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Nucleation of Astral-shaped Microtubules from Latex Beads Conjugated with MTOG Proteins

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ABSTRACT—A model system for the formation of astral-shaped microtubules (Mts) consisting of Latex beads (diameter of 0.2 μm), a protein fraction (p51) comprised of MTOGs (microtubule-organizing granules) and tubulin was established. The Latex beads were first incubated with p51 in the presence of GTP at 0°C, then the purified tubulin dimer fraction was added, resulting in the formation of an aster-like structure observed by dark-field microscopy. On preincubation of the Latex beads with GDP instead of GTP, the asters did not form. Unhydrolyzable GTP analogues such as GTP-γS and GMP-PNP promoted aster formation as did GTP as observed by dark-field microscopy. Polylysine, as representative of basic polymers capable of binding to the surface of the Latex beads, promoted spontaneous Mt assembly, and eventually an aster-like structure without Latex beads in the center formed. Further analyses made by measuring the optical density of the aster-forming system produced the following results. 1) preincubation of the Latex beads with GTP or GMP-PNP supported Mt assembly from the beads showing profiles typical for a site-directed assembly without the lag phase. 2) GTP-γS and GDP inhibited the turbidity increase of the system, causing a decrease in both the initial velocity and the level of steady state of Mt assembly. 3) Anti-p51 monoclonal antibody (HP1) substantially inhibited the aster formation, while anti-γ-tubulin antibody only slightly inhibited assembly.

INTRODUCTION

In the centrosomes of the sea urchin egg, microtubule-organizing granules [MTOGs, (Endo *et al*., 1985)] having a diameter of ~90nm were found surrounding the centrioles (Endo, 1980). In the past ten years, evidence has accumulated that the MTOG-associated 51kDa protein (p51) is responsible for the nucleation of astral and spindle microtubules (Mts) (Toriyama *et al*., 1988; Ohta *et al*., 1988a, b, 1989, 1990; Ohta *et al*., 1999). The experimental results supporting this are as follows.

1) p51 is a major component of the MTOGs prepared from the isolated mitotic apparatus (Toriyama *et al*., 1988) as well as from unfertilized eggs.

2) The p51-enriched fraction formed granular aggregates upon dialysis against a solution of low ionic strength. Addition of purified tubulin dimer fraction to the dialyzed fraction caused asters focused on the granular aggregates to form (Toriyama *et al*., 1988).

3) Both monospecific antibodies against p51 [AP17-1, (Toriyama *et al*., 1988)] and monoclonal antibodies [HP1, (Ohta *et al*., 1988a)] stained the centrosome in the sea urchin egg (Ohta *et al*., 1988b) as well as the centers of the reconstructed asters *in vitro* (Toriyama *et al*., 1988).

4) The monospecific antibodies substantially suppressed aster formation *in vitro* (Toriyama *et al*., 1988).

5) The microinjection of the monoclonal antibodies into the blastomere suppressed the formation of the mitotic apparatus, resulting in inhibition of cleavage (Ohta *et al*., 1988a).

6) p51 was shown to be a GTP-binding protein (Ohta *et al*., 1990). The GTP-saturated form of p51 promoted Mt assembly but the GDP-saturated form of p51 was quite inhibitory for Mt assembly (Ohta *et al*., 1989; Nakazawa *et al*., 1994).

These results suggested that p51 is a component of MTOGs and responsible for the formation of the mitotic apparatus.

Genetic evidence suggested the involvement of another GTP-binding protein, γ-tubulin, in the nucleation of spindle Mts. γ-tubulin has been identified as a new member of the tubulin superfamily in *Aspergillus nidulans* (Oakley and Oakley, 1989) and found to be essential for mitosis (Oakley *et al*., 1990). γ-tubulin has been shown to be localized to the spindle pole body (Oakley *et al*., 1990; Horio *et al*., 1991) and the centrosome (Sterns *et al*., 1991; Zheng *et al*., 1991; Joshi *et al*., 1992). The deduced amino acid sequence of γ-tubulin has

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the GTP binding motif. Furthermore, γ-tubulin was found in ring complexes possibly involved in Mt assembly (Moritz *et al.*, 1995; Zheng *et al.*, 1995; Oegema *et al.*, 1999).

