Septin Ring Assembly Requires Concerted Action of Polarisome Components, a PAK Kinase Cla4p, and the Actin Cytoskeleton in *Saccharomyces cerevisiae*

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Septins are filament-forming proteins that function in cytokinesis in a wide variety of organisms. In budding yeast, the small GTPase Cdc42p triggers the recruitment of septins to the incipient budding site and the assembly of septins into a ring. We herein report that Bni1p and Cla4p, effectors of Cdc42p, are required for the assembly of the septin ring during the initiation of budding but not for its maintenance after the ring converts to a septin collar. In *bni1Δ cla4-75-td* mutant, septins were recruited to the incipient budding site. However, the septin ring was not assembled, and septins remained at the polarized growing sites. Bni1p, a formin family protein, is a member of the polisome complex with Spa2p, Bud6p, and Pea2p. All *spa2Δ cla4-75-td, bud6Δ cla4-75-td*, and *pea2Δ cla4-75-td* mutants showed defects in septin ring assembly. Bni1p stimulates actin polymerization for the formation of actin cables. Point mutants of *BNI1* that are specifically defective in actin cable formation also exhibited septin ring assembly defects in the absence of Cla4p. Consistently, treatment of *cla4Δ* mutant with the actin inhibitor latrunculin A inhibited septin ring assembly. Our results suggest that polisome components and Cla4p are required for the initial assembly of the septin ring and that the actin cytoskeleton is involved in this process.

INTRODUCTION

The development of both unicellular and multicellular organisms requires cells to respond to intracellular and extracellular cues that direct growth and division. These signals regulate polarized cell growth, maintenance of cell shape, cell motility, and cytokinesis (Drubin and Nelson, 1996). Polarized cell growth is a complex process that requires the directed organization of the actin cytoskeleton and the coordinated function of many polarity proteins and signal transduction cascades. Cells of the yeast *Saccharomyces cerevisiae* grow by budding, a process in which a rigid cell wall is locally expanded as a result of polarized secretion (Pruyne and Bretscher, 2000a). Before bud emergence, cells polarize the actin cytoskeleton toward the future bud site and assemble a septin ring at that site.

The septins are a conserved family of filament-forming proteins that play important roles in a variety of cell functions in fungal and animal cells (Longtine et al., 1996; Trimble, 1999; Gladfelter et al., 2001; Longtine and Bi, 2003).

Typical septins have a variable N-terminal region, a conserved core that includes the element of a GTP-binding site, and a variable C-terminal region. Septins were first identified as temperature-sensitive *cdc* mutants in *S. cerevisiae* (Hartwell, 1971). Septins are assembled into a ring before bud formation and remain as a collar subjacent to the plasma membrane at the mother-bud neck for most of the cell cycle. The septin ring is assembled by the copolymerization of the septins Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p into filaments (Frazier et al., 1998; Field and Kellogg, 1999). Purified septins from yeast form filaments of 7–9-nm diameter and of various lengths (Frazier et al., 1998).

One major role for the septins in various organisms is in cytokinesis (Hartwell, 1971; Neufeld and Rubin, 1994; Kinoshita et al., 1997; Bi et al., 1998; Lippincott and Li, 1998). The septins localize to the division site (cleavage furrow) during cytokinesis (Longtine et al., 1996; Trimble, 1999; Nguyen et al., 2000; Westfall and Momany, 2002). The septins recruit a variety of other proteins, whose correct localization to the neck is critical for the performance of their various functions, including chitin deposition (DeMarini et al., 1997), bud site selection (Chant et al., 1995; Sanders and Herskowitz, 1996), and cell cycle control (Barral et al., 1999; Shulewitz et al., 1999; Longtine et al., 2000). Thus, the septins have been proposed to function as a scaffold at the bud neck for protein anchoring and organization (Longtine et al., 1996, 2000; Field and Kellogg, 1999; Gladfelter et al., 2001). Additionally, the septins function as a diffusion barrier at the mother-bud junction to prevent membrane-associated proteins from moving freely between the mother and bud compartments (Barral et al., 2000; Takizawa et al., 2000). In spite of
of our plentiful knowledge of the functions of septins, little is known as to how the septin ring is assembled during budding initiation.

Reorganization of both actin and septins requires the Rho type small GTPase Cdc42 (Pringle et al., 1995). Conditional cdc42 mutant cells are defective in assembly of the septin ring and polarized reorganization of the actin cytoskeleton. Therefore, these cells are defective in bud emergence and localized cell surface growth, and they become arrested as large, multinucleate, un budded cells at the restrictive growth temperature. Isolation of cdc42 mutants, which specifically display defects in septin ring assembly, and analysis of the effect of Cdc42 GTPases on septin ring assembly suggest that cycles of GTP loading and hydrolysis by Cdc42 play an important role in septin ring assembly (Gladelter et al., 2002; Smith et al., 2002; Caviston et al., 2003).

Although significant effort has been dedicated to deciphering the Cdc42 effector pathways important for actin polarization, little is known about how Cdc42 mediates septin ring assembly. Several effectors of Cdc42 have been identified, including the p21-activated kinase (PAK)-like kinases Ste20p, Cla4p, and Skm1p, a formin family protein Bni1p, and Gic1p and Gic2p (Pruyne and Bretscher, 2000a). Among these effectors, Cla4p is required for normal septin function (Cvrckova et al., 1995; Weiss et al., 2000; Schmidt et al., 2003). However, cla4Δ mutants can still assemble a septin ring. Bni1p and its related protein Bnr1p assemble actin cables, which play a pivotal role in the polarized transport of secretory vesicles as a track for type V myosin, Myo2p (Evangelista et al., 2002; Sagot et al., 2002a; Schott et al., 2002). Bni1p can stimulate polymerization of actin in vitro independent of the Arp2/3 complex (Pruyne et al., 2002; Sagot et al., 2002b). Bni1p, together with Spa2p, Bud6p, and Pea2p, constitutes a large complex termed the 12S polarisome (Sheu et al., 1998; Pruyn and Bretscher, 2000b).

