The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis

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SUMMARY

Cytokinesis in budding yeast involves an actomyosin-based ring which assembles in a multisteped fashion during the cell cycle and constricts during cytokinesis. In this report, we have investigated the structural and regulatory events that occur at the onset of cytokinesis. The septins, which form an hour-glass like structure during early stages of the cell cycle, undergo dynamic rearrangements prior to cell division: the hourglass structure splits into two separate rings. The contractile ring, localized between the septin double rings, immediately undergoes contraction. Septin ring splitting is independent of actomyosin ring contraction as it still occurs in mutants where contraction fails. We hypothesize that septin ring splitting may remove a structural barrier for actomyosin ring to contract. Because the Tem1 small GTPase (Tem1p) is required for the completion of mitosis, we investigated its role in regulating septin and actomyosin ring dynamics in the background of the net1-1 mutation, which bypasses the anaphase cell cycle arrest in Tem1-deficient cells. We show that Tem1p plays a specific role in cytokinesis in addition to its function in cell cycle progression. Tem1p is not required for the assembly of the actomyosin ring but controls actomyosin and septin dynamics during cytokinesis.

Key words: Tem1p, GTPase, Cytokinesis, Budding yeast, Actomyosin, Septin

INTRODUCTION

Actomyosin-based contractile rings have been implicated in cell division in eukaryotic organisms as diverse as yeast and man (for reviews, see Field et al., 1999; Fishkind and Wang, 1995; Schroeder, 1990). To ensure that the chromosomes are partitioned equally between the daughter cells, contractile ring activity must be coupled spatially and temporally with chromosome segregation. Whereas many studies have indicated that astral microtubules and spindle-associated components are important for the correct positioning of the cleavage furrow (for reviews, see Field et al., 1999; Oegema and Mitchison, 1997), less is known about how the timing of cytokinesis is controlled. Studies in both animal cells and yeast have shown that the onset of cytokinesis requires destruction of mitotic cyclins, which leads to inactivation of the mitotic kinase CDK1 (for reviews, see Balasubramanian et al., 2000; Field et al., 1999). Presumably, phosphate removal from a Cdk1 target is required for the activation of the contractile apparatus. However, the identity of this target is currently unknown. Furthermore, it remains unclear how the timing of Cdk1 inactivation is in turn controlled to ensure the temporal coordination between chromosome segregation and cytokinesis.

Model organisms such as yeast are valuable for the elucidation of the structural and regulatory pathways that lead to successful cell division. In the budding yeast Saccharomyces cerevisiae, the completion of anaphase events is regulated by a number of genes, collectively called the mitotic exit network (MEN), because mutations in these genes cause arrest in telophase with elongated spindles, segregated chromosomes and a high level of the mitotic cyclin, Clb2p (Jaspersen et al., 1998). A key upstream component of this pathway is Tem1p, a GTPase belonging to the Ras superfamily (Shirayama et al., 1998). GTP-bound Tem1p somehow leads to the activation of several MEN kinases such Cdc15p and Dbf2p, which eventually results in the release of the phosphatase Cdc14p from its resident compartment, the nucleolus (Jaspersen et al., 1998; Shou et al., 1999; Visintin et al., 1999). Cdc14p dephosphorylates and thus activates or stabilizes two negative regulators of the Cdk1/Clb kinase complexes (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). These findings strongly suggest that the MEN functions to coordinate Cdk1 inactivation with chromosome segregation.

