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

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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

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*Abbreviations used in this paper: GAP, GTPase-activating protein; Ts, temperature sensitive.

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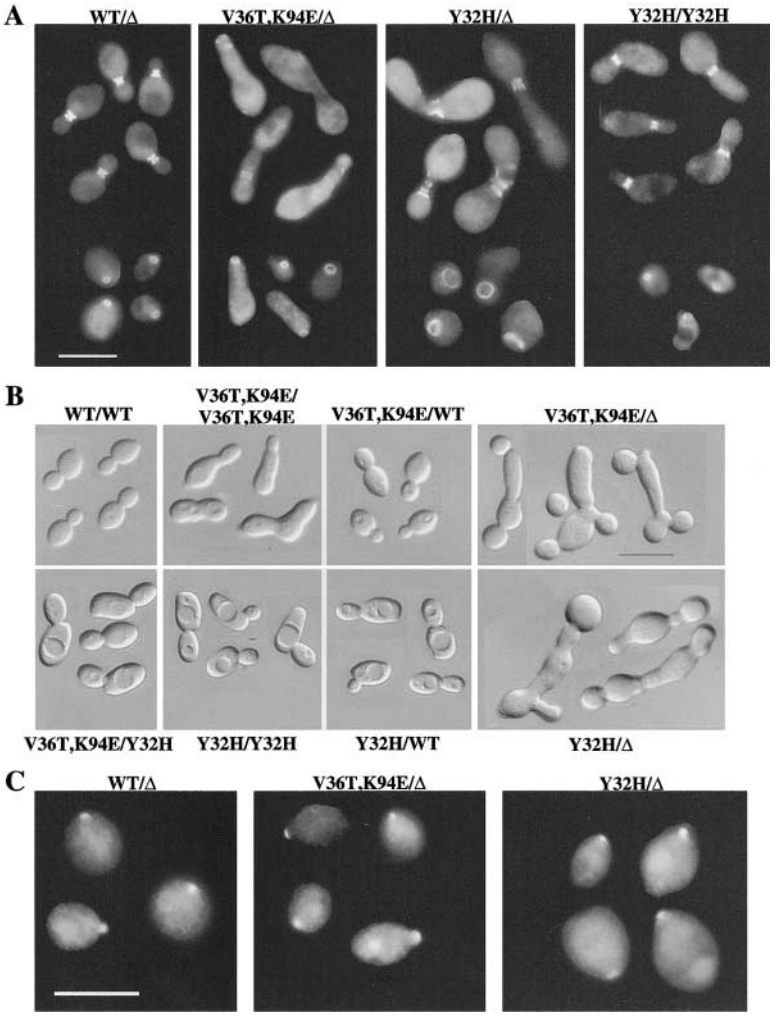

Figure 1. **Septin defects in *cdc42* mutants.** (A) Strains DLY5461 (*CDC42*/*GAL1p-CDC42*), DLY4223 (*cdc42*^{V36T,K94E}/*GAL1p-CDC42*), DLY4224 (*cdc42*^{Y32H}/*GAL1p-CDC42*), and DLY5470 (*cdc42*^{Y32H}/*cdc42*^{Y32H}) were processed to visualize septins. Control Western blots confirmed that hemizygous strains contained approximately twofold less Cdc42p than homozygous strains in all cases. (B) Strains DLY5 (*CDC42*/*CDC42*), DLY5080 (*cdc42*^{V36T,K94E}/*cdc42*^{V36T,K94E}), DLY5082 (*cdc42*^{V36T,K94E}/*CDC42*), DLY4223 (*cdc42*^{V36T,K94E}/*GAL1p-CDC42*), DLY5471 (*cdc42*^{V36T,K94E}/*cdc42*^{Y32H}), DLY5470 (*cdc42*^{Y32H}/*cdc42*^{Y32H}), DLY5461 (*cdc42*^{Y32H}/*CDC42*), and DLY4224 (*cdc42*^{Y32H}/*GAL1p-CDC42*) were visualized by DIC microscopy to evaluate cell morphology. (C) Strains DLY5461 (*CDC42*/*GAL1p-CDC42*), DLY4223 (*cdc42*^{V36T,K94E}/*GAL1p-CDC42*), and DLY4224 (*cdc42*^{Y32H}/*GAL1p-CDC42*) were processed to visualize Cdc42p. In all cases, cells were grown to exponential phase in YEPD at 30°C and documented after >30 h of growth to allow complete depletion of *GAL1p*-regulated Cdc42p. Bars, 10 μm.

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.

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*^{V36T,K94E}, that displayed relatively mild septin and cell morphology phenotypes (Gladfelter et al., 2001a). We also noted that the

Table I. **Septin defects in *cdc42* mutants**

	Septin staining in budded cells					Septin staining in unbudded cells[b]	
Strain[a]	Wild-type	Aberrant neck	In bud	Bud tip	Absent	Normal	Large
				%			
CDC42/cdc42Δ	97	3	0	0	0	89	11
cdc42^{V36T,K94E}/*cdc42*^{V36T,K94E}	12	43	25	15	5	86	14
cdc42^{V36T,K94E}/*cdc42Δ*	2	16	33	29	20	87	13
cdc42^{Y32H}/*cdc42*^{Y32H}	96	4	0	0	0	87	13
cdc42^{Y32H}/*cdc42Δ*	4	61	16	7	12	29	71
CDC42^{Q61L}	17	78	4	0	1	24	76
cdc42^{K186R}	5	23	32	23	17	39	61

[a]The strains employed were (in order): DLY5461, DLY5080, DLY4223, DLY5470, DLY4224, DLY5240, and CCY3-3B.
[b]Only unbudded cells with septin rings were scored.