Polarisome components are required for apical growth (Sheu et al., 2000); in their absence, vegetative buds grow as spheres rather than ellipsoids. In the course of our study on the genetic interactions between effectors of Cdc42, we found that a bni1Δ mutation shows a synthetic lethal interaction with the cla4Δ mutation. The bni1Δ cla4Δ-75-tl mutant showed abnormal morphogenesis, which was caused by a defect in the assembly of the septin ring during budding initiation. Mutations in other polisome components also showed a similar synthetic defect with the cla4Δ mutation in septin ring assembly. Interestingly, actin cables were suggested to be involved in the septin ring assembly, downstream of Bni1p.

**MATERIALS AND METHODS**

**Media and Genetic Techniques**

Unless otherwise specified, strains were grown in rich medium YPDA (1% yeast extract [Difco Laboratories, Detroit, MI], 2% bacto-peptone [Difco], 2% glucose, and 0.01% adenine). Strains carrying plasmids were selected in the presence of 5-fluoro-orotic acid to select for the loss of the URA3-containing plasmid. These Ura- cells were replicated onto two sets of SD-Leu plates that were incubated at 24 and 37°C, respectively, to allow the identification of the temperature-sensitive (ts) mutants. The plasmid carrying the bni1-ts allele was isolated and the bni1-ts ORF was sequenced by a standard protocol.

**Microscopic Observations**

Cells were observed using a Nikon ECFIPS E800 microscope (Nikon Intoc, Tokyo, Japan) equipped with HB-10103AF super-high-pressure mercury lamp and 1.4NA Plan Apo oil immersion objective (Nikon Intoc) with appropriate fluorescence-filter sets (Nikon Intoc) or differential interference contrast (DIC) optics. Images were acquired using a cooled digital CCD camera (Dage-95-12NR, Hamamatsu Photonics K.K.) and AQUACOSMOS software (Hamamatsu Photonics K.K.). To observe Cdc12p-GFP, cells were fixed for 5 min at room temperature by direct addition of commercial 37% formaldehyde stock (Wako Pure Chemical Industries, Osaka, Japan) to a final concentration of 3.7% in the medium and washed twice with phosphate-buffered saline before mounting on a glass microscope slide. Fixed cells were observed using a GFP bandpass filter set (excitation, 460–490 nm; dichroic mirror, 505 nm; emission, 515–545 nm).

Time-lapse analyses of cell morphology and septin ring assembly were carried out as follows. Cells were grown to an early logarithmic phase in SC medium at 25°C, harvested by brief centrifugation, washed once with SC, and resuspended in SC. The cell suspension was spotted onto a thin layer of 9C medium containing 1% agarose on a glass microscope slide, which was quickly covered with a coverslip. Around the edges of the coverslip, a small amount of vaseline (Wako) was applied for sealing. An image at each time point was acquired as described above. During observation, the sample was kept at 37°C by Thermo stage (Tokai Hit Co., Fujinomiya, Japan).

Initial septin ring assembly was monitored by observing Cdc12p-GFP in cells exiting from cell cycle arrest. Cells were synchronized in the G1 phase of the cell cycle by the addition of α-factor and released from the block by removal thereof. In brief, cells were grown to a logarithmic phase, pelleted, and resuspended in YPDA containing 1 μg/ml α-factor (Sigma Chemical, St. Louis) at 0.3–0.5 × 10⁷ cells/ml. When cells were observed to have shmoos, cells were washed with 10% yeast extract twice with phosphate-buffered saline before mounting on a glass microscope slide. Fixed cells were observed using a GFP bandpass filter set (excitation, 400–490 nm; dichroic mirror, 505 nm; emission, 515–545 nm).
examine d G1-arrested cells were treated with 100 μM LAT-A (added to the medium from a 20 mM stock in DMSO) as described by Ayscough et al. (1997). As a control, an equal volume of DMSO alone was added. At least 100 cells containing polarized Cdc12p-GFP were observed in each experiment, where >90% of them showed a uniform phenotype, unless otherwise mentioned. A representative image of the cells is shown in each figure.

To measure septin ring diameter, cells were released from G1 arrest for 30 min and fixed before bud emergence. The diameter of the septin ring, which was defined as the maximum distance across the septin ring, was measured from the center between outer and inner edges of the septin ring to the opposite center between the edges using Adobe illustrator version 9.0 (Adobe Systems, San Jose, CA). For each determination of the average diameter of septin rings, 100 cells were chosen randomly and measured.

**Isolation of Multicopy Suppressors of the bni1-116 cla4Δ mutant**

The bni1-116 cla4Δ strain (YKT530) was transformed with a yeast genomic DNA library constructed in the multicopy plasmid YEp24. After transformation, cells were incubated on SD-Ura plates at 25°C for 48 h to allow recovery replica-plated onto fresh YPDA plates, and then incubated at 35°C for 3 d. About 50,000 transformants were screened, and 12 transformants that reproducibly grew at 35°C were obtained. From each of the transformants, plasmids were recovered for further analysis. PCR revealed that five plasmids contained BNI1 ORF, but no plasmid contained CLA4 ORF. All of the remaining seven plasmids conferred temperature-resistant growth on YKT530. The genes present in the seven plasmids were identified by sequencing both ends of the inserts. The suppressor gene in those was identified by testing individual subcloned fragments for suppressing activity. Two of them were BEH5171-113 and RGA1Δl-32. The other five genes will be described elsewhere.

