *4*). Instead, when the *byr4* null allele was complemented by a truncated version of the *byr4* gene, some protein bands that are likely to be degradation products of *byr4* were present. These results indicate that the 97-kD protein recognized by the immune serum derives from the *byr4* gene. These results also show that the *byr4* protein from *S. pombe* cells migrates slower and is more diffuse than the recombinant protein produced in *E. coli*, suggesting that the *byr4* protein is post-translationally modified in *S. pombe*.

As a first step in localizing the *byr4* protein, its behavior in crude subcellular frationation was determined. Yeast cells were lysed and divided into a high-speed pellet and a high-speed supernatant. If detergents were omitted from the lysis buffer, almost all the *byr4* protein was found in the pellet fraction (Fig. 6 *B*). If 0.2% Triton X-100 was included in the lysis buffer, then the *byr4* protein was approximately equally divided between the pellet and supernatant fractions (Fig. 6 *B*). The addition of 250 mM NaCl to the lysis buffer did not affect its fractionation profile

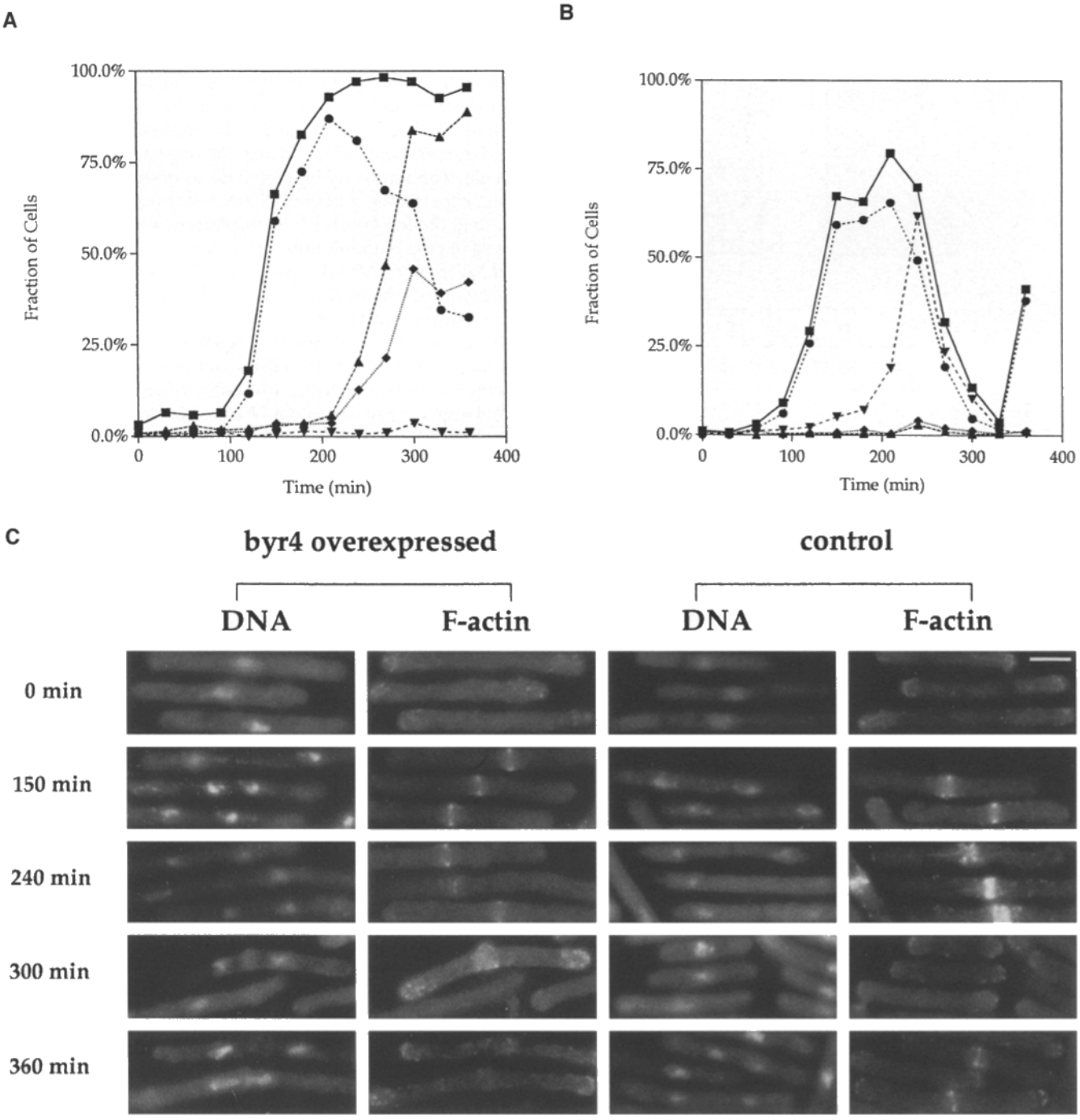


Figure 5. Overexpression of *byr4* in cell cycle–synchronized cells. (*A* and *B*) Cells overexpressing *byr4* (*A*) or control cells (*B*) were synchronized at the G2/M border using the *cdc25-22* allele. Samples were collected at 30 min intervals, fixed, and stained with DAPI and rhodamine-phalloidin. The fraction of cells with two nuclei (■), medial F-actin (●), lysed cells (◆), bumps (▲), and septum (▼) were determined by counting 250–300 cells per time point. (*C*) The location of DNA and F-actin in synchronized cells overexpressing *byr4* (left panels) or control cells (right panels) were determined as before. The time corresponds to the minutes since release of the G2/M block imposed with the *cdc25-22* allele. See text for further discussion. Bar, 5 μm.

(data not shown). These results suggest that a fraction of the *byr4* protein is membrane bound.

Since *byr4* affects cytokinesis and septation, we tested whether mutations in other genes that affect these processes alter the electrophoretic mobility or subcellular localization of the *byr4* protein. Strains containing mutations in *cdc3*, *cdc4*, *cdc8*, *cdc12*, *cdc15*, *cdc7*, *cdc11*, *cdc14*, or *cdc16* were grown to log-phase in rich media. One-half of the culture was incubated for 4 h to 35°C, the restrictive temperature for these mutations, and the other half of the culture was maintained at 25°C, the permissive temperature. Lysates were prepared from these cells using 0.2%