GTP-binding proteins have a common regulatory mechanism for their function. The GDP-binding form is made active by a GDP/GTP exchange reaction (Kaziro, 1978). This general rule led us to assume that p51 has a control mechanism in its putative function for the nucleation of astral and spindle Mts. Therefore, we examined the effect of preincubation of centrosomal fragments with guanine nucleotides on their ability to initiate Mt assembly. The result supports the idea that p51 has the properties of G protein. The effect of GDP on the aster forming activity of MTOGs was preliminarily examined by Ohta *et al.* (1989), who found that the aster formation was inhibited when p51 was saturated with GDP, and recently that p51 has a Kd of 1.1 μM for GTP and 0.15 μM for GDP (Ohta *et al.*, 1999). That Mt assembly is inhibited by GDP-saturated p51 was later confirmed by Nakazawa *et al.* (1994).

It was further shown that p51 purified by GTP affinity column chromatography was incapable of forming asters (Ohta *et al.*, 1990). This result may suggest the presence of co-factors collaborating with p51 to form asters, although the possibility still remains that the dilution of glycerol from 50 to 5% caused denaturation of p51 (Ohta *et al.*, 1990). A model system of the assembly of asters was needed in order to identify the co-factors in the solubilized MTOG fraction. We then constructed such a system using Latex beads (polystyrene beads, with a diameter of 0.2 μm), a crude p51 fraction saturated with GTP and purified tubulin dimers. The present paper describes the nucleation of Mts from the Latex beads and the turbidimetric measurements of astral Mt nucleation, which is further evidence that nuclei on tubulin polymerization are formed on the surface of the beads.

MATERIALS AND METHODS

Preparation of a tubulin dimer fraction

Extraction of Mt proteins from porcine brains and purification of tubulin dimers has been described previously (Maekawa *et al.*, 1992; Nakazawa *et al.*, 1994). Purified tubulin divers (ca. 2–3 mg/ml), mostly free of MAPs, were dissolved in an assembly buffer solution containing 0.1 M 1,4-piperazine-diethane sulfonic acid (PIPES), 1 mM ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM $MgCl_2$ and 1 mM GTP at pH 6.8 and stored at –80°C after being divided into 0.5 ml portions. The frozen samples were thawed and centrifuged at 100,000×g for 10 min to remove a small amount of tubulin aggregate before use. Identification of tubulin was carried out by immunoblotting in a mini trans-blot apparatus (Bio-Rad) using anti-tubulin antibody (monoclonal antibody, YL1/2, from Sera Lab. Sussex, England) and HRP-conjugated secondary antibody (Cappel, Organon Teknika Corp., Durham, NC).

Preparation of the MTOG fraction

The MTOG fraction was prepared from the unfertilized eggs of *Clypeaster japonicus* as described before (Toriyama *et al.*, 1988), except that solubilization of the fraction was carried out in the presence of 1 mM GTP. We omitted the use of 50% glycerol because GTP was found to stabilize the aster-forming activity of the p51-enriched fraction prepared by phosphocellulose column chromatography (Nakazawa *et al.*, 1994). The MTOG fraction (hereafter referred to as p51) was stored at –80°C after being divided into 0.05 ml portions. In this paper, microtubule structures initiated from the Latex beads are referred to as MtSt.

Assembly of MtSt *in vitro* and observation by dark-field contrast microscopy and measurement of microtubule assembly from Latex beads

p51 (15 μl, ca. 1 mg/ml) was incubated first with 5 μl of 100 mM guanine nucleoside triphosphate at 0°C for 30 min and then with Latex beads (5 μl), which had been washed in the assembly buffer solution by suspension and centrifugation, then 500 μl of tubulin solution at a final concentration of over 2 mg/ml was added and incubation was continued at 37°C to assemble MtSt. The final amount of the latex beads was adjusted to an OD_{350} value of 1.0. Preliminary analyses indicated that increase in the amount of p51 causes the number of MtSt to increase. However, a standard system for construction of MtSt was used as described above, mainly due to the restricted amount of p51.

Mts are usually observed in a microscope equipped with a mercury arc lamp. However, we recently found that it is much easier to use an Olympus BX60 and a halogen lamp (Muraoka *et al.*, 1999).