**RESULTS**

**Loss of Bni1p Causes Defective Septin Organization in the Absence of Cla4p**

The effectors of Cdc42p are involved in cellular polarization through the cell cycle, but the details of their functions are unknown. To further explore these functions, we examined the genetic interactions between effectors of CDC42. Previous reports showed that a cell lacking both Cla4p and Ste20p
Bni1p and Cla4p are required for the assembly of the septin ring during budding initiation.

Firstly, we extended this study and found that, as described below, Bni1p and Cla4p are redundantly involved in septin organization.

Whereas our study was in progress, Sprague and colleagues reported a similar genetic interaction between BNI1 and CLA4 (Goehring et al., 2003). Our results are consistent with their data, in which they also showed that loss of Bni1p or its interacting proteins (Spa2p, Bud6p, and Pea2p; see below) in a claiΔ mutant resulted in lethality and caused cells to form elongated buds with mislocalized septin rings. Furthermore, they showed that Bni1p is a Ste20p-dependent phosphoprotein and may be directly regulated by Ste20p. We extended this study and found that, as described below, Bni1p and Cla4p are required for the assembly of the septin ring during budding initiation.

Bni1p and Cla4p Are Required for the Initial Septin Ring Assembly, but Not for the Maintenance of Septin Collar

We performed time-lapse observations of a bni1Δ claiΔ mutant expressing Cdc12p-GFP. When unbudded bni1Δ claiΔ mutant cells that had not yet assembled a septin ring were shifted to 37°C, these cells could not assemble normal septin ring like that of wild-type cells (Figure 2A). Their septins, instead, accumulated as a cap at the incipient budding site and remained at the polarized growing site. In contrast, when budding bni1Δ claiΔ mutant cells that had formed a septin collar were shifted to 37°C, these cells could accomplish cytokinesis normally without disorganizing their septin collar (Figure 2B). However, the resulting mother and daughter cells showed the defect in septin ring assembly in the next cell cycle. To observe the initial septin assembly in detail, we performed time-lapse analyses with the 5-min time points. We observed 16 individual bni1Δ claiΔ mutant cells that had assembled a septin ring at 37°C, suggesting that this mutant cannot assemble a septin ring even with a transient manner (Figure 2C). These results indicate that the bni1Δ claiΔ double mutation causes a defect in the initial assembly of septin ring but not in the maintenance of the formed septin collar.

Because the septin defects have not been described in the bni1Δ claiΔ mutant, we investigated the initial assembly of septin ring. We performed time-lapse observations of a bni1Δ claiΔ ts-factor–induced G1 arrest. The G1-arrested t-factor–induced G1-arrested cells (Figure 3A), as described (Evangelista et al., 1997). Septins formed an array of bars at the base of the projection in G1-arrested cells (Figure 3A), as described (Longtine et al.,
Assembly in the Absence of Cla4p

The Effects of Bni1p Truncation Mutants on Septin Ring Assembly in the Absence of Cla4p

Bni1p is a member of a protein family that is characterized by formin homology (FH) domains, FH1 and FH2. The proline-rich FH1 domain binds to profilin as well as to peptide-recognition modules such as SH3 and WW domains (Wasserman, 1998; Ridley, 1999; Zeller et al., 1999). The FH2 domain is highly conserved, and both FH1 and FH2 are involved in actin filament assembly (Evangelista et al., 2002; Sagot et al., 2002a). Bni1p also has a Rho-binding domain (RBD), which interacts with Rho1p and Cdc42p, in its amino-terminal region (Kohno et al., 1996; Evangelista et al., 1997), a Spa2p-binding domain (SBD) in the middle region (Fujivara et al., 1998), and a Bud6p-binding domain (BBD) at the carboxyl-terminal region (Evangelista et al., 1997). The bni1Δ mutation shows a synthetic lethal interaction with the bnr1Δ mutation due to defects in actin cable assembly (Evangelista et al., 2002; Sagot et al., 2002a). To examine the effects of Bni1p truncation mutants on cell growth in the absence of Cla4p, we constructed five truncation mutations of BNI1 and tested these for the ability to complement the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutation in glucose medium, in which the activity of GAL1 promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutant at all tested temperatures (Figure 4B). We also examined assembly of the septin ring upon exit from G1 arrest induced by a-factor block and tested these for the ability to complement the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutation in glucose medium, in which the activity of GAL1 promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutant at all tested temperatures (Figure 4B). We also examined assembly of the septin ring upon exit from G1 arrest induced by a-factor block and tested these for the ability to complement the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutation in glucose medium, in which the activity of GAL1 promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutant at all tested temperatures (Figure 4B). We also examined assembly of the septin ring upon exit from G1 arrest induced by a-factor block and tested these for the ability to complement the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutation in glucose medium, in which the activity of GAL1 promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutant at all tested temperatures (Figure 4B). We also examined assembly of the septin ring upon exit from G1 arrest induced by a-factor block and tested these for the ability to complement the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutation in glucose medium, in which the activity of GAL1 promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutant at all tested temperatures (Figure 4B). We also examined assembly of the septin ring upon exit from G1 arrest induced by a-factor block and tested these for the ability to complement the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutation in glucose medium, in which the activity of GAL1 promoter is repressed (Figure 4A). ΔFH1 and ΔFH2 could not restore the growth of either the P_GAL1-BNI1 bnr1Δ or P_GAL1-BNI1 cla4Δ mutant at all tested temperatures (Figure 4B).
ture, which was much fainter and thinner than the septin ring in wild-type cells, but the septins became diffused and formed a cap-like structure on the bud tip by 60 min (unpublished results). Our results suggest that actin cables, which are formed by the action of Bni1p, are required for septin ring assembly in the absence of Cla4p.

