Structural Analysis of Septin 2, 6, and 7 Complexes*

Claudia Low† and Ian G. Macara
From the Center for Cell Signaling, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Mammalian septins comprise a family of 13 genes that encode GTP-binding proteins. Specific combinations of septins can hetero-oligomerize and form filaments in vivo and in vitro, by mechanisms that are not understood. Using fluorescence resonance energy transfer, size exclusion chromatography, and multi-angle light scattering techniques, we have characterized the conformation of a complex of filamentous human septins, Sept2, Sept6, and Sept7. We now show that Sept6 and Sept7 interact through a parallel coiled-coil, and that Sept2 interacts with Sept6 through their C-terminal domains. We have also been able to produce soluble, stable individual septins that behave as rod-like monomers and dimers. Taken together, these observations suggest that polymerized filaments could be comprised of laterally arranged septin core subunits.

Mammalian septins form a conserved family of cytoskeletal GTP-binding proteins that have diverse roles in protein scaffolding, cytokinesis, and vesicle trafficking (1–3). There are thirteen mammalian septins known to date, and many are expressed as multiple splice variants. The loss of or a reduction in the expression of specific septins leads to diverse effects on the actin cytoskeleton (4, 5) microtubules (6–8), SNARE protein expression of specific septins leads to diverse effects on the actin cytoskeleton (4, 5) microtubules (6–8), SNARE protein exchange or hydrolysis. Strikingly, only one report describes the assembly of filaments from a single septin, XSept2, which displayed GTP-dependent polymerization (26). In all cases, it remains to be established whether the septin filaments are polarized or are longitudinally symmetric.

Given the diversity of complexes that have been described, and the fact that mammalian septins do not have obvious orthologs of septins in lower eukaryotes (27), it is possible that many distinct oligomeric septin structures have arisen during eukaryote evolution, that nevertheless retain a similar core organization. It is important, therefore, to address directly the assembly instructions for a well characterized mammalian septin complex. Previous studies (4, 16) of Sept2/6/7 showed that this complex could polymerize into filaments in vitro, and suggested that the interaction between Sept6 and Sept7 requires the C-terminal coiled-coil. In this study, we examine the conformation of the Sept2/6/7 complex and its individual constituent septins using FRET, size exclusion chromatography, and multi-angle light scattering. We propose that a core unit consists of multiple types of septin, and that many distinct oligomeric septin structures have arisen during eukaryote evolution, that nevertheless retain a similar core organization. It is important, therefore, to address directly the assembly instructions for a well characterized mammalian septin complex. Previous studies (4, 16) of Sept2/6/7 showed that this complex could polymerize into filaments in vitro, and suggested that the interaction between Sept6 and Sept7 requires the C-terminal coiled-coil. In this study, we examine the conformation of the Sept2/6/7 complex and its individual constituent septins using FRET, size exclusion chromatography, and multi-angle light scattering. We propose that a core unit consists of a parallel coiled-coil interaction between Sept6 and Sept7. The C terminus of Sept2 also interacts specifically with the C terminus of Sept6, and this interaction requires full-length Sept2 protein. When Sept6, Sept7, and Sept2 are individually expressed in Escherichia coli, they behave as rod-like monomers and dimers. These observations suggest a lateral arrangement of core subunits within a polymerized filament.

From the Center for Cell Signaling, University of Virginia School of Medicine, Charlottesville, Virginia 22908

† To whom correspondence should be addressed: Box 800577, HSC, University of Virginia, Charlottesville, VA 22908-0577. Tel.: 434-924-1236; E-mail: ccl3j@virginia.edu.

