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Role of a Cdc42p Effector Pathway in Recruitment of the Yeast Septins to the Presumptive Bud Site[D] [V]

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The septins are GTP-binding, filament-forming proteins that are involved in cytokinesis and other processes. In the yeast *Saccharomyces cerevisiae*, the septins are recruited to the presumptive bud site at the cell cortex, where they form a ring through which the bud emerges. We report here that in wild-type cells, the septins typically become detectable in the vicinity of the bud site several minutes before ring formation, but the ring itself is the first distinct structure that forms. Septin recruitment depends on activated Cdc42p but not on the normal pathway for bud-site selection. Recruitment occurs in the absence of F-actin, but ring formation is delayed. Mutant phenotypes and suppression data suggest that the Cdc42p effectors Gic1p and Gic2p, previously implicated in polarization of the actin cytoskeleton, also function in septin recruitment. Two-hybrid, in vitro protein binding, and coimmunoprecipitation data indicate that this role involves a direct interaction of the Gic proteins with the septin Cdc12p.

INTRODUCTION

Bud formation in the yeast *Saccharomyces cerevisiae* has served as an important model for studies of eukaryotic cell polarization (Hall, 1992; Nelson, 2003). In wild-type cells, nonrandom bud sites are selected by a system that involves cortical marker proteins and a signaling pathway based on the Ras-type GTPase Rsr1p (Pringle *et al.,* 1995; Kang *et al.,* 2004; Pruyne *et al.,* 2004). GTP-bound Rsr1p then promotes the localization and/or localized activation of Cdc24p, the guanine-nucleotide-exchange factor (GEF) for the Rho-type GTPase Cdc42p, and of Cdc42p itself; the localized activation of Cdc42p then causes polarization of the cytoskeletal and secretory systems, which leads to the polarized growth of the bud (Pringle *et al.,* 1995; Kozminski *et al.,* 2003; Pruyne *et al.,* 2004; Shimada *et al.,* 2004).

Among the proteins recruited early to the presumptive bud site are the septins. This widely conserved family of GTP-binding, filament-forming proteins functions in cytokinesis and other processes, many of which involve the organization of specialized regions of the cell cortex (Longtine *et al.,* 1996; Gladfelter *et al.,* 2001b; Longtine and Bi, 2003; Hall and Russell, 2004). *S. cerevisiae* has seven septins, five of which (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p) are expressed in vegetative cells, where they form hetero-oligomeric complexes and localize interdependently to the bud site (Kim *et al.,* 1991; Longtine *et al.,* 1996; Frazier *et al.,* 1998; Mortensen *et al.,* 2002; Versele *et al.,* 2004; Vrabioiu *et al.,* 2004). About 10 min before bud emergence, the septins form a ring at the cell cortex. The bud then emerges through this ring, which concurrently reorganizes into an hourglass-shaped collar that spans the mother-bud neck. This reorganization coincides with a major decrease in the exchangeability of septin subunits, presumably reflecting the formation of more stable higher-order structures at this time (Caviston *et al.,* 2003; Dobbelaere *et al.,* 2003; Versele and Thorner, 2004). The septin collar remains at the neck until cytokinesis, when it splits into two rings as the actomyosin ring contracts and the septum forms (Kim *et al.,* 1991; Lippincott *et al.,* 2001); the exchangeability of the septin subunits increases again at this time. Understanding the mechanisms involved in the recruitment and organizational transitions of the septins is key to understanding their apparent roles as a scaffold for other proteins that assemble in various patterns at the neck during the cell cycle (Gladfelter *et al.,* 2001b; Kozubowski *et al.,* 2005) and as a diffusion barrier that restricts the mobility of membrane-associated proteins (Barral *et al.,* 2000; Takizawa *et al.,* 2000; Dobbelaere and Barral, 2004).

It is generally presumed that septin recruitment to the bud site, like that of most other proteins, depends on activated Cdc42p. However, although some published data support this hypothesis (Cid *et al.,* 2001; Gladfelter *et al.,* 2001a), no definitive test has been presented. In addition, it has not

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been clear whether the septins are recruited directly into a ring or instead into some precursor structure, which then reorganizes to form the ring. Some evidence in support of the latter model has emerged from studies of certain septin mutants and of mutants defective in other proteins that are involved in septin organization, including Cdc42p, its GTPase-activating factors (Bem3p, Rga1p, and Rga2p), Bni5p, Nap1p, the formin Bni1p, and the protein kinases Cla4p, Gin4p, and Elm1p. In these mutants, the septins display a variety of abnormal arrangements, including more-or-less distinct caps on unbudded cells and/or at the tips of abnormally elongated buds (Cvrckova *et al.*, 1995; Richman *et al.*, 1999; Bouquin *et al.*, 2000; Longtine *et al.*, 2000; Weiss *et al.*, 2000; Gladfelter *et al.*, 2001a, 2002, 2004; Lee *et al.*, 2002; Roh *et al.*, 2002; Smith *et al.*, 2002; Caviston *et al.*, 2003; Goehring *et al.*, 2003; Kadota *et al.*, 2004; Versele and Thorner, 2004). Because these caps can sometimes reorganize into normal-looking rings/collars as the cells continue to grow, it has seemed possible that the normal pathway for septin-ring formation also involves the initial formation of a cap (Longtine and Bi, 2003; Versele and Thorner, 2004).

Another major outstanding issue is to identify the effectors that are responsible for septin recruitment and for the subsequent steps in septin organization. The septins and elements of the actin cytoskeleton are able to polarize independently of each other (Adams and Pringle, 1984; Ford and Pringle, 1991; Ayscough *et al.*, 1997; Harkins *et al.*, 2001), although recent evidence indicates that establishment and/or maintenance of a mature septin ring/collar may require actin function (Goehring *et al.*, 2003; Kadota *et al.*, 2004; Kozubowski *et al.*, 2005). In addition, although many other factors have been shown to be involved in establishing normal septin organization (see above), all of these factors appear to function in the initial formation of the septin ring, in its reorganization into a stable collar, or both, and not (except for Cdc42p) in the initial recruitment to the presumptive bud site.

In this study, we have used morphological, genetic, and biochemical approaches to examine the initial recruitment of the septins to the bud site and its dependence on Cdc42p, Cdc24p, and other factors, including the Cdc42p effectors Gic1p and Gic2p.

MATERIALS AND METHODS

Strains, Plasmids, Growth Media, and Genetic Methods

Yeast strains used in this study are listed in Table 1; strain constructions are described below or in the table. Standard media, genetic methods, and molecular biological methods were used except where noted (Guthrie and Fink, 1991; Ausubel *et al.*, 1998). For some experiments, yeast were grown in YM-P, a rich, buffered liquid medium (Lillie and Pringle, 1980). To induce expression from the *CUP1* promoter, copper acetate was added to media at a final concentration of 0.5 mM. To depolymerize actin, latrunculin A (LatA; courtesy of Dr. P. Crews, University of California, Santa Cruz) was made up as a 20 mM stock solution in dimethyl sulfoxide (DMSO) and diluted 100-fold into growth medium. Oligonucleotide primers for PCR (Supplementary Table 1) were purchased from Integrated DNA Technologies (Coralville, IA).

Plasmid YCplac111-CDC3-GFP (*CEN LEU2*) was constructed by subcloning an ~5.3-kb *Eco*RI-*Sal*I fragment carrying GFP^{S65T}*-CDC3* from plasmid pRS316-CDC3-GFP (Caviston *et al.*, 2003) into *Eco*RI/*Sal*I-digested YCplac111 (Gietz and Sugino, 1988). Plasmid pRS314-CDC42 (*CEN TRP1*) was constructed by subcloning an ~1.7-kb *Bam*HI-*Sal*I fragment carrying *CDC42* from pRS316-CDC42 (Bi and Pringle, 1996) into *Bam*HI/*Sal*I-digested pRS314 (Sikorski and Hieter, 1989). Plasmids pCC904 and pCC967 (both 2 μm, *URA3*) carry *GIC1* and *GIC2*, respectively, in plasmid pSM217 (Bi *et al.*, 2000). Plasmids pSM217-GIC1-GFP and pSM217-GIC2-GFP were constructed by tagging the 3′-ends of *GIC1* and *GIC2* in pCC904 and pCC967 with a $GFP^{F64L,S65T}$*:kanMX6* module using the PCR method (Longtine *et al.*, 1998b) with plasmid pFA6a-GFP(F64L/S65T)-kanMX6 (Caviston *et al.*, 2003) as template.

Plasmid pM-6 (integrative, *LEU2*) contains the C-terminal 351 codons of *SHS1* with $GFP^{F64L,S65T}$ sequences fused to its 3′-end (Iwase and Toh-e, 2001). Plasmid YIplac-HA-CDC42 (integrative, *URA3*) was constructed as described for YIplac-GFP-CDC42 (Bi *et al.*, 2000), except that the *Not*I *GFP* fragment immediately downstream of the *CDC42* start codon was replaced by a *Not*I *3HA* fragment (Tyers *et al.*, 1993). Plasmid YEp352-GFP-CDC42 (2 μm, *URA3*) was constructed by subcloning an ~2.4-kb *Hin*dIII-*Eco*RI fragment from YIplac-GFP-CDC42 (Bi *et al.*, 2000) into *Hin*dIII/*Eco*RI-digested YEp352 (Hill *et al.*, 1986). Plasmid pPW66R-CDC42-td (integrative, *URA3*) was constructed by cloning an ~580-base pair fragment carrying the *CDC42* ORF into the *Hin*dIII site of pPW66R (Dohmen *et al.*, 1994); flanking *Hin*dIII sites were included in the PCR primers used to amplify the fragment from a *CDC42* plasmid template. pPW66R-CDC42-td contains *CDC42* tagged at its 5′-end with sequences encoding both a modified dihydrofolate reductase (the "ts-degron") and a single copy of the HA epitope under control of the copper-inducible *CUP1* promoter.

Strains expressing Shs1p-GFP were constructed in two ways. In some cases (*SHS1-GFP:LEU2*), strains were transformed with plasmid pM-6 after cutting with *Bgl*II (within the *SHS1* ORF); this creates a partial duplication of *SHS1* in which the full-length copy is tagged with $GFP^{F64L,S65T}$ and expressed from the chromosomal *SHS1* promoter. In other cases (*SHS1-GFP:His3MX6*), the PCR method was used with plasmid pFA6a-GFP(S65T)-His3MX6 as template (Longtine *et al.*, 1998b) to tag the chromosomal copy of *SHS1*. The PCR method with templates pFA6a-GFP(F64L/S65T)-kanMX6 (see above), pFA6a-TRP1 (Longtine *et al.*, 1998b), and pKT355 (also named pFA6a-link-mCherry-*SpHIS5*; it carries an improved version of monomeric red fluorescent protein ([RFP]; Shaner *et al.*, 2004; Sheff and Thorn, 2004) was also used to introduce the *SPC42-GFP:kanMX6*, *swe1*Δ*::TRP1*, and *CDC3-mCherry:SpHIS5 (CDC3-RFP)* alleles into the appropriate strains. Strain YEF1515 was constructed by sporulating strain YEF1155 (Table 1) after integrating *Eco*RV-digested YIplac-HA-CDC42 at its *ura3* locus. Strain YEF2958 was constructed as follows. First, strain YEF1155 was transformed with plasmid pRS314-CDC42 and then sporulated to generate a *cdc42*Δ*::HIS3* [pRS314-CDC42] strain. *Nco*I-digested pPW66R-CDC42-td was integrated at the *ura3* locus of this strain by selecting stable Ura^+ transformants at 25°C, and repetitive streaking on YPD plates then yielded strain YEF2958, which lacks pRS314-CDC42. (As observed previously by Holly and Blumer [1999], YPD medium without added copper can support the growth of strains carrying some P_{CUP1}-controlled, td-tagged essential genes at 25°C.) The success of these strain constructions was confirmed by PCR and by tetrad and phenotypic analysis.