In budding yeast, cytokinesis involves the function of two ring structures around the bud neck: the septin ring and the actomyosin ring (for a review, see Field et al., 1999). The septins are a family of GTP-binding proteins conserved in eukaryotic organisms (Longtine et al., 1996). The yeast septins localize to a broad ring, made up of 10 nm filaments, around the bud neck through most of the cell cycle (Byers and Goetsch, 1976; Longtine et al., 1996). Morphologically, the septin ring is distinct from the actomyosin ring: the latter covers a narrower region of the bud neck and contracts during cytokinesis whereas the septin ring maintains the same diameter (Lippincott and Li, 1998a). Besides providing a structural scaffold for the localization of many proteins to the bud neck (Longtine et al., 1998), the function of the...
bacterial peptone (Difco Laboratories Inc., Detroit, MI, USA). YPGR described elsewhere (Sherman et al., 1986). Yeast extract, peptone, yeast cell culture and genetic techniques were carried out by methods Media and genetic manipulations and actomyosin rings during cytokinesis. Furthermore, we provide evidence that the Tem1 small GTPase plays a specific role in regulating the dynamics of the septin and a Zeiss 100 oil objective. Image acquisition was carried out with a cooled RTE/CCD 782Y Interline camera (Princeton Instruments).

**Fluorescence microscopy**

Cells were mounted and observed by time-lapse video microscopy on either a DeltaVision or a Nikon E600 microscope system as previously described (Lippincott and Li, 1998a). To generate the flattened three-dimensional view of the cells, multiple Z-sections (nine 350 nm sections or thirteen 200 nm sections) were deconvolved and compressed for each time point using DeltaVision software.

**Table 1. Yeast strains used in this study**

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To examine the effect of Tem1p shutoff on actomyosin ring contraction, RLY652 (Myo1-GFP net1-1 GAL1-UPL-TEM1) cells cultured overnight in medium containing 0.05 μg/ml α-factor and 2% glucose, then washed 3x with sterile water and resuspended in glucose-containing media. Cells were placed on an agarose pad as described (Waddle et al., 1996). Living cells were imaged at room temperature using a Nikon Eclipse E600 microscope with a 100x1.40 oil DIC objective (Nikon Inc.) Images were collected every 2 minutes with 0.1 second exposure to fluorescence using a cooled RTE/CCD 782Y Interline camera (Princeton Instruments). The shutter was controlled automatically using a D122 shutter driver (UniBlitz) and WinView 1.6.2 software (Princeton Instruments) with custom software (courtesy of Aneil Mallavarapu, Harvard Medical School).

**Analysis of cell morphology**

For analysis of the morphology of Tem1-deficient cells, RLY635, 636, 637 and 638 were grown overnight in YPGR. Cells were then arrested with 0.05 μg/ml α-factor and glucose was added to 2%. After 3 hours, cells were released from arrest by washing 3x in sterile water, then resuspended in YPD and grown for 4 or 6 hours. Cells were fixed by addition of formaldehyde to 5% with gentle shaking at room temperature for 1 hour. Cells were treated with zymolyase in sorbitol buffer as previously described (Lippincott and Li, 1998a) before counting.

**RESULTS**

**Septins localize to an hourglass structure, which resolves into two distinct rings**

We first characterized the dynamics of the septin ring during the cell cycle using a DeltaVision 3-dimensional deconvolution microscope system. When observed under conventional fluorescence microscopes, especially by immunofluorescence staining, the septin-containing structure often appears as a double ring around the bud neck. However, when observed using wide-field deconvolusion three-dimensional microscopy (Agard et al., 1989), the septin ring is in fact a continuous hourglass shaped structure around the bud neck, consistent with the images obtained by electron microscopy (Byers and Goetsch, 1976). To observe the dynamics of this structure, we performed time-lapse optical sectioning microscopy on cells expressing GFP-tagged Cdc12p, a subunit of the septins. The septin structure maintains the hourglass shape through most of the cell cycle; however, this structure splits abruptly (within 1-2 minutes, between 2’ to 4’ time points for the top cell, 31’ to 33’ for the bottom cell) from the middle in large budded cells, resulting in two distinct rings (Fig. 1). This splitting event is specific to the septins because Cyk2p, which colocalizes with the septins during most of the cell cycle, actually tightens into a narrow ring around the center of the bud neck and contracts within 2-3 minutes thereafter in a myosin II-dependent manner (Lippincott and Li, 1998a; Lippincott and Li, 2000).