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EXPERIMENTAL PROCEDURES

Fluorescence Spectroscopy—Septin N- and C-terminal fragments were expressed as CFP or YFP fusion proteins in COS-7 cells by transient transfection using calcium phosphate or FuGENE 6 (Roche Applied Science) reagent. Cells were lysed after 48 h in 25 mM HEPES pH 7.5 and 100 mM MgSO₄ on a Spex Fluorolog-tau-3 spectrofluorometer, using a 5-mm rectangular quartz cuvette (25°C). Excitation occurred at 433 nm (slit width 4 nm), and emission was monitored between 450–550 nm (slit width 4 nm).

Western Blots—Proteins to be detected by Western blot were transferred onto nitrocellulose and blocked in Tris-buffered saline, 0.05% Tween-20, and 5% dry milk for at least 1 h. YFP and CFP fusion proteins were detected by blotting with a monoclonal GFP antibody (Covance) at 1:1000 dilution and horseradish peroxidase-conjugated secondary anti-mouse antibody (Jackson ImmunoResearch) used at 1:5000 dilution. Visualization was enhanced by chemiluminescence (KPL). Endogenous Sept2 was visualized using polyclonal antibodies generated and described previously (7), used at 1:1000 dilution.

In Vitro Binding Assays—Septins were produced by in vitro transcription translation, using the TNT-coupled Wheat Germ Extract System (Promega) according to the manufacturer’s instructions. Myc epitopes were immunoprecipitated by incubating monoclonal 9E10 antibody with wheat germ extracts expressing 35S-labeled Myc-Sept6cc or Myc-Sept7cc for 1 h at 4°C, followed by incubation with protein-G-Sepharose for 1 h at 4°C. Beads were washed with 200 volumes of wash buffer (50 mM HEPES pH 7.5, 200 mM MgSO₄, 200 mM (NH₄)₂SO₄, 150 mM sucrose). Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE, and enhanced for fluorography using Amplify (GE Biosciences).

For pull-down assays, wheat germ extracts expressing 35S-labeled Myc-Sept6cc or Myc-Sept7cc were incubated with GST or GST-Sept2-coated glutathione-Sepharose 4B (Amersham Biosciences) beads for 1 h at 4°C. Beads were washed with 200 volumes of wash buffer (above), bound proteins eluted with SDS sample buffer, separated by SDS-PAGE, and enhanced for fluorography using Amplify.

Recombinant Protein Expression and Affinity Purification—Vectors for expression of GST fusion proteins were transformed into BL21(DE3), cultured in Luria Broth (LB) or Terrific broth were obtained by diluting lysates in buffer containing 25 mM HEPES pH 7.5 and 100 mM MgSO₄ on a Spex Fluorolog-tau-3 spectrofluorometer, using a 5-mm rectangular quartz cuvette (25°C). Excitation occurred at 433 nm (slit width 4 nm), and emission was monitored between 450–550 nm (slit width 4 nm).

The abbreviations used are: CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GFP, green fluorescent protein; GST, glutathione S-transferase; FRET, fluorescence resonance energy transfer.
Broth (TB), and expressed by induction at OD = 0.6 with 1 mM isopropyl-1-thio-β-D-galactopyranoside overnight at 18 °C. Cells were resuspended in binding buffer containing 50 mM HEPES pH 7.5, 200 mM MgSO₄, 200 mM (NH₄)₂SO₄, 150 mM sucrose. His-S-Sept6 was resuspended in buffer containing 50 mM HEPES pH 7.5 and 600 mM NaCl. His-S-Sept7 was resuspended in buffer containing 50 mM HEPES pH 7.5 and 300 mM MgSO₄. His-S-Sept2 was resuspended in buffer containing 50 mM HEPES pH 7.5 and 150 mM NaCl or 150 mM MgSO₄. Subsequently, cells were lysed in a French Press, and affinity-purified on a HisTrap affinity column (GE Biosciences) installed on a fast protein liquid chromatography system (FPLC). Proteins were eluted in the presence of an imidazole gradient and stored in elution buffer.

