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Overexpression of truncated γ-tubulins disrupts mitotic aster formation in *Xenopus* oocyte extracts

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Mechanisms of spindle pole formation rely on minus-end-directed motor proteins. γ-Tubulin is present at the centre of poles, but its function during pole formation is completely unknown. To address the role of γ-tubulin in spindle pole formation, we overexpressed GFP (green fluorescent protein)-fused γ-tubulin (γ-Tu-GFP) in *Xenopus* oocytes and produced self-assembled mitotic asters in the oocyte extracts. γ-Tu-GFP associated with endogenous α-, β- and γ-tubulin, suggesting that it acts in the same manner as that of endogenous γ-tubulin. During the process of aster formation, γ-Tu-GFP aggregated as dots on microtubules, and then the dots were translocated to the centre of the aster along microtubules in a manner dependent on cytoplasmic dynein activity. Inhibition of the function of γ-tubulin by an anti-γ-tubulin antibody resulted in failure of microtubule organization into asters. This defect was restored by overexpression of γ-Tu-GFP, confirming the necessity of γ-tubulin in microtubule recruitment for aster formation. We also examined the effects of truncated γ-tubulin mutants, which are difficult to solubly express in other systems, on aster formation. The middle part of γ-tubulin caused abnormal organization of microtubules in which minus ends of microtubules were not tethered, but dispersed. An N-terminus-deleted mutant prevented recruitment of microtubules into asters, similar to the effect of the anti-γ-tubulin antibody. The results indicate possible roles of γ-tubulin in spindle pole formation and show that the system developed in the present study could be useful for analysing roles of many proteins that are difficult to solubly express.

Key words: dynein, microtubule, mitotic aster formation, mitotic extract, γ-tubulin, *Xenopus* oocyte.

INTRODUCTION

Microtubules are cylindrical polymers assembled from α/β-tubulin heterodimers. Microtubules consist primarily of 13 protofilaments of the laterally associated heterodimers, which are inherently polar structures: α-tubulin is at the minus end of the polymer, and β-tubulin is at the plus end. γ-Tubulin, a member of the tubulin superfamily, is highly conserved among all eukaryotes so far studied [1–4]. Its function is thought to be required for nucleation of microtubules at the centrosome. Indeed, genetic or cell-biological studies have demonstrated the requirement of γ-tubulin for nucleation of microtubules in several organisms [4]. Furthermore, biochemical studies have indicated that the γTuRC (γ-tubulin ring complex) isolated from the *Xenopus* egg or *Drosophila* embryo nucleates microtubules *in vitro* [5,6]. Monomeric γ-tubulin translated in reticulocyte lysate also nucleates microtubules [7]. In addition to its nucleating function, γTuRC caps the minus ends of microtubules [8,9], preventing both further growth and depolymerization at the ends of microtubules [10,11]. γTuRC consists of approx. 10–13 γ-tubulin molecules and six to seven additional proteins named grip (gamma ring protein), at least two of which interact directly with γ-tubulin [12].

Although there have been many studies aimed at characterizing proteins that interact with γ-tubulin itself or with the γ-tubulin complex, little is known about which region of γ-tubulin is involved in its function. Structural models have indicated the regions that might interact with α- and β-tubulin or induce self-assembly of γ-tubulin [13]. A systematic search using the SPOT peptide technique indicated possible tubulin-interacting regions on γ-tubulin [14]. Overexpression of mutant γ-tubulins in mammalian cells showed that both the N- and C-termini of human γ-tubulin are necessary for its localization at the centrosome [15]. Alanine-scanning mutations or deletion of the C-terminus in γ-tubulin caused various defects in microtubule organization in yeast cell division [16–18]. Because of its insolubility, however, there has been no detailed biochemical study aimed at determining the functions of distinct regions of γ-tubulin.

In extracts prepared from *Xenopus* eggs arrested at meiotic metaphase II, spindles can form around sperm nuclei with a centrosome at each pole [19]. Spindles are also organized around chromatin beads without a centrosome [20]. Furthermore, poles form in the absence of chromatins after addition of a microtubule-stabilizing agent, such as taxol or DMSO, to the extracts [21,22]. The mechanisms of polar organization in these three cases are similar [23]. In all of the cases, microtubule organization into poles is dependent on the minus-end-directed translocation of microtubules by the cytoplasmic dynein–dynactin–NuMA (nuclear mitotic apparatus) complex [20,21,23–30]. Immunofluorescence studies have revealed that γ-tubulin is always present in the centre of spindle poles [22,23], suggesting an important function of γ-tubulin during pole focusing. Indeed, *Drosophila* null mutants showed the requirement of γ-tubulin for proper spindle formation, but not for microtubule nucleation, in both female and male meiosis [31,32]. How then is γ-tubulin involved in spindle pole formation? To date, no information on the role of γ-tubulin during spindle pole formation has been provided.

