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Inactivation of Mitotic Kinase Triggers Translocation of MEN Components to Mother-Daughter Neck in Yeast

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Chromosome segregation, mitotic exit, and cytokinesis are executed in this order during mitosis. Although a scheme coordinating sister chromatid separation and initiation of mitotic exit has been proposed, the mechanism that temporally links the onset of cytokinesis to mitotic exit is not known. Exit from mitosis is regulated by the mitotic exit network (MEN), which includes a GTPase (Tem1) and various kinases (Cdc15, Cdc5, Dbf2, and Dbf20). Here, we show that Dbf2 and Dbf20 functions are necessary for the execution of cytokinesis. Relocalization of these proteins from spindle pole bodies to mother daughter neck seems to be necessary for this role because *cdc15-2* mutant cells, though capable of exiting mitosis at semipermissive temperature, are unable to localize Dbf2 (and Dbf20) to the "neck" and fail to undergo cytokinesis. These cells can assemble and constrict the actomyosin ring normally but are incapable of forming a septum, suggesting that MEN components are critical for the initiation of septum formation. Interestingly, the spindle pole body to neck translocation of Dbf2 and Dbf20 is triggered by the inactivation of mitotic kinase. The requirement of kinase inactivation for translocation of MEN components to the division site thus provides a mechanism that renders mitotic exit a prerequisite for cytokinesis.

INTRODUCTION

Chromosome segregation, inactivation of mitotic kinase, and cytokinesis are three major tasks cells undertake during mitosis. Coordination between these events is essential for chromosome stability and a cell's fitness and survival. In general, a regulatory order exists between these events. Although failure to separate chromosomes impairs initiation of mitotic exit and cytokinesis, the inability to exit mitosis prevents the onset of cytokinesis but not chromosome segregation (Balasubramanian *et al*., 2000).

In budding yeast, equal partitioning of chromosomes requires coordinated interplay between effectors that regulate spindle dynamics, spindle checkpoint, anaphase-promoting complex (APC), and cohesin stability (Zachariae and Nasmyth, 1999; Pellman and Christman, 2001). Mitotic exit, on the other hand, is under the control of the mitotic exit network (MEN), which includes Tem1 (a GTPase), Lte1 (a GTP/GDP exchange factor), Ser/Thr kinases (Cdc15, Cdc5, Dbf2, and Dbf20) and Cdc14 (a phosphatase) (Morgan, 1999; Lee *et al*., 2001). It has been shown that some of these components (Tem1, Cdc15, Cdc5, and Mob1) localize to the spindle pole body (SPB) at the onset of mitosis. MEN activation eventually leads to Cdc14 release from the nucleolus (Shou *et al*., 1999; Visintin *et al*., 1999), which in turn activates Hct1/Cdh1 (Cdc20 homolog and, like Cdc20, an APC activator) via dephosphorylation. Both APC^{Cdc20} and APC^{Hct1} complexes mediate mitotic cyclin destruction (Schwab *et al*., 1997; Shirayama *et al*., 1999; Baumer *et al*., 2000; Jaspersen and Morgan, 2000; Yeong *et al*., 2000). The septum initiation network (SIN) of fission yeast is similar to the budding yeast MEN (McCollum and Gould, 2001), but it mainly regulates the initiation of septum formation.

Control and execution of cytokinesis involve septins, actomyosin ring constituents, chitin building enzymes and proteins that regulate localization, assembly and functions of these components (Longtine *et al*., 1996; Tolliday *et al*., 2001). The establishment of cytokinesis site in budding yeast (mother-daughter neck) occurs simultaneously with bud emergence as cells traverse START. In late G1, septins are assembled at the base of the emerging bud (Gladfelter *et al*., 2001). Actomyosin ring construction also begins at G1/S with localization of Myo1 (myosin II) in form of a ring but actin is not recruited until telophase (Lippincott and Li, 1998a). Although recruitment of F-actin to Myo1 ring requires Cyk1, the stability of actomyosin ring depends on Cyk2 (Lippincott and Li, 1998b; Shannon and Li, 1999). Contraction of actomyosin ring is believed to guide inward deposition of chitin to form the primary septum (Schmidt *et al*., 2002).

What binds major mitotic events in a temporal sequence? Although a tentative scheme for coordinating chromosome segregation and mitotic exit has been proposed (Bardin *et al*., 2000; Bloecher *et al*., 2000; Pereira *et al*., 2000), it is not known what makes execution of mitotic exit a prerequisite for initiation of cytokinesis. That the expression of proteolytic resistant mitotic cyclin Clb2 prevents not only mitotic exit but also execution of cytokinesis (Surana *et al*., 1993), suggests a link between mitotic kinase inactivation and initiation of cytokinesis. Recently, a number of observations have implicated MEN components in cytokinesis: 1) overexpression of truncated versions of Cdc15 and Cdc5 can lead to cytokinetic defect (Cenamor *et al*., 1999; Song *et al*., 2000;

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Table 1. Strains used in this study

Strain	Genotype	Source
US356	*MATa ade2-1 trp1-1 can1-100 leu2-3 his3-11 bar1:hisG*	US laboratory
US1318	*MATa bar1Δcdc15-2 ura3 trp1 his3 leu2*	US laboratory
US1363	*MATa bar1Δura3 trp1 his3 leu2*	US laboratory
US1998	*MATa dbf2-2 ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*	This study
US2802	*MATa bar1Δcdc15-2-YFP-URA3 SPC29-CFP-KANMX ura3 leu2 trp1*	This study
US2809	*MATa dbf2-2 dbf20ΔTRP1 ade2-1 can1-100 leu2-3 his3-11 ura3*	This study
US2810	*MATa bar1ΔCDC15-YFP-URA3 SPC29-CFP-KANMX trp1 leu2 his3 4X GAL-SIC1-cmyc3-URA3*	This study
US2880	*MATa bar1ΔDBF20-YFP-HIS3 SPC29-CFP-KANMX ura3 leu2 trp1*	This study
US2888	*MATa bar1ΔDBF2-YFP-HIS3 SPC29-CFP-KANMX 4X GAL-SIC1-cmyc3-URA3 leu2 trp1*	This study
US2892	*MATa bar1ΔDBF20-YFP-HIS3 SPC29-CFP-KANMX 4X GAL-SIC1-cmyc3-URA3*	This study
US2944	*MATa dbf20ΔTRP1 bar1ΔURA3 ade2-1 trp1-1 can1-100 leu2-3 his3-11 ura3*	This study
US2998	*MATa bar1Δcdc15-2 YFP-DBF2-TRP1 his3 leu2 ura3*	This study
US3122	*MATa bar1Δcdc15-2 MYO1-GFP (S65T)-KANMX trp1 his3 leu2 ura3*	This study
US3145	*MATa bar1ΔMYO1-GFP (S65T)-KANMX his3 leu2 trp1 ura3*	This study
US3495	*MATa bar1Δcdc15-2 his3 trp1 ura3 pGAL-SIC1-cmyc3/LEU2/CEN*	This study

Menssen *et al*., 2001); 2) under some experimental conditions, Cdc15 (Xu *et al*., 2000), Cdc5 (Song *et al*., 2000), Dbf2 and Mob1 (Frenz *et al*., 2000; Luca *et al*., 2001) can localize to the mother-bud junction; and 3) *cdc15* mutant fails to assemble a stable actomyosin ring (Lippincott and Li, 1998a). However, the regulatory elements that temporally link cytokinesis to mitotic exit remain elusive.

