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

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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) kinase cascade (Herskowitz, 1995; Marshall, 1995). A second ras effector may be phosphatidylinositol-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 rasGDS, a positive regulator of the ras GTPase (Hofer et al., 1994; Kikuchi et al., 1994; Spaargaren and Bischoff, 1994). Activated ras 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., 1986a,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-

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 scdl protein (Chang et al., 1994). scdl has sequence similarity to GDP dissociation stimulators for the cdc42 GTPase. Null alleles of scdl 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-sccl-cdc42 pathway is very similar to the RSRI-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, 1994b). 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, 1994b). 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 HphI 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., 1986a). Stable ura4 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 CAS/SPSU. CA76 was made by transforming SPSU with a linear fragment containing the entire coding sequence of the byr2 gene, where serine 240 and 142 were changed to alanines (Wang et al., 1991). After overnight growth in 10 ml of YE media supplemented with adenine and uracil, 106 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 pWH1 (P. Young, Queens University, Kingston, Kingston, Ontario, Canada). The transformants were plated on MM-glu plates at a density of about 2,000 colonies per 15-cm plate. The colonies were stained with iodine at 3 min intervals for 3 min (C. Crowe). After staining, the colonies 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- were 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 (independant 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, p13, was subcloned using standard techniques (Sambrook et al., 1989). A deletion of a 2.7-kb PstI fragment yielding p122Pst 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 216-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 x 106 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 DNA 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 days of growth at 25°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.

The Journal of Cell Biology, Volume 133, 1996 1308
Table I. S. pombe Strains Used in This Study

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Construction of byr4 Null Allele

To construct a null allele of byr4, a BclI site was added at the stop codon of pS17APst by PCR mutagenesis. The coding sequence and 426 bp of 5' untranslated region were then removed by deleting the 2.4-kb BclI fragment from this vector. The resulting 2.4-kb HindIII fragment from this vector was cloned into pBSK+ (Stratagene, La Jolla, CA) and the ura4 gene containing BamHII ends was inserted into the BclI site (Grimm et al., 1988; Leses Miller et al., 1992). The 4.1-kb HindIII to Smal 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 glus 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<sup>6</sup> 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 (Pau-
For Western analysis, approximately 2.5 × 10^6 cells from mid-log growth were harvested by centrifugation, washed twice with PBS, 50 mM NaF, 1 mM Na2EDTA, 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 MgCl2, 15 mM EGTA, 0.1 mM NaN3, and 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 metabisulfitte). 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 bead beater (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 their samples were adsorbed with a high-speed protein fraction were separated by SDS polyacrylamide electrophoresis and transferred to nitrocellulose membranes.

**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). pbyr4/REP41 was introduced into various strains to overexpress the byr4 protein. An NH2-terminal truncation of the byr4 protein was generated by site-directed mutagenesis. Using pbyr4/REP41, the sequence coding the NH2-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 expression. MM-glutamate 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 the cdcl2-22 temperature-sensitive allele (Moreno et al., 1984). The resulting strains were grown to mid-log phase in MM with thiamine at 25°C, washed twice in thiamine-free media, and grown for 24-33 h in thiamine-free media at 25°C. The culture was shifted to the restrictive temperature, 35°C, for 4 h to impose the cdcl2-22 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 cdcl2-22 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 F-actin staining, 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 a lysozyme enzyme (L-2205, 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-o-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 Axiosvert with 100x 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 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 sequence from the other end of the genomic insert that was identified as a novel yeast gene from S. cerevisiae, designated YJR053W, that was identified as a result of the genomic 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...
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 NH2-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).

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
To test the consequence of byr4 overexpression, we introduced pbyr4/REP41 into wild-type cells and induced byr4 overexpression 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.

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 KG444, 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...
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 fractionation 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 pre-immune 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 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 scdl**

To better understand the relationship between byr4 signaling and ras1 signaling pathways, we overexpressed byr4 in strains containing null alleles of ras1, byr2, scdl, or byr2 and scdl. 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 scdl, but not byr2, accumulated two or more nuclei (Fig. 7 and Table III). ras1 and scdl 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 scdl 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 scdl strains to byr4 overexpression was not due to an indirect effect of ras1 and scdl on the nmt1 promoter because these strains expressed amounts of byr4 that were 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.
Table III. Binucleate Cells in Strains with and without byr4 Overexpression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>Partial genotype</th>
<th>Binucleate cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP870</td>
<td>pbyr4/REP41</td>
<td>wild-type</td>
<td>6.5</td>
</tr>
<tr>
<td>SP870</td>
<td>pREP41</td>
<td>wild-type</td>
<td>6.4</td>
</tr>
<tr>
<td>CA5</td>
<td>pbyr4/REP41</td>
<td>ras1::ura4</td>
<td>33.4</td>
</tr>
<tr>
<td>CA5</td>
<td>pREP41</td>
<td>ras1::ura4</td>
<td>28.8</td>
</tr>
<tr>
<td>SPSU</td>
<td>pbyr4/REP41</td>
<td>byr2::ura4</td>
<td>6.8</td>
</tr>
<tr>
<td>SPSU</td>
<td>pREP41</td>
<td>byr2::ura4</td>
<td>3.1</td>
</tr>
<tr>
<td>CA110</td>
<td>pbyr4/REP41</td>
<td>scdl::ura4</td>
<td>28.2</td>
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<tr>
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<td>5.1</td>
</tr>
<tr>
<td>CA115</td>
<td>pbyr4/REP41</td>
<td>byr2::ura4 scdl::ura4</td>
<td>23.7</td>
</tr>
<tr>
<td>CA115</td>
<td>pREP41</td>
<td>byr2::ura4 scdl::ura4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*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.

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.

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, 1994a; 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, 1994b; 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 sdc1. These results suggest a positive role for the ras1-sdc1 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 exocinzyme 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-sdc1 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 GTase but not other members of the rho-family GTases from S. cerevisiae (Cvckova et al., 1995).

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1318