Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p

Amy S. Gladfelter, Indrani Bose, Trevin R. Zyla, Elaine S.G. Bardes, and Daniel J. Lew

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710

At the beginning of the budding yeast cell cycle, the GTPase Cdc42p promotes the assembly of a ring of septins at the site of future bud emergence. Here, we present an analysis of cdc42 mutants that display specific defects in septin organization, which identifies an important role for GTP hydrolysis by Cdc42p in the assembly of the septin ring. The mutants show defects in basal or stimulated GTP hydrolysis, and the septin misorganization is suppressed by overexpression of a Cdc42p GTPase-activating protein (GAP). Other mutants known to affect GTP hydrolysis by Cdc42p also caused septin misorganization, as did deletion of Cdc42p GAPs. In performing its roles in actin polarization and transcriptional activation, GTP-Cdc42p is thought to function by activating and/or recruiting effectors to the site of polarization. Excess accumulation of GTP-Cdc42p due to a defect in GTP hydrolysis by the septin-specific alleles might cause unphysiological activation of effectors, interfering with septin assembly. However, the recessive and dose-sensitive genetic behavior of the septin-specific cdc42 mutants is inconsistent with the septin defect stemming from a dominant interference of this type. Instead, we suggest that assembly of the septin ring involves repeated cycles of GTP loading and GTP hydrolysis by Cdc42p. These results suggest that a single GTPase, Cdc42p, can act either as a ras-like GTP-dependent “switch” to turn on effectors or as an EF-Tu-like “assembly factor” using the GTPase cycle to assemble a macromolecular structure.

Introduction

Prior to bud emergence in Saccharomyces cerevisiae, cells polarize the actin cytoskeleton toward the future bud site and assemble a septin ring at that site. Reorganization of both actin and septins requires the small GTPase Cdc42p and its exchange factor Cdc24p (for review see Pringle et al., 1995). Whereas significant effort has been dedicated to deciphering the Cdc42p effector pathways important for actin polarization, little is known about how Cdc42p mediates septin ring assembly.

The septins are a conserved family of filament-forming proteins that play important roles in cytokinesis in fungal and animal cells (for review see Longtine et al., 1996; Trimble, 1999; Gladfelter et al., 2001b). Septins were first identified as temperature-sensitive (Ts)* cdc mutants in S. cerevisiae (Hartwell, 1971) and are required for localized chitin deposition, bud site selection, cell cycle control, and plasma membrane compartmentalization in addition to cytokinesis. 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 scaffold recruits a variety of other proteins, whose correct localization to the neck is critical to perform their various functions.

How does Cdc42p promote the assembly of a septin ring? To begin to address this question, we sought to identify cdc42 mutants with specific defects in septin ring assembly. We recently identified such alleles (Gladfelter et al., 2001a), and we now present a genetic and biochemical characterization of two cdc42 mutants that affect the diameter and the stability of the septin ring. Unexpectedly, these studies reveal an important role for GTP hydrolysis by Cdc42p in septin ring assembly. Current models of Cdc42p function are based on the paradigm established for ras in signal transduction and invoke a switch-like “activation” of Cdc42p via exchange of bound GDP for GTP followed by interaction with downstream effectors to promote various outcomes. According to this view, GTP hydrolysis by Cdc42p serves only to terminate signaling, and a defect in GTP hydrolysis would simply cause accumulation of excess GTP-Cdc42p. If this were responsible for the observed septin defects, then the effect of the mutants should be dominant, and increasing...
The Journal of Cell Biology | Volume 156, Number 2, 2002

Figure 1. **Septin defects in cdc42 mutants.** (A) Strains DLY5461 (CDC42/GAL1p-CDC42), DLY4223 (cdc42<sup>V36T,K94E</sup>/GAL1p-CDC42), DLY4224 (cdc42<sup>Y32H</sup>/GAL1p-CDC42), and DLY5470 (cdc42<sup>Y32H</sup>/cdc42<sup>Y32H</sup>) were processed to visualize septins. Control Western blots confirmed that hemizygous strains contained approximately twofold less Cdc42p than homozygous strains in all cases. (B) Strains DLY5 (CDC42/CDC42), DLY5080 (cdc42<sup>V36T,K94E</sup>/cdc42<sup>V36T,K94E</sup>), DLY5082 (cdc42<sup>V36T,K94E</sup>/CDC42), DLY4223 (cdc42<sup>V36T,K94E</sup>/GAL1p-CDC42), DLY5471 (cdc42<sup>V36T,K94E</sup>/cdc42<sup>V36T,K94E</sup>), DLY5470 (cdc42<sup>Y32H</sup>/cdc42<sup>Y32H</sup>), DLY5461 (cdc42<sup>Y32H</sup>/CDC42), and DLY4224 (cdc42<sup>Y32H</sup>/GAL1p-CDC42) were visualized by DIC microscopy to evaluate cell morphology. (C) Strains DLY5461 (CDC42/ GAL1p-CDC42), DLY4223 (cdc42<sup>V36T,K94E</sup>/GAL1p-

