De novo formation of basal bodies in *Naegleria gruberi*: regulation by phosphorylation

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The de novo formation of basal bodies in *Naegleria gruberi* was preceded by the transient formation of a microtubule (MT)-nucleating complex containing γ-tubulin, pericentrin, and myosin II complex (GPM complex). The MT-nucleating activity of GPM complexes was maximal just before the formation of visible basal bodies and then rapidly decreased. The regulation of MT-nucleating activity of GPM complexes was accomplished by a transient phosphorylation of the complex. Inhibition of dephosphorylation after the formation of basal bodies resulted in the formation of multiple flagella. 2D-gel electrophoresis and Western blotting showed a parallel relationship between the MT-nucleating activity of GPM complexes and the presence of hyperphosphorylated γ-tubulin in the complexes. These data suggest that the nucleation of MTs by GPM complexes precedes the de novo formation of basal bodies and that the regulation of MT-nucleating activity of GPM complexes is essential to the regulation of basal body number.

Introduction

We have been using the differentiation of *Naegleria gruberi* amoebae into swimming flagellates as a model system to explore the mechanism of de novo basal body formation. We noticed that the initial events that occur during de novo basal body formation in *N. gruberi* (Suh et al., 2002) are very similar to the events during de novo formation of centrosomes in CHO cells (Khodjakov et al., 2002). First, γ-tubulin and pericentrin are concentrated at a discrete "spot" before the formation of centrioles (or basal bodies) in both systems. Second, the concentration of γ-tubulin is accomplished without the formation of microtubules (MTs); and third, centrioles (or basal bodies) are formed at the site of the γ-tubulin concentration. These similarities suggested to us that the formation of a protein complex containing γ-tubulin and pericentrin might be a common event in the de novo formation of centrioles and basal bodies.

Based on these ideas, we developed biochemical procedures for the purification of γ-tubulin–containing complexes from *N. gruberi* and examined the possible role of these complexes in the formation of basal bodies. In this report, we present evidence that: (a) a protein complex containing γ-tubulin, pericentrin, and myosin II complex (GPM complex) is transiently formed during the differentiation; (b) in vitro nucleation of MT by this complex is dependent on γ-tubulin; (c) the MT-nucleating activity of GPM complexes is tightly regulated during basal body formation by phosphorylation; (d) inhibition of the regulation resulted in the formation of multiple flagella; and (e) γ-tubulin is one of the targets for the phosphorylation.

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Abbreviations used in this paper: Ab, antibody; GPM complex, γ-tubulin, pericentrin, and myosin II complex; MT, microtubule; PI, phosphatase inhibitors.

The online version of this article contains supplemental material.

Results and discussion

Identification of a protein complex containing γ-tubulin and pericentrin

We prepared *N. gruberi* extracts from cells at 40 min after initiation of the differentiation when the percentage of cells with a concentrated spot of γ-tubulin was maximal (Suh et al., 2002). We fractionated the extracts by density-gradient centrifugation. After Western blotting with anti–γ-tubulin Ab, the fractions were pooled into three groups; group 1 (fractions that sedimented faster than the γ-tubulin–containing fractions), group 2 (γ-tubulin–containing fractions), and group 3 (fractions that sedimented more slowly than the γ-tubulin–containing fractions). The γ-tubulin in the group 2 fractions was present in 1–3-μm particles (Fig. 1 A). Pericentrin was present in the same particles (Fig. 1 B). The presence of γ-tubulin in the complex was further supported by a competition assay using *Naegleria*-γ-tubulin (GenBank/EMBL/DDBL accession no. AY919610) synthesized

