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CHAPTER 2: Preparing recombinant yeast septins and their analysis by electron microscopy

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Abstract

Septins are highly conserved and essential eukaryotic cytoskeletal proteins that interact with the inner plasma membrane. They are involved in essential functions requiring cell membrane remodeling and compartmentalization, such as cell division and dendrite morphogenesis, and have been implicated in numerous diseases. Depending on the organisms and on the type of tissue, a specific set of septins genes are expressed, ranging from 2 to 13. Septins self-assemble into linear, symmetric rods that can further organize into linear filaments several microns in length. Only a subset of human septins has been described at high resolution by X-ray crystallography (Sirajuddin et al., 2007). Electron microscopy (EM) has proven to be a method of choice for analyzing the molecular organization of septins. It is possible to localize each septin subunit within the rod complex using genetic tags, such as maltose-binding protein or green fluorescent protein, to generate a visible label of a specific septin subunit in EM images that are processed using single-particle EM methodology.

In this chapter we present, in detail, the methods that we have used to analyze the molecular organization of budding yeast septins (Bertin et al., 2008). These methods include purification of septin complexes, sample preparation for EM, and image processing procedures. Such methods can be generalized to analyze the organization of septins from any organism.

INTRODUCTION

Septins were discovered several decades ago following a cell division cycle mutants screen (Hartwell, 1971). However, septins have only recently been studied in depth and now are referred to as the fourth cytoskeletal system in eukaryotes (Mostowy & Cossart, 2012). Indeed, septins are present in all eukaryotes, with the exception of some plants, and are involved in multiple biological processes. They have been shown to play a role in cell division (Menon & Gaestel, 2015), neuron morphogenesis (Ewers et al., 2014; Xie et al.,

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2007), cell motility (Gilden et al., 2012), spermiogenesis (Kuo et al., 2015; Lin et al., 2011), building diffusion barriers (Ewers et al., 2014; Hu et al., 2010; Shcheprova et al., 2008; Takizawa et al., 2000), or in bacterial invasion (Mostowy et al., 2009, 2010). Septins also interact with other cytoskeletal proteins [actin (Mavrakakis et al., 2014; Smith et al., 2015) and microtubules (Bai et al., 2013)] to alter their organization and function. Not surprisingly, septins have now been implicated in multiple diseases, including cancers (Connolly et al., 2011; Peterson & Petty, 2010) or neurodegenerative diseases like Alzheimer and Parkinson (Choi et al., 2003; Kinoshita et al., 1998; Takehashi et al., 2004).

Depending on the organism and tissue, a specific number of septins are expressed and organize into a repeating unit that can polymerize into long filaments (Versele & Thorner, 2005). The primary sequence of septins includes a basic N-terminal domain at their N-terminus, a GTP-binding domain, and a coiled coil region at their C-terminus. The structure and molecular organization of septins started being investigated at the beginning of the century. A subset of human septins (Sept2, Sept6, Sept7) have been crystallized and described at 4 Å resolution in different conditions (Sirajuddin et al., 2007, 2009). Being filamentous, it is possible to obtain highly relevant information on their molecular organization also by electron microscopy (EM) methods. A number of structural studies by EM have been performed for different septin species (*Caenorhabditis elegans* (John et al., 2007), *Saccharomyces cerevisiae* (Bertin et al., 2008), human (Lukoyanova, Baldwin, & Trinick, 2008; Sellin et al., 2011)). All of the structural studies tend to demonstrate that septins, in their minimal assembly, organize into palindromic rodlike structures. The interaction between adjacent subunits alternates between so-called N-C interfaces and G binding interfaces. The G interface is along the nucleotide (GTP or GDP) binding site. N-terminal alpha helices and C terminal alpha helices of neighboring septin subunits interact to form the N-C interfaces.

In this chapter we present, in details, the methods that we have used to analyze the molecular organization of budding yeast septins, including their purification and their study by EM.

1. METHODS

1.1 PREPARATION OF RECOMBINANT BUDDING YEAST SEPTIN COMPLEXES

The four budding yeast septin subunits (Cdc3, Cdc10, Cdc11, and His₆-Cdc12) are coexpressed in BL21 DE3 cells. To this end, two plasmids carrying two septin subunits each are used. For affinity purification purposes, Cdc12 is labeled with a histidine 6-mers tag. The purification process has to be performed within the same day to enhance the yield. From 1.5 L of cells about 1 mg of septin complexes is produced.