In order to measure the time course of Mt growth from the Latex beads (Fluoresbrite™ carboxy YG 20 micron micro-spheres, 0.202± 0.012 μm (SD), Polysciences, Inc. Washington) *in vitro*, spectrophotometric monitoring of the optical density was carried out using a Beckman spectrophotometer (DU530) equipped with a Peltier Temperature Control Module (set at 37°C). In this case, crude MTOG fractions which were not chromatographed on phosphocellulose column were used.

Others

GTP-γS, guanylyl imidodiphosphate (GMP-PNP) and antibody against γ-tubulin were obtained from Sigma. GTP and other reagents were commercial products from Wako Pure Chemical Industries, Ltd. They were all of reagent grade. SDS-PAGE, immunoblotting and protein determination were performed as described previously (Maekawa *et al.*, 1992).

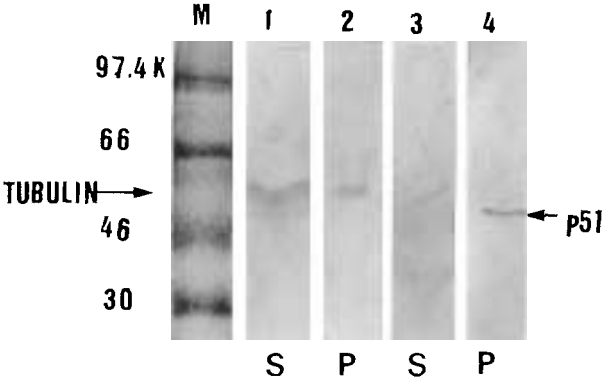


Fig. 1. Binding of p51 and tubulin to Latex beads. Latex beads were incubated with p51 in the presence of GTP for 10 min at 0°C, followed by addition of phosphocellulose-purified tubulin dimer fraction and incubation of the mixture at 37°C for 30 min to construct astral microtubules. For details of the MtSt formation, see MATERIALS AND METHODS. The mixture was then centrifuged at 10,000g for 10 min. Aliquots of the supernatant and the pelleted beads fraction, which was washed once with reassembly buffer solution and treated with the sample buffer for SDS-PAGE to dissolve protein fractions bound to beads, were processed for SDS-PAGE and western blotting (YL1/2 for tubulin, lanes 1 and 2, and anti-p51 for detection of p51, lanes 3 and 4). S and P represent supernatant and pellet of the 1st centrifugation, respectively. M, marker proteins.

RESULTS AND DISCUSSION

Association of p51 with latex beads

First, we measured whether or not p51 binds to the carboxy Latex beads. The Latex beads were incubated with the solubilized MTOG in the presence of GTP. This was followed by washing of the beads with the assembly buffer and solubilization of proteins bound to the beads in the SDS-containing sample buffer solution for SDS-PAGE and western blotting. When the incubated latex beads were centrifuged, the precipitated beads were found to be associated with tubulin (Lanes 1 and 2) and p51 (Lanes 3 and 4). p51 was almost totally associated with beads probably by ionic interaction. This was consistent with the result that non-carboxylated beads did not bind p51 and were incapable of forming asters when combined with tubulin dimers (data not shown). Consistently, p51 and tubulin bound well to Latex beads under this experimental condition.

Western blot analysis indicated that a negligible amount of γ-tubulin was contained in the solubilized MTOG fraction and little amount was detected in the fraction bound to the Latex beads (data not shown) using anti-γ-tubulin antibodies.