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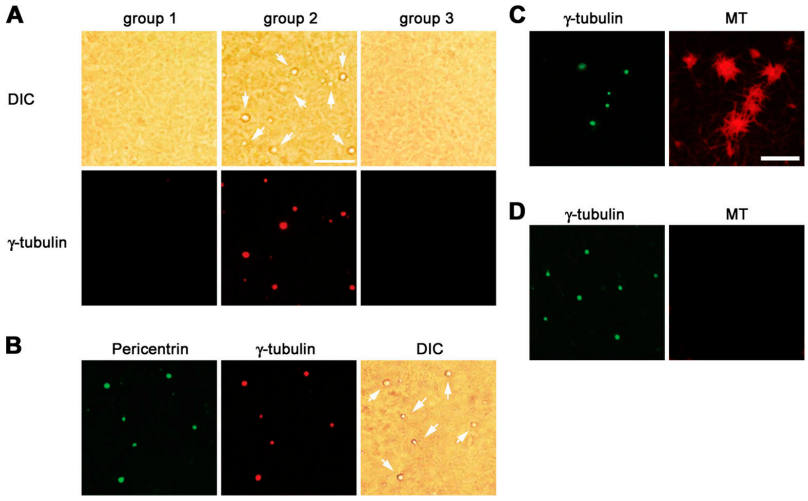

Figure 1. **GPM complexes nucleate MTs in vitro.** (A) A *N. gruberi* extract was prepared from cells at 40 min after initiation of differentiation and fractionated by a sucrose-density gradient centrifugation. After Western blotting with anti–γ-tubulin Ab, the fractions were pooled into three groups based on the γ-tubulin content, see text. 15 μl of each group was transferred onto a round coverslip and stained with monoclonal anti–γ-tubulin Ab (1:500) and Texas red–conjugated anti–mouse-IgG (1:100) second Ab. After observation under DIC optics, the same field was examined under fluorescent optics. Arrows in DIC images indicate the protein complexes. (B) 15 μl of the group 2 extract were transferred onto a round coverslip and stained with rabbit polyclonal anti-pericentrin Ab (1:500) and monoclonal anti–γ-tubulin Ab (1:500). Pericentrin (FITC) and γ-tubulin (Texas red) were visualized with respective second Ab. Arrows in DIC images indicate the protein complexes. (C) MT nucleation was performed using rhodamine-tubulin as described in Materials and methods. The presence of γ-tubulin in the GPM complexes was visualized with anti–γ-tubulin Ab and FITC-conjugated second Ab. (D) A group 2 extract was preincubated with polyclonal anti–γ-tubulin Ab (1:500) for 20 min at RT and used for MT-nucleation with rhodamine-tubulin at 30°C. The presence of γ-tubulin was visualized with FITC-conjugated anti–rabbit IgG Ab. Bars, 10 μm.

in *E. coli* as an MBP-fusion protein (MBP-N-γTub; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200410052/DC1). Additional studies with anti–myosin II Ab showed that the group 2 complexes also contained myosin II (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200410052/DC1). We named these complexes GPM complexes (γ-tubulin, pericentrin, and myosin II complexes). These complexes were not found in group 1 or group 3 fractions.

In vitro MT nucleation from GPM complexes

GPM complexes were capable of nucleating MTs in vitro, Fig. 1 C. Preincubation with anti–γ-tubulin Ab inhibited the formation of MTs from the complexes, Fig. 1 D, although γ-tubulin remained in the complexes after preincubation with anti–γ-tubulin Ab. Preincubation with nonspecific Abs (anti–rabbit IgG Ab or anti–mouse IgG Ab) or addition of anti–γ-tubulin Ab preincubated with MBP-N-γTub had no effect on the formation of MTs from GPM complexes (unpublished data). These data suggest that access to γ-tubulin is required for the in vitro nucleation of MTs from the GPM complexes and that the formation of MTs is not the result of the elongation of prenucleated MTs in the complex.

Changes in the MT-nucleating activity of GPM complexes during the differentiation

The transient concentration of γ-tubulin during the differentiation persists such that ∼30% of cells still have a concentrated γ-tubulin spot at 80 min after the initiation of differentiation, when >80% of cells already have a pair of elongated flagella. In these cells, the γ-tubulin concentration was located at the opposite end of the cell from the flagella and had no MTs associated with it (see Fig. 4 A; Suh et al., 2002). These data suggested that the region of γ-tubulin concentration had lost its MT-nucleating activity after the formation of basal bodies and that the MT-nucleating activity of GPM complex is regulated during the differentiation.