Size Exclusion Chromatography and Estimation of Stokes Radius (Rₛ)—Size exclusion chromatography was carried out at 4 °C on either a Tricorn Superose 6 10/300 GL column (GE Biosciences) or a HiLoad 16/60 Superdex 200 prep grade (GE Biosciences) installed on an FPLC. Both columns were calibrated using both high and low molecular weight calibration kits (GE Biosciences) according to manufacturer's specifications. A standard calibration plot of $(log[K_{av}])^{1/2}$ versus $R_{st}$ where $K_{av} = (V_e - V_o)/(V_t - V_o)$ was used to estimate hydrodynamic radius. The following values were used to calculate $K_{av}$: column void volume, $V_o = 45$ ml, and total bed volume, $V_t = 120$ ml. Eluted fractions were subsequently analyzed for septins by standard SDS-PAGE, with gels either stained with Coomassie Brilliant Blue or probed by Western blot. Intensities of bands were compared using the ImageJ v.1.34s software package (NIH).

Molar Mass Determination—Experiments were carried out at the Macromolecular Interactions Facility (MacInFac) at the University of North Carolina at Chapel Hill, under the guidance of Dr. Ashutosh Tripathy. Fast protein liquid size exclusion chromatography was performed at room temperature on a Tricorn Superose 6 10/300 GL column (GE Biosciences) in 50 mM HEPES pH 7.5, 300 mM MgSO₄, 200 mM (NH₄)₂SO₄. The elution profiles were monitored by a Dawn EOS multiangle laser light scattering system (Wyatt Technologies) at 690 nm, and an Optilab DSP Interferometric Refractometer (Wyatt Technologies). Data acquisition and processing were carried out using ASTRA software (Wyatt Technologies).

FIGURE 2. Full-length Sept2 interacts with the C-terminal Sept6cc. A, GST-Sept2 pulldown of the YFP-Sept6cc + myc-Sept7cc coiled-coil complex. B, anti-GFP Western blot of CFP and YFP Sept2 fusion proteins from COS-7 cell lysates. C–L, corrected emission spectra from cell lysates containing various combinations of Sept2, Sept6, and Sept7 fusions to YFP and CFP. Excitation was at 433 nm.
Coiled-coil Energy Transfer (FRET) to gain further insights into the assembly of Sept6 and Sept7, and ultimately, of septin filaments. We used CFP and YFP as the donor and acceptor fluorophores, respectively. The emission spectrum of CFP overlaps with the excitation spectrum of YFP to provide efficient FRET when the two proteins are close together and are randomly oriented with respect to one another (29). The C-terminal region of Sept6, encompassing residues 283–429, was N-terminally tagged with YFP, and called YFP-Sept6cc, or C-terminally tagged and called Sept6cc-YFP. Sept7-(277–418), was tagged in a similar way with CFP to produce CFP-Sept7cc and Sept7cc-CFP. Initially, the ability of these fluorophore fusion proteins to form complexes was tested in vitro. Myc-tagged C-terminal fragments of the septins were 35S-labeled and expressed together with the YFP or CFP fusions in wheat germ lysate. Plants do not possess septin genes, so this lysate lacks endogenous septins that might otherwise aid in the interaction of exogenously introduced constructs. When co-expressed as a pair with its Myc-tagged binding partner, YFP-Sept6cc and CFP-Sept7cc were co-immunoprecipitated with Myc-Sept7cc and Myc-Sept6cc, respectively, in an approximate 1:1 ratio (Fig. 1A). These results confirmed that the isolated coiled-coil regions of Sept6 and Sept7 can interact with one another.

