Morphogenesis in the Yeast Cell Cycle: Regulation by Cdc28 and Cyclins

Daniel J. Lew and Steven I. Reed
Department of Molecular Biology, Scripps Research Institute, La Jolla, California 92037

Abstract. Analysis of cell cycle regulation in the budding yeast *Saccharomyces cerevisiae* has shown that a central regulatory protein kinase, Cdc28, undergoes changes in activity through the cell cycle by associating with distinct groups of cyclins that accumulate at different times. The various cyclin/Cdc28 complexes control different aspects of cell cycle progression, including the commitment step known as START and mitosis. We found that altering the activity of Cdc28 had profound effects on morphogenesis during the yeast cell cycle. Our results suggest that activation of Cdc28 by G1 cyclins (Cln1, Cln2, or Cln3) in unbudded G1 cells triggers polarization of the cortical actin cytoskeleton and secretory vesicles to these sites, but they do not address the question of how the growth sites are chosen during the cell cycle so as to produce a bud with the same shape as the mother cell.

The generation of specialized and often elaborate cell shapes is crucial to the function of most cell types. Development of many of these shapes, be they neuronal processes or yeast buds, involves polarization of secretion towards a defined subdomain of the plasma membrane. In yeast cells, which are surrounded by a cell wall, the force that drives growth is generated by an osmotic gradient across the plasma membrane (Harold, 1990). This force, or turgor pressure, is isotropic, but growth is channeled to specific sites by vectorial delivery of wall modifying enzymes and new cell wall constituents to these sites (Harold, 1990). To understand the generation of cell shape, we must find out how this delivery is targeted and how the sites of cell growth are chosen. The actin cytoskeleton has been implicated in these processes in the yeast *Saccharomyces cerevisiae*. Cortical structures containing actin filaments cluster at sites of cell growth during the cell cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Furthermore, mutations in the single actin gene (*ACT1*) (Novick and Botstein, 1985) or in genes encoding actin binding proteins (*MYO2*, unconventional myosin [Johnston et al., 1991]; *TPMI*, tropomyosin [Liu and Bretscher, 1992]; *SAC6*, fimbrin [Adams et al., 1991]; *CAP2*, capping protein [Amatruda et al., 1990]; *PFYI*, profilin [Haarer et al., 1990]) disrupt vectorial secretion and lead to more uniform expansion. These observations suggest that the actin cytoskeleton may play a role in specifying target sites on the plasma membrane and delivering secretory vesicles to these sites, but they do not address the question of how the growth sites are chosen during the cell cycle so as to produce a bud with the same shape as the mother cell.

Growth of *S. cerevisiae* cells by budding involves a number of dramatic rearrangements of the cytoskeleton and secretory apparatus of the cell during the cell cycle (see Fig. 1A) (Chant and Pringle, 1991). First, a small region of the surface of an unbudded cell in G1 assembles into a “pre-bud” site. This involves a polarization of the actin cytoskeleton such that cortical actin-rich patches congregate in a ring at the prebud site (Kilmartin and Adams, 1984). Near the G1/S transition, secretion becomes polarized to the region demarcated by the ring of cortical actin patches and the bud emerges. The cortical actin patches then redistribute into the bud. During bud growth in S phase and G2, secretion is directed into the bud and the cortical actin patches stay exclusively in the bud (Tkacz and Lampen, 1972; Parkas et al., 1974; Adams and Pringle, 1984; Kilmartin and Adams, 1984). The shape of the bud (and hence of the daughter cell) is determined by the balance between apical growth (directed towards the tip of the bud) and isotropic bud growth during this period. Apical growth predominates during the early budded phase, whereas isotropic bud growth predominates in the late budded phase (Farkas et al., 1974). During mitosis, cortical actin patches transiently redistribute throughout the surface of both mother and bud portions of the cell, and then congregate on both sides of the neck region at the time of cytokinesis (Kilmartin and Adams, 1984). Coincident with this, secretion is redirected towards the neck region in order to form a specialized chitin-rich septum (Tkacz and...
Cyclins were discovered through their strikingly periodic variation in association with positive regulatory subunits known as cyclins. In S. cerevisiae, cdc28 is responsible for activating Cdc28 at different times in the cell cycle to regulate DNA replication and spindle assembly in G1/S and G2, as well as mitotic events (Richardson et al., 1992). Thus, the sequential activation of the Cdc28 regulator by distinct groups of cyclins, which presumably confer different functional properties on the kinase, results in the orderly progression of events in the nuclear cycle. Do these same regulators control the morphogenetic events of the cytoplasmic cycle?

In this report we have examined the role played by Cdc28 and cyclins in the control of actin distribution and polarized secretion during the yeast cell cycle. Based on the effects of mutational inactivation or hyperactivation of different Cdc28/cyclin complexes in cells at different stages of the cell cycle, we conclude that morphogenetic events in S. cerevisiae are indeed controlled by the same regulators that govern the nuclear cycle. Changes in Cdc28 activity appear to trigger polarization of cortical actin in G1, depolarization of cortical actin and secretion in G2, and redistribution of cortical actin to the neck region after mitosis. Cdc28 activation can trigger cytoskeletal reorganization in the absence of de novo protein synthesis, suggesting that the control of cell polarity occurs post-translationally. Our results also suggest that activation of Cdc28 by different cyclins can have opposing effects on polarization of both the cytoskeleton and the secretory apparatus, providing direct evidence for specialized roles for different cyclins.

Materials and Methods

Yeast Strains, Media and Growth Conditions

All yeast strains used in this study were derivatives of BF264-15DU: MATα ade1 his2 leu2-3,112 trpl1-1 ura3ΔΔ (Richardson et al., 1989). The relevant genotypes of strains used in this study are shown in Table I. Standard genetic procedures for yeast were used to construct the strains (Sherman et al., 1982). Null cln alleles were as described by (Cross and Tinkelenberg, 1991), GALI:CLN constructs as described by (Cross and Tinkelenberg, 1991), Lew et al., 1991), null clb alleles as described by (Richardson et al., 1992), and GALI:CLB constructs as described by C. S. Stueland, D. J. Lew, and S. I. Reed (submitted for publication).

Centrifugal Elutriation and Flow Cytometry

1-2 liters of cells were grown at 25° or 30°C to a density of 107 cells/ml, then chilled to 4°C and sonicated to disperse clumps. The cells were loaded into an elutriator rotor (Beckman Instruments, Inc., Fullerton, CA) at 4,000 rpm, 4°C. The pump speed was adjusted so that the smallest cells flowed through the elutriator and were collected in bottles on ice, whereas larger cells remained in the rotor. When enough cells were collected (≥5% of the population, collected over 10-15 min) they were concentrated by centrifugation, resuspended in fresh media at 2 x 106 cells/ml, and warmed to the appropriate temperature. Elutriations comparing cells of different genotypes (as in the experiments of Figs. 2 and 10) were performed on the same day: both cultures were chilled at the same time and one culture was elutriated, so that both sets of cells spent the same amount of time on ice (usually ≥ h for such double elutriations). Cdc28 activity appears to trigger polarization of cortical actin in G1, depolarization of cortical actin and secretion in G2, and redistribution of cortical actin to the neck region after mitosis. Cdc28 activation can trigger cytoskeletal reorganization in the absence of de novo protein synthesis, suggesting that the control of cell polarity occurs post-translationally. Our results also suggest that activation of Cdc28 by different cyclins can have opposing effects on polarization of both the cytoskeleton and the secretory apparatus, providing direct evidence for specialized roles for different cyclins.