Fig. 2 illustrates the differences in the MT-nucleating activities of GPM complexes prepared before (20 and 40 min; GPM20 and 40 complexes) and after (60 and 80 min; GPM60 and 80 complexes) basal body formation. Although particles from 20 and 80 min cells contain γ-tubulin, they did not nucleate MT. Numerous MTs formed from all of the GPM40 complexes. In contrast only ∼20% of GPM60 complexes were able to nucleate MTs and the number of MTs nucleated from these complexes was significantly decreased based on the intensity of the fluorescence. These data demonstrate that the MT-nucleating activity of GPM complexes is maximal at 40 min, just before the appear-

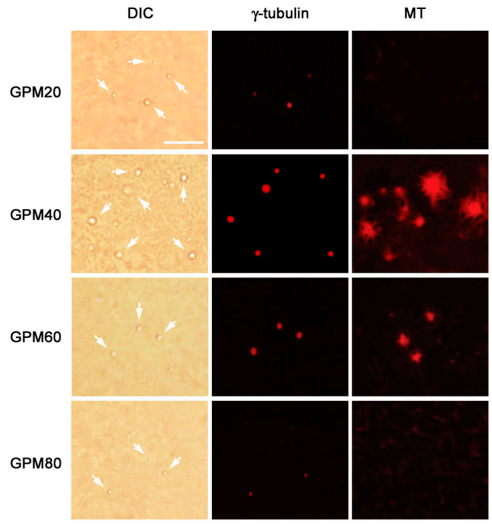

Figure 2. **The MT-nucleating activity of GPM complexes changes during the differentiation.** (Left and middle) GPM complexes were prepared from cells at 20, 40, 60, and 80 min after initiation of differentiation and incubated with anti–γ-tubulin Ab and Texas red–conjugated second Ab and examined under DIC (left) and epifluorescence (γ-tubulin) optics as described in Fig. 1 A. GPM complexes (arrows) from different stages of the differentiation contain γ-tubulin. (Right) In vitro MT-nucleation was performed with GPM complexes from 20, 40, 60, and 80 min cells with rhodamine-tubulin and observed under an epifluorescence microscope. Bar, 10 μm.

