The Majority of the *Saccharomyces cerevisiae* Septin Complexes Do Not Exchange Guanine Nucleotides*  

We show here that affinity-purified *Saccharomyces cerevisiae* septin complexes contain stoichiometric amounts of guanine nucleotides, specifically GTP and GDP. Using a $^{15}$N-dilution assay read-out by liquid chromatography-tandem mass spectrometry, we determined that the majority of the bound guanine nucleotides do not turn over in vivo during one cell cycle period. In vitro, the isolated *S. cerevisiae* septin complexes have similar GTP binding and hydrolytic properties to the *Drosophila* septin complexes (Field, C. M., al-Awar, O., Rosenblatt, J., Wong, M. L., Alberts, B., and Mitchison, T. J. (1996) *J. Cell Biol.* 133, 605–616). In particular, the GTP turnover of septins is very slow when compared with the GTP turnover for Ras-like GTPases. We conclude that bound GTP and GDP play a structural, rather than regulatory, role for the majority of septins in proliferating cells as GTP does for α-tubulin.

Septins are a family of proteins initially identified in *Saccharomyces cerevisiae* and later found in most eukaryotic organisms (1–4). They are important for cytokinesis (2, 4) and membrane trafficking (5–7). The *S. cerevisiae* septins play additional roles in bud morphogenesis (1, 8), chitin deposition (9), and spindle positioning (10). The molecular function of septins in all of the above processes is not well understood. Electron microscopy (11) and immunofluorescence (12–14) experiments in wild-type and temperature-sensitive septin mutants suggest that the *S. cerevisiae* septins assemble into higher-order structures in vivo. One hypothesis is that septins form molecular scaffolds at specific cortical locations and recruit diverse effector proteins (15, 16). Recruitment to the septin scaffold might then catalyze interactions between certain effectors by proximity effects.

Multiple septin proteins (polypeptides) are expressed in each of the studied organisms. When isolated, the septin polypeptides are found in tight complexes with defined stoichiometries (17–19). Five vegetative septins were identified in *S. cerevisiae*: Cdc3, Cdc10, Cdc11, Cdc12, and Shs1 (Sep7) (1) (20). When a peptide antibody against Cdc3 was used for affinity purification, the four Cdc septins were isolated as a defined complex (19). Although the precise stoichiometry of each protein in the complex is not known, an approximate stoichiometry was calculated using gel filtration, sucrose gradients, and quantitation of Coomassie R-250-stained bands (19). These experiments suggest that Cdc3, Cdc10, Cdc11, and Cdc12 are the major constituents of the septin complex, and the approximate stoichiometry of these proteins is 2:2:1:2, respectively (19).

Septins have a characteristic domain structure: an N terminus with variable length and sequence, a conserved central region, and with a few exceptions, a C terminus predicted to form coiled-coils (15). The conserved central region contains Ras-like GTP-binding motifs (4). Indeed, Field et al. (17) showed that *Drosophila* septin complexes copurify with tightly bound GTP and GDP and have GTPase activity in vitro. However, GTP binding/exchange of the *Drosophila* septin complex was very slow in vitro when compared with the activities of generic Ras-like small GTPases (21). In contrast, individual recombinant septins (*Xenopus* (22), mammalian (4, 23)) and reconstituted partial septin complexes (mammalian (23)) show considerably faster GTP binding/exchange kinetics.

The role of septins’ GTP binding and hydrolysis is not well understood, and it constitutes the subject of this report. GTP might turn over rapidly and regulate the function of septins, as in the cases of β-tubulin (24, 25), FtsZ (26), and small GTPases. Alternatively, GTP might incorporate once during folding or complex assembly and play no further role in the function of septins, analogous to GTP bound to α-tubulin (27, 28). In vitro experiments implicate GTP binding in filament formation by an individual *Xenopus* septin (22) and possibly in the interaction between different septin polypeptides (23). However, the *in vivo* significance of these observations has not been tested. The cited papers echo the prevailing assumption in the field that GTP binding plays a regulatory role. Here we use *in vivo* and biochemical assays to test whether GTP plays a regulatory or a structural role. Our data favor the latter model, which should trigger a significant change in the direction of the field.