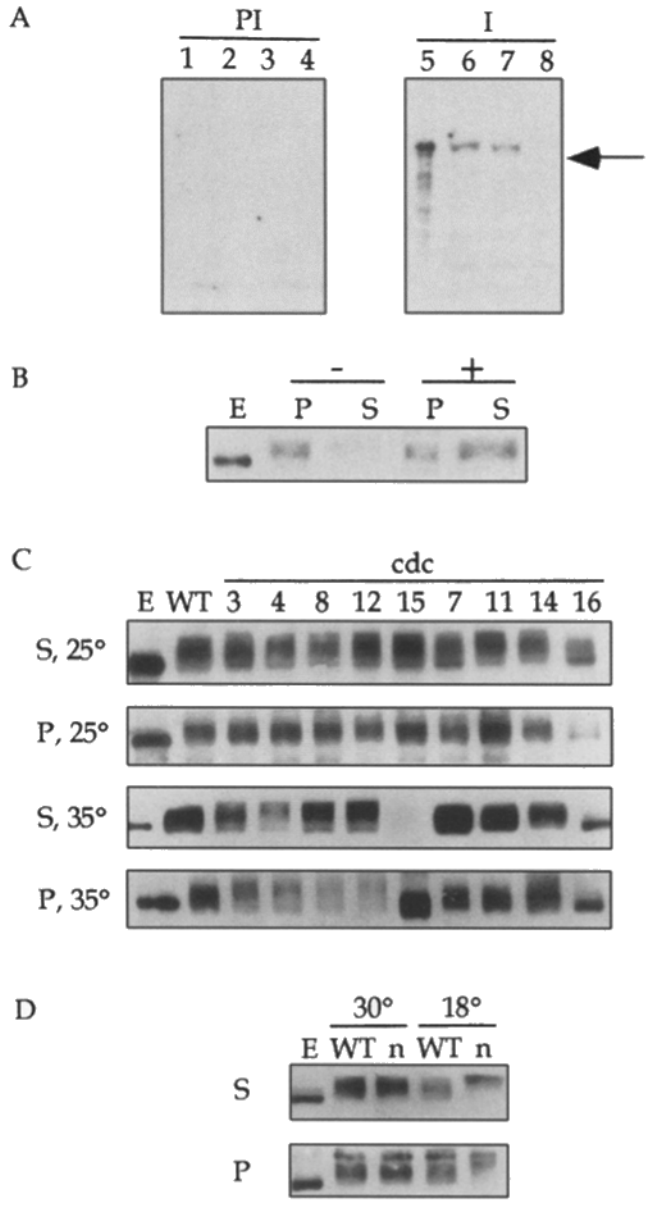


Figure 6. Analysis of the *byr4* protein. (*A*) Identification of the *byr4* protein by Western analysis. Recombinant *byr4* protein from *E. coli* (lane *1* and *5*), total protein from wild-type yeast SP870 (lane *2* and *6*), total protein from a strain with *byr4* null complemented with pbyr4/REP41 (lane *3* and *7*), or from this strain complemented with pbyr4-409/REP41 (lane *4* and *8*) were analyzed by Western analysis. Lanes *1–4* were probed with preimmune serum (PI) and lanes *5–8* were probed with immune serum (I). An arrow indicates the location of the 97-kD protein resulting from the full-length *byr4* gene. (*B*) Subcellular fractionation of the *byr4* protein. Wild-type yeast (KGY247) were lysed in the absence (−) or presence (+) of 0.2% Triton X-100. The lysates were centrifuged at 350,000 *g* for 15 min to yield a high-speed pellet (*P*) and a high-speed supernatant (*S*). The resulting fractions were analyzed by Western blotting. A sample of the recombinant *byr4* protein from *E. coli* is included for comparison (*E*). The protein recovery in the sample without Triton X-100 is less than that recovered with Triton X-100 due to losses in solubilizing the pellet. (*C*) Fractionation and mobility of *byr4* protein in cells with mutations that affect cytokinesis. Wild-type cells (*WT*) or cells with a temperature-sensitive allele of the indicated cdc gene were fractionated in the presence of 0.2% Triton X-100 and analyzed by Western analysis as before. The supernatant (*S*) or pellet (*P*) from cells grown at 25°C or 35°C are shown. With the exception of the supernatant fraction at 35°C from cells with the *cdc15-136* allele, any differences in the amount of *byr4* protein between samples are due to differences in the amount of total protein in the sample, as judged by probing these blots with antibodies to β-tubulin (data not shown). (*D*) Fractionation and mobility of *byr4* in cells with a mutation in β-tubulin gene. Strain CA91 was grown at permissive temperature (30°C) or restrictive temperature (18°C) and the *byr4* protein was analyzed as in *C*. *n*, samples from the CA91 strain with the *nda3-KM311* allele.

