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

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ABSTRACT

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 (SPBs) to mother-daughter neck appears to be necessary for this role since cdc15-2 mutant cells, though capable of exiting mitosis at semi-permissive 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 SPB-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. While 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, Dbf20) and Cdc14 (a phosphatase) (Lee et al., 2001; Morgan, 1999). 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 homologue and, like Cdc20, an APC activator) via dephosphorylation. Both APC\textsuperscript{Cdc20} and APC\textsuperscript{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). While 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? While 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 pre-requisite 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: (i) overexpression of truncated versions of Cdc15 and Cdc5 can lead to cytokinetic defect (Menssen et al., 2001; Song et al., 2000), (ii) 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 (iii) *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 (200mg/l).

Strains and Plasmids

A combination of standard molecular biology and molecular genetic techniques such as gene transplacement, gene disruption, PCR-based tagging of endogenous genes and tetrad dissection were used to construct plasmids and strains with various genotypes (shown in 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 BARI cells). After 3 to 3.5 hours of treatment, cells were filtered, washed and resuspended in fresh medium pre-incubated at the appropriate temperature. For the purpose of synchronizing cells in metaphase, cycling culture was treated with nocodazole (15 µg/ml) for 3-6 hrs. 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 TCA was carried out as described before (Yeong et al., 2000). For Western blot analyses, immuno-detection 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 was used for all Western blot analyses according to the manufacturer’s instructions.
Flow Cytometry, Calcofluor Staining and Visualization of GFP Signals

The method described by Lim et al. (1996) was used for flow cytometry. To visualize signals of the CFP or 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 DAPI. The images were captured using a Leica DMRX microscope attached to a Hamamatsu charge-coupled device camera, driven by the Metamorph software (Universal Imaging Corporation). Typically, the exposure time for the Cdc15-YFP, Dbf2-YFP and Dbf20-YFP fusion proteins was 4 seconds and that for the Spc29-CFP was 500 milliseconds. 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 PBS (3X) 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 above. For actin staining, cells fixed in KPF were washed three times with PBS. Alexa 594 phalloidin (Molecular Probes; conc: 0.24 units/µl) 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 FM-464, 1µl of the dye (400µM) was added to 100µl of cold cell suspension in PBS and incubated on ice. After 20 min, sodium azide (1µl of 25mM 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 non-essential 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 if Dbf2 and Dbf20 play an overlapping role in cytokinesis, as they do in mitotic exit, we made use of dbf20Δ 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), dbf20Δ (US2944) and dbf2-2 dbf20Δ double mutant cells (US2809) since growth at 37°C would obscure the defect in cytokinesis due to telophase arrest caused by dbf2-2. While both dbf2-2 and dbf20Δ cells exhibited no cytokinesis defect, dbf2-2 dbf20Δ cells were unable to separate from each other and grew as long chains at 31°C (Fig. 1A, top panel). YZ view of the mother-daughter neck in calcofluor stained cells revealed the absence of septum material in these chains of cells (Fig. 1A, lower panel). However, a few of the older mother-bud junctions do eventually build partial septum. These results suggest that dbf2-2 dbf20Δ 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 semi-permissive temperature to test if other MEN mutants (already known to be defective in mitotic exit at non-permissive temperature) are also defective in cytokinesis. cdc15-2 cells, when grown at semi-permissive 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 (Fig. 1B, middle panel). 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 results). It has been suggested that
the role of Cdc15 in cytokinesis is linked to its localization to spindle-pole bodies (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 (Fig. 1B, lower panel). This suggests that the failure of
Cdc15-2 to execute cytokinesis properly at semi-permissive 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 appears 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 prior to nuclear division
when Clb2 levels are high but later in mitosis, when Clb2 abundance begins to decline it
appears at the mother-daughter neck (Fig. 2). The Dbf20-YFP signal eventually
disappeared from the ‘neck’ as cells enter the next cycle and form new buds (our unpublished results). 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 earlier, 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 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 (4 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 (Fig. 3A, left panel “180 min glu”). Upon induction of SIC1 by addition of galactose, the kinase activity declined to a very low level at the end of 3 hours (Figure 3A, top right panel) 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 appears to be a compact ring (Figure 3A, left panel “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 over-expression, these cells do not undergo cytokinesis even after prolonged incubation. This suggests that while 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 if 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 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 hrs of SIC1 induction (Figure 4, top panel). However, in LAT-A treated cells, Dbf2-YFP was not seen at the neck at any time during the experiment (Figure 4, bottom panel). Interestingly, the Dbf2-YFP signal was also absent from the SPBs in most cells, suggesting that while 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**