**Septin rearrangement occurs at the onset of actomyosin ring contraction**

The change in septin structure described above occurs around the time of cytokinesis because the new bud emerges about 30 to 40 minutes thereafter, and this is roughly the same length of time between actomyosin ring contraction and new bud emergence (Lippincott and Li, 1998a; Lippincott and Li, 1998b). To further compare the timing of septin rearrangement and actomyosin ring contraction, we constructed a yeast strain that simultaneously expresses cyan fluorescent protein-tagged Cdc12p (Cdc12-CFP) and yellow fluorescent protein-tagged Cyk2p (Cyk2-YFP), which was used as a marker for the contractile ring. Since the absorption and emission of the fluorescence markers are distinct, we could simultaneously observe the changes in septin and Cyk2 distribution as the cell cycles.

**Fig. 1.** The septins undergo dramatic structural rearrangement late in the cell cycle. Three-dimensional time-lapse analysis of cells expressing Cdc12-GFP. All images were collected and deconvolved on a DeltaVision microscope system as described in Materials and Methods. To generate the flattened three-dimensional view of the cells, thirteen 200-nm sections were deconvolved and compressed for each time point using Delta Vision software. Bar, 10 μm.
wavelengths of these two GFP variants are separable, it is possible to examine the dynamics of the two proteins in the same cell using different filter sets. We were unable to successfully capture a time-lapse series of these cells because the CFP signal photobleaches extremely rapidly. However, we were able to compare the morphology of these structures in individual cells during various stages of mitosis and cytokinesis. Fig. 2 shows representative cells at different stages. We observed that in more than 100 cells where Cyk2-YFP had merged into a single ring, the septins were still in an un-split hourglass structure (Fig. 2B). Because we did not observe cells (among hundreds of cells observed) where the septin hourglass had split but Cyk2p remained in a broad ring, we conclude that tightening of the Cyk2 ring occurs before septin ring splitting. In 23 cells where where Cyk2-YFP had merged into a single ring (Fig. 2C) or had started contraction (Fig. 2D), the septin hourglass had resolved into two separate rings, demonstrating that septin splitting occurs at or slightly before the onset of actomyosin ring contraction. Furthermore, the contractile ring, as marked by the tightened Cyk2 ring, was located exactly in the space between the two septin rings (Fig. 2C,D).

Fig. 3. Septin ring splitting is unaffected in Cyk1-deficient cells. RLY 1334 cells (expressing Cdc12-GFP with the sole source of Cyk1p under the control of the Gal1 promoter) were depleted of Cyk1p by arresting with α-factor for 3 hours in the presence of 2% glucose to repress Gal1-Cyk1p expression. The cells were then released into glucose-containing medium and observed on a DeltaVision microscope at various time points as described in Materials and Methods. Bar, 1 μm.
cytokinesis does not occur. Cyk1p is a component of the contractile ring required for both the assembly and contraction of the actomyosin ring (Lippincott and Li, 1998b; Shannon and Li, 1999). We showed previously that the Myo1 ring does not contract in cells lacking Cyk1p (Shannon and Li, 1999). As shown in Fig. 3A, the septin hourglass splits with normal dynamics in Cyk1-deficient cells (splitting occurred abruptly approx. 30 minutes before the cell budded again). Conversion of Cyk2-GFP to a tight single ring also occurs with normal timing in Cyk1-deficient cells and, as expected, this single ring never contracted and remained localized at the bud neck (data not shown). This result suggests that septin hourglass splitting is not simply a consequence of actomyosin ring contraction and cytokinesis.

