The Septins Are Required for the Mitosis-specific Activation of the Gin4 Kinase

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Abstract. In budding yeast, a protein kinase called Gin4 is specifically activated during mitosis and functions in a pathway initiated by the Clb2 cyclin to control bud growth. We have used genetics and biochemistry to identify additional proteins that function with Gin4 in this pathway, and both of these approaches have identified members of the septin family. Loss of septin function produces a phenotype that is very similar to the phenotype caused by loss of Gin4 function, and the septins are required early in mitosis to activate Gin4 kinase activity. Furthermore, septin mutants display a prolonged mitotic delay at the short spindle stage, consistent with a role for the septins in the control of mitotic events. Members of the septin family bind directly to Gin4, demonstrating that the functions of Gin4 and the septins must be closely linked within the cell. These results demonstrate that the septins in budding yeast play an integral role in the mitosis-specific regulation of the Gin4 kinase and that they carry out functions early in mitosis.

Key words: septins • Gin4 • cyclin-dependent kinase • mitosis • morphogenesis

The eukaryotic cell cycle is driven by the combined activity of two families of conserved proteins called cyclins and cyclin-dependent kinases (Nasmyth and Hunt, 1993; Pines, 1993). Cells express different cyclins that appear at specific times during the cell cycle to bind and activate cyclin-dependent kinases, thereby leading to the induction of specific cell cycle events. Simple organisms are able to use a single cyclin-dependent kinase to control all events of the cell cycle, indicating that the same kinase can induce different events when activated at different stages of the cell cycle. The molecular mechanisms underlying the ability of cyclin-dependent kinases to induce specific cell cycle events at different times during the cell cycle remain largely unknown.

The control of bud growth during mitosis in the budding yeast *S. cerevisiae* provides an excellent model system in which to study the pathways used by cyclins and cyclin-dependent kinases to control cell cycle events. As the division cycle begins, a new daughter cell emerges from the mother cell as a bud, which undergoes polar growth at its tip during interphase. Upon entry into mitosis, a switch in the pattern of bud growth occurs such that the bud grows over its entire surface, leading to the formation of a round bud. This switch from polar to isotropic bud growth is induced by the appearance of the mitotic cyclins. Defects in the pathway used by the mitotic cyclins to control this switch lead to the formation of highly elongated buds due to prolonged polar bud growth during mitosis (Lew and Reed, 1993, 1995*b*). This pathway can be dissected genetically because the switch from polar to isotropic bud growth is not required for viability, and cells with highly elongated buds form colonies with a rough morphology that is easily identified (Altman and Kellogg, 1997).

We have used a combination of biochemistry and genetics to identify proteins that function in the pathway used by the mitotic cyclin Clb2 to control bud growth during mitosis (Kellogg et al., 1995; Kellogg and Murray, 1995; Altman and Kellogg, 1997). These experiments have led to the identification of two proteins called Nap1 and Gin4, which are required for the proper control of mitotic events by Clb2. Gin4 is a protein kinase that is specifically activated during mitosis in a Nap1-dependent manner, and Nap1 is able to bind to both Clb2 and Gin4. Nap1 is a member of a highly conserved family of proteins, indicating that other eukaryotic cells are likely to use Nap1-dependent mechanisms to control mitotic events.

We first identified the Gin4 kinase in a genetic screen for mutations that disrupt the mitosis-specific control of bud growth (Altman and Kellogg, 1997). In the same

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screen we isolated a number of additional mutations that cause a phenotype that strongly resembles the phenotype caused by deletion of the genes for Gin4 or Nap1. In this report, we demonstrate that one of these mutations is in the *CDC11* gene, a previously identified member of the septin family of proteins.

The septins comprise a conserved family of proteins found in organisms as divergent as yeast and humans (Neufeld and Rubin, 1994; Sanders, 1994; Cooper and Kiehart, 1996; Longtine et al., 1996; Kinoshita et al., 1997). Four different septins were originally identified in budding yeast, called Cdc3, Cdc10, Cdc11, and Cdc12 (Hartwell, 1971). All of these are localized to a ring of 10-nm filaments found at the bud neck, and mutations in any of the septin genes result in rapid loss of both the 10-nm filaments and the localization of the other septins at the bud neck, suggesting that the septins are components of, or are closely associated with, the 10-nm filaments (Byers, 1976; Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991). In support of this, members of the septin family in both *Drosophila* and budding yeast have been found to associate with each other as tight complexes that are capable of forming filaments in vitro (Field et al., 1996; Frazier et al., 1998). Septin mutants in budding yeast display a uniform cell cycle arrest with highly elongated buds and multinucleate cells. Loss of septin function also causes the formation of multinucleate cells in *Drosophila* and in mammalian cell lines (Neufeld and Rubin, 1994; Fares et al., 1995; Kinoshita et al., 1997). These data have led to a proposed role for the septins in cytokinesis. Recent data, however, suggests that the septins also function in bud site selection, response to mating pheromones, sporulation, and the localized deposition of chitin (Ford and Pringle, 1991; Kim et al., 1991; Flescher et al., 1993; Chant, 1995; Ozsarac, 1995; Fares et al., 1996; DeMarini et al., 1997).

Although the septins are clearly involved in a variety of cellular processes, the molecular mechanisms that underlie septin function remain a mystery. Furthermore, it is unclear why septin mutations in budding yeast cause the formation of highly elongated buds. In this report, we demonstrate that the budding yeast septins are required for the mitosis-specific regulation of the Gin4 kinase and are components of a signaling pathway that is used to control polar bud growth during mitosis.

Materials and Methods

Strains Used in This Study

All strains are in the W303 strain background (*leu2-3,112 ura3-52 can1-100 ade2-1 his3-11 trp1-1 ssd1*), with the exception of the JB811 protease–deficient strain. The *cdc11* allele in the CC3 strain is the mutant allele identified in this report. The CC4 strain was created by crossing the *cdc12-6* allele into the W303 strain background three times. The additional features of the strains used in this study are listed below.