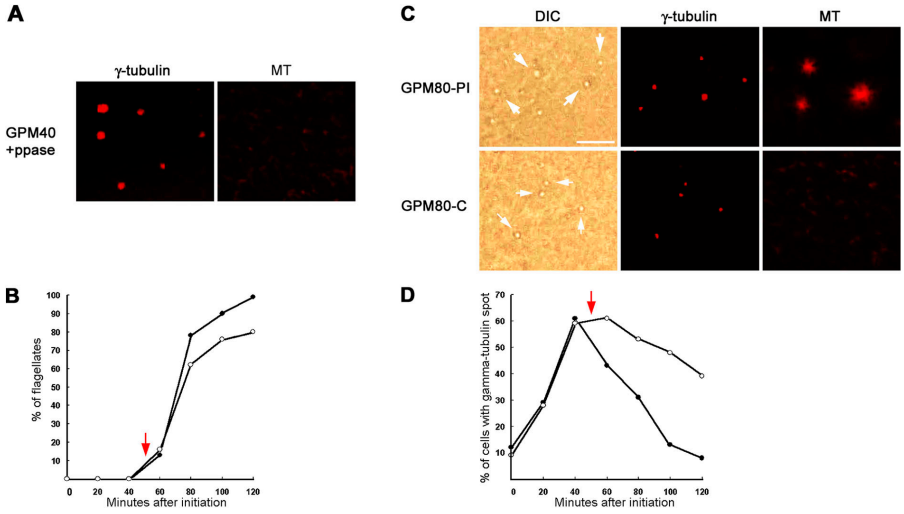

Figure 3. **The effects of phosphatase or PIs on the activity of GPM complexes.** (A) GPM40 complexes were treated with phosphatase for 30 min and used for a MT nucleation assay. The phosphatase-treated complexes still contained γ-tubulin but completely lost MT-nucleation activity. (B) *N. gruberi* amoebae were induced to differentiate. At 50 min after the initiation of differentiation, PI were added. Closed circle, control differentiation; open circle, PI treated; arrow, the time of PI addition. (C) Cell differentiation and PI treatment were performed as described in B. GPM complexes were prepared at 80 min after initiation from control (GPM80-C) and PI-treated cells (GPM80-PI). A portion of each sample was incubated with anti–γ-tubulin Ab and Texas red–conjugated second Ab and observed under DIC or epifluorescence microscope to confirm the presence of γ-tubulin–containing complexes (left and middle). The remaining portion of each sample was used for MT-nucleation assay with rhodamine-tubulin and observed under epifluorescence microscope (right). (D) Cell differentiation and PI treatment were performed as described in B. Cells were taken at 20-min intervals, fixed, and immunostained with anti–γ-tubulin Ab and Texas red–conjugated second Ab. At each time point, 100 cells were examined at random and scored for the presence of a concentrated γ-tubulin spot. Closed circle, control differentiation; open circle, PI treated; arrow, the time of PI addition. Bar, 10 μm.

ance of visible basal bodies and that the activity decreases rapidly after this point. It is also apparent that the diameter of GPM complex decreased by 80 min. The decrease both in the diameter and the intensity of γ-tubulin Ab staining of GPM80 complexes suggests that GPM complexes are being disassembled at this stage of the differentiation (Fig. 3 D; Suh et al., 2002).

Regulation of MT-nucleating activity of GPM complexes by phosphorylation

Phosphatase-treated GPM40 complexes still contained γ-tubulin but completely lost their MT-nucleating activity (Fig. 3 A). The inactivation of GPM40 complexes by phosphatase was not observed when phosphatase inhibitors (PI; final concentrations; 1 mM *o*-vanadate, 5 mM NaF, and 20 mM β-glycerophosphate) were added to GPM40 complexes before the phosphatase treatment (unpublished data). These data suggest that the observed inactivation of GPM complexes after the formation of basal bodies might be the result of dephosphorylation of one or more components. To test this hypothesis, we added PI to differentiating cells at 50 min after initiation of the differentiation a time when the MT-nucleating activity of GPM complexes might be expected to begin to be inactivated (Fulton, 1977; Walsh, 1984). PI had a very limited effect on the formation of flagella. At the end of differentiation 83% of the cells had visible flagella (Fig. 3 B). However, the inhibitor had a noticeable effect on the number of cells with multiple flagella (Table I). At the end of differentiation, ~23% of the PI-treated cells formed multiple flagella. On the contrary, <4% of flagellated cells in the control had more than one pair of flagella. PI treatment had no effect either on the transient accumulation of α-tubulin mRNA or on the synthesis of the tubulin (unpublished data).

These data suggested that the formation of multiple flagella was a result of continuous MT-nucleation from GPM complexes that were not dephosphorylated due to the inhibitor treatment. This conclusion is supported by two experiments. First, we performed in vitro MT-nucleation assays with GPM

Table I. **Phosphatase inhibitors induce the production of multiple flagella**

	Phosphatase inhibitor				Control			
Time (min)	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**
0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0
60	17 ± 0.7	2.3 ± 0.2	0	15 ± 2.7	13 ± 0.7	0	0	0
80	52.3 ± 1.6	10 ± 0.7	0.7 ± 0.2	18.2 ± 0.9	76.7 ± 0.2	1.3 ± 0.1	0	1.7 ± 0.3
100	59.3 ± 2.9	13.3 ± 0.2	1.6 ± 0.2	20.8 ± 0.3	88.3 ± 0.9	2.3 ± 0.2	0	2.6 ± 0.2
120	64 ± 0.7	16.3 ± 0.7	2.3 ± 0.2	22.5 ± 0.5	95.3 ± 0.9	3.3 ± 0.2	0	3.4 ± 0.3

N. gruberi amoebae were divided into two flasks and differentiated at the same time. At 50 min after initiation, PI were added into one of the flasks. At 20-min intervals, 200 μl of differentiating cells were taken, fixed with Lugol's iodine, and observed under phase contrast optics. The percentage of flagellated cells and the number of flagella per flagellated cell were determined by randomly counting 100 cells at each time point. The numbers in Table I are the average of three independent experiments.