1. Transform *Escherichia coli* BL21 DE3 cell with both the derivative of pACYCDuet-1 holding Cdc3 and Cdc11 and the derivative of pETDUET-1 carrying CDC10 and His₆-Cdc12, using a standard transformation method (heat shock or electroporation). One microliter of each plasmid can be pre-mixed before being added to the cells. Plate 50–200 µL of cells into LB agar plates enclosing both ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL). It is recommended that bacteria be

transformed with plasmids just prior to purification rather than using bacteria previously transformed with the plasmids that have been thawed from freezer stocks.

Note: pACYCDuet is resistant to chloramphenicol while pETDUET is resistant to ampicillin. After the transformation, a few colonies are observed (about 10). The rather low transformation efficiency results from the dual antibiotic resistance.

2. The next day, grow a 50-mL preculture after picking up a single colony from the agar plate.
3. After an overnight preculture, dilute the cells into two 2-L flasks containing 750 mL LB each containing both chloramphenicol and ampicillin. A starting optical density of 0.05 is appropriate. Grow the cells at 37°. Once the OD₆₀₀ reaches 0.5, lower the temperature of the incubator down to 16°. Induce the protein expression using IPTG (0.1 mM) at an OD₆₀₀ of 0.8.
4. After about 20 h of expression at 16°, harvest the cells by pelleting them for 20 min at 6000 g. Resuspend the cells in lysis buffer (300 mM NaCl, 2 mM MgCl₂, 40 μM GDP, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.5% Tween 20, 12% glycerol) and flash freeze the resuspended cells in liquid nitrogen.

Note: After induction, the frozen cells can be stored in a –80° freezer for a few months or used at once. A freeze–thaw cycle is useful to facilitate the lysis of the cells.

5. After adding lysis buffer and a protease inhibitor (Complete EDTA free, Roche) to the frozen cells to get a 50-mL volume, thaw the cells on ice. Add lysozyme (0.5 mg/mL) and DNase to the thawing solution and wait 30 min.
6. Sonicate the cells using five pulses (of a tip sonicator) of 30 s, until the solution gets clearer and fluid. The solution should be kept on ice during sonication.

Note: the solution should not be viscous anymore, otherwise it is difficult to proceed to the following steps.

7. Spin down the cells at 10,000 g to get rid of cell debris.
8. Incubate the supernatant with nickel affinity beads (4-mL slurry) preequilibrated with lysis buffer for about 1 h in a cold room and on a swirling apparatus.
9. Pour a column (Econo-column, 2.5 × 20 cm, Bio-Rad) with the lysate plus nickel beads.

- 10.** Collect the flowthrough and pour 100 mL wash buffer (Tris-HCl 50 mM, TCEP 0.1 mM, NaCl 300 mM, Imidazole 10 mM) into the column without disturbing the beads.
- 11.** After washing the beads, add elution buffer (Tris-HCl 50 mM, TCEP 0.1 mM, NaCl 300 mM, Imidazole 500 mM) and collect 500 μ L fractions.
- Note: Steps 8–11 have to be done in a cold room. The elution buffer contains a rather high NaCl concentration to prevent septin filament formation.
- 12.** Analyze the fractions by detecting the protein peak with a Coomassie plus protein assay reagent and run a 10% SDS-PAGE gel to analyze the content of the fractions (see Fig. 1A).
- Note: Usually, the eluted protein is gathered within fractions 4–9.
- 13.** To remove the Imidazole, use a PD10 desalting column (GE healthcare).
- 14.** Run a preequilibrated size exclusion column (superdex 200 HiLoad 16/600 (GE Healthcare) in a 50 mM Tris-HCl, pH 8; NaCl 300 mM, TCEP 0.1 mM) at a 0.8 mL/min flow rate.
- 15.** Analyze the fractions using a 10% SDS-PAGE gel (See Fig. 1B). Pool the fractions of highest purity.
- 16.** Use a desalting PD10 column to lower the ionic strength before running an ion exchange column. To this end, the PD10 column is equilibrated in 50 mM Tris-HCl pH 8.
- Note: At this stage the septin complexes self-assemble into filaments. It is best to proceed as fast as possible after desalting the sample and load the sample onto the Resource Q column. Otherwise, much of the sample could be lost at this stage.
- 17.** Run a resource Q ion exchange column (1 mL, GE Healthcare life science). The ion exchange step is performed at a flow rate of 4 mL/min using a gradient run within 20 column volumes from a low ionic strength buffer (50 mM Tris-HCl, pH8, TCEP 0.1 mM) and a high salt buffer (50 mM Tris-HCl, pH 8, NaCl 500 mM, TCEP 0.1 mM). The purification profile and electrophoresis analysis is displayed in Fig. 1C).
- 18.** Finally, measure the protein concentration using a nanodrop and appropriate molecular weights and extinction coefficients. The protein can be flash frozen in liquid nitrogen without using glycerol. Usually, from 1 L of LB, one should get about 1 mg of pure septin complex.
- Note: In case the protein needs to be concentrated, the membranes of the concentrators need to be passivated with triton, otherwise septins would stick to the membrane and ~80% of the yield would be lost.