G0-Release Experiments

Except as noted below or in the figure legends, G0-release experiments were conducted as follows. A dense lawn of cells was spread on SC plates and incubated for 5 d at 25°C. Cells were then scraped from the plates, suspended in SC medium without glucose, and subjected to two rounds of centrifugation (20 s at 800 rpm), retaining the top ~50% of the supernatant each time, to enrich for unbudded cells. The supernatant from the second spin was diluted to $\sim 5 \times 10^6$ cells/ml with SC medium without glucose and incubated at 37°C for 1.5 h. Glucose was then added to a final concentration of 2%, and the incubation was continued at 37°C. Samples taken just before glucose addition and at appropriate times thereafter were fixed by adding formaldehyde to 3.7% and incubating for 10 min at 37°C and 15 min at 25°C, washed twice with phosphate-buffered saline (PBS), and examined by differential interference contrast (DIC) and fluorescence microscopy. For the experiment of Figure 4, copper was added to the SC plates (to induce expression of the *HA-CDC42-td* allele) but not to the liquid media used in the subsequent steps. For the experiment of Figure 6, YPD plates and YM-P liquid medium were used. For the experiment of Figure 7, SC-Ura plates and liquid medium were used, the cells were incubated on the plates for 10 d at 25°C, the centrifugations were omitted, the incubations in liquid medium (before and after glucose addition) were at 33.5°C, and DMSO or LatA in DMSO was added at the time of glucose addition.

Microscopy

Except where noted, microscopy was performed with a computer-controlled Eclipse E800 microscope (Nikon, Tokyo, Japan) and a high-resolution CCD camera (model C4742–95; Hamamatsu Photonics, Bridgewater, NJ). For the time-lapse experiments of Figures 1A and 2, exponentially growing cells were spotted onto slides spread with a thin layer of medium solidified with 25% gelatin (Acros Organics, Morris Plains, NJ; Cat. No. 41087-5000) (Yeh *et al.*, 1995). Cells were then imaged at intervals of 2–5 min using a DeltaVision Spectris microscope system (Applied Precision, Issaquah, WA) and a CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ). For each time point, 30 images were acquired at 0.3-μm increments, deconvolved, and reconstructed into a 3D image. The relative fluorescence intensities of the nascent septin assemblies were quantified using NIH Image version 1.62 software (http://rsb.info.nih.gov/nih-image/Default.html). Each value was calculated by dividing the boxed fluorescence intensity at that time point (minus the background intensity from the same image) by the boxed fluorescence intensity at the last time point (minus the background intensity from this image). For the time-lapse experiments of Figure 1B, a Nikon Eclipse 600 FN microscope (Melville, NY) and ORCA II CCD camera were used essentially as described by Schenkman *et al.* (2002). For these experiments, exponentially growing cells

Table 1. Yeast strains used in this study

Strain	Genotype	Source/reference
YEF311	**a** *cdc24-4 his3 leu2 trp1 ura3*	This study[a]
YEF319	α *cdc24-11 ade2 his3 his4 leu2 lys2 trp1 ura3*	Bi *et al.* (2000)
YEF473	**a**/α *his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Bi and Pringle (1996)
YEF473A	**a** *his3 leu2 lys2 trp1 ura3*	Segregant from YEF473
YEF1155	As YEF473 except *cdc42Δ:HIS3/CDC42*	This study[b]
YEF1515	**a** *cdc42Δ::HIS3 ura3:3HA-CDC42:URA3 his3 leu2 lys2 trp1*	See text
YEF1963	**a** *cdc42-22 his3 leu2 lys2 trp1 ura3*	Caviston *et al.* (2002)
YEF2194	**a** *cdc42-13 his3 leu2 lys2 trp1 ura3*	This study[c]
YEF2958	**a** *cdc42Δ::HIS3 ura3:HA-CDC42-td:URA3 his3 leu2 lys2 trp1*	See text
CCC114	YEF473 carrying plasmid pRS316-CDC3-GFP	This study
LSY389	As YEF473 except *rsr1Δ:TRP1/rsr1Δ:TRP1*	L. Schenkman[d]
5011-HO1	**a**/α *cdc24-1/cdc24-1*	Adams and Pringle (1984)
JPT19-HO5	**a**/α *cdc24-4/cdc24-4*	Adams and Pringle (1984)
CCY1042-12B	α *gic1Δ::LEU2 gic2Δ::TRP1 his3 leu2 lys2 trp1 ura3*	Bi *et al.* (2000)
PJ69-4A	**a** *his3-200 leu2-3,112 trp1-901 ura3-52 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al.* (1996)
PJ69-4α	α *his3-200 leu2-3,112 trp1-901 ura3-52 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al.* (1996)
Masa1005	**a** *cdc42Δ::HIS3 ura3:3HA-CDC42:URA3 SHS1-GFP:LEU2 his3 leu2 lys2 trp1*	This study[e]
Masa1006	**a** *cdc42Δ::HIS3 ura3:HA-CDC42-td:URA3 SHS1-GFP:LEU2 his3 leu2 lys2 trp1*	This study[e]
Masa1145	α *gic1Δ::LEU2 gic2Δ::TRP1 SHS1-GFP:His3MX6 his3 leu2 lys2 trp1 ura3*	This study[f]
Masa1147	**a** *SHS1-GFP:His3MX6 his3 leu2 lys2 trp1 ura3*	This study[f]
Masa1167	**a** *cdc42Δ::HIS3 ura3:3HA-CDC42:URA3 SHS1-GFP:LEU2 SPC42-GFP:kanMX6 his3 leu2 lys2 trp1*	This study[g]
Masa1183	**a** *cdc42Δ::HIS3 ura3:HA-CDC42-td:URA3 SHS1-GFP:LEU2 SPC42-GFP:kanMX6 his3 leu2 lys2 trp1*	This study[g]
Masa1212	α *gic1Δ::LEU2 gic2Δ::TRP1 SHS1-GFP:His3MX6 SPC42-GFP:kanMX6 his3 leu2 lys2 trp1 ura3*	This study[g]
Masa1234	**a** *cdc24-4 SHS1-GFP:LEU2 SPC42-GFP:kanMX6 his3 leu2 trp1 ura3*	This study[h]
Masa1246	**a** *cdc24-4 swe1Δ::TRP1 SHS1-GFP:LEU2 SPC42-GFP:kanMX6 his3 leu2 trp1 ura3*	This study[i]
Masa1323	α *cdc42-1 SHS1-GFP:LEU2 gal2 his4 leu2 trp1 ura3*	This study[e]
Masa1327	**a** *cdc42-201 SHS1-GFP:LEU2 his3 leu2 lys2 trp1 ura3*	This study[e]
Masa1329	**a** $cdc42^{V36G}$ *SHS1-GFP:LEU2 his3 leu2 lys2 trp1 ura3*	This study[e]
Masa1486	As YEF473 except *SHS1-GFP:LEU2/SHS1*	This study[e]
JGY1326	**a** *CDC3-mCherry:SpHIS5 his3 leu2 lys2 trp1 ura3*	This study[j]

[a] Constructed as described previously for YEF313 (Bi *et al.*, 2000).

[b] The entire *CDC42* ORF was deleted using the method and PCR primers described by Bi *et al.* (2000).

[c] Constructed as described previously for YEF1963.

[d] The entire *RSR1* ORF was deleted using the method and PCR primers described for LSY388 (Schenkman *et al.*, 2002).

[e] *SHS1-GFP:LEU2* was introduced into strains YEF1515, YEF2958, DJTD2–16D (Johnson and Pringle, 1990), YEF1961 (Zhang *et al.*, 2001), YEF2921 (Caviston *et al.*, 2003), and YEF473 as described in the text.

[f] *SHS1-GFP:His3MX6* was introduced into strains CCY1042–12B and YEF473A as described in the text.

[g] *SPC42-GFP:kanMX6* was introduced into strains Masa1005, Masa1006, and Masa1145 as described in the text.

[h] *SHS1-GFP:LEU2* and *SPC42-GFP:kanMX6* were introduced into strain YEF311 as described in the text.

[i] *swe1Δ::TRP1* was introduced into strain Masa1234 as described in the text.

[j] *CDC3-mCherry:SpHIS5* was introduced into strain YEF473A as described in the text.

were mixed with molten (but cooling) medium containing 18% gelatin, spotted onto a slide, and covered with an unsealed coverslip. Of the many cells filmed, a few provided clear en face views of the nascent septin ring and bud. For the time-lapse experiments of Figures 4–6, exponentially growing cells were spotted onto slides spread with a thin layer of medium solidified with 3% agarose (Invitrogen, Carlsbad, CA; Cat. No. 15510-027), and the samples were then held at 37°C using an Objective Heater (Bioptechs, Butler, PA). At appropriate intervals, individual DIC and GFP images were acquired and processed (including contrast enhancement in some cases) using Image-ProPlus software (Media Cybernetics, Silver Spring, MD) and PhotoShop Version 7.0 (Adobe Systems, San Jose, CA). For the colocalization studies in Figure 9, DIC, GFP, and RFP images were acquired using the Image-ProPlus software, pseudocolored and overlaid using MetaMorph Version 6.2r4 (Universal Imaging, Downingtown, PA), and processed using the imaging software and PhotoShop Version 7.0.

Immunofluorescence and staining of actin with rhodamine- or Alexa-conjugated phalloidin (Molecular Probes, Eugene, OR) were performed essentially as described by Pringle *et al.* (1989) using (for Figure 5B) a Leitz Orthoplan microscope (Rockleigh, NJ) and Kodak T-MAX film (Eastman Kodak, Rochester, NY). Antibodies used were the rat monoclonal anti-tubulin YOL1/34 (Kilmartin *et al.*, 1982), affinity-purified rabbit polyclonal anti-Cdc3p (Kim *et al.*, 1991) and anti-Cdc10p (S. R. Ketcham and J. R. Pringle, unpublished results), rhodamine-labeled goat anti-rat-IgG, and FITC-labeled goat anti-rabbit-IgG.

Immunoblotting

Proteins were separated by 10% SDS-PAGE, transferred electrophoretically to PVDF membranes, and analyzed by standard methods (Ausubel *et al.*, 1998). Primary antibodies included a rabbit anti-Cdc42p (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal anti-HA (Covance, Princeton, NJ), a rabbit antibody to the mitochondrial-outer-membrane protein porin (courtesy of Dr. D. Pain, UMDNJ—New Jersey Medical School, Newark, NJ), a mouse monoclonal anti-GFP (Covance), an affinity-purified rabbit anti-Cdc3p (see above), a rabbit anti-Cdc11p (Santa Cruz Biotechnology), and an affinity-purified rabbit antibody to the C-terminal 14 amino acids of Cdc12p (Sigma-Genosys, The Woodlands, TX). Secondary antibodies were horseradish-peroxidase-conjugated goat anti-rabbit-IgG and anti-mouse-IgG (Jackson

ImmunoResearch, West Grove, PA), which were detected using the ECL system (Amersham Biosciences, Buckinghamshire, United Kingdom).

Two-Hybrid Assays

Two-hybrid assays were conducted as described previously (Drees *et al.*, 2001). To test for interactions, strain PJ69–4α carrying a DNA-binding domain (DBD) plasmid was mated to strain PJ69–4A carrying an activation domain (AD) plasmid. Diploids were selected on SC-Leu-Trp plates and replica-plated onto SC-Leu-Trp-Ade plates.