**Results**

**Increased penetrance and severity of septin defects in cdc42 hemizygotes**

To address the role of Cdc42p in septin ring assembly, we have focused on two cdc42 mutants that cause defects in septin localization without overt effects on actin organization. In an earlier study, we described a mutant, cdc42<sup>V36T,K94E</sup>, that displayed relatively mild septin and cell morphology phenotypes (Gladfelter et al., 2001a). We also noted that the mutant gene dosage should exacerbate the phenotype. However, the septin-specific cdc42 alleles were fully recessive, and a twofold increase in gene dosage significantly ameliorated the mutant phenotype. These data argue strongly that GTP hydrolysis by Cdc42p plays a positive role in septin ring assembly. Based on the paradigm established for the GTPase EF-Tu in protein translation, we suggest that cycles of GTP loading and GTP hydrolysis by Cdc42p mediate the proper assembly of the septin ring.

| Strain<sup>a</sup> | Septin staining in budded cells | Septin staining in un budded cells<sup>b</sup> |
|-------------------|-------------------------------|----------------------------------|
|                   | Wild-type | Aberrant neck | In bud | Bud tip | Absent | Normal | Large |
| CDC42/cdc42<sup>Δ</sup> | 97  | 3  | 0  | 0  | 0  | 89  | 11  |
| cdc42<sup>V36T,K94E</sup>/cdc42<sup>V36T,K94E</sup> | 12  | 43  | 25  | 15  | 5  | 86  | 14  |
| cdc42<sup>V36T,K94E</sup>/cdc42<sup>Δ</sup> | 2  | 16  | 33  | 29  | 20  | 87  | 13  |
| cdc42<sup>Y32H</sup>/cdc42<sup>Y32H</sup> | 96  | 4  | 0  | 0  | 0  | 87  | 13  |
| cdc42<sup>Y32H</sup>/cdc42<sup>Δ</sup> | 4  | 61  | 16  | 7  | 12  | 29  | 71  |
| CDC42<sup>Q61L</sup> | 17  | 78  | 4  | 0  | 0  | 24  | 76  |
| cdc42<sup>K186R</sup> | 5  | 23  | 32  | 23  | 17  | 39  | 61  |

<sup>a</sup>The strains employed were (in order): DLY5461, DLY5080, DLY4223, DLY5470, DLY4224, DLY5240, and CCY3-3B.

<sup>b</sup>Only un budded cells with septin rings were scored.
severity of many other cdc42 mutants was greatly affected by gene dosage so that elevated copy number could partially rescue mutant phenotypes (Gladfelter et al., 2001a). Conversely, we reasoned that lowering the gene copy number might reveal more severe phenotypes, which could be useful for characterizing mutants with mild defects. To that end, we examined the effects of reducing mutant gene dosage by generating hemizygous cdc42-6 yeast carrying cdc42Δ mutant diploids. This reduction of gene copy number by a factor of two made the mutant phenotype significantly more penetrant and more severe (Fig. 1 B and Table I). In contrast, control hemizygous CDC42/cdc42Δ diploids were indistinguishable from homozygous CDC42/CDC42 wild-type diploids.

Examination of the phenotypes of hemizygous cdc42/cdc42Δ mutant strains further allowed us to identify septin defects associated with a novel allele, cdc42ΔV32H (Fig. 1 A and Table I). In contrast to the hemizygous cdc42Δ32H/cdc42Δ strain, septin staining appeared completely normal in haploid cdc42Δ32H and homozygous cdc42Δ32H/cdc42Δ32H or heterozygous cdc42Δ32H/CDC42 diploids containing this allele. However, cells from each of these strains displayed a mild elongated bud morphology (Fig. 1 B), indicating that cdc42Δ32H has a slight dominant effect on bud morphology in addition to a recessive defect in septin organization that is detectable at low gene dosage. For the purposes of this report, we have concentrated on the septin defect.

The septin localization defects in hemizygous cdc42Δ32H/cdc42Δ and cdc42Δ32H/cdc42Δ strains appeared quite distinct. cdc42Δ32H/cdc42Δ cells frequently showed faint or even undetectable septin staining at the neck and prominent mislocalized septin rings within the bud (Fig. 1 A and Table I). In contrast, septin staining in cdc42Δ32H/cdc42Δ cells was generally localized to the neck but with aberrant patterns of staining including septin “bars” running along the mother bud axis (similar to those observed in gin4 mutants [Longtime et al., 1998]) or irregular septin zones (Fig. 1 A and Table I). Occasional cdc42Δ32H/cdc42Δ cells displayed additional septin-containing patches in the bud, particularly when cells were grown in minimal medium. Septin staining in heterozygous cdc42Δ32H/K04E/cdc42Δ32H cells was indistinguishable from that of wild-type Cdc42p as judged by immunofluorescence microscopy (unpublished data). Thus, these two cdc42 alleles displayed distinct recessive defects in septin localization that were ameliorated upon raising the gene dosage.

Initial assembly of the septin ring in cdc42 mutants

Examination of the septin rings that initially formed in unbudded cells revealed further differences between the cdc42Δ32H/cdc42Δ and cdc42Δ32H/cdc42Δ mutants. The cdc42Δ32H/cdc42Δ septin rings were quite similar to those in wild-type cells, although occasional cells displayed fainter or wider rings (Fig. 1 A). However, the initial rings formed in cdc42Δ32H/cdc42Δ cells had strikingly larger diameters than those in wild-type cells (Fig. 1 A). Interestingly, cdc42Δ32H/cdc42Δ cells subsequently developed unusually broad necks, whereas cdc42Δ32H/cdc42Δ cells generally had narrow and sometimes “stretched”-appearing necks (Fig. 1 B). Thus, the diameter of the initial septin ring was not correlated with the width of the subsequent neck in these mutants.