2. MATERIALS

Refrigerated incubator

Two-liter Erlenmeyer flasks for cell culture

Centrifuge

Ni-NTA beads: Nickel sepharose high performance (GE healthcare)

Econo-column (2.5 × 20 cm), Bio-Rad

PD10 desalting columns (GE Healthcare)

Superdex 200 HiLoad 16/600 (GE Healthcare)

Resource Q 1-mL column (GE Healthcare)

2.1 SAMPLE PREPARATION FOR ELECTRON MICROSCOPY

Septin filaments and octamers can be analyzed by either negative stain or cryo-EM methods. For negative stain, either uranyl acetate or uranyl formate can be used. However, uranyl formate staining seems to result in a “finer” stain grain.

2.1.1 Sample preparation for negative stain samples

1. Glow discharge a continuous carbon grid (carbon side up) at an intensity of 2–5 mA for 30 s.

Note: Continuous carbon grids can either be “homemade” or purchased from commercial sources (CF300-Cu, Electron Microscopy Sciences). The effect of glow discharge lasts for about an hour.

2. Prepare uranyl acetate or uranyl formate in water at 2%.

Uranyl acetate preparation: Dissolve uranyl acetate in water at a 2% concentration. Syringe filter using a 0.2 µm cut off. Wrap the tube with aluminum foil to avoid light exposure. The solution can then be aliquoted and stored in the –20°C.

Uranyl formate preparation: Boil some water and let it cool down at room temperature. Add 20 mg of uranyl formate powder into 1 mL of water.

Vortex for 5 min. Add 10 µL of NaOH 1M. Vortex again for 5 min. Syringe filter using a 0.2 µm cut off. Wrap the tube with aluminum foil.

Note: uranyl acetate can be prepared in advance and stored in –20°C. Uranyl formate has to be prepared fresh.

3. To look at octameric yeast septin structures, dilute a septin sample in high salt buffer (50 mM Tris–HCl, pH 8, NaCl 300 mM) at a 0.02 mg/mL

concentration. To examine septin filaments, dilute a septin sample into a Tris–HCl buffer (Tris–HCl, pH 8) at 0.05–0.1 mg/mL.

4. Adsorb a 4 μ L drop of the septin solution onto the grid for 30 s to 1 min.
Note: antipillary self-closing tweezers are used to keep the liquid drop on the grid.
5. Remove most of the solution using a filter paper.
6. Add a drop of stain solution.
7. After 60 s, remove the stain with a filter paper and let the grid dry for at least 5 min on the bench. Images of negatively stained samples are shown in Fig. 2A and C.

2.1.2 Sample preparation for thin film frozen hydrated samples—One can use an automated device to vitrify the grids in liquid ethane (EMGP, Leica or Vitrobot, FEI) or a manual plunge freezing apparatus.

1. Glow discharge holey grids: either quantifoil-like (quantifoil) or lacey grids (Ted Pella).
2. Adsorb 4 μ L of septins, at 0.5 mg/mL, onto the grid and plunge freeze the sample after it has been thoroughly blotted with a filter paper. Images of plunge-frozen septins are displayed in Fig. 2B and D.