In this report we have examined the role played by Cdc28 and cyclins in the control of actin distribution and polarized secretion during the yeast cell cycle. Based on the effects of mutational inactivation or hyperactivation of different Cdc28/cyclin complexes in cells at different stages of the cell cycle, we conclude that morphogenetic events in S. cerevisiae are indeed controlled by the same regulators that govern the nuclear cycle. Changes in Cdc28 activity appear to trigger polarization of cortical actin in G1, depolarization of cortical actin and secretion in G2, and redistribution of cortical actin to the neck region after mitosis. Cdc28 activation can trigger cytoskeletal reorganization in the absence of de novo protein synthesis, suggesting that the control of cell polarity occurs post-translationally. Our results also suggest that activation of Cdc28 by different cyclins can have opposing effects on polarization of both the cytoskeleton and the secretory apparatus, providing direct evidence for specialized roles for different cyclins.

Materials and Methods

Yeast Strains, Media and Growth Conditions

All yeast strains used in this study were derivatives of BF264-15DU: MATα ade1 his2 leu2-3,112 trpl1-1 ura3ΔΔ (Richardson et al., 1989). The relevant genotypes of strains used in this study are shown in Table I. Standard genetic procedures for yeast were used to construct the strains (Sherman et al., 1982). Null cln alleles were as described by (Cross and Tinkelenberg, 1991), GALI:CLN constructs as described by (Cross and Tinkelenberg, 1991), Lew et al., 1991), null clb alleles as described by (Richardson et al., 1992), and GALI:CLB constructs as described by C. S. Stueland, D. J. Lew, and S. I. Reed (submitted for publication).

Centrifugal Elutriation and Flow Cytometry

1-2 liters of cells were grown at 25° or 30°C to a density of 107 cells/ml, then chilled to 4°C and sonicated to disperse clumps. The cells were loaded into an elutriator rotor (Beckman Instruments, Inc., Fullerton, CA) at 4,000 rpm, 4°C. The pump speed was adjusted so that the smallest cells flowed through the elutriator and were collected in bottles on ice, whereas larger cells remained in the rotor. When enough cells were collected (≥5% of the population, collected over 10-15 min) they were concentrated by centrifugation, resuspended in fresh media at 2 x 106 cells/ml, and warmed to the appropriate temperature. Elutriations comparing cells of different genotypes (as in the experiments of Figs. 2 and 10) were performed on the same day: both cultures were chilled at the same time and one culture stayed on ice while the other was elutriated, so that both sets of cells spent the same amount of time on ice (usually ≥ h for such double elutriations). Cdc28 activity appears to trigger polarization of cortical actin in G1, depolarization of cortical actin and secretion in G2, and redistribution of cortical actin to the neck region after mitosis. Cdc28 activation can trigger cytoskeletal reorganization in the absence of de novo protein synthesis, suggesting that the control of cell polarity occurs post-translationally. Our results also suggest that activation of Cdc28 by different cyclins can have opposing effects on polarization of both the cytoskeleton and the secretory apparatus, providing direct evidence for specialized roles for different cyclins.

Materials and Methods

Yeast Strains, Media and Growth Conditions

All yeast strains used in this study were derivatives of BF264-15DU: MATα ade1 his2 leu2-3,112 trpl1-1 ura3ΔΔ (Richardson et al., 1989). The relevant genotypes of strains used in this study are shown in Table I. Standard genetic procedures for yeast were used to construct the strains (Sherman et al., 1982). Null cln alleles were as described by (Cross and Tinkelenberg, 1991), GALI:CLN constructs as described by (Cross and Tinkelenberg, 1991), Lew et al., 1991), null clb alleles as described by (Richardson et al., 1992), and GALI:CLB constructs as described by C. S. Stueland, D. J. Lew, and S. I. Reed (submitted for publication).

Centrifugal Elutriation and Flow Cytometry

1-2 liters of cells were grown at 25° or 30°C to a density of 107 cells/ml, then chilled to 4°C and sonicated to disperse clumps. The cells were loaded into an elutriator rotor (Beckman Instruments, Inc., Fullerton, CA) at 4,000 rpm, 4°C. The pump speed was adjusted so that the smallest cells flowed through the elutriator and were collected in bottles on ice, whereas larger cells remained in the rotor. When enough cells were collected (≥5% of the population, collected over 10-15 min) they were concentrated by centrifugation, resuspended in fresh media at 2 x 106 cells/ml, and warmed to the appropriate temperature. Elutriations comparing cells of different genotypes (as in the experiments of Figs. 2 and 10) were performed on the same day: both cultures were chilled at the same time and one culture stayed on ice while the other was elutriated, so that both sets of cells spent the same amount of time on ice (usually ≥ h for such double elutriations).
cytometry as described (Lew et al., 1992). Cell size was measured by forward angle light scattering as described (Lew et al., 1992).

Staining of Cells and Microscopy

For visualization of F-actin, cells were fixed by addition of 37% formaldehyde directly to the culture to a final concentration of 3.7%, and incubation at 25°C for 1-2 h. In some cases the fixed cells were left at 4°C overnight. Washing with PBS and staining with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) was as described (Adams and Pringle, 1991). Staining of fixed cells with calcofluor (Sigma Chemical Co., St. Louis, MO) was as described (Pringle, 1991).

For cell wall staining, live cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl at 5-10 × 10^7 cells/ml and sonicated to disperse clumps. FITC-ConA (E-Y Laboratories, Inc., San Mateo, CA) was added to a final concentration of 50-100 μg/ml, and the cells were incubated for 10 min at 25°C in the dark. After labeling, the cells were harvested by centrifugation and resuspended in appropriate media at the indicated temperature. After the indicated return-to-growth interval, the cells were fixed as above.

Stained cells were resuspended in a drop of mounting medium (Pringle et al., 1991) and observed using a Zeiss Axiophot photomicroscope with a 100× objective (Carl Zeiss, Inc., Thornwood, NY).

Results

Timing of Pre-bud Site Assembly in G1

Establishing the timing of pre-bud site formation in G1 is important because it would indicate whether pre-bud site formation, or the subsequent step of redirecting the secretory apparatus to the pre-bud site, is the step regulated by execution of START. We therefore attempted to map the timing of pre-bud site formation relative to START using cells synchronized by centrifugal elutriation. This method of synchronization has the advantage that the smallest G1 cells are selected from a relatively unperturbed asynchronous population, as opposed to induction synchrony protocols that produce extensive physiological changes in addition to G1 arrest. Furthermore, we have mapped the timing of START transit in our strains using this procedure to be 20 min before bud emergence, providing a landmark with which to compare the timing of pre-bud site assembly (Lew et al., 1992).

