Borg/Septin Interactions and the Assembly of Mammalian Septin Heterodimers, Trimers, and Filaments*

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Peter J. Sheffield‡, Carey J. Oliver‡, Brandon E. Kremer‡, Sitong Sheng§, Zhifeng Shao§, and Ian G. Macara†‡¶

From the ‡Department of Microbiology and Center for Cell Signaling and the ¶Department of Physiology and Biophysics, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Septins constitute a family of guanine nucleotide-binding proteins that were first discovered in the yeast Saccharomyces cerevisiae but are also present in many other eukaryotes. In yeast they congregate at the bud neck and are required for cell division. Their function in metazoan cells is uncertain, but they have been implicated in exocytosis and cytokinesis. Septins have been purified from cells as hetero-oligomeric filaments, but their mechanism of assembly is unknown. Further studies have been limited by the difficulty in expressing functional septin proteins in bacteria. We now show that stable, soluble septin heterodimers can be produced by co-expression from bicistronic vectors in bacteria and that the co-expression of three septins results in their assembly into filaments. Pre-assembled dimers and trimers bind guanine nucleotide and show a slow GTPase activity. The assembly of a heterodimer from monomers in vitro is accompanied by GTP hydrolysis. Borg3, a downstream effector of the Cdc42 GTPase, binds specifically to a septin heterodimer composed of Sept6 and Sept7 and to the Sept2/6/7 trimer, but not to septin monomers or to other heterodimers. Septins associate through their C-terminal coiled-coil domains, and Borg3 appears to recognize the interface between these domains in Sept6 and Sept7.

Septins form a class of eukaryotic guanine nucleotide-binding proteins that were first identified in the budding yeast, Saccharomyces cerevisiae, and congregate in a ring at the bud neck during cell division (1–4). Temperature-sensitive mutations in four of the septin genes produce cell cycle arrest and cytokinesis defects when the yeast is grown at the non-permissive temperature. One essential function of septins at the bud neck may be as a diffusion barrier that helps segregate mother and daughter cell membrane components (5–7). The septin ring also provides a structural scaffold for the localization of many proteins to the bud neck (3). Septins purified from yeast form filaments of variable length that are 7–9 nm in diameter, although the physiological significance of the filament structure remains unclear (8). The filaments show a periodicity of about 32 nm, which may represent an octamer comprising two copies of the four different septin subunits. Similar filaments have also been isolated from Drosophila and mammalian brain tissue (9, 10). However, the mechanism by which the filaments assemble from septin monomers is completely unknown. The Drosophila filaments appear to be composed of three different septins. Two of these septins, Pnut and Sep1, have been localized to the cleavage furrow in dividing cells, and Pnut has been implicated in cytokinesis, although only in certain cell types, and RNA interference of Pnut expression has an unusually low penetrance (11–13).

At least 10 mammalian septins have been identified to date, but most have not been studied in any detail. (Note that many have been given multiple different names, which can cause confusion in the literature and, for this reason, we will use a standard nomenclature in this report, in accordance with the Human Genome Organization (HUGO) and Mouse Genome Nomenclature (MGN) committee guidelines; see Ref. 14.) The murine Sept2 (previously called Nedd5) localizes to the contractile ring of dividing cells, and the injection of anti-Sept2 antibodies partially inhibits cytokinesis (15). Mammalian septins have also been implicated in exocytosis and are associated with the exocyst proteins Sec6 and Sec8 (10). Sept5 (previously called Cdcrel-1 or Pnut1) binds syntaxin and copurifies with synaptic vesicles (16). Sept5 also copurifies with syntaxin-4 in a complex from platelets, and platelets from a Sept5 knockout mouse show misregulation of secretion and aggregation (17), although the mice appear otherwise normal (18).

In interphase cells, mammalian septins are organized into filamentous structures that often partially colocalize with actomyosin stress fibers (15). Sept2, Sept6, and Sept7 each colocalize with one another, suggesting that they are part of a complex, and these proteins also can be copurified in a 1:1 stoichiometry using the Borg3 protein as an affinity matrix (19). Borg3 is a small adapter protein that is part of a family of downstream effectors for the small GTPase Cdc42 (20, 21). A short, conserved motif within the Borgs (residues 83–110 in Borg3), called the BD3 domain, is both necessary and sufficient for binding septins, and, when expressed in cells, this domain induces a pronounced aggregation of septin filaments (19). Full-length Borg3 induces a less pronounced reorganization of septins, and this effect is inhibited by a gain-of-function mutant of Cdc42. Therefore, Borgs can function as adapters that link the Ras-like GTPase Cdc42 to the septin GTP-binding proteins. It is of interest that in budding yeast, which does not contain any Borg-like genes, Cdc42 has also been implicated in cytokinesis defects when the yeast is grown at the non-permissive temperature (22, 23).