In Vitro Protein-binding Assays

Gic1p and Gic2p were expressed in *Escherichia coli* as fusions to maltose-binding protein (MBP). To construct the needed plasmids, the full-length *GIC1* and *GIC2* ORFs were amplified by PCR using pCC904 and pCC967 (see above) as templates, and the *Bam*HI-*Sal*I *GIC1* and *Eco*RI-*Sal*I *GIC2* fragments (restriction sites included in the primers) were cloned into the corresponding sites of plasmid pMAL-c (New England Biolabs, Ipswich, MA). *E. coli* strain BL21 (Promega, Madison, WI) containing pMAL-c, pMAL-GIC1, or pMAL-GIC2 was grown to exponential phase ($OD_{600} \approx 0.4$) at 23°C, and protein expression was then induced for 5 h by the addition of IPTG to a final concentration of 1 mM. Cells were harvested, frozen in liquid nitrogen, and stored at −70°C. To purify MBP and the fusion proteins, each cell pellet was resuspended in tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% Triton X-100 and a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Cells were disrupted by sonication (three rounds of 10 s with 2 min on ice between rounds), and the extract was incubated on ice for 30 min and then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was centrifuged again at 12,000 rpm for 10 min at 4°C, and the resulting supernatant was transferred to a tube containing 200 μl bed-volume of amylose resin (New England Biolabs). After 1 h at 4°C with gentle mixing, the beads were washed three times by centrifugation with 1 ml of TBS containing 5 mM EDTA, 5 mM DTT, and 0.1% Triton X-100, followed by the addition of 200 μl of TBS containing 1% Triton X-100.

To express septin proteins in vitro, full-length *CDC3, CDC11,* and *CDC12* were cloned into *Bgl*II/*Sma*I-digested plasmid pSP64T(SXBBSE), which is pSP64T (Krieg and Melton, 1984) modified by the addition of a multiple-cloning site (courtesy of Dr. R. Matts, Oklahoma State University, Stillwater, OK). For *CDC3,* an ~1.5-kb *Bam*HI-*Sma*I fragment was subcloned from plasmid pGEX-4T/CDC3 (Lee *et al.,* 2002). For *CDC11,* an ~1.3-kb fragment was obtained by performing PCR with primers ML21 and ML22 and genomic DNA as template, digesting with *Sal*I, treating with Klenow fragment to fill in the cut end, and digesting with *Bgl*II (restriction sites included in the primers); sequencing verified that no mutations had been introduced during the PCR. For *CDC12,* an ~1.3-kb fragment was obtained by digesting plasmid pGEX-4T/CDC12 (Lee *et al.,* 2002) with *Not*I, filling in the ends, and digesting with *Bam*HI. ^{35}S-labeled septin proteins were then synthesized using a coupled in vitro transcription/translation system from rabbit reticulocytes (Craig *et al.,* 1992) and precleared by incubating with amylose-resin-bound MBP for 20 min at 23°C and then centrifuging for 30 s at 12,000 rpm.

For binding assays, 20-μl samples of each precleared septin sample were transferred to tubes containing ~2 μg of amylose-resin-bound MBP, MBP-Gic1p, or MBP-Gic2p. After incubation for 1 h at 23°C with gentle mixing, the samples were centrifuged for 3 min at 6000 rpm, and a 5-μl sample of each supernatant was collected as the "unbound" fraction. The beads were then washed four times with 1 ml of TBS containing 5 mM EDTA, 5 mM DTT, and 0.1% Triton X-100, yielding the "bound" fraction. Bound and unbound fractions were separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by staining with Ponceu S, autoradiography, and immunoblotting (see above). Ponceu S staining conditions were identical for all blots, and for each septin, the figure shows identical exposures of each autoradiogram and immunoblots prepared using identical conditions and exposures.

Immunoprecipitation

One hundred OD_{600} units of cells from a culture growing exponentially in SC-Ura medium at 25°C were chilled and disrupted by vortexing (12 rounds of 30 s with 30 s on ice between rounds) with ~0.5-mm glass beads in 700 μl of PBS containing 0.1% NP40 and a cocktail of protease inhibitors (Sigma, St. Louis, MO). After centrifugation at 4°C to remove cell debris, a sample of the supernatant was saved as the "input" fraction, and 500 μl of supernatant was used for immunoprecipitation with 50 μl of anti-Cdc11p (see above). After incubation overnight at 4°C, 50 μl of protein G-agarose beads (Invitrogen) was added to each sample, and incubation was continued for 3 h at 4°C. The samples were then centrifuged for 5 min at 14,000 rpm, and a sample of the supernatant was saved as the "unbound" fraction. The beads were then washed six times with 200 μl per wash of the same buffer as used for lysis. Proteins bound to the beads were eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting (see above).

RESULTS

Septin Recruitment and Ring Formation in Wild-Type Cells

In a variety of abnormal situations, the septins have been observed to form a distinct caplike structure at the presumptive bud site and/or at the tip of the growing bud, rather than the normal ring and collar structures (see *Introduction*). Because these caps can sometimes convert to normal-looking septin rings or collars, it has seemed possible that the initial formation of the septin ring might normally involve the formation of a cap that is then converted to a ring before bud emergence. Previous studies have not addressed this possibility clearly, mainly because nascent septin rings have mostly been viewed from the side, so that it was not possible to distinguish between caps and rings. Thus, we examined septin recruitment and ring formation in wild-type cells expressing GFP-tagged septins using time-lapse microscopy in which the initial septin assemblies were viewed en face, 3D image reconstruction, and quantitation of the fluorescence signals. We found that GFP-septin fluorescence could almost always be detected at the presumptive bud site 3–5 min before a distinct septin ring was visible (Figures 1 and 2A; Supplementary Movies 1–6). However, the initial septin assemblies appeared disorganized and variable from cell to cell, and they rarely, if ever, took the form of a discrete cap. Moreover, the septin assemblies typically continued to increase in total fluorescence (implying the continuing recruitment of subunits) until some time after a distinct ring structure could be discerned. We conclude that although septins are recruited to the presumptive bud site over a period of several minutes before the appearance of a distinct septin ring, the ring itself is the first distinct structure that forms.

Requirement for F-Actin, but Not for the Bud-Site-Selection Pathway, for Efficient Septin-Ring Formation

In wild-type cells, the new septin ring and bud normally form at a site that is determined by a signaling pathway based on the Ras-like GTPase Rsr1p, which transmits the position of a cortical marker to the polarity-establishment machinery (Pringle *et al.,* 1995; Pruyne and Bretscher, 2000). Thus, it seemed possible that efficient formation of the septin ring would depend on the function of this pathway. However, we could detect no difference in the kinetics of septin-ring formation in wild-type versus *rsr1*Δ cells (Figure 2, A and B).

Although the septin ring can form in the absence of F-actin (Ayscough *et al.,* 1997; Harkins *et al.,* 2001), recent evidence suggests that the efficient formation and/or maintenance of the septin ring depends on F-actin (Goehring *et al.,* 2003; Kadota *et al.,* 2004; Kozubowski *et al.,* 2005). Using the methods described here, we also observed that the septin ring formed at least 10 min more slowly and typically appeared less well organized when F-actin was eliminated by growth of cells in medium containing LatA (Figure 2C).

Dependence of Septin Recruitment on Cdc42p and Its Activator Cdc24p

It is generally believed that the initial recruitment of septins to the presumptive bud site depends on Cdc42p and Cdc24p (see *Introduction*). However, no rigorous examination of this hypothesis has been presented, in part because of the leakiness and complex phenotypes (reflecting the multitude of Cdc42p functions) of most temperature-sensitive *cdc42* alleles. To investigate this issue further, we constructed strains containing a "ts-degron" allele (Dohmen *et al.,* 1994) of *CDC42.* In these strains, the sole copy of *CDC42* is a chromosomal locus expressed under the control of the copper-

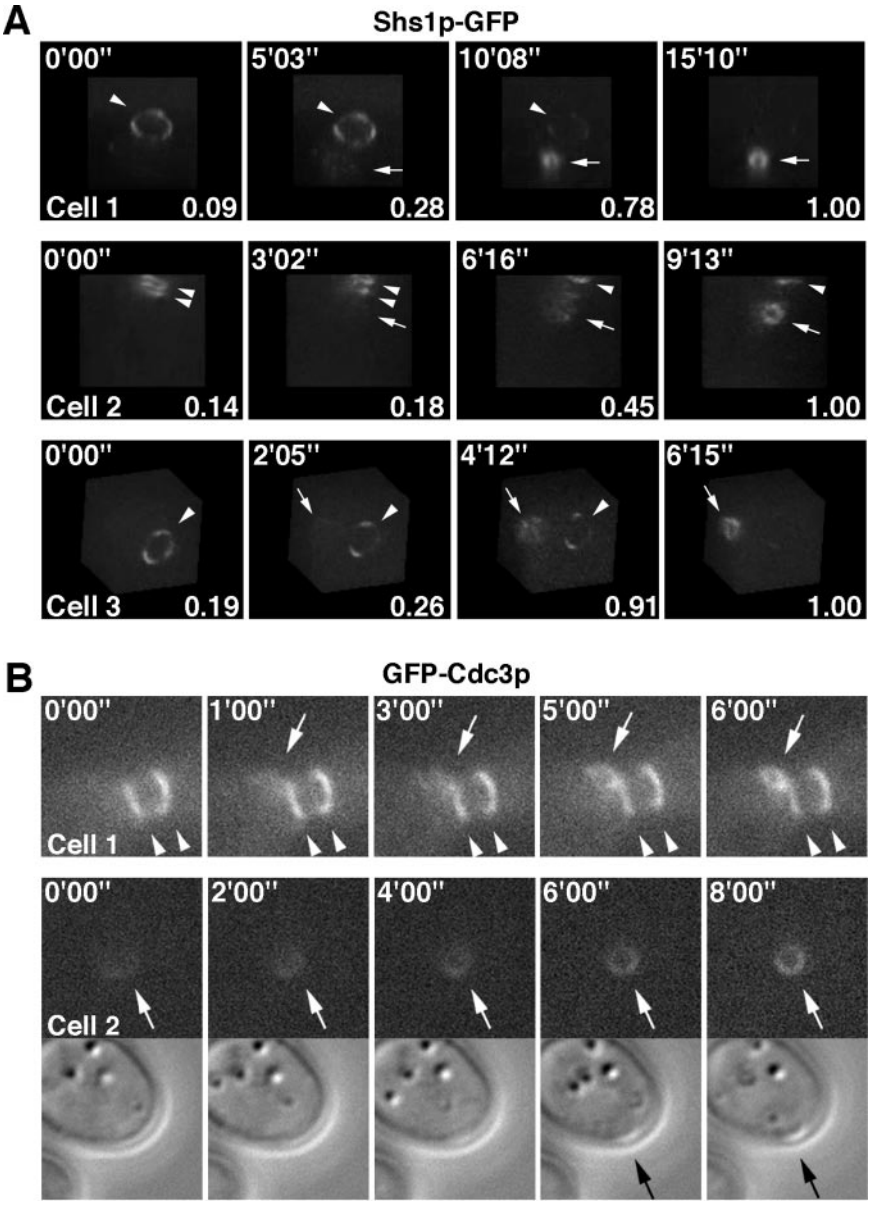

Figure 1. Septin recruitment and ring formation in wild-type cells. Diploid strains (A) Masa1486 (*SHS1-GFP*) and (B) CCC114 (*GFP-CDC3*) were grown to exponential phase in SC-Leu and SC-Ura medium, respectively, and then observed by time-lapse microscopy (see *Materials and Methods*) in SC-Leu and YM-P medium, respectively, at ~23°C. Selected images are shown; times are given in minutes and seconds after an arbitrary starting point. Arrowheads, the septin ring(s) from the preceding cell cycle (note that these typically disassemble as the new rings form); white arrows, the nascent septin rings; black arrows, the emerging bud (inconspicuous in the *en face* view). Images in A were generated by deconvolution and 3D image reconstruction (see *Materials and Methods*). Views of the 3D images from particular angles are shown; full rotating views of cell 2 at 6′16″ and 9′13″ and of cell 3 at 4′12″ and 6′15″ are provided as Supplementary Movies 1–4. The relative fluorescence intensities of the nascent septin-GFP structures were determined as described in *Materials and Methods* and are indicated in the lower right of each panel. Results shown are representative of those obtained with 15 cells. Images in B were generated by conventional fluorescence and DIC microscopy. The complete time-lapse series are provided as Supplementary Movies 5 and 6.

inducible *CUP1* promoter, and the Cdc42p that is produced is fused both to an HA-epitope tag and to a modified form of dihydrofolate reductase that serves as a temperature-dependent (active at high temperature) signal for rapid ubiquitination-mediated proteolysis. Control experiments showed that the ts-degron strains behaved as expected. They could grow on medium containing copper at 25°C but not on medium without copper at 37°C, and the growth defect was rescued by a low-copy plasmid containing wild-type *CDC42* (Figure 3A). On shift of medium and temperature, Cdc42p was rapidly depleted, being essentially gone by 90 min (Figure 3B), and most cells (87%; n = 300) arrested as large, round, unbudded cells (Figure 3C).