Early G1 cells were inoculated into YEPdextrose at either 25 or 37°C. At various intervals, samples were fixed and stained with rhodamine-phalloidin (which selectively binds to F-actin [Cooper, 1987]) to visualize the cortical actin distribution (Fig. 1). Fig. 1 A shows a schematic of the patterns observed. To assess the timing of pre-bud site assembly (b in Fig. 1 A), we compared the cumulative proportion of cells that had polarized their cortical actin with the cumulative proportion of cells that had budded at each time point (Fig. 1 B). This comparison showed that actin polarization preceded bud emergence by only 7-10 min at both temperatures, thus clearly placing pre-bud site assembly after START, which occurs 20 min before bud emergence in this strain (Lew et al., 1992).

It should be noted that elutriation is not a completely non-perturbing technique, as the cells are chilled to 4°C for 30-60 min during elutriation and then rewarmed. We have noticed that warming of the cells after elutriation results in a transient randomization of the cortical actin distribution. Thus, the newborn daughter cells isolated by elutriation contain randomly distributed cortical actin patches at the beginning of the incubation. It could be argued, therefore, that this experiment is not an accurate reflection of events in the unperturbed cell cycle but rather an artifact of the elutriation, such that the timing of actin polarization merely reflects recovery from the randomizing effects of the temperature.

Table I. Strains list

| Strain | Relevant genotype |
|--------|-------------------|
| DLY005 | MATa/α             |
| DLY104 | MATa/α cdc28-4/cdc28-4 |
| DLY105 | MATa/α cdc28-13/cdc28-13 |
| DLY106 | MATa/α cdc28-9/cdc28-9 |
| DLY112 | MATa/α cdc28-13/cdc28-13 GAL1:CLN2(LEU2)/leu2 |
| DLY113 | MATa/α cdc28-13/cdc28-13 GAL1:CLB1Δ152(LEU2)/leu2 |
| DLY224 | MATa/α ctn1/ctn1 ctn2/ctn2 ctn3/ctn3 GAL1:CLN3(LEU2)/leu2 GAL1:CLN3(Trp1)/trp1 |
| DLY257 | MATa/α GAL1:CLN2(LEU2)/leu2 |
| DLY372 | MATa/α ctnb1::URA3/cnb1::URA3 |
| DLY373 | MATa/α ctnb2::LEU2/cnb2::LEU2 |
| DLY374 | MATa/α ctnb3::TRP1/cnb3::TRP1 |
| DLY375 | MATa/α ctnb4::HIS2/cnb4::HIS2 |
| DLY376 | MATa/α ctnb1::URA3/cnb1::URA3 ctnb3::TRP1/cnb3::TRP1 |
| DLY377 | MATa/α ctnb1::URA3/cnb1::URA3 ctnb4::HIS2/cnb4::HIS2 |
| DLY378 | MATa/α ctnb1::URA3/cnb1::URA3 ctnb3::TRP1/cnb3::TRP1 ctnb4::HIS2/cnb4::HIS2 |
| DLY379 | MATa/α GAL1:CLB1(LEU2)/leu2 ctnb1::URA3/cnb1::URA3 ctnb2::LEU2/cnb2::LEU2 ctnb3::TRP1/cnb3::TRP1 ctnb4::HIS2/cnb4::HIS2 |
| DLY384 | MATa/α GAL1:CLB1(LEU2)/leu2 ctnb1::URA3/cnb1::URA3 ctnb2::LEU2/cnb2::LEU2 ctnb3::TRP1/cnb3::TRP1 ctnb4::HIS2/cnb4::HIS2 |
| DLY328 | MATa/α GAL1:CLB1Δ152(LEU2)/leu2 |
| DLY333 | MATa/α GAL1:CLB1(LEU2)/leu2 |
| DLY385 | MATa/α GAL1:CLB2(LEU2)/leu2 |
| DLY338 | MATa/α GAL1:CLB3(LEU2)/leu2 |
| DLY389 | MATa/α GAL1:CLB4(LEU2)/leu2 |
| DLY1006 | MATa/α GAL1: wee1+ (URA3) iura3 |
Figure 1. Timing of pre-bud site assembly in G1. (A) Schematic of the distributions of cortical actin patches (black dots) observed during the cell cycle. Actin cables are omitted for simplicity. (B) Timecourse of actin polarization and bud emergence. Wild-type (DLY005) cells were elutriated and incubated at 25°C (left) or 37°C (right) in YEPglucose. At the indicated times, samples were fixed for analysis of the actin distribution by rhodamine-phalloidin staining and fluorescence microscopy. Percent of cells that had polarized the cortical actin patches (morphologies b-g in A) or budded (morphologies c-g in A) were quantitated by counting 400 cells at each time point.

shift. To circumvent this problem, we attempted to determine the timing of actin polarization to the pre-bud site in unperturbed cycling cells.

The major difficulty in this analysis is that the "post-cytokinesis" polarization of cortical actin in newborn cells is morphologically very similar to the polarization of cortical actin to the pre-bud site. In diploid daughter cells, however, it is possible to distinguish these patterns because the bud always forms at the opposite pole of the cell from the site of cytokinesis (Chant and Pringle, 1991). G1 daughter cells with a polarized cortical actin distribution can be scored as newborn (actin patches at the site of cytokinesis) or prebudding (actin patches at the opposite pole from the site of cytokinesis).

Upon cell abscission, a chitin-rich bud scar is left in the cell wall of the mother cell, while a less characterized birth scar marks the site of cytokinesis in the daughter cell wall (Barton, 1950; Beran, 1968). Bud scars can be stained with calcofluor (Pringle, 1991), and we report here that birth scars stain brightly with FITC-Con A (Fig. 2). The lectin Con A binds to mannose residues, which are abundant on the "mannan" or glycoproteins which form a major component of the yeast cell wall (Ballou, 1981). Thus, the entire surface of the cell is stained upon exposure to FITC-Con A. The bright FITC-Con A labeled patch marks the birth scar, as shown by the fact that daughter cells (cells without bud scars) always budded at the opposite pole of the cell from the patch (Fig. 2 and data not shown).

Wild-type diploid cells growing exponentially in rich medium were fixed and stained with calcofluor, FITC-Con A, and rhodamine-phalloidin. G1 daughter cells (unbudded without bud scars) were assigned to one of three categories based on the actin distribution. Of these, 48% were scored as "post-cytokinesis" (cortical actin patches clustered at the same pole as the FITC-Con A labeled birth scar), 26% were random (cortical actin patches distributed with no clear polarity), and 26% were prebudding (cortical actin patches clustered at the opposite pole from the birth scar). Assuming that all daughter cells were born with a post-cytokinesis actin distribution which was then randomized and reassembled into a pre-bud site, these data indicate that pre-bud site assembly occurred about three quarters of the way through G1 in an unperturbed rapidly proliferating population. This is fully consistent with the analysis of elutriated cells presented above, strongly suggesting that actin polarization to the pre-bud site occurs in late G1 after START.