However, this type of experiment does not distinguish parallel from anti-parallel interactions of the coiled-coil dimers (Fig. 1B). Therefore, YFP and CFP pairs were co-expressed in COS-7 cells, and the soluble cell extracts tested for FRET by exciting CFP at 433 nm and monitoring the emission between 450–550 nm. All raw emission spectra were adjusted to account for varying protein expression levels. Spectra were also obtained for the CFP-Sept7cc and Sept7cc-CFP. The YFP spectrum was subtracted from the FRET spectrum to correct for spectral overlap, and the emission of CFP fusion protein alone, excited at 433 nm, is plotted for comparison (Fig. 1C). The combinations of YFP-Sept6cc and CFP-Sept7cc, both N-terminally tagged, and Sept6cc-YFP and Sept7cc-CFP, both C-terminally tagged, resulted in efficient FRET (Fig. 1E and F). Furthermore, the FRET signal generated by two C-terminally tagged constructs improved when using full-length septins, Sept6FL-CFP and Sept7FL-YFP (Fig. 1G). This result suggested that the two proteins formed a parallel coiled-coil. To test this conclusion, we next used a combination of YFP-Sept6cc and Sept7cc-CFP, in which the Sept6 is N-terminally tagged, and the Sept7 is C-terminally tagged. This donor-acceptor pair would be predicted to generate a FRET signal only if the coiled-coils are arranged head-to-tail. How-

Calculation of the Perrin Factor (F)—The frictional ratio ($f/f_0$) was calculated using Equation 1,

$$f/f_0 = R_f/(3
\nu M_s/4\pi N_A)^{1/3}$$  (Eq. 1)

which compares an ideal sphere in its hydrated versus anhydrous state. $R_f$ is the observed hydrodynamic radius (in units of cm), $\nu$ is the partial specific volume (0.73 ml/g was assumed), $M_s$ is the molecular weight predicted by protein sequence, and $N_A$ is Avogadro's number, 6.02 × 10$^{23}$. The partial specific volume for most proteins is between 0.69 and 0.75 ml/g; the average of 0.73 ml/g was assumed. The resulting values of $f/f_0$ were then used to calculate the Perrin factor (F) using Equation 2,

$$F = f/f_0(1 + \delta/\rho \nu)^{-1/3}$$  (Eq. 2)

where $\delta$ is the protein hydration (0.35 ml/g was assumed), $\rho$ is the solvent density (1.0 was assumed), and $\nu$ is the partial specific volume mentioned previously. Hydration values of 0.3 to 0.4 ml/g account for the hydrodynamic behavior of typical proteins; the average of 0.35 ml/g was assumed for these calculations.

RESULTS

Mammalian Sept6 and Sept7 Interact through a Parallel Coiled-coil—Previously, it has been shown that a C-terminal fragment is sufficient for the heterodimerization of Sept6 and Sept7 (16). The COILS algorithm (28) predicts that the C-terminal regions of Sept6 and Sept7 each contain coiled-coil regions that extend for about 100 amino acid residues. However, the relative orientation of these coiled-coils was not established. Therefore, we decided to use Fluorescence Resonance Energy Transfer (FRET) to gain further insights into the assem-

FIGURE 3. Sept6cc interacts with the GTP binding domains of Sept6 (Sept6gtp) and Sept7 (Sept7gtp). A, anti-GFP Western blot of CFP and YFP Sept6gtp and Sept7gtp fusion proteins from COS-7 cell lysates transfected. B, FRET between YFP-Sept6cc + CFP-Sept7FL. C, FRET between YFP-Sept6cc + Sept7gtp-CFP. D, FRET between YFP-Sept6cc + CFP-Sept7gtp. E, FRET between YFP-Sept6cc + Sept7gtp-CFP. F, FRET between Sept6cc-YFP + Sept7gtp-CFP.
ever, this pair did not yield any FRET (Fig. 1H), supporting the idea that the septin coiled-coils are parallel. To confirm protein expression, all soluble cell extracts were Western-blotted using anti-GFP (Fig. 1D). Together, these results argue strongly that Sept6cc and Sept7cc interact through a parallel coiled-coil.