CC3: *Mat**a** Δbar1 cdc11*
CC4: *Mat**a** Δbar1 cdc12-6*
CC10: *Mat**a** Δbar1 SEP7-3XHA::URA3*
RA24: *Mat**a** Δbar1 ΔSep7::URA3*
RA25: *Mat**a** Δbar1 ΔSep7::URA3 Δclb1 Δclb3::TRP1 Δclb4::HIS3*
DK186: *Mat**a** Δbar1*
DK212: *Mat**a** Δbar1 Δclb1 Δclb3::TRP1 Δclb4::HIS3*
DM1: *Mat**a**/α CDC11/Δcdc11::LEU2*
JB811: *Mat**a** prb1 pep4-3 trp1 leu2-3,112 ura3-52*

Cloning of CDC11

To clone the gene corresponding to the mutation identified in the genetic screen we transformed mutant cells with a genomic library carried in a CEN-containing vector and screened for rescue of the temperature sensitivity and elongated bud phenotypes of the original mutation. To demonstrate that the mutation is allelic to the *CDC11* gene we mated the strain carrying the original mutation to a wild-type strain in which the *URA3* nutritional marker had been integrated immediately downstream of the *CDC11* locus. Tetrad analysis of this diploid revealed that the mutation segregated opposite the *URA* marker in 15/15 tetrads dissected. Additionally, we demonstrated that the mutation was not able to complement a *CDC11* gene deletion.

Purification of Gin4 and Gin4-binding Proteins

A bacterial expression vector was created in which Gin4 is fused to glutathione-S-transferase (GST)[1] at its NH_2 terminus, and to six histidines at its COOH terminus. Cells carrying this plasmid are grown at room temperature to an optical density of 1.0, and expression is induced for 3 h by the addition of 0.2 mM IPTG. Cells are harvested by centrifugation and immediately scraped out of the bottles and frozen on liquid nitrogen as chunks and stored at −80°C. To purify GST-Gin4 fusion protein, the frozen cell chunks are ground under liquid nitrogen in a mortar and pestle until a fine powder is obtained. The powder is transferred to a beaker, allowed to warm briefly at room temperature, and is then rapidly resuspended in five volumes (wt/vol) of PBS containing 1.0 M NaCl, 0.5% Tween-20, and 2 mM PMSF at room temperature. The rapid harvesting, freezing, and lysis of the Gin4-expressing cells in buffer containing high concentrations of salt dramatically improved the yield of full-length Gin4 fusion protein, and similar results have been obtained with four other fusion proteins in our laboratory. After resuspension of the cells, the lysate is centrifuged at 100,000 *g* for 1.5 h and the Gin4 fusion protein is purified by glutathione affinity chromatography as previously described (Kellogg et al., 1995), with the exception that the wash buffer contains 1.0 M NaCl, and the column is eluted with a buffer containing 50 mM phosphate, pH 8.0, 0.4 M NaCl, and 5 mM glutathione. The eluted GST-Gin4-6×His fusion protein is loaded directly onto a 2 ml Ni-NTA column, which is washed with 10 column volumes of 50 mM phosphate, pH 8.0, 0.5 M NaCl, 10 mM imidazole followed by 10 column volumes 50 mM phosphate, pH 6.0, 0.5 M NaCl. The Ni-NTA column is eluted with 50 mM phosphate, pH 7.0, 0.5 M NaCl, 0.25 M imidazole. The yield from 6 liters of culture is ~6 mg of full-length Gin4 protein. Peak fractions are pooled and concentrated to 3 mg/ml using a Centricon concentrator, and are frozen on liquid nitrogen and stored at −80°C. Before coupling to Affigel 10, the protein is passed over a desalting column pre-equilibrated with 50 mM Hepes-KOH, pH 7.6, 0.5 M KCl.

Gin4 affinity chromatography is carried out as previously described for Nap1 affinity chromatography (Altman et al., 1997), except that the extract and wash buffers contain 150 mM KCl, and the column is eluted in steps of 100 mM KCl.

Identification of Proteins by Mass Spectrometry

Protein bands were excised from a gel similar to the one shown in Fig. 2 and in-gel trypsin digestion was carried out following the method of Shevchenko et al. (1996). Proteins were identified by micro column high performance liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry. A 100 × 200 mm fused silica capillary (Polymetrics, Inc., Phoenix, AZ) was used for the micro-column as described by Kennedy and Jorgensen (1989). The column was packed to a length of ~15 cm with 10 mm POROS 10 R2 reverse phase material (PerSeptive Biosystems, Framingham, MA). The fritted end of the column was inserted into the needle of the ion source. The sample was directly loaded onto the micro-column by helium pressurization of the sample in a stainless steel bomb (Yates et al., 1994). The amount of sample loaded during pressurization was measured with a glass capillary by displacement of the solution at the fritted end of the column. Liquid chromatography was carried out using a dual syringe pump (PE Applied Biosystems, Fos-

1. *Abbreviations used in this paper*: GST, glutathione-S-transferase; ORF, open reading frame.

ter City, CA). A 100:1 precolumn split was used to deliver a flowrate of 1–1.5 μl/min through the column. Solvent A consisted of 0.5% acetic acid and solvent B consisted of 80:20 acetonitrile/water containing 0.5% acetic acid. The HPLC pump was programmed to ramp solvent B from 0% to 60% in 30 min. Electrospray ionization was carried out at voltage of 4.6 kV. Tandem mass spectra were acquired automatically during the entire gradient run (Link et al., 1997).

Tandem mass spectra were searched against an *S. cerevisiae* protein database using the SEQUEST program (Eng et al., 1994). This database was obtained from the *Saccharomyces* Genome Database (Stanford University, Palo Alto, CA), which represents the complete genome sequence. Sequences for bovine trypsin and human keratin were included to facilitate identification of potential contaminants. Every sequence with high scores that matched to a tandem mass spectrum was manually verified to insure the match was correct.

Gin4 Activation and Hyperphosphorylation Assays

Cell cycle time courses and analysis of Gin4 hyperphosphorylation were carried out as previously described (Altman and Kellogg, 1997), except that *cdc11* cells were synchronized by treatment with α-factor for 3 h at room temperature, and then released by washing two times with media prewarmed to 37°C. Gin4 kinase assays were carried out as previously described (Altman and Kellogg, 1997). For mitotic arrest, cells were grown to log phase at 23°C and were then incubated for 2.5 h in the presence of 20 μg/ml nocodazole and 30 μg/ml benomyl at 37°C.