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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*^{V36T,K94E}/*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, *cdc42*^{Y32H} (Fig. 1 A and Table I). In contrast to the hemizygous *cdc42*^{Y32H}/*cdc42Δ* strain, septin staining appeared completely normal in haploid *cdc42*^{Y32H} and homozygous *cdc42*^{Y32H}/*cdc42*^{Y32H} or heterozygous *cdc42*^{Y32H}/*CDC42* diploids containing this allele. However, cells from each of these strains displayed a mild elongated bud morphology (Fig. 1 B), indicating that *cdc42*^{Y32H} has a slight dominant effect on bud morphology in addition to a recessive defect in septin organization that is only detectable at low gene dosage. For the purposes of this report, we have concentrated on the septin defect.

The septin localization defects in hemizygous *cdc42*^{V36T,K94E}/*cdc42Δ* and *cdc42*^{Y32H}/*cdc42Δ* strains appeared quite distinct. *cdc42*^{V36T,K94E}/*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 *cdc42*^{Y32H}/*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 [Longtine et al., 1998]) or irregular septin zones (Fig. 1 A and Table I). Occasional *cdc42*^{Y32H}/*cdc42Δ* cells displayed additional septin-containing patches in the bud, particularly when cells were grown in minimal medium. Septin staining in heterozygous *cdc42*^{V36T,K94E}/*cdc42*^{Y32H} cells was indistinguishable from that of wild-type cells (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 *cdc42*^{V36T,K94E}/*cdc42Δ* and *cdc42*^{Y32H}/*cdc42Δ* mutants. The *cdc42*^{V36T,K94E}/*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 *cdc42*^{Y32H}/*cdc42Δ* cells had strikingly larger diameters than those in wild-type cells (Fig. 1 A). Interestingly, *cdc42*^{V36T,K94E}/*cdc42Δ* cells subsequently developed unusually broad necks, whereas *cdc42*^{Y32H}/*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.

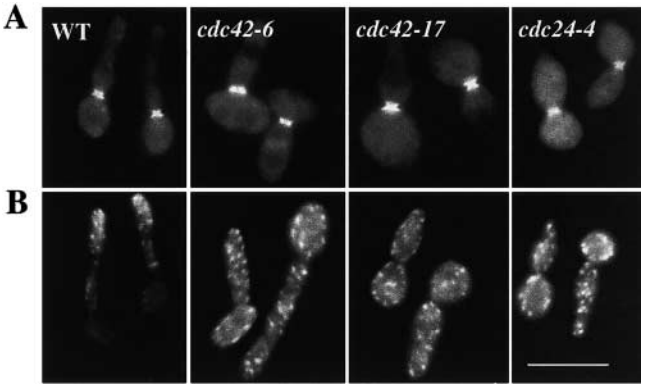

Figure 2. **Cdc42p is required for maintenance of actin polarization but not septin organization in budded cells.** Strains RSY136 (*GAL1p-SWE1*), DLY5079 (*cdc42–6 GAL1p-SWE1*), DLY4849 (*cdc42–17 GAL1p-SWE1*), or DLY5078 (*cdc24–4 GAL1p-SWE1*) were grown to exponential phase in sucrose-containing medium 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.

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 *cdc42*^{Y32H}/*cdc42Δ* cells would be that Cdc42p^{Y32H} 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 Cdc42p^{Y32H} 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 Cdc42p^{Y32H}.

In contrast to *cdc42*^{Y32H}/*cdc42Δ* cells, the apparently normal initial rings in most *cdc42*^{V36T,K94E}/*cdc42Δ* cells suggest that *cdc42*^{V36T,K94E}/*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 *cdc42-27*) 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-

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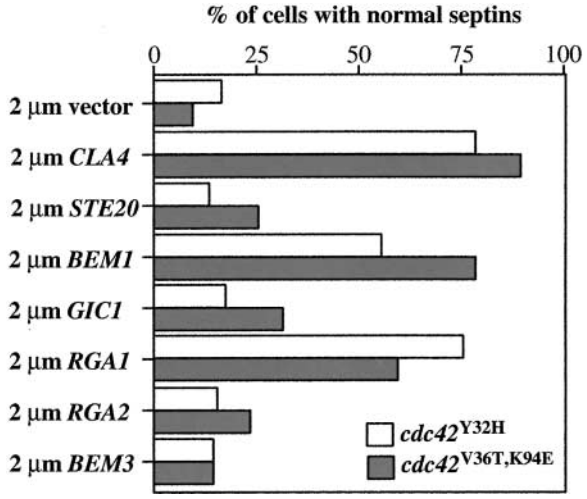

Figure 3. **Effect of overexpressing Cdc42p effectors and GAPs on the septin defects of *cdc42* mutants.** Strains DLY4223 (*cdc42*^{V36T,K94E}/*GAL1p-CDC42*) and DLY4224 (*cdc42*^{Y32H}/*GAL1p-CDC42*) were transformed with pDLB722 (*CLA4*), pDLB723 (*STE20*), pDLB678 (*BEM1*), pMOSB229 (*GIC1*), pDLB1537 (*RGA1*), pDLB1981 (*RGA2*), pPB547 (*BEM3*), or an empty vector. Transformants were grown to exponential phase in dextrose-containing medium at 30°C, and cell morphology and septin localization were documented after >30 h of growth to allow depletion of *GAL1p*-regulated Cdc42p.