**EXPERIMENTAL PROCEDURES**

*Saccharomyces cerevisiae* Strains

The YEF473 strain (a/α ura3–52/ura3–52 lys2–801/lys2–801 leu2–Δ1/leu2–Δ1 his3–Δ200/his3–Δ200 trpl–Δ63/trpl–Δ63) (29) was used for isolating septin complexes for the *in vitro* GTP-binding experiments. The YEF473 strain was a gift from Prof. Mark Longtine (Oklahoma State University). FY4 strain (a prototrof, S288C background) was used for the *in vivo* nucleotide turnover experiment. The FY4 strain was obtained from Prof. Fred Winston’s laboratory (Harvard Medical School).

**Reagents**

Anti-Cdc3 antibody was raised against a synthetic peptide corresponding to the C terminus of the Cdc3 protein ([C(NHS)PVTKKK]FLLR), as described previously (19). Anti-Shs1 antibody was a gift from Prof. Douglas Kellogg (University of California at Santa Cruz) (30). ($^{15}$N)H$_2$SO$_4$, 98+ atom % $^{15}$N was purchased from Aldrich Chemical Co.

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The results of six independent experiments are shown. The yeast extract was obtained either by head beat or by the mortar and pestle method, as indicated. The septin complexes were precipitated either by incubation with PEG 8000 for 2 h at 4 °C followed by 25 min centrifugation at 390,880 × g, or by 2-h dialysis to 75 mM KCl at 4 °C followed by 15 min centrifugation at 390,880 × g. Dialysis promotes the association of septin complexes into long filaments that can be pelleted using the above conditions. The bound nucleotide was quantified using co-injection with ATP standards into the SMART system (see “Nucleotide Content Analysis”). The septin protein retrieved from the 30-kDa cut-off filter (see “Nucleotide Content Analysis”) was quantified by running it on a 10% polyacrylamide gel together with BSA standards of known concentrations. Quantity One software was used for analysis. The protein concentration is probably underestimated by our method because of the limitations of the technique (using BSA as a standard) and to the multiple inherent manipulations (spinning through the 30-kDa cut-off filter, transferring to tubes) before the final measurement. The nucleotide concentration is internally controlled using ATP and therefore much more accurate. Thus, the molar ratio of total nucleotide to protein is overestimated. The first number in the Nucleotide/protein ratio columns were determined using the 2:2:1:2 stoichiometry, whereas the second one of each set was determined using the 1:2:2:2:2 stoichiometry (see “Experimental Procedures”).

### Table I

**Summary of nucleotide content analysis experiments**

| Experiment                      | Total nucleotide | Total protein | Nucleotide/protein ratio | GDP/GTP |
|---------------------------------|------------------|---------------|--------------------------|--------|
| PEG precipitation/Bead beating  | 101.7            | 80.5/103.5    | 1.26/0.98                | 2.17   |
| PEG precipitation/Bead beating  | 114              | 95.2/122.4    | 1.2/0.93                 | 2.19   |
| PEG precipitation/Bead beating  | 146              | 97.5/125.3    | 1.5/1.16                 | 2.12   |
| PEG precipitation/Bead beating  | 136.5            | 225/289.5     | 1.32/1.04                | 2.24   |
| PEG precipitation/Bead beating  | 140/180          | 1.70/3.5      | 2.28                     |        |

### Septin Complex Purification

*S. cerevisiae* septin complexes were purified using a variation of a purification method published previously (19). Briefly, YEF473 frozen yeast was pulverized with a liquid N2-chilled mortar and pestle. The resulting yeast powder was thawed shortly at room temperature. All of the following steps were performed at 4 °C. Extract buffer (50 mM Tris-HCl, pH 7.8, 250 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, and 0.2% Triton X-100, 1 mM Dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM chymostatin, and 1 mM pepstatin) was added to the thawed powder in a ratio of 1:1.3 (mass/ml). The mixture was gently rotated for 1 h and then spun for 25 min at 390,880 g. The supernatant was supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM chymostatin, and 1 mM pepstatin and incubated for ~210 min with (anti-Cdc3)-protein A beads (Affi-Prep Protein A Support, Bio-Rad; previously incubated with anti-Cdc3 antibody for 2 h at 4 °C). The beads were pelleted, and the supernatant depletion of Cdc3, as assessed by Western blotting analysis, was ~80%. The beads were washed four times with eight volumes of wash buffer (50 mM Tris-HCl, pH 7.8, 250 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, and 0.2% Triton X-100) over a period of ~90 min. The beads were filtered through nitrocellulose and washed as above. Urea solution (8 M, 50 mM Tris-HCl, pH 7.8, 75 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, 1 mM Dithiothreitol, and 4% sucrose, and the resulting septin filaments (most of the septin protein) were then pelleted at 390,880 g for 15 min. The septin pellet was resuspended in 8 mM urea, and the nucleotides were isolated as above. For nucleotide quantitation, we added known amounts of ATP to the septin pellet prior to urea denaturation, injected the total isolated nucleotides on the SMART system, and monitored the absorbance at 254 nm. The ATP signal was corrected for the difference in extinction coefficient between ATP and guanine nucleotides and was used to calculate the absolute amounts of GTP and GDP.