In this study, we focus on Dbf2 and Dbf20, the two downstream components of MEN. We show that Dbf2 and Dbf20 are together essential for cytokinesis. The appearance of both proteins at the "neck" is consistent with this role. Interestingly, the translocation of both Dbf2 and Dbf20 from SPB to the mother-daughter junction requires inactivation of the mitotic kinase. We propose that this requirement makes the execution of cytokinesis critically dependent on mitotic exit.

MATERIALS AND METHODS

Yeast Media and Reagents

All strains used in this study were haploid and were congenic to the wild-type strain W303. Cells were routinely grown in yeast-extract peptone (YEP) or selective medium supplemented with 2% glucose (+Glu) or raffinose-galactose (+Raff+Gal). Kanamycin-resistant strains were selected on plates containing G418 (200 mg/l).

Strains and Plasmids

A combination of standard molecular biology and molecular genetic techniques such as gene transplacement, gene disruption, polymerase chain reaction-based tagging of endogenous genes, and tetrad dissection were used to construct plasmids and strains with various genotypes (Table 1). Southern blot analysis was performed to confirm gene disruptions and transplacements.

Synchronization Procedures

For experiments requiring synchronous cultures, exponential phase cells were grown in medium at 24°C containing either 1 μg/ml α factor (for *bar1Δ* cells) or 5 mg/ml (for *BAR1* cells). After 3–3.5 h of treatment, cells were filtered, washed, and resuspended in fresh medium preincubated at the appropriate temperature. For the purpose of synchronizing cells in metaphase, cycling culture was treated with nocodazole (15 μg/ml) for 3–6 h. Samples were then taken at specific intervals for measurement of H1 kinase activity, Western blot analysis, flow cytometry, and immunofluorescent staining.

Cell Extracts, Kinase Assays, Immunoprecipitation, and Western Blot Analysis

For determining Clb2-Cdc28 kinase activity, cells were harvested by centrifugation at 4°C, washed with ice-cold stop mix, and used for the preparation of cell extract (Surana *et al*., 1993). For the determination of kinase activities, immunoprecipitation of Cdc28-Clb2 was carried out using polyclonal antibodies against Clb2 at 1:60 dilution. The kinase assays were performed as described in Surana *et al.* (1993). For preparation of crude extracts for Western blot analysis, precipitation of proteins by trichloroacetic acid was carried out as described previously (Yeong *et al*., 2000). For Western blot analyses, immunodetection of Cdc28 and Clb2 was performed using anti-Cdc28 polyclonal antibodies (1:1000 dilution) and anti-Clb2 polyclonal antibodies (1:1000 dilution), respectively. Enhanced chemiluminescence kit from Santa Cruz Biotechnology (Santa Cruz, CA) was used for all Western blot analyses according to the manufacturer's instructions.

Flow Cytometry, Calcofluor Staining, and Visualization of Green Fluorescent Protein (GFP) Signals

The method described by Lim *et al.* (1996) was used for flow cytometry. To visualize signals of the cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) fusion proteins, cells collected at various time points were frozen immediately on dry ice without fixation and stored until further use. Cells were later thawed and mounted on slides with Vectorshield containing 4,6-diamidino-2-phenylindole (DAPI). The images were captured using a DMRX microscope (Leica, Wetzlar, Germany) attached to a charge-coupled device camera (Hamamatsu, Bridgewater, NJ) driven by the MetaMorph software (Universal Imaging, Downingtown, PA). Typically, the exposure time for the Cdc15-YFP, Dbf2-YFP, and Dbf20-YFP fusion proteins was 4 s and that for the Spc29-CFP was 500 ms. To obtain deconvolved images, 36–40 optical sections spaced 0.2 μm apart were captured for each sample and processed using the Autodeblur software from AutoQuant Imaging.

For calcofluor staining, cells were fixed in KPF (Surana *et al*., 1993). The fixative was washed off using phosphate-buffered saline (PBS) (3×), and calcofluor was added to cells at the final concentration of 1 μg/ml. Samples were mounted in Vectorshield (without DAPI) and images were captured as described above. For actin staining, cells fixed in KPF were washed three times with PBS. Alexa 594 phalloidin (concentration 0.24 U/μl; Molecular Probes, Eugene, OR) was added at 1:100 dilution and suspension was incubated overnight at 4°C. Cells were then washed twice with PBS, mounted in Vectorshield containing DAPI, and images captured as described above. For staining with FM4-64, 1 μl of the dye (400 μM) was added to 100 μl of cold cell suspension in PBS and incubated on ice. After 20 min, sodium azide (1 μl of 25 mM stock) was added to this suspension. Cells were collected by centrifugation, washed once with cold PBS/sodium azide, and mounted in Vectorshield (without DAPI) for microscopic observations.

RESULTS

Requirement of Dbf2 and Dbf20 for Cytokinesis

Although both *DBF2* and *DBF20* are nonessential genes, in combination they exhibit synthetic lethal behavior in that *dbf2Δ dbf20Δ* cells are unable to exit mitosis and consequently arrest in telophase (Toyn and Johnston, 1994). Although Dbf2 localization to the mother-bud neck during late mitosis suggests that it may play a role in cytokinesis (Frenz *et al*., 2000), thus far no cytokinesis defect has been clearly associated with *dbf2* mutants. To determine whether Dbf2

