

# When Overexpressed, a Novel Centrosomal Protein, RanBPM, Causes Ectopic Microtubule Nucleation Similar to $\gamma$ -Tubulin

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**Abstract.** A novel human protein with a molecular mass of 55 kD, designated RanBPM, was isolated with the two-hybrid method using Ran as a bait. Mouse and hamster RanBPM possessed a polypeptide identical to the human one. Furthermore, *Saccharomyces cerevisiae* was found to have a gene, YGL227w, the COOH-terminal half of which is 30% identical to RanBPM. Anti-RanBPM antibodies revealed that RanBPM was localized within the centrosome throughout the cell cycle. Overexpression of RanBPM produced multiple spots which were colocalized with  $\gamma$ -tubulin and acted as ectopic microtubule nucleation sites, resulting in a reorganization of microtubule network. RanBPM cosedimented with the centrosomal fractions by sucrose-density gradient centrifugation. The formation of

microtubule asters was inhibited not only by anti-RanBPM antibodies, but also by nonhydrolyzable GTP-Ran. Indeed, RanBPM specifically interacted with GTP-Ran in two-hybrid assay. The central part of asters stained by anti-RanBPM antibodies or by the mAb to  $\gamma$ -tubulin was faded by the addition of GTP $\gamma$ S-Ran, but not by the addition of anti-RanBPM antibodies. These results provide evidence that the Ran-binding protein, RanBPM, is involved in microtubule nucleation, thereby suggesting that Ran regulates the centrosome through RanBPM.

**Key words:** centrosome •  $\gamma$ -tubulin • Ran • RanBPM • YGL227w

RAN is a Ras-like nuclear small GTPase (Bischoff and Ponstingl, 1991a). The hydrolysis of GTP-Ran is enhanced by the RanGTPase-activating protein, RanGAP1/Rna1p (Bischoff et al., 1994, 1995) and the nucleotide exchange on Ran is carried out by RCC1 (Bischoff and Ponstingl, 1991b). RanGAP is located within the cytoplasm (Matunis et al., 1996; Mahajan et al., 1997), whereas RCC1 is localized on the chromatin (Bischoff and Ponstingl, 1991a; Ohtsubo et al., 1989). Therefore, GTP-Ran created by the aid of RCC1 in the nucleus must be transferred to the cytoplasm in order to hydrolyze the GTP of Ran, although there still exists the possibility that RanGAP proteins are present within the nucleus (Cheng et al., 1995; Traglia et al., 1996). The notion that Ran shuttles between the nucleus and the cytoplasm is consistent

with the finding that Ran functions as a carrier for nucleus/cytosol exchange of macromolecules (for review see Moore and Blobel, 1994; Melchior and Gerace, 1995, 1998; Görlich and Mattaj, 1996; Nigg, 1997; Görlich, 1998; Wozniak et al., 1998).

In addition to nucleocytoplasmic transport, Ran is thought to be involved in ribosomal RNA processing (Mitchell et al., 1997), and cell cycle regulation (for review see Dasso, 1993; Seki et al., 1996). The tsBN2 cell line, a temperature-sensitive (ts)<sup>1</sup> *rcc1* mutant of the hamster BHK21 cell line, shows either G1 arrest or premature chromatin condensation, depending on the phase of the cell cycle at which cultures start to be incubated at the nonpermissive temperature (Nishimoto et al., 1978; Nishi-

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1. *Abbreviations used in this paper:*  $\gamma$ -TuRC,  $\gamma$ -tubulin ring complex; ORF, open reading frame; PCM, pericentriolar material; RACE, rapid amplification of cDNA ends; SPB, spindle pole body; ts, temperature sensitive.

tani et al., 1991). A ts mutant of the *Schizosaccharomyces pombe* *RCC1* homologue, *pim1-D1*, is arrested with condensed chromatin at the nonpermissive temperature (Sazer and Nurse, 1994). On the other hand, a ts mutant of the *Saccharomyces cerevisiae* *RCC1* homologue *PRP20*, *srm1-1*, which was isolated as a mutant involved in the mating signal transduction pathway (Clark and Sprague, 1989) is arrested in the G1 phase at the nonpermissive temperature, whereas other *prp20* alleles have a defect in mRNA export and do not demonstrate the cell cycle-specific arrest (Aebi et al., 1990; Kadowaki et al., 1993). It is a matter of debate as to how to explain the cell cycle defects of *rcc1/pim1-D1/srm1-1*.