Triton X-100 in the lysis buffer and fractionated into pellets and supernatants (Fig. 6 *C*). The *cdc16-116* mutation resulted in a dramatic increase in the *byr4* protein electrophoretic mobility to a mobility very similar to that of the *byr4* protein made in *E. coli*. This increased mobility was partially realized at 25°C and fully realized at 35°C. The *cdc15-136* mutation resulted in an increase in the *byr4* protein electrophoretic mobility and a loss of the *byr4* protein in the supernatant fraction. A small but reproducible decrease in the *byr4* protein electrophoretic mobility was observed in cells with mutations in either *cdc3, cdc4, cdc8*, or *cdc12* (Fig. 6 *C*). Mutations in *cdc7* and *cdc11* did not noticeably affect the *byr4* protein electrophoretic mobility or fractionation properties.

Since the *byr4* null allele resulted in abnormal DAPI staining and might affect karyokinesis, we investigated whether a mutation in the β-tubulin gene, *nda3-KM311*, would affect the *byr4* protein. We found that this arrest resulted in a decrease in the *byr4* protein electrophoretic mobility (Fig. 6 *D*).

byr4 Overexpression Phenotypes Are More Severe in Cells without ras1 or scd1

To better understand the relationship between *byr4* signaling and *ras1* signaling pathways, we overexpressed *byr4* in strains containing null alleles of *ras1*, *byr2, scd1*, or *byr2* and *scd1*. When *byr4* was overexpressed using pbyr4/REP41 without thiamine in the media, all of these strains accumulated multiple nuclei (as shown in Fig. 3) and had decreased growth rates (data not shown). We noticed, however, that even with thiamine in the media to repress the attenuated *nmt1* promoter, strains containing null alleles of *ras1* or *scd1*, but not *byr2*, accumulated two or more nuclei (Fig. 7 and Table III). *ras1*$^-$ and *scd1*$^-$ strains with pbyr4/REP41 and repressing conditions contained about 24–33% binucleate cells, compared to 3–7% for these strains with control plasmids or the other strains tested (Table III). A value of 3–7% binucleate cells in a culture is consistent with the fraction of cells that would normally be found in late mitosis in an actively growing culture (Hagan and Hyams, 1988). The binucleate cells in *ras1*$^-$ and *scd1*$^-$ strains were larger than haploid cells, suggesting that these cells were binucleate because they entered the next cell cycle without cytokinesis and not because they were delayed in mitosis (Fig. 7). The hypersensitivity of the *ras1*$^-$ and *scd1*$^-$ strains to *byr4* overexpression was not due to an indirect effect of *ras1* and *scd1* on the *nmt1* promoter because these strains expressed amounts of *byr4* that were