Cdc42p polarizes to a tight patch at the presumptive bud site (Ziman et al., 1993), and it is presumably this localized pool of Cdc42p that triggers the assembly of the concentric septin ring. Thus, one possible explanation for the increased diameter of the septin ring in cdc42Δ32H/cdc42Δ cells would be that Cdc42pV32H polarizes to a larger diameter patch, which assembles septins correspondingly farther away from the center of the patch. However, we found that the localization of Cdc42pV32H was indistinguishable from that of wild-type Cdc42p as judged by immunofluorescence microscopy (Fig. 1 C). This result suggests that the defect in the initial assembly of the septin ring arises from impaired function, rather than impaired localization, of Cdc42pV32H.

In contrast to cdc42Δ32H/cdc42Δ cells, the apparently normal initial rings in most cdc42Δ32H/K04E/cdc42Δ cells suggest that cdc42Δ32H/K04E/cdc42Δ mutants are primarily defective in maintaining septins at the neck during bud growth and not in assembling a septin ring before bud formation. This surprising observation raised the possibility that Cdc42p may act after bud emergence to stabilize the septin collar at the neck, as well as promoting initial septin ring assembly.

Cdc42p-independent maintenance of septin organization

To ask directly whether Cdc42p and its exchange factor, Cdc24p, were required to maintain septin localization after bud emergence, we inactivated conditional cdc24 and cdc42 alleles in budded cells. The Ts alleles of these genes analyzed to date were identified based on their homogeneous unbudded terminal phenotype, which may have biased the screen in favor of alleles that were still capable of contributing to septin maintenance in budded cells even at restrictive temperature. To avoid this problem, we used two new Ts alleles (cdc42-6 and cdc24-27) that were selected only for lethality rather than for any particular terminal phenotype (see Materials and methods). To provide as much time as possible for the inactivation of mutant gene products after bud forma-

Figure 2. Cdc42p is required for maintenance of actin polarization but not septin organization in budded cells. Strains RSY136 (GAL1p-SWE1), DLY5079 (cdc42-6 GAL1p-SWE1), DLY4849 (cdc42-17 GAL1p-SWE1), or DLY5078 (cdc24-4 GAL1p-SWE1) were grown to exponential phase in sucrose-containing medium at 24°C, galactose was added to 2% to induce Swe1p expression, and after 2 h cells were shifted to 37°C to inactivate Cdc24p/Cdc42p. After a further 4 h, cells were processed to visualize septin (top row) or actin (bottom row) distribution. Bar, 10 μm.
tion, we arrested mutant cells in G2 by overexpressing Swe1p at the permissive temperature. We then shifted the cells to 37°C and maintained the G2 arrest for 4 h to allow ample time for Cdc42p inactivation. In both cdc42 and cdc24 mutants, septins remained localized to the neck with no observable diminishment in the intensity of staining (Fig. 2A). In contrast, the mutants failed to maintain a polarized actin distribution under these conditions (Fig. 2B), indicating that long-term maintenance of actin polarity does in fact require continued Cdc42p function. These results suggest that Cdc42p was effectively inactivated after shift to the restrictive temperature but that this did not affect maintenance of septins at the neck. Thus, Cdc42p appears not to be required for maintenance of septin organization.

These results appear to rule out the possibility that the mislocalization of the septin rings in budded cdc42V36T,K94E/cdc42Y32H/V36T,K94E mutants is due to a defect in a “septin maintenance” function of Cdc42p. Rather, it appears that subtle defects in the initial assembly of the septin ring in cdc42V36T,K94E/cdc42Δ mutants is due to a defect in a “septin maintenance” function of Cdc42p. Rather, it appears that subtle defects in the initial assembly of the septin ring in cdc42V36T,K94E/cdc42Δ mutants cause septins to disassemble gradually from the neck after a bud has formed, subsequently reassembling at ectopic locations (this phenotype will be described in more detail elsewhere). In summary, the two septin-specific cdc42 alleles that we examined display distinct defects in the initial assembly of the septin ring. The cdc42V36T,K94E/cdc42Δ mutant forms an unstable ring, whereas the cdc42V32H/cdc42Δ mutant forms a more stable but much larger diameter septin ring.

Molecular pathways underlying the cdc42 septin defects

One way to identify the molecular pathways that are impaired in the septin-defective cdc42 mutants is to identify suppressors that restore septin organization. We began by asking whether overexpression of known Cdc42p effectors could rectify the defect in our mutants. As reported previously for haploid strains (Gladfelter et al., 2001a), overexpression of the Cdc42p-activated kinase Cla4p or the scaffold protein Bem1p but not of Ste20p or other effectors suppressed the septin misorganization phenotype of hemizygous cdc42V36T,K94E/cdc42Δ mutants, and we observed a similar pattern for cdc42V32H/cdc42Δ mutants (Fig. 3). Cla4p and Bem1p participate in a feedback loop that regulates the phosphorylation state of Cdc24p (Gulli et al., 2000; Bose et al., 2001), and moderate overexpression of these proteins suppresses many cdc42 mutants with varied defects (Gladfelter et al., 2001). Thus, suppression in these cases may reflect a global enhancement of Cdc42p function rather than a specific compensation of impaired septin organization pathways.
Like effectors, GTPase-activating proteins (GAPs) recognize specifically the GTP-bound form of small G proteins. There are three proteins currently thought to act as Cdc42p-directed GAPs in yeast: Rga1p, Rga2p, and Bem3p (Zheng et al., 1993; Stevenson et al., 1995). Strikingly, we found that overexpression of Rga1p (though not of Rga2p or Bem3p) effectively suppressed the septin defects of both cdc42V36T,K94E/cdc42Δ and cdc42V332H/cdc42Δ mutants (Fig. 3; see Fig. 5 C).