Deletion of the SEP7 Gene and Generation of an HA-tagged Sep7

The *SEP7* gene was deleted by using oligos to amplify the *URA* marker from pRS306 with regions of homology to the *SEP7* gene on each end (oligos: ATGAGCACTGCTTCAACACCGCCAATTAACTTATTTC-GTAGAAAGAAAGAACATAAACGTGGGATCACATACACACG-GCATCAGAGCAGATTG and TCAATCTCTACCCGATGCAATA-GAGGCTAAATCAGTATACGTGTCATTTTTTTTAATTTGGCTC-CTTGTGCTCTGGTGCGGTATTTCACACCG). The *SEP7* gene disruption was confirmed by PCR. The disruption of the *SEP7* gene was first carried out in a strain that carries a deletion of the *URA* gene to minimize the background of transformants resulting from gene conversion of the *URA* gene. Once a deletion was obtained in this background, oligos were used to amplify the deletion with 500 bases of homology to the *SEP7* gene region on each side, and the PCR product was used to disrupt the *SEP7* gene in the W303 strain background. The *SEP7* gene was tagged with three copies of the HA epitope by amplifying a region of the *SEP7* open reading frame (ORF) (oligos: GCGGGATCCCACTTCATTAGAACT-TGGGG and GCGGGTACCATCTCTACCCGATGCAATAG) followed by insertion of this region into the BamHI and KpnI sites of plasmid pDK51 to create plasmid pCC4, which contains the 3′ end of Sep7 fused to three repeats of the HA epitope. pCC4 was linearized with SacI to target integration at the *SEP7* gene and then transformed into strain DK186 to create strain CC10. Successful integration at the *SEP7* gene was confirmed with a monoclonal antibody specific for the HA epitope.

Generation of Antibodies that Recognize Cdc11 and Immunofluorescence Methods

The full-length Cdc11 ORF was amplified by PCR (primers: CGCA-GATCTATGTCCGGAATAATTGACG and GCGCCTCGAGTCAT-TCTTCCTGTTTGATTT) and cloned into the BamHI and XhoI sites of pGex4T-3 (Pharmacia Biotech, Inc., Piscataway, NJ) to generate plasmid pCC2. GST-Cdc11 fusion protein was purified using the same procedure used to purify GST-Gin4 and 2 mg of the fusion protein was used to immunize a rabbit. To affinity purify the anti-Cdc11 antibodies, an MBP-Cdc11 fusion protein was constructed by cloning a full-length Cdc11 fragment into the pMALc vector, and the resulting fusion protein was purified using amylose affinity chromatography. Antibodies were affinity purified using a full-length Cdc11 maltose-binding protein fusion as previously described (Kellogg and Alberts, 1992). Fixation and staining of cells with antibodies was carried out as previously described (Pringle et al., 1991). For the data shown in Fig. 4 *B*, a short spindle was defined as a short, thick bar of staining ≤1.5 times the diameter of the nucleus.

Results

Identification of an Allele of the CDC11 Gene in a Screen for Mutations That Disrupt the Control of Bud Growth during Mitosis

In a genetic screen for mutations that cause an elongated bud phenotype we isolated a temperature-sensitive mutation that causes the formation of elongated buds (see Altman and Kellogg, 1997 for a description of the genetic screen). When cells carrying this mutation are grown at 23°C they appear wild type, but at 30°C, the cells display a highly elongated bud phenotype that is identical to the phenotype seen for other mutations that disrupt the mitotic control of bud growth (Fig. 1). The mutant cells also display an elongated bud phenotype when grown at 37°C but are inviable. To clone the gene corresponding to this mutation, a low copy plasmid library was used to rescue both viability and the elongated bud phenotype at 37°C, and two independent rescuing plasmids were identified. Partial sequencing of these plasmids indicated that both plasmids contained a common ORF corresponding to the *CDC11* gene. Meiotic linkage and complementation analysis confirmed that the mutation is allelic to the *CDC11* gene (see Materials and Methods).

The Septins Bind to Gin4

The elongated bud phenotype caused by septin mutations suggests that the septins function in the pathway used by Clb2, Nap1, and Gin4 to control bud growth during mitosis (Kellogg and Murray, 1995; Altman and Kellogg, 1997). Further evidence for such a role has come from affinity chromatography experiments that we are using to identify proteins that interact with the Gin4 kinase. For these experiments, we use a purified Gin4-GST fusion protein to construct a Gin4 affinity column. An extract made from log phase yeast cells is loaded onto the Gin4 affinity column, and the column is eluted with a 0.2–1 M KCl gradient. We found that a number of proteins bind to the Gin4 affinity column (Fig. 2 *A*), whereas no proteins were detected binding to a GST control column (not shown). To begin to characterize the proteins that bind to Gin4, we have used mass spectrometry to identify the major pro-

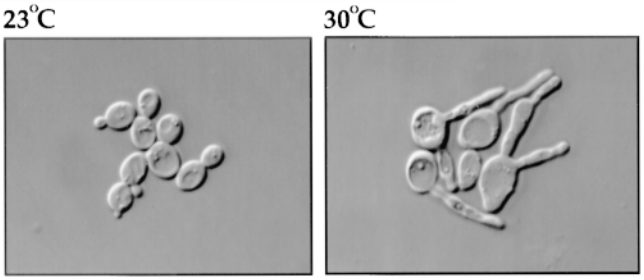

Figure 1. The temperature-sensitive elongated bud phenotype of the mutant strain identified in a screen for mutations that cause the formation of elongated buds. The mutant strain was grown overnight to log phase at room temperature and then either left at room temperature or shifted to 30°C for 6 h and photographed by Nomarski optics.