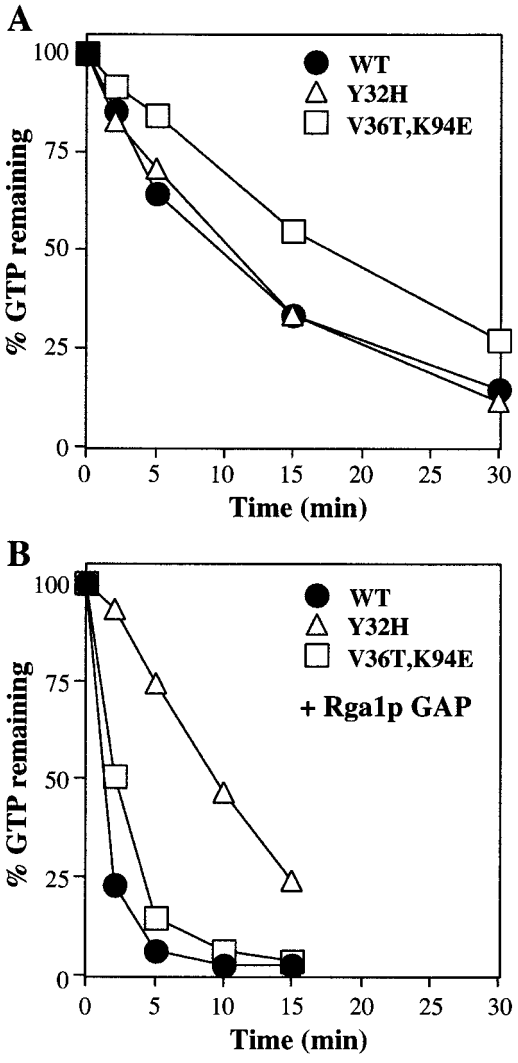

Figure 4. **GTP hydrolysis by Cdc42p^{V36T,K94E} and Cdc42p^{Y32H}.** GST-Cdc42p (●), GST-Cdc42p^{Y32H} (△), and GST-Cdc42p^{V36T,K94E} (□) were prebound to [γ-^{32}P]GTP and incubated with buffer alone (A) or with a 1:10 molar ratio of recombinant GST-Rga1p GAP domain (B), and radioactivity remaining bound to Cdc42p is plotted against time of incubation. Control experiments indicated that the Rga1p GAP domain did not stimulate release of ^{32}P from Cdc42p prebound with [α-^{32}P]GTP, indicating that Rga1p stimulates GTP hydrolysis and not release.

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. 2 A). In contrast, the mutants failed to maintain a polarized actin distribution under these conditions (Fig. 2 B), 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 *cdc42*^{V36T,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 *cdc42*^{V36T,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 *cdc42*^{V36T,K94E}/*cdc42Δ* mutant forms an unstable ring, whereas the *cdc42*^{Y32H}/*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 *cdc42*^{V36T,K94E}/*cdc42Δ* mutants, and we observed a similar pattern for *cdc42*^{Y32H}/*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., 2001a). Thus, suppression in these cases may reflect a global enhancement of Cdc42p function rather than a specific compensation of impaired septin organization pathways.

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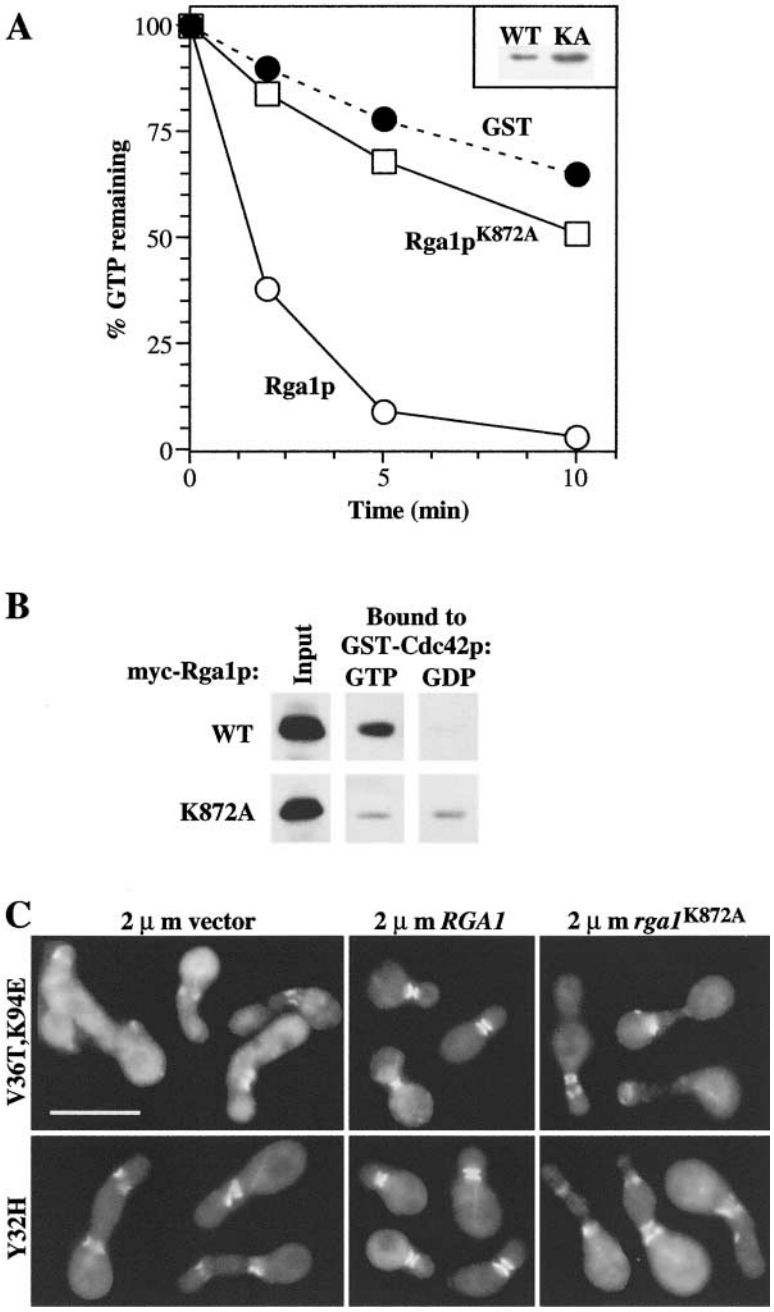

