NAP1 Acts with Clb2 to Perform Mitotic Functions and to Suppress Polar Bud Growth in Budding Yeast

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Abstract. NAP1 is a 60-kD protein that interacts specifically with mitotic cyclins in budding yeast and frogs. We have examined the ability of the yeast mitotic cyclin Clb2 to function in cells that lack NAP1. Our results demonstrate that Clb2 is unable to carry out its full range of functions without NAP1, even though Clb2/$p34^{CDC28}$-associated kinase activity rises to normal levels. In the absence of NAP1, Clb2 is unable to efficiently induce mitotic events, and cells undergo a prolonged delay at the short spindle stage with normal levels of Clb2/$p34^{CDC28}$ kinase activity. NAP1 is also required for the ability of Clb2 to induce the switch from polar to isotropic bud growth. As a result, polar bud growth continues during mitosis, giving rise to highly elongated cells. Our experiments also suggest that NAP1 is required for the ability of the Clb2/$p34^{CDC28}$ kinase complex to amplify its own production, and that NAP1 plays a role in regulation of microtubule dynamics during mitosis. Together, these results demonstrate that NAP1 is required for the normal function of the activated Clb2/$p34^{CDC28}$ kinase complex, and provide a step towards understanding how cyclin-dependent kinase complexes induce specific events during the cell cycle.

THE cyclins are a large family of related proteins that were originally identified by their cyclic appearance during the cell cycle (Evans et al., 1983) and were later shown to play a central role in cell cycle control. Cyclins associate with members of a family of catalytic subunits called cyclin-dependent kinases to form active kinase complexes that induce the events of the cell cycle (for review see Murray and Hunt, 1993; Norbury and Nurse, 1992; Nasmyth, 1993). Mitosis is induced by kinase complexes that contain B-type cyclins and a cyclin-dependent kinase known as $p34^{CDC28}$ in budding yeast and $p34^{cdc2}$ in fission yeast and other eukaryotes. This activity is often referred to as maturation or M-phase promoting factor (MPF).[1] In budding yeast, there are four different B-type cyclins that appear during mitosis, called Clb1, Clb2, Clb3, and Clb4, while in vertebrates there are three B-type cyclins called cyclins B1, B2, and B3 (Gallant and Nigg, 1994; Pines, 1993). Additional members of the cyclin family appear earlier in the cell cycle and activate $p34^{cdc2}$ or $p34^{cdc2}$-like proteins to induce passage through Start and the events of S phase (for review see Reed, 1992).

How do cyclin-dependent kinases induce mitotic events? It is generally assumed that cyclin-dependent kinases act to phosphorylate a range of substrates, which thereby become activated to induce the events of mitosis. However, this simple model raises a number of problems. First, although many proteins have been identified that are substrates of cyclin-dependent kinases in vitro, it has been very difficult to establish unambiguously that any of these are also in vivo substrates (Moreno and Nurse, 1990; Nigg, 1993). Second, the substrate specificity of cyclin B/$p34^{cdc2}$ kinase complexes overlaps with that of MAP kinases, which are also thought to be activated during mitosis, making it difficult to determine which kinase phosphorylates a given substrate in vivo (Gotoh et al., 1991; Heider et al., 1994; Minshull et al., 1994; Nigg, 1993; Peter et al., 1992). Third, the $p34^{CDC28}$ catalytic subunit is also activated during interphase by association with the G1 class of cyclins (called Cln proteins in budding yeast), but induces events that lead to DNA replication and budding rather than mitosis. It is unclear how the same catalytic subunit is able to induce different events when activated at different times during the cell cycle.

In the preceding paper, we used affinity chromatography to purify proteins that interact specifically with B-type cyclins, with the goal of identifying proteins that play a role in the ability of the B-type cyclins to induce mitotic events. We identified a 60-kD protein called NAP1 that interacts specifically with B-type cyclins. Evolution has conserved this interaction, since NAP1 homologs in both budding yeast and *Xenopus* interact specifically with members of the cyclin B family. NAP1 in budding yeast interacts

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1. *Abbreviation used in this paper*: MPF, M-phase promoting factor.

with Clb2, and deletion of the *NAP1* gene in a strain that is dependent upon Clb2 for survival produces cells that are temperature sensitive. Even at a permissive temperature, these cells form large clumps of interconnected and highly elongated cells. This phenotype is largely suppressed by the presence of other members of the Clb family that can compensate for a lack of Clb2 function, indicating that the phenotype of these cells is due to a defect in Clb function.

We have attempted to determine how NAP1 participates in the function of Clb2. The functions of B-type cyclins include associating with $p34^{cdc2}$ during mitosis to form an active kinase complex, and acting as substrates for specific proteolysis at the end of mitosis (for review see Nasmyth, 1993; Norbury and Nurse, 1992). In budding yeast, Clb2 is known to be involved in the assembly of the mitotic spindle, stimulation of *CLB2* transcription in a positive feedback loop, repression of *CLN2* transcription, and suppression of polarized bud growth (Amon et al., 1993; Lew and Reed, 1993; Richardson et al., 1992). B-type cyclins have also been implicated in the control of microtubule stability in *Xenopus* embryo extracts (Verde et al., 1992). By examining the role of NAP1 in cells that are dependent on Clb2, we show that NAP1 plays important roles in suppressing polarized bud growth and other events induced by activation of $Clb2/p34^{CDC28}$ kinase complexes.

Materials and Methods

Buffers and Media

LPC Protease Inhibitor: 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml chymostatin dissolved in dimethylsulfoxide. Lysis buffer: 50 mM Hepes, pH 7.6, 1 M KCl, 1 mM EGTA, 0.2% Tween-20, 1 mM PMSF, 2× LPC protease inhibitor mix. Wash buffer: 50 mM Hepes, pH 7.6, 1 mM EGTA, 0.1% Tween-20, 1× LPC protease inhibitor mix. H1 Kinase Assay buffer: 50 mM Hepes, pH 7.6, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 300 μM ATP, 50 μg/ml histone H1, 0.1 mCi/ml [γ^{32}P]ATP (10 mCi/ml, 3,000 Ci/mmol). 4× Sample buffer: 260 mM Tris-HCl, pH 6.8, 12% sodium dodecyl sulfate (SDS), 20% β-mercaptoethanol, 40% glycerol, bromphenol blue. NETS buffer: 10 mM Tris (pH 7.5), 0.3 M NaCl, 1 mM EDTA, 0.2% SDS. YPD media is prepared as previously described (Sambrook et al., 1989).

Cell Cycle Synchronization

Cells are grown overnight at room temperature to an optical density of 0.3–0.8, and are then diluted down to an OD of 0.3 (strains AFS34, DK131, and K2652) or 0.35 (DK97). Alpha factor (Bio-Synthesis, Lewisville, TX) is then added to 20 μg/ml, and the cells are incubated at room temperature for 3–3.25 h. This causes greater than 90% of the cells to arrest with schmoos in G1. The α factor is removed by washing the cells twice with YPD media at room temperature, and the cells are shifted to 30°C 10 min after removal of the α factor. All time courses were carried out at 30°C, with the exception of the benomyl time course shown in Fig. 10, which was carried out at 25°C because benomyl gives a better arrest at lower temperatures.

Antibodies, Immunoprecipitation, and H1 Kinase Assays

Antibodies that recognize the Clb2 protein were raised by immunizing rabbits with a Clb2-GST fusion protein, and were affinity purified as previously described (Kellogg and Alberts, 1992).

Yeast cultures are arrested in G1 with α factor as described above. At each time point after release from the arrest, 1.6 ml of culture are removed and the cells are pelleted for 1 min in a microfuge at room temperature. After removal of the supernatant, the cell pellet is frozen in liquid nitrogen.