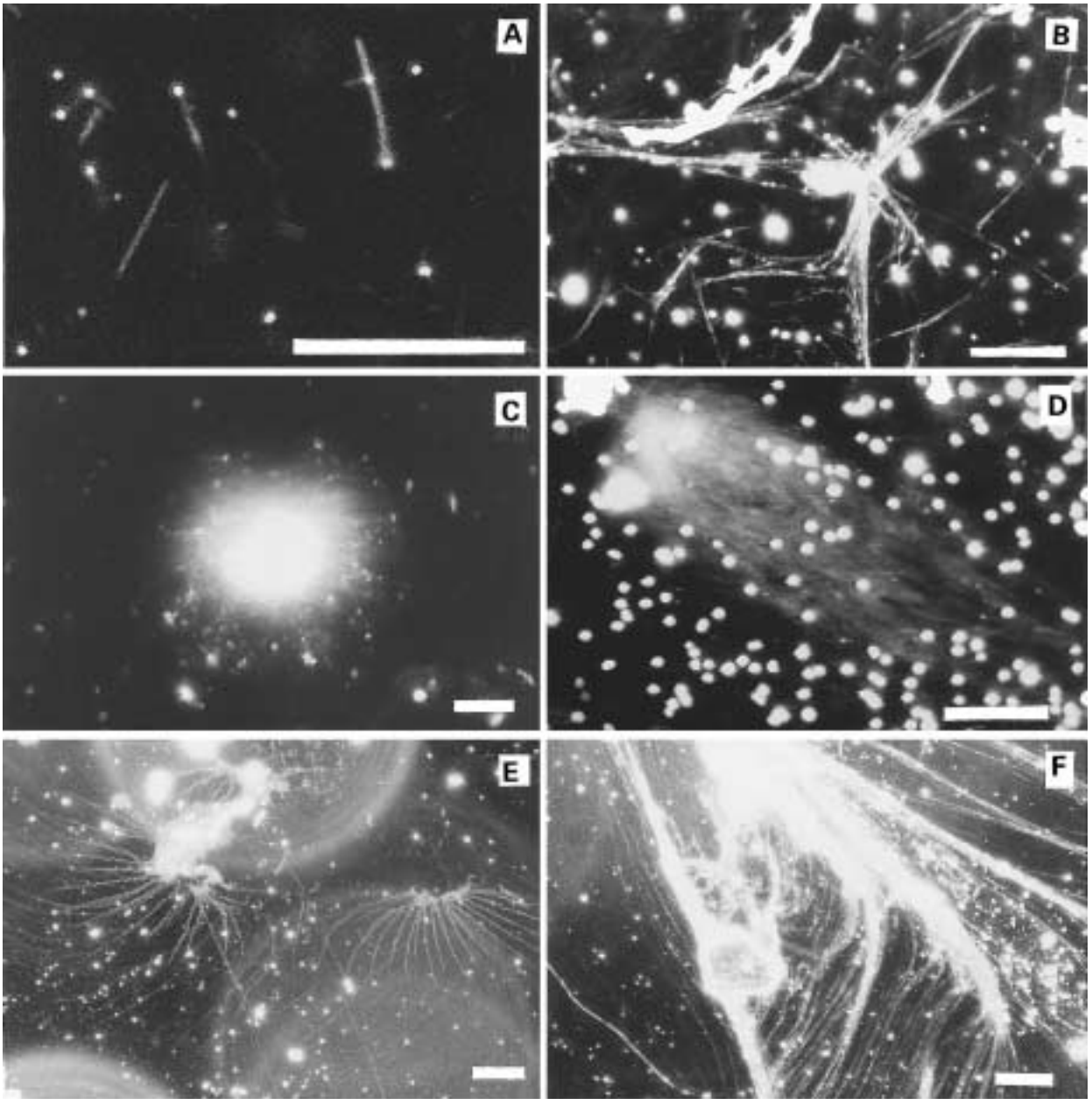


Fig. 2. Dark-field micrographs of various types of MtSt constructed in the mixture of Latex beads, p51, and tubulin. A: Single Mt initiated from a single bead B: Fragmental astral structure initiated from oligomeric beads. C: Typical astral Mts initiated from a large aggregate of Latex beads. D: Mts were frequently unidirectionally arrayed by the flow of assembly buffer in the space between the coverslip and glass-slide. E and F: Fanwise MtSt initiated from belt-shaped aggregates of beads. Bars, 10 μm.

Formation of MtSt from Latex beads, p51 and tubulin dimers

Since p51 is a basic protein, we chose Latex beads associated with carboxy groups to make beads coated with p51. We expected this kind of beads to serve as a nucleus for Mt assembly. This was the case as indicated in Figure 2. Individual beads initiated one or two Mts, which looked like relaxed spermatozoa (Fig. 2A) although their dimensions quite differed. Small aggregates of beads caused the formation of a deformed aster-shaped structure with several Mts (Fig. 2B). Large aggregates of beads formed a typical aster-like structure (Fig. 2C), although most of them were deformed by the flow of assembly buffer solution in the space between the glass-slide and coverslip as shown in Figure 2D. Figures 2E and F show some belt-like aggregates of beads initiating fanwise Mts.

Electron microscopic observation of the negatively stained specimen of the assembly system revealed that Mt formation is initiated at the surface of the beads (Fig. 3), although the fine structure of the nucleated site could not be seen. Therefore, we judged that p51 molecules in GTP-bound form attached to the surface of the beads form nucleation sites for Mt assembly.