Since severe cytokinesis defect of dbf2-2 dbf20Δ 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). Since Cdc15 is an upstream effector in MEN hierarchy, we asked if 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 to 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’ (Fig. 5, upper and middle panels). YFP-Dbf2 was clearly seen at the SPBs (Fig. 5, bottom panel); 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,
re-budded and exhibited cytokinesis defect (Fig. 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. Taken 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 (Fig. 6A, left panel 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 (Fig. 6B, lower left panel). Calcofluor staining revealed that these cells had deposited the septum and the aperture between the mother and daughter had been sealed (Fig. 6B, upper left panel). *cdc15-2* cells also formed the Myo1 and actin rings which progressively constricted (Fig. 6A, right panel and the graph). However, compared to 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 cytoplasm, as revealed by FM-464 staining (Fig. 6B, lower right panel). However, calcofluor staining showed that these cells had failed in septum deposition (Fig. 6B, upper right panel, YZ view). The *dbf2-2 dbf20Δ* cells, though defective in cytokinesis when grown at 31°C, also showed clear separation of mother and daughter cytoplasms (Fig. 6C). These results suggest that the cytokinesis defect in *cdc15-2* and *dbf2-2 dbf20Δ* 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 quite 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 6hr and were stained with calcofluor as before. Despite Sic1 over-expression, these cells remained defective in cytokinesis (Fig. 6D, photomicrographs) suggesting that the inability of cdc15-2 cells to undergo cytokinesis is not due to insufficient kinase inactivation. The over-expression of Sic1 also failed to alleviate the cytokinesis defect of dbf2-2 dbf20Δ cells grown at 31°C (our unpublished results).

DISCUSSION
Though 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 which ensures that final steps of cytokinesis are 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
One of the most down-stream effectors in SIN is sid2, the homologue 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 (Fig. 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 $\text{dbf2-2 dbf20}^{\Delta}$ double mutant exhibits a severe cytokinesis defect at semi-permissive temperature (Fig. 1). Like Dbf2, Dbf20 also first localizes to the SPBs and then appears at the ‘neck’ in late telophase, in the form of what appears 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 $\text{cdc15-2}$ 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 since $\text{cdc15-2}$ 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. As 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 echoes the behavior and functional relationships of their respective homologues in fission yeast, i.e. sid2 and Cdc7 (McCollum and Gould, 2001).

While the activation of Dbf2 by Cdc15 may be of some importance in priming its eventual translocation, the inactivation of mitotic kinase Cdc28/Clb appears 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 (Fig 3). In synchronous cultures, translocation of Dbf2 (our unpublished results) and Dbf20 to the ‘neck’ also correlates well with the decline in Clb2 abundance (Fig 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 (Fig. 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 towards 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 over-expression 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 like Dbf2.