We then examined a ts-degron strain that expressed both Shs1p-GFP and Spc42p-GFP; the latter protein is a spindle-pole-body (SPB) component that allows monitoring of nuclear-cycle progression (Adams and Kilmartin, 1999). In one experiment, this strain and a congenic wild-type strain were released from G0 arrest (see *Materials and Methods*) into fresh medium without copper at 37°C. As expected, within a few hours, the wild-type cells had formed new septin rings and new buds and were progressing through the nuclear cycle (Figure 4, A and B, left panels). In contrast, although the ts-degron strain progressed into the nuclear cycle as judged by the separation of SPBs, neither new buds nor distinct cortical septin structures were formed (Figure 4, A and B, right panels). In other experiments, the same strains were grown to exponential phase, shifted to Cdc42p-depleting conditions, and observed by time-lapse microscopy. The wild-type cells showed the expected progression of cell-cycle completion, formation of new septin rings and new buds, and progression through the nuclear cycle (Figure 4D, left panel). In contrast, although the ts-degron cells were able to divide and progress into the next nuclear cycle and sometimes could complete one or even two seemingly normal cell cycles (presumably reflecting cell-to-cell differences in the times needed to deplete Cdc42p), they always terminated development as unbudded cells that lacked new cortical septin structures (Figure 4D, right panel). Western blotting showed that the failure to form new septin structures

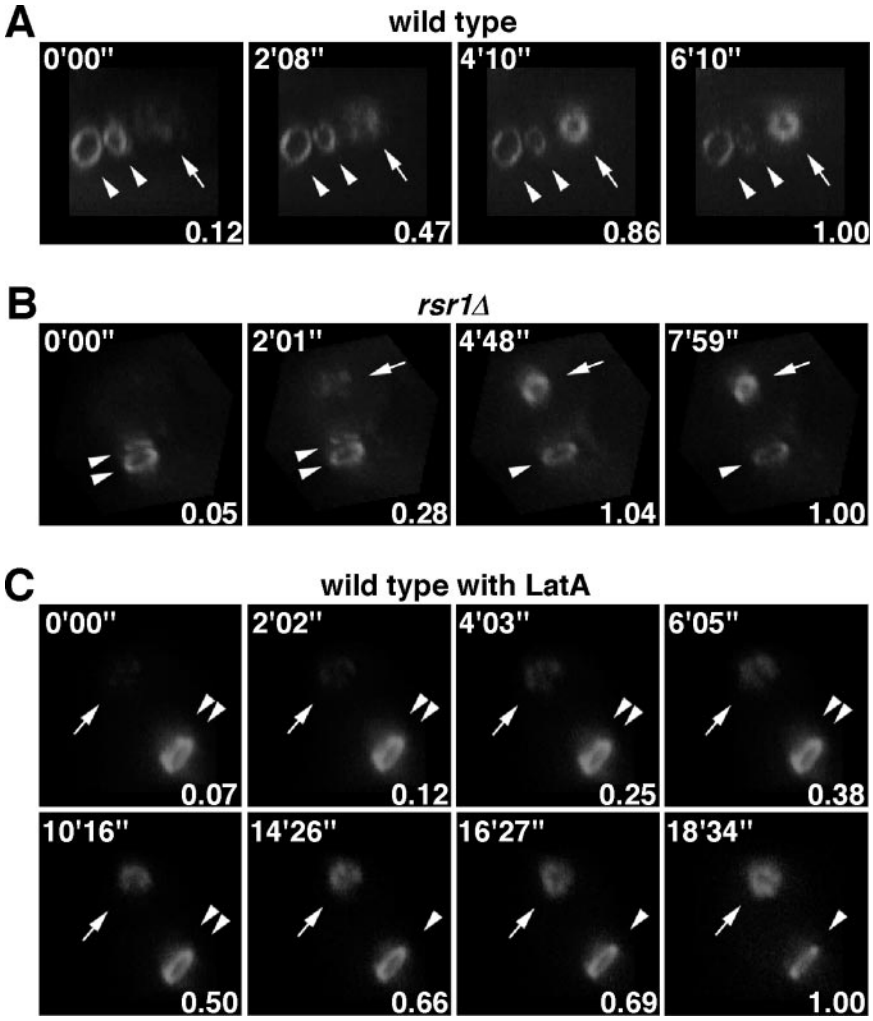

Figure 2. Delayed formation of the septin ring in LatA-treated cells but not in *rsr1*Δ cells. Cells were examined and data are displayed as described for Figure 1A. All strains carried plasmid YCplac111-CDC3-GFP. (A and C) Diploid wild-type strain YEF473 examined in the absence of drug (A) or in the presence of 200 μM LatA (C); treatment with LatA began 20 min before the start of time-lapse observations. Staining with Alexa-phalloidin verified that actin was indeed fully depolymerized after 20 min of LatA treatment. (B) Homozygous diploid *rsr1*Δ strain LSY389. Results in each panel are representative of those obtained with six cells.

did not reflect a major change in the total level of Shs1p-GFP upon depletion of Cdc42p (Figure 4C). Taken together, these results indicate that Cdc42p is required for the initial recruitment of the septins to the presumptive bud site. In contrast, Cdc42p is apparently not required for the maintenance of the septin ring at the bud neck once it has formed (see also Gladfelter *et al.*, 2002).

To ask if septin recruitment depends on the activation of Cdc42p by its guanine-nucleotide-exchange factor Cdc24p (Zheng *et al.*, 1994), we performed similar experiments using temperature-sensitive *cdc24* mutant strains. In G0-release experiments, the cells of each strain progressed through the nuclear cycle, as shown by monitoring either Spc42p-GFP localization (Figure 5A) or spindle formation using anti-tubulin antibodies (Figure 5B). However, cortical septin structures were not detected either by monitoring Shs1p-GFP fluorescence (Figure 5A) or by using immunofluorescence to localize endogenous Cdc3p or Cdc10p (Figure 5B). We also performed time-lapse experiments on exponentially growing *cdc24* mutant cells after a shift from 25 to 37°C. To avoid possible complications due to activation of the morphogenesis checkpoint by the loss of Cdc24p function (Lew and Reed, 1995), the strain also was deleted for *SWE1*. As expected (Sloat *et al.*, 1981), budded cells present at the time of shift appeared to complete division despite the failure of the buds to attain normal size (Figure 5C; note the normal-looking disappearance of the old septin ring). Although the cells typically then proceeded through one or more nuclear cycles, no new cortical septin structures were detected by monitoring Shs1p-GFP fluorescence (Figure 5C). In an experiment like that of Figure 4C, the levels of Shs1p-GFP did not appear to be affected by the loss of Cdc24p function (unpublished data). Thus, Cdc24p function also appears to be required for the initial recruitment of the septins to the presumptive bud site.

Role of Gic1p and Gic2p in Septin Recruitment

Several Cdc42p effectors and other proteins have been shown to be involved in organizing the normal septin ring and collar (see *Introduction*). However, the Cdc42p effectors presumed to be responsible for the initial recruitment of the septins to the presumptive bud site have remained elusive. A clue was provided by the detection of two-hybrid interactions between the septin Cdc12p and the Gic proteins (Drees *et al.*, 2001; and see below). Gic1p and Gic2p are structurally related proteins that bind to Cdc42p-GTP and appear to have overlapping roles in cell polarization (see *Discussion*). Their role seems to be particularly important in haploid cells and at higher temperatures, so that a *gic1*Δ *gic2*Δ double-mutant haploid is temperature sensitive for growth.

To test for a possible role of the Gic proteins in septin recruitment, we first shifted exponentially growing *gic1*Δ

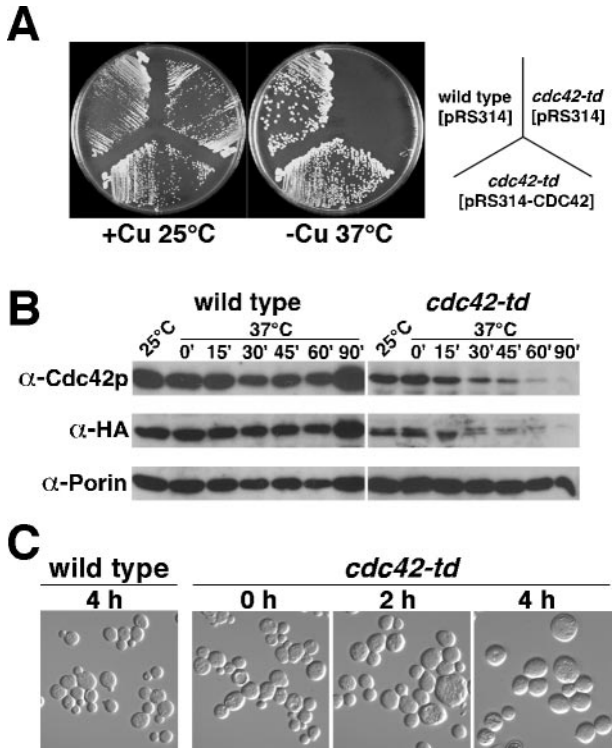

Figure 3. Characterization of the Cdc42p-depletion strain. (A) Temperature-sensitive growth and rescue by low-copy *CDC42*. Strain YEF1515 (*cdc42*Δ *ura3:3HA-CDC42*) carrying plasmid pRS314 (vector) and strain YEF2958 (*cdc42*Δ *ura3:HA-CDC42-td*) carrying plasmid pRS314 or pRS314-CDC42 were streaked onto SC-Trp plates with copper at 25°C (left) or without copper at 37°C (right) and incubated for 3 d. (B) Rapid disappearance of HA-Cdc42p-td after medium and temperature shift. Strains YEF1515 and YEF2958 were grown to exponential phase in SC-Ura medium with copper at 25°C and then washed twice and incubated further in SC-Ura medium without copper at 37°C. Samples taken at the indicated times after the shift were analyzed by immunoblotting using antibodies to Cdc42p, the HA epitope, and porin (as a loading control). (C) Loss-of-polarity phenotype. Strains YEF1515 and YEF2958 were grown, washed, and incubated further as described for B. Samples taken at the indicated times after the shift were fixed with 3.7% formaldehyde and observed by DIC microscopy.