$\Delta$SBD and $\Delta$BBD restored the growth of the $P_{\text{GAL1}}$-BNII $\text{bni1}\Delta$ mutant at all temperatures tested, suggesting that the interaction of Bni1p with either Spa2p or Bud6p is not required for formation of the actin cables that are sufficient for polarized cell growth. Consistently, a $\text{bnr1}\Delta$ mutation did not show a synthetic lethal interaction with either a $\text{spa2}\Delta$ or $\text{bud6}\Delta$ mutation (unpublished results). Interestingly, neither $\Delta$SBD nor $\Delta$BBD restored the growth of $P_{\text{GAL1}}$-BNII $\text{cla4}\Delta$ mutant in glucose medium at high temperatures, suggesting that the interaction of Bni1p with either Spa2p or Bud6p plays an important role for growth in the absence of Cla4p (Figure 4B). Assembly of the septin ring was examined in the $\text{bni1}\Delta$ $\text{cla4-75-td}$ mutant expressing either $\Delta$SBD or $\Delta$BBD upon exit from G1 arrest at 37°C. When grown for 30 min,
Assemble a septin ring upon exit from G1 arrest (Figure 4C).

We also examined the terminal phenotype of a polarisome component. The cell morphology of 

\( \text{pea2}^{\Delta} \) mutant at 25°C (unpublished results). Therefore, the interaction of Bni1p with either Spa2p or Bud6p seems to be required for normal septin ring assembly in the absence of Cla4p. 

\( \Delta \text{RBD} \) restored the growth of \( P_{\text{GAL1}} \cdot \text{BNI1} bni1^{\Delta} \) mutant in glucose medium at all temperatures tested. Interestingly, \( \Delta \text{RBD} \) could not restore the growth of \( P_{\text{GAL1}} \cdot \text{BNI1} \) mutant in glucose medium at high temperatures, suggesting that the interaction between Bni1p and Rho-GTPase is also important for growth in the absence of Cla4p (Figure 4B). 

The \( \text{bni1}\Delta \) mutant expressing \( \Delta \text{RBD} \) could not assemble a septin ring upon exit from G1 arrest (Figure 4C). These results suggest that all of the functional domains of Bni1p, which have been identified so far, are required for septin ring assembly during budding initiation, especially at high temperatures in the absence of Cla4p.

### Loss of Polarisome Components Causes Defective Septin Ring Assembly in the Absence of Cla4p

If the interaction between Bni1p and a polisome component is important for the growth in the absence of Cla4p, polisome genes would also show the genetic interaction with \( \text{CLA4} \). The \( \text{cla4}^{\Delta} \) mutant was crossed with \( \text{bud6}^{\Delta}, \text{spa2}^{\Delta}, \text{or pea2}^{\Delta} \) mutant, and the resulting diploid cells were sporulated and dissected for tetrad analysis. The \( \text{bud6}^{\Delta} \) and \( \text{spa2}^{\Delta} \) double mutants exhibited a synthetic lethality at 25°C, while the \( \text{pea2}^{\Delta} \) double mutant exhibited a poor growth phenotype at 25°C (unpublished results). 

We also examined the terminal phenotype of \( \text{cla4}^{\Delta} \) mutants lacking a polisome component. The cell morphological properties of 80% of cells expressing \( \Delta \text{SBD} \) and <10% of cells expressing \( \Delta \text{BBD} \) could form a septin ring-like structure at the incipient budding site. This septin ring-like structure appeared fainter and thinner than a normal septin ring in wild-type cells. However, the septins in both mutants became diffused and formed a cap-like structure on the bud tip after a 60-min incubation (unpublished results). 

These results suggest that the presence of a polisome component and its interaction with Bni1p is required for initial septin ring assembly in the absence of Cla4p, and can stimulate actin polymerization in vitro (Pruyne et al., 2002; Sagot et al., 2003). To further examine whether a polisome component is required for initial septin ring assembly in the absence of Cla4p, we analyzed the assembly of the septin ring upon exit from G1 arrest at 37°C (Figure 5A). 

In each strain, Cdc12p-GFP localized as a cap at the polarized growing site or as a patch around the wide bud neck. These results are consistent with the recent report by Sprague and colleagues (Goehring et al., 2003). To further examine whether a polisome component is required for initial septin ring assembly in the absence of Cla4p, we analyzed the assembly of the septin ring upon exit from G1 arrest at 37°C (Figure 5B). When grown for 30 min, both \( \text{spa2}^{\Delta} \) and \( \text{pea2}^{\Delta} \) mutants could not assemble a normal septin ring as well as \( \text{bni1}\Delta \) mutant carrying \( \Delta \text{RBD} \). However, \( \text{bni1}\Delta \) mutant expressed \( \Delta \text{SBD} \) could form a septin ring-like structure at the incipient budding site. This result suggests that the actin-nucleating activity of Bni1p may be required for septin ring assembly in the absence of Cla4p.