To clarify the downstream events of RanGTPase cycle, a series of Ran-binding proteins has been identified (Sazer, 1996; Avis and Clarke, 1996). The family of proteins possessing the RanBP1 domain (Dingwall et al., 1995), and the importin  $\beta$  family (Görlich et al., 1997) are currently well known. Both RanBP1 (Coutavas et al., 1993) and its yeast homologue, Yrb1p, which are functionally exchangeable (Noguchi et al., 1997), are located within the cytoplasm (Schlenstedt et al., 1995; Richards et al., 1996). RanBP2 which possesses four RanBP1 domains is located on the cytoplasmic filament of the nuclear pore complexes (Melchior et al., 1995; Wu et al., 1995; Yokoyama et al., 1995). Yrb2p, yet another member of the *S. cerevisiae* RanBP1 family (Noguchi et al., 1997; Taura et al., 1997), and its human homologue RanBP3 (Mueller et al., 1998), are localized within the nucleus. Recently, Yrb2p was shown to be required for nuclear protein export (Taura et al., 1998) (Noguchi, E., Y. Saitoh, S. Sazer, and T. Nishimoto, manuscript in preparation). The importin  $\beta$  family (Görlich et al., 1997) could be divided into importins and exportins (for review see Ullman et al., 1997; Weis, 1998). The former is required for nuclear protein import and the latter for nuclear protein export. Ntf2/p10, which binds to GDP-Ran, is the other Ran-binding protein required for nuclear protein import (Paschal and Gerace, 1995; Corbett and Silver, 1996; Nehrbass and Blobel, 1996).

On the other hand, Dis3p which binds to GTP- and GDP-bound Ran (Noguchi et al., 1996; Shiomi et al., 1998), does not seem to be involved in nuclear pore transport function. Dis3p was originally isolated as a cold-sensitive mutation involved in mitotic progression (Ohkura et al., 1988; Kinoshita et al., 1991). The finding that Dis3p exists in an oligomeric form (Kinoshita et al., 1991; Noguchi et al., 1996) is consistent with the recent report that Dis3p corresponds to Rrp44p, which comprises the exosomes that are required for the 3' processing of the 5.8S rRNA (Mitchell et al., 1997). Indeed, *S. cerevisiae* Dis3p is localized within the nucleolus (Shiomi et al., 1998). In this study, we found another Ran-binding protein, designated as RanBPM. Surprisingly, RanBPM was localized within the centrosome throughout the cell cycle.

The centrosome organizes microtubules during both the interphase and mitosis (for review see Kalt and Schliwa, 1993). It consists of a pair of centrioles (Lange and Gull, 1995), surrounded by a complex collection of proteins known as the pericentriolar material (PCM) (Kellogg et al., 1994). The criterion for the classification as a centrosomal component is basically its localization within the central body. The  $\gamma$ -tubulin (Oakley and Oakley, 1989) is one of