Pre-bud Site Assembly Requires Activation of the Cln/Cdc28 Kinase

Because assembly of the pre-bud site followed START, it seemed plausible that activation of Cdc28 by G1 cyclins (Clns) could be the trigger for actin polarization. To test this hypothesis, homozygous cdc28-13 diploid cells were elutriated to obtain G1 daughter cells, which were inoculated into rich medium at 37°C (the restrictive temperature for this mutant). These cells grew in size at the same rate as wild-type cells at 37°C (assayed by forward angle light scattering in a flow cytometer: data not shown), but did not pass START or

Figure 2. Birth scars are stained by FITC-ConA. Wild-type cells (DLY005) growing in YEPglucose were fixed and stained with calcofluor to visualize bud scars (A), and with FITC-ConA to stain mannan (B). Examples of birth scars (stained with FITC-ConA but not calcofluor) are indicated by arrowheads; bud scars (stained by calcofluor and faintly by FITC-ConA) by arrows. Bar, 10 μm.
form buds. As before, cells were fixed and stained at various intervals to monitor actin distribution (Fig. 3). Comparing cdc28-13 mutants and wild-type cells at 37°C, it was clear that cortical actin polarization was greatly delayed in the mutants. Similar results were obtained with the cdc28-9 allele (data not shown). In the mutant cells, cortical actin patches remained randomly distributed until the cells were much larger than their wild-type counterparts (Fig. 3, B and C). These results are consistent with a role for Cdc28 in triggering pre-bud site assembly.

Figure 4. Actin polarization in G1 requires Clns. (A) Flow cytometric analysis of DNA content of cln1 cln2 cln3 GAL1: CLN3 (DLY224) cells. Cells were grown in YEPgalactose and glucose was added to terminate Cln3 synthesis. At the indicated times, samples were fixed and stained with propidium iodide to monitor DNA content. Left peak, G1 cells; right peak, G2/M cells. The rightward drift of the G1 peak at the 3 h time point is due to increased autofluorescence and mitochondrial DNA as the cells enlarge. (B) Actin distribution in the same cells 2 h after glucose addition: the cells were arrested in G1 (A) with dispersed cortical actin patches. Bar, 10 μm.
As an alternative approach, we monitored the actin distribution in cells upon removal of Cln function. \( \text{cln}1 \text{ cln}2 \text{ cln}3 \text{ GAL1:CLN3} \) cells are viable when grown in galactose media (which induces the \( \text{GAL1} \) promoter) but arrest at \( \text{START} \) in the first cycle upon addition of glucose (which represses the \( \text{GAL1} \) promoter). Homozygous diploid \( \text{cln1 cln2 cln3 GAL1:CLN3} \) cells were grown in galactose medium and glucose was added to a final concentration of 2\% to terminate Cln3 synthesis. At various times the distribution of cells in the cell cycle was monitored by flow cytometry and the actin distribution was visualized as above. As shown in Fig. 4 \( A \), the cells were all arrested in \( \text{G1} \) by 2 h after glucose addition. Microscopic examination of stained cells showed that 2 or 3 h after glucose addition the cells displayed a random distribution of cortical actin patches (Fig. 4 \( B \)). This experiment did not involve any changes in the incubation temperature of the cells (30°C), ruling out potential temperature shift artifacts. Together, the experiments of Figs. 1–4 suggest that polarization of cortical actin patches to the pre-bud site is triggered by activation of the Cln/Cdc28 kinase at \( \text{START} \).

At long incubation times, the \( \text{cdc28-13} \) cells exhibited some polarization of cortical actin patches (Fig. 3 \( D \)). Eventually, this cytoskeletal polarization was reflected in a polarization of secretion, giving rise to the characteristic “shmoo” morphology that has been noted in \( \text{cdc28} \) mutants. Prolonged incubation (>3 h) without Clns also led to the development of shmoo (data not shown). The basis for this polarization is unclear. We do not think that it is the result of residual Cdc28 activity in temperature sensitive \( \text{cdc28} \) strains, because the timing and extent of actin polarization in these strains was unaffected by overexpression of either Cln2 or ClblA152 (data not shown). Possible explanations include a slow Cdc28-independent pathway for pre-bud site assembly, or simply an artificial consequence of prolonged

---

Figure 5. Cln/Cdc28 activation triggers actin polarization in the absence of de novo protein synthesis. (A) \( \text{cdc28-13 GAL1:CLN2} \) (DLY112) \( \text{G1} \) daughter cells were isolated by centrifugal elutriation and incubated at 37°C (to maintain a \( \text{cdc28} \) arrest) in YEPgalactose to induce Cln2 synthesis for 3 h. At time zero, the cells were shifted to 25°C to activate the Cln2/Cdc28 kinase. Cycloheximide (CHX) was added to the indicated culture 5 min before the temperature shift at a final concentration of 10 \( \mu \)g/ml. Cells were fixed at the indicated times for analysis of DNA content by flow cytometry. (B) The percent of cells displaying a polarized distribution of cortical actin patches was quantitated at various times after shiftdown by counting at least 300 cells. Control cultures were maintained at 37°C as indicated. (C) Actin distribution at time zero. (D) Actin distribution at 30 min for cells shifted to 25°C without cycloheximide. (E) Actin distribution at 30 min for cells shifted to 25°C with cycloheximide. Bar, 10 \( \mu \)m.
Cln/Cdc28 Activation Triggers Actin Polarization in the Absence of De Novo Protein Synthesis

Regulation of actin polarization by the Cln/Cdc28 kinase could occur in various ways, from the most direct route (perhaps involving direct phosphorylation of actin binding proteins) to a very indirect route (perhaps involving induction of new transcripts whose products somehow affect the actin distribution). As a first step towards characterizing this regulatory pathway, we tested whether actin polarization could be triggered by kinase activation in the absence of de novo protein synthesis. To do this, we again employed the cdc28-13 allele, which is easily reversible upon return to 25°C after arrest at 37°C. cdc28-13 cells arrested at 37°C accumulate very low levels of Cln1 and Cln2, but upon shift-down to 25°C there is a massive accumulation of these Clns through a positive feedback mechanism whereby CLN1 and CLN2 transcription is stimulated by the Cln/Cdc28 kinase (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). This burst of Cln accumulation leads to a rapid activation of high levels of Cln/Cdc28 activity, and hence passage through START. We reasoned that if cdc28-13 cells were arrested and then shifted down to 25°C in the presence of cycloheximide, the positive feedback loop would be interrupted (because Cln synthesis would be blocked) and only very low levels of Cln/Cdc28 kinase would be activated. To circumvent this problem, we used a strain containing an integrated GAL1:CLN2 construct. This allowed the cells to induce the GAL1 promoter and accumulate Cln2 during the cdc28 arrest at 37°C, so that high levels of Cln/Cdc28 kinase activity could be attained upon shift-down to 25°C even in the presence of cycloheximide.

cdc28-13 GAL1:CLN2 cells in early G1 were isolated by centrifugal elutriation and incubated for 3 h in galactose based medium at 37°C to allow time for Cln2 accumulation. At this time, the cells were still uniformly arrested in G1 (Fig. 5 A) and 93% of the cells displayed a random distribution of cortical actin patches (Fig. 5 C). Upon shift-down to 25°C, the cells not treated with cycloheximide rapidly polarized their actin, budded, and replicated their DNA (Fig. 5, A, B, and D). In the presence of 10 µg/ml cycloheximide (a concentration sufficient to block 96–97% of cellular protein synthesis [Marini and Reed, 1992]), the cells did not bud or replicate their DNA upon shift-down, but ~50% of the cells polarized their actin after a short (15 min) lag (Fig. 5, B and E). By focusing up and down, it could be seen that the cortical actin patches formed a ring, as observed at a normal pre-bud site. Thus, activation of Cln/Cdc28 induced actin polarization in a rapid, post-translational manner.