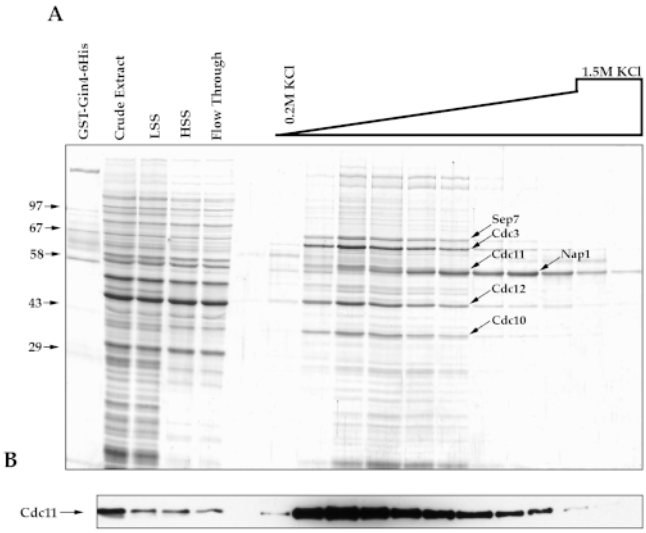

Figure 2. (*A*) Affinity purification of Gin4 binding proteins. The lane marked GST-Gin4-6His shows the purified fusion protein used to construct a Gin4 affinity column. The major band migrating above the 97-kD marker is the full-length fusion protein, while the other bands represent breakdown products. A Gin4 affinity column was loaded with a crude extract made from log-phase yeast cells, washed with buffer, and then eluted with a salt gradient. Eluted fractions were precipitated with TCA, resuspended in gel sample buffer, and then resolved on a 12.5% SDS-polyacrylamide gel as previously described (Kellogg et al., 1995). The gel is stained with Coommassie blue. *LSS*, the low speed supernatant (12,000 *g*, 5 min); *HSS*, the high speed supernatant that was loaded onto the column (100,000 *g*, 90 min); *Flow Through*, shows the extract after it passed through the column. (*B*) A Western blot confirming that the Cdc11 septin binds to the Gin4 affinity column. The same elution fractions shown in *A* were subjected to Western blotting using an anti-Cdc11 antibody. For the Western blot, we loaded only 1/10 the amount of sample that was loaded onto the gel shown in *A* so that the signal would not be too strong.

teins that elute from the affinity column. We found that one of these proteins is Nap1, as expected from previous experiments in which we demonstrated a tight and specific interaction between Gin4 and Nap1 (Altman and Kellogg, 1997). The other major proteins are members of the septin family, including Cdc3, Cdc10, Cdc11, Cdc12, and a new member of the septin family identified by the yeast genome sequencing project that we have named Sep7 (ORF designation YDL225W, see below). Western blotting confirmed that the Cdc11 septin binds to the Gin4 affinity column (Fig. 2 *B*). Note that the septins co-elute, suggesting that they associate with each other as a complex in the same way that has been observed for the *Drosophila* septins (Field et al., 1996). When a crude extract from a *Δnap1* strain is loaded onto a Gin4 affinity column the septins are still found to bind, demonstrating that Nap1 is not required for septin binding (not shown). Since the septins are the major proteins that bind to the Gin4 affinity column it is likely that they do so directly. Immunofluorescence experiments have demonstrated that Gin4 is localized to the bud neck in a septin-dependent manner, consistent with a direct interaction between these two proteins (Longtine et al., 1998; Carroll, C., and D. Kellogg, unpublished observations).

The Septins Are Required for the Mitosis-specific Activation of the Gin4 Kinase

The septins are amongst the major proteins that bind to Gin4, and thus are likely to be closely involved in Gin4 function. We therefore tested whether septin function is required for the mitosis-specific activation of Gin4. In previous work, we demonstrated that the Gin4 kinase is activated during mitosis by hyperphosphorylation, which leads to a shift in the electrophoretic mobility of Gin4 that can be detected by Western blotting (Altman and Kellogg,

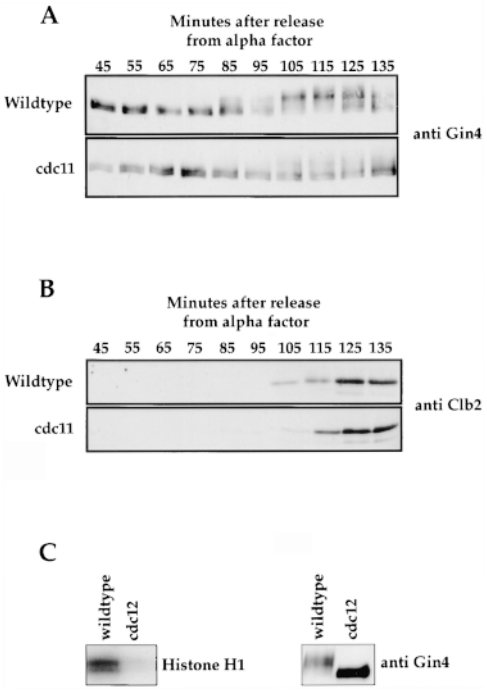

Figure 3. (*A*) Septin function is required for the mitosis-specific activation of the Gin4 kinase. Wild-type and *cdc11* cells were arrested in G1 by the addition of α-factor, and were then released from the arrest to begin the cell cycle at the restrictive temperature for *cdc11*. At each of the indicated time points, samples were taken and the mitosis-specific activation of Gin4 was assayed using Western blotting to detect the hyperphosphorylation of Gin4 that leads to activation. (*B*) The mitotic cyclin Clb2 appears with normal kinetics in cells lacking septin function. Wild-type and *cdc11* cells were synchronized and released at the restrictive temperature as in Fig. 3 *A*, and the appearance of the Clb2 cyclin was assayed by Western blotting. This experiment was carried out independently of the experiment shown in Fig. 3 *A*, and the cells entered mitosis slightly later than the cells shown in Fig. 3 *A*. (*C*) Septin function is required for the activation of Gin4 kinase activity. Log phase cultures of wild-type and *cdc12-6* were arrested in mitosis at 37°C with 20 μg/ml nocodazole and 30 μg/ml benomyl for 2.5 h. Gin4 was then immunoprecipitated and assayed for its ability to phosphorylate histone H1 in vitro (*left*). In addition, Western blotting was used to assay Gin4 hyperphosphorylation in each sample (*right*). Similar results were obtained using the allele of *cdc11* that we isolated in our screen.