To each tube containing frozen cells, 150 μl of acid washed beads are added, followed by 170 μl of lysis buffer. The tubes are then placed in a Biospec MultiTube bead beater for 50 s at top speed. The tubes are removed and cooled in an ice water bath before a second pulse of 50 s. The lysed cells are spun for 5 min in a microfuge at top speed, and 65 microliters of the supernatant is transferred to a 0.5-ml microfuge tube on ice. 1 μg of anti-Clb2 antibody is added to each tube, and the tubes are incubated on ice for 1 h. A slurry of protein A beads in lysis buffer is added to each tube to give 10 μl of beads/tube, and more lysis buffer is added to give a final volume of 300 μl. The tubes are mixed on a rotator at 4°C for 1 h, and the beads are then washed twice with lysis buffer and twice with wash buffer. The beads are transferred to a fresh tube after the third wash. After the final wash, the supernatant is completely removed and 20 μl of H1 kinase assay buffer is added. The tubes are vortexed gently and incubated at 30°C for 30 min, with gentle vortexing every 10 min. The reaction is stopped by the addition of 10 μl of 4× gel sample buffer, and 10 μl are loaded onto each lane of a 15% polyacrylamide-SDS gel. H1 kinase activity is quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) to scan the dried polyacrylamide gel.

For Western blotting, 15 μl of the lysate used for immunoprecipitation are diluted into 200 μl of 10% TCA and incubated on ice for 10 min. The precipitate is collected by centrifugation in a microfuge for 5 min, the supernatant is removed, and 40 μl of 1× gel sample buffer is added. The samples are neutralized with the vapors from a cotton tip soaked in NH_4OH and then incubated at 100°C for 3 min. 7.5 μl of each sample are used for Western blotting.

Immunofluorescence and Measurement of Bud Growth

Staining of actin, tubulin, and DNA was carried out as previously described (Pringle et al., 1991). To measure bud growth, we used a video camera and monitor to generate large images of cells, and then measured the ratio of the bud length to width in 20 randomly chosen cells at each time point. The length of the bud was measured from the bud neck to the bud tip, while the width was measured as the longest distance between the cell walls along a line perpendicular to the line connecting the bud neck to the bud tip.

Northern Blotting, Western Blotting, and Polyacrylamide Gel Electrophoresis

To prepare total yeast RNA, we used a scaled down version of the procedure described by Cross and Tinklenburg (1991). At each time point during the cell cycle a 1.6-ml aliquot of culture is taken, the cells are pelleted, and the sample is frozen on liquid nitrogen. Added to each frozen sample is 150 μl of acid–washed beads, 350 μl phenol/chloroform, and 350 μl of NETS buffer. The samples are then immediately placed in a Biospec Multibeater-8 at full speed for 2 min. The samples are spun at room temperature in a microfuge for 5 min, and 300 μl of the aqueous phase are transferred to a new tube. The RNA is precipitated by addition of 1 ml of ethanol, followed by a 5-min spin in a microfuge. After removal of the supernatant, 40 μl of TE buffer containing 0.2% SDS is added to each pellet, and the samples are incubated at 65°C for 10 min. Formaldehyde-agarose gel electrophoresis and Northern blotting are carried out as previously described (Ausubel et al., 1987; Cross and Tinkelenberg, 1991). We load approximately 1/10 of each sample on each lane.

Polyacrylamide gel electrophoresis and Western blotting were done as previously described (Anderson et al., 1973; Harlow and Lane, 1988; Towbin et al., 1979). For the Cln2 Western blot shown in Fig. 8, we prepared samples in the same way that we did for the Clb2 immunoprecipitations and Western blots.

Yeast Strains

All of the strains used in these experiments are in the W303 strain background (*MATa, his3-11, leu2-3,112, trp1-1, ura3-1, ade2-1, can1-100*). DK131 carries a deletion of the *NAP1* gene marked with *LEU2* (Kellogg et al., 1994). The Clb2-dependent strain that we used is K2652 (*clb1::URA3, clb3::TRP1, clb4::HIS3*) (Fitch et al., 1992), and DK97 is the same as K2652, but carries a deletion of the *NAP1* gene marked with *LEU2* (Kellogg et al., 1994). For the Western blots shown in Fig. 8, we integrated a triple HA tagged *CLN2* gene (Tyers and Futcher, 1993) into K2652 and DK97 using pDK31, which carries the *ADE2* marker, to produce strains DK159 and DK160, respectively.

Results

We have studied the role of NAP1 in Clb2 activities primarily in a strain that is dependent upon Clb2 for survival. Such a strain is generated by deleting the genes for the mitotic cyclins Clb1, Clb3, and Clb4, which share functional overlap with Clb2 and can compensate for an absence of Clb2 activity (Fitch et al., 1992). By studying the role of NAP1 in a Clb2-dependent background, we can focus on Clb2 function without the complication of additional B-type cyclins. Throughout this paper we refer to a strain in which only the *NAP1* gene is deleted as *Δnap1*, while a strain carrying a deletion of the NAP1 gene in a Clb2-dependent background is referred to as *Δnap1 Δclb1 Δclb3 Δclb4*.

Deletion of the NAP1 Gene Causes a Delay in the Appearance of Clb2-associated Kinase Activity

Genetic interactions demonstrate that NAP1 is required for Clb2 function (Kellogg et al., 1995). A simple explanation for this requirement would be that NAP1 is required to generate normal levels of the Clb2/p34^{CDC28} kinase complex. For example, NAP1 could function in the positive feedback loop that allows Clb2 to amplify its own transcription (Amon et al., 1993), or in the posttranslational events that lead to formation of active kinase complexes (for reviews see Murray and Hunt, 1993; Norbury and Nurse, 1992). To test these possibilities, we synchronized cells in G1 with the mating pheromone α factor, released the cells from the arrest, and took samples every 10 min. We followed accumulation of the Clb2 protein by Western blotting, and formation of active Clb2/p34^{CDC28} kinase complexes by assaying for histone H1 kinase activity in anti-Clb2 immunoprecipitates.

Fig. 1 shows the effect of deleting *NAP1* in a cell that has a full complement of mitotic cyclins. In the *NAP1* control cells, Clb2-associated H1 kinase activity peaks at 70 min after release from α factor, and then rapidly disappears, as expected. In the *Δnap1* cells, we see that Clb2-associated H1 kinase activity rises to normal levels and rapidly disappears, although the activity peaks 10 min later than in the control cells. The Western blot demonstrates that Clb2 protein levels rise with similar kinetics in the *Δnap1* strain and the control strain, and that the Clb2 protein levels remain high slightly longer in the *Δnap1* strain. These experiments demonstrate that NAP1 is not required for the activation of Clb2/p34^{CDC28} kinase activity to normal levels, or for the ability of Clb2 to stimulate *CLB2* transcription, since a defect in this pathway should lead to a defect in accumulation of the Clb2 protein. The delay in the activation of Clb2/p34^{CDC28} kinase activity seen in *Δnap1* cells may be due to a defect in the ability of the Clb2/p34^{CDC28} kinase to stimulate its own activation in a positive feedback loop. Such a positive feedback loop has been observed in the activation of cyclin B/p34^{cdc2} kinase activity in the *Xenopus* embryo (Masui and Markert, 1971; for reviews see Murray and Hunt, 1993; Norbury and Nurse, 1992). The fact that Clb2 protein levels stay high slightly longer in the *Δnap1* strain is perhaps due to the delay in the activation of H1 kinase levels: if Clb2/p34^{CDC28} kinase complexes normally carry out an important mitotic event required for the exit from mitosis, the delay in the activation of this complex would produce a corresponding delay in the destruction of Clb2, as the cells wait for the completion of this event.