The basis for the 15 min lag in the cycloheximide-treated cells is unclear, as is the reason why only half of these cells polarized their cortical actin patches. Possibly there is a parallel pathway for actin polarization in response to Cln/Cdc28 activation that does involve protein synthesis. Alternatively, the pleiotropic effects of cycloheximide treatment may indirectly affect the actin distribution (this is supported by the observation that the cells kept at 37°C in the presence of cycloheximide all randomized their cortical actin patches, unlike the controls that gradually increased actin polarization: Fig. 5).

Activation of Cln/Cdc28 in G2 Results in Hyperpolarization of Cortical Actin and Secretion

Normally, Cln accumulation is periodic in the cell cycle, peaking in late G1 and decaying in S phase (at least for Cln1 and Cln2; Cln3 is present in very low abundance and its periodicity is unclear) (Wittenberg et al., 1990; Tyers et al., 1992). We used a wild-type strain containing an integrated GAL1:CLN2 construct to test whether deregulated expression of Cln2 would have any effects on the actin distribution of cells in G2. Wild-type cells in G2 contain cortical actin patches spread over the entire bud surface (Fig. 1 A, Fig. 6, top). As shown in Fig. 6, budded cells in an asynchronous culture induced to express Cln2 by addition of galactose displayed a remarkable hyperpolarization of the cortical actin to the bud tip. Furthermore, the buds became very elongated, suggesting that secretion had also become hyperpolarized towards the tip of the bud in these cells (see below). The degree of hyperpolarization was dependent on the level of Cln2 expression. Similar effects were observed with deregulated Cln1 expression, but the effects of deregulated Cln3 expression were much less severe (data not shown).

Figure 6. Ectopic Cln2 expression in budded cells causes hyperpolarization of the cortical actin patches. Wild-type (DLY005) or GAL1:CLN2 (DLY257) cells were grown in YEPsucrose and galactose was added for 2 h. (Left) Rhodamine-phalloidin staining. (Right) Phase contrast of same cells. Bar, 10 µm.
Thus, activation of the Cln/Cdc28 kinase apparently results in polarization of cortical actin and secretion in G2 as well as G1.

**Activation of the Clb/Cdc28 Kinase is Required for Depolarization of Cortical Actin in G2**

Once the bud has formed, the cortical actin patches are redistributed into the bud. As the bud grows, the cortical actin patches spread out over the bud surface, so that the patch-to-patch distance increases. In mitosis, the cortical actin patches redistribute randomly throughout both the mother and bud, and then cluster on either side of the neck region (Adams and Pringle, 1984; Kilmartin and Adams, 1984).

The Cdc28 kinase is activated by at least four B-type cyclins (Clb1-Clb4) in the budded portion of the cell cycle (Ghiara et al., 1991; Surana et al., 1991). To determine whether the Clb/Cdc28 kinase was important for morphogenesis in the budded portion of the cell cycle, we used the cdc28-4 allele, which has been shown to be defective in both G1 and G2 functions at 37°C (Reed and Wittenberg, 1990). We reasoned that a shift of cdc28-4 budded cells to 37°C would result in inactivation of the Clb/Cdc28 forms of the kinase. If these forms of the kinase regulated the actin distribution during the budded portion of the cell cycle, then the cells would be expected to display abnormalities in the actin distribution upon temperature shift. Homozygous diploid cdc28-4 cells and wild type controls were grown at 25°C, shifted to 37°C, and fixed at various times to determine the actin distribution in budded cells. After a transient (30–60 min) depolarization of cortical actin patches similar to that seen upon temperature shift of wild-type cells (data not shown), cdc28-4 budded cells displayed a striking clustering of cortical actin patches at the tip of the bud (Fig. 7 A). After 2 h, the buds became elongated (presumably as a result of hyperpolarized secretion: see below), but many of the cells were still able to go through mitosis, randomize their actin, refocus the cortical actin patches to the neck region and undergo cell separation. In some of the cdc28-4 cells at 37°C, a fraction of the cortical actin patches clustered at a site on the surface of the mother portion of the cell distal to the bud, as well as at the bud tip (Fig. 7 A, right). These cells acquired a rather bizarre morphology with elongated projections in both mother and bud.

These results suggested that the Clb/Cdc28 kinase might be responsible for depolarizing the cortical actin within the bud. Further support for this hypothesis was obtained using two other methods to inactivate the Clb/Cdc28 kinase. Cells lacking Clb1-Clb4 are inviable but can be rescued by overexpression of Clb1 using a GAL1:CLB1 construct. clb1,2,3,4 GAL1:CLB1 cells are viable when grown on galactose based medium, but arrest in the budded phase of the cell cycle.
Figure 8. Contribution of the different Clbs to the actin- and secretion-depolarizing activity in G2 cells. (A and B) Wild-type cells were stained with FITC-ConA and returned to growth in YEP-glucose at 30°C for 90 min before fixation. Cells originating from labeled buds were identified by the unlabeled birth scars (arrowheads), and showed two staining patterns: uniform staining (e.g., cell 1) or staining that faded out towards one end (e.g., cell 2). (C) Cells containing the indicated clb null alleles (DLY005, 372-379) were grown in YEP-glucose and analyzed by FITC-ConA staining and return to growth as above. The percent of cells displaying the fade-out staining pattern indicative of bud-tip-directed growth was quantitated by counting at least 200 cells of each strain. A higher percent of tip directed growth indicates a delayed switch to isotropic bud growth. (D) clb2 (DLY-373) cells were grown in YEP-glucose, fixed, and stained with rhodamine-phalloidin to visualize the actin distribution. (E) Phase contrast of same field. Bar, 10 μm.
that had undergone apical growth displayed intense labeling at the pole adjacent to the unlabeled birth scar which diminished towards the opposite pole, while cells from buds that grew isotropically were uniformly labeled over their surface except for the birth scar (Fig. 8, A and B). Depending on the growth medium 35–50% of the new cells from a wild-type population exhibited apical growth, suggesting that the switch to isotropic secretion occurred one third to one half of the way through the budded phase (corresponding to early G2 in this strain).

The hyperpolarized secretion observed upon inactivation of the Clb/Cdc28 forms of the kinase (Fig. 7) suggested that one or more of these were responsible for triggering the depolarizing switch in wild type cells. To determine which Clbs were involved in this switch, we repeated the FITC-Con A labeling and return to growth experiment with cells carrying individual null mutations in the four CLB genes, as well as the viable multiple clb null combinations (Fig. 8 C). It was clear from this experiment that Clb2 was the major contributor to the actin- and secretion-depolarizing activity of Clb/Cdc28 complexes. clb2 cells retained an apical secretion pattern through most of the budded interval, resulting in elongated buds and hence elongated cells. In addition, the actin distribution in these cells was hyperpolarized, with most cells displaying cortical actin patches clustered at the tip of the bud (Fig. 8, D and E).