Figure 5. **Rga1p GAP activity and *cdc42* suppression.** (A) Cdc42p prebound to [γ-^{32}P]GTP was incubated with GST (●), GST-Rga1p GAP domain (○), or the same domain containing the K872A change (□), and radioactivity remaining bound to Cdc42p is plotted against time of incubation. The inset shows that somewhat less wild-type than mutant GAP domains were used in the assay. (B) Recombinant myc-tagged Rga1p or Rga1p^{K872A} GAP domains were incubated with bead-bound recombinant GST-Cdc42p^{Q61L} (GTP-bound) or GST-Cdc42p^{T17N} (GDP-bound) to assess binding. (C) Strains DLY4223 (*cdc42*^{V36T,K94E}/*GAL1p-CDC42*) and DLY4224 (*cdc42*^{Y32H}/*GAL1p-CDC42*) were transformed with YEplac195 (vector), pDLB1537 (*RGA1*), or pDLB1580 (*rga1*^{K872A}). Transformants were grown for >30 h in selective dextrose-containing medium at 30°C and processed to visualize septin distribution. Bar, 10 μm.

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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 *cdc42*^{V36T,K94E}/*cdc42Δ* and *cdc42*^{Y32H}/*cdc42Δ* mutants (Fig. 3; see Fig. 5 C).

GTP hydrolysis by Cdc42p^{V36T,K94E} and Cdc42p^{Y32H}

The finding that overproduction of a Cdc42p GAP could ameliorate the septin defects of *cdc42*^{V36T,K94E}/*cdc42Δ* and *cdc42*^{Y32H}/*cdc42Δ* mutants raised the possibility that the septin misorganization arose due to a defect in the ability of Cdc42p^{V36T,K94E} and Cdc42p^{Y32H} 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-^{32}P]GTP. GTP hydrolysis was followed by monitoring loss of the ^{32}P associated with Cdc42p using a filtration assay. As shown in Fig. 4 A, GTP hydrolysis by Cdc42p^{V36T,K94E} was ~40% slower than GTP hydrolysis by wild-type Cdc42p, whereas GTP hydrolysis by Cdc42p^{Y32H} 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,