### Actin Polymerization Is Required for Septin Ring Assembly in the Absence of Cla4p

The FH2 domain of Bni1p is required for actin filament assembly in vivo (Evangelista et al., 2002; Sagot et al., 2002a) and can stimulate actin polymerization in vitro (Pruyne et al., 2002; Sagot et al., 2002b). As described above, \( \Delta \text{FH2} \) could not restore the growth of the \( \text{bni1}\Delta \text{cla4}\Delta \) double mutant. This result suggests that the actin-nucleating activity of Bni1p may be required for septin ring assembly in the absence of Cla4p. However, it is also possible that the FH2 domain possesses a distinct function in septin ring assembly. To discriminate these possibilities, we isolated a ts-allele of \( \text{BNI1}, \text{bni1}-116 \), which causes amino acid substitutions...
(V1475A, K1498E, and D1511N) in the FH2 domain. In cells carrying bni1-116 bnr1/H9004 mutations, actin cables visualized by rhodamine-phalloidin disappear in 2 min after upshift to 37°C (unpublished results). This effect on actin cables was not due to degradation of Bni1-116p, because Bni1-116p-GFP was localized to a bud tip in cells grown at 37°C (unpublished results). The bni1-116 cla4/H9004 mutant showed growth defects above 35°C and abnormally elongated buds with wide necks, results similar to bni1/H9004 cla4-75-td mutant cells (unpublished results). We examined assembly of the septin ring upon exit from G1 arrest at 37°C in the bni1-116 cla4Δ mutant. When grown for 30 min, bni1-116 cla4Δ mutant as well as bni1Δ cla4-75-td mutant could not assemble normal septin ring (Figure 6A). bni1-11 (amino acid substitutions: D1511G and K1601R; Evangelista et al., 2002) and bni1-FH2#1 (amino acid substitutions: R1528A and R1530A; Sagot et al., 2002a) are previously characterized mutations in the FH2 domain. Both mutations also cause rapid disassembly of actin cables at 37°C. We confirmed that bni1-11 cla4Δ and bni1-FH2#1 cla4Δ mutants showed the defects in cell growth and septin ring assembly as bni1Δ cla4-75-td mutant (unpublished results). These results suggest that actin polymerization mediated by the FH2 domain of Bni1p is required for the assembly of the septin ring during budding initiation. A previous study suggested that assembly of the septin ring is independent of the actin cytoskeleton (Ayscough et al., 1997). They showed that the actin assembly inhibitor
LAT-A did not affect septin ring assembly in wild-type cells. The results described above suggest that LAT-A may inhibit septin ring assembly in the \( \text{cla4}\Delta \) mutant. We analyzed septin ring assembly upon exit from \( G_1 \) arrest at 25°C in the presence or absence of LAT-A in the wild-type, \( \text{bni1}\Delta \), and \( \text{cla4}\Delta \) mutants. When grown for 30 min after release from \( G_1 \) arrest in the absence of LAT-A, each strain assembled a septin ring. An extended diameter and jagged morphology of the septin ring were observed in \( \text{bni1}\Delta \) mutant as shown in Figure 3A, and the periphery of the septin ring in \( \text{cla4}\Delta \)
mutant looked fainter compared with wild-type (Figure 6B). After an additional 30 min, each strain formed a small bud and the septin collar localized to the bud neck (unpublished results). The diameter of the septin collar in the \textit{bni1Δ} mutant was slightly greater than that in wild-type. Less than 10% of the \textit{cla4Δ} mutant cells localized the septins on the bud tip (unpublished results). When grown for 30 min in the presence of LAT-A, each strain localized the septins at the incipient budding site, but did not assemble a septin ring (unpublished results). An additional 30 min later, >70% of the wild-type and \textit{bni1Δ} mutant cells assembled a septin ring (Figure 6B), suggesting that LAT-A treatment caused a delay in septin ring assembly. However, the \textit{cla4Δ} mutant could not assemble a septin ring (Figure 6B), even after a 6-h

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**Figure 5.** Defective septin ring assembly in \textit{cla4-75-td} mutants lacking a polarisome component. (A) YKT649 (\textit{bni1Δ cl\textit{a4-75-td CDC12-GFP}}, YKT664 (\textit{bud6Δ cl\textit{a4-75-td CDC12-GFP}}, YKT666 (\textit{spa2Δ cl\textit{a4-75-td CDC12-GFP}}, and YKT668 (\textit{pea2Δ cl\textit{a4-75-td CDC12-GFP}) were grown in YPDA medium at 25°C and then shifted to 37°C for 2 h. Cells were fixed and observed by DIC and fluorescence microscopy. (B) Cells of each strain were G1-arrested with α-factor at 25°C and released into fresh medium at 25°C or 37°C. After a 30-min incubation, cells were fixed and observed by DIC or fluorescence microscopy. Bars, 5 μm.
incubation (unpublished results). Previous study reported that actin perturbation by LAT-A triggers a morphogenesis checkpoint and induces a delay of nuclear division (McMillan et al., 1998). To assess some aspect of cell cycle progression in the LAT-A–treated cells, we monitored the mitotic cell cycle delay. The extent of the cell cycle delay, however, was similar between the wild-type, bni1Δ, and cla4Δ mutant cells. The extent of cell cycle delay was not only in the cells that assembled a mitotic spindle decreased from 70 to 40% compared with the untreated cells in each strain (unpublished results), suggesting that the LAT-A treatment induces the cell cycle delay. The extent of the cell cycle delay, however, was similar between the wild-type, bni1Δ, and cla4Δ mutants, suggesting that the cla4Δ mutant cannot assemble a septin ring in the presence of LAT-A independent of the cell cycle delay. These results are consistent with our observations that Bni1p-dependent actin polymerization is required for initial septin ring assembly in the absence of Cla4p.