*gic2*Δ haploid cells from 25 to 37°C. Septin rings present at the time of the shift did not appear to be affected and disassembled only at the normal time as the cells divided (Figure 6, A and C; Supplementary Movie 7). However, new septin rings (or other cortical septin structures) did not appear, even as the cells progressed into the following nuclear cycle (Figure 6C); thus, the fraction of cells with septin rings gradually declined over time (Figure 6A). The failure to form new septin structures did not appear to result from a loss of septin protein after the temperature shift (Figure 6B) and thus presumably reflected a failure of septin recruitment. As a further test, we released *gic1*Δ *gic2*Δ cells from G0 arrest into fresh medium at 37°C. Although the loss-of-polarity phenotype was slightly leaky under these conditions, very few cells acquired detectable septin rings or other cortical septin structures (Figure 6, D and E). Thus, it appears that the Gic proteins are essential for septin recruitment at 37°C although not at 25°C.

Additional evidence for a role of Gic1p in septin recruitment was obtained from dosage-suppression experiments. We found that a multicopy plasmid carrying *GIC1* under its own promoter could suppress the growth defect of either of two temperature-sensitive *cdc24* alleles, *cdc24-4* and *cdc24-11*, at temperatures of 33–35°C (Figure 7A and unpublished data). As expected, suppression of the growth defect was accompanied by a restoration of bud formation (implying also more-or-less normal actin polarization) and of septin recruitment and ring formation (Figure 7B, top and middle panels, and 7C, top panels). Suppression of the septin-recruitment defect did not depend on either actin polarization or bud formation, because it was observed also in LatA-treated cells (Figure 7, B and C, bottom panels). Remarkably, the buds produced by the suppressed cells were of approximately normal shape (Figure 7B, middle panel); because most perturbations of septin organization or function cause the production of abnormally elongated buds (Hartwell, 1971; Adams and Pringle, 1984; Gladfelter *et al.*, 2005 and references cited therein), this suggests that septin function is essentially normal in these cells. It was also noteworthy that bud emergence lagged considerably behind the appearance of cortical septin structures in the suppressed cells (Figure 7C, top right panel). This contrasts with a typical lag of ~10 min in normal cells (Kim *et al.*, 1991; Figure 4B, left panel) and suggests that overexpression of Gic1p actually suppresses the septin-recruitment defect of the *cdc24* mutant more effectively than it suppresses the actin-polarization defect.

Despite its good suppression of *cdc24* mutations, multicopy *GIC1* suppressed several temperature-sensitive *cdc42* alleles only weakly (strains YEF2194 and YEF1963) or not at all (strains Masa1323, Masa1327, and Masa1329; unpublished data). In addition, multicopy *GIC2* under its own promoter could not suppress either *cdc24-4* or *cdc24-11* at temperatures of 35°C (strain YEF311: Figure 7A) or 33°C (strain YEF319: unpublished data), and it actually inhibited the growth of each of the five *cdc42* strains just mentioned at temperatures below their normal minimal restrictive temperatures (unpublished data; see *Discussion*). Importantly, however, other *cdc42* alleles have been found previously to be suppressed by multicopy *GIC1* and/or *GIC2* (Kozminski *et al.*, 2000; Gladfelter *et al.*, 2001a).

To ask if the apparent role of Gic1p and Gic2p in septin recruitment involves a direct interaction with the septins, we performed three kinds of experiments. First, by two-hybrid analysis, we confirmed the finding of Drees *et al.* (2001) that Gic1p and Gic2p both interact well with Cdc12p; we also observed an interaction with Cdc11p, but not with the other septins (Figure 8A). Second, to test for direct physical interactions, we asked whether septins produced by transcription and translation in vitro could interact with MBP-Gic fusion proteins purified from *E. coli*. By this assay, we detected significant binding of Cdc12p to both Gic1p and Gic2p (Figure 8B, bottom section). Cdc3p also appeared to bind the Gic proteins weakly, but no binding of Cdc11p was detected (Figure 8B, top two sections). Finally, to ask if Gic1p and Gic2p interact with the septins in vivo, we performed coimmunoprecipitation experiments. Because the vegetatively expressed septins are known to form a tight complex (Frazier *et al.*, 1998; Mortensen *et al.*, 2002), we used anti-Cdc11p antibodies to precipitate the septin complex and associated proteins from cells expressing Gic1p-GFP or Gic2p-GFP. Cdc11p, Cdc3p, and Cdc12p were efficiently coprecipitated; comparison of input and unbound samples indicated that ~60–70% of each protein had been precipitated (unpublished data). In addition, immunoblotting with anti-GFP antibody showed that both Gic1p-GFP and Gic2p-GFP were significantly enriched in the immunoprecipitates (Figure 8, C and D, bottom sections). Taken together, the

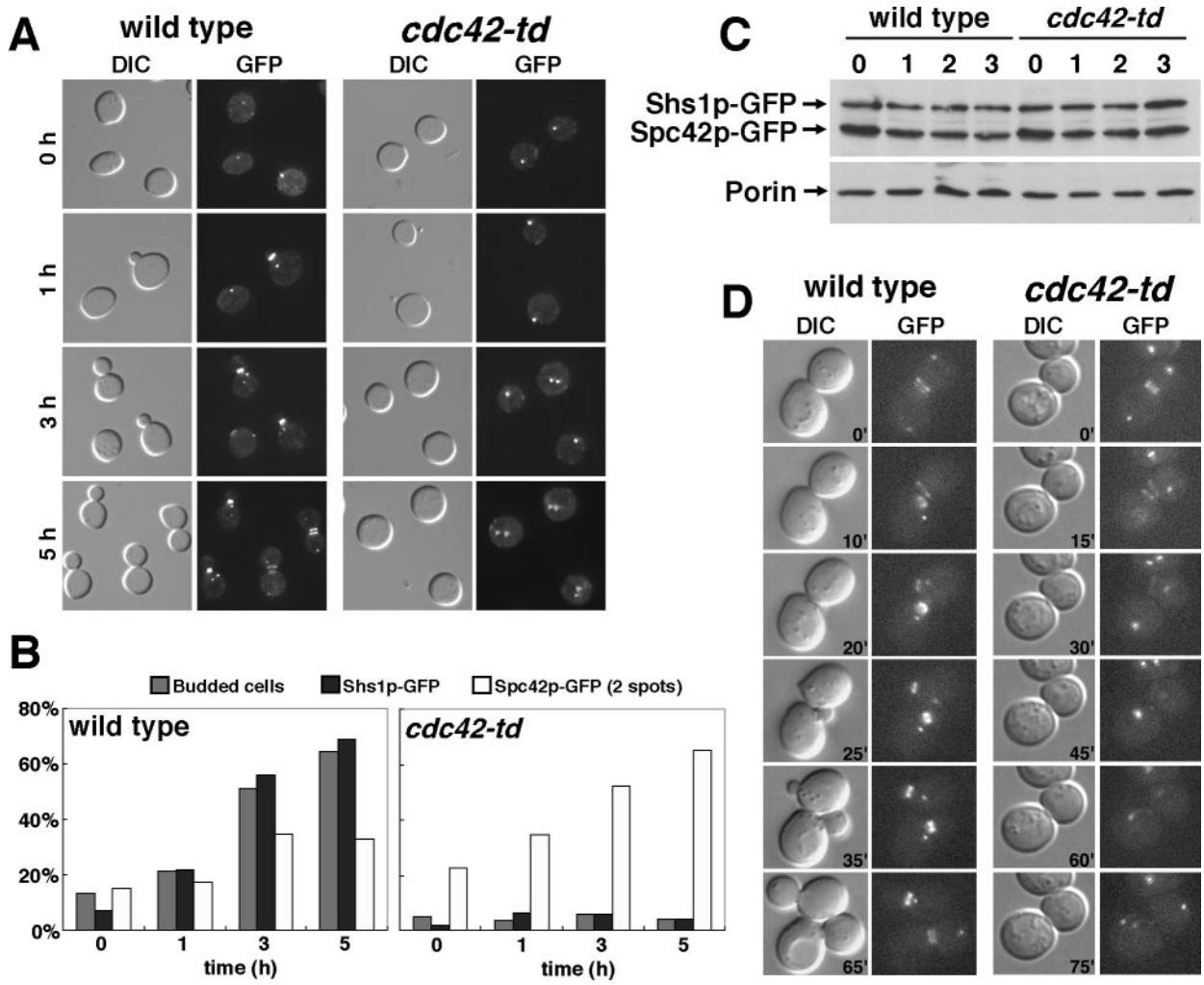

Figure 4. Cdc42p dependence of septin recruitment. Strains Masa1167 (*cdc42Δ ura3:3HA-CDC42 SHS1-GFP SPC42-GFP*) and Masa1183 (*cdc42Δ ura3:HA-CDC42-td SHS1-GFP SPC42-GFP*) were examined. (A and B) Cells were fixed and examined at the indicated times after release from G0 in medium without copper at 37°C (see *Materials and Methods*). (A) Images of representative cells. (B) Quantitation of cells with buds, with detectable cortical Shs1p-GFP structures (rings or patches), and with separated SPBs. Two hundred cells were counted for each time point. (C) Expression levels of Shs1p-GFP and Spc42p-GFP in wild-type and Cdc42p-depleted cells. Cells were grown, washed, and incubated further under Cdc42p-depleting conditions as described for Figure 3B. Samples collected at the indicated times (in hours) after the shift were analyzed by immunoblotting using antibodies to GFP and porin (as a loading control). (D) Cells grown as described for C were examined by time-lapse microscopy at 37°C beginning 25 (Masa1167) or 75 (Masa1183) min after the shift to Cdc42p-depleting conditions. Individual cells that were late in the cell cycle or had recently divided were identified and followed for 2–3 h. Times are indicated in minutes; the results shown are representative of those obtained with 88 wild-type cells and 38 ts-degron cells.

results suggest that Gic1p and Gic2p may interact directly with Cdc12p and perhaps also, more weakly, with Cdc3p and Cdc11p, to promote septin recruitment to the bud site.

Consistent with the apparent role of Gic1p and Gic2p in mediating Cdc42p-dependent septin recruitment and with the other evidence for a septin-Gic protein interaction, Cdc42p (Figure 9A), Gic1p (Figure 9B), Gic2p (Supplementary Figure 1), and Cdc3p (Figure 9, A and B, and Supplementary Figure 1) all colocalized at the presumptive bud site. In addition, both Gic1p and Gic2p colocalized with Cdc3p at the bud neck in medium- and large-budded cells (Figure 9B and Supplementary Figure 1); in contrast, Cdc42p was concentrated at the bud neck only in large-budded cells and was always sandwiched by Cdc3p (Figure 9A). Interestingly, in tiny- and small-budded cells, Gic1p and Gic2p did not colocalize with the septins and behaved somewhat differently from each other. In tiny-budded cells, both Gic proteins localized to the bud cortex, presumably reflecting a continued association with Cdc42p (Figure 9B, row 3; Supplementary Figure 1, row 3). However, Gic1p appeared to form a discrete cap at the tip of the bud that had little or no overlap with the septins, and such caps could often be seen also in small-budded cells (Figure 9B, row 4). In contrast, Gic2p appeared to localize to the entire cortex of tiny buds as well as to the neck, and the portion of Gic2p not at the neck appeared to disappear quickly as the bud grew (Supplementary Figure 1, rows 4 and 5). This difference in localization may reflect some differentiation in the functions of Gic1p and Gic2p during polarized growth.