**Overproduction of Clbl and Clb2 Accelerates the Switch to Depolarized Secretion in the Bud**

If the switch to isotropic secretion is indeed triggered by activation of Cdc28 by Clbs, then expression of Clbs earlier in the cell cycle than usual should result in premature depolarization of secretion. To test this, we used diploid cells containing various GAL1:CLB constructs integrated into the genome. Transfer of these cells to galactose medium induced high levels of Clb synthesis and (in the case of Clbl, Clb2, and Clb3) delayed or blocked exit from mitosis (C. S. Stueland, D. J. Lew, and S. I. Reed, submitted for publication). We transferred these strains and wild-type controls to galactose for 60 min to induce the GAL1 promoter, stained the cells with FITC-Con A, and returned them to galactose medium for 90 min. Buds and new cells were scored for apical growth as above. As shown in Fig. 9 A, overexpression of Clbl or Clb2 accelerated the transition to depolarized growth. ClblΔ152, a truncated derivative of Clbl which is more stable than full-length Clbl (Ghiara et al., 1991), was even more effective in depolarizing secretion (Fig. 9 A). Surprisingly, however, Clb3 overexpression had the opposite effect: the cells retained an apical secretion pattern for longer than the wild-type cells. Clb4 overexpression had no effect. In all cases, overproduction of the Clb protein was confirmed with appropriate antibodies (N. Grandin and S. I. Reed, unpublished observations).

![Figure 9. Effects of Clb overexpression on secretion and actin distribution of budded cells.](image)

(A) Cells containing the indicated GALI:CLB alleles (DLY005,333,385,328,338) were grown in YEP-sucrose, galactose was added for 1 h, and the cells were stained and analyzed by FITC-ConA return to growth. The percent of cells displaying the fade-out staining pattern indicative of bud-tip-directed growth was quantitated by counting at least 200 cells of each strain. A lower percent of tip directed growth indicates an accelerated switch to isotropic bud growth. (B and C) GALI:CLB1 (DLY333) cells were grown in YEP-sucrose, galactose was added for 3 h, and the cells were fixed and stained with rhodamine-phalloidin (B). (C) Phase contrast of same field. Similar results were obtained with GALI:CLB2 (DLY385) (data not shown). (D and E) GALI:CLB3 (DLY338) cells were grown in YEP-sucrose, galactose was added for 3 h, and the cells were fixed and stained with rhodamine-phalloidin (D). (E) Phase contrast of same field. Bar, 10 μm.
The actin distribution in these cells was monitored 3 h after transfer of the cells to galactose to induce Clb expression (Fig. 9, B and C). A high proportion of the cells overproducing Clbl or Clb2 displayed cortical actin patches spread evenly over the entire bud surface, and some cells also had patches in the mother portion of the cell. Cells with cortical actin patches at the tip of the bud were virtually absent from these populations. The buds formed by these cells were spherical (Fig. 9 C), rather than the normal oval shape for diploids (inset), consistent with a premature depolarization of secretion. Cells overexpressing ClblΔ152 had a similar morphology, and almost all the budded cells had cortical actin patches delocalized over the entire cell surface (mother and bud: see Fig. 11 A). These cells were arrested in mitosis. Cells overexpressing Clb3 were also arrested in mitosis, but many of these cells had elongated buds and cortical actin patches clustered at the tip of the bud (Fig. 9, D and E). Clb4 overexpression had no effect (data not shown).

These results suggest that depolarization of the actin cytoskeleton and secretory apparatus in G2 is triggered by activation of the Clbl/Cdc28 and Clb2/Cdc28 kinase. The strikingly different results obtained with Clb3 suggest that the Clb3/Cdc28 kinase may have different targets than the Clbl/Cdc28 and Clb2/Cdc28 forms of the kinase.

Overexpression of ClblΔ152 in G1 Accelerates Cell Cycle Progression but Prevents Actin Polarization and Budding

Previous experiments showed that ectopic expression of Clns in G2 resulted in hyperpolarization of the actin cytoskeleton and secretion (Fig. 6), counteracting the depolarizing effects of Clbl and Clb2. We tested whether ectopic Clb expression in G1 would have any effect on the cytoskeleton or secretion. To address this issue, we used diploid cells containing a GALI:CLBIΔ152 construct integrated into the genome, because previous experiments suggested that ClblΔ152 was most effective in activating the depolarizing functions of the kinase (Fig. 9 A). GI cells were isolated by centrifugal elutriation and inoculated into galactose medium to induce ClblΔ152 synthesis from the GALI promoter. At various intervals, samples were taken for analysis of DNA content and actin distribution (Fig. 10). Cells expressing ClblΔ152 initiated and completed DNA replication more rapidly than wild type cells (Fig. 10 A), suggesting that ClblΔ152 promoted accelerated passage through START. However, none of the cells expressing ClblΔ152 were able to polarize their cortical actin patches to the pre-bud site or form a bud (Fig. 10 B). This suggests that although Clbs and Clns may both be able to trigger events leading to DNA replication, they affect different targets with opposite effects in the control of morphogenesis.

Inactivation of Cdc28 May Be Required for Actin Assembly at the Cytokinesis Site

During mitosis, wild-type cells transiently randomize the distribution of cortical actin patches throughout the surface of mother and bud, and then both cortical actin and secretion are redirected to the neck region for cytokinesis and septum formation. Cells arrested in mitosis by activation of the ClblΔ152/Cdc28 kinase display a random distribution of cortical actin patches (Fig. 11 A). Thus, it seemed plausible that redistribution of cortical actin patches to the cytokinesis site might normally require inactivation of the mitotic Cdc28 kinase. To address this issue we constructed a homozygous diploid cdc28-13 strain containing an integrated GALI:CLBLΔ152 construct. CDC28 cells containing the same con-
struct were used as a control. Cells were arrested in mitosis by incubation in galactose medium for 4 h at 25°C, and then the kinase was inactivated by shifting up to 37°C (Fig. 11). 1 h after temperature shift, a significant fraction (10–40% in different experiments) of the budded cdc28 cells displayed clustered cortical actin patches in the neck region (Fig. 11C), while all of the CDC28 cells retained a random distribution (Fig. 11B). The clustering of cortical actin in the neck region was transient, as the cells progressed through cytokinesis and arrested as unbudded cells. By 2 h after temperature shift, 60–80% of the budded cdc28 cells had progressed through cytokinesis and cell separation, while all of the budded CDC28 cells were still budded. These results are consistent with the hypothesis that clustering of cortical actin patches in the neck region at cytokinesis is triggered by inactivation of the mitotic Cdc28 kinase.

Discussion

Activation of Cdc28 by G1 Cyclins Triggers Polarization of the Cortical Actin Cytoskeleton to the Pre-bud Site

Assembly of the pre-bud site involves not only polarization of the cortical actin cytoskeleton but also formation of 10-nm filament rings (composed of the CDC3, CDC10, CDC11, and CDC12 gene products) and a cap containing Spa2 protein at the same site (Chant and Pringle, 1991; Madden et al., 1992). Previous studies showed that polarization of cortical actin patches, 10 nm filament rings, and the Spa2 cap all occur simultaneously, but there was some disagreement as to whether this occurred in early or late G1 (Ford and Pringle, 1991; Kim et al., 1991; Snyder et al., 1991). Using cells synchronized by starvation or mating pheromone, Kim et al. (1991) concluded that pre-bud site assembly of 10-nm filament rings occurred ~15 min before bud emergence, in late G1. Using mating pheromone synchronized cells but observing the second cell cycle to avoid possible artifacts from the synchronization technique, Snyder et al. (1991) concluded that pre-bud site assembly of the Spa2 cap occurred immediately after cell separation, in early G1. Our results using cells synchronized by centrifugal elutriation and unperturbed cycling cells are in agreement with those of Kim et al. (1991), and demonstrate that pre-bud site assembly occurs after the regulatory START step late in G1.