The mechanism by which Borgs bind to and regulate septins is not understood and has been frustrated by the lack of information on septin oligomerization and filament assembly. Moreover, it has not been clear whether the Borgs bind to one specific septin, to all septins, or rather to a particular supramolecular assembly of septins.
To address this issue, we have developed methods for the expression of septin heterodimers and trimers in bacteria. We find that, whereas monomers are very unstable, the dimers and trimers are soluble and stable and can bind guanine nucleotides. The coiled-coil domains of the septins are required for these interactions. Interestingly, only the Sept6/Sept7 heterodimer can bind Borg3. Septin trimers can assemble into filaments of a diameter similar to that seen in native structures isolated from yeast and flies. We find that septin dimerization is associated with GTP hydrolysis but that Borg3 does not regulate this assembly step.

**MATERIALS AND METHODS**

**Septin Expression in Escherichia coli**—Individual septins were cloned into the BamHI site of the Novagen pET30 vector so as to attach in-frame His6 + S-peptide tags (HS)1 to their N termini. The septin open reading frames were then excised by digestion with Necl/NotI and subcloned into a second bacterial vector called ptaeNT, which contains an ampicillin resistance marker, a ptac promoter, and no epitope tag (NT). To create a bicistronic vector, the pET30-Sept constructs were ligated into a compatible vector that contained a p15A origin of replication and chloramphenicol and tetracycline resistance genes (pACYC184, from New England Biolabs). Expression cassettes for the septins were ligated into compatible vector that contained a p15A origin of replication and chloramphenicol and tetracycline resistance genes (pACYC184, from New England Biolabs).

**Dynamic Light Scattering**—Protein solutions (~1.0 mg/ml) were cleared by centrifugation at 14,000 *g* for 10-fold dilution into a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.5 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, and 0.1% bovine serum albumin.

**Dynamic Light Scattering**—Protein solutions (~1.0 mg/ml) were cleared by centrifugation at 14,000 × *g* for 10 min immediately prior to measurements. Light scattering was performed using a DynaPro-MSX instrument, with DYNAMICS control and analysis software (Proteins Solutions, Inc.).

**Electron Microscopy**—The purified Septin complex (Sept2-Sept6-Sept7) was diluted to 100 μg/ml in a buffer containing 50 mM HEPES, pH 7.4, 250 mM NaCl, and 5 mM MgCl2. The protein solution was applied onto glow-discharged, carbon-coated grids and incubated for 5

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1 The abbreviations used are: HS, His6 + S-peptide tag; NT, no epitope tag; MOPS, 4-morpholinepropanesulfonic acid.
**Fig. 3. Guanine nucleotide binding and hydrolysis by septins.** Proteins were produced as described in the Fig. 1 legend and under “Materials and Methods.” A, binding was performed at 30 °C, with [35S]labeled guanine nucleotide (3000 Ci/mmol). B, off-rates were determined after dilution into buffer containing 1 mM GTP plus 1 mM GDP. Data are means of duplicate assays. Curves were fit assuming a single exponential decay. C, hydrolysis of bound [α-32P]GTP was measured after the removal of free nucleotides from the septins by passage through a Centricon spin column. At intervals, aliquots of the labeled protein were bound to nitrocellulose filters and denatured with SDS and EDTA. Released nucleotides were separated by thin layer chromatography and quantified on a PhosphorImager. Data are presented as percentage of total bound nucleotide and are means of duplicate assays. D, binding was performed as described for panel A, but using septin monomers mixed in the presence of the nucleotide.

min. The sample was then washed with the dilution buffer and stabilized in the same buffer with 0.5% glutaraldehyde for 2 min. After rinsing with water, the grid was stained with 1% uranyl acetate in 30% ethanol and examined in an electron microscope (Philips CM12). For rotary shadowing of the protein complex, the specimen was prepared as described above but on a native mica surface, and the sample was directly shadowed without staining.