DISCUSSION

In this study, we have addressed three major issues. First, does the septin ring at the presumptive bud site form by

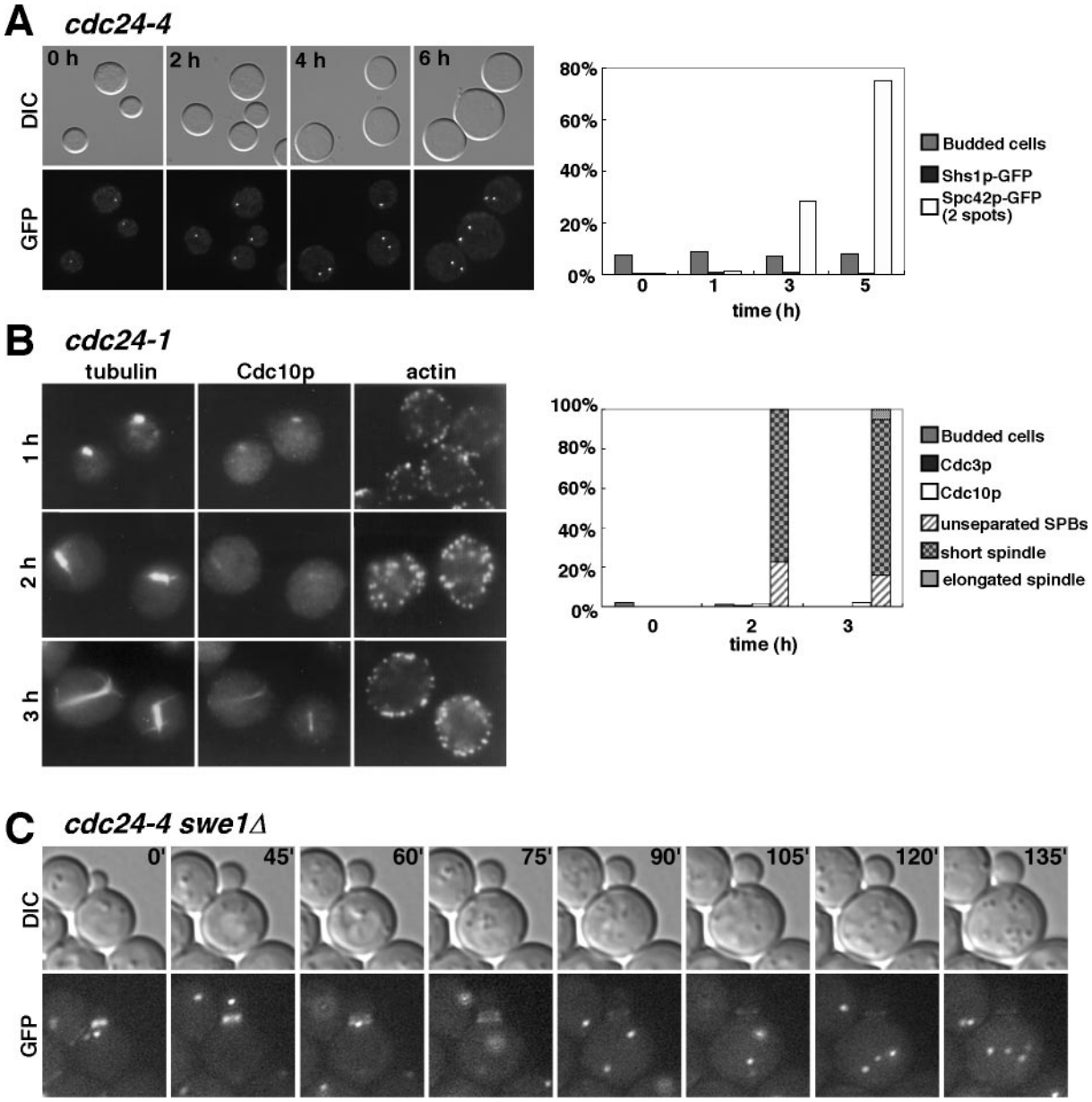

Figure 5. Cdc24p dependence of septin recruitment. (A) Strain Masa1234 (*cdc24-4 SHS1-GFP SPC42-GFP*) was grown and analyzed as described for Figure 4, A and B (except that no copper was added during the preparation of G0 cells). (B) Strain 5011-HO1 (*cdc24-1*) was grown to stationary phase in YM-P medium at 23°C and released into fresh YM-P medium at 37°C. After 0, 1, 2, and 3 h, samples were fixed with 3.7% formaldehyde and examined by immunofluorescence using antibodies to tubulin, Cdc3p, and Cdc10p; other samples were stained with rhodamine-phalloidin to visualize actin organization. Left, images of representative cells that were either double-stained for tubulin and Cdc10p (note some bleed-through of the anti-tubulin [rhodamine] signal into the anti-Cdc10p [FITC] channel with the optics used here) or stained for actin. Right, quantitation of the results (essentially as in Figure 4B; 100 cells were counted for each time point). Essentially identical results were obtained with the *cdc24-4* strain JPT19-HO5 (unpublished data). Because of difficulties in digesting stationary-phase cell walls (Deutch and Parry, 1974; Werner-Washburne *et al.*, 1993), we were unable to obtain consistent immunofluorescence staining of cells from the 0- and 1-h time points with either strain. (C) Strain Masa1246 (*cdc24-4 swe1Δ SHS1-GFP SPC42-GFP*) was grown to exponential phase in SC-Leu medium at 25°C and examined by time-lapse DIC and fluorescence microscopy at 37°C. Times are indicated in minutes; time zero was the time of shift to 37°C. The results shown are representative of those obtained with 23 cells.

direct recruitment of subunits into a ring, or does some other structure (such as a cap) form initially and then rearrange into a ring? Second, does the initial recruitment of the septins really depend on activated Cdc42p, as has long been thought? Third, if so, through which effector(s) does Cdc42p operate during this initial recruitment (as distinct from the subsequent steps in the organization of the septin ring and collar)? The results appear to provide unambiguous answers to the first two questions and a partial answer to the third.

Initial Recruitment of Septins to the Bud Site

Studies of various mutants had suggested that formation of the septin ring might normally proceed via the prior formation of a cap (see *Introduction*). However, our analysis by

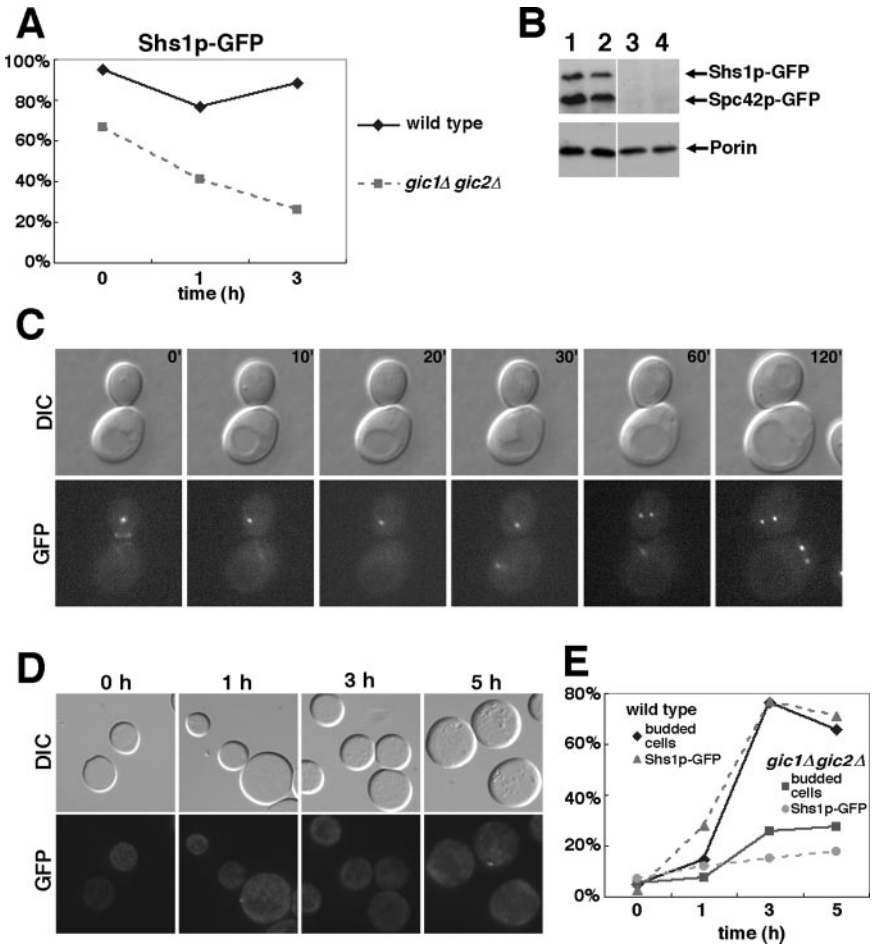

Figure 6. Defective septin recruitment in *gic1 gic2* mutants. (A–C) Loss of Shs1p-GFP localization, but not Shs1p-GFP protein, in *gic1*Δ *gic2*Δ cells at 37°C. (A) Strains Masa1147 (*GIC1 GIC2 SHS1-GFP*) and Masa1145 (*gic1*Δ *gic2*Δ *SHS1-GFP*) were grown to exponential phase in YM-P medium at 25°C and shifted to 37°C for the indicated times before quantitation of the cells with detectable cortical Shs1p-GFP structures (rings or patches). Two hundred cells (both unbudded cells and cells with buds of various size) were counted for each sample. (B) Strains Masa1212 (*gic1*Δ *gic2*Δ *SHS1-GFP SPC42-GFP*; lanes 1 and 2) and YEF473A (wild type; lanes 3 and 4) were grown to exponential phase in YM-P medium at 25°C and shifted to 37°C. Extracts prepared from cells before (lanes 1 and 3) and 3 h after (lanes 2 and 4) the shift were analyzed by immunoblotting using antibodies to GFP and porin. (C) Strain Masa1212 was grown to exponential phase in SC-Leu medium at 25°C and then examined by time-lapse microscopy in the same medium at 37°C. Times are indicated in minutes; time 0 as shown here was 50 min after the shift to 37°C. The images from earlier time points showed a seemingly normal progression from a tight septin collar to split septin rings (see Supplementary Movie 7). The Spc42p-GFP fluorescence shows the progression of the nuclear cycle. The cell shown is representative of 13 cells examined. (D and E) Inability of *gic1*Δ *gic2*Δ cells to localize Shs1p-GFP at 37°C. Cells of strains Masa1147 and Masa1145 were released from G0 at 37°C and examined as described in *Materials and Methods.* (D) Images of representative cells. (E) Quantitation of the cells with buds and with detectable cortical Shs1p-GFP structures (rings or patches). Two hundred cells were counted for each time point.

time-lapse microscopy, 3D image reconstruction, and quantitation of GFP-septin fluorescence shows that this is not the case: although the septins typically begin to concentrate near the presumptive bud site several minutes before a ring structure can be discerned, there is no sign of a distinct cap or other precursor structure that precedes the ring itself. Instead, the GFP-septins first appear as a diffuse and variable cloud that increases progressively in brightness (implying the continuing recruitment of subunits) and continues to do so after a ring structure can be discerned. When the actin cytoskeleton is disrupted by treatment with LatA, septin recruitment occurs, but the formation of a well-defined ring is significantly delayed. Taken together with other recent results (Caviston *et al.,* 2003; Dobbelaere *et al.,* 2003; Goehring *et al.,* 2003; Gladfelter *et al.,* 2004; Kadota *et al.,* 2004; Versele and Thorner, 2004; Rodal *et al.,* 2005), these observations suggest that the formation of the mature septin collar of the budded cell involves at least three distinguishable steps: recruitment, ring assembly, and ring maturation (Figure 10A).

This model leaves open the interesting question of whether the ring is templated (e.g., by forming at the periphery of the cap of Cdc42p) or assembles spontaneously once recruitment has increased the local septin concentration to a threshold level. The latter possibility appears to be supported by the ability of the septin caps in some mutant strains to reorganize into normal-looking rings/collars (Caviston *et al.,* 2003), the formation of ectopic septin rings of ~0.7-μm diameter in a strain overexpressing the septin-interacting protein Bni4p (Kozubowski *et al.,* 2003), and the ability of mammalian fibroblast septins to form rings of ~0.6-μm diameter both when incubated under appropriate conditions in vitro and when actin filaments are disrupted in vivo (Kinoshita *et al.,* 2002). However, further studies will be needed to resolve this issue.