The Tem1 small GTPase plays a specific role in cytokinesis

To understand how septin and actomyosin structural changes are initiated at the onset of cytokinesis, we sought to identify regulatory proteins that are important for these changes. A key regulator of post-anaphase events is Tem1p, a Ras family small GTPase (Morgan, 1999). The observation that Tem1p binds the GAP-related domain of Cyk1p first led us to speculate that Tem1p may directly regulate the onset of cytokinesis. However, in order to know whether Tem1p has a specific role in cytokinesis, it was necessary to bypass the requirement for Tem1p in cell cycle progression. To do this we took advantage of the net1-1 allele (Shou et al., 1999). In wild-type cells, Net1p binds and sequesters the Cdc14 protein phosphatase in the nucleolus, and Tem1p is required for Cdc14p to be released from the nucleolus and trigger mitotic exit (Shou et al., 1999; Visintin et al., 1999). However, net1-1 has a reduced affinity for Cdc14p, so in this mutant background Tem1p is no longer required for Cdc14p release and mitotic exit (Shou et al., 1999). It was noticed in the same report that the Tem1-deficient cells that had bypassed the cell cycle arrest in this manner appeared as chains. To further test if this indicated a cytokinesis defect and if so, which step of cytokinesis was affected, we used a net1-1, GAL1-UPL-TEM1 strain. UPL, which stands for ubiquitin-proline-LacI, acts as a destabilization signal so that Tem1p is quickly degraded after transcription is shut off (Shou et al., 1999). This strain, a net1-1 TEM1

![Fig. 4. Tem1p plays a specific role in cytokinesis.](image)

(A) Bypass of the requirement for Tem1p in cell cycle progression reveals a role for Tem1p in cytokinesis. RLY635 (Tem1+), 636 (Tem1 net1-1), 637 (net1-1) and 638 (wt) cells (see Table 1) were synchronized with α-factor and release into the cell cycle as described in Materials and Methods. The numbers of cells that were unbudded, small budded, large budded, or in chains of three or more cell bodies were counted after zymolyase treatment (n \(\geq\) 100 for each strain). Their percentages of the total cell population were calculated and presented as histograms. The experiment was repeated three times with similar results. (B) Morphology of Tem1+ (RLY636; a) and Tem1 net1-1 (RLY637; b) cells 6 hours after release from the G1 arrest in the above experiment. (C) Phalloidin staining of large-budded RLY636 (Tem1+) cells after Tem1p depletion. Arrows point to the actin ring. Bar, 10 µm.
of Tem1p-deficient cells arrested in telophase (Fig. 4C), suggesting that Tem1p is not required for recruitment of F-actin to the ring. Similarly, myosin II localization was also unaffected in Tem1p-deficient cells as visualized using GFP-tagged Myo1p (Fig. 5).

**DISCUSSION**

**Septin dynamics during cytokinesis**

We have described experiments that were aimed at understanding the structural and regulatory events required for the initiation of cytokinesis. First, we found that prior to cytokinesis, the septins abruptly split into two separate rings, leaving a space that is occupied predominantly by the actomyosin ring. This change in septin distribution is immediately followed by and occurs independently of actomyosin ring contraction and cytokinesis. Electron microscopy studies have shown that the septins form 10 nm filaments around the bud neck during most of the cell cycle (Byers and Goetsch, 1976), and these filaments provide scaffolding for the localization of many neck components, most notably Myo1p, the budding yeast myosin II (Bi et al., 1998; Lippincott and Li, 1998b). Time-lapse microscopy studies revealed that the septin structure does not constrict during cytokinesis, unlike the actomyosin ring (Lippincott and Li, 1998a), and this could be due to the rigidity of the septin filaments. If this is true, then the actomyosin ring must free itself from the septins in order to undergo contraction. Thus, septin ring splitting may be an important structural change that enables the actomyosin ring to contract (Fig. 7). This is an appealing possibility as these two events are coupled both temporally and spatially. However, without a means of specifically inhibit septin ring splitting, we cannot directly test the significance of this structural change.