*cde15-2* 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 (Fig 1B and Fig. 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 (Fig 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 *cde15-2* 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. Taken 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 while 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℃, even though they are proficient in mediating mitotic exit (Fig. 5 and 6). What role may Dbf2 and Dbf20 play in cytokinesis upon reaching the ‘neck’? It is known that Chitin synthase II (Chs2), 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 Chs2 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 while 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 (Fig. 3). Clearly, further experiments are required to uncover additional regulatory elements.
In conclusion, our results suggest a mechanism which ensures that the final stages of cytokinesis commence only after the mitotic kinase is inactivated. The three critical elements that together impose such a co-ordination are: (i) the requirement of MEN components (like Dbf2 and Dbf20) in the final execution of cytokinesis (ii) their SPB-to-‘neck’ translocation and (iii) triggering of this translocation by the inactivation of mitotic kinase. Given the general similarity in the macromolecular assemblies and effectors (mitotic kinase constituents Cdc2/Cdc28 and cyclin B, septins, microtubules, actomyosin ring, actin cytoskeleton, IQGAPs etc) 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.

Acknowledgements

We thank Yeast Resource Centre for SPC29-CFP strain, pDH3 (CFP construct) and pDH5 (YFP construct). We are grateful to Drs Leeland Johnston, John Pringle, Simonetta Piatti for providing some of the strains and plasmids used in this study, and to Drs Alan Munn and Mohan Balasubramaniam for useful discussions. This work was supported by the Biomedical Research Council of Singapore.
REFERENCES


• Toyn, J.H., and Johnston L.H. (1994). The Dbf2 and Dbf20 protein kinases of budding yeast are activated after the metaphase to anaphase cell cycle transition. EMBO J. 13, 1103-1113.


FIGURE LEGENDS

**Fig. 1.** (A) Cytokinesis defect in dbf2-2 dbf20Δ double mutant. dbf2-2 (US1998), dbf20Δ (US2944) and dbf2-2 dbf20Δ (US2809) mutant cells were grown at 31°C (semi-permissive temperature) and were stained with DAPI to visualize the nuclei. The dbf2-2 dbf20Δ (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 semi-permissive temperature of 31°C and stained with calcofluor to visualize the state of the septa (upper panel). 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.

**Fig. 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.

**Fig. 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 panel). H1 kinase
assays were performed to ascertain that Clb2/ Cdc28 kinase activity was inactivated upon SIC1 induction (top right panel). The graph (right panel) 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 panel) shows the proportion of cells with Dbf20-YFP at the SPBs or the mother daughter junction. We suspect that these numbers are underestimated since Dbf20-YFP signal is much weaker compared to Dbf2-YFP.

**Fig. 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 2 halves. While galactose (2%) was added to both halves to induce SIC1 transcription, Latrunculin A (LAT-A; 100μM) was added to only one of them to disrupt actin cytoskeleton. After 2 hrs, samples were collected and fluorescence signals were visualized as described before.

**Fig. 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 panel). YFP-Dbf2 was visualized at various time points using fluorescence microscopy as before (lower panel).

**Fig. 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 hrs and stained with FM4-64 as 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 panel: *cdc15-2* cells carrying *GAL-SIC1-cmyc3* were grown in raffinose or raffinose+galactose for 6 hrs at 31°C. Cells were stained with calcofluor to visualize the septum. YZ views across the cytokinesis sites are shown on the right.
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B

cdc15-2 (31°C)

Nomarski calcofluor deconvolved

YZ view a b c d e

cdc15-2-YFP SPC29-CFP (31°C)

Nomarski DAPI Spc29-CFP Cdc15-2-YFP
A  
DBF2-YFP SPC29-CFP GAL-SIC1  
Nomarski  Spc29-CFP Dbf2-YFP

NOC
180' glu
180' gal

B  
DBF20-YFP SPC29-CFP GAL-SIC1  
Nomarski  Spc29-CFP Dbf20-YFP

NOC
180' glu
180' gal

% cells with Dbf2-YFP signals

Glu (neck)
Glu (SPB)
Gal (neck)
Gal (SPB)
DBF2-YFP SPC29-CFP GAL-SIC1

Nomarski Spc29-CFP Dbf2-YFP

120' (- LAT-A)

120' (+ LAT-A)

Gal

Blank NOC 0.5 hr 1 hr 1.5 hr 2 hr

H1