Mammalian Sept2 Interacts with the C-terminal Coiled-coil of Sept6—Previously it has been shown that Sept6, Sept7, and Sept2 form a ternary complex that self-assembles into filaments when the three proteins are co-expressed simultaneously in vitro (16). We were therefore interested in how Sept2 associates with the Sept6/7 heterodimer. To test whether Sept2 can bind to the coiled-coils of Sept6cc and Sept7cc, we used GST-Sept2 expressed in E. coli and YFP-Sept6cc and Myc-Sept7cc that were co-expressed in a wheat germ lysate. GST-Sept2 immobilized on beads was able to bind the Sept6/7 coiled-coils, while GST alone did not (Fig. 2A), showing that the isolated coiled-coil regions of Sept6 and Sept7 can interact directly with Sept2. However, this type of experiment does not distinguish whether Sept2 binds at the N or C terminus of the coiled-coil dimer. To address this question, N- and C-terminal-tagged YFP and CFP fusion proteins of full-length Sept2FL, Sept2cc (residues 283–362, containing the putative coiled-coil), and Sept2gtp (resi-
dues 1–283, containing the isolated GTP-binding domain) were constructed. Whereas the coiled-coils of Sept6 and Sept7 were predicted to be 100 residues long, Multicoil (30) and COILS (28) predict that the coiled-coil of Sept2 includes only 50 residues. In addition, COILS assigned a high probability score to the formation of a coiled-coil, whereas Multicoil assigns the same residues a low probability score. Despite the disagreement in the probability of coiled-coil formation, we could use FRET to distinguish whether the interaction between Sept2 and the coiled-coils of Sept6 and Sept7 occurred at the N or C terminus. Initially, fluorophore fusion proteins were transiently transfected into COS-7 cells, and protein expression was verified by Western blot using anti-GFP (Fig. 2B).

Next, full-length Sept2 fluorophore fusion proteins were co-expressed with Sept6cc or Sept7cc fluorophore fusion proteins in COS-7 cells, and the soluble cell lysates were tested for FRET. Surprisingly, the only combination that gave an efficient FRET signal was Sept2FL-CFP with Sept6cc-YFP, in which both constructs were C-terminally tagged with fluorophore (Fig. 2C). FRET was not detected when an N- and C-terminal combination, YFP-Sept6cc and Sept2FL-CFP, was used (Fig. 2D). We then asked whether the C-terminal region of Sept2 alone could mediate the FRET interaction. However, neither Sept2cc-CFP nor a CFP-Sept2cc could generate a FRET signal equal to that of Sept2FL-CFP and Sept6cc-YFP (Fig. 2E and F). Similarly, the N-terminal GTP binding region of Sept2 alone, Sept2gtp-CFP,

**TABLE 2**

Summary of tested FRET combinations

| INTERACTING PARTNER A | SEPT7 FUSIONS | SEPT6 FUSIONS | SEPT2 FUSIONS |
|-----------------------|---------------|---------------|---------------|
| Y-gtp                 | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| C-gtp                 | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| gtp-Y                 | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| gtp-C                 | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| Y-ce Y-ce C-cc C-cc C-gtp | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| C-cc C-cc C-gtp Y-gtp | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| FL-C                  | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| C-FL                  | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| Y-FL Y-FL             | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| FL-Y                   | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |
| FL-C                   | Y-ce C-cc C-gtp Y-gtp | gtp-Y gtp-C | gtp-C |

Y = YFP  C = CFP  ce = C-terminal coiled-coil  gtp = N-terminal GTP binding domain  FL = full-length protein
and CFP-Sept2gtp, were unable to generate any detectable FRET interaction with Sept6cc-YFP (Fig. 2, G and H). In addition, no FRET was observed between Sept2FL-YFP and Sept7cc-CFP or Sept2FL-YFP and CFP-Sept7cc, suggesting that FRET between the Sept2FL-CFP and Sept6cc-YFP combination is specific for the coiled-coil of Sept6 (Fig. 2, I and J).