### GTP Hydrolysis Assay

This experiment was performed in parallel to the filter-binding assay. Two extra samples were taken at each time point. These samples were filtered through nitrocellulose and washed as above. Urea solution (8 M, 50 mM ATP) was added immediately to each filter to denature the bound protein. Samples of the nucleotides released (25 μl) were spotted onto a TLC plate (Baker-flex, cellulose RF, together with ATP and GDP standards) and spray-stained with Coomassie G-250 (31). The amount of Cdc3 in the sample was determined using a Filter-binding assay.

### Filter-binding assay

Septin complexes in 1 mM KCl (as eluted from the antibody beads) were dialyzed for 2 h at 4 °C against 20 mM Tris-HCl, pH 7.8, 75 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, 1 mM Dithiothreitol, and 4% sucrose. The dialyzed septin complexes were supplemented with [32P]-GTP (at a final concentration of 2 μM (specific activity (SA), 150–300 Ci/mmols). The samples were incubated at 30 °C. Aliquots were withdrawn at specific times, diluted in ice-cold wash buffer, and applied to 1.3 cm × 1.3 cm nitrocellulose filters (Schleicher & Schuell, 0.45 μm) connected to a vacuum system. Two to three aliquots were used for every time point. The filters were then washed with 5 ml of wash buffer (20 mM Tris-HCl, pH 7.8, 100 mM KCl, 0.5 mM EGTA, and 2 mM MgCl2) and dissolved in 250 μl of acetone. Scintillation mixture (ScintiSafe Plus 50%, Fisher Scientific, 5.6 ml) was added to each of them. The mixture was briefly vortexed and counted for 2 min.

### Nucleotide Content Analysis

The nucleotide content of the septin proteins was determined using a variation of a procedure published previously (17, 32, 33). The following manipulations were performed at 4 °C. Poly(ethylene glycol) (PEG)1 8000 was added to the septin complex preparation at a final concentration of 10%. The mixture was incubated for 2 h and then spun for 25 min at 390,880 × g using a TLA 100 rotor and a Beckman Coulter Optima TL Ultracentrifuge. Most of the septin protein were pelleted in this way. The pellet was resuspended in 20 μl of 8 mM urea, heated at 100 °C for 1 min, and diluted with 20 μl of water. The resulting 40 μl were spun through a 30-kDa cut-off filter (Microcon, Millipore) at 10,000 × g. Water (40 μl) was then added to the top of the filter, and the spin was repeated. The total filtrate was applied to a Pharmacia Biotech SMART system MonoQ PC 1.6/5 column. The nucleotide bound to the column was eluted using a gradient of NH4HCO3 (from 100 to 500 mM over 3 ml). The supernatant of the PEG 8000-pelleted septins was supplemented with 8 mM urea, heated 1 min at 100 °C, diluted to 2 ml in water, and then applied to the MonoQ column. We found that the supernatant contained only trace amounts of nucleotides. Alternatively, the septin complex was dialyzed for 2 h at 4 °C against 20 mM Tris-HCl, pH 7.8, 75 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, 1 mM Dithiothreitol, and 4% sucrose, and the resulting septin filaments (most of the septin protein) were pelleted at 390,880 × g for 15 min. The septin pellet was resuspended in 8 mM urea, and the nucleotides were isolated as above. For nucleotide quantitation, we added known amounts of ATP to the septin pellet prior to urea denaturation, injected the total isolated nucleotides on the SMART system, and monitored the absorbance at 254 nm. The ATP signal was corrected for the difference in extinction coefficient between ATP and guanine nucleotides and was used to calculate the absolute amounts of GTP and GDP.