Also unresolved are the related questions of the timing and roles of septin filament formation. Like the septins of *Drosophila* (Field *et al.,* 1996), *Xenopus* (Mendoza *et al.,* 2002), and mammals (Kinoshita *et al.,* 2002; Sheffield *et al.,* 2003), the yeast septins can form extended filaments in vitro (Frazier *et al.,* 1998; Versele *et al.,* 2004; Versele and Thorner, 2004). The septin rings observed by Kinoshita *et al.* (2002) in vitro consist of circumferentially oriented filaments, and it was long presumed that the "neck filaments" visualized in yeast by EM (Byers and Goetsch, 1976; Byers, 1981) also represent a circumferential array. However, more recent results have suggested that yeast septin filaments might instead run longitudinally through the mother-bud neck (Field *et al.,* 1996; Longtine *et al.,* 1998a, 2000; Kozubowski *et al.,* 2005) or form gauzelike structures that wrap around the neck (Rodal *et al.,* 2005), and it is also unclear whether filament formation is actually essential for septin function (Frazier *et al.,* 1998; Longtine *et al.,* 1998a). If the heterooligomeric septin complexes do assemble into longer filaments

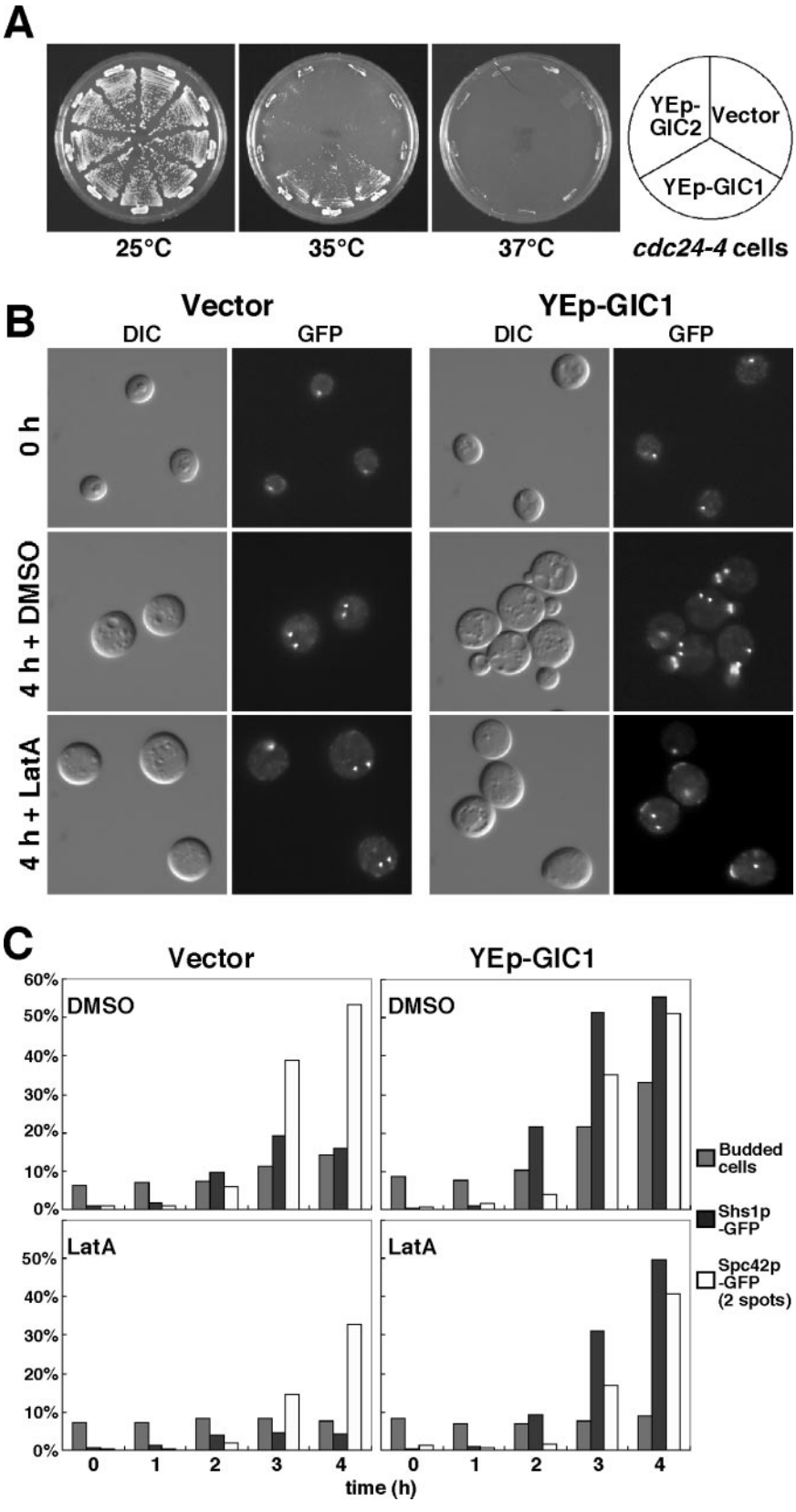

Figure 7. Suppression of the *cdc24* septin-recruitment defect by multicopy *GIC1*. (A) Strain YEF311 (*cdc24-4*) was transformed with plasmid pSM217 (vector), pCC904 (YEp-GIC1), or pCC967 (YEp-GIC2), streaked onto SC-Ura plates, and incubated at 25, 35, or 37°C for 3 d. (B and C) Strain Masa1234 (*cdc24-4 SHS1-GFP SPC42-GFP*) carrying plasmid pSM217 or pCC904 was released from G0 at 33.5°C in the presence of 1% DMSO or 200 μM LatA and 1% DMSO (see *Materials and Methods*). (B) Representative images of cells from the 0- and 4-h time points. (C) Quantitation of cells with buds, with detectable cortical Shs1p-GFP structures (rings or patches), and with separated SPBs at the indicated times after release. Three hundred cells were counted for each time point.

in vivo, it will be important to determine when this occurs; it could conceivably be before ring formation, during ring assembly, or only later when the ring matures into the collar at the time of bud formation. At present, the third possibility seems most likely, both because of the dramatic decrease in septin exchangeability that occurs coincident with collar

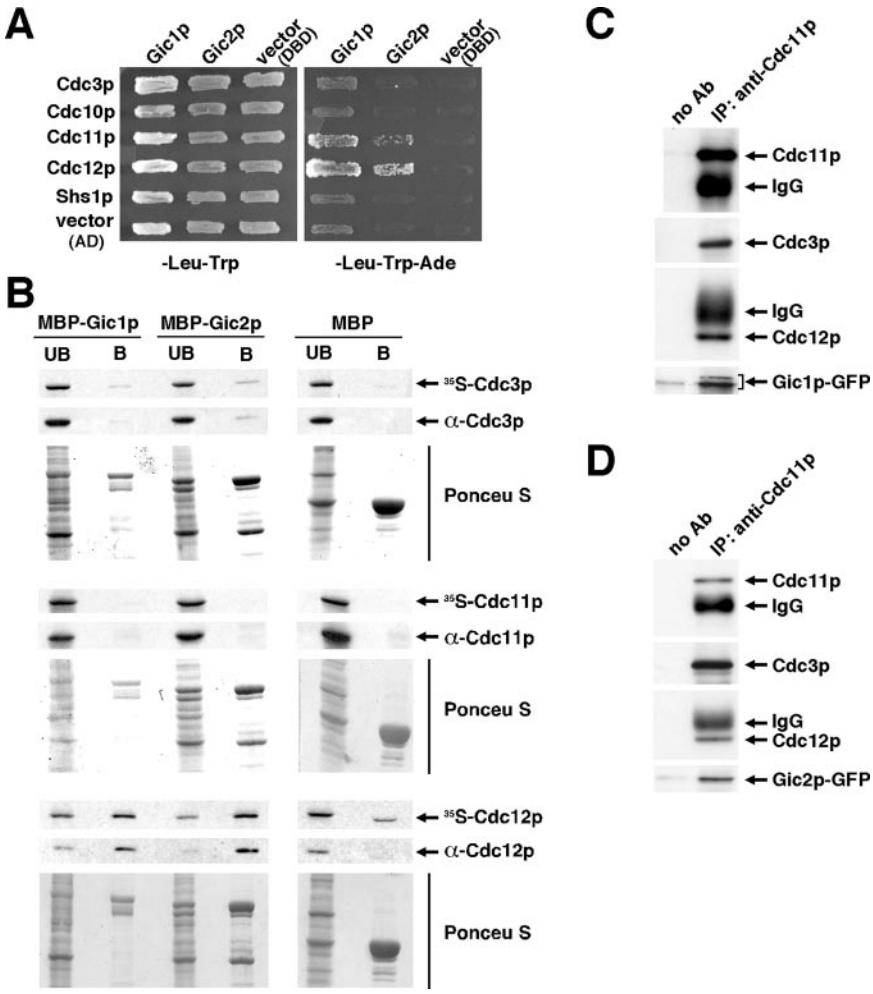

Figure 8. Physical interactions of Gic1p and Gic2p with septins. (A) Two-hybrid assays were conducted as described in *Materials and Methods*; growth on the -Ade plate (photographed after 9 d at 25°C) indicates interaction between the DBD and AD fusion proteins. (B) In vitro protein binding. MBP and fusions of MBP to full-length Gic1p or Gic2p were expressed in *E. coli* and purified, and ^{35}S-labeled full-length septins were synthesized in vitro and incubated with resin-bound MBP or MBP-fusion protein (see *Materials and Methods*). Unbound (UB) and bound (B) fractions were analyzed by SDS-PAGE followed by autoradiography and immunoblotting with antibodies to Cdc3p, Cdc11p, and Cdc12p. Ponceau S staining indicated comparable amounts of bound MBP and MBP-fusion proteins. It is not clear whether the band visible by autoradiography in approximately the Cdc12p position in the MBP, the B lane is actually Cdc12p (note its slightly different motility and lack of detection by immunoblotting, at least at the exposure used here). In any case, the ratios of band intensities in the UB and B lanes indicate that Cdc12p interacts with the Gic portions of the MBP-Gic fusion proteins. (C and D) Coimmunoprecipitation from yeast extracts. Extracts were prepared from strain YEF473 carrying pSM217-GIC1-GFP (C) or pSM217-GIC2-GFP (D) and analyzed by immunoprecipitation using anti-Cdc11p antibodies (see *Materials and Methods*). A mock immunoprecipitation was also performed using protein G-agarose beads but no antibodies; procedures and solution volumes were the same as in the real immunoprecipitation. The relative amounts of Cdc11p, Cdc3p, Cdc12p, and Gic1p-GFP or Gic2p-GFP in the mock and real immunoprecipitates were determined by immunoblotting.

formation (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003) and because the "neck filaments," which presumably represent some sort of higher-order structure, were never observed by EM in unbudded cells but only during and after bud formation (Byers and Goetsch, 1976; Byers, 1981).

Dependence of Septin Recruitment on Activated Cdc42p

Our results with the *cdc42*-ts-degron strain appear to show that the initial recruitment of the septins does indeed depend on Cdc42p. Moreover, the parallel results obtained with temperature-sensitive *cdc24* mutants imply that, as expected, Cdc42p function in septin recruitment depends on its activation by GTP binding. However, these results do not discriminate between a model in which GTP-bound Cdc42p, like Ras, binds effectors that then recruit the septins and a model in which Cdc42p, like the elongation factor EF-Tu in protein synthesis, must continuously cycle between GTP-bound and GDP-bound forms to effect septin recruitment. For later steps in the organization of the septin ring and collar, studies of *cdc42* mutants defective in GTP hydrolysis and of mutants defective in the Cdc42p GAPs support the latter model (Gladfelter *et al.*, 2002; Smith *et al.*, 2002; Caviston *et al.*, 2003). However, because these same mutants can recruit the septins to the presumptive bud site (albeit into abnormally organized structures), it seems likely that the Ras model may apply to the initial recruitment step.