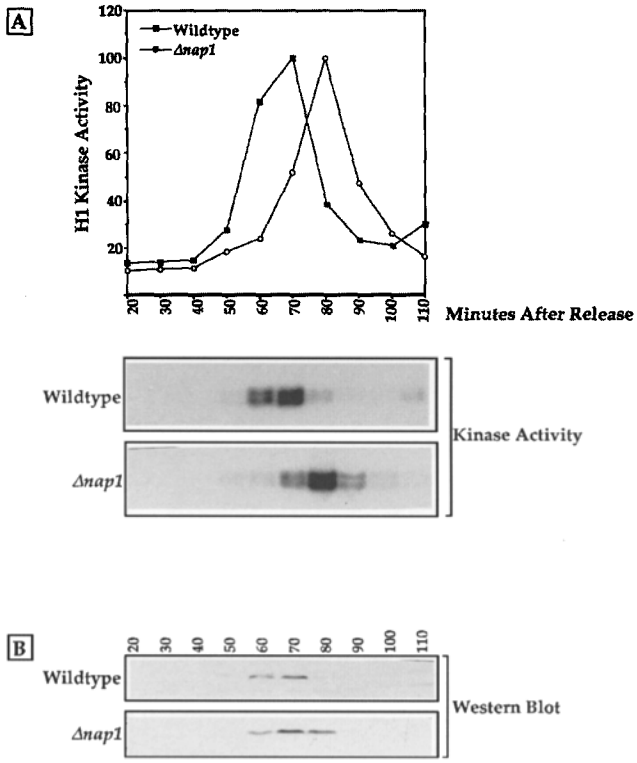

Figure 1. Behavior of the Clb2 protein and Clb2-associated kinase activity in *Δnap1* cells and isogenic control cells. (*A*) Clb2-associated kinase activity was assayed as described in the Materials and Methods section. The lower panels show an exposure of the gel used to detect histone H1 phosphorylation, while the graph shows the results of quantification of the same gel. Kinase activity is expressed as a percentage of the maximal value reached in each strain. (*B*) A Western blot showing the behavior of the Clb2 protein in the same samples used for the Clb2-associated kinase assay in *A*.

NAP1 Plays an Important Role in the Ability of the Clb2/p34^{CDC28} Kinase to Direct Mitotic Events

We next wanted to determine how deletion of the *NAP1* gene affects Clb2 behavior in cells that lack the other Clbs that can compensate for defects in Clb2 function. Fig. 2 shows Clb2 protein levels and activation of Clb2/p34^{CDC28} kinase activity during a synchronous cell cycle in a *Δnap1 Δclb1 Δclb3 Δclb4* strain and a *Δclb1 Δclb3 Δclb4* strain. As in the case of *Δnap1* cells, we find that Clb2/p34^{CDC28} kinase activity is activated to normal levels in this Clb2-dependent background, and that the kinase activity reaches peak levels slightly later than the control strain. We also see that Clb2 protein levels rise with identical kinetics. However, in the *Δnap1 Δclb1 Δclb3 Δclb4* cells, the fall in the levels of Clb2 protein and Clb2-associated kinase is profoundly delayed. By the time the control strain has passed through interphase with low Clb2 levels and entered a second mitosis, the *Δnap1 Δclb1 Δclb3 Δclb4* strain still has relatively high levels of Clb2 protein and Clb2/p34^{CDC28} kinase activity.

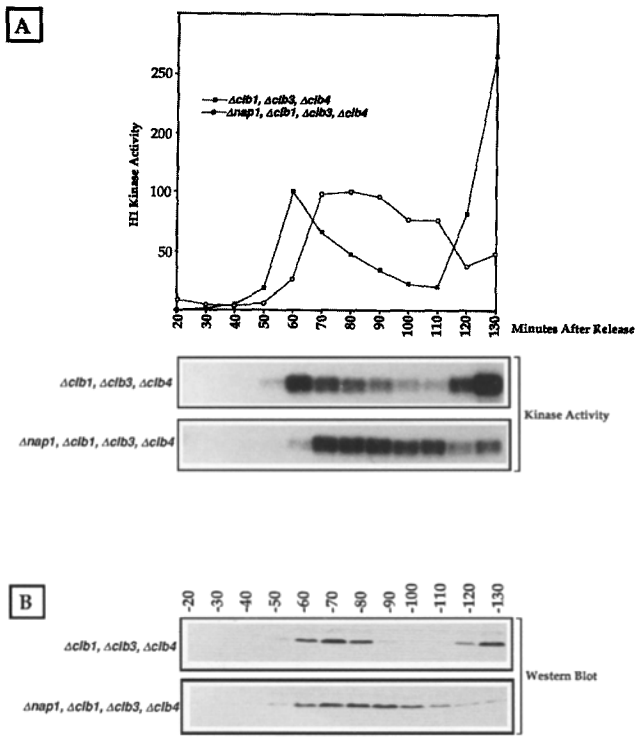

Figure 2. Behavior of the Clb2 protein and Clb2-associated kinase activity in *Δnap1 Δclb1 Δclb3 Δclb4* cells and in *Δclb1 Δclb3 Δclb4* control cells. (*A*) Clb2-associated kinase activity was assayed as described in the Materials and Methods section. The lower panels show an exposure of the gel used to detect histone H1 phosphorylation, while the graph shows the results of quantitation of the same gel. Kinase activity is expressed as a percentage of the maximal value reached during the first mitosis in each strain. In this time course, we observed that kinase levels peak at 60 min, but more often we observe kinase levels peaking at 70 min. The slight variability is probably due to the fact that the first 10 min of the time course occur at room temperature (due to the washes used to remove the α factor), and there is considerable variability in room temperature. We consistently see that CLB2-associated kinase activity does not drop as abruptly in *Δclb1 Δclb3 Δclb4* cells as it does in wild-type cells (compare Fig. 2 *A* and Fig. 1 *A*). (*B*) A Western blot showing the behavior of the Clb2 protein in the same samples used for the Clb2-associated kinase assay in *A*.

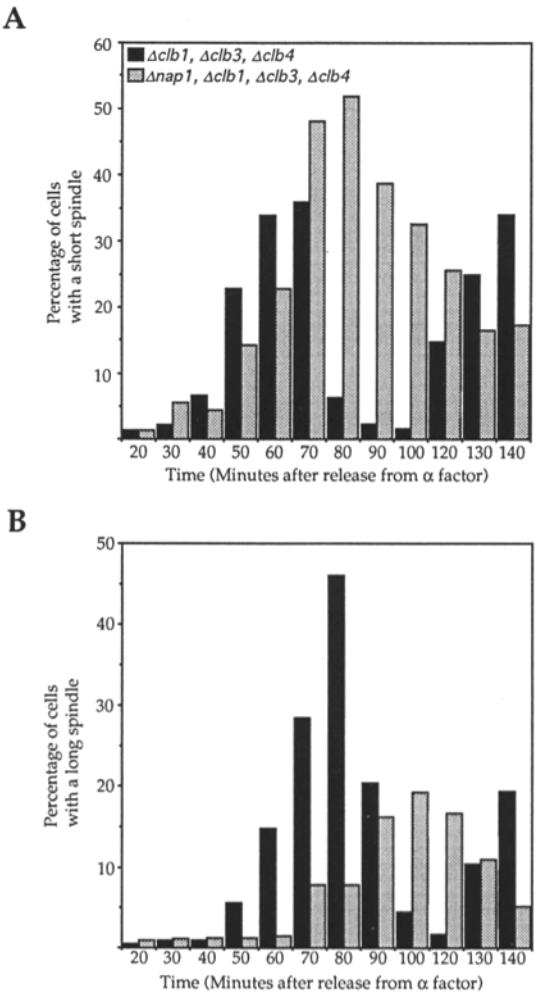

Figure 3. The progress of mitotic spindle assembly in *Δnap1 Δclb1 Δclb3 Δclb4* cells and in *Δclb1 Δclb3 Δclb4* control cells. Cells were fixed every 10 min after release from an α factor arrest, and then stained with anti-tubulin. Short spindles were defined as spindles whose length was less than 1.5× the diameter of the nucleus. Long spindles included spindles associated with dumb-bell shaped nuclei as well as spindles associated with nuclei that had already divided.