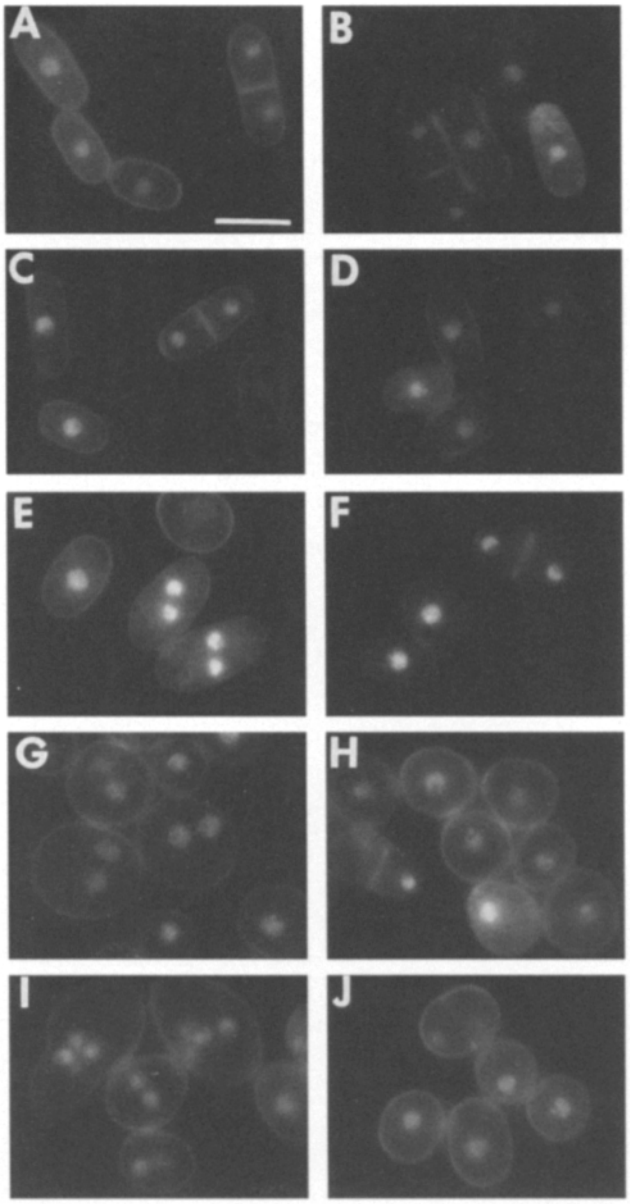


Figure 7. Effect of mutations in *ras1* signaling pathways on *byr4* overexpression phenotypes. pbyr4/REP41 (left) or pREP41 (right) were introduced into yeast SP870 (*A* and *B*), SPSU (*C* and *D*), CA5 (*E* and *F*), CA110 (*G* and *H*), or CA115 (*I* and *J*). These strains contain null alleles of *byr2* (SPSU), *ras1* (CA5), *scd1* (CA110), or *byr2* and *scd1* (CA115). Expression of the *byr4* gene from the attenuated *nmt1* promoter in pREP41 was repressed by thiamine in the media. Cells were collected, fixed, and stained with DAPI to reveal the number of nuclei per cell. Bar, 10 μm.

indistinguishable from wild-type and *byr2*$^-$ strains containing pbyr4/REP41 (data not shown).

Discussion

A novel gene, designated *byr4*, that can perturb three aspects of the mitotic cycle was isolated. First, null alleles of *byr4* permit repeated rounds of cytokinesis and septation. This suggests that *byr4* is needed to limit cytokinesis and septation to a single occurrence per cell cycle and for entry into the next cell cycle. Second, cells without *byr4* frequently arrest with two nuclei that do not stain equally with DAPI. While the mechanism of this effect is unclear, the only other case where this phenotype was observed resulted from the missegregation of individual chromosomes (Takahashi et al., 1994; Ohkura et al., 1988; Yamamoto et al., 1996). Third, cells overexpressing *byr4* fail to undergo cytokinesis, leading to the formation of multinucleate cells. Medial F-actin accumulates in these cells at the usual time in mitosis, suggesting the early events of the septation pathway occur normally. However, the later steps of the septation pathway, including contraction of the F-actin ring, septation, and proper rearrangement of the medial F-actin after mitosis, rarely occur.

Table III. Binucleate Cells in Strains with and without byr4 Overexpression

Strain	Vector	Partial genotype	Binucleate cells*
			%
SP870	pbyr4/REP41	wild-type	6.5
SP870	pREP41	wild-type	6.4
CA5	pbyr4/REP41	*ras1::ura4*	33.4
CA5	pREP41	*ras1::ura4*	2.8
SPSU	pbyr4/REP41	*byr2::ura4*	6.8
SPSU	pREP41	*byr2::ura4*	3.1
CA110	pbyr4/REP41	*scd1::ura4*	28.2
CA110	pREP41	*scd1::ura4*	5.1
CA115	pbyr4/REP41	*byr2::ura4 scd1::ura4*	23.7
CA115	pREP41	*byr2::ura4 scd1::ura4*	3.9

*Cells were grown on plates with thiamine in the medium, harvested, and stained with DAPI. 400–600 cells were counted to determine the fraction with two nuclei.