**GTP hydrolysis by Cdc42pV36T,K94E and Cdc42pV332H**

The finding that overexpression of a Cdc42p GAP could ameliorate the septin defects of cdc42V36T,K94E/cdc42Δ and cdc42V332H/cdc42Δ mutants raised the possibility that the septin misorganization arose due to a defect in the ability of Cdc42pV36T,K94E and Cdc42pV332H to hydrolyze GTP. To test directly whether such a defect existed, mutant and wild-type versions of Cdc42p were produced as recombinant GST fusion proteins in E. coli, purified using glutathione-sepharose, and loaded with [γ-32P]GTP. GTP hydrolysis was followed by monitoring loss of the 32P associated with Cdc42p using a filtration assay. As shown in Fig. 4 A, GTP hydrolysis by Cdc42pV36T,K94E was ~40% slower than GTP hydrolysis by wild-type Cdc42p, whereas GTP hydrolysis by Cdc42pV332H was indistinguishable from wild-type.

To investigate whether GTP hydrolysis by the mutants was appropriately stimulated by GAPs, we produced recombinant Rga1p GAP homology domain (comprising the COOH-terminal 224 residues) as a GST fusion protein in E. coli. After purification on glutathione-sepharose beads,
this domain effectively stimulated GTP hydrolysis by Cdc42p and by Cdc42pV36T,K94E in vitro (Fig. 4 B). However, Cdc42pV36T,K94E was almost completely insensitive to the Rga1p GAP domain (Fig. 4 B). Thus, both of these mutants affect Cdc42p GTP hydrolysis but in different ways:

Cdc42pV36T,K94E displays a slower intrinsic GTPase activity that is still responsive to the Rga1p GAP, whereas Cdc42pV36T intrinsic GTPase activity is normal but cannot be greatly stimulated by the Rga1p GAP.

It is curious that overexpression of Rga1p was able to suppress the septin defects of the cdc42Y32H/cdc42Δ mutant despite the fact that the Rga1p GAP domain was unable to stimulate GTP hydrolysis by Cdc42pV36T in vitro. It is possible that full-length Rga1p retains significant GAP activity for Cdc42pV36T in vivo and that suppression occurs by enhancing this residual activity. However, these findings could also indicate that Rga1p can somehow improve septin organization in vivo without acting as a GAP for Cdc42p.

**Suppression of cdc42 septin defects by Rga1p requires a functional GAP domain**

To address whether the effect of Rga1p on septin organization depends on its GAP activity, we generated a point mutant form of Rga1p that lacked GAP activity. Previous studies identified a lysine residue conserved among Rho-GAPs (Lys 872 in Rga1p) that is essential for activation of the Rho A GTPase by mammalian Rho-GAP (Li et al., 1997). Mutation of Lys 872 to Ala in the GST-Rga1p-GAP domain construct similarly eliminated GAP activity (Fig. 5 A). In addition, interaction of the Rga1pK872A GAP domain with Cdc42p was greatly diminished and no longer sensitive to GTP/GDP status (Fig. 5 B). When this mutation was introduced into full-length RGA1, overexpression of rga1K872A no longer suppressed the septin defects of either of our cdc42 mutants (Fig. 5 C), suggesting that Rga1p GAP function, and by extension Cdc42p GTP hydrolysis, is in fact important for septin organization.

**Dominant effect of GTPase-defective Cdc42p on septin organization**

The results described above establish a correlation between the effects of certain cdc42 alleles on septin organization and alterations in GTP hydrolysis by the encoded mutant proteins. However, it remained possible that the correlation was entirely coincidental and that the septin organization defects of these mutants were unrelated to their altered GTP hydrolysis. To test whether preventing Cdc42p GTP hydrolysis itself would affect septin organization, we turned to the CDC42Q61L allele that was generated by homology to the corresponding oncogenic allele of ras and has been characterized extensively as showing an essentially complete defect in GTP hydrolysis. Previous studies showed that moderate or high level expression of CDC42Q61L in yeast is lethal (Ziman et al., 1991), whereas low level expression can be tolerated (Mosch et al., 1996). We constructed strains expressing CDC42Q61L from a crippled version of the GAL1 promoter in addition to wild-type CDC42 expressed from its own promoter. These cells were able to proliferate well on galactose-containing medium, and the level of Cdc42pQ61L expression was roughly comparable to that of endogenous wild-type Cdc42p (Fig. 6 B). However, there were striking defects in septin organization in these cells: unbudded cells displayed large and faint initial rings similar to those observed in cdc42V36T/cdc42Δ mutants, and budded cells displayed mis-
organized and diffuse septin staining at the neck (Fig. 6A and Table I). In addition, cells expressing Cdc42pK186R frequently had broad necks similar to those observed in cdc42V36T,K94E/cdc42Δ mutants. Thus, preventing GTP hydrolysis by Cdc42p leads to dominant effects on septin localization and neck morphology.