### Determination of Septin Complex Concentrations

The purified septin complexes together with bovine serum albumin (BSA) standards were run on a 10% SDS-PAGE and stained with Coomassie G-250 (31). The amount of Cdc12 in the sample was determined based on the Coomassie G-250 staining intensity of the BSA standards using Quantity One software. The precise stoichiometry of the septin complex is not known. Two extreme stoichiometries were assumed for determining the molarity of the GTP-binding sites (Table I: one was 2 Cdc3/2 Cdc10:1 Cdc11:2 Cdc12; and the other was 1 Shs1:1.2 Cdc3:2 Cdc10:2 Cdc11:2 Cdc12). The molar concentration of the septin complex is the same in both cases using our analysis method, but the molarity of the GTP-binding sites is different by a factor of 9/7 = 1.28. The molar concentration of the septin complexes in our preparations ranged from ~100 to 300 nm.
Dynamics phosphorimager screen. The screen was analyzed using a Bio-Rad Molecular Imager and Quantity One software.

UV Cross-linking

Samples of the dialyzed septin complexes were incubated with [α-32P]GTP (3 μM GTP SA, 300 CPM/mol) and 300 μM ATP at 30 °C with or without competing GTP at 2 μM. The samples (25 μl) were taken at each time point and used as follows. Three samples of 5 μl each were counted using the filter-binding assay. The remaining 10 μl were mixed with additional ATP and 2-mercaptoethanol at a final concentration of 300 μM ATP and 1% 2-mercaptoethanol. The resulting mixture was incubated on ice for 1 min and then cross-linked on ice for five periods of 3 min each, with a 1 min pause between each cross-linking period. The samples were then run over a SMART Dynamics phosphorimager screen. The screen was analyzed using a Bio-Rad Molecular Imager and Quantity One software.

Nucleotide Content Analysis

Sections of gel corresponding to Cdc3, Cdc10, Cdc11, Cdc12 and Cdc13 migration at each time point were excised individually and stained with Coomassie R-250. The septin protein bands were cut out of the overnight culture each. The cultures were grown at 30 °C until yeast nitrogen base without amino acids and (NH₄)₂SO₄, and 20 g/l ice-cold 1 M formic acid saturated in 1-butanol to lyse the cells, precipitating the septins with PEG 8000, denaturing them with 8 M urea, transferred to new flasks containing 650 ml of chase medium (5 g/l (15NH₄)₂SO₄, 1.7 g/l yeast nitrogen base without amino acids and (NH₄)₂SO₄, 0.06 g/l guanosine, and 20 g/l glucose) each. The doubling times were Lyophilized and analyzed by mass spectrometry. The supernatant was frozen in liquid N₂ and lyophilized. The small molecules were lyophilized and analyzed by mass spectrometry.