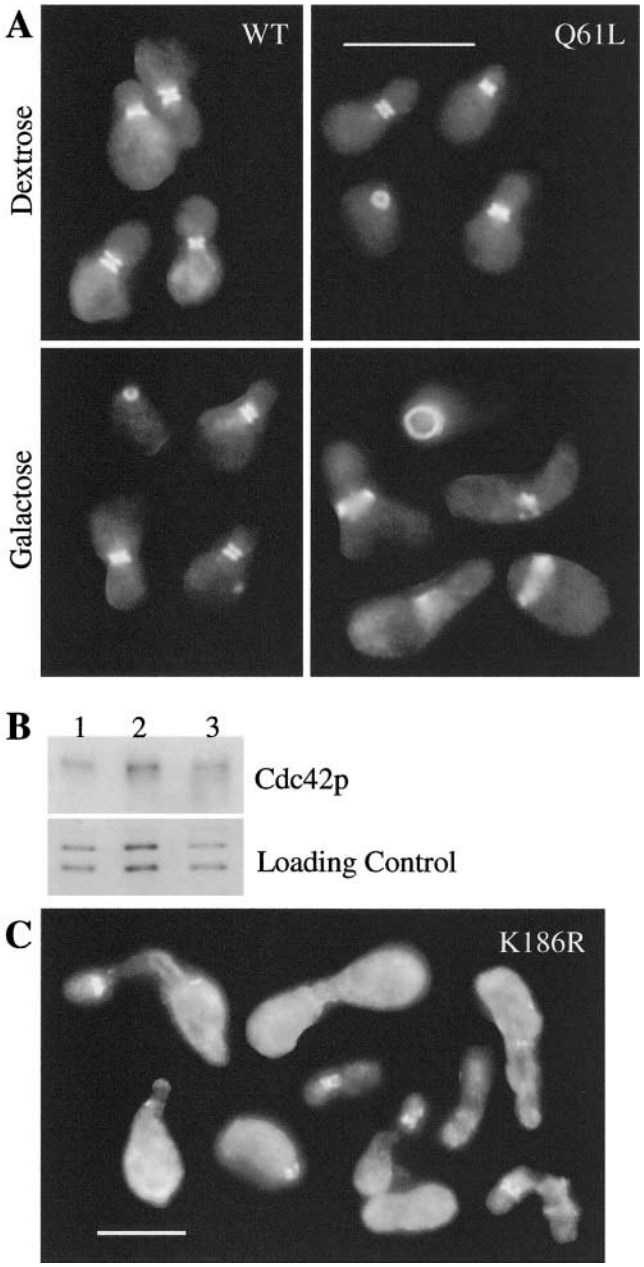

Figure 6. **Effect of blocking or accelerating Cdc42p GTP hydrolysis on septin organization.** (A) Strains DLY5237 (*CDC42 EG43p-CDC42*) and DLY5240 (*CDC42 EG43p-CDC42*^{Q61L}) were grown to exponential phase in either YEPD or YEP with galactose to induce *CDC42* or *CDC42*^{Q61L} expression and processed to visualize septins. (B) Anti-Cdc42p Western blot of strains shown in A. Lane 1 contains lysate from DLY1 (WT control), lane 2 from DLY5237, and lane 3 from DLY5240, all grown in galactose-containing medium. The same blot was probed with anti-PSTAIRE (which detects Cdc28p and Pho85p) as a control for loading. (C) Strain CCY3-3B (*cdc42*^{K186R}) was grown to exponential phase in YEPD at 30°C and then processed to visualize septins. Bars, 10 μm.

this domain effectively stimulated GTP hydrolysis by Cdc42p and by Cdc42p^{V36T,K94E} in vitro (Fig. 4 B). However, Cdc42p^{Y32H} 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: Cdc42p^{V36T,K94E} displays a slower intrinsic GTPase activity that is still responsive to the Rga1p GAP, whereas Cdc42p^{Y32H} 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 *cdc42*^{Y32H}/*cdc42Δ* mutant despite the fact that the Rga1p GAP domain was unable to stimulate GTP hydrolysis by Cdc42p^{Y32H} in vitro. It is possible that full-length Rga1p retains significant GAP activity for Cdc42p^{Y32H} 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 Rga1p^{K872A} 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 *rga1*^{K872A} 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 *CDC42*^{Q61L} 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 *CDC42*^{Q61L} in yeast is lethal (Ziman et al., 1991), whereas low level expression can be tolerated (Mosch et al., 1996). We constructed strains expressing *CDC42*^{Q61L} 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 Cdc42p^{Q61L} 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 *cdc42*^{Y32H}/*cdc42Δ* mutants, and budded cells displayed mis-

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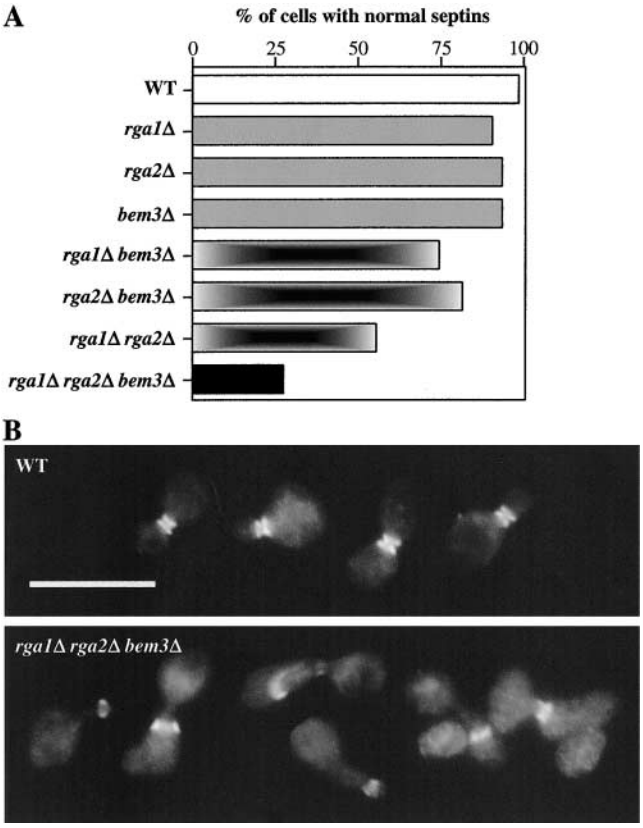

Figure 7. **Cdc42p GAPs and septin organization.** (A) Strains DLY1 (WT), DLY3344 (*rga1Δ*), DLY3353 (*rga2Δ*), DLY3346 (*bem3Δ*), DLY3341 (*rga1Δ bem3Δ*), DLY3361 (*rga2Δ bem3Δ*), DLY3347 (*rga1Δ rga2Δ*), and DLY2723 (*rga1Δ rga2Δ bem3Δ*) were grown to exponential phase in YEPD at 30°C and processed to visualize septin distribution. (B) Photographs of strains DLY1 (WT) and DLY2723 (*rga1Δ rga2Δ bem3Δ*) above. Bar, 10 μm.

organized and diffuse septin staining at the neck (Fig. 6 A and Table I). In addition, cells expressing Cdc42p^{Q61L} frequently had broad necks similar to those observed in *cdc42*^{V36T,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 Lys 186 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 *cdc42*^{K186R} strains displayed dramatic defects in septin organization (Fig. 6 C 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 *cdc42*^{V36T,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 Cdc42p^{V36T,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, Cdc42p^{Y32H} 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 (Cdc42p^{Q61L}) or to accelerate GTP hydrolysis (Cdc42p^{K186R}) 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