Effect of increasing the intrinsic GTPase activity of Cdc42p

Yeast Cdc42p has an unusually slow intrinsic GTPase activity compared with several homologues from other organisms. This appears to be due to an “arginine finger” motif present in fly or mammalian Cdc42p but absent from yeast Cdc42p that accelerates GTP hydrolysis. Mutation of Lys186 to Arg introduces a similar arginine finger into yeast Cdc42p and correspondingly increases its intrinsic rate of GTP hydrolysis (Zhang et al., 1999). We found that cdc42K186R strains displayed dramatic defects in septin organization (Fig. 6C and Table I), suggesting that overly rapid GTP hydrolysis also impairs septin assembly.

A role for Cdc42p GAPs in septin organization

The association between defects in Cdc42p GTP hydrolysis and defects in septin organization suggests that proper assembly of the septin ring requires proper regulation of GTP hydrolysis. If this is true, then mutational inactivation of Cdc42p GAPs Rga1p, Rga2p, and Bem3p might be expected to perturb septin organization also. Although none of the single mutants displayed striking septin defects, double mutants (particularly rga1Δ rga2Δ) were somewhat defective, and the triple mutants showed a strong defect comparable to that of cdc42V36T,K94E/cdc42Δ mutants (Fig. 7), indicating that these proteins share a role in septin organization.

Discussion

A connection between septin organization and GTP hydrolysis by Cdc42p

We have described two cdc42 mutants that display defects in septin organization, assembling unstable or large diameter septin rings. Unexpectedly, we found that overexpression of the Cdc42p GAP, Rga1p, could largely suppress the septin defects. Biochemical characterization of the mutant proteins revealed that Cdc42pV36T,K94E had a slower intrinsic GTPase activity than wild-type Cdc42p, although the GTPase could still be stimulated by the Rga1p GAP domain in vitro. In contrast, Cdc42pY32H intrinsic GTPase activity was similar to that of wild-type Cdc42p but could no longer be effectively stimulated by the Rga1p GAP domain. Below, we discuss three possible hypotheses to explain the observed correlation between defects in septin ring assembly and defects in GTP hydrolysis by Cdc42p.

First, the correlations between Cdc42p GTP hydrolysis and septin organization may not reflect a functional link between the two. Suppression of the septin defects by overexpression of the GAP might reflect a role for Rga1p as a classical effector of Cdc42p for septin organization independent of its GAP activity, and the GTP hydrolysis defects may be unrelated to the septin defects exhibited by the cdc42 mutants. Several findings argue against this “pure coincidence” interpretation. First, a point mutation in the Rga1p GAP domain that abrogated GAP activity also eliminated suppression of the septin defects. Second, combined deletion of the three genes thought to encode Cdc42p GAPs (RGA1, RGA2, and BEM3) caused severe defects in septin organization even in cells containing wild-type Cdc42p. Deletion of only two of these GAPs produced a much milder septin phenotype, indicating that all three GAPs contribute to septin organization despite the fact that there is little or no homology between Bem3p and Rga1p/Rga2p outside of the catalytic domain. These findings suggest a general requirement for GAP activity to promote proper septin assembly. Third, Cdc42p variants that were shown previously to either prevent GTP hydrolysis (Cdc42pK186R) or to accelerate GTP hydrolysis (Cdc42pY32H) were also found to impair septin organization. In aggregate, these results provide compelling evidence that correct regulation of Cdc42p GTP hydrolysis is important for septin organization.

Second, a defect in Cdc42p GTP hydrolysis (or in Cdc42p-directed GAP activity) may cause accumulation of excess GTP-Cdc42p, leading to unphysiological hyperactivation of some effector(s), which then interferes with normal septin assembly. This hypothesis makes the strong prediction that the septin defects caused by the GTPase-defective
The detailed organization of septins within the septin ring is unknown, although the documented ability of septins to polymerize makes it likely that the ring is comprised of septin filaments (Byers and Goetsch, 1976; Byers, 1981; Frazier et al., 1998). Fig. 8 presents a speculative model illustrating how cycles of GTP loading and GTP hydrolysis by Cdc42p may contribute to septin ring assembly. This model is based on the paradigm established for the role of another small G protein, the translation elongation factor EF-Tu (EF-1α in eukaryotes), in protein synthesis (Thompson, 1988).

EF-Tu acts as part of the “ternary complex” in protein synthesis. After GTP loading, EF-Tu binds to aminoacyl tRNA, allowing docking of the complex to the ribosome. However, incorporation of the amino acid into the nascent protein is prevented until GTP hydrolysis by EF-Tu releases the G protein from the ribosome. GTP hydrolysis in this instance serves to introduce a delay between aminoacyl tRNA docking and peptide chain elongation, which allows for a “kinetic proofreading” mechanism. If the codon–anticodon pairing for the particular tRNA is incorrect, then the EF-Tu–tRNA complex will usually dissociate from the ribosome before GTP hydrolysis occurs, whereas correct pairing stabilizes the tRNA–ribosome interaction and provides time for GTP hydrolysis by EF-Tu, allowing accurate peptide chain elongation. Altering the rate of GTP hydrolysis by EF-Tu can result in slower translation (if hydrolysis is delayed) or inaccurate translation (if hydrolysis is accelerated).
Table II. Yeast strains used in this studya