Abbreviations used: Cdc2, cell division control 2; GFP, green fluorescent protein; ORF, open reading frame; γ-Tu-C, C-terminal part of γ-tubulin (amino acids 303–451) fused with GFP at the C-terminus; γ-Tu-Full, full-length γ-tubulin (amino acids 1–451) fused with GFP at the C-terminus; γ-Tu-GFP, γ-tubulin fused with GFP; γ-Tu-M, middle part of γ-tubulin (amino acids 151–302) fused with GFP at the C-terminus; γ-Tu-MC, C-terminal part of γ-tubulin (amino acids 151–451) fused with GFP at the C-terminus; γ-Tu-N, N-terminal part of γ-tubulin (amino acids 1–150) fused with GFP at the N-terminus; γ-Tu-NM, N-terminal part of γ-tubulin (amino acids 1–302) fused with GFP at the N-terminus; γTuRC, γ-tubulin ring complex.

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In the present study, we overexpressed GFP (green fluorescent protein)-fused γ-tubulin (γ-Tu-GFP) and its truncated mutants in *Xenopus* oocytes and induced oocyte maturation *in vitro* to investigate the formation of asters in a cell-free system. Time-lapse microscopy revealed that γ-Tu-GFP was translocated to the centre of mitotic aster along microtubules in a manner that was dependent on cytoplasmic dynein motility. Anti-γ-tubulin antibody prevented mitotic aster formation, and overexpressed γ-Tu-GFP rescued the defect, confirming the requirement of γ-tubulin in microtubule recruitment for aster formation. Overexpression of an N-terminus-deleted mutant of γ-tubulin also inhibited microtubule recruitment into aster formation. The middle part of γ-tubulin prevented tethering of microtubules at the minus ends. These results show that γ-tubulin possibly plays a role in recruitment and tethering of microtubules for spindle pole formation, and that the system developed in the present study allows us to examine roles of insoluble proteins in other systems.

EXPERIMENTAL

Animals and oocytes

Xenopus laevis was purchased from Hamamatsu Seibutsu Kyozai (Shizuoka, Japan). Full-grown immature oocytes at stage VI were isolated as described previously [33]. Meiotic metaphase-II-arrested mature oocytes were obtained by incubating the oocytes with 10 μg/ml progesterone.

Preparation of γ-Tu-GFP

γ-Tu-GFP was produced as follows. Using pEGFP-N1 (Clontech) as a template, the ORF (open reading frame) of GFP was PCR-amplified with two sets of primers, one introducing BglII and BamHI sites (5′-GAAGATCTACCATGGTGAGCAAGGGCGAG-3′ and 5′-ACGGATCCTTGTACAGCTCGTCCATGCC-3′) and another introducing XbaI and SpeI sites (5′-GCTCTAGAAATGGTGAGCAAGGGCGAG-3′ and 5′-GACTAGTTACTTGTACAGCTCGTCCAT-3′). After digestion with the set of BglII and BamHI or XbaI and SpeI, the PCR products were ligated into the BamHI or XbaI site of pCS2$^+$ [33]. Correctly oriented clones were selected and named pCS2$^+$/GFP-N (BamHI site) or pCS2$^+$/GFP-C (XbaI site). The full ORF of *Xenopus* γ-tubulin [3] was amplified by PCR with a primer set that introduces EcoRI and XhoI sites (5′-GGAATTCACCATGCCACGGGAGATTATC-3′ and 5′-CCGCTCGAGTTTATCCTGGGTTCCCCA-3′). The PCR product was digested with EcoRI and XhoI, and ligated into the same cloning site of pCS2$^+$/GFP-C to construct γ-Tu-Full (full-length γ-Tu-GFP). Fragments of γ-tubulin [γ-Tu-NM (amino acids 1–302), -C (amino acids 303–451), -N (amino acids 1–150), -M (amino acids 151–302) and -MC (amino acids 151–451); see Figure 4] were PCR-amplified from the full ORF γ-tubulin cDNA using the following primer sets: 5′-GGAATTCAATGCCACGGGAGATTATCACC-3′ and 5′-GGAATTCAATGCCACGGGAGATTATCACC-3′ for γ-Tu-NM, 5′-GGAATTCCACCATGGTTATGGTATCCACTG-3′ and 5′-CCGCTCGAGTTTATCCTGGGTTCCCCA-3′ for γ-Tu-C, 5′-GGAATTCAATGCCACGGGAGATTATCACC-3′ and 5′-CCGCTCGAGCCCTAATCCAGAGCCTGT-3′ for γ-Tu-N, 5′-GGAATTCCACCATGTCTTACCTTTTAGAGA-3′ and 5′-GGAATTCAATGCCACGGGAGATTATCACC-3′ for γ-Tu-M, and 5′-GGAATTCCACCATGTCTTACCTTTTAGAGA-3′ and 5′-CCGCTCGAGTTTATCCTGGGTTCCCCA-3′ for γ-Tu-MC. The resulting PCR products were digested with EcoRI and XhoI and ligated into the EcoRI/XhoI site of pCS2$^+$/GFP-N (γ-Tu-NM and -N) or pCS2$^+$/GFP-C (γ-Tu-C, -M and -MC).

Microinjection

cDNAs for GFP-fused γ-tubulins were linearized with NotI and transcribed using an mMESSAGE mMACHINE SP6 kit (Ambion). Oocytes were isolated manually with forceps and injected with 25 ng of γ-Tu-Full, -NM, -N, -M, -MC or -C mRNA. The oocytes were treated with progesterone to induce maturation *in vitro*.