[a]The percentage of cells with one pair of flagella.
[b]The percentage of cells with two pairs of flagella.
[c]The percentage of cells with three (or more) pairs of flagella
[d]The percentage of cells with excess flagella (b + c)/(a + b + c).

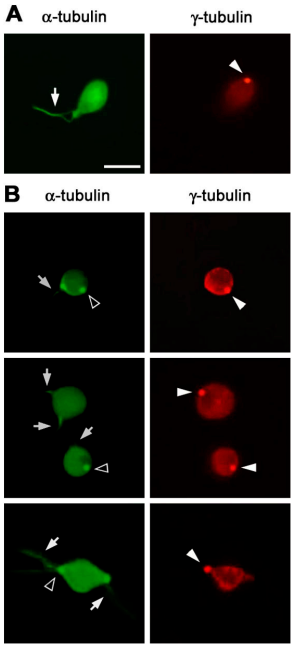

Figure 4. **Phosphatase inhibitor treatment induced the formation of multiple flagella.** Cells were taken at 80 min after initiation, fixed, and immunostained with rabbit polyclonal anti–γ-tubulin Ab and monoclonal anti–α-tubulin Ab. GPM complexes (Texas red) and MTs (FITC) were visualized by respective second Ab. (A) Control cells. (B) PI-treated cells. Arrows indicate flagella. Closed arrowheads indicate GPM complexes recognized by anti–γ-tubulin Ab. Open arrowheads indicate GPM complexes recognized by both anti–γ-tubulin and anti–α-tubulin Ab. Bar, 10 μm.

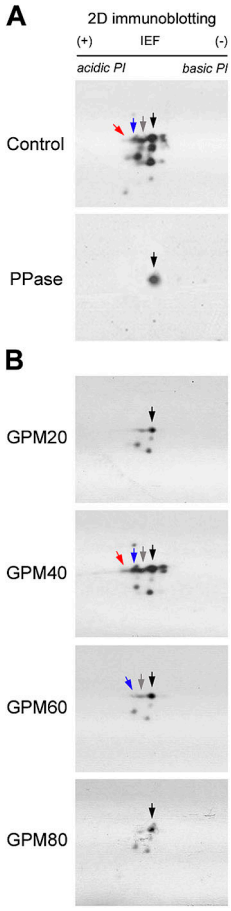

Figure 5. **Hyperphosphorylated γ-tubulin in GPM40 complexes.** (A) GPM40 complexes were prepared as described in Materials and methods. After 2D-gel electrophoresis, Western blots were performed with anti–γ-tubulin Ab. The background spots that were detected with the second Ab alone were used as internal markers to align γ-tubulin spots. Control: GPM40 complexes without phosphatase treatment. PPase: GPM40 complexes treated with phosphatase. (B) GPM complexes were prepared at 20, 40, 60, and 80 min after initiation of the differentiation. After 2D-gel electrophoresis and Western blotting with anti–γ-tubulin Ab, γ-tubulin spots were aligned as described above.

complexes prepared from PI-treated cells. PI were added to differentiating cells at 50 min after initiation and GPM complexes were prepared at 80 min. Fig. 3 C shows that GPM80 complexes prepared from the inhibitor-treated cells were still capable of nucleating MTs, whereas GPM80 complexes from control cells had lost this activity. Second, we examined the effect of PI on the formation of GPM complexes and on MT-nucleation from the complexes by immunostaining the differentiating cells. PI treatment significantly delayed the disappearance of the γ-tubulin spot (Fig. 3 D). In control cells, the presence of a concentrated spot of γ-tubulin peaked at 40 min (60%) than decreased rapidly as reported previously (Suh et al., 2002). In contrast, >40% of PI-treated cells still had a concentrated spot of γ-tubulin at 120 min. These data suggest that dephosphorylation is also involved in the disassembly of GPM complexes.