Furthermore, full-length Sept7FL did not yield any FRET with full-length Sept2FL (Fig. 2, K and L). Taken together, these data suggest that Sept2 does not interact directly with Sept7, and that only full-length Sept2 can interact with the coiled-coil of Sept6cc, with the interaction being mediated through the C-terminal region of the Sept6 coiled-coil.

**Mammalian Septin GTP Binding Domains Interact with the Coiled-coil of Sept6**—Our data suggest that the C-terminal regions in mammalian Sept6, Sept7, and Sept2 play key roles in mediating their interactions. To determine whether the GTP binding domains of septins are involved in complex formation, we developed N- and C-terminal YFP and CFP-labeled Sept6gtp (residues 1–255), and Sept7gtp (residues 1–272) constructs containing the GTP binding domain. These fusion proteins were individually transfected into COS-7 cells, and protein expression was verified by Western blot using anti-GFP (Fig. 3A). For FRET analysis, YFP and CFP constructs were co-transfected into COS-7 cells, and soluble cell lysates were produced. Interestingly, five positive pairings all implicated interactions between the coiled-coil of Sept6 and the GTP-binding domains of both Sept6 and Sept7 (Fig. 3, B–F). The remaining forty-nine of the fifty-four tested pairings resulted in no FRET (Tables 1 and 2), including all cases that asked whether the coiled-coil of Sept7 interacted in proximity to the GTP binding domains of Sept6 or Sept7. Compared with the rigid linearity of coiled-coils, the globular nature of GTP binding domains precludes conclusions about its orientation in these complexes. However, the proximity of the GTP binding domains to the Sept6 coiled-coil implies that the coiled-coil domain of Sept6 is a key element in septin filament polymerization.

**Recombinant Septins Expressed Individually in E. coli Are Asymmetric Monomers and Dimers**—Size-exclusion chromatography (SEC) was used to gain insights into septin filament assembly. Previously, it has been shown that when Sept2, Sept6, and Sept7 are co-expressed in E. coli and affinity-purified on a nickel affinity column, filaments can be visualized by electron microscopy using negative stain (16). The same preparation of septins analyzed by electron microscopy was also analyzed by SEC. The septin trimer eluted from a Superose 6 column between 10–15 ml, which was determined by SDS-PAGE staining with Coomassie Brilliant Blue (Fig. 4A). This elution volume corresponds to a mean hydrodynamic radius much larger than that expected for a trimer or hexamer. Endogenous septins in HeLa cell lysates showed similar elution behavior on a Superose 6 column, eluting between 9 and 13 ml, as detected by Western blot (Fig. 4B). An overlay of the elution profiles for endogenous and in vitro purified septins suggest that they polymerize into multimeric complexes with similar hydrodynamic radii (Fig. 4C).

To address whether polymerization is a property unique to the Sept2+Sept6+Sept7 complex, the hydrodynamic radii of individually expressed septins were determined. His-S-Sept2, His-S-Sept7, and His-S-Sept6 were expressed individually in E. coli, and affinity-purified on a nickel affinity column, filaments can be visualized by electron microscopy using negative stain (16). The same preparation of septins analyzed by electron microscopy was also analyzed by SEC. The septin trimer eluted from a Superose 6 column between 10–15 ml, which was determined by SDS-PAGE staining with Coomassie Brilliant Blue (Fig. 4A). This elution volume corresponds to a mean hydrodynamic radius much larger than that expected for a trimer or hexamer. Endogenous septins in HeLa cell lysates showed similar elution behavior on a Superose 6 column, eluting between 9 and 13 ml, as detected by Western blot (Fig. 4B). An overlay of the elution profiles for endogenous and in vitro purified septins suggest that they polymerize into multimeric complexes with similar hydrodynamic radii (Fig. 4C).
The highest concentrations of protein were further analyzed by SEC on a Superdex 200. The absorbance at 280 nm and intensity of bands resolved on an SDS-PAGE gel of individual septins are plotted on the same graph for clarity (Fig. 5, A–C). Below each graph, SDS-PAGE gels represent total protein content in each fraction at the indicated elution volume. The expected hydrodynamic radius of a globular protein with a molecular mass of 50 kDa is 3.2 nm. However, the hydrodynamic radii ($R_h$) calculated from the peak elution volumes for His-S-Sept6, His-S-Sept7, and His-S-Sept2 correspond to 5.0 ± 0.1, 5.65 ± 0.15, and 3.65 ± 0.09 nm, respectively (Table 3). SEC cannot distinguish whether the larger measured hydrodynamic radii of the individually expressed septins are caused by elongated protein shape or oligomerization state. Thus, the absolute molar mass was determined on a size-exclusion chromatography multi-angle light scattering instrument (SEC-MALS) using a Superose 6 column. His-S-Sept7 behaved as a mixture of monomer and dimer, with ~4-fold more dimer present than monomer (Fig. 5D). Presumably, dimer formation is highly favorable because of the presence of the C-terminal coiled-coil, which would normally interact with the coiled-coil of Sept6. His-S-Sept2 also behaved as a heterogeneous mixture, but with predominantly monomeric composition (Fig. 5E). The concen-