To determine where in mitosis the *Δnap1 Δclb1 Δclb3 Δclb4* cells are arresting, we stained cells at each time point with anti-tubulin to follow the progress of mitotic spindle assembly, and we then determined the percentage of cells at each time point that have either a short bipolar spindle, or an elongated anaphase spindle. The results are shown in Fig. 3. Over the course of the experiment, the control cells synchronously formed short bipolar spindles, which then converted to anaphase spindles. The cells then passed through interphase and started a second mitosis. In contrast, the *Δnap1 Δclb1 Δclb3 Δclb4* cells show a slight delay in the formation of short bipolar spindles, and then enter a prolonged delay at the short bipolar spindle stage. By the time the control cells have passed through interphase and started a second mitosis, a significant fraction of the *Δnap1 Δclb1 Δclb3 Δclb4* cells still have short bipolar spindles. The time-integrated number of cells that contain short bipolar spindles is increased 2.5-fold in the *Δnap1 Δclb1 Δclb3 Δclb4* cells, suggesting that the duration of this part of mitosis is substantially increased. The observation that the anaphase cells in the *Δnap1 Δclb1 Δclb3 Δclb4* strain are found over a longer time period than those in the *Δclb1 Δclb3 Δclb4* strain reveals that the extent of the mitotic lag in the *Δnap1 Δclb1 Δclb3 Δclb4* is rather variable, and observation of the cells during the time course suggests that some of the *Δnap1 Δclb1 Δclb3 Δclb4* cells never leave the short spindle stage.

Two observations argue strongly that the mitotic delay is not due to a requirement for NAP1 in the degradation of Clb2. First, *Δnap1* cells do not undergo a significant mitotic delay (see Fig. 1), indicating that the mitotic delay phenotype is recessive in the presence of other Clbs. Second, *Δnap1 Δclb1 Δclb3 Δclb4* cells delay at the short spindle stage rather than in anaphase. A defect in Clb2 destruction would be expected to produce a dominant anaphase arrest

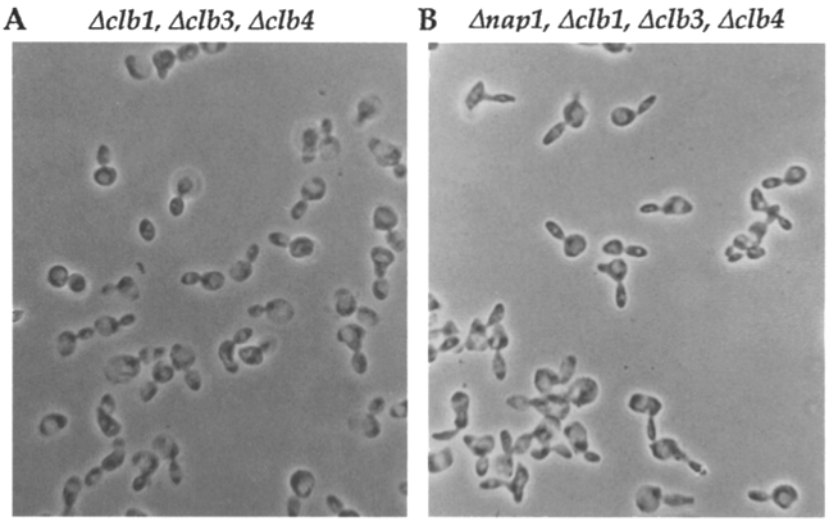

Figure 4. Bud morphology in *Δnap1 Δclb1 Δclb3 Δclb4* cells and control cells. Phase contrast views of cells 85 min after release from α factor at 30°C. The scale bar represents 20 microns.

with elongated spindles and separated chromosomes (Surana et al., 1993; Holloway et al., 1993).

NAP1 appears to be required only for progression through mitosis, as expected for a protein that interacts with B-type cyclins. For example, there is no delay in the appearance of the CLB2 protein in *Δnap1* cells (Figs. 1, 2, and 10), nor is there a delay in bud emergence (not shown), suggesting that the cell cycle progresses normally before mitosis. These observations show that NAP1 plays a specific role in the function of mitotic cyclins, rather than playing a more general role in the functions of all cyclins.

NAP1 Is Required for Clb2-induced Suppression of Polarized Bud Growth

One function of the Clb cyclins in yeast is to suppress activities induced earlier in the cell cycle by the G1 cyclins (Cln1, Cln2, and Cln3) (Amon et al., 1993; Lew and Reed, 1993). The G1 cyclins induce polarized bud growth, and when Clb1, Clb2, Clb3, and Clb4 appear later in the cell cycle they induce a switch from polarized to isotropic bud growth, giving rise to the normal oval-shaped buds seen in wildtype yeast. In the absence of Clb1, Clb2, Clb3, and Clb4, polar bud growth continues, giving rise to highly elongated cells (Amon et al., 1993; Lew and Reed, 1993; Richardson et al., 1992).

Δnap1 Δclb1 Δclb3 Δclb4 cells are highly elongated and similar in appearance to the previously described phenotype of cells that lack the activity of Clb1, Clb2, Clb3, and Clb4. We therefore tested whether NAP1 is required for Clb2 to induce the switch from polarized to isotropic bud growth by following bud growth in a population of *Δnap1 Δclb1 Δclb3 Δclb4* cells after release from alpha factor arrest. Treatment of the *Δnap1 Δclb1 Δclb3 Δclb4* cells with zymolyase after fixation breaks down the cell wall and causes the large clumps observed in this strain to break up, which allowed us to observe bud morphology easily. As expected, we found that the control cells form oval shaped buds, while the buds in the *Δnap1 Δclb1 Δclb3 Δclb4* cells continue polar growth, giving rise to elongated buds. Examples of cells from each strain 85 min after release from alpha factor are shown in Fig. 4. To quantify polar bud growth, we plotted the ratio of bud length to bud width during bud growth in sychronized populations of wild-type cells, *Δclb1 Δclb3 Δclb4* cells, and *Δnap1 Δclb1 Δclb3 Δclb4* cells (Fig. 5). These measurements demonstrate that the ratio of bud length to width in wild-type cells changes very little during the time course, indicating that there is little or no polar bud growth after bud emergence in these cells. In *Δclb1 Δclb3 Δclb4* cells, polar bud growth continues to some extent. This observation is consistent with previous experiments indicating that Clb2 is the primary cyclin responsible for inducing the switch from polarized to isotropic bud growth, while Clb1, Clb3, and Clb4 play a minor role (Lew and Reed, 1993). A dramatic result is seen in *Δnap1 Δclb1 Δclb3 Δclb4* cells, which continue strong polar bud growth throughout the

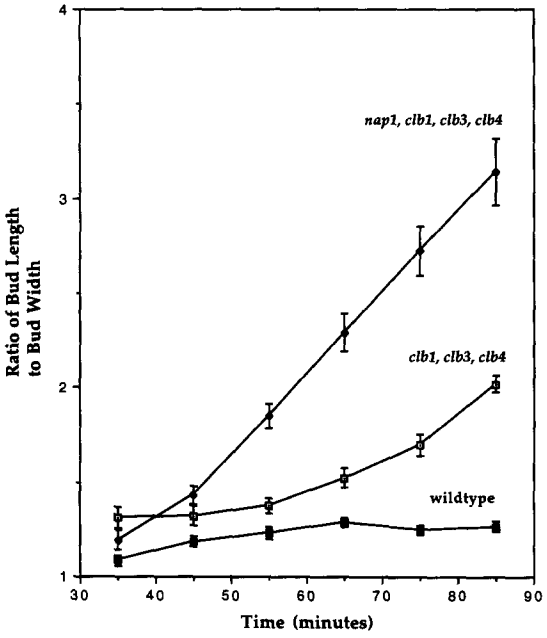

Figure 5. Polar bud growth in *Δnap1 Δclb1 Δclb3 Δclb4* cells. The ratio of bud length to bud width was measured in populations of cells going through the cell cycle after release from an α factor arrest. Beyond 85 min it became difficult to unambiguously identify buds.