1997). The mitosis-specific activation of Gin4 can therefore be conveniently assayed by Western blotting. To determine whether the septins play a role in Gin4 activation, we synchronized *cdc11* and wild-type cells in G1 with the mating pheromone α-factor. The cells were then released from the cell cycle block at the restrictive temperature and Gin4 activation was assayed as the cells progressed through a single cell cycle. We found that the mitosis-specific activation of Gin4 fails to occur in the *cdc11* mutant strain that we isolated in our genetic screen (Fig. 3 *A*). We obtained similar results using the *cdc12-6* mutation (not shown). The Clb2 protein appears with normal kinetics demonstrating that the lack of Gin4 activation in septin mutants is not due to a failure to enter mitosis (Fig. 3 *B*).

To confirm that Gin4 kinase activity is dependent upon septin function, we used microtubule inhibitors to arrest *cdc12-6* and wild-type cells in mitosis at the restrictive temperature for 2.5 h. We then immunoprecipitated Gin4 and assayed its kinase activity in vitro. As expected, the Gin4 protein from wild-type cells displays high kinase activity, consistent with previous work demonstrating that Gin4 is active during mitosis (Altman and Kellogg, 1997). In contrast, the Gin4 protein from septin mutant cells shows dramatically reduced kinase activity in vitro. Western blotting of these samples confirms that the Gin4 protein is hyper-phosphorylated in the control cells, but not in the *cdc12-6* cells (Fig. 3 *C*). These results demonstrate that the mitosis-specific activation of Gin4 is dependent upon septin function in vivo.

Loss of Septin Function Causes a Mitotic Delay

Loss of function of Gin4 or Nap1 in a Clb2-dependent strain causes cells to undergo a prolonged delay in mitosis at the short spindle stage with high levels of the Clb2 cyclin (Kellogg and Murray, 1995; Altman and Kellogg, 1997). To determine whether the same is true for septin mutants, we grew *cdc12-6* and wild-type cells to log phase at the permissive temperature and then shifted the cells to the restrictive temperature and added α-factor. Since α-factor can only act to arrest cells during G1, cells that are able to pass through mitosis will become arrested when they arrive in G1 and will contain no Clb2 cyclin, while cells that arrest in mitosis will be unaffected by the α-factor and will remain in mitosis with high levels of the Clb2 cyclin. One can therefore determine whether cells arrest in mitosis at the restrictive temperature by following Clb2 levels after the addition of α-factor. We found that the Clb2 protein in wild-type cells rapidly disappears after addition of α-factor, as expected. In contrast, Clb2 protein levels in the *cdc12-6* cells increase slightly during the time course and then remain constant, consistent with many of the cells entering a mitotic arrest (Fig. 4 *A*). To determine the stage of mitosis at which the septin mutant cells arrest, we assayed the percentage of cells with a mitotic spindle as a function of time after the addition of α-factor. The wild-type cells rapidly arrest in G1 with no mitotic spindle, whereas 60% of the cells carrying the *cdc12-6* mutation arrest with a short mitotic spindle (Fig. 4 *B*). We observed a similar mitotic delay with elevated Clb2 levels and short spindles using the allele of *cdc11* identified in our screen (not shown). Previous analysis of septin mutants has shown they form multibudded, multinucleate cells after prolonged incubation at restrictive temperatures, suggesting that they are eventually able to break through the mitotic arrest (Hartwell, 1971).

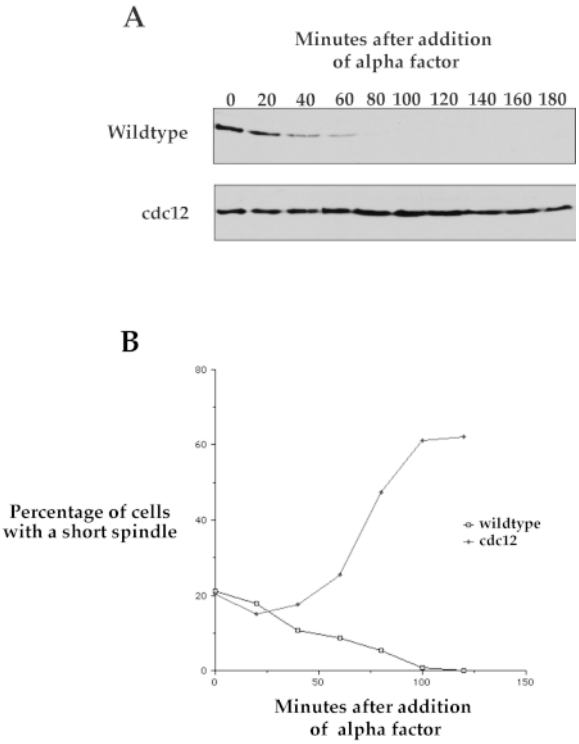

Figure 4. (*A*) Loss of septin function results in a mitotic delay. Log phase cultures of *cdc12-6* and wild-type cells were grown at 23°C and were then shifted to 33°C and α-factor was added. Samples were taken at the indicated time points after the addition of α-factor, and Western blots were performed using anti-Clb2 antibodies. (*B*) Septin mutants arrest at the short spindle stage of mitosis. A time course was carried out as described for *A*, except that samples were taken at each time point and cells were stained with an anti-tubulin antibody. The percentage of cells with a short spindle at each time point was quantitated, counting at least 200 cells for each time point.

Characterization of Sep7, a Novel Septin Involved in Mitotic Control

One of the proteins that binds to a Gin4 affinity column is a previously uncharacterized septin that we have named Sep7. The major budding yeast septins range in size from 322 to 551 amino acids in length; however, all contain a conserved central region that includes a predicted GTP-binding domain and a predicted coiled-coil domain. An alignment of the conserved central region of Sep7 with the analogous regions of the other major yeast septins is shown in Fig. 5. Sep7 differs from the other septins in that it has a COOH-terminal extension of ~200 amino acids that shows no homology to any known proteins.

