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In Vitro Analysis of Microtubule Nucleation at the Fission Yeast Spindle Pole Body.

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I. Introduction

The yeast spindle pole body (SPB), the structure responsible for promoting microtubule nucleation and spindle formation, is the functional equivalent of the animal centrosome. In the fission yeast *Schizosaccharomyces pombe*, the SPB is an oblate ellipsoidal structure that resides on the cytoplasmic surface of the nuclear envelope during interphase (see Chapter I-3). As the cell enters mitosis, the nuclear envelope invaginates beneath the SPB and forms an opening into which the SPB settles (Ding et al., 1997). Microtubules are then nucleated at the nuclear surface of the SPB for spindle assembly. During anaphase, the SPBs move back into the cytoplasm.

Microtubule-nucleating activity of the *S. pombe* SPB is regulated during the cell cycle (Hagan and Hyams, 1988; Masuda et al., 1992), although γ-tubulin, a protein responsible for microtubule nucleation, is localized at the SPB throughout the cell cycle (Horio et al., 1991; Masuda et al., 1992). Although some γ-tubulin is at the cytoplasmic face of the SPB, most γ-tubulin is found on the osmiophilic material that lies near the inner surface of the nuclear envelope immediately adjacent to the SPB (Ding et al., 1997).). During interphase, a few microtubules attach to or terminate at the region close to the cytoplasmic face of the SPB. The γ-tubulin associated with the osmiophilic material is not active for microtubule nucleation during interphase. At the onset of mitosis, the γ-tubulin on the osmiophilic material begins to nucleate spindle microtubules. At metaphase through telophase, the cytoplasmic face of the SPB nucleates cytoplasmic microtubules that are required for nuclear positioning (Hagan and Yanagida, 1997).

The activity of the centrosome in animal cells has been studied using permeabilized cells (Snyder and McIntosh, 1975; Joshi et al., 1992) and isolated centrosomes (Kuriyama and Borisy, 1981; Mitchison and Kirschner, 1984; Bornens et al., 1987). For preparation of permeabilized cells, mammalian cultured cells are treated with microtubule inhibitors to depolymerize microtubules, followed by permeabilization with non-ionic detergents to remove nuclear and cytoplasmic soluble proteins including α/β-tubulin heterodimers.

The activity of the centrosome in the permeabilized cell can be monitored by addition of □/□-tubulin dimers purified from bovine or porcine brains. Centrosomes also can be isolated from cultured cells, after incubation of the cells in drugs that disrupt the cytoskeleton. After this treatment, the centrosome is no longer anchored to other components of the cytoplasm, and subsequent lysis in hypotonic solution releases the centrosomes into the medium.

These same kinds of approaches can be used for studying the activity of the yeast SPB *in vitro*. In *Saccharomyces cerevisiae*, the SPB is a disk-shaped, multilayered structure that is embedded into the nuclear envelope throughout the cell cycle. The isolation procedure requires fractionation and lysis of the nuclei to release the SPB from the nuclear envelope (Rout and Kilmartin, 1990; see Chapter I-1). These isolated preparations were used for defining the composition (Rout and Kilmartin, 1990; Wigge et al., 1998) and the structure of the SPB (Bullitt et al., 1997). Microtubule-nucleating activity of the budding yeast SPB has also been studied using the partially purified SPB (Byers et al., 1978; Hyams and Borisy, 1978). An isolation procedure for purifying fssion yeast SPBs has not yet been developed.

We have developed a procedure to permeabilize *S. pombe* cells for studying the mechanisms of spindle elongation (Masuda et al., 1990) and of microtubule nucleation at the SPB (Masuda et al., 1992, 1996; Takada et al., 2000). Using permeabilized cells we have shown that the mitotic SPB, but not the interphase SPB is capable of nucleating microtubules *in vitro*. This result indicates that □-tubulin localized at the SPB is in an inative form during interphase. The interphase SPB can be activated for microtubule nucleation by incubation in mitotic extracts prepared from unfertilized *Xenopus* eggs. This activation seems to occur through interaction of an activator present in the extracts with □-tubulin located at the SPB. Finally, the activator has been isolated from the extracts based on the *in vitro* activity, and identified as the ribonucleotide reductase, large subunit R1.