the important centrosomal residents which is conserved through evolution (Kalt and Schliwa, 1993; Kellogg et al., 1994; Stearns and Winey, 1997). Several proteins interacting directly or indirectly with the  $\gamma$ -tubulin-like Tub4p of *S. cerevisiae* have been identified to comprise the spindle pole body (SPB), the functional equivalent of the centrosome in *S. cerevisiae* (Rout and Kilmartin, 1990; Osborne et al., 1994; Geissler et al., 1996; Kilmartin and Goh, 1996; Bullitt et al., 1997; Knop et al., 1997; Knop and Schiebel, 1998). The SPB of *S. cerevisiae* is a multilayered cylinder embedded in the nuclear envelope (Bullitt et al., 1997) and the Tub4p locates at both cytoplasmic and nuclear sides (Knop and Schiebel, 1998). In animal cells, two centrioles in the centrosome is suggested to be surrounded by an intricate lattice structure containing pericentrin (Dictenberg et al., 1998). The  $\gamma$ -tubulin is localized in the cytoplasm as the form of the  $\gamma$ -tubulin ring complex ( $\gamma$ -TuRC) which is recruited to the centrosome scaffolds and acts as an active microtubule-nucleating unit (Stearns and Kirschner, 1994; Zheng et al., 1995; Moritz et al., 1995a,b). Thus, the centrosome is comprised of the materials, such as  $\gamma$ -TuRC, which can be removed by salt, and the salt-stripped scaffolds possessing a lattice structure. The salt-stripped centrosome scaffolds recovers microtubule organizing potential when treated with high-speed oocyte extracts (Schnackenberg et al., 1998). Moritz et al. (1998) suggested that a factor in addition to the  $\gamma$ -TuRC is necessary for reassembly of the functional centrosomes. Such a factor could induce an ectopic microtubule nucleation like  $\gamma$ -tubulin (Shu and Joshi, 1995). Our results showed that RanBPM can induce an ectopic microtubule nucleation when overexpressed. The centrosome controls cell reproduction which is fundamental to the life. Such activity depends upon precise control of its own duplication (Kalt and Schliwa, 1993). The questions as to how many proteins comprise the centrosome and how the centrosome regulates cell division and its own duplication remain a mystery. Our present results suggest that RanBPM is a novel centrosomal protein and that Ran regulates the centrosomal function through RanBPM.

## Materials and Methods

### Cells and Cell Culture

The HeLa cell line is derived from human uterine cervical carcinoma, and MRC5 is a primary human cell culture. COS7 cells expressing the SV-40 early gene are derived from a green monkey cell line CV1 (Gluzman, 1981). CHO-k1 cells are derived from the Chinese hamster. Cells were cultured in Dulbecco's modified Eagle's medium (DME) (NIPRO Hymedium; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma Chemical Co.) in a humidified atmosphere containing 10% CO<sub>2</sub>.

Cells on glass coverslips cultured in dishes (35-mm-diam) were transfected with 1  $\mu$ g of plasmid DNA using the Lipofectamine protocol (GIBCO BRL, Gaithersburg, MD). The efficiency of transfection was usually ~5%.

### Construction of RanBPM Plasmids

1.5 kb of human *RanBPM*-ORF carried in the plasmid containing *RanBPM* cDNA cloned was amplified by PCR using as the 5' primer: 5' AAGGTCGACACATGAATAGACTACCAGGTTGG 3', and as the 3' primer: 5' CGCAAGCTTTTCAAATCAGCAGAGCTAGTC 3'. The resultant DNA fragment was digested with the restriction enzymes SalI and HindIII, and inserted into the SalI/HindIII sites of pET28, resulting

in pET28-RanBPM, which express RanBPM tagged with T7 at the NH<sub>2</sub> terminus.

pcDEB-T7-RanBPM: 1.7 kb of *T7-RanBPM* was excised from pET28-RanBPM with the restriction enzymes XbaI and HindIII, then inserted into the XbaI and HindIII sites of the pcDEB vector (Hayakawa et al., 1990) which had been digested with XbaI and HindIII enzymes, resulting in pcDEB-T7-RanBPM.

pAS404-RanBPM: 1.5 kb of *RanBPM* containing the open reading frame (ORF) was amplified by PCR using as the primers, 5' AGGGTC-GACCATGAATAGACTACCAGGTTGG 3', and 5' CGCAAGCTT-TTCAAATCAGCAGAGCTAGTC 3', digested with the HincII enzyme, and then inserted into the SmaI site of the pAS404 vector derived from pAS1 (Durfee et al., 1993).

pACTII-RanG19V/pACTII-RanT24N: 1.3 kb of the fragments containing *Ran* were excised with the restriction enzymes NcoI and BamHI from pET8c-RanG19V and pET8c-RanT24N (Dasso et al., 1994), and inserted into the NcoI and BamHI sites of pACTII (Durfee et al., 1993).