Fig. 4 A shows a control cell fixed at 80 min after initiation of differentiation. This cell has a pair of flagella and a heavy concentration of α-tubulin at the base of flagella (basal bodies). This cell also contains a concentrated spot of γ-tubulin (Suh et al., 2002) but the γ-tubulin spot is not detected with anti–α-tubulin Ab suggesting that it is not capable of nucleating MT. Fig. 4 B shows cells fixed at 80 min that were treated with PI at 50 min. Many of these cells contain a concentrated spot of γ-tubulin that is also recognized by anti–α-tubulin Ab suggesting the presence of a GPM complex capable of nucleating MT. In some cells an extra pair of flagella is seen emanating from the GPM complex.

A parallel relationship between the MT-nucleating activity and phosphorylation of γ-tubulin in GPM complexes

It has been reported that the phosphorylation of γ-tubulin plays important roles in MT nucleation from the spindle pole body of *Saccharomyces cerevisiae* (Vogel et al., 2001). Based on this observation, we examined the possible phosphorylation of the γ-tubulin in GPM complexes. GPM40 complexes contained four differently modified γ-tubulins, Fig. 5 A (spots 1 to 4 indicated by red, blue, gray, and black arrows, respectively; from acidic to basic side of the gel). After phosphatase treatment of isolated GPM40 complexes, the three acidic forms of γ-tubulin were not detected. Only the most basic form of γ-tubulin (spot 4) was found (Fig. 5 A). This change was not observed when PI were present in the reaction (not depicted). 2D-gel electrophoresis and Western blotting with anti–γ-tubulin Ab of GPM complexes prepared from cells at 20, 40, 60, and 80 min after the initiation of differentiation showed that there is a parallel relationship between the change in MT-nucleating activities of GPM complexes from different stages of differentiation and the degree of phosphorylation of γ-tubulin in these complexes (Fig. 2 and Fig. 5 B).

Our data demonstrate that de novo formation of basal bodies in *N. gruberi* is preceded by the formation of a protein complex (GPM complex) that contains two well-known components of pericentriolar material (γ-tubulin and pericentrin). GPM complexes acquire their MT-nucleating activity around 40 min

after initiation of the differentiation just before the appearance of visible basal bodies (Fulton, 1977; Walsh, 1984; Suh et al., 2002). After the formation of basal bodies, the GPM complexes lose MT-nucleating activity and are no longer detected by anti–γ-tubulin Ab suggesting the disassembly of the complexes.

Combined with our in vitro data, the formation of extra pairs of flagella in PI-treated cells (Table I and Fig. 5) strongly suggest that MT-nucleation from GPM complexes is essential for the de novo formation of basal bodies and that the GPM complexes are the site of de novo basal body assembly. Our data also suggest that the inactivation of GPM complex after basal body formation is involved in regulating the number of basal bodies per cell. This hypothesis is supported by recent studies (Marshall et al., 2001; Khodjakov et al., 2002; Wong and Stearns, 2003; La Terra et al., 2005) that suggest the presence of a centriole inhibits the unregulated assembly of new centrioles.

The phosphorylation of γ-tubulin in GPM complexes is correlated with the MT-nucleating activity of the complex. In *Saccharomyces cerevisiae*, Vogel et al. (2001) showed that phosphorylated Tub4p had an augmented activity in MT assembly and suggested that the phosphorylation of Tub4p in G1 phase might be related to the replication of the spindle pole body. The similar effect of γ-tubulin phosphorylation on MT formation observed in budding yeast and *N. gruberi* suggests the presence of a similar mechanism in other organisms.

Materials and methods

Growth and differentiation of *N. gruberi*

Growth and differentiation of *N. gruberi* NB-1 has been described elsewhere (Fulton, 1977; Walsh, 1984; Suh et al., 2002). For PI treatment, a stock solution of a PI cocktail was added at 50 min after initiation of differentiation (final concentrations; 1 mM *o*-vanadate, 5 mM NaF, and 20 mM β-glycerophosphate; Vogel et al., 2001).