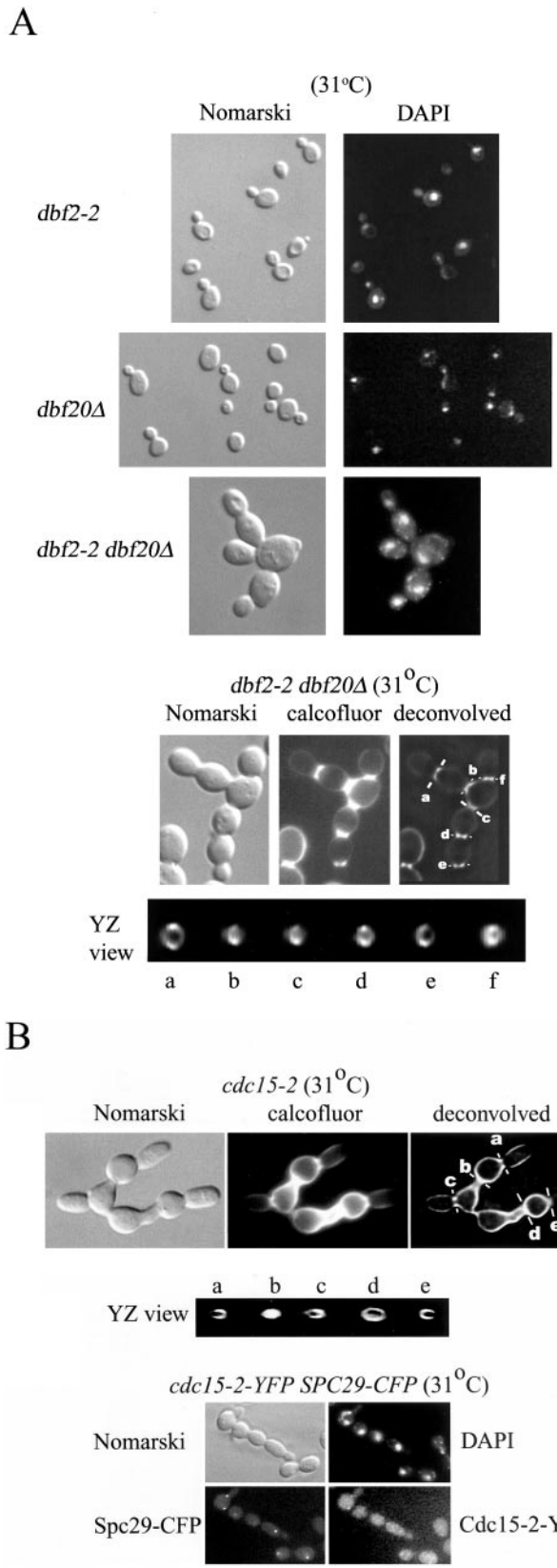

Figure 1. (A) Cytokinesis defect in *dbf2-2 dbf20*Δ double mutant. *dbf2-2* (US1998), *dbf20*Δ (US2944), and *dbf2-2 dbf20D* (US2809) mutant cells were grown at 31°C (semipermissive temperature) and were stained with DAPI to visualize the nuclei. The *dbf2-2 dbf20D* (US2809) double mutant was stained with calcofluor to visualize the septum material. The YZ views across the mother-bud neck are included to show the presence or absence of primary septum. (B) Cytokinesis defect of *cdc15-2* mutant cells and the localization of the Cdc15-2 mutant protein. *cdc15-2* (US1318) cells were grown at semipermissive temperature of 31°C and stained with calcofluor to visualize the state of the septa (top). To determine the localization of Cdc15-2 protein, mutant cells (US2802), in which the endogenous *cdc15-2* gene had been fused with the fluorescence tag *YFP*, were grown at 31°C and analyzed by fluorescence microscopy.

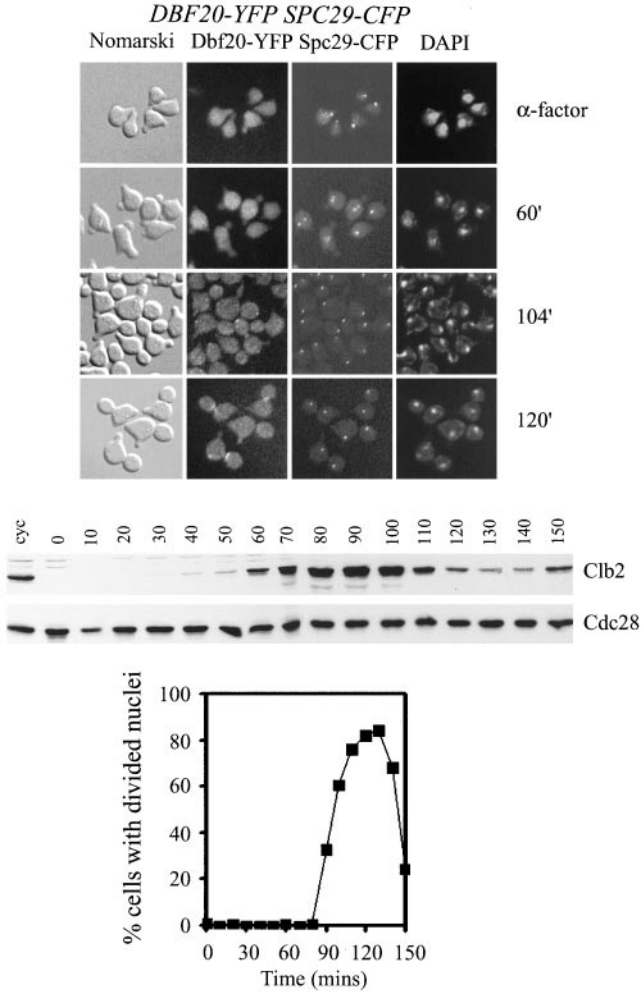

Figure 2. Localization of Dbf20-YFP during cell cycle. Cells (US2880) carrying both *DBF20-YFP* and *SPC29-CFP* (native promoter driven constructs, each integrated at its respective locus) cells were released from α-factor induced G1 arrest into fresh medium at 24°C. Samples were collected at the indicated time points and analyzed.

and Dbf20 play an overlapping role in cytokinesis, as they do in mitotic exit, we made use of *dbf20*Δ and the *dbf2-2* mutation (a temperature-sensitive allele of *DBF2* that causes arrest at 37°C but is normal in exiting mitosis at 31°C). We used growth at 31°C to test for cytokinesis defect in *dbf2-2* (US1998), *dbf20*Δ (US2944), and *dbf2-2 dbf20*Δ double mutant cells (US2809) because growth at 37°C would obscure the defect in cytokinesis due to telophase arrest caused by