Oocyte extraction and mitotic aster formation

Thirty oocytes injected with mRNA and treated with progesterone were washed three times with microtubule assembly buffer (BRB80) (80 mM potassium Pipes, 5 mM EGTA and 1 mM $MgCl_2$, pH 6.8) supplemented with 1 mM dithiothreitol, 300 μM (*p*-amidinophenyl)methanesulphonyl fluoride, 3 μg/ml leupeptin, 1 μM taxol and 10 μg/ml cytochalasin B. After removing excess BRB80, 1 μl of new BRB80 with protease inhibitors, taxol and cytochalasin B was added. The oocytes were homogenized with a pestle and centrifuged at 15000 ***g*** for 20 min at 4 °C. The supernatant was collected and incubated with 0.2 mg/ml Rhodamine–tubulin (Molecular Probes) for 30–60 min at 24 °C. For inhibition of the dynein activity, a final concentration of 0.1 mg/ml anti-dynein mouse antibody (clone 70.1; Sigma) was added. The extracts were mounted on to a glass slide and observed using a Bio-Rad MicroRadiance confocal microscope. For inhibition of γ-tubulin function, 80 mature oocytes were homogenized as described above (8 μl of BRB80 being used for extraction). After adding Rhodamine–tubulin, the supernatant was incubated with 0.4 mg/ml anti-γ-tubulin rabbit antibody (Sigma), 0.2 mg/ml anti-γ-tubulin rabbit antibody (Santa Cruz Biotechnology) or 0.4 mg/ml anti-His-probe rabbit antibody (Santa Cruz Biotechnology). The supernatant (3 μl) was analysed for Cdc2 (cell division control 2) kinase activity, and the remainder (5 μl) was observed using the confocal microscope.

Immunoblotting and immunoprecipitation

Anti-γ-tubulin rabbit antibody (Sigma), anti-α-tubulin mouse antibody (DM1A, Sigma) and anti-β-tubulin mouse antibody (TUB2.1, Sigma) were used to detect *Xenopus* γ-, α- and β-tubulin respectively. An anti-GFP mouse antibody (Roche) was used to detect and precipitate γ-Tu-GFP. To precipitate γ-tubulin, we used four different antibodies, anti-γ-tubulin rabbit and mouse antibodies (Sigma), which react to the N-terminus of γ-tubulin, and anti-γ-tubulin rabbit and goat antibodies (Santa Cruz Biotechnology), which react to the C-terminus of γ-tubulin. Anti-α-tubulin antibody (DM1A) was used to precipitate α-tubulin. Immunoblotting and immunoprecipitation were performed as described previously [33,34].

Cdc2 kinase assay

The activity of Cdc2 kinase was measured with histone H1 as a substrate (typeIII-S from calf thymus; Sigma), according to the procedures described previously [35].

RESULTS

Binding of γ-Tu-GFP to the minus ends of microtubules

To analyse the role of γ-tubulin during spindle pole formation, we developed an *in vitro* system in which γ-Tu-GFP was overexpressed in *Xenopus* oocytes. Injection of 25 ng of mRNA caused the expression of a 78-kDa γ-Tu-GFP, the concentration of which was approx. 3-fold higher than that of endogenous γ-tubulin (2.92 ± 0.33; $n = 5$) (Figure 1A). Immunoprecipitation