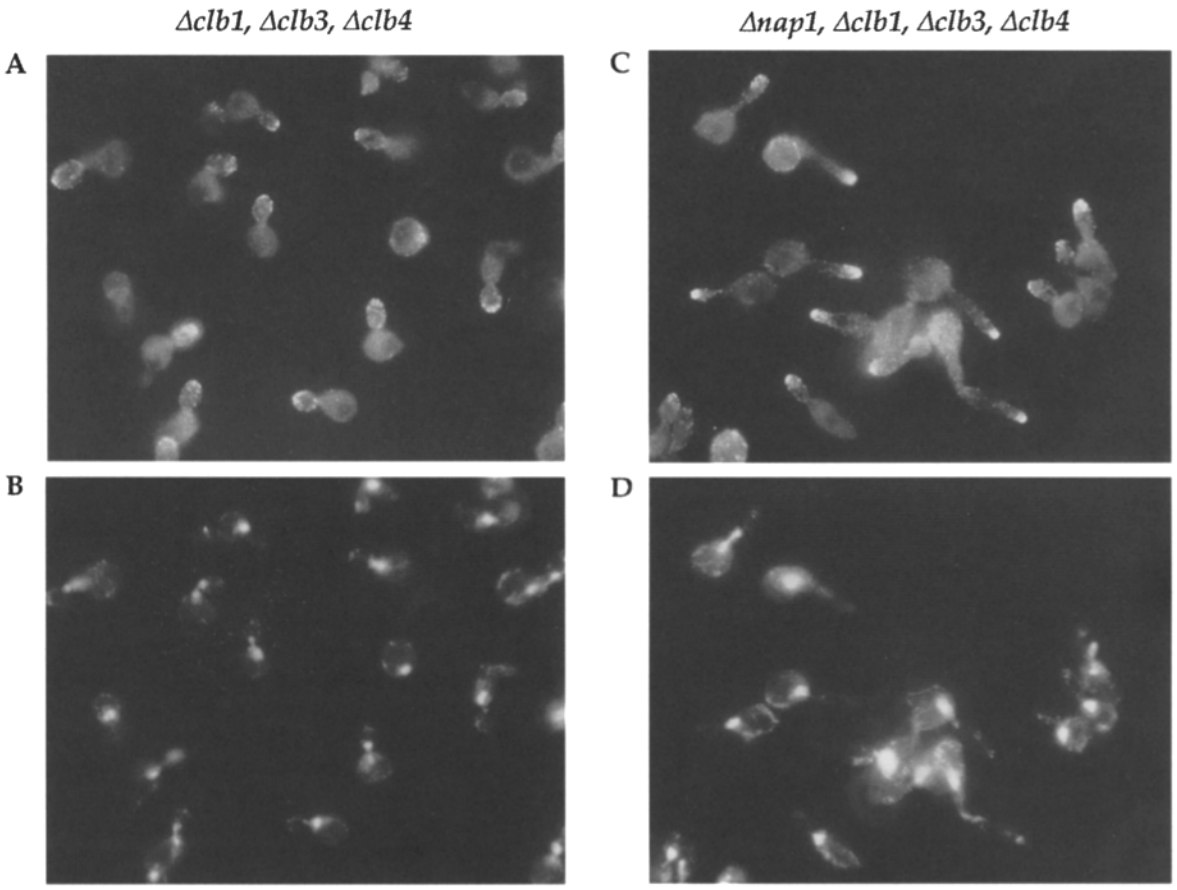

Figure 6. Polarization of the actin cytoskeleton in *Δnap1 Δclb1 Δclb3 Δclb4* cells. Cells at 75 min after release from α factor were fixed and stained with rhodamine-labeled phalloidin and the DNA-binding dye DAPI. (*A*) *Δclb1 Δclb3 Δclb4* cells stained with rhodamine-labeled phalloidin. (*B*) DNA staining in the same field shown in *A*. (*C*) *Δnap1 Δclb1 Δclb3 Δclb4* stained with rhodamine-labeled phalloidin. (*D*) DNA staining in the same field shown in *C*.

duration of the time course. Comparing the time course of bud growth with the activation of Clb2/p34^{CDC28} kinase activity (Fig. 2 *A*) reveals that polar bud growth in the *Δnap1 Δclb1 Δclb3 Δclb4* cells continues even after Clb2/p34^{CDC28} kinase levels have peaked.

We also fixed and stained the cells at each time point to determine the distribution of actin, a key element in directing the protein secretion required for bud growth (for review see Welch et al., 1994). Actin is concentrated toward the bud tip during polar growth, and then becomes delocalized over the entire surface of the bud during isotropic growth (Adams and Pringle, 1984; Kilmartin and Adams, 1984). We found that the *Δnap1 Δclb1 Δclb3 Δclb4* cells fail to depolarize their actin cytoskeleton, as expected for cells that cannot switch to isotropic growth (Fig. 6). The same phenotype is seen in cells that lack the activity of Clb1, Clb2, Clb3, and Clb4 (Lew and Reed, 1993). The most extreme examples that we observed were generally found at later time points in cells that remained arrested at the short spindle stage, indicating that polarized bud growth continues at this stage. We never observed polarization of the actin cytoskeleton in cells that have passed through anaphase, suggesting that reorganization of the actin cytoskeleton at this stage does not require NAP1. This reorganization may involve formation of the actin ring during cytokinesis, which appears to occur normally in *Δnap1 Δclb1 Δclb3 Δclb4* cells. Since the cell clumps in this strain can be broken up with enzymes that break down the cell wall, it seems likely that the clumping is due to a problem in septation, rather than in cytokinesis.

These results indicate that NAP1 is required for the ability of Clb2 to suppress polarized bud growth. A simple explanation for this observation would be that NAP1 is required for the ability of Clb2 to suppress *CLN* transcription (Amon et al., 1993), and that the continued presence of the Cln proteins causes continued polar bud growth. We tested this possibility by using Northern blotting to measure the levels of Cln2 mRNA during the cell cycle in *Δnap1 Δclb1 Δclb3 Δclb4* cells. Fig. 7 demonstrates that Cln2 mRNA levels rise and fall with nearly identical kinetics in *Δnap1 Δclb1 Δclb3 Δclb4* cells and control cells, ruling out the possibility that NAP1 is required for the ability of Clb2 to suppress *CLN2* transcription. Another possibility is that NAP1 is required for the short half life of the Cln2 protein (Wittenberg et al., 1990; Salama et al., 1994). We tested this idea by using Western blotting to follow the behavior of the Cln2 protein (Fig. 8). Like Cln2 mRNA, we find that the Cln2 protein is degraded with identical kinetics in *Δnap1 Δclb1 Δclb3 Δclb4* cells and control cells. We see a slightly higher basal level of the Cln2 mRNA and

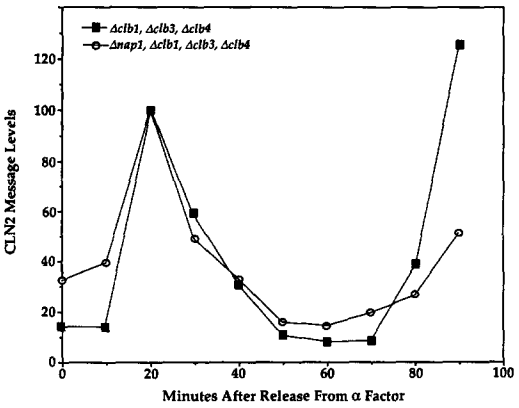

Figure 7. Behavior of the Cln2 mRNA in *Δnap1 Δclb1 Δclb3 Δclb4* cells and *Δclb1 Δclb3 Δclb4* control cells. A Northern blot was carried out using RNA samples from cells going through the cell cycle after release from an α factor arrest, and the amount of Cln2 message at each time point was quantified using a phosphorimager. Cln2 levels are expressed as a percentage of the maximal value reached by each strain. The absolute levels of the Cln2 mRNA are always similar in the two strains.

the Cln2 protein in the *Δnap1 Δclb1 Δclb3 Δclb4* cells. This is probably due to a greater degree of asynchrony in these cells, which have a considerably longer cell cycle time and do not give as tight an arrest with α factor. The low basal level of unregulated Cln expression is unlikely to cause the polarized growth phenotype, since Clns must be expressed at very high levels from the galactose promoter to cause morphological defects (Lew and Reed, 1993; Lew, D., personal communication). The most likely explanation for the continued polar growth of *Δnap1 Δclb1 Δclb3 Δclb4* cells is that the Cln proteins initiate a polarized budding program that can continue even in the absence of Cln/p34^{CDC28} activity, and that NAP1 is required for Clb2 to turn this program off.