**Guanine Nucleotide Binding—**Septins were passed over a PD-10 column (Pharmacia) to exchange them into binding buffer (50 mM MOPS, pH 7.4, 150 mM NaCl, 2 mM β-mercaptoethanol, 5 mM MgCl2, 100 μg/ml bovine serum albumin). The proteins (20 μg) were incubated with [α-32P]GTP or [α-32P]GDP (3000 Ci/mmol) for the specified times, at 30 °C. Binding was assessed by filtering through nitrocellulose membranes as described previously (20). To assess guanine nucleotide binding and hydrolysis to mixed monomers, 30 μg of each freshly prepared HS-tagged monomer was mixed in the presence of 2 μl of either [α-32P]GTP or [γ-32P]GTP (3000 Ci/mmol) in 1 ml of binding buffer and incubated at 30 °C. Aliquots (50 μl) were removed and washed through nitrocellulose filters at intervals.

GTP hydrolysis was measured after loading 20 μg of heterotrimeric septins with [α-32P]GTP for 3 h and then passing them through a Centricon spin column equilibrated with binding buffer to remove free nucleotides. The septins were then incubated for various times, and samples were analyzed for guanine nucleotide state by thin layer chromatography on polyethylenimine-cellulose plates. EDTA (10 mM) and SDS (2%) were added to the samples to release the nucleotides. Plates were developed in 0.75 M potassium phosphate, pH 3.4, as described previously (24), and radioactive spots were detected and quantified using an Amersham Biosciences PhosphorImager.

**RESULTS**

**Expression of Recombinant Septin Monomers, Dimers, and Trimmers in E. coli**—A fundamental problem in studying septins has been the absence of systems for their expression as stable, soluble proteins in bacteria. We were able to express small quantities of each of the Sept2, 6, and 7 monomers (Fig. 1A), but the majority of the protein in each case remained in the bacterial pellet after lysis under all conditions tested, and the proteins in the soluble fraction tended to precipitate out of solution after purification. However, using a bicistronic vector, we were able to co-express pairs of septins that were soluble and could be purified over Ni2+-agarose in a 1:1:1 stoichiometry when a HS epitope tag was attached to one member of each pair (Fig. 1B). All three pairs of Sept2, Sept6, and Sept7 could be expressed in this manner. Binding of the untagged partner did not represent nonspecific association with the Ni2+-agarose, because enterokinase treatment to cleave the HS tag resulted in the loss of both proteins from the beads (e.g. Fig. 1C). Finally, by co- transformation of the bacteria with a bicistronic vector plus a second, compatible plasmid that expresses the third septin, we could copurify all three proteins on Ni2+-agarose in a 1:1:1 stoichiometry. An example is shown in Fig. 1D, where Sept2 was tagged and the Sept6 and Sept7 were untagged.

When the purified septin trimer was analyzed by dynamic light scattering, two distinct peaks were observed, i.e. a major one corresponding to a hydrodynamic radius of ~14 nm and a minor peak corresponding to a radius of ~74 nm (Fig. 2A). The major peak is consistent with a molecular size of about 256 kDa, assuming a spherical shape. This peak may correspond to a Sept hexamer, which would have a calculated molecular size of about 280 kDa. The other peak represents very large particles (>5 MDa) and may represent aggregates or filaments. In contrast, a Sept6-Sept7 complex displayed a single peak radius of ~4 nm corresponding to a molecular mass of ~87 kDa, which is consistent with the two proteins forming a heterodimer.

To determine whether the septin trimer preparation contains filaments, the purified proteins were visualized by electron microscopy. The proteins were negatively stained on carbon film (Fig. 2B and D) or were metal-shadowed (Fig. 2C). In each field, long filaments were visible. These filaments are ~10 nm in diameter. The filament shown in Fig. 2D is ~850 nm in length. A larger field is shown at lower magnification in Fig. 2B, which contains filaments with a variety of lengths. No filaments were observed when a Sept6-Sept7 dimer was examined after similar treatment. For comparison, the septin fila-
ments from yeast are 7–9 nm in diameter (8). Those from Drosophila are also ~7 nm in diameter, and those from mammalian brain tissue are ~8.25 nm in diameter (9, 10). A significant difference between the recombinant and purified septin filaments is that the latter show a periodicity along the length of the filament with a unit length of about 25–30 nm, whereas our recombinant Sept2/6/7 filaments do not. This difference and the low percentage of trimers that assemble into filaments in bacteria together suggest that we are lacking a stabilizing component. One possibility is that this component is a fourth septin, such as MSF or E-septin (now called Sept9), which was detected, although at low stoichiometry, in our initial pull-down experiments with GST-Borg3 (19). Nonetheless, our data show that septin filaments can form spontaneously when expressed in bacteria. Moreover, septin monomers are highly unstable but can form stable heterodimers.