**Actin Cable-dependent Transport May Be Required for Septin Ring Assembly in the Absence of Cla4p**

We examined actin cable-related factors for their involvement in septin ring assembly. We constructed cla4Δ mutants combined with actin cable-related ts mutations such as bni1-116 bnr1Δ, tpm1-2 tpm2Δ, and myo2-20 (Pruyne et al., 1998; Schott et al., 1999). Tropomyosins (Tpm1p and Tpm2p) are actin-cable-stabilizing proteins. Myo2p, a type V myosin, has a direct role in secretory vesicle targeting using actin cables as tracks for transport. We analyzed septin ring assembly upon exit from G1 arrest at 37°C. When grown for 60 min, >80% of bni1-116 bnr1Δ, tpm1-2 tpm2Δ, and myo2-20 mutants assembled a septin ring (Figure 7). In contrast, bni1-116 bnr1Δ cla4Δ, tpm1-2 tpm2Δ cla4Δ, and myo2-20 cla4Δ mutant cells could not assemble septin rings (Figure 7). Deletion of only BNR1 from cla4Δ mutant affected neither growth nor morphology of the cells (unpublished results). Because loss of Bni1p is known to cause depolarization of cortical actin patches (Kohno et al., 1996; Evangelista et al., 1997), we examined the involvement of an actin patch-related protein in septin ring assembly. We constructed the cla4Δ mutants combined with the arp2-2 and myo3Δ myosin-1 ts mutations (Geli and Riezman, 1996; Madania et al., 1999). Arp2p is a subunit of the Arp2/3 complex, which is the major known contributor to actin nucleation in vivo (Yamada, 1999). Myo3/5p, type I myosins, are known as regulators of the Arp2/3 complex (Evangelista et al., 2000; Lechler et al., 2000). Both arp2-2 and myo3Δ myosin-1 mutants showed normal septin ring assembly, and depletion of Cla4p in these mutants did not affect this assembly (Figure 7). These results suggest that actin cable-dependent transport, but not actin patch function, is required for the assembly of a normal septin ring.

**Cdc42p and Its GAPs May Be Involved in the Regulation of Septin Ring Assembly by Bni1p and Cla4p**

To investigate genes involved in septin ring assembly mediated by Bni1p and Cla4p, we isolated multicopy suppressors of the ts growth phenotype of bni1-116 cla4Δ mutant. We isolated truncated fragments of BEM3 and RGA1 that encode GAP-activating protein (GAP) for Cdc42p (Zheng et al., 1993; Stevenson et al., 1995; Johnson, 1999). Both of the isolated fragments, BEM3Δ1-114 and RGA1Δ1-632, contained a region encoding a GAP domain (Figure 8A). Multicopy BEM3Δ1-114 and RGA1Δ1-632 also suppressed the growth defect of the bni1Δ cla4-75-td mutant (Figure 8B). The full-length BEM3 suppressed the growth defect of the bni1Δ cla4-75-td mutant, but to a lesser extent than that of BEM3Δ1-114, whereas the full-length RGA1 did not, suggesting that an N-terminal region of Rga1p and Bem3p may possess a negative regulatory role for GAP activity (unpublished results). We also examined the assembly of the septin ring upon exit from G1 arrest at 37°C in bni1Δ cla4-75-td mutants carrying multicopy BEM3Δ1-114 or RGA1Δ1-632 (Figure 8C). The cells carrying BEM3Δ1-114 or RGA1Δ1-632 did not assemble septin rings either after 30-min (Figure 8C) or 60-min growth (unpublished results). Surprisingly, after 3 h, they often formed ring-like structures comprised of septins around the bud neck and eventually accomplished cytokinesis (Figure 8C). These results suggest that the defects in septin assembly in bni1Δ cla4-75-td mutant can be alleviated during polarized growth after budding has occurred.

Multicopy BEM3Δ1-114 and RGA1Δ1-632 also suppressed the growth defects of bude6Δ cla4-75-td, spa2Δ cla4-75-td, and pea2Δ cla4-75-td mutants, but not that of bni1-116 bnr1Δ mutant (unpublished results), suggesting that they suppressed the defects caused by the cla4Δ mutation. Because the Cdc42p GAPs seem to down-regulate Cdc42p, expression of a dominant active version of Cdc42p may cause lethality in bni1Δ mutant. However, expression of

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**Figure 6.** Perturbation of actin polymerization causes defective septin ring assembly in the absence of Cla4p. (A) Point mutant in the FH2 domain of BNII, characterized as that defective in actin cable assembly, causes the defects in septin ring assembly in the absence of Cla4p. YKT638 (bni1-116 cla4Δ CDC12-GFP) was G1-arrested with α-factor at 25°C and released into fresh medium at 25 or 37°C. After a 30-min incubation, cells were fixed and observed by DIC and fluorescence microscopy. (B) LAT-A inhibits septin ring assembly in cla4Δ mutant. YKT637 (CDC12-GFP), YKT676 (bni1Δ CDC12-GFP), and YKT171 (cla4Δ CDC12-GFP) were G1-arrested with α-factor and released into fresh medium at 25°C in the absence (DMSO only) or presence of LAT-A. After 30- (DMSO) and 60 (LAT-A)-min incubations, cells were fixed and observed by DIC or fluorescence microscopy. Bars, 5 μm.
Cdc42G12Vp caused lethality in wild-type cells (unpublished results). Overexpression of a GFP-tagged dominant active version of Cdc42p, GFP-Cdc42G12Vp, did not inhibit the growth of wild-type cells (Figure 9A), suggesting that its activity is relatively weaker than that of Cdc42G12Vp. GFP-Cdc42G12Vp inhibited the growth of the bni1/H9004 mutant (Figure 9A), but not the wild-type (Figure 9A) or cla4/H9004 mutant (unpublished data). This growth inhibition appears to be due to GTP-bound Cdc42p, because GFP-Cdc42p did not inhibit the growth of the bni1Delta mutant. The bni1Delta mutant overexpressing GFP-Cdc42G12Vp also displayed a wide bud neck, although hypopolarized growth was not remarkable compared with bni1Delta cla4-75-td mutant (Figure 9B). In these cells, the septins formed a broad ring around the bud neck, leading to defects in cytokinesis. These results suggest that the cla4Delta mutation induces effects similar to those caused by accumulation of Cdc42p-GTP and that this results in the synthetic defects in septin ring assembly with the bni1Delta mutation.