In Vivo GTP Exchange Assay

Yeast Growth, Collection, and Processing—An FY4 yeast strain was grown in liquid minimal medium containing 2.5 g/l (NH₄)₂SO₄, 1.7 g/l yeast nitrogen base without amino acids and (NH₄)₂SO₄, and 20 g/l glucose. The culture was grown for ~34 h to a final A₅₆₅ of 7. Four flasks containing 600 ml of 15N minimal medium were inoculated with 45 ml of the overnight culture each. The cultures were grown in liquid minimal medium containing 2.5 g/l (NH₄)₂SO₄, 1.7 g/l yeast nitrogen base without amino acids and (NH₄)₂SO₄, 0.06 g/l guanosine, and 20 g/l glucose) each. The doubling time was ~3 h under these conditions. The cultures were grown for 0, 1, 2, and 3 h in the chase medium, respectively. From each culture, 600 ml were spun at 2,600 g for 25 min. The pelleted yeast was frozen in liquid N₂. The remaining 45 ml were filtered through a 47-mm Millipore BV, Millipore), and the filters were lyophilized and analyzed by liquid chromatography-selected ion monitoring. The pooled fractions were lyophilized and used as follows. Three samples of 5 μl each were counted using the filter-binding assay. The remaining 10 μl were mixed with additional ATP and 2-mercaptoethanol at a final concentration of 300 μM ATP and 1% 2-mercaptoethanol. The resulting mixture was incubated on ice for 1 min and then cross-linked on ice for five periods of 3 min each, with a 1 min pause between each cross-linking period. The samples were then run over a SMART Dynamics phosphorimager screen. The screen was analyzed using a Bio-Rad Molecular Imager and Quantity One software.
ods with a Q1 resolution of 0.4 FWHM; for all (M-
H) ions, the selected ion monitoring scan time was 700 milliseconds with a Q1 resolution of 0.2 FWHM. Each range was scanned in alternation. Cdc3: SDILTDEEILSKF (14 nitrogens), (M-
H) ions with a Q1 resolution of 0.4 FWHM; for all (M-
H) ions, the selected ion monitoring scan time was 700 milliseconds with a Q1 resolution of 0.2 FWHM. Each range was scanned in alternation. Cdc3: SDILTDEEILSKF (14 nitrogens), (M-
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H) ions with a Q1 resolution of 0.4 FWHM; for all (M-
H) ions, the selected ion monitoring scan time was 700 milliseconds with a Q1 resolution of 0.2 FWHM. Each range was scanned in alternation.

We determined that there was no significant in vivo exchange of the septin-bound GTP and GDP. Because yeast actively grew during our chase period (approximately one cell cycle), the 15N-labeled amino acids and nucleotides were diluted with the newly synthesized 14N material. If a particular protein and/or nucleotide pool did not turn over, the 15N to 14N ratio would be 0.5 by the time the cells doubled once. This is approximately what we observe for both septin-bound GTP + GDP (data for GDP (M-H) - shown in Fig. 2A, second row, and Fig. 2B, solid squares); similar data not shown for GTP (M-H) - and the septin proteins (data for the SWDPIIK Cdc3 peptide shown in Fig. 2A, third row; data for peptides from other septin proteins shown in Fig. 2B, open squares and solid squares). Thus, septin-bound GTP and GDP do not turn over during one cell cycle period. Because the yeast was not synchronized, each cell would have visited every cell cycle state at the end of the chase period (approximately one cell cycle). Thus, we can rule out the possibility that a certain cell cycle transition could cause rapid GTP turnover in the bulk population of septins. This scenario would have resulted in an almost complete turnover of the labeled GTP by the end of the chase period. Because this is a bulk measurement, we would, however, miss fast GTP turnover in a small subpopulation of septin complexes, or in a single, special GTP-binding site within the septin complex (see “Discussion”).

| Column I, protein | Column II, % total protein (mol) | Column III, % time-dependent cross-links | Column IV, % normalized (to % mol of protein, Column II) time-dependent cross-links |
|-------------------|---------------------------------|------------------------------------------|--------------------------------------------------------------------------------|
| Shs1 (Sep7)       | 14.27                           | 17.7                                     | 25.35                                                                          |
| Cdc3              | 20.32                           | 6.8                                      | 6.85                                                                           |
| Cdc11             | 17.1                            | 11.2                                     | 13.39                                                                          |
| Cdc12             | 24.98                           | 31.1                                     | 25.35                                                                          |
| Cdc10             | 23.32                           | 33.1                                     | 29.04                                                                          |

Table II: Quantiﬁcation of the UV cross-linking experiment

The Coomassie G-250-stained gel shown in Fig. 5 was scanned, and the protein bands were quantiﬁed using Quantity One software. The intensity of each band after background subtraction was normalized by the molecular weight of that particular protein, and percentages for each of the normalized intensities from the total normalized intensities were calculated (Column II). The intensity of the time-dependent UV cross-links was calculated as follows. Intensities of each band of the phosphorimager scan were calculated for both the 0-h and the 5-h time points using Quantity One software. The 0-h intensities were subtracted from their respective 5-h intensities. A percentage was calculated for the adjusted intensity from the sum of the total adjusted intensities (Column III). The percentage of time-dependent cross-links normalized to the molar amount of protein is shown in Column IV. For Shs1, the protein quantitation data represent the sum of the Shs1 bands that do not overlap with Cdc3, whereas the UV cross-linking quantitation data represent the slower migrating form that signiﬁcantly cross-links GTP.