What might cause the septins to disappear from the middle? One possibility is filament disassembly, as it was reported that the septin filaments were not observed by electron microscopy in late anaphase cells undergoing cytokinesis (Byers and Goetsch, 1976). If the actomyosin ring, which occupies the
middle region of the bud neck, only interacts with filamentous septin, septin filament disassembly may reduce the interaction with the contractile ring and promote septin subunits to dissociate from the neck region, although this does not explain why the septins remain associated with the cortical regions flanking the contractile ring. Alternatively, septin clearance from the middle of the bud neck may be due to localized protein degradation or modification. The APC-dependent degradation machinery is active during cytokinesis, but there is no data to suggest that septin components are degraded by this pathway. The septins are known to be sumoylated late in the cell cycle, however, the importance of septin sumoylation is not yet clear (Johnson and Blobel, 1999; Takahashi et al., 1999).

The role of Tem1p in cytokinesis

Recent studies reported an exciting role for the Tem1p small GTPase in ensuring that the spindle is correctly oriented prior to cytokinesis (for a review, see Li, 2000). Tem1p is preferentially localized on the spindle pole body that migrates into the daughter cell and is activated following spindle penetration into the bud, presumably due to the presence of Lte1, a putative nucleotide exchange factor for Tem1p. Once activated, Tem1p is thought to activate the mitotic exit network, consisting of Cdc5, Cdc15 and Dbf2/20 kinases and a non-kinase protein, Mob1p, eventually leading to exit from mitosis and cell cycle re-entry (Morgan, 1999). In this study, we showed that Tem1p also controls actomyosin and septin dynamics during cytokinesis, a role that is only unmasked when its requirement in anaphase progression is circumvented. Tem1p is not required for the assembly of actin and myosin to the bud neck but is required for the contraction of the actomyosin ring.

This role is similar to that described for the fission yeast homolog of Tem1p, Spg1p. Spg1p, spg1 mutant cells do not exhibit any cell cycle defect but are deficient in the final steps of cytokinesis (Schmidt et al., 1997). Our results suggest that Tem1p has dual roles in budding yeast, one in cell cycle...
regulation, and the other in the onset of cytokinesis. The latter role is likely to be conserved with that carried out by Spg1p. Still, it remains a question as to whether the mitotic exit network proteins, several of which are homologous to those acting downstream of Spg1p (Balasubramanian et al., 2000), are also involved in the cytokinesis function of Tem1p. Preliminary results suggest that the mitotic exit network proteins are required for cytokinesis when their cell cycle function is circumvented (K. Shannon and R. Li, unpublished).

However, several studies have shown that these mutants are defective in actin ring formation at the non-permissive temperature (Frenz et al., 2000; Jimenez et al., 1998; Lippincott and Li, 1998b), whereas most of the Tem1 deficient cells can assemble an F-actin ring. This implies either that Tem1p regulates a step different from that from by other MEN proteins, or that the other MEN proteins have a role in actin ring formation in addition to their participation in the Tem1p-dependent function in regulating contraction. However, it is also possible that the cell cycle arrest at high temperature in the MEN mutants causes destabilization of the actin ring. This is likely since we have noticed that the percentage of Tem1p-deficient cells that contained actin rings decreased to approx. 50% if the cells were grown at 37°C, and the actin rings that were present appeared significantly fainter than those in cells grown at room temperature (data not shown).

What might be the ultimate target of Tem1p in the final stages of cytokinesis? We have observed that both septin splitting and actomyosin ring contraction are defective in Tem1p-deficient cells. If former event is a prerequisite for the latter, then the key target of Tem1p in cytokinesis may be the septins. However, we previously reported that Tem1p can bind directly to the GRD domain of Cyk1p, and this domain, like Tem1p, is required for actomyosin ring contraction, not assembly (Shannon and Li, 1999). These observations suggest that an interaction between Tem1p and the GRD of Cyk1p may trigger actomyosin ring contraction. If this is true, because septin splitting occurs dependent function in regulating contraction. However, it is Tem1p regulates a step different from that by other MEN cells can assemble an F-actin ring. This implies either that

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REFERENCES