DISCUSSION

The Role of Polarisome Components and Actin Cytoskeleton in Septin Ring Assembly

We showed that the polarisome components (Bni1p, Spa2p, Bud6p, and Pea2p) are required for septin ring assembly in the absence of Cla4p. In double mutants carrying mutations in either of polarisome components and CLA4, Cdc12p-GFP was recruited to the budding site, but a septin ring was not assembled and Cdc12p-GFP was instead localized to the polarized site. Time-lapse analyses indicated that a polari-
some component and Cla4p are not required for the stable maintenance of the septin collar, once a septin collar has been formed at the permissive temperature, but that they are specifically required for the initial assembly step of the septin ring. On the contrary, Goehring et al. (2003) reported that the bni1Delta cla4Delta mutant carrying the YCp-cla4-75 plasmid, which contains the original cla4-75 allele, not the cla4-75-ts degron allele, formed a normal initial septin ring, with septin abnormalities only appearing later. We also observed that ~30% of the bni1Delta cla4Delta mutant cells carrying YCp-cla4-75 initially assembled a septin ring-like structure with deformed morphology, which decayed to a cap as the bud began to grow (unpublished results). The residual activity of Cla4-75p, caused by the lack of rapid degradation and the increased expression from YCp plasmid, may partially promote the initial septin ring assembly in the bni1Delta cla4Delta mutant.

Deletion analysis of Bni1p revealed the importance of Rho-, Spa2p-, and Bud6p-binding domains for septin ring assembly, a clear contrast to the results that these domains are not required for the suppression of growth defects of bni1Delta bnr1Delta mutant. These results suggest that the integrity
of the polarisome complex is required for septin ring assembly, in addition to the presence of each component.

We showed that a bni1Δ mutant assembles a septin ring with a wider diameter than that of wild-type cells. The polarisome component mutants display defects in apical growth because they fail to confine the polarized growth site to a small region at the bud tip (Sheu et al., 2000). If polarisome mutants also display this defect during bud site assembly and if the area of bud site assembly determines the width of septin ring, depletion of a polarisome component would result in the assembly of a wider septin ring.

Interestingly, LAT-A treatment restored a septin ring with normal diameter and morphology in bni1Δ mutant. In bni1Δ mutant, Bnr1p assembles actin cables, and thus polarized growth still occurs. The LAT-A treatment completely abolishes polarized growth by inhibiting the actin polymerization. This may allow the bni1Δ mutant to have sufficient time to assemble a septin ring with normal morphology (Figure 7). However, in the absence of CLA4, the bni1-116 bnr1Δ mutant did not assemble a septin ring, suggesting that the septin ring in bni1Δ mutant is altered not only morphologically, but also functionally. This is consistent with previous observations that bni1 and spa2 mutations show synthetic growth defects with cdc12 (Zahner et al., 1996) and cdc10 (Flescher et al., 1993) mutations, respectively.

Figure 8. Suppression of the growth, morphological and septin organization defects of bni1Δ cla4-75-td mutants by multicopy RGA1Δ1-632 or BEM3Δ1-114. (A) Structures of Rga1(Δ1-632)p and Bem3(Δ1-114)p. Each number indicates the amino acid residue. Both of the isolated fragments contained a region encoding a GAP domain. (B) Suppression of the growth defect. YKT811 (bni1Δ cla4-75-td CDC12-GFP) was transformed with plasmid YEp24 (vector), pKT1364 (YEp24-BEM3Δ1-114), pKT1365 (YEpplac195-RGA1Δ1-632), or pKT1254 (pRS316-CLA4). The transformants were streaked on a YPDA plate, which was incubated at 25 or 35°C for 2 d. (C) Suppression of the septin assembly defect. Each transformant was G, arrested with α-factor at 25°C and released into fresh medium at 35°C. After 30-min and 3-h incubations, cells were fixed and observed by DIC and fluorescence microscopy. Arrows indicate the cells in cytokinesis. Bar, 5 μm.
A Bni1 mutant protein, which is defective in actin cable formation, also shows a defect in septin ring assembly in the absence of Cla4p, suggesting that the actin cytoskeleton is involved in septin ring assembly. Consistently, LAT-A treatment inhibited septin ring assembly in the \textit{cla4}/H9004 mutant. Because LAT-A does not inhibit septin ring assembly in wild-type cells, the Cla4p functions in septin assembly appear to be independent of the actin cytoskeleton. Actin cables serve as a track for a type V myosin, Myo2p. Our results that \textit{myo2}-20 \textit{cla4}/H9004, \textit{tpm1}-2 \textit{cla4}/H9004, and \textit{bni1}-116 \textit{bnr1}/H9004 \textit{cla4}/H9004 mutants showed a defect in septin ring assembly suggest that Myo2p may be required for transport of a factor specifically required for septin ring assembly to the bud emergence site. Consistent with this, even in the presence of \textit{CLA4}, the assembly of septin ring was delayed by LAT-A treatment (Figure 6) and in the \textit{bni1}-116 \textit{bnr1}/H9004 \textit{cla4}/H9004 mutants showed a defect in septin ring assembly suggesting that Bnr1p can form actin cables. The Bni1p-specific actin cables may guide polarized growth to a focused region to assemble a septin ring with normal diameter and morphology. Another interesting possibility is that Bni1p provides an actin-based structure, which is different from the actin cables used for polarized transport. Interestingly, in mammalian systems, actin bundles can serve as a template for septin assembly via an actin-binding protein, anillin (Kinoshita \textit{et al.}, 2002). It is an intriguing possibility that the actin cytoskeleton, formed by the action of Bni1p, plays a more direct role in septin assembly in conjunction with polarisome components.