### TABLE 3

Measured and calculated quantities used to estimate the Perrin factor

| Septin  | $R_h$ (nm) | $M_w$ | $f/f_0$ | F   |
|---------|------------|-------|---------|-----|
| Sept6 (dimer) | 5.0 ± 0.1 | 97,744 | 1.64 ± 0.03 | 1.44 ± 0.03 |
| Sept7 (dimer) | 5.65 ± 0.15 | 97,316 | 1.85 ± 0.05 | 1.63 ± 0.05 |
| Sept2 (monomer) | 3.65 ± 0.09 | 41,526 | 1.59 ± 0.04 | 1.40 ± 0.04 |

The hydrodynamic radius ($R_h$), molecular weight ($M_w$), frictional ratio ($f/f_0$), and Perrin factor (F) are reported for Sept6, Sept7, and Sept2. $R_h$ was estimated on a Superdex 200 calibrated using globular protein standards, and $M_w$ was predicted based on the protein sequence. $R_h$, $f/f_0$, and F were calculated using equations described under "Experimental Procedures." A ± 1 ml error in the determination of elution volume from gel filtration was used to calculate these values.

**FIGURE 5.** Recombinant septins expressed in *E. coli* are mixtures of monomers and dimers. A–C, His-S-Sept7, his-S-Sept2, and His-S-Sept6, respectively, affinity-purified from *E. coli* and applied onto a Superdex 200 gel filtration column. The total protein content of each fraction was separated on an SDS-PAGE gel, and the gel band intensity plotted against elution volume. D and E, SEC-MALS analysis of His-S-Sept7 and His-S-Sept2, respectively. Molecular mass (MM) and refractive index (RI) are plotted as a function of elution volume.
The concentration of His-S-Sept6 was insufficient to gather highly reliable light scattering data. However, analysis suggested that it may form a dimer similar to His-S-Sept7.

Using the observations made from SEC and SEC-MALS, the frictional coefficient \( f/f_0 \) and Perrin factor \( F \) were calculated to estimate the extent to which His-S-Sept2, His-S-Sept6, and His-S-Sept7 deviate from perfect spheres (Table 3). A sphere would have \( f/f_0 = 1.2 \) and \( F = 1 \). Using the estimated hydrodynamic radii from gel filtration and molar masses calculated from SEC-MALS, \( f/f_0 \) values for His-S-Sept6, His-S-Sept7, and His-S-Sept2 were 1.64 ± 0.03, 1.85 ± 0.05, and 1.59 ± 0.04, respectively. These values give Perrin factors of 1.44 ± 0.03, 1.63 ± 0.05, and 1.40 ± 0.04, respectively, which correspond to axial ratios of 6–10 for prolate ellipsoids (31). Taken together, these data strongly suggest that septins expressed individually behave as highly asymmetric proteins in solution, and that rod-like asymmetric proteins with lengths of 11.5–19 nm and maximum diameters of 4 nm (Fig. 6A). This representation is supported by our hydrodynamic and molecular mass data; the calculated Perrin factors indicate a high ratio (6–10) between the long and short axes of the molecules, which would match well with the mean lengths and diameters we expect from structural considerations.