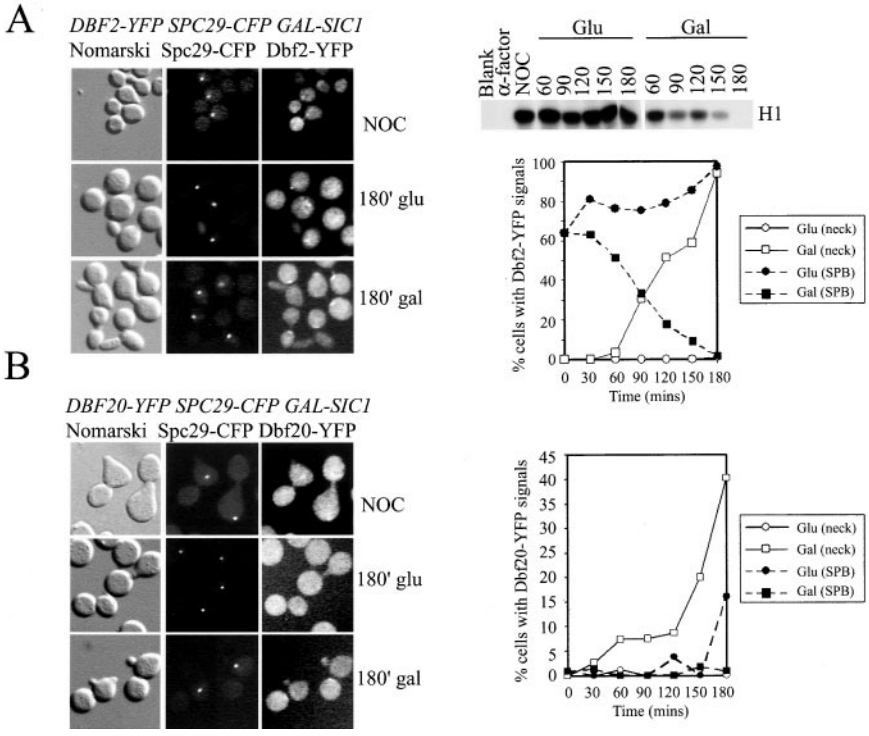

Figure 3. Dbf2-YFP and DBF20-YFP localization in response to the inactivation of mitotic kinase. (A) Cells (US2888) harboring a single copy of *DBF2-YFP* and *SPC29-CFP* at their respective loci, and four copies of *GAL-SIC1*, integrated at the *TRP1* locus were arrested in metaphase by treatment with nocodazole (NOC; 15 μg/ml) in raffinose medium. Glucose (2%) was added to one-half to repress any spurious transcription from *GAL1* promoter and galactose (2%) to the other half to induce *SIC1* transcription. Samples were collected and signals were visualized as before (left). H1 kinase assays were performed to ascertain that Clb2/Cdc28 kinase activity was inactivated upon *SIC1* induction (top right). The graph (right) shows the proportion of cells with Dbf2-YFP at the SPBs or the mother-daughter neck in different samples. (B) Dbf20-YFP localization in response to the inactivation of mitotic kinase. Cells (US2892) carrying a single copy of *DBF20-YFP* and *SPC29-CFP* at their respective loci, and four copies of *GAL-SIC1*, integrated at the *TRP1* locus were subjected to an experimental regime identical to that described in A and were analyzed similarly. The graph (right) shows the proportion of cells with Dbf20-YFP at the SPBs or the mother daughter junction. We suspect that these numbers are underestimated because Dbf20-YFP signal is much weaker compared with Dbf2-YFP.

dbf2-2. Although both *dbf2-2* and *dbf20*Δ cells exhibited no cytokinesis defect, *dbf2-2 dbf20*Δ cells were unable to separate from each other and grew as long chains at 31°C (Figure 1A, top). YZ view of the mother-daughter neck in calcofluor-stained cells revealed the absence of septum material in these chains of cells (Figure 1A, bottom). However, a few of the older mother-bud junctions do eventually build partial septum. These results suggest that *dbf2-2 dbf20*Δ mutant cells are largely defective in executing cytokinesis properly and that Dbf2 and Dbf20 have an overlapping function in cytokinesis.

We used the strategy of growth at semipermissive temperature to test whether other MEN mutants (already known to be defective in mitotic exit at nonpermissive temperature) are also defective in cytokinesis. *cdc15-2* cells, when grown at semipermissive temperature (31°C), undergo repeated rounds of cell division but the progeny cells fail to separate (Figure 1B). YZ views of the mother-daughter neck in calcofluor-stained cells showed the absence of septum material at most mother-bud junctions (Figure 1B, middle). It should be noted that a small proportion of junctions do form septa eventually, implying that septum formation is extremely inefficient, if not entirely defective, in this mutant. Similarly, other MEN mutants, namely, *cdc14-3*, *cdc5-1* and *tem1-1*, though able to undergo repeated rounds of nuclear division when grown at 31°C, were also defective in cytokinesis (our unpublished data). It has been suggested that the role of Cdc15 in cytokinesis is linked to its localization to SPBs (Menssen *et al*., 2001). To test whether the cytokinetic defect was due to the failure of Cdc15-2 mutant protein to reach the SPBs, we tagged the chromosomal *cdc15-2* mutant gene with YFP (US2802). When cells carrying *cdc15-2-YFP* and *SPC29-CFP* (a SPB component) were grown at 31°C, they exhibited cytokinesis defect and showed Cdc15-2-YFP fluorescence on SPBs (Figure 1B, bottom). This suggests that the failure of Cdc15-2 to execute cytokinesis properly at semipermissive temperature is not connected to its ability to reach SPBs

Localization of Dbf20 during Cell Cycle

Consistent with its role in both mitotic exit and cytokinesis, Dbf2 is known to first localize to the SPBs in early mitosis and then occurs at the mother-daughter neck in late telophase (Frenz *et al*., 2000). Although expected to be similar to that of Dbf2, the cellular localization of Dbf20 has not yet been reported. To determine localization behavior of Dbf20, cells carrying native promoter-driven *DBF20-YFP* and *SPC29-CFP* (US2880), each integrated at its respective locus, were released from α-factor induced G1 arrest into fresh growth medium. Dbf20 localized to both SPBs before nuclear division when Clb2 levels are high but later in mitosis, when Clb2 abundance begins to decline it occurs at the mother-daughter neck (Figure 2). The Dbf20-YFP signal eventually disappeared from the neck as cells enter the next cycle and form new buds (our unpublished data). It should be noted that the Dbf20-YFP is weakly visible at the SPBs, however, for some reason the signal becomes more clearly visible upon localization to the neck. This pattern of cellular distribution is very similar to that described for Dbf2 (Frenz *et al*., 2000).

Dbf2 and Dbf20 Localization to the Mother-Daughter Neck Requires Inactivation of Mitotic Kinase

As we had noted above, the appearance of Dbf20 at the mother-daughter neck correlates with the decline in Clb2 abundance. This raised the possibility that Clb2 proteolysis (mitotic kinase inactivation) may be an important prerequisite for the localization of these proteins to the neck. We therefore tested whether untimely inactivation of the Cdc28/Clb kinase could trigger premature translocation

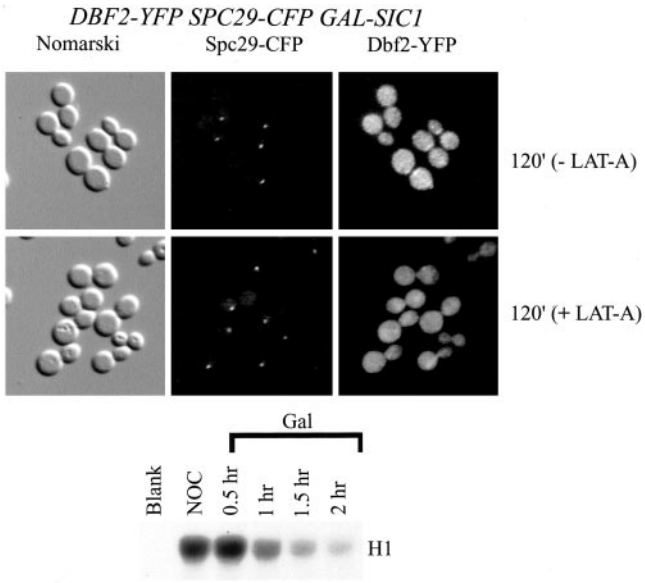

Figure 4. Involvement of actin cytoskeleton in the translocation of Dbf2-YFP. Cells (US2888) harboring a single copy of *DBF2-YFP* and *SPC29-CFP* at their respective loci, and four copies of *GAL-SIC1*, integrated at the *TRP1* locus were first synchronized in metaphase by treatment with nocodazole (NOC; 15 μg/ml) in raffinose medium. The culture was divided into two halves. Although galactose (2%) was added to both halves to induce *SIC1* transcription, LAT-A (100 μM) was added to only one of them to disrupt actin cytoskeleton. After 2 h, samples were collected and fluorescence signals were visualized as described in text.