II. Protocol for Permeabilization

A. Solutions

YPD: 1% yeast extract, 2% polypeptone, 2% glucose
YPDS: YPD + 1M sorbitol
0.1 M Tris-H_2SO_4 (pH9.4): 0.1 M Tris, adjusted to pH9.4 with H_2SO_4
SSD: 0.67% yeast nitrogen base without amino acid (DIFCO), 2% glucose, 1 M sorbitol, adjusted to pH7.2 with NaOH
MDS: 0.1 M MES (pH6.5), 5 mM EDTA, 1 mM spermidine, 0.5 mM spermine, 20% DMSO, 1 M sorbitol, 0.1 mM trolox (Fluka Chemical Co.), 5 mM DTT, 0.2 mM PMSF, 1 ug/ml aprotinin, and 1/200 of PI
MD: MDS without sorbitol
PI (protease inhibitor cocktail): 10 mg/ml BAME, 10 mg/ml TPCK, 10 mg/ml TAME, 1 mg/ml leupeptin, 1 mg/ml pepstatin A in DMSO

B. Cell Culture

Wild type cells (h^-972) are grown at 26 ~33°C in rich media such as YPD and YEA to a density at 2 ~4 x 10^6/ml. 40 ml of cells (0.8~1.6 x 10^8 cells) are used for one preparation. Since the composition of the cell wall is known to vary with growth conditions, cells grown at early log phase are recommended. Synthetic media such as EMM2 may be used instead of rich media, although we have not tested it.

Wild type cells treated with drugs, or cell division cycle mutants arrested at the restrictive temperatures, are used to obtain cells that are in a similar stage of the cell cycle. Wild type cells are arrested at S phase by incubation at 29°C for 3-4 hr in the presence of 10 mM hydroxyurea. Mutant strains arrested in M phase at the restrictive temperatures are used for obtaining mitotic cells. *Nuc2* cells (nuc2-663) are used for obtaining mitotic cells arrested at metaphase (Hirano et al., 1988). They have a defect in APC/C or cyclosome function at the restrictive temperature (36°C) (Yamada et al., 1997). *Nuc2* cells are grown at 26°C in YPD to a density of 2-4 x10^6 cells/ml and then incubated at 36°C for 4 hr. *Nda3* cells (nda3-KM311) are used for obtaining mitotic cells arrested in mitosis without spindle assembly. They have a mutation on the □-tubulin gene and show a defect in microtubule nucleation at early stages of mitosis at the restrictive temperature (20°C) (Hiraoka et al., 1984). They are grown at 30°C in YPD to the density of 2-3 x10^6 cells/ml and then incubated at 20°C for 6 hrs and a further 2 hrs in the presence of 15 □g/ml methyl benzimidazole carbamate (MBC), a microtubule depolymerizing drug. MBC is dissolved in 100% DMSO at 1.5 mg/ml, and added 1/100 vol. to the culture medium. The addition of MBC is required for obtaining permeabilized mitotic cells without

microtubule assembly. We found that a short spindle is formed even at the restrictive temperature during the period when the cell wall is being digested for spheroplasting.

C. Digestion of the Cell Wall

Digestion of the cell wall with lytic enzymes is required prior to permeabilization of the cells with a non-ionic detergent. The cell wall consists mainly of polysaccharides of glucose, mannose and N-acetylglucosamine (Cabib et al., 1982). In addition, chitin constitutes the primary septum. We use Zymolyase-100T (Seikagaku Corp.) for digestion. An essential enzyme responsible for lysis in the product is β-1,3-glucan laminaripentaohydrolase, which hydrolyses linear glucose polymers with β-1, 3-linkages.

1. Wash cells briefly in 40 ml of 0.1 M Tris-H_2SO_4 (pH9.4) prewarmed to 29°C (36°C for nuc2, and 20°C for nda3). Incubate cells with 0.1 M Tris-H_2SO_4 (pH9.4) containing 10 mM DTT for 4-5 min at 29°C (36°C for nuc2, and 20°C for nda3). For nda3, 15 μg/ml of MBC is added to both solutions.