The phenotype of *byr4*$^-$ cells is similar to the terminal phenotype of *cdc16*$^-$ cells and *cdc7*-overexpressing cells in that they arrest in late mitosis and undergo repeated rounds of septation (Fankhauser et al., 1993; Fankhauser and Simanis, 1994*a*; Minet et al., 1979). The effect of *cdc16-116* on the electrophoretic mobility of the *byr4* protein further suggests a connection between these genes. In cells with the *cdc16-116* allele, a large fraction of the *byr4* protein is in a rapidly migrating form at permissive temperature and all of the *byr4* protein is in this form at restrictive temperature. Since the *byr4* protein electrophoretic mobility is perturbed at permissive temperature, this effect is probably not due to the cell cycle arrest caused by loss of *cdc16* but reflects a more specific interaction, such as *byr4* and *cdc16* functioning in the same signaling pathway. The similarity of the *byr4* repeat sequence to a region of the *cdc7* protein kinase also supports a connection between *byr4* and *cdc7*. One possible model is that the *byr4* and *cdc7* proteins bind a common protein that is required for cytokinesis. While *cdc16*$^-$ and *cdc7*-overexpressing cells do not share the abnormal DAPI staining phenotype of *byr4*$^-$ cells, *cdc16* may participate in the control of karyokinesis. The *S. cerevisiae* homologue of *cdc16* is probably *BUB2* (Fankhauser et al., 1993). *BUB2* and *cdc16* are required for cell cycle arrest at the metaphase–anaphase transition in response to damage to the mitotic spindle (Hoyt et al., 1991; Fankhauser et al. 1993). Consistent with a role for *byr4* in this process, the electrophoretic mobility of the *byr4* protein decreases in response to the cell cycle arrest caused by mutations in the β-tubulin gene. Whether this decrease in *byr4* mobility is specific to de-

fects in the mitotic spindle or whether it reflects cell cycle arrest at the metaphase–anaphase transition is under investigation. Hence, *byr4* and *cdc16* may be part of a signaling pathway that coordinates cytokinesis and karyokinesis.

There may also be connections between *byr4* and the early septation genes, which include *cdc7*, *cdc11*, *cdc14*, and *cdc15*. Cells overexpressing *byr4* or with loss-of-function mutations in the early septation genes arrest as elongated cells with multiple nuclei due to the absence of cytokinesis (Nurse et al., 1976). Like cells overexpressing *byr4*, cells with mutations in *cdc7*, *cdc11*, and *cdc14* form a medial F-actin ring at the restrictive temperature and cells with the *cdc14-114* allele arrest with nuclei clustered in the cell center (Hagan and Hyams, 1988; Fankhauser et al., 1995). Unlike cells overexpressing *byr4*, cells with a mutation in *cdc15* do not form an F-actin ring, cells with a mutation in *cdc11* are able to properly relocalize their medial F-actin following mitosis, and cells with mutations in *cdc7*, *cdc11*, and *cdc15* do not cluster their nuclei at the arrest point (Marks and Hyams, 1985; Fankhauser and Simanis, 1994*b*; Fankhauser et al., 1995). The effect of the *cdc15-136* mutation on the *byr4* protein also suggests a possible interaction between *cdc15* and *byr4*. We found an increased *byr4* electrophoretic mobility and a lack of *byr4* in the supernatant fraction in cells arrested by *cdc15-136*. These effects are probably not due to perturbation of the medial F-actin ring by *cdc15-136* since other mutations that perturb F-actin rings, including *cdc3-124*, *cdc4-8*, *cdc8-110*, and *cdc12-112*, did not cause similar changes in the *byr4* protein mobility and localization.

The *byr4* gene was identified in a search for genes that can partially bypass the need for *ras1* in conjugation. While *byr4* does not restore conjugation to strains with null alleles of *ras1*, it does show two distinct genetic interactions with *ras1*. First, *byr4* overexpression can partially bypass the need for *ras1* in sporulation. Since the genes that are known to bypass the need for *ras1* in sporulation affect the *byr2-byr1* kinase cascade, it is possible that *byr4* activates or shares common downstream signaling components with this MAP kinase cascade in the sporulation pathway. The recent observation that the *BEM1* protein of *S. cerevisiae* binds the *STE20* kinase is consistent with a coupling between the MAP kinase cascade required for mating and the cell polarity pathway (Leeuw et al., 1995). Second, the inhibition of cytokinesis due to *byr4* overexpression is worse in cells without *ras1* and *scd1*. These results suggest a positive role for the *ras1-scd1* pathway in controlling cytokinesis.