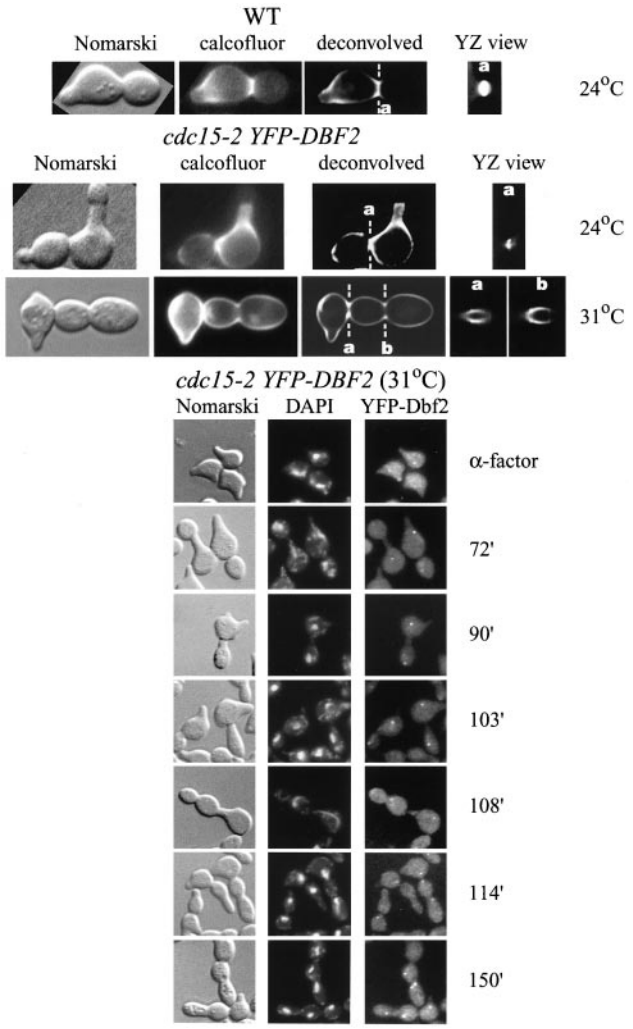

Figure 5. YFP-Dbf2 localization in *cdc15-2* cells. *cdc15-2* cells, carrying a single copy of *YFP-DBF2* at its native locus (US2998), were released from α-factor arrest into fresh medium at 24°C or 31°C. The middle panels show cells in the 110-min sample, stained with calcofluor to visualize septum material. The YZ views across the mother-bud neck are included (a and b) to show the presence or absence of primary septum. Wild-type cells (US356) are shown for comparison (top). YFP-Dbf2 was visualized at various time points by using fluorescence microscopy as before (bottom).

from SPB to the mother-daughter neck of Dbf2 and Dbf20 proteins. Cells carrying *DBF2-YFP* and *SPC29-CFP* at their respective native loci (driven by their respective native promoters; US2888) and *GAL-SIC1* (four copies) integrated at *URA3* locus, were arrested in metaphase by nocodazole treatment. As expected, the Cdc28/Clb2 kinase was high in these cells and Dbf2-YFP was seen at the SPBs (Figure 3A, left, 180 min glu). On induction of *SIC1* by addition of galactose, the kinase activity declined to a very low level at the end of 3 h (Figure 3A, top right) and as expected, cells formed new, elongated buds even though the nucleus remained undivided (Padmashree and Surana, 2001). The Dbf2-YFP signal was clearly visible at the daughter-side of the neck in almost all cells, as what seems to be a compact ring (Figure 3A, left, 180 min gal and graph). We also performed similar experiments with cells expressing Dbf20-YFP and Spc29-CFP (US2892), each expressed from their respective native promoter. The localization pattern of Dbf20-YFP, before and after *SIC1* expression, was found to be similar to that of Dbf2-YFP (Figure 3B), although the signals were not as clearly visible as Dbf2-YFP. These results suggest that inactivation of the mitotic kinase is sufficient to trigger translocation of at least some of the MEN components (Dbf2 and Dbf20 in the present case) to the mother-daughter neck. It should be noted that although Dbf2 and Dbf20 translocate to the neck in response to Sic1 overexpression, these cells do not undergo cytokinesis even after prolonged incubation. This suggests that although localization of Dbf2 and Dbf20 to the mother-bud junction may be a prerequisite for the execution of cytokinesis, this event by itself is not sufficient.

Involvement of Actin Cytoskeleton in Translocation of Dbf2 to Mother-Daughter Neck

How do MEN components such as Dbf2 reach mother-daughter neck from the SPBs? The fact that lowering of mitotic kinase allows Dbf2 and Dbf20 to translocate from SPB to the neck in nocodazole-treated cells suggests that the translocation is not dependent on microtubules. Therefore, we asked whether actin cytoskeleton is important for this change in locale. Hence, cells (US2888) containing a chromosomally integrated copy of *DBF2-YFP* (at its native locus) and four copies of *GAL-SIC1*, integrated at *URA3* locus, were first synchronized in metaphase by treatment with nocodazole in raffinose medium. Latrunculin-A (LAT-A), an actin cytoskeleton-destabilizing agent, was then added to one-half of the culture. After 15