To determine whether Sep7 is colocalized with the other members of the septin family at the bud neck, we generated a version of Sep7 tagged with three tandem copies of the HA epitope. We found that the Sep7-HA fusion pro-

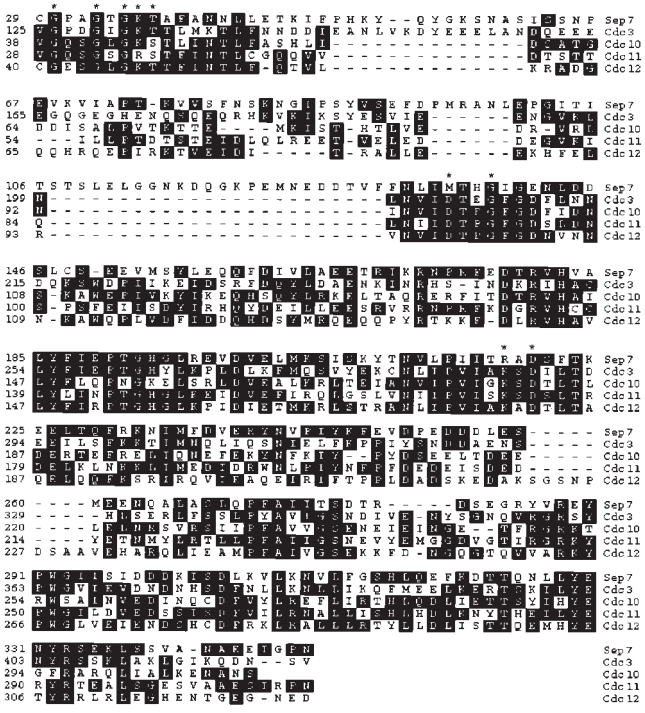

Figure 5. An alignment of the septins expressed in vegetative budding yeast cells. Conserved residues found in the GTP-binding domains G1, G3, and G4 are marked with asterisks (for a discussion of GTP-binding consensus sequences see Bourne et al., 1991).

tein colocalizes with Cdc11 (Fig. 6 *A*). To learn more about the function of Sep7, we deleted the corresponding gene. We found that the *SEP7* gene is not required for viability, and that deletion of the *SEP7* gene in a wild-type strain background causes only a mild elongated bud phenotype (Fig. 6 *B*). This phenotype is similar to the phenotype caused by deletion of the genes for *NAP1* or *GIN4* in a wild-type background. In the case of *NAP1* and *GIN4*, the relatively mild phenotype caused by deletion of these genes in a wild-type background appears to be due to the existence of Nap1- and Gin4-independent pathways that work through mitotic cyclins other than Clb2. In support of this idea, deletion of the genes for either *NAP1* or *GIN4* in a genetic background where cells are dependent upon Clb2 for the control of mitosis causes a much more severe elongated bud phenotype and a prolonged mitotic delay at the short spindle stage (Kellogg and Murray, 1995; Altman and Kellogg, 1997). Interestingly, we found that deletion of the *SEP7* gene also causes a much more severe elongated bud phenotype in a Clb2-dependent background (Fig. 6 *B*). The severity of the *ΔSep7* phenotype in the Clb2-dependent background suggested that Sep7 may be required for activation of the Gin4 kinase. By assaying Gin4 hyperphosphorylation in *ΔSep7* cells, we found that Sep7 is indeed required for the full mitosis-specific activation of the Gin4 kinase, as expected from the elongated bud phenotype (Fig. 6 *C*). We observe the same result in both a wild-type background and in the Clb2-dependent background (not shown).

Discussion

In this report, we describe the use of both biochemical and genetic approaches to identify proteins that function in a pathway used by the mitotic cyclin Clb2 to control bud growth during mitosis. Both of these approaches have led to the identification of members of the septin family of proteins. We identified an allele of the Cdc11 septin in a screen for mutations that cause the formation of highly elongated buds because of a failure in the mitotic switch from polar to isotropic bud growth. In addition, we found that members of the septin family bind directly to a protein kinase called Gin4 that is required for the mitosis-specific control of bud growth. Furthermore, the septins are required in vivo for the mitosis-specific regulation of Gin4. These results demonstrate that the septins play an integral role in events that occur during mitosis.

The Septins Are Required for the Proper Control of Bud Growth during Mitosis

Four members of the septin family were originally identified in budding yeast, called CDC3, CDC10, CDC11, and CDC12 (Hartwell et al., 1970). Temperature-sensitive mutations in any of these genes cause cells to arrest with highly elongated buds and defects in cytokinesis at the restrictive temperature (Hartwell, 1971). All four of these septins are localized to the bud neck, and loss of function of any one septin causes the other septins to lose their localization at the bud neck (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991). The localization of the yeast septins and the phenotypes of septin mutations have led to the idea that the septins function in cytokinesis. Evidence from other organisms has supported this idea. For example, the *Drosophila* septins *pnut* and *sep1* are localized to the cleavage furrows of dividing cells during late anaphase, and mutations in the *pnut* gene lead to the formation of multinucleate cells in larval tissues (Neufeld and Rubin, 1994; Fares et al., 1995). A human septin called NEDD5 is also localized to cleavage furrows, and injection of anti-NEDD5 antibodies into telophase cells causes a disruption of cytokinesis in many of the injected cells (Kinoshita et al., 1997).

Although the septins appear to be required for cytokinesis, their exact role has remained unclear, and it appears that they carry out additional functions as well. For example, the budding yeast septins have been implicated in bud site establishment (Chant, 1995), formation of the mating projection (Ford and Pringle, 1991; Kim et al., 1991), sporulation-specific events (Ozsarac, 1995; Fares et al., 1996) and in the localized deposition of chitin (DeMarini et al., 1997). In addition, the septins have been observed to be localized to sites other than the cleavage furrow, including the bud tip, the cell cortex, stress fibers, neurons, and the leading edge of migrating animal cells (Neufeld and Rubin, 1994; Fares et al., 1995; Kinoshita et al., 1997). In budding yeast, septin mutations cause the formation of highly elongated buds in which the actin cytoskeleton is abnormally polarized to the bud tip, suggesting a role for the septins in bud growth control that is independent of a role in cytokinesis. In all cases, septin localization and/or function appears to be closely tied to the actin cytoskeleton.