Actin polarization to the pre-bud site in G1 was dependent on both Cdc28 and Clns (Fig. 12A). Thus, actin polarization to the pre-bud site requires Cln/Cdc28 activity and occurs immediately following Cln/Cdc28 activation in the cell cycle. Actin polarization can be triggered by Cln/Cdc28 activation even in the absence of de novo protein synthesis (Fig. 4), showing that, like transcriptional induction after Cln/Cdc28 activation (Marini and Reed, 1992) but unlike DNA replication or budding, this response occurs post-translationally. Furthermore, we have shown that premature Cln accumulation results in premature bud emergence in G1 (Lew et al., 1992). Together, these results argue strongly that Cln/Cdc28 activation is the regulatory trigger for pre-bud site assembly.

Activation of Cdc28 by the Mitotic Cyclins Clb1 and Clb2 Causes Depolarization of the Cortical Actin Cytoskeleton and Secretory Apparatus in G2

Monitoring the deposition of new cell wall as a marker for the polarity of secretion in budded cells, we confirmed the conclusion of (Farkas et al., 1974) that a switch occurred in G2 from a highly polarized pattern of secretion to the bud tip to a more general pattern of secretion to the entire bud surface. We cannot tell from our experiments whether the switch was gradual or abrupt, but this question has been quantitatively addressed in the dimorphic yeast Candida albicans by Soll and co-workers (Staebell and Soll, 1985). They found that in the budding mode, cell wall growth in small buds results from 70% bud tip growth and 30% isotropic (uniformly distributed) bud growth. However, later in the budded phase the tip-directed growth was abruptly terminated, leaving only the isotropic pathway. The timing of this switch may be a critical determinant of cell shape, such that cells that switch early would be more spherical, whereas those that switch late would be more tubular.

Inactivation of the Clb/Cdc28 kinase in any of three ways (shift-up of temperature sensitive cdc28 mutants, elimination of Clbs, or inhibitory phosphorylation of Cdc28 by the S. pombe wee1 kinase) prevented the switch to uniform bud growth in G2 and resulted in hyperpolarization of cortical actin patches and secretion to the tip of the bud (Fig. 12B). This was not an indirect consequence of cell cycle arrest, be-
Figure 12. Summary. (A) Wild-type G1 cells polarize their cortical actin patches to a ring at the pre-bud site. Elimination of Cln/Cdc28 activity causes these cells to retain a dispersed pattern of cortical actin patches, and they grow uniformly to a size much larger than normal G1 cells. Hyperactivation of Cln/Cdc28 results in premature actin polarization and budding at a smaller than normal size, and the cortical actin patches in the bud are hyperpolarized to the bud tip. (B) Wild-type budded cells depolarize their cortical actin patches in G2. Elimination of Clb/Cdc28 activity causes these cells to hyperpolarize their cortical actin patches to the bud tip. Hyperactivation of Clb1,2/Cdc28 results in premature depolarization of cortical actin patches and secretion in S phase, leading to generation of a spherical bud. (C) Wild-type cells redistribute their cortical actin patches to the neck region for cytokinesis (e). (D) Model for the role of Cdc28 and cyclins in the control of morphogenetic transitions in the cell cycle. Cln/Cdc28 activation at START triggers polarization of cortical actin patches to the pre-bud site (a, a'). Clb1,2/Cdc28 activation in G2 triggers depolarization of cortical actin patches and secretion (c). Inactivation of Cdc28 at the end of mitosis triggers redistribution of cortical actin patches to the neck region for cytokinesis (e).

cause neither Cdc28 impairment by incubating a temperature sensitive cdc28 mutant at elevated temperature nor induction of wee1 inactivated enough of the Clb/Cdc28 kinase to block progression through mitosis. Furthermore, cell cycle arrest induced by hydroxyurea (which blocks DNA replication) or nocodazole (which blocks spindle assembly) resulted in a completely random distribution of cortical actin patches (data not shown).

The four B-type cyclins studied, Clb1–Clb4, fall into two categories based on sequence homology and time of expression in the cell cycle (Surana et al., 1991; Richardson et al., 1992). Clb1 and Clb2 are closely related and are expressed in G2. Clb3 and Clb4 form a distinct class and are expressed at the beginning of S phase. For both classes, activation of Cdc28 follows closely upon Clb induction (N. Grandin and S. I. Reed, submitted for publication). Analysis of strains bearing null mutations in the different CLB genes demonstrated that Clb2 played the greatest role in the depolarizing function of Cdc28 (Fig. 8). Thus, depolarization of the cortical actin cytoskeleton and secretory apparatus in G2 requires Clb1,2/Cdc28 activity and occurs immediately after Clb1,2/Cdc28 activation in the cell cycle. In addition, deregulated expression of Clb1 or Clb2 (but not Clb3 or Clb4) resulted in premature transition to uniform bud growth (Fig. 9). These results strongly suggest that Clb1,2/Cdc28 activation is the regulatory trigger for the depolarizing switch that determines the shape of the bud (Fig. 12, B and D).

This conclusion may have implications for the dimorphic
switch in C. albicans. These cells can be induced to switch from the yeast mode to a mycelial mode of growth by incubation in medium of appropriate pH. Soll and co-workers found that budded yeast cells could form mycelia upon pH change, but only if they had not yet made the switch to isotropic bud growth (Soll et al., 1985). If this transition had passed, the cells would complete the cell cycle in the yeast mode and only switch to the mycelial mode in the next cell cycle. Thus, a developmental option is closed after switching to general bud growth. This situation is analogous to the developmental options (budding, mating, or entrance to stationary phase) available to cells in G1. Activation of Cln/Cdc28 to pass START constitutes a decision in favor of budding, and closes off the other options until the next cell cycle. Perhaps activation of the Candida equivalents of Clbl,2/Cdc28 to switch to isotropic bud growth is similarly a decision in favor of budding, and closes off the mycelial option until the next cell cycle.

Inactivation of Cdc28 after Mitosis May Trigger Actin Redistribution to the Cytokinesis Site

At the end of mitosis, cortical actin patches cluster on both sides of the neck region. Recently, it has been shown that capping protein, an actin-binding protein that colocalizes with cortical actin patches during most of the cell cycle, is absent from the patches that cluster in the neck region (Amatruda and Cooper, 1992). Thus, this redistribution also involves a change in the composition of the actin-rich patches. We found that sustained Cdc28 activation using a nondestructible derivative of Clbl (ClblΔ152) prevented actin redistribution to the cytokinesis site (Fig. 12, C). This was not a nonspecific consequence of Clb/Cdc28 inactivation, because the redistribution could be triggered in the arrested cells by Cdc28 inactivation (achieved by shift-up of a temperature sensitive cdc28 mutant). Thus, actin redistribution to the cytokinesis site requires Cdb/Cdc28 inactivation and occurs immediately after Cdb destruction in the cell cycle, suggesting that inactivation of Cdc28 may be the regulatory trigger for this rearrangement (Fig. 12, C and D).