Mannan is depolymerized by mild alkaline treatment. In addition, digestion of the cell wall by lytic enzymes is facilitated by preincubation with sulfhydryl compounds (Cabib et al., 1982).

2. Wash cells twice with 10 ml of YPDS. Resuspend cells in 2 ml of YPDS and add 60-100 μl of 20 mg/ml Zymolyase 100T (final concentration 0.6-1.0 mg/ml). Incubate at 29°C (36°C for nuc2) with shaking. Check the extent of digestion every ten minutes under a microscope by diluting a small amount of the cell suspension into water. Incubate a further 30 min, after more than half of the cells diluted burst in water. We usually incubate for 40-60 min total. For nda3 cells, wash cells twice with 10 ml of SSD at 20°C. Incubate cells at 20°C with 1.5 mg/ml Zymolyase 100T in SSD (pH7.2) containing MBC, for 40-60 min.

The digestion requires the presence of 1 M sorbitol for preventing cells from bursting due to the osmotic pressure. The solubility of Zymolyase is limited. Add the enzyme using the 20 mg/ml suspension in H_2O. The quality of the enzyme depends on the product batches. The optimal pH of the enzyme activity at 35°C is pH7.5, whereas the pH of YPD is around 6. Digestion of the cell wall at 20°C requires increasing the pH of culture media to near the opitimal pH for enzymatic activity.

D. Permeabilization with Triton X-100

1. Wash cells three times with 7 ml of ice-cold MDS. Resuspend cells in 4 ml of MDS and add 4 ml of MDS containing 1% Triton X-100. Incubate cells for 7-10 min on ice.

Addition of DMSO, Trolox, and protease inhibitors (PI) to the medium is critical for preserving the structure and function of the SPB**.** Trolox is a water-soluble derivative of Vitamin E, which acts as a radical scavenger. For studying the mechanism of spindle elongation, spindle microtubules are preserved by washing twice with MDS at room temperature, followed by a wash with cold MDS. The quality of permeabilization needs to be checked by an *in vitro* activation assay of microtubule nucleation (see section III).

2. Wash cells three times with 10 ml of MD. Resuspend cells in MD at 2.5 x 10^7/ml. Freeze cells as aliquots in liquid N_2, and store at –80°C.

Permeabilized cells used for SPB activation are stored at –80°C up to 1 year. For spindle elongation, use of freshly prepared permeabilized cells is required. Freezing cells may inactivate the motor proteins required for spindle elongation.

III. Protocol for Microtubule Nucleation

A. Solutions

Make 2x solutions and freeze as aliquots. Before use, dilute with H_2O and mix with additives.

Wash: 75 mM HEPES, 2.5 mM MgAcetate, 5 mM EGTA, 10% DMSO, adjusted to pH7.6 with KOH; add 1 mg/ml ovalbumin, 3 mM GTP, 5 mM DTT, and 1/200 vol. of PI before use.

XEB: 10 mM HEPES, 5.9 mM MgCl2, 9.5 mM EGTA, 24 mM □-glycerophosphate, 35 mM sucrose, 0.1 mM trolox, adjusted to pH7.6 with KOH; add 1 mg/ml ovalbumin, 3 mM GTP, 4.8 mM glutathione, 5 mM DTT, and 1/200 vol. of PI before use.

TG: 70 mM PIPES, 19 mM MgAcetate, 9 mM EGTA, 80 mM □-glycerophosphate, 0.2 mM trolox, adjusted to pH6.8 with KOH; add 6 mM GTP, 10 □M taxol, 5 mM DTT, and 1/200 vol. of PI, and centrifuge before use.

PME: 80 mM PIPES, 1 mM MgSO4, 1 mM EGTA, adjusted to pH6.8 with KOH

PMEG: 75 mM PIPES, 10 mM MaAcetate, 5 mM EGTA, 40 mM □-glycerophosphate, 0.1 mM trolox, adjusted to pH6.8 with KOH; add 3 mM GTP, 10% DMSO, 10 □M taxol, 5 mM DTT, and 1/200 vol. of PI before use.