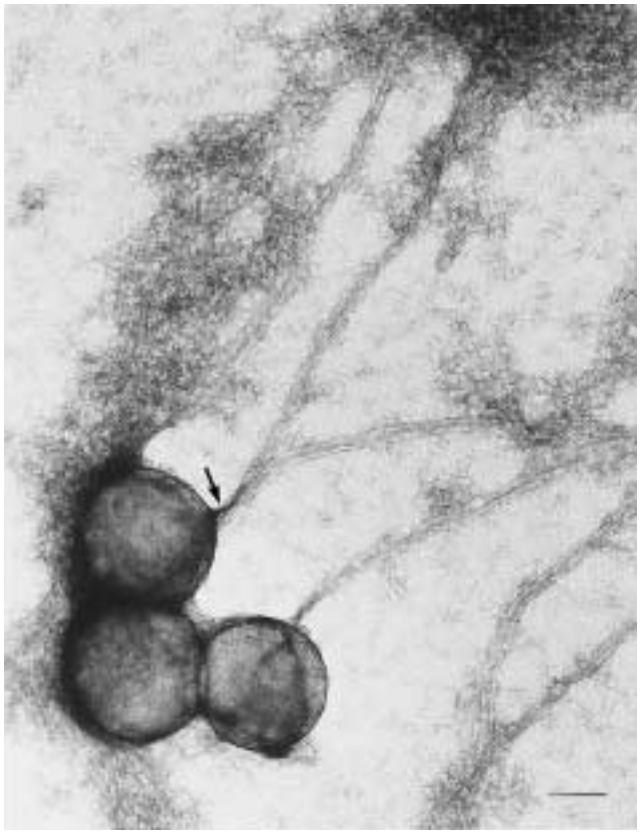


Fig. 3. Electron micrograph of an Mt initiated from the surface of a Latex bead which was incubated first with p51 in the presence of GTP and then with tubulin. Bar, 100 nm.

The effect of polylysine as a representative of non-specific basic polymers possibly associated with carboxylated Latex beads

To determine whether or not the MtSt-forming activity of p51 is due to its non-specific basic character, we examined the action of polylysine as a representative of non-specific basic polymers on the Latex beads. Carboxylated Latex beads suspended in standard assembly buffer solution deprived of GTP (OD_{350} 1.0, 1 μl) were incubated first with 1–10 μl of 1 mg/ml polylysine (5, 000–15, 000 MW) for 10 min at 0°C, and then with 50 μl of tubulin (a final concentration of ca. 2.4 mg/ml) at 37.5°C for 30 min. Samples were fixed with glutaraldehyde at a final concentration of 1%, and observed by dark-field microscopy.

Figure 4 shows that the incubation with 5 μl of polylysine resulted in occasional formation of MtSt (astral structures). However, Latex beads were not found in the center of the MtSt. Increasing the concentration of polylysine up to 10 μl caused amorphous large aggregates of Mts to form (photograph not shown). Lowering the polylysine concentration, by contrast, caused a decrease in the number of MtSt. These results suggested that polylysine associated with the Latex beads did not construct nuclei of tubulin polymerization, although it promoted initiation of spontaneous Mt assembly. It seems likely, therefore, that p51 specifically works in the formation of the nucleus for tubulin polymerization on the surface of the Latex beads.