NAP1 May Play a Role in Controlling Microtubule Stability

Studies on animal cells have demonstrated that entry into mitosis is accompanied by a dramatic change in microtubule dynamics. During interphase, microtubules are relatively long and stable, whereas in mitosis microtubules become short and very unstable (Belmont et al., 1990; Verde et al., 1990, 1992). This transition is thought to allow the rapid remodeling of the interphase microtubule array into the mitotic spindle. Adding sea urchin cyclin B to interphase *Xenopus* embryo extracts destabilizes microtubules, whereas addition of bovine cyclin A does not (Verde et al., 1992), even under conditions where the two cyclins cause a similar increase in H1 kinase activity. These results suggest that B-type cyclins play a specific role in regulating microtubule dynamics during mitosis.

Since NAP1 interacts with B-type cyclins but not with A cyclins, it represents a good candidate for a protein involved in the mitotic destabilization of microtubules. We therefore tested whether deletion of the *NAP1* gene in wild-type cells alters the properties of yeast microtubules. We examined the growth of *Δnap1* cells in the presence of the drug benomyl and at low temperatures, two conditions that destabilize microtubules (Dustin, 1984). Fig. 9 shows that *Δnap1* cells grow on concentrations of benomyl that almost completely block the growth of wild-type cells and also grow considerably better than the wild-type cells at low temperatures. We obtained identical results in two different strain backgrounds (W303 and S288c).

Since NAP1 interacts with Clb2 to mediate mitotic events, one might expect NAP1 to alter microtubule stability during mitosis. To test this idea, we arrested *Δnap1* and wild-type cells with α factor and released the cells from the arrest into media containing 11 μg/ml benomyl. We then used Western blotting to follow Clb2 levels as a way of assessing passage through mitosis (Fig. 10). In the wild-type cells, we observed that Clb2 protein levels rise normally but then fail to drop as the cells arrest in mitosis because they are unable to assemble a functional mitotic spindle. In the *Δnap1* cells, however, Clb2 levels rise and fall, and the cells do not exhibit a benomyl-induced mitotic delay. This experiment supports the idea that deletion of the *NAP1* gene either increases microtubule stability or reduces the requirements for microtubule function during mitosis.

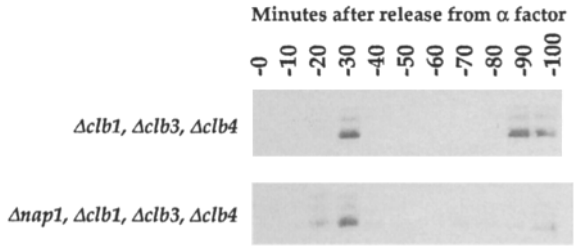

Figure 8. Behavior of the Cln2 protein in *Δnap1 Δclb1 Δclb3 Δclb4* cells and *Δclb1 Δclb3 Δclb4* control cells. A Western blot was carried out using samples of cells going through the cell cycle after release from an α factor arrest. We used strains DK159 and DK160, which carry Cln2 marked with a triple HA tag, and the blot was probed with the 12CA5 anti-HA monoclonal antibody (Tyers and Futcher, 1993).

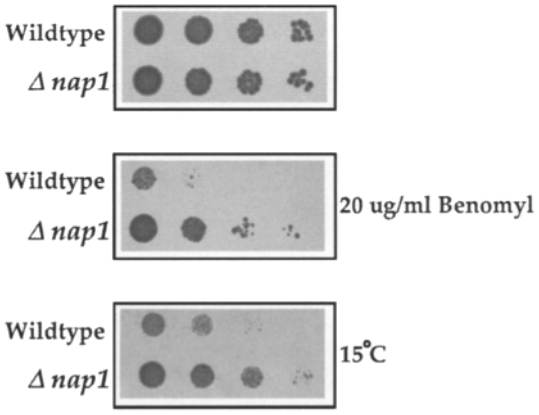

Figure 9. Deletion of the *NAP1* gene causes cells to become resistant to conditions that destabilize microtubules. A series of 10-fold dilutions of the *Δnap1* cells and wild-type control cells were spotted onto plates containing YPD or YPD + 20 μg/ml benomyl and grown at 25°C (*top two panels*) or 15°C (*lower panel*).

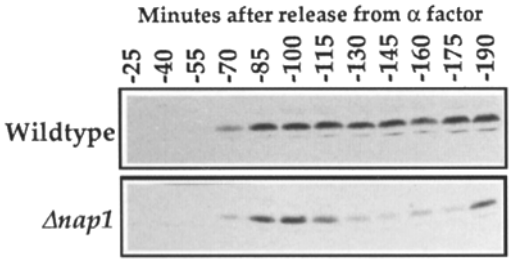

Figure 10. *Δnap1* cells are able to pass throughout mitosis in the presence of benomyl. Cells were released from an α factor arrest into YPD media containing 11 μg/ml benomyl at 25°C, and samples were taken every 10 min and used for an anti-Clb2 Western blot. Since this time course was carried out at a lower temperature, the Clb2 protein takes longer to appear. Notice that the Clb2 protein is again found to appear with identical kinetics in the two strains.

Discussion

In this study we show that Clb2 is unable to carry out its normal range of activities in the absence of NAP1, a protein that interacts specifically with members of the cyclin B family (Kellogg et al., 1994). Since Clb2/p34^{CDC28}-associated kinase activity is activated to normal levels in the absence of NAP1, we suggest that NAP1 facilitates the function of the activated Clb2/p34^{CDC28} kinase complex. These observations provide a starting point for understanding how cyclin-dependent kinase complexes carry out different events at different times during the cell cycle.

NAP1 and the Induction of Mitotic Events

Although NAP1 is not required for activation of Clb2/p34^{CDC28}-associated kinase activity to normal levels, we reproducibly observe a slight delay in the appearance of Clb2/p34^{CDC28}-associated kinase activity in *Δnap1* cells. This delay is likely to be due to a defect in ability of the kinase complex to amplify its own formation. A positive feedback loop that amplifies cyclin B/p34^{cdc2} kinase activation exists in the *Xenopus* embryo and results in a rapid rise in kinase levels as cells enter mitosis (for reviews see Murray and Hunt, 1993; Norbury and Nurse, 1992). It seems likely that a similar mechanism exists in other organisms.

Deletion of the *NAP1* gene in a strain that is dependent upon Clb2 function causes cells to undergo a prolonged delay in mitosis with normal levels of Clb2/p34^{CDC28}-associated kinase activity. It is of particular interest that the cells arrest with high Clb2/p34^{CDC28} kinase activity. This indicates that high kinase activity is not sufficient to allow cells to progress through mitosis, and suggests that NAP1 is required for the ability of the activated Clb2/p34 kinase to induce mitotic events. The observation that high kinase activity is not sufficient for mitotic progression has also been made in cells that carry a temperature sensitive allele of *CDC28* that arrests with high H1 kinase activity (Surana et al., 1991).

The *Δnap1 Δclb1 Δclb3 Δclb4* cells are blocked at the short spindle stage, suggesting that the Clb2/p34^{CDC28}-associated kinase is unable to induce events required for assembly or function of the mitotic spindle without the help of NAP1. Cells that are unable to assemble a normal mitotic spindle would be expected to delay in mitosis due to checkpoint controls that prevent exit from mitosis before spindle assembly is complete (Hoyt et al., 1991; Li and Murray, 1991; Minshull et al., 1994). A number of genes have been identified in budding yeast that are required for the mitotic spindle assembly checkpoint that is activated by drugs that prevent mitotic spindle assembly (called the MAD genes and the BUB genes) (Hoyt et al., 1991; Li and Murray, 1991). We have been unable to test whether the mitotic delay seen in *Δnap1 Δclb1 Δclb3 Δclb4* cells is dependent upon the MAD2 gene because *Δmad2* is synthetically lethal with *Δclb3 Δclb4* (Kellogg, D., unpublished). It is possible that the spindle assembly defect seen in *Δnap1 Δclb1 Δclb3 Δclb4* cells is not detected by the known spindle assembly checkpoint genes, or that the mitotic delay is not due to a direct requirement for NAP1 in spindle function. For example, one might imagine that the highly polarized bud growth observed in *Δnap1 Δclb1 Δclb3 Δclb4* cells produces a bud neck that is too narrow for the nucleus to fit through, and that cells detect this problem and induce a mitotic delay by activating the same checkpoint that is activated by the failure of bud emergence (Lew and Reed, 1995*a*).