Guanine Nucleotide Binding by Recombinant Septins—To test the functionality of the recombinant septins, we assayed guanine nucleotide binding. Initially, septins were incubated with \([\alpha-32P]GTP\) or \([\alpha-32P]GDP\) and then bound to nitrocellulose filters. Under a variety of conditions (± MgCl₂ or EDTA at 4 °C or 30 °C) we could not detect \(32P\) binding to Sept6 but found a low level of binding to Sept7 and Sept2 (not shown). Others have also reported GTP-binding to Sept2 and indicated that the nucleotide has a high off-rate (15). These data are consistent with our conclusion that the septin monomers are unstable and may be largely unfolded. The three septin heterodimers and the trimers all bound \([\alpha-32P]GTP\) in a time-dependent fashion (Fig. 3A). When diluted into a solution containing 1 μM unlabeled GTP, the \(32P\) was slowly released with a similar half-time for all the complexes of ~150 min at 30 °C (Fig. 3B). Nucleotide binding to the trimer continued for at least 24 h at 30 °C, by which time the complex was labeled to a stoichiometry of about 10% (assuming that each subunit is capable of binding nucleotide). This result is consistent with guanine nucleotide binding data for purified septins from Drosophila (9). A competition assay using cold GTP or GDP showed that the septin dimers do not discriminate between dinucleotides and trinucleotides (data not shown). Remarkably, neither the binding nor release of guanine nucleotide was affected by the addition of excess EDTA to complex the magnesium ions. This behavior is very different from that of most other GTP-binding proteins, which require magnesium ions to stabilize the association of the nucleotide with the protein.

To test for GTPase activity, we incubated the septins with \([\alpha-32P]GTP\) for 2 h and then passed the complex through a size exclusion column to remove unincorporated nucleotide. At intervals the septins were then denatured with SDS, and the bound nucleotides were separated by thin layer chromatography. Under these conditions, we observed a time-dependent fall in the level of bound \([\alpha-32P]GTP\) coupled to a rise in the level of bound \([\alpha-32P]GDP\) (Fig. 3C). This reciprocal change strongly supports the hypothesis that the septin complex does possess a GTPase activity, although it is extremely inefficient.

Finally, we wished to determine whether GTP hydrolysis might play a role in the assembly of septin heterodimers or filaments from monomers. Because the monomers are unstable, we were unable to detect efficient association into dimers when they were mixed and could not directly test the effects of slowly hydrolyzable analogs of GTP on dimerization. Therefore, to address this issue, we prepared small quantities of pure monomers and rapidly mixed them in the presence of either \([\alpha-32P]GTP\) or \([\gamma-32P]GTP\). We reasoned that if GTP hydrolysis occurs on dimerization, then we would not detect bound \(32P\) when using the \([\gamma-32P]GTP\), but with \([\alpha-32P]GTP\), \(32P\) would be retained on the septin heterodimer as \([\alpha-32P]GDP\). On the other hand, if GTPase activity was not involved in dimerization, both the \([\gamma-32P]GTP\) and \([\alpha-32P]GTP\) should bind equally well. The appearance of bound \(32P\) in the experiment would also provide an indirect measure of dimerization, because the monomers bind nucleotide very inefficiently. The results of this type of experiment are shown in Fig. 3D. When \([\alpha-32P]GTP\) was added to Sept6 or Sept7 alone, only a small amount of binding to the Sept7 monomer was detected and none to Sept6. However, when the Sept6 and Sept7 were mixed at the same time as the nucleotide was added, bound counts accumulated over time, suggesting that a fraction of the monomers was combining into stable dimers that could bind nucleotide. When the same experiment was performed but in the presence of an equal amount of \([\gamma-32P]GTP\), significantly less protein-bound \(32P\) accumulated. This result suggests that GTP hydrolysis is occurring at a rate that is equal to or greater than the rate of dimer formation.