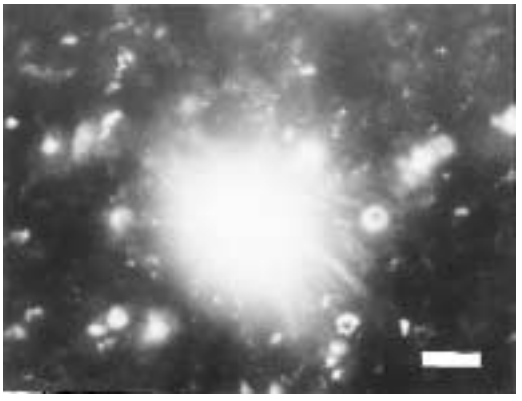
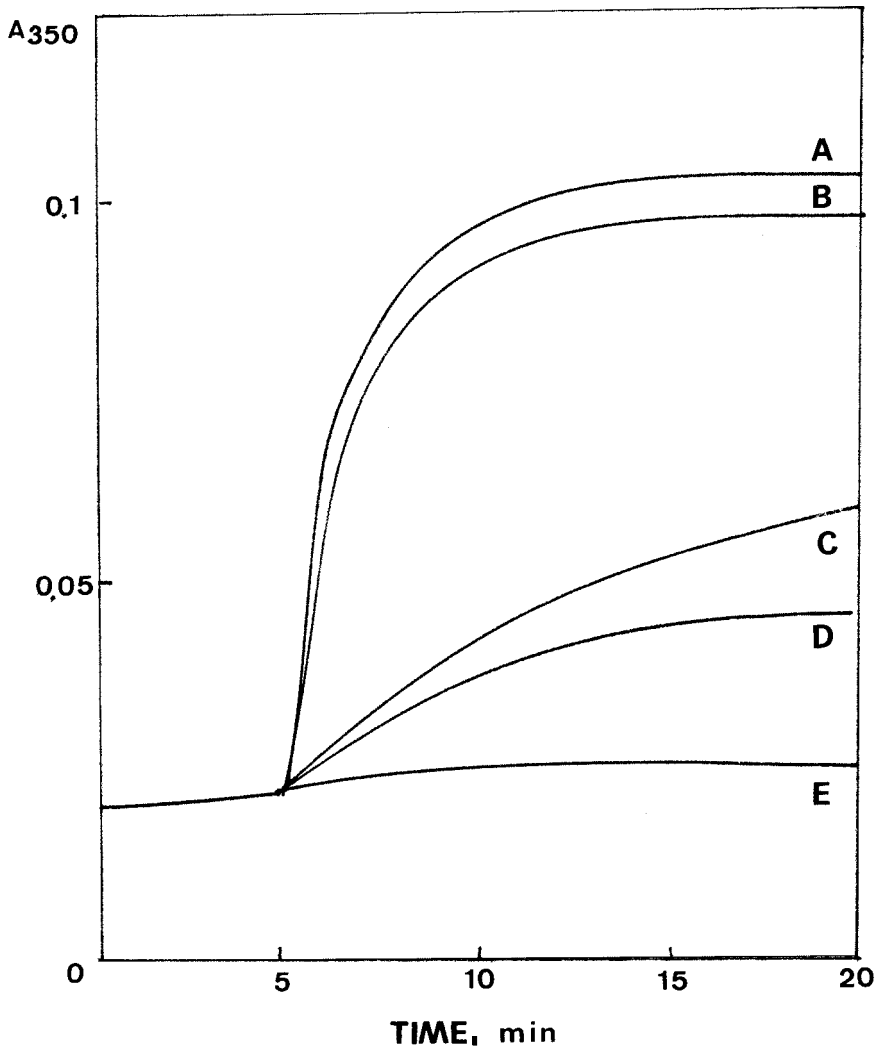


Fig. 4. Dark-field micrograph of astral shaped MtSt constructed in the mixture of Latex beads, polylysine, and tubulin. Latex beads are usually seen as bright spots. However, no beads are observed in the center of this MtSt. Bar, 10 μm.

Turbidimetric analyses of the bead-directed assembly system

In order to obtain details on the bead-directed assembly of Mts, turbidimetric measurement of the process was carried out using a 0.5 ml buffer system containing Latex beads and the solubilized MTOG fraction (crude p51 fraction, ca. 1 mg/ml, 15 μl) preincubated with GTP or GTP analogues, and tubulin dimer (final 2–3 mg/ml).

Figure 5 shows the time course of turbidity in the assembly system. Incubation of tubulin dimers with beads alone