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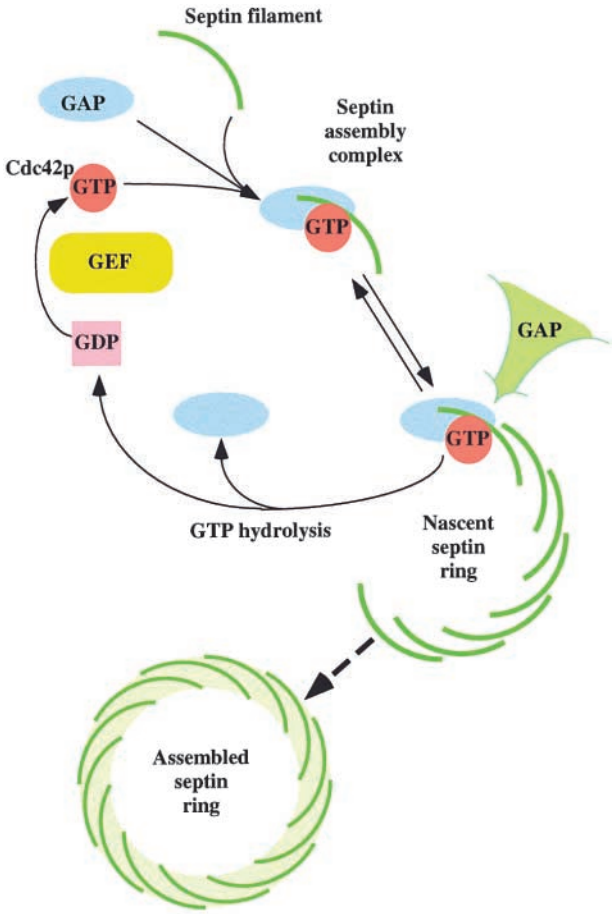

Figure 8. **Model for the role of Cdc42p GTP hydrolysis in septin ring assembly.** GTP-bound Cdc42p (red) is proposed to bind to septin filaments (green) in a complex together with other assembly factors (blue), which may include the Cdc42p GAPs Rga1p, Rga2p, or Bem3p. Interaction of the assembly complex with the ring is reversible, and we imagine that improper interactions would lead to dissociation of the complex from the ring before GTP hydrolysis. Upon proper docking of the septin assembly complex to the growing septin ring, the GAPs promote GTP hydrolysis by Cdc42p (green arrow), triggering disassembly of the complex and departure of GDP-Cdc42p (pink) and the assembly factors, leaving the septin filament incorporated into the ring. Exchange of bound GDP for GTP catalyzed by the GEF Cdc24p allows repeated cycles of Cdc42p-mediated septin recruitment, resulting in a fully assembled septin ring. The organization of septin filaments within the ring is purely speculative, but the model does not depend on the details of this organization as long as the ring is composed of repeated units (which could equally well be septin complexes rather than polymerized filaments). GAP action is hypothesized to occur upon docking of the assembly complex to the ring, but the timing of GAP association could either be early (if the GAPs are themselves assembly factors; blue) or late (if the GAPs only recognize the docked complex; green arrow). We stress that this model only aims to account for the findings reported here regarding the importance of Cdc42p GTP hydrolysis in septin ring assembly. Cdc42p may well have additional roles in septin ring assembly (e.g., directing the location at which the ring assembles) that were not perturbed by the mutants we analyzed and are not addressed by the model.

cdc42 mutants should be dominant, since hyperactivation of effectors would occur even in the presence of a wild-type copy of *CDC42*. In addition, the septin defects should be exacerbated by increasing the mutant gene dosage, which should lead to even greater hyperactivation of effectors. However, both of the septin-specific *cdc42* mutants were fully recessive with regard to their septin defects. Furthermore, a twofold increase in mutant gene dosage significantly ameliorated (rather than exacerbating) the septin defects. Given the genetic behavior of these *cdc42* alleles, the septin defects cannot be a simple consequence of effector hyperactivation. In addition, suppression of the septin defects by overexpressed Rga1p cannot simply be due to downregulation of Cdc42p to prevent effector hyperactivation. Thus, the results are inconsistent with the "hyperactive signaling" hypothesis and indicate that GTP hydrolysis by Cdc42p is playing a positive role in septin organization.

Third, GTP hydrolysis by Cdc42p may be a requisite step in the normal assembly of the septin ring. This hypothesis predicts that defects in (or misregulation of) GTP hydrolysis by Cdc42p should cause defects in septin ring assembly, as observed. However, unlike the "hyperactive signaling" hypothesis, this hypothesis does not predict that the mutant effects will be dominant: appropriate GTP hydrolysis by wild-type Cdc42p could enable septin ring assembly even in the presence of the misregulated mutants. Therefore, this hypothesis is consistent with all of our findings, but it is starkly at odds with the prevailing view of Cdc42p action, which follows the paradigm established for *ras* signaling in which the G protein acts as a simple GTP-dependent switch to activate effectors. Below, we consider how GTP hydrolysis by Cdc42p might actively contribute to septin ring assembly.

What is the role of Cdc42p GTP hydrolysis in septin ring assembly?

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).

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Table II. **Yeast strains used in this study**[a]