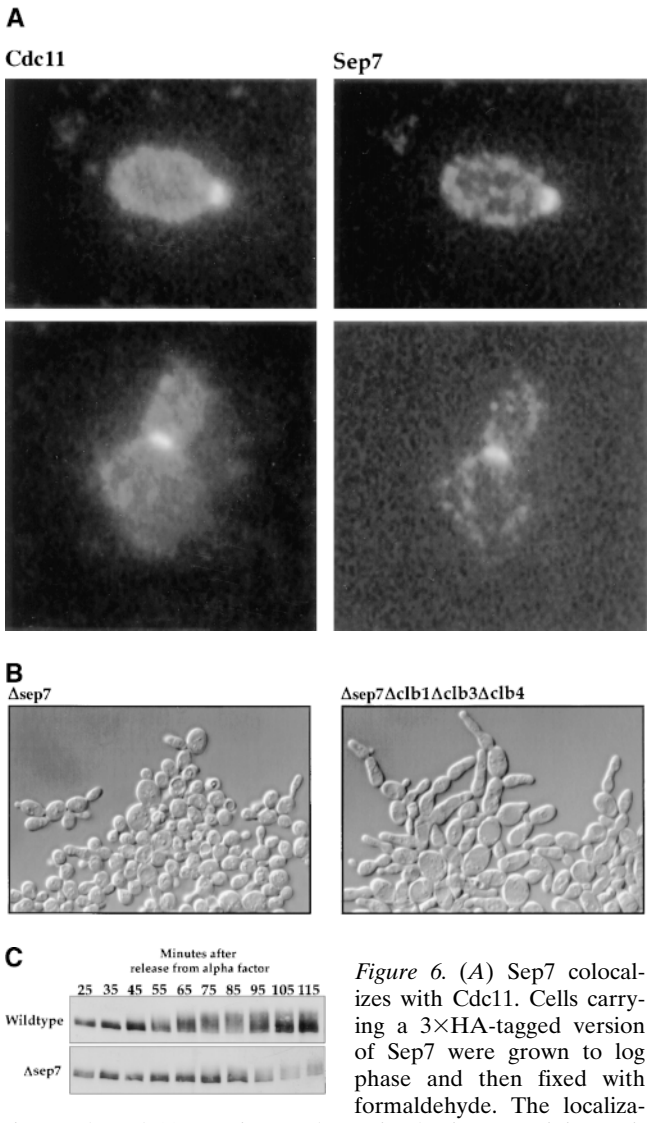

Figure 6. (*A*) Sep7 colocalizes with Cdc11. Cells carrying a 3×HA-tagged version of Sep7 were grown to log phase and then fixed with formaldehyde. The localization of the Cdc11 protein was determined using an affinity-purified polyclonal anti-Cdc11 antibody, while the localization of the Sep7 protein was determined using the 12CA5 anti-HA mAb. (*B*) Deletion of the *SEP7* gene in Clb2-dependent cells causes the formation of elongated buds. The indicated strains were grown overnight to log phase at 30°C and were then photographed with Nomarski optics. (*C*) Sep7 is required for the mitosis-specific activation of the Gin4 kinase. A wild-type strain and a *ΔSep7* strain were arrested in G1 with α-factor for 2.5 h, and then released from the arrest to begin the cell cycle at 30°C. At each of the indicated time points, samples were taken and the mitosis-specific activation of Gin4 was assayed by Western blotting. Both strains used in this experiment are in the Clb2-dependent background, and identical results are obtained in a wild-type background.

Our results demonstrate that the septins carry out functions early in mitosis, before cytokinesis occurs, and that they function in a pathway used by the mitotic cyclins to control polar bud growth during mitosis. Previous work has demonstrated that the mitotic cyclins initiate a pathway that leads to a reorganization of the actin cytoskeleton and a switch in the pattern of bud growth as cells enter mitosis (Lew and Reed, 1993; Kellogg and Murray, 1995; Altman and Kellogg, 1997). When a newly formed bud emerges during interphase, the actin cytoskeleton is polarized towards the bud tip where it directs growth of the bud. When the mitotic cyclins appear, they cause the actin cytoskeleton to redistribute over the entire bud surface, leading to uniform growth over the surface of the bud. The Clb2 mitotic cyclin induces this reorganization of the actin cytoskeleton by initiating a signaling pathway that includes the Gin4 kinase and a protein called Nap1. Inactivation of this pathway causes polar bud growth to continue during G2/M, leading to the formation of highly elongated buds.

A number of experimental results suggest that the septins play a critical role in the pathway that controls polar bud growth during mitosis. First, we identified an allele of the *CDC11* septin gene in a screen for mutations that inactivate the mitotic bud growth control pathway, and we found that the elongated bud phenotype caused by septin mutations is identical to the phenotype caused by mutations that inactivate other proteins that function in this pathway (Kellogg and Murray, 1995; Altman and Kellogg, 1997). Furthermore, we found that members of the septin family bind directly to the Gin4 kinase, which plays a critical role in the pathway used by Clb2 to control bud growth. Finally, we found that septin function is required for the mitosis-specific activation of the Gin4 kinase.

The fact that the septins are required for the switch from polar to isotropic bud growth makes it difficult to make firm conclusions about the role of the septins in cytokinesis in budding yeast. In cells that fail to make this switch, the majority of the actin in the cell remains localized to the tip of the elongated bud. Since cytokinesis in budding yeast is likely to be an actin-dependent process, it is therefore possible that the cytokinesis defect observed in septin mutants is due more to an inability of cells to relocalize the actin network from the hyperpolarized bud tip to the site of cytokinesis, rather than to a direct role for the septins in the process of cytokinesis. The finding that septin mutants arrest early in mitosis at the short spindle stage adds a further complication to the interpretation of cytokinesis defects observed in septin mutations, since the cell cycle is arresting significantly before cytokinesis occurs.