Septins Interact through Their Coiled-coil Domains—Most septins consist of a variable N-terminal region followed by a GTP-binding domain and terminate in a C-terminal coiled-coil domain. To determine which regions of the septins are required for dimerization, we co-expressed either the isolated C-terminal regions or the N-terminal regions (variable domain plus GTP-binding domain) in bacteria, with an HS tag on only one of the components and asked if the untagged component co-purified on Ni²⁺-agarose beads. The isolated N terminus of Sept6 was mostly insoluble, and its solubility was not increased by the co-expression of Sept7 (data not shown; also see below and Fig. 5D). However, when the C-terminal coiled-coil domains of each were expressed, the HS-tagged Sept7 co-purified with the untagged C terminus of Sept6 (Fig. 4). Note also that the stability of the Sept7 fragment appears to be increased by the presence of Sept6-(283–429). We conclude that at least two of the septins, Sept6 and Sept7, likely associate into a heterodimer through their coiled-coil domains.

Borg3 Binds Specifically to the Sept6-Sept7 Heterodimer—Affinity purification of cell lysates, using GST-Borg3 attached to beads, can isolate a complex of at least three septins from cell lysates. We have shown previously that a small domain of about 28 amino acids (residues 83–110), which is conserved within the Borg family, is both necessary and sufficient to bind septins (19). We named this sequence the BD3 domain. To identify the minimum requirement for binding of septins to this BD3 domain, we tested three septin monomers, their various heterodimers, and the trimer for association with a GST-BD3. As shown in Fig. 5A, none of the monomers were capable of binding to the BD3 domain. Additionally the Sept2-Sept6 het-
erodimer did not bind the BD3 domain. However, the Sept6-Sept7 complex and the septin trimer were efficiently captured by the BD3 domain. In a second experiment, a GST fusion of the full-length Borg3 was used, and all three heterodimers were tested using detection of the S-peptide tag on one of the septins as a more sensitive assay for bound protein (Fig. 5B).

Again, only the Sept6-Sept7 heterodimer bound to the GST-BD3 beads. This result supports the idea that individual septin polypeptides are unable to recognize the BD3 domain of Borg3 and that epitopes provided by both Sept6 and Sept7 are required. As a further test of this hypothesis, we used a yeast three-hybrid conjugation assay. Yeast (yDW-12a) was produced that expresses Sept7 and/or a fusion protein comprising Sept6 attached to the LexA-DNA binding domain (the bait). These strains were mated with yeast that expresses a fusion protein of Borg3 plus the activation domain of VP16 (the prey). If the Borg3 binds to Sept6, it induces the expression of the His3 gene and allows survival of diploid yeast on selective medium lacking Leu, Trp, and Ura. Interaction of the Borg3 with the Sept6 was tested on a selective medium also lacking His and containing a histidine synthesis inhibitor, 3-aminotriazole. D, the C-terminal coiled-coil region of Sept6 is sufficient for binding of Borg3 to the Sept6/7 dimer. HS-tagged Sept7 was expressed together with either the N-terminal region (1–282) or the C terminus (283–429) of Sept6, purified on Ni2+-agarose beads, and then incubated with GST-BD3 or GST on glutathione beads. After washing, the bound proteins were eluted with glutathione, analyzed by SDS-PAGE, and stained with Coomassie Brilliant Blue.

To begin to identify the regions of these septins that are required for the interaction with Borg3, we used the isolated C-terminal coiled-coil region of Sept6 and full-length Sept7. Although Borg3 did not bind to either of these proteins alone, it did bind to the paired proteins (Fig. 5D). Note that this result supports the observation (Fig. 4) that the C-terminal domain of Sept6, which lacks the GTP-binding domain, is sufficient to bind Sept7. The N-terminal region of Sept6 is mostly insoluble and was not captured in a complex with Sept7 by GST-BD3 on beads (Fig. 5D). We conclude, therefore, that Borg3 binding to septins requires the Sept6-Sept7 heterodimer and that it does not require both GTP binding domains. We have been unable thus far to detect Borg3 binding to the dimerized coiled-coil domains of Sept6-Sept7, however, suggesting that the BD3 binding domain may extend further into the N-terminal region than do the dimerization motifs.