| Strain               | Relevant genotype                                      | Source        |
|----------------------|-------------------------------------------------------|---------------|
| CCY3-3B              | a cdc42::TRP1 ura3::cdc42V36L::URA3                  | Zhang et al., 1999 |
| DLY1                 | a bar1                                                | Sia et al., 1996 |
| DLY5                 | a/a                                                   | Lew and Reed, 1993 |
| DLY2723              | a rga1::TRP1 rga2::URA3 bem3::LEU2                    | This study    |
| DLY3341              | a rga1::TRP1 bem3::LEU2                               | This study    |
| DLY3344              | a rga1::TRP1                                         | This study    |
| DLY3346              | a bem3::LEU2                                         | This study    |
| DLY3347              | a rga1::TRP1 rga2::URA3                               | This study    |
| DLY3353              | a rga2::URA3                                          | This study    |
| DLY3361              | a rga2::URA3 bem3::LEU2                               | This study    |
| DLY4223              | a/a cdc42V36L::CDC42::LEU2::GAL1p-CDC42               | This study    |
| DLY4224              | a/a his2::cdc42V36L::CDC42::LEU2::his2 cdc42::URA3    | This study    |
| DLY4831              | a his2::cdc42V36L::CDC42::LEU2::GAL1p-CDC42           | This study    |
| DLY4849              | a cdc42-17 GAL1p-SWE1myc::URA3                       | This study    |
| DLY5078              | a cdc42-4 GAL1p-SWE1myc::URA3                        | This study    |
| DLY5079              | a cdc42-6 GAL1p-SWE1myc::URA3                        | This study    |
| DLY5080              | a/a cdc42V36L::HE4CDC42:LEU2                         | This study    |
| DLY5082              | a cdc42V36L::HE4CDC42                                 | This study    |
| DLY5237              | a his2::EG43-CD42::HIS2                               | This study    |
| DLY5240              | a his2::EG43-CD42V36L::HIS2                          | This study    |
| DLY5461              | a/a CDC42/cdc42::LEU2::GAL1p-CDC42                   | This study    |
| DLY5470              | a/a his2::cdc42V36L::HIS2::his2::cdc42V36L::HIS2 cdc42::LEU2::GAL1p-CDC42::LEU2::GAL1p-CDC42 | This study |
| DLY5471              | a/a his2::cdc42V36L::HIS2::his2 cdc42::LEU2::GAL1p-CDC42::LEU2::GAL1p-CDC42 | This study |
| RSY136               | a bar1GAL1p-SWE1myc::URA3                            | Sia et al., 1998 |

aAll strains except CCY3-3B are in the BF264-15Du (Richardson et al., 1989) background (ade1 his2 leu2-3,112 trp1-1). CCY3-3B is in the Y604 background (ade2-101 his3Δ200 lys2-801 trp1Δ1 ura3-52).

By analogy to EF-Tu, we speculate that GTP-Cdc42p interacts with septin filaments as part of a “septin assembly complex,” which allows docking of the complex to the assembling septin ring (Fig. 8). However, incorporation of the filament into the nascent ring cannot occur until GTP hydrolysis by Cdc42p releases the Cdc42p from the ring (Fig. 8). Conceivably, GTP hydrolysis by Cdc42p may provide a fidelity mechanism for septin assembly, occurring only when each arriving septin filament is properly positioned within the larger structure. Impairment of this mechanism could lead to incorporation of misoriented or improperly docked filaments into the ring, possibly generating the sorts of unstable or large diameter rings observed in the cdc42 mutants analyzed here.

One aspect of our model that differs from the EF-Tu paradigm is the participation of Cdc42p GTPases in septin ring assembly. We suggest that the GTPases help to couple proper docking of the incoming septin complex to GTP hydrolysis by Cdc42p. The GTPases may form part of the hypothesized “septin assembly complex,” or they may interact with the properly docked complexes to promote hydrolysis (Fig. 8).

A single GTPase can act as a signaling switch or as an assembly factor

GTPases are generally thought to act either as ras-like “signaling switches” or as EF-Tu-like “assembly factors.” Cdc42p exhibits well-characterized “switch”-like behavior with respect to several effectors, including the PAK family kinases (Bagrodia and Cerione, 1999; Tu and Wigler, 1999) and WASP (Bi and Zigmond, 1999), leading to the expectation that its mode of action will be similarly switch-like for all of its functions. However, there is no logical or structural argument that we are aware of that prevents a single G protein from acting as either a “signaling switch” or an “assembly factor,” depending on the particular target proteins with which it interacts to carry out various functions. We suggest that Cdc42p can operate in both ways, recruiting and activating PAKs and other effectors to promote actin polarization and acting as an assembly factor in conjunction with the GTPases to promote assembly of the septin ring. It remains to be determined whether septin ring assembly also employs signaling-type effector pathways and whether other Cdc42p functions also involve assembly factor-like roles.

Experimental approaches to examine GTPase function and to identify presumed effectors often rely on the assumption that the GTPase acts exclusively as a switch so that GTPase-defective mutants can faithfully mimic “activation” of the GTPase. Our results suggest that it would be profitable to expand such studies to look for functions in which the GTPase acts as a signaling switch or an assembly factor-like roles.

Materials and methods

Strains, plasmids, and PCR manipulations

Standard media and methods were used for plasmid and yeast manipulations (Guthrie and Fink, 1991; Ausubel et al., 1995). S. cerevisiae strains are listed in Table II, plasmids are listed in Table III, and primers are listed in Table IV.