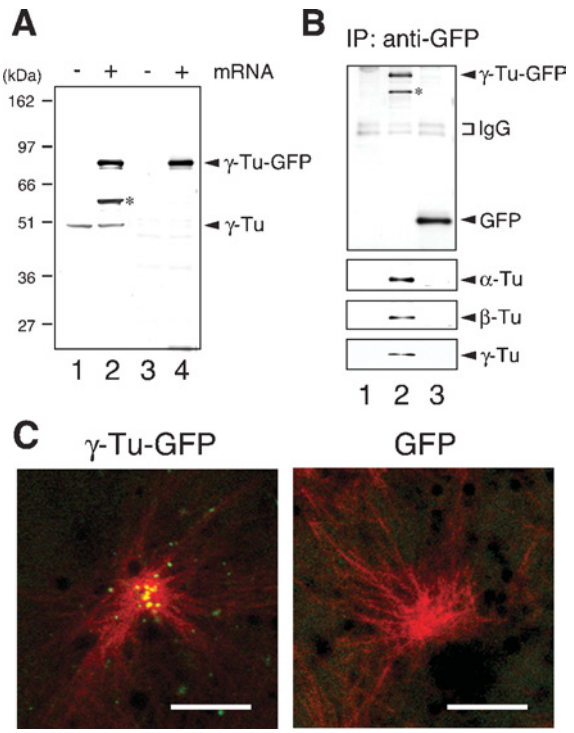

Figure 1 Overexpression of γ-Tu-GFP

(**A**) Immunoblotting of γ-Tu-GFP. Extracts from oocytes injected with distilled water (−) or 25 ng of γ-Tu-GFP mRNA (+) were immunoblotted with anti-γ-tubulin antibody (lanes 1 and 2) or anti-GFP antibody (lanes 3 and 4). Molecular-mass sizes are indicated in kDa. (**B**) Co-immunoprecipitation of α-, β- and γ-tubulin with γ-Tu-GFP. Oocytes injected with distilled water (lane 1), γ-Tu-GFP mRNA (lane 2) or GFP mRNA (lane 3) were immunoprecipitated (IP) with anti-GFP antibody and immunoblotted with anti-GFP antibody (γ-Tu-GFP and GFP), anti-α-tubulin antibody (α-Tu), anti-β-tubulin antibody (β-Tu) or anti-γ-tubulin antibody (γ-Tu). (**C**) Behaviour of γ-Tu-GFP in the mitotic aster. Meiotic metaphase-II-arrested extracts from oocytes injected with γ-Tu-GFP mRNA (left) or GFP mRNA (right) were incubated with taxol and observed using a confocal microscope. Rhodamine–microtubules are shown in red, and GFP is shown in green. Co-localization of Rhodamine–microtubules and GFP is shown in yellow. Scale bars, 10 μm. Similar results were obtained from five independent experiments. Note that a proteolysed 58-kDa γ-Tu-GFP (asterisk in **A**) contains the full-length γ-tubulin sequence, since the remaining 20-kDa GFP moiety was detected by immunoblotting with anti-GFP antibody (results not shown) and that a truncated γ-Tu-GFP (asterisk in **B**) is produced during the immunoprecipitation procedure since it is not detected in (**A**).

with an anti-GFP antibody showed that γ-Tu-GFP, but not GFP alone, bound to α- and β-tubulin (Figure 1B), as endogenous γ-tubulin did (results not shown). The binding of α-tubulin and γ-Tu-GFP, but not GFP, was also confirmed by immunoprecipitation with an anti-α-tubulin antibody (see Figure 4B). We elucidated further that endogenous γ-tubulin was co-immunoprecipitated with γ-Tu-GFP (Figure 1B), suggesting that γ-Tu-GFP was incorporated in γ-tubulin multimers formed in oocytes. To obtain meiotic metaphase-II-arrested extracts, these oocytes were treated with progesterone to induce oocyte maturation, and the mature oocytes were homogenized and centrifuged. The supernatant was incubated with taxol to induce formation of mitotic asters. Immunofluorescence of an anti-γ-tubulin antibody revealed the existence of γ-tubulin at the centre of mitotic asters in the extracts prepared from non-injected mature oocytes (results not shown) [22,23]. Consistent with endogenous γ-tubulin, γ-Tu-GFP was localized at the centre of mitotic asters, while GFP did not show any localization (Figure 1C), indicating the binding of γ-Tu-GFP to the minus ends of microtubules. Moreover, interestingly, they aggregated to form dot-like structures (Figure 1C). This finding, and the result of immunoprecipitation (Figure 1B),

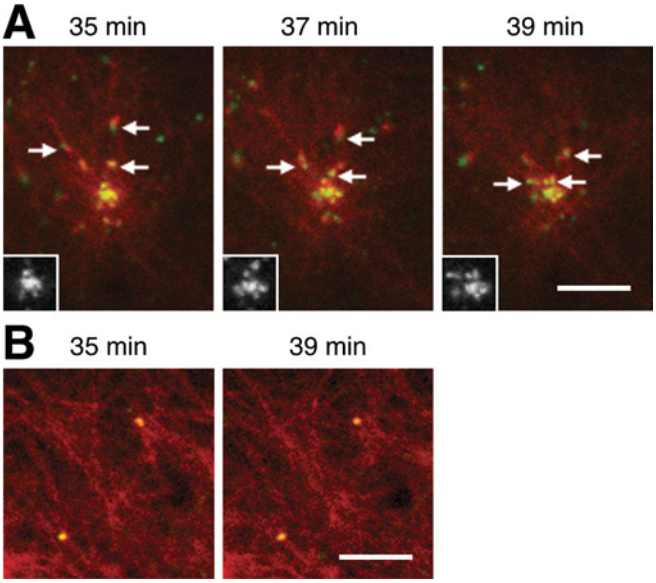

Figure 2 Dependence of translocation of γ-Tu-GFP on dynein motility

Rhodamine–microtubules are shown in red, and γ-Tu-GFP is shown in green. (**A**) Time-lapse microscopy of γ-Tu-GFP during mitotic aster formation. The arrows indicate dots of γ-Tu-GFP, which translocate along microtubules. The insets show fluorescence of γ-Tu-GFP at the aster. (**B**) Inhibition of dynein activity by anti-dynein antibody. Scale bars, 5 μm. Similar results were obtained from two independent experiments.

suggests a formation of γ-tubulin multimer at the minus ends of microtubules.

Necessity of γ-tubulin for the recruitment of microtubules for mitotic aster formation

The role of γ-tubulin during spindle pole formation was examined by observing the process of aster formation in γ-Tu-GFP-expressed oocyte extracts. Time-lapse microscopy provided evidence that dots of γ-Tu-GFP moved along microtubules to the centre of the aster (Figure 2A). In addition, the fluorescence intensity of γ-Tu-GFP at the centre of the aster increased 1.2-fold between 35 and 39 min after the start of incubation at 24 °C (Figure 2A, insets). Induction of the formation of mitotic asters by taxol requires dynein motility [21]. Thus it seems likely that the movement of γ-Tu-GFP is also dynein-dependent. To verify this possibility, we examined the effect of an anti-dynein antibody on γ-Tu-GFP movement. This antibody reacts to the dynein intermediate chain and inhibits dynein function in *Xenopus* egg extracts [20,23,36]. Consistent with results reported previously, the anti-dynein antibody prevented mitotic aster formation. Furthermore, dots of γ-Tu-GFP did not move at all in the presence of the antibody (Figure 2B). These results therefore indicate that the movement of γ-Tu-GFP is dependent on dynein motility. We have observed dynamic changes in γ-tubulin localization during spindle formation in *Xenopus* oocytes and showed that the translocation of γ-tubulin requires dynein activity [37]. Therefore movement of γ-tubulin *in vivo* is also dependent on dynein.