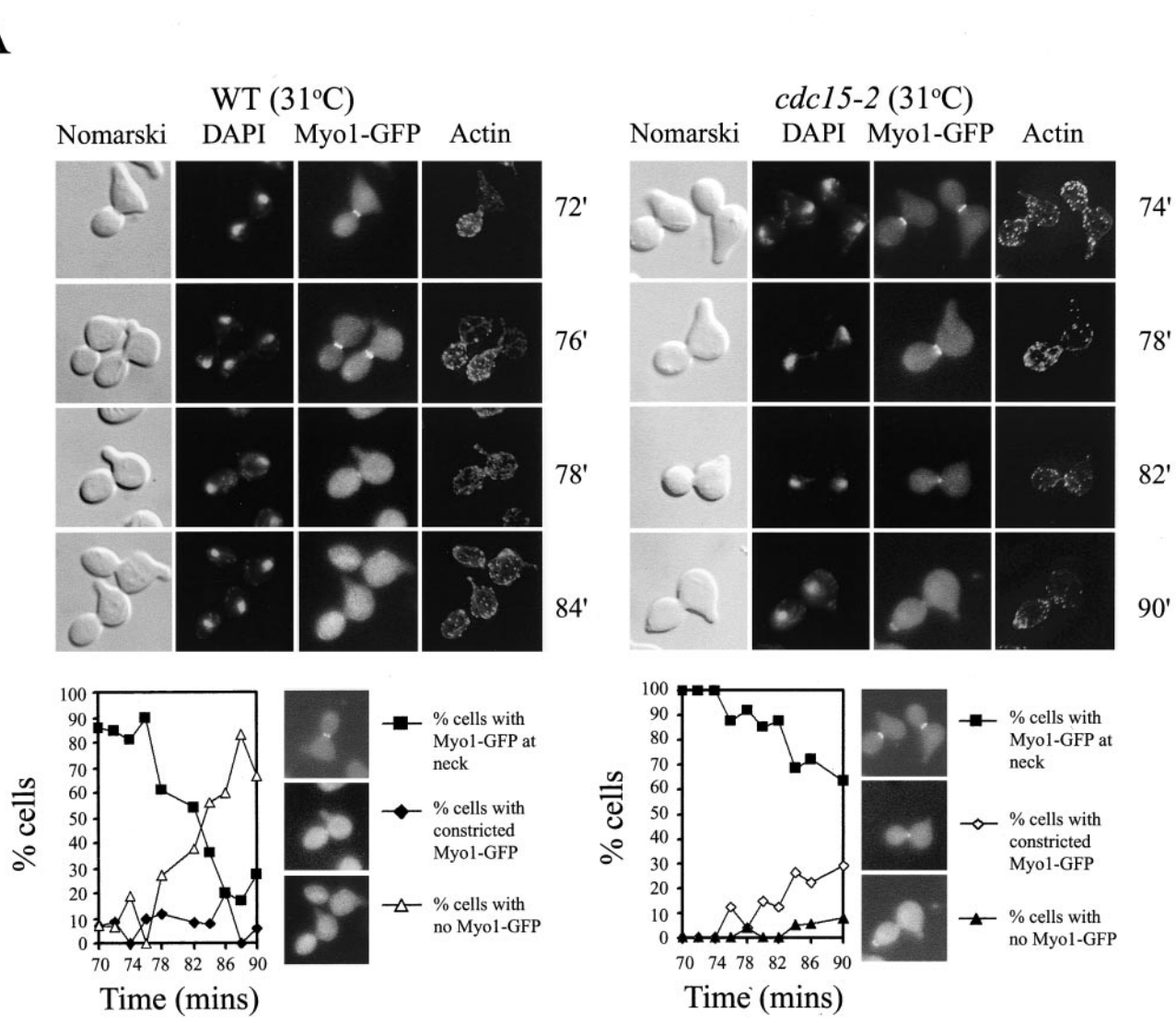

Figure 6. Actomyosin ring constriction and septum formation in cdc15-2 cells at 31°C. (A) Wild-type (US3145) and cdc15-2 (US3122) cells carrying native promoter driven MYO1-GFP (at its native locus) were synchronized in G1 by α factor treatment and then allowed to resume cell cycle progression at 31°C. Samples drawn at various times were analyzed for the presence of Myo1 ring and stained with phalloidin to visualize actin rings. The graphs show the proportion of cells with Myo1 rings at various stages of constriction. (B) Cells were stained with calcofluor and FM4-64 for visualizing primary septum and the cytoplasmic membrane, respectively. (C) dbf2–2 dbf20Δ cells (US2809) were grown in YEPD at 31°C for 6 h and stained with FM4-64 as described above. (D) Clb2 degradation in wild-type and cdc15-2 strains. Cells were synchronized in G1 by α factor treatment and then allowed to resume cell cycle progression at 31°C. Samples drawn at various times were analyzed for Clb2, Cdc28 protein levels and stained with Vectorshield containing DAPI to visualize nuclei. Bottom, cdc15-2 cells carrying GAL-SIC1-cmyc$_3$ were grown in raffinose or raffinose+galactose for 6 h at 31°C. Cells were stained with calcofluor to visualize the septum. YZ views across the cytokinesis sites are shown on the right.

min, galactose was added to both halves to induce *SIC1* expression. As expected, in cells not treated with LAT-A, Dbf2-YFP translocated to the mother-daughter neck within 2 h of *SIC1* induction (Figure 4, top). However, in LAT-A–treated cells, Dbf2-YFP was not seen at the neck at any time during the experiment (Figure 4, bottom). Interestingly, the Dbf2-YFP signal was also absent from the SPBs in most cells, suggesting that although Dbf2 dislodges from SPB in response to the inactivation of mitotic kinase, it is unable to translocate to the mother-daughter neck in the absence of an organized actin cytoskeleton.

Significance of Dbf2 and Dbf20 Localization to the Mother-Daughter Junction

Because severe cytokinesis defect of *dbf2-2 dbf20*Δ double mutant and the appearance of both Dbf2 and Dbf20 at the neck are both strongly suggestive of their role in cytokinesis, we asked whether localization of Dbf2 and Dbf20 to the neck is necessary for their role in cytokinesis. As mentioned above, we found that *cdc15-2* mutant cells, though able to exit mitosis at 31°C, exhibit cytokinetic defect (Figure 1B). Because Cdc15 is an upstream effector in MEN hierarchy, we