**Functional Interactions between Polarisome Components, Cla4p and Cdc42 GAPs for Septin Ring Assembly**

Cla4p has also been implicated in the initial assembly of the septin ring (Cvrckova \textit{et al.}, 1995; Weiss \textit{et al.}, 2000). Fluorescence-recovery-after-photobleaching (FRAP) studies have shown that septin ring is labile during budding initiation and mitotic exit and are stable during S, G2, and M phases (Caviston \textit{et al.}, 2003; Dobbelzaere \textit{et al.}, 2003). Cla4p is required for this immobilization of the septin ring (Dobbelaere \textit{et al.}, 2003). The defects in septins in the \textit{cla4} mutant, in conjunction with the spatial defects in polarisome mutants as to where septin rings are assembled, may result in severe defects in the assembly of the septin ring. Deletion of \textit{SWE1}, which encodes a protein kinase thought to be part of the formin family, also results in defects in septin ring assembly. These results suggest that Cla4p and formins are involved in the regulation of septin ring assembly.}

Figure 9. Constitutive activation of Cdc42p is sufficient to cause defective septin organization in the absence of Bni1p. (A) YKT637 (CDC12-GFP), YKT676 (\textit{bni1}/CDC12-GFP), YKT891 (\textit{PGAL1-GFP-CDC42 CDC12-GFP}), YKT892 (\textit{PGAL1-GFP-CDC42G12V CDC12-GFP}), YKT893 (\textit{PGAL1-GFP-CDC42 bni1}/CDC12-GFP), and YKT894 (\textit{PGAL1-GFP-CDC42G12V bni1}/CDC12-GFP) were streaked on a YPDA or a YPGA plate, which was incubated at 25°C for 3 d. (B) The strains used in A were released from stationary phase into YPGA medium at 25°C. After a 6-h incubation, cells were fixed and observed by DIC and fluorescence microscopy. Cytosolic and plasma membrane staining in GFP-Cdc42p–expressing cells appear to be due to fluorescence of GFP-Cdc42p. Bar, 5 μm.
of a morphogenesis checkpoint that negatively regulates Cbl1, 2p-Cdc28p activity, restores normal bud morphology in *cla4*-mutant (Longtine et al., 2000; Weiss et al., 2000; Mitchell and Sprague, 2001). Goehring et al. (2003) reported that deletion of *SWE1* restores the localization of septins to the mother-bud neck in unsynchronized cultures of *bni1Δ cla4-75* mutant. However, we observed septin ring assembly defects in *bni1Δ cla4-75-td swe1Δ* cells upon upshift from G1 arrest (unpublished results), suggesting that Cla4p possesses specific functions for septin ring assembly.

We showed that overexpression of *BEM3Δ1-114* and *RGAΔ1-632*, which encode truncated versions of Cdc42p GAPs, suppressed the growth defects of the *bni1Δ cla4-75-td* mutant. It was recently reported that a mutant in Cdc42p GAPs (*bem3Δ rga1Δ rga2Δ*) showed severe defects in the assembly of the septin ring (Caviston et al., 2003). Therefore, it seems that a similar molecular defect underlies the *bni1Δ cla4-75-td* and *bem3Δ rga1Δ rga2Δ* mutants. Our results suggest that Cdc42p GAPs are involved in the Cla4p-related pathway for septin ring assembly. Because one of the plausible functions of Cdc42p GAPs is to down-regulate Cdc42p-GTP, Cdc42p-GTP may accumulate in the *cla4Δ* mutant. Consistently, we showed that a dominant active version of Cdc42p caused a septin ring assembly defect in *bni1Δ* mutant. Cla4p phosphorylates Cdc24p, resulting in the dissociation of Bem1p from Cdc24p and the subsequent down-regulation of Cdc24p interaction (Bose et al., 2001). Based on the observation that hyper activation of Cdc42p and depletion of Cdc42p GAPs caused defects in septin organization, Gladfelter et al. (2002) proposed that Cdc42p cycling between a GTP-bound “ON” state and GDP-bound “OFF” state is required for normal septin ring assembly. Septin ring assembly defects in the *cla4Δ* mutant may be partly due to hyper activation of Cdc42p.

We showed that overexpression of Cdc42p GAPs restored septin ring assembly in the *bni1Δ cla4-75-td* mutant. This suppression appeared to occur during polarized growth, rather than at bud emergence. Similar observations were made in the Cdc42p GAP triple mutant and in *cdc24Δ* GAP mutant (Caviston et al., 2003). They showed that, by a time-lapse analysis, the septin can be converted to a normal ring structure next to bud tip at some point during bud growth and that a bud of normal shape began to form distal to the septin ring. Therefore, a septin collar can be constructed, not only at the initiation of budding, but also during polarized growth. Caviston et al. (2003) proposed that septin ring formation consists of at least two steps: recruitment of septin proteins and their assembly into the septin ring. In this model, both steps depend on Cdc42p, whereas the Cdc42p GAPs and other “assembly factor,” such as Cla4p, Gin4p, and Elm1p, function in the assembly step. How do the truncated Cdc42p GAPs induce the septin collar formation in the *bni1Δ cla4-75-td* mutant after budding has occurred? The truncated Cdc42p GAPs may partially suppress the defects in the initial septin assembly, to an extent that the effects on the septins cannot be visualized under the microscope, resulting in the formation of a septin collar later on. It is also possible that, due to deregulation by truncation, they could induce septin assembly even during polarized growth. Caviston et al. (2003) proposed that Cdc42p GAPs function as effectors of Cdc42p for septin ring assembly. According to this model, the Cdc42p GAPs-related function of Cla4p may be to interact more directly with septins, rather than to regulate the nucleotide-binding state of Cdc42p. It was recently shown that Cla4p interacts directly with and phosphorylates septins in vitro and in vivo (Versele and Thorner, 2004). Currently, we favor a hypothesis that Cla4p regulates the septin assembly through both direct interactions with septins and regulation of the nucleotide binding state of Cdc42p.

Our observations suggest that polarisome components also function in the assembly step together with Cdc42p GAPs and Cla4p. Further identification of a protein, which directs septin ring assembly through direct interactions with septins under the control of polarisome, Cla4p and Cdc42p GAPs, is an important step toward understanding of the molecular mechanisms of septin ring assembly.

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