To elucidate γ-tubulin function during aster formation, mitotic extracts were incubated with an anti-γ-tubulin antibody, which is able to immunoprecipitate γ-tubulin molecules (Figure 3A) and hence is expected to disrupt its function. The anti-γ-tubulin antibody prevented mitotic aster formation in a way similar to that in the case of the anti-dynein antibody (Figure 3C, compare with Figure 3D), while an anti-His-probe antibody did not affect aster

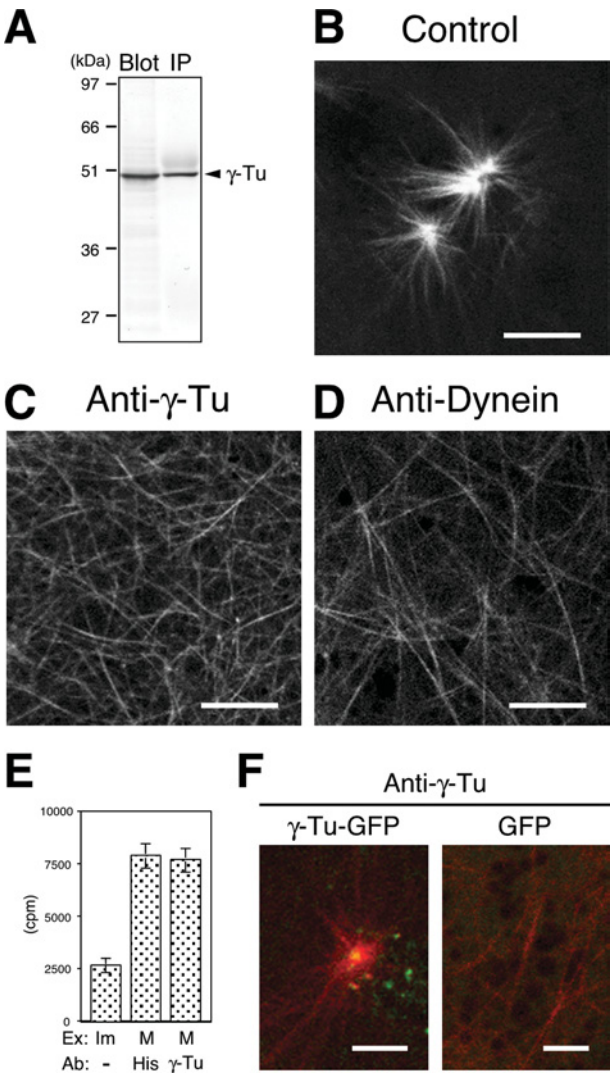

Figure 3 Effect of anti-γ-tubulin antibody on mitotic aster formation

(**A**) Immunoprecipitation of γ-tubulin. Crude oocyte extracts (left) and immunoprecipitation of the extracts with anti-γ-tubulin rabbit antibody (right) were immunoblotted with anti-γ-tubulin mouse antibody. Molecular-mass sizes are indicated in kDa. (**B**–**D**) Inhibition of mitotic aster formation by anti-γ-tubulin antibody or anti-dynein antibody. Mitotic extracts were incubated with anti-His-probe antibody (**B**), anti-γ-tubulin antibody (**C**) or anti-dynein antibody (**D**) and were observed using a confocal microscope. Similar results were obtained from three independent experiments. (**E**) Cdc2 kinase activities in the extracts. The kinase activities in the immature (Im) or mature (M) extracts incubated without antibody (−), with anti-His-probe antibody (His), or with anti-γ-tubulin antibody (γ-Tu) were measured with histone H1. Results are means ± S.D.; $n = 3$. (**F**) Effect of overexpressed γ-Tu-GFP (left) or GFP (right) in the extracts incubated with anti-γ-tubulin antibody. Rhodamine–microtubules are shown in red and GFP is shown in green. Scale bars in (**B**–**D**), 10 μm; scale bars in (**F**), 5 μm.

formation (Figure 3B). We confirmed that another anti-γ-tubulin antibody, which also immunoprecipitates γ-tubulin, caused a similar defect in microtubule organization (results not shown). The defect in aster formation is not due to a reduction in the level of Cdc2 kinase activity, because both extracts had similar levels of Cdc2 kinase activity (Figure 3E). We examined further whether the defect in extracts treated with the anti-γ-tubulin antibody could be restored by the overexpression of γ-Tu-GFP. A 3-fold higher expression level of γ-Tu-GFP than that of endogenous γ-tubulin (Figure 1A) was able to rescue mitotic aster formation (Figure 3F, left), whereas GFP alone could not (Figure 3F, right). It is therefore concluded that the inhibition of aster formation by the anti-γ-tubulin antibody is due to impairment of γ-tubulin function. These results demonstrate that γ-tubulin is required for the organization of microtubules into mitotic asters and suggest that γ-tubulin interacts with the dynein complex, which consists of several molecules in the cytoplasm, to recruit microtubules for mitotic aster formation.