A role for rho-family GTPases in cytokinesis is suggested by data from higher eukaryotic systems. Inhibition of rho GTPases with exoenzyme C3 of *Clostridium botulinum* or rhoGDI can prevent cytokinesis (Rubin et al., 1988; Kishi et al., 1993). Enlarged, multinucleate cells, resulting from a lack of cytokinesis, are also found in mammalian cells transformed by the dbl oncogene (Ron et al., 1991). The dbl protein functions as a guanine nucleotide dissociation stimulator for the cdc42 and rhoA GTPases, raising the possibility that dbl is functioning in a pathway analogous to the *ras1-scd1* pathway of *S. pombe* to inhibit cytokinesis (Hart et al., 1991; Hart et al., 1994). Studies from *S. cerevisiae* also suggest a need for rho-family GTPases in cytokinesis. Cells without *CLA4*, a *STE20*-like kinase, fail to undergo cytokinesis (Cvckova et al., 1995). The *CLA4* protein binds the *CDC42* GTPase but not other members of the rho-family GTPases from *S. cerevisiae* (Cvckova et al., 1995).

We would like to thank Dr. M. Wigler and Dr. E. Chang (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) for the generous gift of *S. pombe* strains, Dr. P.G. Young for a generous gift of the genomic DNA library in pWH5, Dr. J. Fikes for generous gift of the cDNA library, Drs. K. Gould, D. McCollum, and M. Balasubramanian, and other members of the Gould laboratory (Vanderbilt University, Nashville, TN) for the generous gifts of plasmids, strains, and technical advice, Dr. Jim Price for assistance with the FACS analysis, Dr. J. Henikoff for assistance with the BLOCKs search of the conserved sequences in *byr4*, Michelle Reader for technical assistance, and Drs. Ron Wisdom, Jeff Flick, Patricia Bauman, and Qiu-chen Cheng for helpful discussions and comments on the manuscript.

This work was funded by grants from the Lucille P. Markey Charitable Trust and Boehringer-Ingelheim Pharmaceuticals, Inc.

Received for publication 12 October 1995 and in revised form 12 April 1996.

References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

Basi, G., E. Schmid, and K. Maundrell. 1993. TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not transcription start point or thiamine repressibility. *Gene.* 123:131–136.

Becker, D.M., J.D. Fikes, and L. Guarente. 1991. A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. *Proc. Natl. Acad. Sci. USA.* 88:1968–1972.

Boguski, M.S., and F. McCormick. 1993. Proteins regulating *ras* and its relatives. *Nature (Lond.).* 366:643–654.

Booher, R.N., C.E. Alfa, J.S. Hyams, and D.H. Beach. 1989. The fission yeast *cdc2/cdc13/suc1* protein kinase: regulation of catalytic activity and nuclear localization. *Cell.* 58:485–497.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (Lond.).* 348:125–132.

Cantor, S., T. Urano, and L. Feig. 1995. Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. *Mol. Cell. Biol.* 15:4578–4584.

Chang, E.C., M. Barr, Y. Wang, V. Jung, H.-P. Xu, and M. Wigler. 1994. Cooperative interaction of *S. pombe* proteins required for mating and morphogenesis. *Cell.* 79:131–141.

Chant, J. 1994. Cell polarity in yeast. *Trends in Genet.* 10:328–333.

Cvckova, F., C. DeVirgilio, E. Manser, J. Pringle, and K. Nasmyth. 1995. Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes & Dev.* 9:1817–1830.

Fankhauser, C., and V. Simanis. 1994*a*. The *cdc7* protein kinase is a dosage dependent regulator of septum formation in fission yeast. *EMBO J.* 13:3011–3019.

Fankhauser, C., and V. Simanis. 1994*b*. Cold fission: splitting the *S. pombe* cell at room temperature. *Trends Cell Biol.* 4:96–101.

Fankhauser, C., J. Marks, A. Reymond, and V. Simanis. 1993. The *S. pombe cdc16* gene is required both for maintenance of $p34^{cdc2}$ kinase activity and regulation of septum formation: a link between mitosis and cytokinesis? *EMBO J.* 12:2697–2704.

Fankhauser, C., A. Reymond, L. Cerutti, S. Utzig, K. Hofmann, and V. Simanis. 1995. The *S. pombe cdc15* gene is a key element in the reorganization of F-actin at mitosis. *Cell.* 82:435–444.

Fukui, Y., and M. Yamamoto. 1988. Isolation and characterization of *Schizosaccharomyces pombe* mutants phenotypically similar to *ras1*−. *Mol. & Gen. Genet.* 215:26–31.