The generation of cdc42::GAL1p-CDC42::LEU2 (Gladfelter et al., 2001a), GAL1p-SWE1myc::URA3 (McMillan et al., 1998), rga1::TRP1, and bem3::LEU2 (Bi et al., 2000) alleles was described previously. The
The rga2::URA3 allele was constructed using the PCR method (Baudin et al., 1993) with plasmid pRS306 (URA3) as template and the primers listed in Table IV. Disruption was confirmed by PCR.

The cdc42

V36T allele was described previously (Gladfelter et al., 2001a), but in the course of checking the hemizygous strains generated for this work we discovered that this allele contained a second mutation that was missed in the previous work (changing lysine 94 to glutamate), and in all subsequent work this mutant is identified as cdc42

V36T,K94E. This does not account for the difference between hemizygous and homozygous diploid strains because we found that the second mutation was present in the parental plasmid from which all of the strains were derived. In addition, the second mutation is unlikely to contribute significantly to the septin phenotype because the cdc42

V36A allele showed a similar (though less penetrant) phenotype (Gladfelter et al., 2001a), and we have confirmed that this allele did not contain any other mutations.

cdc42

Y32H was generated as described for other cdc42 effector loop mutations and integrated at HIS2 in DLY3067 (Gladfelter et al., 2001a). Integrants were screened by Western blot to confirm that Cdc42p Y32H was expressed at levels comparable to endogenous wild-type Cdc42p (DLY4831). DLY4831 was then crossed to an isogenic wild-type strain.

### Table III. Plasmids used in this study

| Plasmid     | Vector         | Insert                          | Source                  |
|-------------|----------------|---------------------------------|-------------------------|
| pDLB659     | YIpEG43-HIS2   | CDC42Q61L                       | This study              |
| pDLB664     | YIpEG43-HIS2   | CDC42                           | This study              |
| pDLB678     | 2 μm URA3      | BEM1                            | Bender and Pringle, 1991|
| pDLB722     | 2 μm URA3      | CLA4                            | Erei Bi               |
| pDLB723     | 2 μm URA3      | STE20                           | Erei Bi               |
| pDLB1030    | pUNI-10        | RGA1 GAP                        | This study              |
| pDLB1131    | pHB2-GST       | RGA1 GAP                        | This study              |
| pDLB1537    | 2 μm URA3      | RGA1                            | This study              |
| pDLB1539    | pUNI-10        | rga1P872A GAP                   | This study              |
| pDLB1552    | pHB2-GST       | rga1P872A GAP                   | This study              |
| pDLB1580    | 2 μm URA3      | rga1P872A GAP                   | This study              |
| pDLB1981    | 2 μm URA3      | RGA2                            | This study              |
| pDLB2083    | pGEX-KG        | CDC42Q61L                       | This study              |
| pDLB2088    | pGEX-KG        | CDC42Q61L                       | This study              |
| pDLB2091    | pGEX-KG        | CDC2                            | This study              |
| pDLB2119    | pHBl-myc3      | rga1P872A GAP                   | This study              |
| pDLB2121    | pHBl-myc3      | RGA1 GAP                        | This study              |
| pDLB2213    | pGEX-KG        | cdc42Y32H                       | This study              |
| pDLB2221    | pGEX-KG        | cdc42Y32H                       | This study              |
| pGEX-KG     |                | GST                             | Guan and Dixon, 1991   |
| pMOSB229    | 2 μm URA3      | GIC1                            | Matthias Peterb        |
| pPB547      | 2 μm TRP1      | BEM3                            | Alan Bendersc          |
| pRS314      | CEN TRP1       |                                  | Sikorski and Hieter, 1989|
| pRS316      | CEN URA3       |                                  | Sikorski and Hieter, 1989|
| pRS426      |                |                                  | Christianson et al., 1992|
| pRS424      | 2 μm TRP1      |                                  | Christianson et al., 1992|
| YEp24       | 2 μm URA3      |                                  | New England Biolabs, Inc.| |
| YEpplac195  | 2 μm URA3      |                                  | Gietz and Sugino, 1988 |

*aUniversity of Pennsylvania, Philadelphia, PA.

*bSwiss Institute for Experimental Cancer Research, Boveresses, Switzerland.

*cIndiana University, Bloomington, IN.

### Table IV. Oligonucleotide primers used in this study

| Primer     | Sequence (5’ to 3’) | Comments      |
|------------|---------------------|---------------|
| RGA1-A     | GTTGTGAAATATTGCTGAC | Mscl; K872A mutation |
| RGA1-M1    | GTTACTGTGGTGTTGCGCCAGATCTTAAGAAGGC | RGA1-M1 reverse complement |
| RGA1-M2    | GTTTCTTAAGTATCTGGCCAAACACCAGTAAC | RGA1 reverse complement |
| RGA1-UNI1  | GAATTCTCATAATGGGAGCACTGGTTGCTCAAGTCTC | NdeI; begin GAP domain |
| RGA1-UNI2  | CCCAGCTCTCTAGTCAAGATATTTATGGGATTTTGCCTC | SacI; end GAP domain |
| RGA2-A     | CCAGAGTGCAGACATCACATTAAATCTTGC | SacI; –565 |
| RGA2-B     | CCATTGAACACTTGTTACGGGTCGACTGACGATCTTGC | Sall; –140/+135 overlap |
| RGA2-C     | GCAATGAGATGAGCTACGAGAAGGCTCAAAGTCTCAATGGCC | RGA2-B reverse complement |
| RGA2-D     | CCGCTCGAGAGATTCGAAAGGGCTCAAAGG | XhoI; +650 |
| RGA2-1     | GAAATATAACGTAGCATCTCAAGAGCAAGAGGAGGAGGAGGATGAC | Y32H mutation |
| cdc12      | CATGTTCAACAGTTCGCG | PvuII, distal primer for Y32H mutagenesis |
| cdc13      | GTCAGCTGGAAATTTTCGCG | PvuII, distal primer for Y32H mutagenesis |