To examine whether γ-tubulin molecules interact with dynein, we immunoprecipitated γ-tubulin using four different antibodies (two of them recognizing the N-terminus, and the other two reacting with the C-terminus of a γ-tubulin molecule) and immunoblotted the precipitates with the anti-dynein antibody. In addition, overexpressed γ-Tu-GFP was immunoprecipitated with the anti-GFP antibody and immunoblotted with the anti-dynein antibody. However, dynein was not co-immunoprecipitated with either γ-tubulin or γ-Tu-GFP (results not shown). The results did not show a stable binding of γ-tubulin to dynein, although it is possible that γ-tubulin interacts with dynein weakly, transiently or via another protein, and this interaction is easily dissociated by sample preparation in the immunoprecipitation assays.

Inhibition of mitotic aster formation by overexpression of truncated-γ-tubulins

To determine which part of the γ-tubulin molecule is important for the recruitment of microtubules and hence for mitotic aster formation, we examined behaviours of truncated mutants of γ-tubulin during aster formation. First, γ-Tu-GFP containing either the N-terminal (γ-Tu-NM) or the C-terminal (γ-Tu-C) part was expressed (Figures 4A and 4B). Immunoprecipitation with an anti-α-tubulin antibody showed that both γ-Tu-NM and γ-Tu-C bound to α-tubulin (Figure 4B). In the extracts incubated with taxol, γ-Tu-NM was associated with the minus ends of microtubules organized into asters (Figure 4C). In contrast, γ-Tu-C was found on stable and long bundles of microtubules (Figures 4D and 4E). Furthermore, mitotic aster formation was prevented in γ-Tu-C-expressed oocytes. In budding yeast, the C-terminus of γ-tubulin is not required for localization of the spindle pole body, but is required for the proper organization of microtubules [18]. In addition, phosphorylation of a conserved tyrosine residue within the C-terminus is important for the assembly of microtubules [38]. Further analysis is required to determine the critical function of the C-terminus of γ-tubulin.

Secondly, to examine in more detail the behaviour of the N-terminal part, we dissected γ-Tu-NM into γ-Tu-N and γ-Tu-M (Figures 4A and 4F). Immunoprecipitation with the anti-α-tubulin antibody showed that both γ-Tu-N and γ-Tu-M bound to α-tubulin (Figure 4F). In taxol-induced mitotic asters, γ-Tu-N behaved almost the same as γ-Tu-Full and NM (Figure 4G). In less than 25 % of the cases, however, γ-Tu-N induced abnormal microtubule organization, in which some of the microtubule-minus ends were focused, but not organized into asters (Figure 4H). These results suggest that γ-Tu-N associates with microtubule minus ends and rarely affects mitotic aster formation. In contrast, γ-Tu-M was associated with the minus ends of microtubules, but the microtubules were not strictly focused into asters (Figure 4I). Detailed observations showed that the γ-Tu-M-associated minus ends of microtubules were uniformly distributed in the extracts without tethering (Figure 4J). This finding suggests that γ-Tu-M does not affect recruitment of the associating microtubules along other microtubules to the minus ends, but prevents tethering of the microtubules at their minus ends, the ends therefore being dispersed. To determine whether dynein is involved in the recruitment of γ-Tu-M-associated microtubules to the minus ends, dynein activity was inhibited by an anti-dynein antibody. As expected, microtubule arrays remained unchanged under the conditions of inhibition of dynein activity