HMEG: 75 mM HEPES, 10 mM MaAcetate, 5 mM EGTA, 40 mM □-glycerophosphate, 0.1 mM trolox, adjusted to pH7.4 with KOH; add 6 mM ATP, 5 mM DTT, and 1/200 vol. of PI before use.

Fixative: 75 mM PIPES, 10 mM MaAcetate, 5 mM EGTA, 40 mM □-glycerophosphate, 0.1 mM trolox (adjusted to pH6.8 with KOH); add 10% DMSO, 4% paraformaldehyde, 0.2% glutaraldehyde.

PEMBL: 0.1 M PIPES (pH7.2), 1 mM EGTA, 1 mM MgSO4, 1.83% L-lysine, 1% BSA, and 0.1% sodium azide.

B. Tubulin Preparation

The protocol for tubulin isolation from *S. pombe* cells has not been developed yet. Instead, neuro-tubulin is purified from bovine or porcine brains using DEAE-Sepharose or phosphocellulose. Purified tubulin dissolved in PME and 1 mM GTP is frozen in liquid N_2 as aliquots and stored at –80°C.

C. Preparation of *Xenopus* Egg Mitotic Extracts

Mitotic crude extracts are prepared from *Xenopus laevis* unfertilized eggs according to the method of Murray (1991) with slight modifications (Masuda et al., 1992; Masuda and Shibata, 1996). The unfertilized eggs are crushed at 16°C by centrifugation at 10,000 rpm for 10 min in XEB containing 100 □g/ml cytochalasin B and protease inhibitors (10 □g/ml leupeptin, chymostatin, and pepstatin). High-speed extracts (HSE) are prepared by diluting the crude extract 1.5-fold with XEB containing protease inhibitors and energy mixture (7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, 1 mM $MgCl_2$, pH7.7), followed by centrifugation at 80,000 rpm for 60 min with Beckman TLA100 rotor. The clear central zone of the supernatant is collected as HSE, frozen in liquid N_2, and stored at -80°C.

D. Microtubule Nucleation

1. Thaw frozen permeabilized cells on ice, and aliquot 10^6 cells per reaction in 500 □l eppendorf tubes. Wash cells with 70 □l of ice-cold Wash solution. For nuc2 cells, depolymerize microtubules by incubating for 10 min with HMEG containing 6 mM ATP at room temperature. For SPB activation in interphase cells, incubate cells with 20 □l of HSE prepared from Xenopus unfertilized eggs or purified fractions from HSE for 5 min at room temperature.

2. Wash with 50 □l of XEB, and resuspend in 15 □l of TG. Add 15 □l of 10-25 □M tubulin in PME, and incubate for 10 min at room temperature. Centrifuge at 5,000 rpm for 40 sec, discard the sup and add 50 □l of PMEG (Do not vortex or pipet). Centrifuge at 5,000 rpm for 40 sec, discard the sup, add 400 □l of fixative, and incubate for 20 min. Wash 4 x with 70 □l of PEMBL.

E. Immunofluorescence

Incubate permeabilized cells for 90-120 min in 20 □l of PEMBL containing mouse monoclonal anti-□-tubulin (Sigma, DM1A or B-5-1-2; diluted to 1/250) and affinity-purified rabbit anti-*S. pombe* □-tubulin at room temperature. Wash 3 x with 70 □l of PEMBL. Incubate for 90-120 min with FITC (or

Alexa488)-labeled anti-mouse IgG and Cy3-labeled anti-rabbit IgG (Jackson ImmunoResearch). Wash 3 x with 70 □l of PBS containing 1 □g/ml DAPI, and resuspend in 50 □l of H_2O. Put a small amount of cell suspension on multiwell coverslips, dry them out completely and seal in 90% glycerol, 10% PBS, and 100 mg/ml 1, 4-diazabicyclo(2.2.2)octane.