Since the mitotic delay occurs at the short spindle stage, and the delay is rescued by the presence of other Clbs, it is unlikely that it is due to a specific defect in Clb2 destruction, since previously characterized defects in B-type cyclin destruction produce a dominant block late in mitosis (Holloway et al., 1993; Murray et al., 1989; Surana et al., 1993). Nevertheless, it is difficult to completely rule out the possibility that the mitotic delay is due to a problem with Clb2 destruction. For instance, one could imagine that there are two pathways for inducing the destruction of Clb2—one that is dependent on NAP1 and another that is independent of NAP1. The normal destruction of Clb2 seen in wild-type cells could be due to a NAP1-independent pathway that works through the other Clbs, while the delay seen in the *Δnap1 Δclb1 Δclb3 Δclb4* cells could be due to a defect in the Clb2- and NAP1-dependent pathway. To explain the observation that the cells arrest early in mitosis, one would have to suggest that NAP1 is also required for the destruction of proteins other than Clb2 that must be destroyed before cells can separate sister chromosomes and elongate the spindle (Holloway et al., 1993; Surana et al., 1993). The pleiotropic phenotype of *Δnap1 Δclb1 Δclb3 Δclb4* cells cannot be entirely due to a defect in Clb2 destruction, since such a defect could not account for the delay in the appearance of Clb2/p34^{CDC28}-associated kinase, the hyperpolarized bud growth, and the apparent increase in microtubule stability during mitosis.

How might NAP1 be involved in spindle function? One possibility is that NAP1 is required for the destabilization of microtubules that occurs as cells enter mitosis (Belmont et al., 1990; Verde et al., 1990). This change in microtubule dynamics is thought to play an essential role in assembly of the mitotic spindle, and appears to be induced specifically by B-type cyclins (Verde et al., 1992). Since NAP1 interacts specifically with B-type cyclins, it represents a good candidate for a protein that could play a role in the destabilization of microtubules. The finding that *Δnap1* cells are resistant to conditions that destabilize microtubules is con-

sistent with the idea that NAP1 has a microtubule destabilizing activity. In the absence of NAP1, microtubules should be more stable and therefore more resistant to microtubule destabilizing conditions. In mammalian cells, mitotic arrest can be produced by low doses of microtubule-binding drugs that do not affect the overall structure of the spindle or the monomer-polymer equilibrium of tubulin in the cell (Jordan et al., 1992, 1993; Toso et al., 1993; Wendell et al., 1993).

NAP1 was originally identified as a protein that promotes nucleosome assembly in vitro (Ishimi et al., 1984). In this study, we have found that *Δnap1* cells enter mitosis at the same time as control cells (as judged by the timing of the appearance of the Clb2 protein), indicating that *Δnap1* cells show no delay in passage through S phase. In contrast, drugs that inhibit DNA synthesis (i.e., hydroxyurea) cause a long delay in S phase and a corresponding delay in the appearance of the Clb2 protein (Kellogg, D., unpublished results). These observations, combined with the finding that NAP1 is primarily a cytoplasmic protein (Kellogg et al., 1995), suggest that NAP1 does not play an essential role in nucleosome assembly during DNA replication in vivo.

NAP1 and Polarized Bud Growth

Clb activity is required to turn off the polarized growth of buds that is initiated at Start by the G1 cyclins (Lew and Reed, 1993). Our results demonstrate that NAP1 plays an essential role in the ability of Clb2 to turn off polarized bud growth. In *Δnap1 Δclb1 Δclb3 Δclb4* cells, polar bud growth continues during mitosis, giving rise to highly elongated cells. A similar phenotype is seen in cells defective in Clb1, Clb2, Clb3, and Clb4 (Amon et al., 1993; Lew and Reed, 1993; Richardson et al., 1992). Since NAP1 interacts with Clb2, but not with Clb3, our results agree well with the previous finding that Clb2 is the primary cyclin responsible for suppression of polarized bud growth, and that Clb3 plays little or no role in this process (Lew and Reed, 1993).

Previous experiments have not defined how the Clbs act to induce the switch from polarized to isotropic bud growth. The Clb proteins could act directly to turn off the activity of the Cln, either at the transcriptional level or by causing inactivation of Cln/p34^{CDC28} complexes. Our results argue against this possibility, since continued polar bud growth occurs in the apparent absence of Cln2 protein. An alternative possibility is that the Cln initiate a polarized budding program that then continues, independent of Cln/p34^{CDC28} activity, until it is turned off by Clb activity. This kind of model is supported by the observation that hyperpolar bud growth occurs when Cdc28 activity is switched off in budded cells using temperature sensitive *cdc28* alleles, suggesting that continued activity of Cln/p34^{CDC28} complexes is not required for polar bud growth (Lew and Reed, 1993; Lew, D., personal communication). Our results also support this kind of model, and point to NAP1 as playing an important role in inducing the switch from polarized to isotropic bud growth.

Many proteins involved in polar bud growth have been identified (for reviews see Chant, 1994; Lew and Reed, 1995*b*), but the mechanisms by which the activities of these proteins are coordinated with the basic cell cycle regulators are completely unknown. The finding that NAP1 is required for the ability of the Clb2/p34^{CDC28}-associated kinase to suppress polar bud growth suggests strategies for identifying the molecules whose activity is required for this process.

NAP1 and the Function of Clb2/p34^{CDC28} Kinase Complexes

Our results demonstrate that NAP1 plays an important role in Clb2 function, and that the presence of other Clbs (Clb1, Clb3, and Clb4) can compensate for a NAP1 deficiency. These observations suggest that as well as a NAP1-dependent pathway that works through Clb2 to carry out mitotic events, there are alternative NAP1-independent pathways that can work through Clb1, Clb3, and/or Clb4. Our finding that there is an additional NAP1-like protein in *Xenopus* (the SET protein) (Kellogg et al., 1995) suggests the existence of additional NAP1-like proteins in budding yeast that might interact with the other Clbs. Since there is no detectable NAP1-like protein interacting with Clb3 (Kellogg et al., 1995), and Clb3 is not able to support the viability of yeast cells in the absence of other cyclins (Fitch et al., 1992; Richardson et al., 1992), it seems possible that NAP1-like proteins may be required for the ability of Clbs to carry out mitotic events. The finding of redundant pathways for spindle function is not surprising, given the high degree of redundancy found in other cell cycle events in budding yeast.

We have found that NAP1 plays an important role in activities that appear to be carried out specifically by B-type cyclins. Since Clb2/p34^{CDC28}-associated kinase activity rises to normal levels in *Δnap1* cells, it seems that NAP1 is required for specific functions of the activated Clb2/p34^{CDC28} kinase complex. One possible explanation for this requirement is that Clb2/p34^{CDC28} kinase complexes specifically phosphorylate NAP1, which then becomes activated to induce specific mitotic events. This kind of model is supported by the finding that Xenopus NAP1 is a specific substrate of cyclin B/p34^{cdc2} kinase complexes in vitro (Kellogg et al., 1995). Another possibility is that NAP1 is part of a system that determines the substrate specificity of the activated kinase complex, and plays a role in targeting kinase activity to specific substrates. It remains formally possible that the kinase activity of p34^{CDC28} is not directly involved in the induction of some cell cycle events. In this view, the Clb2/p34^{CDC28} complex might interact with NAP1 to form a complex that carries out functions independently of p34^{CDC28} kinase activity, perhaps by inducing the activity of other kinases.