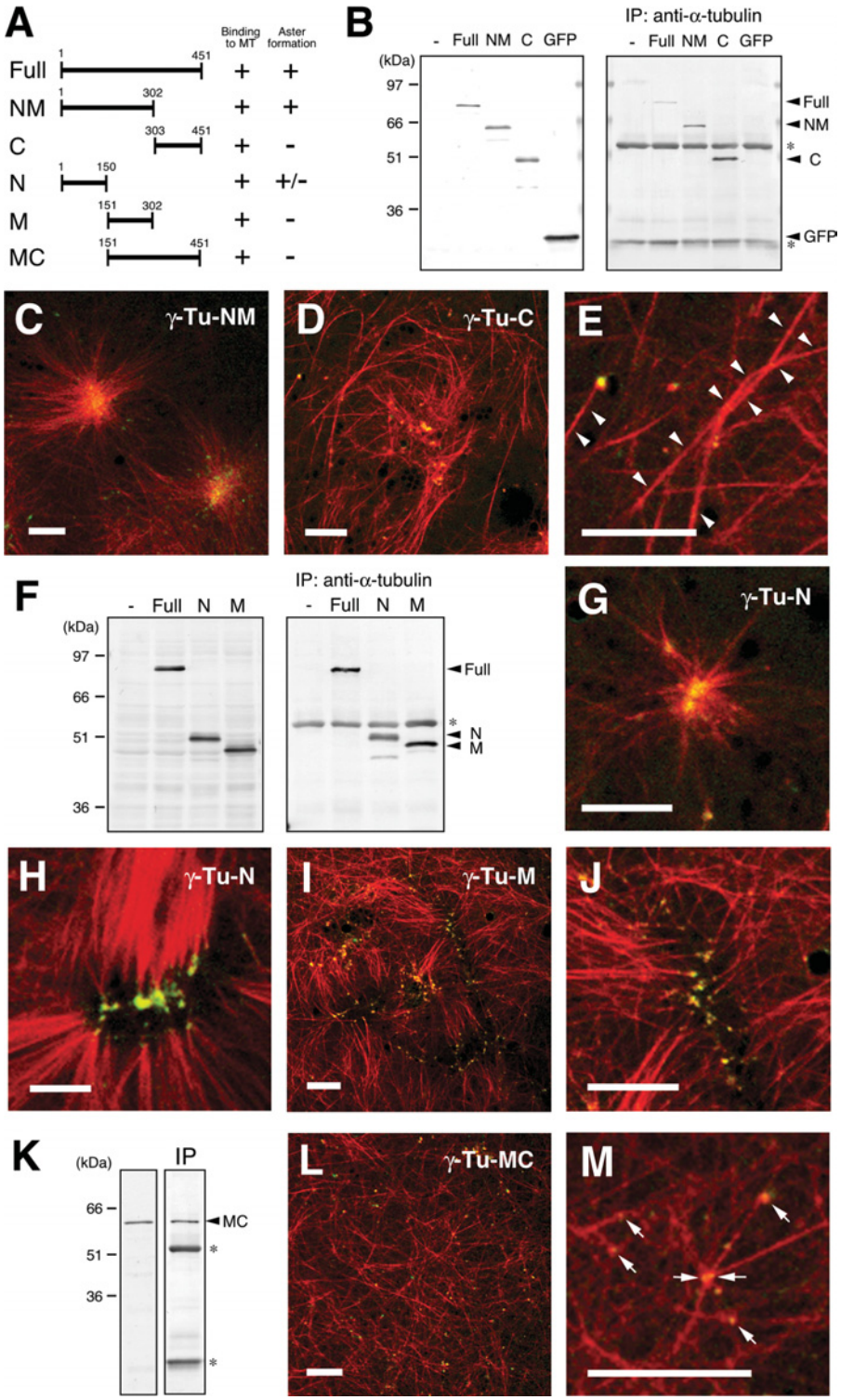

Figure 4 Behaviour of γ-Tu-GFP mutants

(**A**) Schematic diagrams showing the fragments used in the experiments. The activity of each molecule to bind to microtubules and to form mitotic asters is summarized on the right. (**B**) Expression of γ-Tu-Full, -NM and -C mutants, and binding of the mutants to α-tubulin. Extracts from oocytes injected with distilled water (−), γ-Tu-Full (Full), γ-Tu-NM (NM) or γ-Tu-C (C) mRNAs were immunoblotted with anti-GFP antibody (left). The same extracts were immunoprecipitated (IP) with anti-α-tubulin and immunoblotted with anti-GFP antibody (right). (**C**) Behaviour of γ-Tu-NM. (**D**) Behaviour of γ-Tu-C. (**E**) Enlarged view of (**D**). Arrowheads indicate stable microtubule bundles associated with γ-Tu-C. (**F**) Expression of γ-Tu-Full (Full), -N (N) and -M (M) mutants, and binding of the mutants to α-tubulin. Extracts from oocytes injected with distilled water (−), γ-Tu-Full (Full), γ-Tu-N (N) or γ-Tu-M (M) mRNAs were immunoblotted with anti-GFP antibody (left). The same extracts were immunoprecipitated with anti-α-tubulin and immunoblotted with anti-GFP antibody (right). (**G**–**H**) Behaviour of γ-Tu-N. (**I**) Behaviour of γ-Tu-M. (**J**) Enlarged view of (**I**). (**K**) Expression of γ-Tu-MC mutant and binding of the mutant to α-tubulin. Extracts from oocytes injected with γ-Tu-MC mRNA were immunoblotted with anti-GFP antibody (left). The same extracts were immunoprecipitated with anti-α-tubulin and immunoblotted with anti-GFP antibody (right). (**L**) Behaviour of γ-Tu-MC. (**M**) Enlarged view of (**L**). Arrows indicate dots of γ-Tu-MC. Rhodamine–microtubules and γ-Tu-GFP mutants are shown in red and green respectively. Scale bars, 10 μm. Similar results were obtained from two independent experiments. Asterisks in (**B**), (**F**) and (**K**) show IgG of the anti-α-tubulin antibody. Molecular-mass sizes are indicated in kDa.

(see Supplementary Figure A at http://www.BiochemJ.org/bj/389/bj3890611add.htm). We also examined whether endogenous γ-tubulin contributes to this recruitment by specifically preventing its function with an anti-γ-tubulin antibody, because this antibody recognizes the N-terminus of γ-tubulin (amino acids 38–53) and thereby does not react with γ-Tu-M. The anti-γ-tubulin antibody did not affect the movement of γ-Tu-M-associated microtubules (see Supplementary Figure B at http://www.BiochemJ.org/bj/389/bj3890611add.htm), indicating that endogenous γ-tubulin is not involved in these events.