To determine turnover rates of nucleotide and protein in the same experiment, we used a 15N-dilution assay. Speciﬁcally, we labeled the nucleotide and protein pools with 15N by growing yeast in medium containing (15NH4)2SO4 as the sole nitrogen source. The label was then chased with medium containing exclusively 14N nitrogen sources. Subsequently, samples were analyzed by mass spectrometry, and the ratio of 15N to 14N was measured in three samples: total cellular GTP + GDP (to determine how fast the label was diluted in the pool), septin-bound GTP + GDP, and septin polypeptides (Fig. 2B). The turnover rate of the cellular GTP + GDP pool was not measured precisely. However, we found that the 15N GDP (M-H) - ion (that originates from both GTP and GDP) was almost completely turned over during the ﬁrst hour of the chase (−1/3 of the cell cycle under these growth conditions) (Fig. 2A, ﬁrst row, and Fig. 2B, solid circles). This is sufﬁciently fast to allow turnover analysis for septin-bound GTP and GDP. The turnover rate for the soluble amino acids pool was not measured, but we assume that it is at least as fast as the total guanine nucleotide pool. Note that because we did not make this measurement, we cannot exactly compare the turnover rates of protein and bound nucleotides.

We determined that there was no signiﬁcant in vivo exchange of the septin-bound GTP and GDP. Because yeast actively grew during our chase period (approximately one cell cycle), the 15N-labeled amino acids and nucleotides were diluted with the newly synthesized 14N material. If a particular protein and/or nucleotide pool did not turn over, the 15N to 14N ratio would be 0.5 by the time the cells doubled once. This is approximately what we observe for both septin-bound GTP + GDP (data for GDP (M-H) - shown in Fig. 2A, second row, and Fig. 2B, solid squares); similar data not shown for GTP (M-H) - and the septin proteins (data for the SWDPIIK Cdc3 peptide shown in Fig. 2A, third row; data for peptides from other septin proteins shown in Fig. 2B, open squares and solid squares). Thus, septin-bound GTP and GDP do not turn over during one cell cycle period. Because the yeast was not synchronized, each cell would have visited every cell cycle state at the end of the chase period (approximately one cell cycle). Thus, we can rule out the possibility that a certain cell cycle transition could cause rapid GTP turnover in the bulk population of septins. This scenario would have resulted in an almost complete turnover of the labeled GTP by the end of the chase period. Because this is a bulk measurement, we would, however, miss fast GTP turnover in a small subpopulation of septin complexes, or in a single, special GTP-binding site within the septin complex (see “Discussion”).
To confirm the lack of GTP exchange in the bulk septin population and to test for the presence of faster exchanging guanine nucleotide sites, we examined GTP exchange and GTP hydrolysis in vitro. We isolated *S. cerevisiae* septin complexes by immunoprecipitation and peptide elution, incubated them with [α-32P]GTP, and filtered them through nitrocellulose at

![In vivo turnover analysis of cellular GTP + GDP, septin-bound GTP + GDP, and septin polypeptides.](image-url)