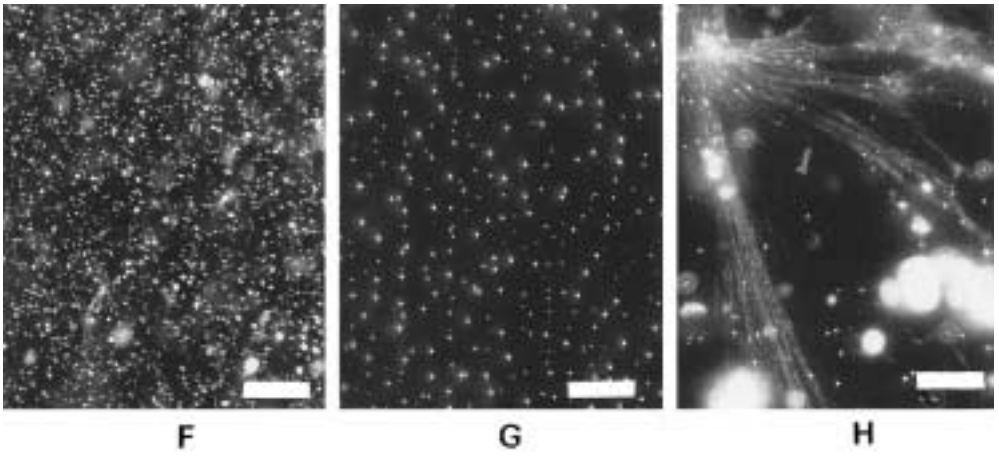


Fig. 5. Turbidimetric measurement of MtSt formation with Latex beads and p51 previously saturated with GTP(curve A), GMP-PNP(B), GTP-γS(C) and GDP(D). The tubulin solution alone was first incubated for 5 min, showing little increase in the optical density, followed by the addition of the mixture of Latex beads incubated with each p51 fraction. Curve E shows only a slight increase in turbidity of the mixture of beads and tubulin alone. Its dark-field micrograph is shown in inset F. Inset G represents a micrograph of the mixture of beads and MTOG fraction alone, while inset H shows an astral-shaped MtSt observed in A. Bars, 10 μm.

caused little turbidity to develop (Fig. 5, curve E and inset F). Incubation of beads with the MTOG fraction alone did not induce aggregation of beads (Fig. 5, inset G). Five min after incubation with tubulin alone, the addition of Latex beads coated with the MTOG fraction which was saturated with GTP resulted in a remarkable assembly of MtSt that appeared to be site-directed and without a lag phase (Fig. 5, curve A and inset H). Almost the same results were obtained with GMP-PNP (Fig. 5, curve B).

The next step was to examine the effect of GDP on this assembly system. By dark field microscopy, the number of asters in the assembly buffer containing the beads pre-incubated with p51 which was saturated with GDP was found to have decreased substantially. Turbidity measurement confirmed the observation (Fig. 5, curve D).

Previous results indicated that preincubation of p51 with non-hydrolyzable GTP analogues such as GTP-γS or GMP-PNP little affected the number of reconstructed asters. However, turbidimetric measurement indicated that GTP-γS inhibited Mt assembly substantially not only initially but in the steady state of assembly (Fig. 5, curve C) for reasons so far unexplained.

Hamel and Lin (1984) reported that GTP-γS inhibits Mt assembly especially in the presence of microtubule-associated proteins (MAPs). In the present study, we did not use MAPs for Mt assembly. This may be why Figure 5 shows a weaker inhibition by GTP-γS than GDP. In a previous study (Nakazawa *et al.*, 1994), the number of asters was not clearly distinguishable among assembly systems containing GTP, GTP-γS, or GMP-PNP. Therefore, the inhibition of the MtSt formation by GTP-γS in the present study would suggest that the inhibition was due not to the Mt nucleation but to the Mt growth.

When substoichiometric amounts of microtuble toxins such as 0.1 mM colchicine or podophyllotoxin were added to the assembly system, beads formed large aggregates, pre-