Despite its dependence on Cdc24p and Cdc42p, septin recruitment was not detectably delayed by an absence of Rsr1p, which normally determines the site of budding by interacting with Cdc24p and Cdc42p (see *Introduction*). This observation is consistent with other recent evidence that in the absence of the normal positional cue, the localization and activation of Cdc42p can occur efficiently by one or more positive-feedback processes (Butty *et al.*, 2002; Irazoqui *et al.*, 2003; Kawasaki *et al.*, 2003; Wedlich-Soldner *et al.*, 2003).

Role of the Gic Proteins as Cdc42p Effectors for Septin Recruitment

Regardless of whether a Ras- or an EF-Tu-type model for Cdc42p function in septin recruitment proves to be correct, it will be important to determine the proteins with which Cdc42p interacts during this process. Of the many proteins known previously to participate in septin organization (see *Introduction*), all appear to be involved in the assembly of the ring, its maturation into the collar, or both (Figure 10A). In contrast, we have here presented evidence that in the absence of the structurally related Gic1p and Gic2p proteins, septin recruitment does not occur in haploid cells at 37°C.

The Gic proteins have previously been implicated in polarization of the actin cytoskeleton (Brown *et al.*, 1997; Chen *et al.*, 1997; Bi *et al.*, 2000; Jaquenoud and Peter, 2000) and in the stabilization of Cdc42p-effector complexes at the pre-

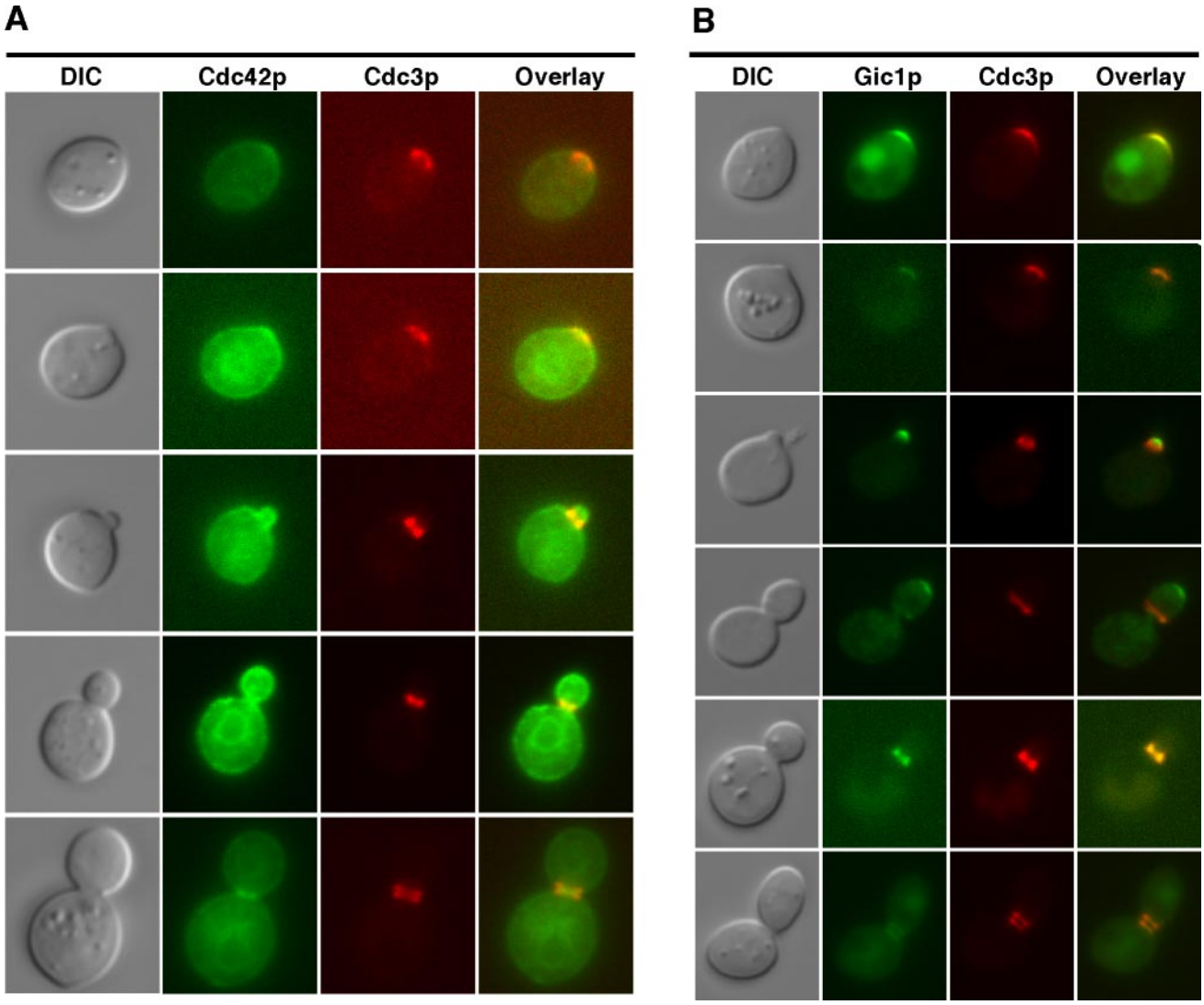

Figure 9. Localization of Cdc42p, Gic1p, and Cdc3p through the cell cycle. (A) Cells of strain JGY1326 (*CDC3-RFP*) carrying plasmid YEp352-GFP-CDC42 were grown to exponential phase in SC-Ura medium at 25°C, fixed with formaldehyde, and observed by DIC and fluorescence microscopy. (B) Strain JGY1326 carrying plasmid pSM217-GIC1-GFP was observed as described for A.

sumptive bud site (Kawasaki *et al.*, 2003). Moreover, although actin function is not required for the initial polarization of Cdc42p to the presumptive bud site (Ayscough *et al.*, 1997), it does appear to be necessary to maintain Cdc42p polarization (Irazoqui *et al.*, 2005). Thus, it is conceivable that the effects of *GIC* gene deletion or overexpression on septin recruitment are indirect consequences of the other roles of the Gic proteins in cell polarization. However, there are several arguments against this interpretation. First, the complete loss of F-actin and of bud formation only slightly retards septin recruitment (Ayscough *et al.*, 1997; Harkins *et al.*, 2001; Figure 2C). Second, the suppression of the septin-recruitment defect in *cdc24* mutants by overexpression of *GIC1* appears to be very efficient (indeed, more efficient than the accompanying suppression of the actin-polarization defect) and does not depend on F-actin or bud formation (Figure 7, B and C). Finally, two-hybrid, in vitro-binding, coimmunoprecipitation, and colocalization analyses all indicate that the Gic proteins interact directly with the septins (Figures 8 and 9). Thus, it seems most likely that the Gic proteins play a direct role in septin recruitment.

However, it is also clear that the Gic proteins cannot be the only Cdc42p effectors involved in septin recruitment. The *gic1*Δ *gic2*Δ double-mutant haploid recruited septins and grew well at 25°C, and, at least in some strain backgrounds, *gic1*Δ *gic2*Δ double-mutant diploids grow reasonably well even at 37°C (Bi *et al.*, 2000). Thus, there must be at least one other pathway involved in septin recruitment (Figure 10A). Other remaining mysteries include the observations that despite its efficient suppression of *cdc24* mutations, multicopy *GIC1* suppressed *cdc42* mutations poorly or not at all, and that multicopy *GIC2* suppressed neither *cdc24* nor *cdc42* mutations and indeed exacerbated the temperature sensitivity of the latter. Although further studies will be needed to clarify these issues, it is probably relevant that the level of Gic2p in the cell is normally tightly controlled at bud emergence by ubiquitin-dependent, SCF^{Grr1}-mediated degradation and that overexpression of Gic2p in some genetic backgrounds causes severe defects in cell growth and morphology (Jaquenoud *et al.*, 1998; Jaquenoud and Peter, 2000).

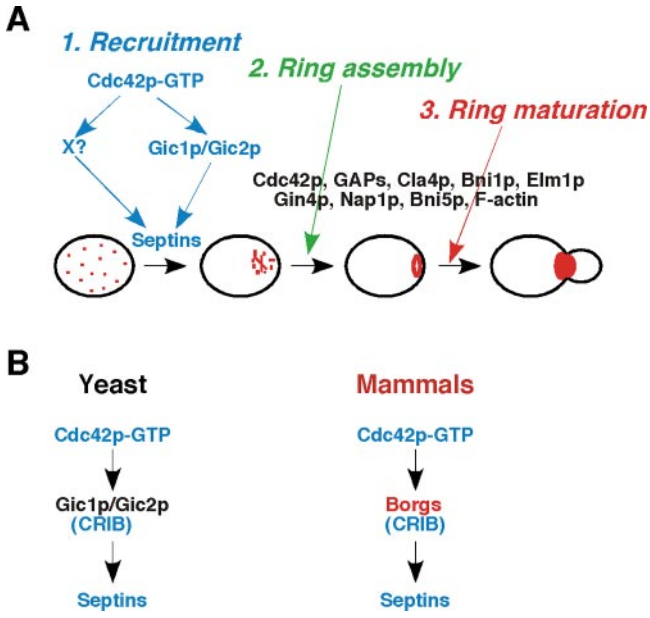

Figure 10. Cdc42-dependent septin assembly. (A) In yeast, assembly of the septin ring appears to occur in at least three steps: (1) recruitment of the septins to the presumptive bud site, which depends on activated Cdc42p and at least two partially redundant effector pathways; (2) initial assembly of the septins into a cortical ring in which the subunits are still highly exchangeable; (3) maturation of the initial ring into the mature septin collar at the bud neck, in which the subunits do not exchange freely. Both steps 2 and 3 appear to depend on the cycling of Cdc42p between GTP- and GDP-bound forms and on a variety of other proteins. These proteins are probably differentially involved in the assembly and maturation steps (Gladfelter *et al.*, 2004 and references cited therein), but we do not address this here. (B) Possible conservation of septin-organization pathways. Both in yeast and in mammalian cells, Cdc42 organizes the septins at least in part through the binding of Cdc42-GTP to CRIB-domain-containing proteins that in turn bind to septins. See text for more details.

Are Cdc42p-controlled Septin-Organization Pathways Conserved?

Cdc42p appears to effect septin recruitment in part by a direct interaction of septins with Gic1p and Gic2p, both of which contain a CRIB site that binds to Cdc42p-GTP (Brown *et al.*, 1997; Chen *et al.*, 1997). No close homologues of Gic1p and Gic2p have been found except in closely related fungi. However, the mammalian Cdc42 effector Borg3 also contains a CRIB site and interacts directly with the septin complexes containing Sept6 and Sept7 (Joberty *et al.*, 2001; Sheffield *et al.*, 2003). In in vitro protein-interaction assays, Cdc42 inhibited the interaction between Borg3 and Sept6/7 complexes, but it is not known how Cdc42 regulates septin organization through Borg3 in vivo (Joberty *et al.*, 2001). No homologues of Borg3 have been found in evolutionarily distant species such as yeast and *Drosophila*. Thus, it appears that the highly conserved small GTPase Cdc42p regulates septin organization in different organisms through proteins that contain a conserved Cdc42p-binding site but otherwise are species-specific in structure (Figure 10B). These structural features presumably allow specific regulatory mechanisms to coordinate septin organization with other species-specific processes.

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