Fig. 2. *In vivo* turnover analysis of cellular GTP + GDP, septin-bound GTP + GDP, and septin polypeptides. A prototrophic yeast strain was grown in minimal medium containing (15NH4)2SO4 as the only nitrogen source. The 15N labeling of the cellular GTP + GDP and septin pools was ~98% as determined by mass spectrometry. The label was then chased by changing the yeast to medium containing (14NH4)2SO4. The yeast was collected at 0, 1, 2, and 3 h after the start of the chase. The total cellular GTP + GDP, the septin proteins, and the septin-bound GTP + GDP were purified from the collected yeast. Each of the isolated fractions was analyzed by mass spectrometry. A, sample spectra for each of the analyzed pools are shown. Each row represents a particular ion (as indicated), and each column represents a particular time point of the chase (as indicated). Of the protein pool, a Cdc3 peptide, SWDPIIK (M-H) 858.47 m/z, is shown. The 15N SWDPIIK (M-H) is detected at 867.47 m/z. All other Cdc3 peptides analyzed and the other peptides of the septins analyzed have similar turnover to SWDPIIK (see B). The 15N GDP (M-H) is detected at 442 m/z; and the 15N GDP (M-H) is detected at 447 m/z. Both are detected in a linked scan as precursors containing a phosphate product ion at 79.0 m/z. GTP (M-2H) was also analyzed for the septin-bound GTP + GDP pool and has similar turnover to GDP (M-H). The positions of the 14N and 15N signal are indicated at the top of each column. B, the intensity of the 15N signal and of the total (14N + 15N) signal was calculated by integrating the area corresponding to each peak using XCalibur QualBrowser (ThermoFinnigan). The ratio 15N/(14N + 15N) was plotted as a function of time for each of the analyzed nucleotide or peptide ions. ○, GDP (M-H) from the cellular GTP + GDP pool; ■, GDP (M-H) from the septin-bound GTP + GDP pool, and the average signal for the Cdc12 peptides ALLLR (M-2H) and ANLIPVIAK (M-H); □, the average signal for the Cdc10 peptides LTELIEPIVIGK (M-2H) and LDVEALK (M-H), and the average signal for the Cdc11 peptides HQYDEILLEESR (M-2H) and VQQELLLK (M-2H); ○, average signal for the Cdc3 peptides SWDPIIK (M-H) and SDILTDEEILSFK (M-2H); For the septin proteins, results are given as the average of two different peptides. The highest and the lowest values of the averaged peptides for each septin protein are within the height of the symbols representing their average.

To confirm the lack of GTP exchange in the bulk septin population and to test for the presence of faster exchanging guanine nucleotide sites, we examined GTP exchange and GTP hydrolysis in vitro. We isolated *S. cerevisiae* septin complexes by immunoprecipitation and peptide elution, incubated them with [α-32P]GTP, and filtered them through nitrocellulose at
specific time points. Because the nitrocellulose filters retain the septin complexes, bound radioactive nucleotides can be measured. We found that the isolated septin complex binds GTP in vitro (Fig. 3A, solid diamonds). The GTP-binding activity of the S. cerevisiae septins is competed by excess cold GTP (Fig. 3A, solid triangles) but not by excess cold ATP (Fig. 3A, solid squares). The maximum binding of ~0.5 mol of guanine nucleotide per mol of septin complex was achieved after 8 h of incubation at 30 °C (Fig. 4). This binding represents ~7% of the binding sites of the complexes given that one mol of the septin complex contains 7–9 mol of GTP-binding sites (depending on the stoichiometry of the complex, see “Experimental Procedures”), and assuming that all binding sites are equally susceptible to this reaction. Note that given the sub-stoichiometric nature of the GTP-binding reaction and our results described in Fig. 4, we cannot discriminate between binding attributable to exchange of the already bound GTP and GDP and binding attributable to the presence of empty septin GTP-binding sites (see “Discussion”).

To test whether the S. cerevisiae septins hydrolize the GTP they bind, we analyzed the radioactive nucleotides retained on the nitrocellulose filter by the septin complexes using TLC for 0.5–8-h incubation periods (Fig. 3B). TLC plate phosphorimager scans show that the rate of GTP-bound GDP formed is ~0.055 h⁻¹, as determined from the equations of the fitted curves. The association rate constant kₐ = (kₐ – kₐoff)GTP is ~10⁵ M⁻¹ s⁻¹ h⁻¹.

GTPases (21), the hydrolytic activity of the septins is limited by their slow GTP-binding or exchange activity (Figs. 3A and 4).

To determine whether the GTP binding we observed in vitro (Fig. 3A) was caused by the exchange of the already bound GTP and GDP that co-purified with the complex, we analyzed the association and dissociation kinetics of the GTP-binding reaction. We incubated septin complexes with [α-32P]GTP, and at time = 4.5 h, we added excess cold GTP to an aliquot of the reaction. We quantified the bound nucleotides at earlier and later time points using the filter-binding assay (Fig. 4). We found that the GTP association and dissociation reactions have different kinetics parameters. The t₉₀ of the association reaction (~3.5 h) is shorter than the t₉₀ of the dissociation reaction (~12.5 h). This may indicate that different rate-limiting steps govern these reactions (see “Discussion”).