3. MATERIALS

Grids for EM: continuous carbon grids for negative stain, holey grids for cryo-EM (quantifoil, C-flats or lacey grids)

Antipillary self-closing tweezers

Glow-discharge apparatus

Plunge freezing apparatus (type EM GP, Leica or Vitrobot, FEI)

Uranyl formate or uranyl acetate

Electron microscope: For negative stain samples, a 120 kV microscope is sufficient

3.1 MICROSCOPY AND IMAGE TWO-DIMENSIONAL ANALYSIS

After choosing a sample of good quality, images are acquired with an electron microscope. To perform 2D analysis of septin complexes a 120 kV microscope is sufficient.

Image analysis is often necessary to check the behavior of a given septin mutant and determine whether it is stable (ie, whether the octamer complex is altered), as well as to localize a tag that is either covalently bound to a subunit [by genetic tagging with a green fluorescent protein (GFP) or maltose-binding protein (MBP), for example] or has been

incubated with the sample (like antibody). The computational process of 2D classification sorts out images from a data set into groups (classes) based on similarity.

Image processing can be performed with any of the available software packages dedicated to image processing for single-particle EM. In our case we used SPIDER (Frank et al., 1996). The method presented later is a possible protocol among a number of other possibilities in terms of choice of algorithm. Before performing image analysis, the quality of the sample has to be checked. If the quality is not good enough in terms of sample concentration or signal to noise ratio, the samples have to be prepared again.

1. Collect data either on film or on a CCD camera depending on the available equipment. For data collected on film, micrographs have to be scanned using a scanner (Nikon Coolscan 8000 scanner, for instance). Beforehand, select areas of the grid where the quality is best in terms of stain quality. For instance, in Fig. 2A, septins (white) are clearly visible on a dark background. Images are collected at a magnification between 30,000 and 50,000.
2. Assess the quality of the data (signal over noise, drift, protein concentration). From contrast transfer function estimation, one can assess the drift of a given image. Besides, the protein should not be too concentrated otherwise it is difficult to select and pick individual particles. Keep the best images.
3. Use software to “pick” particles and box them out of the image. In our case, we have manually picked particles using the Boxer toolbox from the EMAN suite software (Tang et al., 2007). To perform 2D analysis on negative stain sample, a few 1000 particles are required (typically 2000–5000).
4. Normalize the data set (stack of image) using a boxed area of the background of one image (without protein density). An example of raw particles is shown in Fig. 3A.
5. Perform a reference-free alignment and classification to obtain about 200 classes with an average of about 100 particles each (in SPIDER: AP SR command). Some classes obtained after a reference-free alignment are shown in Fig. 3B.

Note: As an initial step, a reference-free alignment algorithm is necessary to avoid biasing the alignment.

6. Use those first classes as new references to perform a multireference alignment (in SPIDER: AP MQ command, for instance).
7. Perform a classification of the aligned data set by correspondence analysis (CA S command in SPIDER, for instance), hierarchical classification (commands CL HC, CL HD, CL HE in SPIDER). After correspondence analysis, appropriate “eigenimages” are chosen. Classes from a first round of alignment-classification are displayed in Fig. 3C.

Note: the commands detailed here in SPIDER can be modified depending on one's choice.

8. From the new classes obtained, select a few representative ones as new references for a further cycle of alignment and classification.
9. Perform as many cycles of alignment and classification necessary until no improvement is visible in the classes obtained. Typically, four cycles are carried out. Classes from a second round of alignment-classification are displayed in Fig. 3D.
10. Classes corresponding to septin rods of different sizes or of different orientation will be obtained. Also, when a tag has been introduced (covalent tag or antibody), it should be visible in specific classes.

4. MATERIALS

Electron microscope equipped with a camera

Scanner: Nikon Coolscan 8000

Workstation or cluster: a personal workstation is sufficient, typically 48 GB of RAM.

4.1 TAGGING STRATEGIES TO LABEL SEPTIN SUBUNITS WITHIN COMPLEXES

To localize a particular septin subunit within the octameric complex, visible extra density can be added to that subunit. Image processing is then used to detect more clearly and with more confidence the presence and location of the extra density. It is possible to combine different tagging strategies for the same septin subunit to make their localization within the complex even more robust.

4.1.1 Antibody labeling—From our experience, several antibodies have to be tested for their interaction with septins. Indeed, for most of the antibodies we tried, the interaction with the protein of interest was barely visible by EM. Such results are not totally unexpected, as antibodies are not covalent labels and the concentrations of protein used in negative stain imaging are very low.