*aRestriction sites are underlined in sequence. Negative numbers refer to sequences upstream of the start codon, and positive numbers refer to sequences downstream of the stop codon for the relevant gene. Primers RGA2-1 and RGA2-2 were used to disrupt RGA2.*
Production of recombinant proteins and GAP assays

Bacterial strains, growth, and lysis conditions were as described (Bose et al., 2001) except that bacteria were shifted to 18°C before induction with IPTG. GST-tagged proteins were isolated by passing the bacterial lysate over a 0.25-mL column of glutathione beads (50% slurry of GST-Sepharose 4B [Amersham Pharmacia Biotech] equilibrated with wash buffer) four times at 4°C. Bound proteins were washed with 30 ml wash buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl₂, 1 mM DTT, and in the case of GST-Cdc42p also 5 μM GDP) and eluted with 10 mM glutathione in wash buffer at 4°C. Yield of recombinant protein was estimated by Coomassie staining after separation by SDS-PAGE.

To load Cdc42p with GTP, it was incubated for 15 min at 20°C with 100 000 cpm/mol [γ-32P]GTP or [α-32P]GTP (New England Nuclear) in binding buffer (25 mM Tris-HCl, pH 7.5, 200 mM [NH₄]SO₄, 5 mM MgCl₂, 2 mM DTT, 1 mM EDTA, 5 μM GTP, 1 mM phenylmethylsulphonyl fluoride, and 10 μg/ml each of peptatin, aprotinin, and leupeptin). GTP-bound Cdc42p was then diluted 10-fold into reaction buffer (as above but lacking [NH₄]SO₄ and supplemented with 1 mM GTP) and incubated at 20°C. The amount of bound GTP remaining in duplicate samples containing 20 pmol GST-Cdc42p was measured by the nitrocellulose filtration method.

We thank Alan Bender, Erife Bi, Mark Longtine, and Doug Johnson for plasmids and strains. We also thank Erife Bi, Mark Longtine, and John Pringle for communicating unpublished results and for many stimulating interactions. Particular thanks to Chandra Theesfeld and members of the Lew lab for many productive discussions and ideas. This work was supported by National Institutes of Health grant GM62300 and American Cancer Society grant RPG-98-046-CCG to D.J. Lew.

Submitted: 20 September 2001
Revised: 19 November 2001
Accepted: 11 December 2001

References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York. 1600 pp.
Bagrodia, S., and R.A. Cerrone. 1999. Pak to the future. Trends Cell Biol. 9:350–355.
Baudin, A., O. Ozier-Kalogeropoulos, A. Desouez, F. Lacroix, and C. Callin. 1993. A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. Nucleic Acids Res. 21:3329–3330.
Bender, A.J., and J.R. Pringle. 1991. Use of a screen for synthetic lethal and multicopy suppressors mutants to identify two new genes involved in morphogenesis in Saccharomyces cerevisiae. Mol. Cell. Biol. 11:1295–1305.
Bi, E., and S.H. Zigmond. 1999. Actin polymerization: where the WASP stings. Curr. Biol. 9:R160–R163.
Bi, E., J.B. Chiavetta, H. Chen, G.C. Chen, C.S. Chan, and J.R. Pringle. 2000. Identification of novel, evolutionarily conserved Cdc42p-interacting proteins and of redundant pathways linking Cdc24p and Cdc42p to actin polarization in yeast. Mol. Biol. Cell. 11:773–783.
Bose, L., J. Irazoqui, J.J. Moskow, T.R. Zyla, and D.J. Lew. 2001. Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cad2p, and the effector Cla4p required for cell cycle regulated phosphorylation of Cad2p. J. Biol. Chem. 276:7176–7186.
Byers, B., 1981. Cytology of the yeast life cycle. In The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, J.N. Straherath, E.W. Jones, and J.R. Broach, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 59–96.
Byers, B., and L. Goetsch. 1976. A highly ordered ring of membrane-associated filaments in budding yeast. J. Cell Biol. 69:717–721.
Christianson, T.W., R.S. Sikorski, M. Dante, J.H. Shero, and P. Hieter. 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene. 110:119–122.
Frazier, J.A., M.L. Wong, M.S. Longtine, J.R. Pringle, M. Mann, T.J. Mitchison, and C. Field. 1998. Purification of polymerized yeast septins: evidence that organized filament arrays may not be required for septin function. J. Cell Biol. 143:737–749.
Gietz, R.D., and A. Sugino. 1988. New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six base pair restriction sites. Gene. 74:527–534.

Microscopy

To deplete wild-type Cdc42p expressed from the GAL1 promoter, cells were grown in dextrose-containing medium for at least 24 h. All staining and microscopic analysis was performed as described (Gladfelter et al., 2001a).