Fukui, Y., T. Kozasa, Y. Kaziro, T. Takeda, and M. Yamamoto. 1986. Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe. Cell.* 44:329–336.

Gotoh, Y., E. Nishida, M. Shimanuki, T. Toda, Y. Imai, and M. Yamamoto. 1993. *Schizosaccharomyces pombe spk1* is a tyrosine phosphorylated protein functionally related to *Xenopus* mitogen-activated protein kinase. *Mol. Cell. Biol.* 13:6427–6434.

Grimm, C., J. Kohli, J. Murray, and K. Maundrell. 1988. Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. *Mol. & Gen. Genet.* 215:81–86.

Gutz, H., H. Heslot, U. Leupold, and N. Loprieno. 1974. Handbook of Genetics. Plenum Press, New York, NY. 395–436.

Hagan, I.M., and J.S Hyams. 1988. The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe. J. Cell Sci.* 89:343–357.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Habor, NY. 313–315.

Hart, M., A. Eva, T. Evans, S. Aaronson, and R. Cerione. 1991. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. *Nature (Lond.).* 354:311–314.

Hart, M., A. Eva, D. Zangrilli, S. Aaronson, T. Evans, R. Cerione, and Y. Zheng. 1994. Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. *J. Biol. Chem.* 269:62–65.

Henikoff, S., J. Henikoff, W.J. Alford, and S. Pietrokovski. 1995. Automated construction and graphical presentation of protein blocks from unaligned sequences. *Gene.* 163:17–26.

Herskowitz, I. 1995. MAP kinase pathways in yeast: for mating and more. *Cell.* 80:187–197.

Hofer, F., S. Fields, C. Schneider, and G. Martin. 1994. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. *Proc. Natl. Acad. Sci. USA.* 91:11089–11093.

Hoyt, M.A., L. Totis, and B. Roberts. 1919. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell.* 66:507–517.

Hu, Q., A. Klippel, A.J. Muslin, W.J. Fantl, and L.T. Williams. 1995. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science (Wash. DC).* 268:100–102.

Hughes, D.A., A. Ashworth, and C.J. Marshall. 1993. Complementation of *byr1* in fission yeast by mammalian MAP kinase requires coexpression of *raf* kinase. *Nature (Lond.).* 364:349–352.

Kikuchi, A., S.D. Demo, Z.-H. Ye, Y.-W. Chen, and L.T. Williams. 1994. ral-GDS family members interact with the effector loop of *ras* p21. *Mol. Cell. Biol.* 14:7483–7491.

Kishi, K., T. Sasaki, S. Kuroda, T. Itoh, and Y. Takai. 1993. Regulation of cytoplasmic division of *Xenopus* embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J. Cell Biol.* 120:1187–1195.

Lees-Miller, J.P., G. Henry, and D.M. Helfman. 1992. Identification of *act2*, an essential gene in the fission yeast *Schizosaccharomyces pombe* that encodes a protein related to actin. *Proc. Natl. Acad. Sci. USA.* 89:80–83.

Leeuw, T., A. Fourest-Lieuvin, C. Wu, J. Chenevert, K. Clark, M. Whiteway, S. Thomas, and E. Leberer. 1995. Pheromone response in yeast: association of BEM1p with proteins of the MAP kinase cascade and actin. *Science (Wash. DC).* 270:1210–1213.

Marks, J., C. Fankhauser, and V. Simanis. 1992. Genetic interactions in the control of septation in *Schizosaccharomyces pombe. J. Cell Sci.* 101:801–808.

Marks, J., and J.S. Hyams. 1985. Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe. Eur. J. Cell Biol.* 39:27–32.

Marshall, C.J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 80: 179–185.

Masuda, T., K. Kariya, M. Shinkai, T. Okada, and T. Kataoka. 1995. Protein kinase *byr2* is a target of *ras1* in the fission yeast *Schizosaccharomyces pombe. J. Biol. Chem.* 270:1979–1982.

Maundrell, K. 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene.* 123:127–130.

Minet, M., P. Nurse, P. Thuriaux, and J. Mitchison. 1979. Uncontrolled septation in a cell division cycle mutant of the fission yeast *Schizosaccharomyces pombe. J. Bacteriol.* 137:440–446.

Moreno, S., A. Klar, and P. Nurse. 1989. Regulation of the p34-cdc2 protein kinase during mitosis. *Cell.* 58:361–372.

Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe. Methods Enzymol.* 194:795–823.

Nadin-Davis, S.A., and A. Nasim. 1988. A gene which encodes a predicted protein kinase can restore some function of the *ras* gene in fission yeast. *EMBO J.* 7:985–993.