Choose antibodies against a specific septin subunit or against a tag present in the septin complex (His₆ tag, for instance).

Make sure the antibody solution is as pure as possible. Otherwise perform a supplementary purification step (ie, protein A or protein G) to get rid of contaminants.

1. In high salt conditions (when septins are octameric), incubate a molar excess (typically 2:1) antibodies with septins for 1 h on ice.
2. Prepare an EM grid with negative stain, as explained in detail previously.
3. After checking the quality of the grid, collect some data.

4. Perform 2D analysis as described previously to pinpoint the presence of the antibody. A representative class of an anti-His tag antibody bound to the septin complex is presented in Fig. 4A (right).
5. In low salt conditions, a large excess of antibodies (fivefold) can also be incubated in the presence of septin preformed bundles. In case antibodies and septin bundles interact, a periodic decoration of the bundles is expected (see Fig. 4A, left).

4.1.2 Covalent bound tags

1. Design constructs to include a tag (MBP, GFP) on one of the septin subunits.
2. Express and purify the septin complex as usual (see previous paragraph).

Note: During this stage one will see whether the tagged complex is well expressed.
3. Analyze the stability of the complex by checking on an EM grid whether the protein can self-assemble into octameric rods and filaments (depending on the salt conditions).
4. Perform 2D analysis as described previously to pinpoint the presence of the tag as an additional density of a size that is comparable to the size of a single septin subunit. Two examples of resulting class averages are displayed in Fig. 4B (MBP tag bound to CDC3) and in Fig. 4C (MBP tag bound to CDC12).

CONCLUSION

Yeast septin complexes that are active for polymerization into long filaments can be purified from bacterial sources and structurally characterized by EM to define their overall architecture, subunit organization, and assembly capabilities. Here we have presented the methodology for expressing and purifying septins, as well as the detailed procedures for making samples for EM. We also summarized a methodology for 2D image processing that allow the generation of 2D class averages that allow the visualization of individual septin subunits and their localization based on extra mass used for labeling. The procedures presented here can be generalized to other septin complexes.

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FIGURE 1.

Budding yeast septins purification profiles. (A) 10% SDS-PAGE electrophoresis acrylamide gel resulting from the nickel affinity purification step. Five lanes are displayed: protein marker, flowthrough (FT), wash (W), elution tube 5 (from 3 to 2.5 mL), and elution tube 7 (from 3.5 to 4 mL). The four septin subunits are visible (from top to bottom: Cdc3, Cdc11, Cdc12, and Cdc10). (B) Elution profile after size exclusion (Superdex 200 column), insert on the left: 10% SDS-PAGE gel displaying the protein composition from elution tubes 43 to 49. (C) Elution profile after ion exchange (Resource Q column). Insert on the left: 10% SDS-PAGE gel displaying the protein composition from elution tubes 30 to 33 (main peak).

FIGURE 2.

Septin images by electron microscopy using negatively stained samples (A and C) or frozen hydrated samples (B and D). Short (about 30 nm long) *rodlike structures* are visible in high salt (NaCl 300 mM) conditions (A and B). Septins assemble into paired filaments in low salt conditions (C and D). Scale bars: A: 100 nm, B: 50 nm, C: 100 nm, D: 20 nm.

FIGURE 3.

2D image analysis for a given septin data set. (A) Some raw picked and selected particles. (B) Some of the classes obtained after reference-free alignment. (C) Some of the classes obtained after a first round of multireference alignment and classification. (D) Final classes obtained after two rounds of multireference alignment and classification. Scale bar: 20 nm.

FIGURE 4.

Septins labeling using either antibodies (A) or covalent tags (B and C). (A) Left: decoration of septin bundles by anti-Cdc11 antibodies, right: class average displaying the labeling of His₆-Cdc12 by an anti-Histidine tag antibody. (B) Class averages of the Cdc11-Cdc12-MBP-CDC3-CDC10 septin complex. One or both maltose-binding protein (MBP) tags are visible as extra blobs on the side of the septin rod. (C) Class averages of the Cdc11-MBP-Cdc12-CDC3-CDC10 septin complex. The MBP tags are also visualized.