The Septins Bind to Gin4 and Are Required for the Mitosis-specific Activation of Gin4 Kinase Activity

In previous work, we demonstrated that the Gin4 kinase is activated specifically during mitosis in a Nap1-dependent manner, and that Nap1 interacts with both Clb2 and Gin4, indicating that these proteins are likely to function close to each other within the cell. In this study, we have found that members of the septin family and Nap1 are the major proteins that bind to a Gin4 affinity column, and that the septins are required for the mitosis-specific activation of Gin4. There are five members of the septin family expressed during vegetative growth in budding yeast, and we found that all of these bind to the Gin4 affinity column, including Cdc3, Cdc10, Cdc11, Cdc12, and a previously uncharacterized member of the septin family that we have named Sep7. In both *Drosophila* and budding yeast, members of the septin family have been found together in tight

complexes (Field et al., 1996) (Frazier, J., submitted manuscript), and the fact that the yeast septins co-elute from the Gin4 affinity column suggests that they do so as a complex. We found that the septins bind to Gin4 in a Nap1-independent manner, and that the septins are the major proteins that bind to the Gin4 affinity column. In addition, Gin4 and the septins are colocalized within the cell, and Gin4 localization is disrupted in *cdc12* mutant cells at the restrictive temperature (Longtine et al., 1998; Carroll, C., and D. Kellogg, unpublished observations). Taken together, these observations strongly suggest that Gin4 binds directly to the septins.

The exact role played by the septins in the mitosis-specific activation of Gin4 is unclear at this point. One possibility is that the septins function to provide a scaffold for the localization and assembly of a multiprotein complex that regulates Gin4 activity. Another possibility is that the septins play a more direct role in the regulation of Gin4 kinase activity. Since the septins are GTP-binding proteins, it is possible that GTP binding and hydrolysis may play an important role in the regulation of Gin4. Interestingly, Gin4 and the septins were both identified as mutations that are synthetically lethal with deletions of the G1 cyclins *CLN1* and *CLN2* (Benton et al., 1993; Cvrckova and Nasmyth, 1993). Although the basis for the lethality is unknown, this finding supports a role for Gin4 and the septins in the control of cell cycle events.

Sep7 Appears to Function Specifically in a Clb2-dependent Mitotic Control Pathway

In addition to Cdc3, Cdc10, Cdc11, and Cdc12, we found that a fifth member of the septin family binds to a Gin4 affinity column. This septin was originally identified by the yeast genome sequencing project as ORF YDL225W, and we have given it the name Sep7. We found that Sep7 is required in vivo for the mitosis-specific activation of the Gin4 kinase. We also found that deletion of the *SEP7* gene in a wild-type background gives essentially no phenotype, whereas deletion of the *SEP7* gene in cells that are dependent upon the Clb2 mitotic cyclin for survival causes a strong elongated bud phenotype. Previous work has demonstrated that both Gin4 and Nap1 have similar genetic interactions with Clb2, and that Nap1 binds specifically to Clb2 (Kellogg et al., 1995; Altman and Kellogg, 1997). One interpretation of these results is that Sep7, Gin4, and Nap1 all function in a pathway that is specifically initiated by Clb2 to control polar bud growth during mitosis, and that the other mitotic cyclins are able to control polar bud growth through redundant pathways that do not require the function of Sep7, Gin4, and Nap1. The fact that loss of function of Cdc3, Cdc10, Cdc11, or Cdc12 causes a severe elongated bud phenotype in wild-type cells that contain all of the mitotic cyclins suggests that all pathways that control polar bud growth during mitosis ultimately work through the septins. An important function of Sep7 may therefore be to help link the Gin4/Nap1 pathway to the other septins. We have found that Gin4 remains localized to the bud neck in *sep7* cells, suggesting that the role of Sep7 in Gin4 activation is not merely to localize Gin4 to the bud neck (Carroll, C., and D. Kellogg, unpublished results).

Septin Function Is Required for Progression through Mitosis

We found that septin mutations cause cells to undergo a prolonged delay in mitosis, demonstrating that septin function is required for proper progression through mitosis in budding yeast. This delay occurs early in mitosis, considerably before cytokinesis occurs. Loss of function of Nap1 or Gin4 can also cause prolonged delays at the short spindle stage, consistent with these proteins being involved in a pathway that includes the septins (Kellogg and Murray, 1995; Altman and Kellogg, 1997). Two general possibilities exist for the nature of this mitotic delay. First, the septins, Gin4, and Nap1 may be directly involved in events essential for the assembly or function of the mitotic spindle. A second possibility is that septin function may be monitored by a checkpoint that causes the cell cycle to arrest early in mitosis at the short spindle stage when septin function is impaired. It is known that a checkpoint monitors the ability of cells to polarize bud growth in G1 and delays entry into mitosis in the absence of bud emergence (Lew and Reed, 1995*a*). Similarly, a mitotic checkpoint may monitor the ability of cells to depolarize growth to ensure the redistribution of actin from the bud tip to other locations where it is required. Therefore, general mechanisms may exist that monitor nuclear and morphological states to ensure the coupled progression of the two through the cell cycle.

The mitotic arrest exhibited by septin mutants is not terminal because septin mutants form cells with multiple nuclei, indicating that cells are eventually able to overcome the arrest and divide their DNA (Hartwell, 1971). It seems likely that cells eventually try to undergo cytokinesis after the long delay, but the failure to carry out cytokinesis properly may then be a secondary defect that results from an earlier defect caused by a loss of septin function. Although the basis for the mitotic arrest displayed by septin mutants remains to be elucidated, our results demonstrate that septin function is clearly essential for the proper control of events that occur during mitosis, including the activation of Gin4 and the switch from polar to isotropic bud growth. Since the septins and Nap1 are both required for the mitosis-specific activation of Gin4, it appears that Gin4 activation is a complex event involving a number of different proteins. Identification and characterization of additional proteins that function in the pathway used by Clb2 to control bud growth should lead to a better understanding of the mitosis-specific activation of Gin4 and the mechanisms used by cyclin-dependent kinases to control mitotic events.

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