Nadin-Davis, S.A., A. Nasim, and D. Beach. 1986a. Involvement of *ras* in sexual differentiation but not in growth control in fission yeast. *EMBO J.* 11: 2963–2971.

Nadin-Davis, S.A., R.C.A. Yang, S.A. Narang, and A. Nasim. 1986b. The cloning and characterization of a RAS gene from *Schizosaccharomyces pombe. J. Mol. Evol.* 23:41–51.

Nurse, P., and Y. Bissett. 1981. Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature (Lond.).* 292:558–560.

Nurse, P., P. Thuriaux, and K. Nasmyth. 1976. Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe. Mol. & Gen. Genet.* 146:167–178.

Ohkura, H., Y. Adachi, N. Kinoshita, O. Niwa, T. Toda and M. Yanagida. 1988. Cold-sensitive and caffeine-supersensitive mutants of the *Schizosaccharomyces pombe dis* genes implicated in sister chromatid separation during mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1465–1473.

Paulovich, A.G., and L.H. Hartwell. 1995. A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell.* 82:841–847.

Rodriguez-Vinciana, P., P.H. Warne, R. Dhand, B. VanHaesebroeck, I. Gout, M.J. Fry, M.D. Waterfield, and J. Downward. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature (Lond.).* 370:527–532.

Ron, D., M. Zannini, M. Lewis, R.B. Wickner, L.T. Hunt, G. Graziani, S.R. Tronick, S.A. Aaronson, and A. Eva. 1991. A region of the proto-dbl essential for its transforming activity shows sequence similarity for a yeast cell cycle gene, CDC24, and the human breakpoint cluster gene, bcr. *New Biol.* 3: 372–379.

Rubin, E., D. Gill, P. Boquet, and M. Popoff. 1988. Functional modification of a 21-kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum. Mol. Cell. Biol.* 8:418–426.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Laboratory Press, Cold Spring Harbor, NY.

Schuler, G.D., S.F. Atshul, and D.J. Lipman. 1991. A workbench for multiple alignment construction and analysis. *Proteins.* 9:180–190.

Spaargaren, M., and J. Bischoff. 1994. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, N-ras, K-ras, and Rap. *Proc. Natl. Acad. Sci. USA.* 91:12609–12613.

Takahashi, K., H. Yamada, and M. Yanagida. 1994. Fission yeast minichromosome loss mutants *mis* cause lethal aneuploidy and replication abnormality. *Mol. Biol. Cell.* 5:1145–1158.

Toda, T., M. Yamamoto, and M. Yanagida. 1981. Sequential alteration in the nuclear chromatin region during mitosis of the fission yeast *Schizosaccharomyces pombe*: Video fluorescence microscopy of synchronously growing wild-type and cold-sensitive *cdc* mutants by using a DNA-binding fluorescent probe. *J. Cell Sci.* 52:271–287.

Toda, T., M. Shimanuki, and M. Yanagida. 1991. Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes & Dev.* 5:60–73.

Van Aelst, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler. 1993. Complex formation between *ras* and *raf* and other protein kinases. *Proc. Natl. Acad. Sci. USA.* 90:6213–6217.

Vassoroti, A., M. Boutry, A.-M. Colson, and A. Goffeau. 1984. Independent loci for the structural genes of the yeast mitochondrial α and β ATPase subunits. *J. Biol. Chem.* 259:2845–2849.

Wang, Y., H.-P. Xu, M. Riggs, L. Rodgers, and M. Wigler. 1991. *byr2*, a *Schizosaccharomyces pombe* gene encoding a protein kinase capable of partial suppression of the *ras1* mutant phenotype. *Mol. Cell. Biol.* 11:3554–3563.

Watanabe, Y., and M. Yamamoto. 1994. *S. pombe mei2+* encodes an RNA-binding protein essential for premeiotic DNA synthesis and meiosis I, which cooperates with a novel RNA species meiRNA. *Cell.* 78:487–498.

Xu, H.P., M. White, S. Marcus, and M. Wigler. 1994. Concerted action of *ras* and G proteins in the sexual response pathways of *Schizosaccharomyces pombe. Mol. Cell. Biol.* 14:50–58.

Xu, H.P., T. Rajavashisth, N. Grewal, V. Jung, M. Riggs, L. Rodgers, and M. Wigler. 1992. A gene encoding a protein with seven zinc finger domains acts on the sexual differentiation pathways of *Schizosaccharomyces pombe. Mol. Biol. Cell.* 3:721–734.

Yamamoto, A., V. Guacci, and D. Koshland. 1996. Pds1p, an inhibitor of anaphase in budding yeast plays a critical role in the APC and checkpoint pathway(s). *J. Cell Biol.* 133:99–110.