An alternative model is that NAP1 somehow acts in a more general way to increase the amount of kinase activity associated with Clb2. Since our kinase assays must be carried out in vitro, we cannot rule out the possibility that NAP1 acts in vivo to increase the kinase activity associated with Clb2/p34^{CDC28} complexes. In a wild-type strain, the multiplicity of the Clbs could allow cells to complete mitosis in the absence of NAP1, while in a strain lacking Clb1, Clb3, and Clb4, the loss of NAP1 would reduce the kinase activity of the remaining mitotic Clb below the level required to complete mitosis. The observation that deletion of the *NAP1* gene affects only a subset of the known activities of Clb2 argues that NAP1 may carry out

more specific functions, rather than being responsible for a general increase in the kinase activity of Clb2/p34^{CDC28} complexes in vivo. Also, genetic interactions demonstrate that there are distinct functional differences between the Clbs that cannot be accounted for by simple differences in kinase levels. For example, deletion of *NAP1* causes a clear phenotype in *Δclb2* cells, but has little effect in *Δclb3 Δclb4* cells. In contrast, inactivation of the spindle assembly checkpoint by deleting *MAD2* has no effect in *Δclb2* cells but is synthetically lethal with the *Δclb3 Δclb4* double deletion (Kellogg et al., 1995; Kellogg, D., unpublished). Thus the biological effects of removing Clb2 on the one hand, and Clb3 and Clb4 on the other, cannot be explained as the effect of different quantitative reductions in a pool of B type cyclins that are functionally equivalent.

Our results provide a starting point for learning more about the specific functions of cyclin-dependent kinase complexes during the cell cycle. By identifying proteins that interact with NAP1, both biochemically and genetically, we hope to identify additional proteins involved in controlling mitotic spindle assembly and polarized bud growth. Furthermore, by identifying proteins that interact with the many other cyclins we should be able to identify and characterize additional proteins involved in cell cycle control.

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References

Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution in wild-type and morphogenetic mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934–945.

Amon, A., M. Tyers, B. Futcher, and K. Nasmyth. 1993. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell.* 74:993–1007.

Anderson, C. W., P. R. Baum, and R. F. Gesteland. 1973. Processing of adenovirus 2-induced proteins. *J. Virol.* 12:241–252.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current Protocols in Molecular Biology. Wiley Interscience, New York. pp. 4.9.1–4.9.12.

Belmont, L. D., A. A. Hyman, K. E. Sawin, and T. J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* 62:579–589.

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

Cross, F. R., and A. H. Tinkelenberg. 1991. A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell.* 65:875–883.

Dustin, P. 1984. Microtubules. Springer-Verlag, New York.

Evans, T., E. T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell.* 33:389–396.

Fitch, I., C. Dahmann, U. Surana, and A. Amon. 1992. Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 3:805–818.

Gallant, P., and E. A. Nigg. 1994. Identification of a novel vertebrate cyclin: cyclin B3 shares homology with both A and B-type cyclins. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:595–605.

Gotoh, Y., K. Moriyama, S. Matsuda, E. Okumura, T. Kishimoto, H. Kawasaki, K. Suzuki, I. Yahara, H. Sakai, and E. Nishida. 1991. *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2661–2668.

Harlow, E., and D. Lane. 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. pp. 313–315.

Heider, H., C. Hug, and J. M. Lucocq. 1994. A 40-kDa myelin basic protein kinase, dinstinct from erk1 and erk2, is activated in mitotic HeLa cells. *Eur. J. Biochem.* 219:513–520.

Holloway, S. L., M. Glotzer, R. W. King, and A. W. Murray. 1993. Anaphase is initiated by proteolysis rather than by the inactivation of MPF. *Cell.* 73: 1393–1402.

Hoyt, M. A., L. Trotis, and B. T. Roberts. 1991. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell.* 66:507–517.

Ishimi, Y., J. Hirosumi, W. Sato, K. Sugasawa, S. Yokota, F. Hanaoka, and M. Yamada. 1984. Purification and initial characterization of a protein which facilitates assembly of nucleosome-like structure from mammalian cells. *Eur. J. Biochem.* 142:431–439.

Jordan, M. A., D. Thrower, and L. Wilson. 1992. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. *J. Cell Sci.* 102:401–416.

Jordan, M. A., R. J. Toso, D. Thrower, and L. Wilson. 1993. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. USA.* 90:9552–9556.

Kellogg, D. R., and B. M. Alberts. 1992. Purification of a multiprotein complex containing centrosomal proteins from the Drosophila embryo by chromatography with low-affinity polyclonal antibodies. *Mol. Biol. Cell.* 3:1–11.

Kellogg, D. R., A. Kikuchi, T. Fujii-Nakata, Y. Ishimi, C. W. Turck, and A. W. Murray. 1995. Members of the NAP/SET family of proteins interact specifically with B-type cyclins. *J. Cell Biol.* 130:661–673.

Kilmartin, J., and A. E. M. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J. Cell Biol.* 98: 922–933.

Lew, D. J., and S. I. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J. Cell Biol.* 120:1305–1320.

Lew, D. J., and S. Reed. 1994. Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Gen. Dev.* In press.

Lew, D. J., and S. Reed. 1995*a*. A cell cycle checkpoint that monitors cell morphogenesis in budding yeast. *J. Cell Biol.* 129:739–749.

Lew, D. J., and S. I. Reed. 1995*b*. Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Gen. Dev.* 5:17–23.

Li, R., and A. W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell.* 66:519–531.

Masui, Y., and C. L. Markert. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* 177:129–145.

Minshull, J., H. Sun, N. K. Tonks, and A. W. Murray. 1994. MAP-kinase dependent mitotic feedback arrest in *Xenopus* egg extracts. *Cell.* 79:475–486.

Moreno, S., and P. Nurse. 1990. Substrates for p34^{cdc2}: in vivo veritas? *Cell.* 61: 549–551.

Murray, A., and T. Hunt. 1993. The Cell Cycle: an Introduction. Oxford University Press, New York.

Murray, A. W., M. J. Solomon, and M. W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature (Lond.).* 339:280–286.

Nasmyth, K. 1993. Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.* 5:166–179.

Nigg, E. A. 1993. Cellular substrates of p34cdc2 and it companion cyclin-dependent kinases. *Trends Cell Biol.* 3:296–301.

Norbury, C., and P. Nurse. 1992. Animal cell cycles and their control. *Annu. Rev. Biochem.* 61:441–470.

Peter, M., J. S. Sanghera, S. L. Pelech, and E. A. Nigg. 1992. Mitogen-activated protein kinases phosphorylate nuclear lanims and display sequence specificity overlapping that of mitotic protein kinase p34cdc2. *Eur. J. Biochem.* 205: 287–294.

Pines, J. 1993. Cyclins and cyclin-dependent kinases: take your partners. *Trends Biochem. Sci.* 18:195–197.

Pringle, J. R., A. E. M. Adams, D. G. Drubin, and B. K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565–602.

Reed, S. I. 1992. The role of p34 kinases in the G1 to S-phase transition. *Annu. Rev. Cell Biol.* 8:529–561.

Richardson, H., D. J. Lew, M. Henze, K. Sugimoto, and S. Reed. 1992. Cyclin B homologs in *Saccharomyces cerevisiae* function in S phase and in G2. *Genes Dev.* 6:2021–2034.

Salama, S. R., and K. B. Hendricks. 1994. G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover. *Mol. Cell. Biol.* 14:7953–7966.

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

Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth. 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1969–1978.

Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell.* 65:145–161.

Toso, R. J., M. A. Jordan, K. W. Farrell, B. Matsumoto, and L. Wilson. 1993. Kinetic stabilization of microtubule dynamic instability in vitro by vinblastine. *Biochemistry.* 32:1285–1293.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 80:4350–4354.

Tyers, M., and B. Futcher. 1993. Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. *Mol. Cell. Biol.* 13:5659–5669.

Verde, F., J.-C. Labbe, M. Doree, and E. Karsenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. *Nature (Lond.)*. 343:233–238.

Verde, F., M. Dogterom, E. Stelzer, E. Karsenti, and S. Leibler. 1992. Control of microtubule dynamics and length by cyclin A and cyclin B-dependent kinases in *Xenopus* egg extracts. *J. Cell Biol.* 118:1097–1108.

Welch, M. D., D. A. Holtzman, and D. G. Drubin. 1994. The yeast actin cytoskeleton. *Curr. Opin. Cell Biol.* 6:110–119.

Wendell, K. L., L. Wilson, and M. A. Jordan. 1993. Mitotic block in HeLa cells by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and in centrosomes. *J Cell Sci.* 104:261–274.

Wittenberg, C., and K. Sugimoto. 1990. G1-specific cyclins of *S. cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with the $p34^{CDC28}$ protein kinase. *Cell.* 62:225–237.