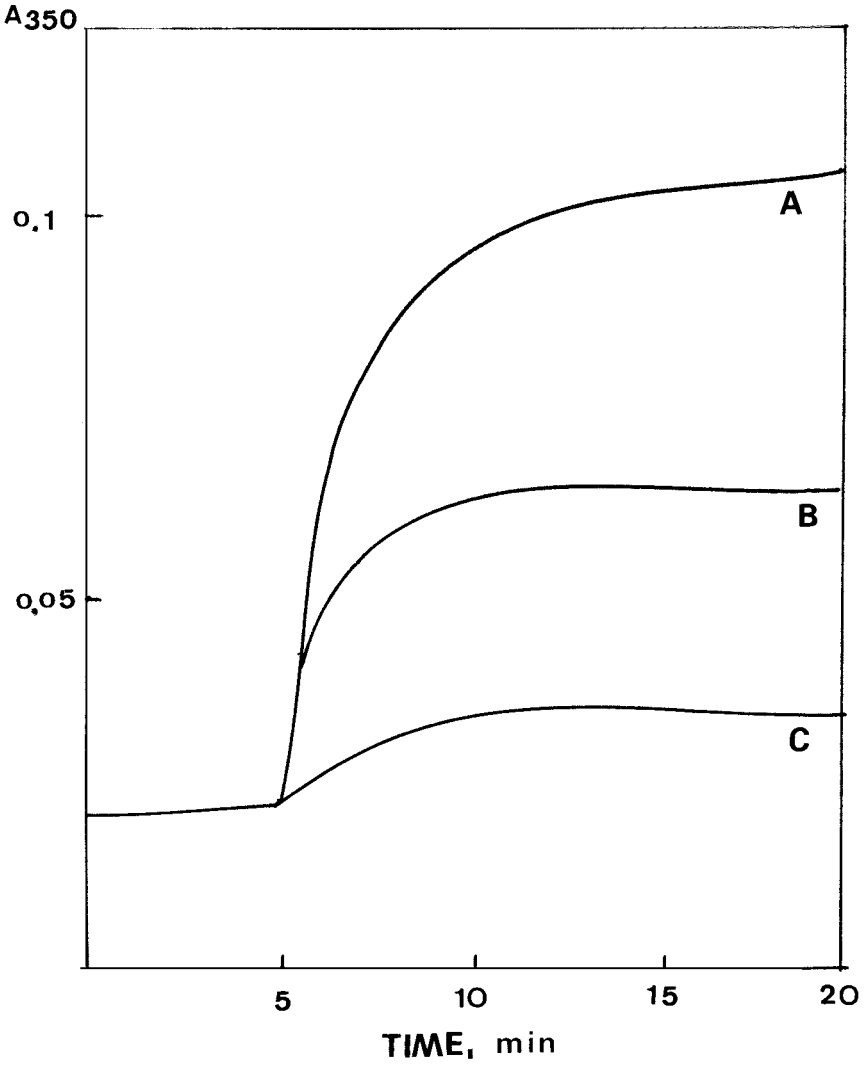


Fig. 6. Effect of antibodies against p51 and γ-tubulin on the construction of MtSt. Curve A, turbidity increase of the standard assembly system containing GTP; B, effect of an excess amount (100 μg) of anti-γ-tubulin; and C, effect of an excess amount (100 μg) of anti-p51(HP 1). In both cases, tubulin alone was first incubated for 5 min, followed by the addition of the mixture of beads and p51 which had previously been incubated with anti-bodies.

venting quantitative measurements. however, MtSt or astral-shaped microtubule structures were no longer observed in either case (data not shown).

We further examined the effect of antibodies made for p51 and γ-tubulin. Figure 6 clearly indicates that anti-p51 antiserum largely inhibited assembly. Dark-field microscopy revealed that latex beads were dispersed evenly like an image as shown in Figure 5, inset F, and no construction of MtSt was observed. On the other hand, anti-γ-tubulin antibody suppressed the assembly less than did anti-p51 antibody. Since we do not know the function of γ-tubulin in the sea urchin eggs, it was necessary at least to know the content of γ-tubulin in the MTOG fraction. Preliminary immunoblot analysis revealed that p51 was a major component of the MTOG fraction as shown by Toriyama *et al* (1988). However, the signal for γ-tubulin detected by anti-γ-tubulin antibodies was almost negligible (unpublished data).

Although the possibility still remains that p51 and γ-tubulin share a role in Mt assembly, p51 would be the major component of centrosomes to form asters in the sea urchin egg. This idea is consistent with the preliminary results that the MTOG did not contain γ-tubulin, and we could not observe signal for γ-tubulin in the protein fraction bound to the carboxylated Latex beads as examined by using anti-γ-tubulin.

Most of the beads could not initiate Mts in this experiment. Some of the independent beads initiated one or two microtubules. This may suggest that the formation of nucleus for tubulin polymerization occurs by chance, and was required for aggregates of beads and aster-shaped micro-tubules to form. The aggregation of beads was probably due to a crosslinking action of p51 as basic protein. The relationship between the amount of p51 and the number of MtSt formed on the Latex beads remains to be elucidated. The establishment of a system for the formation of asters using Latex beads will facilitate identification of the protein factors collaborating with p51 in the formation of Mts from the centrosomes.

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