To ascertain whether Borg3 modulates septin dimerization, we mixed recombinant Sept6 and Sept7 monomers in the presence of [α-32P]GTP with or without GST-Borg3, but no differ-

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**Fig. 5.** A, Borg3 binds specifically to the trimer and Sept6-Sept7 heterodimer. Septins were prepared as described in the Fig. 1 legend and under “Materials and Methods.” GST-BD3 and GST alone (10 µg) were attached to glutathione beads and incubated for 45 min with the septins (20 µg) at 4 °C. The beads were then washed, and bound proteins were eluted with glutathione and analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue. B, binding was performed as described for panel A but to GST or full-length GST-Borg3. Bound HS-tagged protein was detected by immunoblotting with horseradish peroxidase-coupled S-protein. C, yeast three-hybrid conjugation assay. Yeast that expressed LexA-DBD-Sept6 and/or untagged Sept7 (or vector alone) were mated with yeast that expressed VP16-Borg3. Successful mating was tested on a selective medium lacking Leu, Trp, and Ura. Interaction of the Borg3 with the Sept6 was tested on a selective medium also lacking His and containing a histidine synthesis inhibitor, 3-aminotriazole. D, the C-terminal coiled-coil region of Sept6 is sufficient for binding of Borg3 to the Sept6/7 dimer. HS-tagged Sept7 was expressed together with either the N-terminal region (1–282) or the C terminus (283–429) of Sept6, purified on Ni2+-agarose beads, and then incubated with GST-BD3 or GST on glutathione beads. After washing, the bound proteins were eluted with glutathione, analyzed by SDS-PAGE, and stained with Coomassie Brilliant Blue.
ence in nucleotide binding was observed. Similarly, when a preformed Sept6-Sept7 dimer was loaded with \([\gamma\text{-}^{32}\text{P}]\)GTP and exposed to Borg3, no change in the release of \(^{32}\text{P}\) from the protein was observed (data not shown). Therefore, Borg3 does not increase the GTPase activity during or after dimerization.

**DISCUSSION**

Our understanding of septin function depends on knowledge of the biochemistry of these proteins. However, their study has been hampered by difficulties associated with their expression as recombinant proteins in bacteria and the complexity of their interactions with one another. We have developed systems to express multiple septins in *E. coli* and have demonstrated that three septins can together form filaments with dimensions similar to those purified from cells. Understanding their biochemistry remains a challenge, however, because the septin oligomers bind guanine nucleotides and hydrolyze GTP, only very slowly. Moreover, the instability of the monomers complicates investigations of oligomer assembly.

Nonetheless, we can begin to understand the assembly process. First, we conclude that any pair of septins (at least Sept2, Sept6, and Sept7) can form heterodimers, although the Sept6/7 dimer is more stable; second, at least the Sept6/7 dimer associates through the coiled-coil regions in the C terminus of each septin rather than through their GTP-binding domains; and third, GTP hydrolysis accompanies dimer formation (although we cannot prove that hydrolysis is required for dimerization). Finally, we have found that the septin binding protein, Borg3, associates not with individual monomeric septins, but rather that it specifically recognizes the Sept6/7 heterodimer. We speculate that the BD3 domain binds at the coiled-coil interface between the subunits of the dimer.

The mechanism by which Borg3 binding triggers septin reorganization in cells remains obscure, however, because we have been unable to detect any effect on dimerization or on recombinant filament bundling in *in vitro*. One interesting possibility is that it displaces another protein, perhaps another septin such as Sept9, which normally associates with Sept6/7 in the cell via an overlapping binding site. We are currently investigating the effects of Borg3 on septin dynamics, both in *vivo* and *in vitro*. The ability to express recombinant septin filaments and heterodimers will provide powerful new tools in this endeavor, because these proteins can be tagged with fluorescent markers and injected into cells. Speckle microscopy can then be used to analyze the filament dynamics. The ability to express septin dimers and trimers will also be useful for production of affinity ligands, to aid in the identification of septin binding proteins from mammalian cell extracts, and to search for regulatory factors that may modulate GTP binding or hydrolysis. Finally, the ability to produce septin oligomers is an essential step toward the goal of obtaining high resolution x-ray crystallographic structures of the septins.

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