IV. Results

A. Microtubule nucleation at the mitotic SPB

Mitotic and interphase *S. pombe* cells were processed for permeabilization, and microtubule-nucleating activity of the SPB in the cells was tested by addition of brain tubulin. After fixation, microtubules and the SPB were detected by immunofluorescence with anti-□-tubulin and anti-□-tubulin antibody, respectively.

Microtubule nucleation at the SPB was induced in permeabilized mitotic cells (Masuda et al., 1992). The permeabilized *nuc2* cells that retained a metaphase spindle were further incubated with 6 mM ATP at pH7.6 to depolymerize spindle microtubules. After this treatment, most of the SPBs still were able to nucleate microtubules in the presence of exogeneous tubulin (Fig. 1a, b). We also examined the ability of mitotic SPBs in *nda3* cells that arrested in mitosis with no microtubules nucleated at the SPBs. The SPBs also promoted microtubule assembly (Fig. 1c, d).

In contrast, the SPBs in most of cells grown asynchronously, and the SPBs in most of cells arrested at S phase (Fig. 1e, f) did not nucleate microtubules in the presence of exogenous tubulin, showing that microtubule-nucleating activity of the SPB at G2 and S phase is low (Masuda et al., 1992).

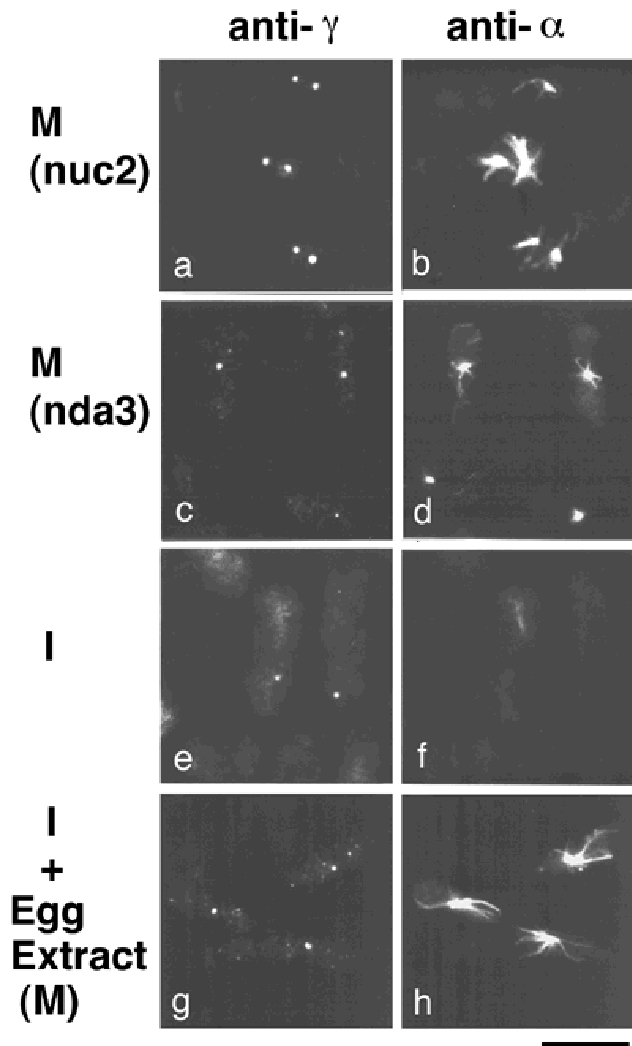

Figure 1. Microtubule nucleation at the SPB in permeabilized *S. pombe* cells. (a, b) *nuc2* cells arrested at metaphase, (c, d) *nda3* cells arrested in mitosis, (e, f) interphase cells arrested at S phase, and (g, h) interphase cells treated with *Xenopus* egg mitotic extracts. Cells were double-stained with anti-*S. pombe* □-tubulin antibody (a, c, e, g), and anti-□-tubulin antibody (b, d, f, h). Bar, 10 □m.