Preparation of *Naegleria* extracts and partial purification of GPM complexes

Cells (~4×10^8) were harvested by centrifugation at different stages of differentiation. The cell pellet was resuspended in 2 vol of cold amoeba extraction buffer (25 mM Hepes, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose) containing 0.5 mM benzamine-HCl and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A (Wilhelm et al., 2000). Cells were lysed by three cycles of freezing and thawing. The cell lysate was centrifuged at 11,000 *g* for 15 min at 4°C. The supernatant was collected and recentrifuged as above. The supernatant from the second centrifugation was stored at −80°C for further study. *Naegleria* extract (700 μl) was mixed with 300 μl of sucrose-free amoeba extraction buffer and applied to a 10–40% sucrose step gradient (seven 1.4-ml steps). Centrifugation was performed using a SW 41 rotor (Beckman Instruments) at 100,000 *g* for 16 h.

Antibodies

Two anti–γ-tubulin Abs were purchased from Sigma-Aldrich (T-3599, polyclonal; T6557, monoclonal). Polyclonal antiserum against *Dictyostelium* myosin II heavy chain was raised in rabbits (Yumura, 1994). mAb against α-tubulin was purchased from Sigma-Aldrich (T5168). pAb against pericentrin was purchased from Babco. Second antibodies were purchased from Santa Cruz Biotechnology, Inc.: Texas red anti–rabbit IgG (sc-2780), Texas red anti–mouse IgG (sc-2781), FITC-anti–rabbit IgG (sc-2012), and FITC-anti–mouse IgG (sc-2010).

In vitro MT nucleation from GPM complexes

In vitro MT nucleation was performed using the method of Moritz and Alberts (1999) with modifications. 20 μl of free tubulin in TDB (2 mg/ml; 1:7 mixture of rhodamine-labeled tubulin and unlabeled tubulin; Cytoskeleton, Inc.) was mixed with 20 μl of *Naegleria* extract (group 2) and incubated for 10 min at 30°C. To fix the formed MTs, 240 μl of 1% glutaraldehyde in BRB 80 was added to each tube and the tubes were incubated at RT for 3 min. For detection of γ-tubulin, pericentrin, or myosin II in the complex, 20 μl of each sample was applied to a round glass coverslip (acid washed and coated with poly-D-lysine) and incubated for 10 min. The coverslips were incubated in cold methanol (−20°C) for 3 min and washed with TBS. The coverslips were incubated in 0.1% sodium borohydride in TBS for 7 min and transferred into blocking solution (3% BSA in TBS). After 5 min, primary Ab was added to each coverslip. After 1 h, the coverslips were washed and incubated for 1 h with desired second Ab. After washing, all samples were mounted in Mowiol mounting solution for microscopy. Buffers for in vitro MT-nucleation assay: BRB 80 (80 mM K-Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM Na_3EGTA, prepared as a 5× stock, diluted to 1× before use; Moritz and Alberts, 1999); GTP stock (0.5 M GTP in 1× BRB 80); tubulin dilution buffer (TDB; 1× BRB 80, 10% glycerol, 1 mM GTP); and TDB washing buffer (TDB-containing BSA [fraction V], 10 mg/ml).

Production of *Naegleria* γ-tubulin in *E. coli*

We recently cloned a γ-tubulin gene from *N. gruberi* (GenBank/EMBL/DDBL accession no. AY919610). The deduced amino acid sequence of the *Naegleria* γ-tubulin is 50–60% identical to γ-tubulins from various other species. *Naegleria* γ-tubulin contains a conserved NH_2-terminal peptide sequence used to raise the antibody that was used in this study (aa 38–53 of human γ-tubulin, EEFATEGTDRKDVFFY; aa 38–54 of *Naegleria* γ-tubulin, EDFAIQGAGDRKDVFFY). We expressed the NH_2-terminal half (aa 1–249) of *Naegleria* γ-tubulin in *E. coli* as an MBP-fusion protein (MBP-N-γTub).

Microscopy

Examinations by differential interference contrast and epifluorescence microscopy were performed at ambient temperature with an Axioplan 2 multipurpose microscope with 63× magnification, Plan-Neofluar 1.25 objective, Axio Cam, and AxioVision 3.1 software (all purchased from Carl Zeiss MicroImaging, Inc.). All images were imported into Adobe Photoshop 6.0 for contrast manipulation and figure assembly.

Online supplemental material

Fig. S1 shows the presence of γ-tubulin in GPM complex: a competition assay. Fig. S2 shows the presence of myosin II in GPM complex. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200410052/DC1.

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