Complexed septins maintain their extended conformations while interacting with one another. Our FRET data suggest that the core subunit of the Sept2/6/7 complex consists of a parallel coiled-coil interaction between Sept6cc and Sept7cc (Fig. 6B). The C terminus of full-length Sept2 specifically interacts with the C terminus of Sept6cc. One model of the Sept2/6/7 complex consisting of all three full-length proteins is also presented, but since the globular GTP binding domains have rotational freedom with respect to each

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**FIGURE 6. Models of individual and complexed septin subunits.** A, individual septins are rod-like proteins –11.5–19 nm long and 4 nm in diameter. GTP-bd indicates the GTP-binding domain. B, model of the septin core subunit and dimensions of the core subunit comprised of full-length septins. C, models suggested by polymerizing core subunits into polar and non-polar filaments.

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**DISCUSSION**

As functional roles for septins continue to be uncovered, septin filament structure will be key to understanding and unifying how they coordinate a myriad of processes. In this study, we examine the conformation of the Sept2/6/7 complex to gather insight on filament polymerization. Tables 1 and 2 summarize the FRET experiments that suggest a model of a core subunit that could polymerize into filaments (Fig. 6B). They also show that the majority of the tested FRET pairs do not give a positive FRET signal, indicating that positive signals are not the result of nonspecific fluorophore aggregation. In Fig. 6, we propose model representations of individual and complexed septins. X-ray crystallographic structures obtained from other coiled-coil proteins help to predict the length of Sept6, Sept7, and Sept2. Assuming a 100 amino acid coiled-coil and a length of 0.15 nm/amino acid for coiled-coil structures (32), the coiled-coils of Sept6 and Sept7 are expected to extend ~15 nm. Sept2 has a predicted coiled-coil of ~50 amino acids, resulting in an estimated length of 7.5 nm. Furthermore, based on crystallographic data, typical small GTP-binding proteins have a diameter of 4 nm (33, 34). Therefore, we model Sept6, Sept7, and Sept2 as...
other, the exact conformation cannot be deduced using this type of experiment. Similarly, FRET analysis summarized in Tables 1 and 2 suggest close interactions between the coiled-coil of Sept6 and the GTP binding domains of Sept6 and Sept7, however, we cannot distinguish whether their proximity arises within one subunit or as a consequence of multiple subunit oligomerization.

The frequency with which FRET was observed using Sept6cc leads us to speculate that the coiled-coil domains could play a critical role in the polymerization of septin filaments. Previously, others have shown that septins may interact through their C-terminal coiled-coils. In budding yeast, Cdc3 and Cdc12 associate through the CTE, or C-terminal extension, which includes the coiled-coiled regions. This association is critical for their function in vivo (23). Biochemical analysis of the Sept7/9b/11 complex reveals that Sept7 and Sept11 also interact through their C-terminal coiled-coil domains (15). However, the relative orientations of the components of these complexes were not determined.

Based on our observations, we could assemble two models for filament polymerization by varying the orientation of the core subunit. Both polar and non-polar filaments can be constructed (Fig. 6C). The polar filament consists of hexamers of two heterotrimers rotated 180° along the vertical axis with respect to each other. A non-polar filament can be modeled by hexamers of two heterotrimers reflected over the horizontal axis. Future work will attempt to differentiate and identify which model most closely resembles septin filaments. A long-term goal is to obtain three-dimensional structures of a septin core subunit. However, much biochemical work remains to fully characterize the relationship and significance between structure and function.

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