Cdc28 Substrates Involved in the Control of Morphogenesis

Our results indicate that changes in Cdc28 activity at three different stages of the cell cycle regulate distinct rearrangements of the cortical actin cytoskeleton and secretory apparatus. Two other rearrangements, the redistribution of cortical actin patches into the small bud in S phase and the redistribution of some cortical actin patches to the mother in mitosis remain to be explained. We have been unable to determine whether Cdc28 activity plays a role in these events.

In the case of pre-bud site assembly after START, we have shown that the actin rearrangement can be triggered by Cln/Cdc28 activation in the absence of de novo protein synthesis. This suggests that the kinase may directly phosphorylate substrates (such as actin-binding proteins) that regulate actin distribution in cells. Alternatively, the target substrates might be “polarity establishment” genes, such as CDC42, CDC42, BEM1, and BEM2 (Sloat et al., 1981; Adams et al., 1990; Johnson and Pringle, 1990; Bender and Pringle, 1991; Chenévert et al., 1992). Mutations in these genes prevent polarization of cortical actin patches, 10-nm filaments, and Spa2 protein to the pre-bud site. Cdc42 is a ras-like GTP binding protein (Johnson and Pringle, 1990), while Cdc24 is related to the oncogene dbi, which catalyzes dissociation of GDP from the human homolog of Cdc42 (Hart et al., 1991). These proteins could potentially participate in a signal transduction pathway whereby Cln/Cdc28 activation triggers assembly of the pre-bud site.

Different Cdc28/Cyclin Kinases Can Have Opposing Effects on Both the Actin Cytoskeleton and the Secretory Apparatus

In principle, the different effects of Cln/Cdc28 activation in G1 and Cdb/Cdc28 activation in G2 could arise without the different forms of the kinase displaying any differences in substrate specificity. In this scenario, distinct substrates would be available to the kinase at different times in the cell cycle, so that activation of essentially the same kinase at different points in the cell cycle would have different effects. However, the results obtained with ectopic activation of specific forms of the kinase at inappropriate times in the cell cycle argue strongly against this model. Expression of Cln1 or Cln2 in G2 resulted in hyperpolarization of cortical actin and secretion to the bud, while expression of ClblΔ152 in G1 prevented polarization of cortical actin to the pre-bud site. These results suggest that similar substrates may be available throughout the cell cycle, and that the different roles of the kinase are enacted by targeting the Cln/Cdc28 and Clb/Cdc28 complexes to different substrates.

Differences were also observed upon overproduction of different Clbs in G2. Specifically, Clbl and Clb2 overproduction had a depolarizing effect, while Clb3 overproduction had a hyperpolarizing effect. This suggests that there may be differences in the substrates of the Clbl,2/Cdc28 and Clb3/Cdc28 complexes. In all cyclin overexpression experiments, there is a possibility that the effects of overexpression are not a direct consequence of activation of the targeted cyclin/Cdc28 complex, but rather an indirect “dominant negative” consequence of inhibition of other cyclin/Cdc28 complexes. In the simplest case, overexpression of one cyclin might result in its binding to all of the cellular Cdc28, leaving none for other cyclins. This does not appear to be the case in our experiments because co-overexpression of Cdc28 together with a cyclin had the same effect as overexpression of the cyclin alone (data not shown). However, we cannot rule out the possibility that some of the cyclin overexpression results arose through dominant negative competition between cyclins for other potentially limiting factors. This does not affect our conclusion that different forms of the Cdc28 kinase are targeted to different substrates because such differences are a prerequisite for any dominant negative model.

Understanding the molecular basis of the regulation of cytoskeletal function is the key not only to morphogenesis in yeast but also to regulation of cell shape and cell motility in nonwalled cells. The structural homology between various yeast genes involved in morphogenesis and their mammalian counterparts (e.g., between CDC42 and G25K, or CDC24 and dbi and bcr [Chant and Pringle, 1991]) suggests that insights gained in the genetically tractable yeast system will have far-reaching implications for other cells.

We thank Sally Kornbluth, Nick Marini, and anonymous reviewers for crit-
Amatruda, J. F., and J. A. Cooper. 1992. Purification, characterization, and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

2. Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

3. Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

4. Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

5. Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

6. Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.

7. Haarer, B. K., S. H. Lillie, A. E. M. Adams, V. Magdonio, W. Bando, and S. S. Brown. 1990. Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells. J. Cell Biol. 110: 105–114.
Richardson, H. E., C. Wittenberg, F. R. Cross, and S. I. Reed. 1989. An essential G1 function for cyclin-like proteins in yeast. Cell. 59:1127-1133.
Russell, P., S. Moreno, and S. I. Reed. 1989. Conservation of mitotic controls in fission and budding yeast. Cell. 57:295–303.
Russell, P., and P. Nurse. 1987. Negative regulation of mitosis by wee1, a gene encoding a protein kinase homolog. Cell. 45:145–153.
Sherman, F., G. Fink, and J. B. Hicks. 1982. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Sloat, B. F., A. E. M. Adams, and J. R. Pringle. 1981. Roles of the CDC24 gene product in cellular morphogenesis during the Saccharomyces cerevisiae cell cycle. J. Cell Biol. 89:395–405.
Snyder, M., S. Gehrung, and B. D. Page. 1991. Studies concerning the temporal and genetic control of cell polarity in Saccharomyces cerevisiae. J. Cell Biol. 114:515-532.
Soll, D. R., M. A. Herman, and M. A. Staebell. 1985. The involvement of cell wall expansion in the two modes of mycelium formation of Candida albicans. J. Gen. Microbiol. 131:2367–2375.
Staebell, M., and D. R. Soll. 1985. Temporal and spatial differences in cell wall expansion during bud and mycelium formation in Candida albicans. J. Gen. Microbiol. 131:1467–1480.
Sudbery, P. E., A. R. Goodey, and B. L. A. Carter. 1980. Genes which control cell proliferation in the yeast Saccharomyces cerevisiae. Nature (Lond.). 288:401–404.
Surana, U., H. Robitsch, C. Price, T. Shuster, I. Fitch, A. B. Fitcher, and K. Nasmyth. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell. 65:145–161.
Tkacz, J. S., and J. O. Lampen. 1972. Wall replication in Saccharomyces species: use of fluorescein-conjugated concanavalin A to reveal the site of mannan insertion. J. Gen. Microbiol. 72:243–247.
Tyers, M., G. Tokiwa, R. Nash, and B. Fitcher. 1992. The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation. EMBO (Eur. Mol. Biol. Organ.). J. 11:1773–1784.
Wittenberg, C., and S. I. Reed. 1988. Control of the yeast cell cycle is associated with assembly/disassembly of the Cdc28 protein kinase complex. Cell. 54:1061–1072.
Wittenberg, C., K. Sugimoto, and S. I. Reed. 1990. G1-specific cyclins of Saccharomyces cerevisiae: cell cycle periodicity, regulation by mating pheromone and association with the p34cdc28 protein kinase. Cell. 62:225–237.
Xiong, Y., and D. Beach. 1991. Population explosion in the cyclin family. Curr. Biol. 1:362–364.