To identify which polypeptides are responsible for the observed in vitro GTP-binding activity, we incubated our septin preparation with [α-32P]GTP for different amounts of time (0 and 5 h) and then UV cross-linked the septin complex to the bound radioactive nucleotide (27). The identity of the cross-linked proteins was determined by SDS-PAGE and phosphorimager analysis (Fig. 5). The septin incubation reaction contained 300 μM ATP throughout the incubation period, and additional 300 μM ATP and 1% 2-mercaptoethanol were added just before the UV irradiation to suppress nonspecific cross-linking. Although all septin polypeptides showed GTP cross-links competed by excess cold GTP (300 μM) (Fig. 5), two of the septins, Cdc10 and Cdc12, were responsible for the majority of time-dependent cross-links (Fig. 5) (Table II, Column III).

**DISCUSSION**

We developed a novel 13N-dilution assay for analysis of nucleotide turnover and determined that the majority of the yeast septin complexes do not turn over GTP in vivo during one cell cycle period (Fig. 2). In vitro, the GTP binding/exchange of septin complexes is sub-stoichiometric and slow when compared with the
S. cerevisiae Septins Do Not Exchange Guanine Nucleotide

The properties of the bound guanine nucleotides of the S. cerevisiae septins are remarkably similar to those of the Drosophila septin complex (17) and of the reconstituted mammalian septin complex (18). In particular, the nucleotide:septin protein ratio (1:1) and the ratio of bound GDP:bound GTP (2.2:1) are highly conserved between these organisms, despite different numbers of septin polypeptides in the complex. This evolutionary conservation suggests that GTP/GDP-related biochemistry is critical for the function of septins. We propose that GTP binding is important for stabilizing the septin polypeptides. GTP hydrolysis by certain septin polypeptides (Cdc10, Cdc12, and Shs1) during complex assembly may control the architecture of the septin complex (for example, by forcing certain polypeptides to be neighbors, or by locking the conformation of the septin complex). Indeed, a defined architecture of the septin complex might be required for septins to accomplish their function as molecular scaffolds (15, 16) so as to bring together diverse effector molecules in a defined spatial pattern.

Previous experiments showed that mutagenesis of Cdc11-conserved amino acids eliminated GTP binding in vitro but failed to reveal a phenotype in vivo at normal temperatures (40). This result agrees with our data and suggests that GTP binding does not play an active, regulatory role for the function of Cdc11. Moreover, this result points toward other mechanisms additional to GTP binding that might stabilize the septin polypeptides.

The caveats of our conclusions pertain to the limitations of the techniques used. Specifically, for the in vivo experiment, we may not have purified a special subpopulation of septin complexes that turn over GTP. Our isolation procedure—immuno-precipitation—defines and thus potentially limits the septin population we analyzed. Also, we may have missed exchange in a single GTP-binding site within the septin complex because of the limited sensitivity of the 15N-dilution experiment. Although the in vitro filter-binding assay has the potential to identify a single, fast-turnover GTP-binding site, it would miss it if additional cofactors (guanine nucleotide exchange factors, for example) were required for stoichiometric binding or exchange.

Of special interest is Shs1 (Sep7). This protein is present in our preparation as multiple bands that partially overlap with Cdc3. Whereas each individual Shs1 band is sub-stoichiometric to the other septin proteins, the sum of the Shs1 bands represents a stoichiometric amount (Table II, Column II). As was shown by Mortensen et al. (30), Shs1 is a phosphoprotein, and the phosphorylated form migrates slower on SDS-PAGE. Interestingly, one of the slowly migrating forms of Shs1 exchanges and cross-links GTP faster than other polypeptides (Table II, Column IV). It may be that Shs1 can turn over GTP more rapidly than the other septins and that this potentially rapid GTP turnover is regulated by phosphorylation.

An intriguing possibility not ruled out by our experiments is that individual septin proteins, not part of the septin complex, function in vivo. Those might turn over GTP rapidly and behave like conventional GTPases, as suggested by the behavior of individual recombinant septin proteins in vitro (22, 23). Given that we used an antibody against one of the septins, Cdc3, to isolate the septin complex, we may not have isolated the other potentially free septin proteins. During our purification procedure, the depletion of Cdc3 from the clarified extract (see “Experimental Procedures”) was >80%. Additionally, we did not detect free Cdc3 protein in our preparation, as evi-
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