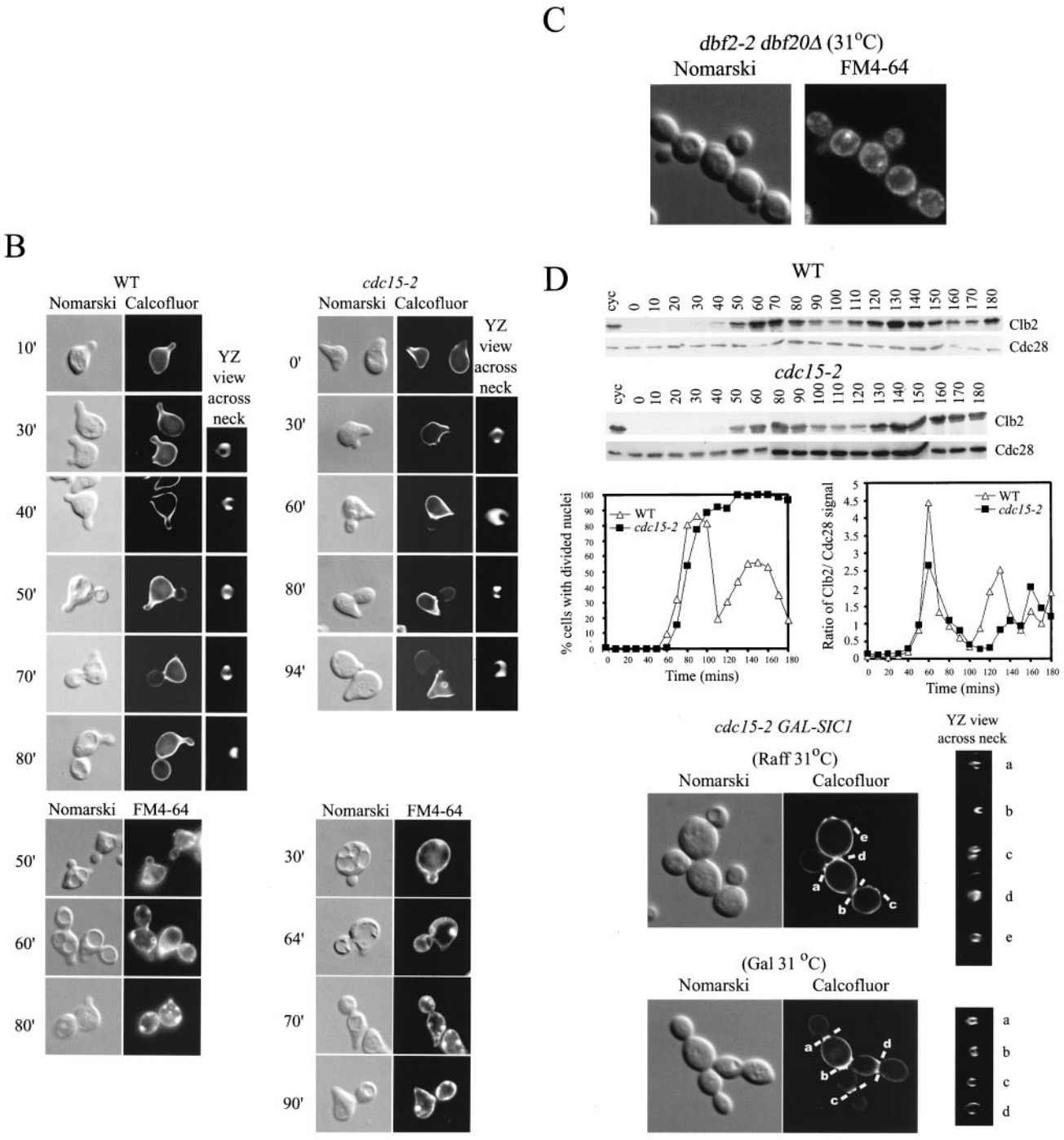

Figure 6. Continued

asked whether the failure of these cells to undergo cytokinesis is due to their inability to localize Dbf2 and Dbf20 to the mother-bud neck. We preferred to use YFP-Dbf2 for this experiment because of the relative ease with which it can be detected compared with Dbf20 fused with fluorescent tags. *cdc15-2* cells carrying native promoter-driven YFP-Dbf2 (integrated at its own locus; US2998) were released at 31°C from α-factor-induced G1 arrest and localization of YFP-Dbf2 was followed. As before, *cdc15-2* cells exhibited cytokinetic defect and a lack of septum at the neck (Figure 5, top and middle). YFP-Dbf2 was clearly seen at the SPBs (Figure 5, bottom); however, it could not be detected at the neck during the entire course of this experiment (samples withdrawn at 3-min interval), although the cells had exited mitosis, rebudded, and exhibited cytokinesis defect (Figure 5, 150 min). Hence, *cdc15-2* cells, though normal with respect to their mitotic exit function at 31°C, are defective in the recruitment of Dbf2 to the neck. Together with the observations described above, these results are consistent with the notion that the localization of Dbf2, and perhaps also Dbf20,

to the mother-daughter neck is dependent on Cdc15 function and that their localization to the neck may be necessary for proper execution of cytokinesis.

cdc15-2 Cells Are Defective in Septum Deposition, Not in Actomyosin Ring Formation and Constriction

Successful separation of mother and daughter requires proper assembly and then progressive constriction of the actomyosin ring. The constriction of the actomyosin ring is believed to direct the inward deposition of septum. A failure to undergo proper cytokinesis can result either from the inability to assemble and constrict the actomyosin ring or from defects in the ability to lay down the septum or both. To determine which of these processes is defective in *cdc15-2* mutant cells at 31°C, *cdc15-2* cells carrying native promoter-driven Myo1-GFP (US3122) were synchronized in G1 by α-factor treatment and then allowed to resume cell cycle progression at 31°C. Cells were analyzed at various times for the state of the Myo1 and actin rings and the presence of the primary septum at the mother-daughter junction. A wild-type strain (US3145) was treated in identical manner as a control. As expected, the wild-type cells formed Myo1 and actin rings, which later constricted to close the mother-daughter junction and then disappeared (Figure 6A, left and graph). The constriction of Myo1 rings was very rapid; hence at any time point only a very small proportion (~10%) of cells could be "caught in the act" of ring constriction. Consistent with this, staining with the dye FM4-64 (in the presence of sodium azide at 0°C, which prevents internalization of the dye but allows staining of the cytoplasmic membrane) showed that the mother and daughter cytoplasmic membranes at the junction were completely "pinched away" from each other by 80 min (Figure 6B, bottom left). Calcofluor staining revealed that these cells had deposited the septum and the aperture between the mother and daughter had been sealed (Figure 6B, top left). *cdc15-2* cells also formed the Myo1 and actin rings that progressively constricted (Figure 6A, right and graph). However, compared with wild type, both rings persisted longer in these cells. The cytoplasmic membrane in these cells had pinched completely at the junction to separate the mother and daughter cytoplasms, as revealed by FM4-64 staining (Figure 6B, bottom right). However, calcofluor staining showed that these cells had failed in septum deposition (Figure 6B, top right, YZ view). The *dbf2-2 dbf20*Δ cells, though defective in cytokinesis when grown at 31°C, also showed clear separation of mother and daughter cytoplasms (Figure 6C). These results suggest that the cytokinesis defect in *cdc15-2* and *dbf2-2 dbf20*Δ cells at 31°C is largely because of their inability to form a septum, and not due to a defect in the formation and constriction of actomyosin ring.

It can be argued that the failure of *cdc15-2* cells to form septum at 31°C is not because Cdc15-2 (and other MEN components) protein is defective in its cytokinesis function. Instead, it may be because *cdc15-2* cells degrade mitotic cyclin less efficiently such that the extent of cyclin destruction in these cells is sufficient for triggering both the assembly and constriction of the actomyosin ring, and entry into the next cycle, but not for the initiation of septum formation. To rule out this possibility, we compared the extent of Clb2 destruction in the wild-type and *cdc15-2* cells, released at 31°C from G1 arrest. As shown in Figure 6C, the progress through the cell cycle is very similar in wild-type and *cdc15-2* cultures as indicated by the almost identical timing of the rise in the proportion of anaphase cells (divided nuclei). As expected, the proportion of such cells eventually declines in wild-type culture, but these cells accumulate in the mutant culture because of the cytokinesis defect (Figure 6D, left graph). The pattern of Clb2 accumulation and degradation in *cdc15-2* cells is also similar to that in wild-type cells. This implies that the cytokinesis defect exhibited by *cdc15-2* cells at 31°C is not due to incomplete inactivation of the mitotic kinase. To rule out the possibility that the cytokinetic defect may result from inadequate inactivation of mitotic kinase complexes other than Cdc28/Clb2, *cdc15-2* cells carrying *GAL-SIC1* on a *CEN* vector were grown at 31°C in raffinose or raffinose+galactose medium for 6 h and were stained with calcofluor as described above. Despite Sic1 overexpression, these cells remained defective in cytokinesis (Figure 6D, photomicrographs) suggesting that the inability of *cdc15-2* cells to undergo cytokinesis is not due to insufficient kinase inactivation. The overexpression of Sic1 also failed to alleviate the cytokinesis defect of *dbf2-2 dbf20*Δ cells grown at 31°C (our unpublished data).

DISCUSSION

Although a possible involvement of MEN components in cytokinesis has been suggested many times in recent years (Lee *et al*., 2001; Tolliday *et al*., 2001), the mechanism that ensures that final steps of cytokinesis is initiated only after the execution of mitotic exit is not known. An important impetus behind the investigation of links between MEN and cytokinesis in budding yeast has been the fact that the fission yeast SIN (a network analogous to budding yeast MEN) plays a key role in the initiation of cytokinesis (McCollum and Gould, 2001). One of the most downstream effectors in SIN is sid2, the homolog of budding yeast Dbf2, which first localizes to SPBs and then relocates to the medial ring to promote septum formation (Sparks *et al*., 1999).