B. Activation of the interphase SPB

The interphase SPB was converted into a state competent for microtubule nucleation by incubation with mitotic extracts prepared from *Xenopus* unfertilized eggs (Fig.1g, h) (Masuda et al., 1992). The conversion was not induced by interphase extracts, but by an interphase extract driven into mitosis by addition of exogenous cyclin B (GST-cyclin B) in the presence of cycloheximide. The conversion was ATP-dependent, and suppressed by protein kinase inhibitors or alkaline phosphatase. Purified, active cdc2/cyclin B complex, however, was not effective for SPB activation. These results suggest that SPB activation occurs through a downstream pathway that is regulated by cdc2 kinase.

C. Role of □-tubulin in SPB activation

To study the role for □-tubulin in SPB activation, we examined the effect of anti-□-tubulin antibodies on microtubule nucleation (Masuda and Shibata, 1996). Preincubation of permeabilized interphase cells with anti-*S. pombe* □-tubulin prior to incubation in the *Xenopus* mitotic extract suppressed microtubule nucleation in the presence of exogenous tubulin, suggesting that □-tubulin also is involved in microtubule nucleation at the activated SPB. Using antibodies that were affinity-purified with an N-terminal, middle, and C-terminal fragment of □-tubulin, we have found that anti-N-terminal fragment antibody was effective in inhibiting microtubule nucleation at the interphase SPB incubated in *Xenopus* mitotic extracts, but not at the mitotic SPB of *nda3* cells. Anti-C-terminal fragment antibody suppressed microtubule assembly at the SPB incubated in *Xenopus* mitotic extracts, and at the mitotic SPB of *nda3* cells. Anti-middle fragment antibody was not effective for inhibiting microtubule nucleation. These results suggest that the N-terminal region of □-tubulin is important for SPB activation, and the C-terminal region is involved in microtubule assembly.

We also examined the effect of recombinant *S. pombe* □-tubulin on SPB activation (Masuda and Shibata, 1996). Addition of full length or an N-terminal fragment of □-tubulin to *Xenopus* mitotic extracts suppressed SPB activation in a concentration-dependent manner.

To partially purify the activator, we first fractionated the mitotic extracts with Q-Sepharose column chromatography (Masuda and Shibata, 1996). The flow-through fraction retained the SPB converting activity. The active fraction did not contain □-tubulin, showing that recruitment of □-tubulin from the extract is not required for SPB activation. The active fraction was further fractionated by affinity column chromatography with an N-terminal fragment of □-tubulin. The bound fraction had the SPB converting activity. Taken together, these results suggest that the SPB activator present in Xenopus mitotic extracts interacts with □-tubulin located on the SPB for SPB activation.

D. Identification of an SPB activator

Based on the *in vitro* activity monitored by the SPB activation assay, we have purified an 85kDa SPB activator from *Xenopus* egg mitotic extracts (Takada et al., 2000). The 85 kDa protein was identified as the ribonucleotide reductase large subunit R1 by amino acid sequence analysis. The ribonucleotide reductase (RNR) consists of a complex formed by a dimer of the large subunit R1 and a dimer of the small subunit R2. It catalyses the synthesis of deoxyribonucleotides, and provides substrates required for DNA synthesis and repair. Recombinant mouse R1 was also effective for SPB activation. In contrast, R2 was not required for SPB activation. SPB activation by R1 was inhibited by antibodies against R1, and by a partial oligopeptide of R1. GST-mouse R1 bound to R2, □-tubulin and □/□-tubulin dimers in *Xenopus* extracts.

R1 also seems to be involved in regulation of the centrosome activity. The partial oligopeptide of R1 inhibited aster formation at the centrosome reconstituted from *Xenopus* sperm centrioles in *Xenopus* egg mitotic extracts (see chapter V-25, 26 for detail). R1 was detected in the animal centrosome by immnofluorescence and immunoblotting with anti-R1 antibodies.

Our results suggest that R1 is a bifunctional protein that acts as the subunit of ribonucleotide reductase and as an activator of the SPB. This bifunctionality may be important for regulating the onset of mitosis. For instance, DNA damage increases the activity of ribonucleotide reductase, which may suppress the SPB activator function of R1 and subsequent spindle formation. Further *in vitro* and *in vivo* studies on

regulation of R1 dual functions will clarify the molecular mechanisms of microtubule nucleation at the SPB, and its regulation during the cell cycle.

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