Strain	Relevant genotype	Source
CCY3-3B	***a*** *cdc42::TRP1 ura3::cdc42^{K186R}::URA3*	Zhang et al., 1999
DLY1	***a*** *bar1*	Sia et al., 1996
DLY5	***a***/α	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***/α *cdc42^{V36T,K94E}/cdc42::LEU2::GAL1p-CDC42*	This study
DLY4224	***a***/α *his2::cdc42^{Y32H}::HIS2/his2 cdc42::URA3/cdc42::LEU2::GAL1p-CDC42*	This study
DLY4831	***a*** *his2::cdc42^{Y32H}::HIS2 cdc42::LEU2::GAL1p-CDC42*	This study
DLY4849	***a*** *cdc42-17 GAL1p-SWE1myc::URA3*	This study
DLY5078	α *cdc24-4 GAL1p-SWE1myc::URA3*	This study
DLY5079	***a*** *cdc42-6 GAL1p-SWE1myc::URA3*	This study
DLY5080	***a***/α *cdc42^{V36T,K94E}/cdc$^{42V36T,\ K94E}$*	This study
DLY5082	***a***/α *cdc42^{V36T,K94E}/CDC42*	This study
DLY5237	***a*** *his2::EG43-CDC42::HIS2*	This study
DLY5240	***a*** *his2::EG43-CDC42^{Q61L}::HIS2*	This study
DLY5461	***a***/α *CDC42/cdc42::LEU2::GAL1p-CDC42*	This study
DLY5470	***a***/α *his2::cdc42^{Y32H}::HIS2/his2::cdc42^{Y32H}::HIS2 cdc42::LEU2::GAL1p -CDC42/cdc42::LEU2::GAL1p-CDC42*	This study
DLY5471	***a***/α *his2::cdc42^{Y32H}::HIS2/his2 cdc42::LEU2::GAL1p-CDC42/cdc42^{V36T,K94E}*	This study
RSY136	***a*** *bar1GAL1p-SWE1myc::URA3*	Sia et al., 1998

[a]All strains except CCY3-3B are in the BF264-15Du (Richardson et al., 1989) background (*ade1 his2 leu2-3,112 trp1-1 ura3Δns*). 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 GAPs in septin ring assembly. We suggest that the GAPs help to couple proper docking of the incoming septin complex to GTP hydrolysis by Cdc42p. The GAPs 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 GAPs Rga1p, Rga2p, and Bem3p 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 operates as an assembly factor, using GTP hydrolysis to perform its function. It will be interesting to explore how widespread such roles may be.

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

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Table III. **Plasmids used in this study**

Plasmid	Vector	Insert	Source
pDLB659	YIpEG43-HIS2	*CDC42*^{Q61L}	This study
pDLB664	YIpEG43-HIS2	*CDC42*	This study
pDLB678	2 μm *URA3*	*BEM1*	Bender and Pringle, 1991
pDLB722	2 μm *URA3*	*CLA4*	Erfei Bi[a]
pDLB723	2 μm *URA3*	*STE20*	Erfei 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	*rga1*^{K872A} *GAP*	This study
pDLB1552	pHB2-GST	*rga1*^{K872A} *GAP*	This study
pDLB1580	2 μm *URA3*	*rga1*^{K872A}	This study
pDLB1981	2 μm *URA3*	*RGA2*	This study
pDLB2083	pGEX-KG	*CDC42*^{Q61L}	This study
pDLB2088	pGEX-KG	*CDC42*^{T17N}	This study
pDLB2091	pGEX-KG	*CDC42*	This study
pDLB2119	pHB1-myc3	*rga1*^{K872A} *GAP*	This study
pDLB2121	pHB1-myc3	*RGA1 GAP*	This study
pDLB2213	pGEX-KG	*cdc42*^{V36T,K94E}	This study
pDLB2221	pGEX-KG	*cdc42*^{Y32H}	This study
pGEX-KG		*GST*	Guan and Dixon, 1991
pMOSB229	2 μm *URA3*	*GIC1*	Matthias Peter[b]
pPB547	2 μm *TRP1*	*BEM3*	Alan Bender[c]
pRS314	CEN *TRP1*		Sikorski and Hieter, 1989
pRS316	CEN *URA3*		Sikorski and Hieter, 1989
pRS426	2 μm *URA3*		Christianson et al., 1992
pRS424	2 μm *TRP1*		Christianson et al., 1992
YEp24	2 μm *URA3*		New England Biolabs, Inc.
YEplac195	2 μm *URA3*		Gietz and Sugino, 1988

[a]University of Pennsylvania, Philadelphia, PA.
[b]Swiss Institute for Experimental Cancer Research, Boveresses, Switzerland.
[c]Indiana University, Bloomington, IN.

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 IV. **Oligonucleotide primers used in this study**

Primer	Sequence (5′ to 3′)	Comments[a]
RGA1-A	GTGGTGAAAATATTGCTGAC	
RGA1-M1	GTTACTGGTGTGTTGGCCAGATACTTAAGAAAGC	MscI; K872A mutation
RGA1-M2	GCTTTCTTAAGTATCTGGCCAACACACCAGTAAC	RGA1-M1 reverse complement
RGA1-UNI1	GGAATTCCATATGGGAAGCAATTTGTATGGTTCAAGTCTC	NdeI; begin GAP domain
RGA1-UNI2	CCGAGCTCCTACGTCAGGATATCTTTGTAGTTCCC	SacI; end GAP domain
RGA2-A	CCGAGCTCCGGACATCACTAATAATCTCTGC	SacI; –565
RGA2-B	CCATTGAACACTTGTTACGGGTCGACTGATCGATCTTCTGGC	SalI; −140/+135 overlap
RGA2-C	GCCAGAAGATCGATCAGTCGACCCGTAACAAGTGTTCAATGG	RGA2-B reverse complement
RGA2-D	CCGCTCGAGAGGATTCGCAAAGGCTCAAAG	XhoI; +650
RGA2-1	GAAATATAACGTAGCATCTCAAGAGCAAGGAGATTTTGATGAA-AAAAATGCGCGTTTCGGTGATGAC	
RGA2-2	TTTAATCTATCCTATGTTTATTTAACTTTTGCAAATCTGTATTATGC-TTGCCCTGATGCG	
cdc12	CATGTTCCAACAGTGTTCG	Y32H mutation
cdc13	GTCAGCTGGAAATTGATTCG	PvuII, distal primer for Y32H mutagenesis

[a]Restriction 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.