Finally, we overexpressed an N-terminus-deleted mutant of γ-tubulin (γ-Tu-MC) (Figures 4A and 4K). Co-immunoprecipitation showed binding of γ-Tu-MC and α-tubulin (Figure 4K). In the extracts incubated with taxol, γ-Tu-MC inhibited microtubule recruitment into asters, similar to the effects of the anti-γ-tubulin and anti-dynein antibodies (Figure 4L). Detailed observations showed that γ-Tu-MC was associated with microtubules at the minus ends of cross-linking microtubules (Figure 4M, arrows). These findings suggest that γ-Tu-MC inhibits the interaction of γ-tubulin with dynein at the minus ends of microtubules and prevents microtubule recruitment for aster formation.

DISCUSSION

In the present study, we obtained the following results: (i) γ-Tu-GFP used as an indicator of the behaviour of endogenous γ-tubulin molecules moved to the centre of a mitotic aster, and this movement was inhibited by an anti-dynein antibody; (ii) an anti-γ-tubulin antibody prevented the organization of microtubules into asters, and this defect was restored by overexpression of γ-Tu-GFP; (iii) all of the three regions of the γ-tubulin molecule examined in the present study, the N-terminus, the middle part and the C-terminus, were associated with microtubules; and (iv) overexpression of N-terminal parts (γ-Tu-NM and γ-Tu-N) did not affect aster formation, whereas N-terminus-deleted parts (γ-Tu-M, γ-Tu-C and γ-Tu-MC) caused abnormal organization of microtubules; the middle part recruited microtubules via dynein activity, but inhibited the tethering of them at the minus ends, the C-terminus induced the formation of stable microtubule bundles and prevented aster formation, and the middle and C-terminal part inhibited the recruitment of microtubules into asters, resulting in a defect similar to that induced by the anti-γ-tubulin antibody. The results suggest that γ-tubulin is involved in recruitment of microtubules and in tethering of their minus ends to form mitotic asters and possibly plays a role in spindle pole formation.

Our findings are fairly consistent with immunofluorescent observations during meiotic spindle formation *in vivo* [37,39], in which γ-tubulin is distributed along the length of spindle microtubules in the early stage of bipolar spindle formation and is finally concentrated at the spindle poles. In support of the results of the present study, we have also demonstrated that inhibition of dynein activity during spindle formation in *Xenopus* oocytes results in failure of spindle formation and distribution of γ-tubulin around the microtubule array [37]. Our observations also contribute to explain the abnormalities in γ-tubulin-depleted *Drosophila* mutants. In spermatocytes of the mutant, a significant number of microtubules form a disordered-mesh array in the cell and never assemble a bipolar spindle, even though centrosomes with astral microtubules divide at prophase [32]. An explanation of this defect is failure of interaction between microtubule-minus ends and dynein complexes via γ-tubulin molecules, dynein therefore being unable to organize microtubules into bipolar spindles, as observed in cells injected with an anti-dynein antibody at prophase [40]. Even when bipolar arrays of microtubules form around chromosomes in maturing oocytes of the mutant, the poles are not focused [31], suggesting that the lack of γ-tubulin molecules results in failure to tether the microtubule-minus ends at the poles.

In the present study, we have shown that overexpression of some truncated mutants of γ-tubulin disrupted mitotic aster formation. There is a possibility that the truncated mutants prevented γTuRC function by binding to its subunits, but did not disrupt the function of endogenous γ-tubulin itself, although it remains unclear whether γTuRC is formed at the minus ends of microtubules during mitotic aster formation. We obtained the following results in this study: (i) all of the mutants bound to α-tubulin (Figures 4B, 4F and 4K); (ii) incubation with the anti-γ-tubulin antibody and overexpression of γ-Tu-MC caused similar defects in microtubule organization (Figures 3C and 4L–4M); and (iii) γ-Tu-M could partially restore the defect induced by the anti-γ-tubulin antibody (see Supplementary Figure B at http://www.BiochemJ.org/bj/389/bj3890611add.htm). These results suggest that overexpression of the truncated mutants disrupts γ-tubulin function itself, but not γ-TuRC subunits.

We have demonstrated that all of the three parts of the γ-tubulin molecule, the N-terminus, the middle part and the C-terminus, can bind to microtubules, although they show distinct behaviours in oocytes. The binding of γ-Tu-N to microtubules (Figure 4F) is an unexpected finding, since a systematic search for tubulin-binding sites using the SPOT peptide technique suggested that the microtubule-interacting region exists in the middle and C-terminus regions of γ-tubulin, but not in the N-terminus [14]. An explanation for this discrepancy is that small peptides corresponding to the N-terminus of γ-tubulin cannot exert affinity for microtubules, whereas the N-terminal region of γ-tubulin used in the present study can retain affinity in *Xenopus* oocytes.

In conclusion, we have demonstrated the involvement of γ-tubulin in taxol-induced pole formation, namely recruitment and tethering of microtubules to the mitotic aster. We have also shown that the protein expression system developed in the present study could be useful for analysing roles of various proteins that are insoluble in other systems. Further investigations, including biochemical study to identify the molecules that bind to the respective regions of γ-tubulin, should make a great contribution to the understanding of the mechanisms of spindle pole formation.

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