In budding yeast, Dbf2 also initially localizes to SPBs and then translocates to the mother-daughter neck (Frenz *et al*., 2000). This is strongly suggestive of Dbf2's involvement in cytokinesis. However, loss of Dbf2 function alone does not lead to any cytokinesis defect (Figure 1). We find that as in mitotic exit where Dbf2 is functionally redundant with Dbf20, Dbf2 and Dbf20 have a redundant function in cytokinesis in that the *dbf2-2 dbf20*Δ double mutant exhibits a severe cytokinesis defect at semipermissive temperature (Figure 1). Like Dbf2, Dbf20 also first localizes to the SPBs and then occurs at the neck in late telophase, in the form of what seems to be a compact ring (Figure 2A). At present, it is not clear whether these proteins anchor to a preexisting scaffold, such as septins or actomyosin ring, or assemble themselves into a ring-like structure independently. The translocation of Dbf2 and Dbf20 to the mother-daughter neck may be important for cytokinesis since *cdc15-2* cells, which exhibit a severe cytokinesis defect at 31°C, also fail to translocate Dbf2 (and also perhaps Dbf20) to the neck (Figure 3B). We make this suggestion cautiously because *cdc15-2* cells grown at 31°C may develop additional defects that contribute significantly to the cytokinesis defect. However, these observations do imply that Dbf2 translocation requires fully functional Cdc15 kinase. Because Cdc15 is known to activate Dbf2 by phosphorylation (most likely at the SPBs when they come into contact) (Mah *et al*., 2001; Visintin and Amon, 2001), it is possible that this activation step is in some way important for the subsequent translocation of Dbf2 to the neck. Thus, Dbf2/Dbf20's localization behavior and Cdc15-dependent translocation to the neck essentially ech-

oes the behavior and functional relationships of their respective homologs in fission yeast, i.e., sid2 and Cdc7 (McCollum and Gould, 2001).

Although the activation of Dbf2 by Cdc15 may be of some importance in priming its eventual translocation, the inactivation of mitotic kinase Cdc28/Clb seems to be a critical factor in SPB-to-neck translocation of Dbf2. That untimely inactivation of mitotic kinase can cause Dbf2 to translocate from SPB to the neck prematurely, strongly supports this notion (Figure 3). In synchronous cultures, translocation of Dbf2 (our unpublished data) and Dbf20 to the neck also correlates well with the decline in Clb2 abundance (Figure 2). Thus, Dbf2 and Dbf20's dependence on mitotic kinase inactivation for their translocation to the cytokinesis site provides a mechanism that would ensure that cytokinesis initiates only after execution of mitotic exit (inactivation of the mitotic kinase). Such spatial movement of proteins from one cellular location to another in response to mitotic kinase inactivation is not unprecedented. It is well established that the movement of transcription factor Swi5 from cytoplasm to nucleus is triggered by the inactivation of mitotic kinase (Moll *et al*., 1991). Similarly, we have previously shown that Spa2, a bud-site component, translocates from mother-daughter neck to the cell cortex in response to mitotic kinase inactivation (Padmashree and Surana, 2001).

How does the mitotic kinase inactivation trigger the movement of proteins such as Dbf2 from SPB to the neck? Our observation that Dbf2 translocates to the neck in response to mitotic kinase inactivation in nocodazole-arrested cells, argues against involvement of microtubules in SPB-to-neck translocation (Figure 3). Experiments involving LAT-A treatment (Figure 4) suggest that SPB-to-neck translocation occurs via actin cytoskeleton. Given that actin cytoskeleton is normally in a disorganized state during M phase (Pruyne and Bretscher, 2000), it may seem puzzling that LAT-A treatment can prevent Dbf2-YFP translocation to the neck. However, it is known that actin cytoskeleton is reorganized and reoriented toward the neck in late telophase, coinciding with the inactivation of mitotic kinase (Amberg, 1998). The inactivation of mitotic kinase may play a significant role in inducing such reorganization. In our experiments, the inactivation of mitotic kinase at metaphase, due to overexpression of Sic1, may allow cells to initiate late telophase events prematurely, such as reorganization of actin cytoskeleton, thereby making possible the translocation of MEN components such as Dbf2.

cdc15-2 cells are unable to translocate Dbf2 (and also perhaps Dbf20) and, though proficient in mitotic exit, are obviously defective in cytokinesis when grown at 31°C (Figures 1B and 5). Our results also show that these cells are not defective in actomyosin ring formation and constriction but they are unable to build primary septum (Figure 6). These observations argue that the assembly and constriction of actomyosin ring does not require the presence of Dbf2 and Dbf20 kinases at the neck. Instead, their translocation to the mother-daughter neck may be important for the formation of the primary septum. The ability of *cdc15-2* cells to form actomyosin ring at 31°C is consistent with the previous suggestion that actomyosin ring assembly occurs after Cdc15 function (i.e., MEN activation) is executed (Lippincott and Li, 1998b). This implies that the inactivation of the mitotic kinase may be a prerequisite for the proper assembly and constriction of the actomyosin ring. Together, these considerations point to a regulatory scheme in which MEN is involved in the process of cytokinesis at two levels. First, MEN catalyzes the inactivation of mitotic kinase (Cdc28/Clb), thereby paving the way for the actomyosin ring assembly. The inactivation of the kinase also triggers translocation of some of MEN components such as Dbf2 and Dbf20 to the mother-daughter junction. Once at the cytokinesis site, the MEN components can participate in the initiation of septum formation. It should be noted that although mitotic kinase inactivation is a prerequisite for the translocation, the activity of Cdc15 (and perhaps other MEN components) is also required for the proper execution of this translocation. This is exemplified by the inability of *cdc15-2* cells to catalyze Dbf2 translocation at 31°C, even though they are proficient in mediating mitotic exit (Figures 5 and 6). What role may Dbf2 and Dbf20 play in cytokinesis upon reaching the neck? It is known that chitin synthase II, the enzyme responsible for the primary septum formation, is synthesized in a zymogen form and localizes to the neck in late mitosis (Chuang and Schekman, 1996). One possibility is that Dbf2 and/or Dbf20 kinases play an important role in the activation of chitin synthase II zymogen, resulting in the initiation of septum deposition. Alternatively, these kinases may mediate the proper assembly and general stabilization of the cytokinesis apparatus. We have recently discussed these notions elsewhere (Surana *et al*., 2002) and some of them are currently being tested. It is important to point out that although kinase inactivation-dependent SPB-neck translocation of Dbf2 and Dbf20 may be a necessary link in temporal ordering of mitotic exit and cytokinesis, the presence of these kinases at the neck alone is not sufficient to trigger cytokinesis. This is highlighted by our observation that mitotic kinase inactivation in metaphase arrested cells can cause translocation of Dbf2 and Dbf20 to the neck but does not initiate cytokinesis (Figure 3). Clearly, further experiments are required to uncover additional regulatory elements.

In conclusion, our results suggest a mechanism that ensures that the final stages of cytokinesis commence only after the mitotic kinase is inactivated. The three critical elements that together impose such a coordination are 1) the requirement of MEN components (such as Dbf2 and Dbf20) in the final execution of cytokinesis, 2) their SPB-to-neck translocation, and 3) triggering of this translocation by the inactivation of mitotic kinase. Given the general similarity in the macromolecular assemblies and effectors (e.g., mitotic kinase constituents Cdc2/Cdc28 and cyclin B, septins, microtubules, actomyosin ring, actin cytoskeleton, IQGAPs [IQ-GTPase activity protein]), which play an important role in mitotic exit and cytokinesis among various organisms, it is possible that some elements of this molecular strategy is operative among other eukaryotes to ensure the dependence of cytokinesis on the completion of mitosis.

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