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(DLY2) to confirm that the elongated bud morphology phenotype was indeed due to the *cdc42* allele. Spores generated from this cross segregated 2:2 His^+/His^-, and elongated cell morphology cosegregated with the His^+ phenotype. In both Leu^-/His^+ colonies (which have wild-type *CDC42* at the *CDC42* locus) and Leu^+/His^+ colonies (which have *GAL1p-CDC42* at the *CDC42* locus), cells were elongated on both dextrose and galactose (although less elongated in Leu^+ strains), indicating that *cdc42*^{Y32H} is dominant for this phenotype.

The Ts *cdc42-6* and *cdc42-17* alleles were generated, integrated at the *CDC42* locus, and characterized as described for *cdc42-6* (Gladfelter et al., 2001a). The minimal restrictive temperature was 33°C for *cdc42-6* and 28.5°C for *cdc42-17*, but we shifted the mutants to 37°C to ensure inactivation of as much residual Cdc42p function as possible.

The crippled *GAL1* promoter used to express *CDC42*^{Q61L} contained only one of the four Gal4p binding sites (the third) in UAS_G upstream of proximal *GAL1* promoter sequences as described in Giniger and Ptashne (1988) (pEG43; Fig. 3, top line). We began with a plasmid (YIpEG43) from the Reed lab (S.I. Reed, The Scripps Research Institute, La Jolla, CA) that has a pUC18 backbone with *URA3* sequences cloned into the HindIII site and the crippled *GAL1* promoter cloned between the SphI and BamHI sites of the polylinker. *CDC42* sequences (the complete ORF plus limited downstream sequences) were excised from pRS315-*CDC42* or pRS315-*CDC42*^{Q61L} (gifts from Doug Johnson, University of Vermont, Burlington, VT) using NdeI, the resulting overhangs were blunted, and the fragments were cloned into the SmaI site of YIpEG43. The *URA3* marker was then excised with HindIII and replaced with a 2-kb HindIII fragment containing the *HIS2* gene from plasmid YIpGAP2 (Sia et al., 1996), yielding pDLB664 and pDLB659. Digestion of these plasmids at the unique HpaI site in *HIS2* was employed to target integration to the *his2* locus in DLY1. His^+ transformants were streaked onto plates containing galactose to induce either *CDC42* or *CDC42*^{Q61L}, and colonies were selected that proliferated well to ensure sublethal amounts of GTP-locked Cdc42p were expressed.

To overexpress *RGA1*, a 3.6-kb XhoI/HindIII fragment extending from 940 bp upstream of the start codon to 300 bp downstream of the stop codon was subcloned into the corresponding sites in YEplac195.

To overexpress *RGA2*, we first amplified sequences upstream (RGA2-A + RGA2-B) and downstream (RGA2-C + RGA2-D) of the ORF by PCR using yeast genomic DNA as template. RGA2-B and RGA2-C contain complementary sequences, allowing a fusion of these fragments by overlap PCR (RGA2-A + RGA2-D). The SacI and XhoI sites incorporated into these primers were used to clone the overlap PCR product into the corresponding sites of pRS426. The overlapping central primers introduce a unique SalI site, which was used to create a "gapped plasmid," which was transformed into wild-type yeast to recover full-length *RGA2* (extending from 565 bp upstream of the start codon to 650 bp downstream of the stop codon) by gap repair.

To express GST-Cdc42p, we subcloned a SacI/EcoRI fragment containing *CDC42* from pUNI-10-CDC42 into the corresponding sites in pGEX-KG. An identical strategy was used for the mutant alleles of *CDC42*.

To express the Rga1p GAP domain, the relevant region (amino acids 785–1008) was amplified by PCR using yeast genomic DNA as template and primers RGA1-UNI1 and RGA1-UNI2, digested with NdeI and SacI (sites in primers), cloned into the corresponding sites of pUNI-10, and then recombined with either pHB2-GST or pHB1-MYC3 (Liu et al., 1998).

The K872A mutation was introduced into the Rga1p GAP domain by overlap PCR. Two initial PCR products (RGA1-UNI1 + RGA1-M2 and RGA1-M1 + RGA1-UNI2) were used for overlap PCR with RGA1-UNI1 + RGA1-UNI2, and the product was cloned into pUNI-10 and recombined with pHB2-GST and pHB1-MYC3. Accurate amplification and introduction of the mutation was confirmed by sequencing.

To overexpress *rga1*^{K872A}, we used gap repair in yeast to transfer the mutant GAP domain into the full-length *RGA1* in pDLB1468. pDLB1468 was digested with BglII and HindIII and gel purified, yielding a gapped plasmid lacking *RGA1* sequences downstream of residue 862. The gap was repaired by cotransformation of this plasmid together with a 670-bp NdeI/SacI fragment from pDLB1539 containing the mutant GAP domain and a 730-bp DraI/PvuII fragment from pDLB1468 containing downstream and vector sequences into yeast. Accurate recombination was confirmed by sequencing.

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).

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 GSH–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 $MgCl_2$, 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 10,000 cpm/pmol [γ-^{32}P]GTP or [α-^{32}P]GTP (New England Nuclear) in binding buffer (25 mM Tris-HCl, pH 7.5, 200 mM $[NH_4]_2SO_4$, 5 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 5 μM GTP, 1 mM phenylmethylsulphonyl fluoride, and 10 μg/ml each of pepstatin, aprotinin, and leupeptin). GTP-bound Cdc42p was then diluted 10-fold into reaction buffer (as above but lacking $[NH_4]_2SO_4$ 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.

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