### 5' Rapid Amplification of cDNA Ends

To amplify the 5' end of RanBPM cDNA, three kinds of the primers consisting of the RanBPM nucleotides 186–212, 219–245, or 907–936 were prepared based on the nucleotide sequence of RanBPM (GenBank/EMBL/DBJ accession number AB008515). Using these primers, human cDNAs were amplified either from mRNA-isolated HeLa cells by AmpliFINDER RACE kit (Clontech, Palo Alto, CA) and then by TaKaRa EX Taq (TaKaRa, Otsu City, Shiga, Japan) or from Human Burkitt Lymphoma cDNA library with Marathon-Ready cDNA kit (Clontech) and then by TaKaRa LA Taq (TaKaRa).

### Northern Analysis

RNA filters were prepared as described previously (Yokoyama et al., 1995) and prehybridized with 100 µg/ml of salmon sperm DNA at 42°C for 2 h in buffer containing 0.5% SDS, 50% formamide, 5× SSPE (0.15 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 1 mM EDTA), 5× Denhardt's solution, and then incubated with <sup>32</sup>P-labeled cDNA for 24–48 h. After hybridization, the filters were washed in the following manner: twice in 2× SSPE plus 0.5% SDS for 30 min at room temperature, twice in 0.1× SSPE plus 0.5% SDS for 30 min at 70°C, and then once in 2× SSPE at room temperature. Finally, filters were dried and subjected to autoradiography.

### Preparation of Anti-RanBPM

The antibodies to RanBPM, against the peptide FDIEDYMREWRTKIQ were prepared in the rabbit as described (Nakashima et al., 1993) and then affinity purified by using antigenic peptide-coupled Sepharose columns.

### Indirect Immunofluorescence Microscopy

Cells on coverslips were washed for 15 s at 37°C with microtubule stabilization buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, 4 M glycerol, and 1 mM GTP) as described (Shu and Joshi, 1995), incubated for 1 min at 37°C in the same buffer containing 0.5% Triton X-100, rinsed with microtubule stabilization buffer, and then plunged into methanol at –20°C for 5 min. After fixation, cells were rehydrated in PBS and doubly stained with the primary antibodies; the mAb to T7-tag (Novagen, Madison, WI), α-tubulin (N356; Amersham Pharmacia Biotech, Piscataway, NJ) or γ-tubulin (T6557; Sigma Chemical Co.), and with rabbit affinity-purified anti-γ-tubulin antibodies (Masuda et al., 1996) or RanBPM. After staining, the antibodies were diluted with PBS-T (PBS containing 0.1% Tween 20) for 1 h at room temperature. After rinsing with PBS-T, cells were stained for 45 min at room temperature with the secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (AMI3408; Biosource) or anti-rabbit IgG (AL13408; Biosource, Camarillo, CA), Texas red-conjugated goat anti-rabbit IgG (55675; Cappel, Malvern, PA), or anti-mouse IgG (N2031; Amersham). Cells were finally stained with Hoechst 33342 and then mounted on Vectashield (Vector, Burlingame, CA).

### Microscopy and Image Analysis

Zeiss Axio Photo (Carl Zeiss Inc., Thornwood, NY) was used with the standard microscopic method. Digital imaging of stained cells was obtained using the laser-scanning microscope, Zeiss LSM 310 (Carl Zeiss Inc.) or Olympus LSM-GB200 system (Tokyo, Japan), and printed by pic-

trography 3000 (Fujix Tokyo, Japan) through Adobe Photoshop™ 3.0J (Adobe Systems Inc., San Jose, CA).

### Isolation of Centrosomes

Isolation of centrosomes from HeLa cells and CHO cells was carried out in accordance with previous method (Bornens et al., 1987) but with some minor modifications. Cultured HeLa or CHO cells were incubated with 10 µg/ml nocodazole and 5 µg/ml cytochalasin B for 2 h, rinsed with isolation buffer (1 mM Tris, pH 8, 0.5 mM EGTA, 0.1% β-mercaptoethanol), and then lysed by swaying the dishes in isolation buffer containing 0.5% NP-40, at 4°C for 10 min. Next, a one-fiftieth volume of PE buffer (0.5 M Pipes, pH 7.2, 0.1 M EGTA) was added to the extract which was then subjected to the discontinuous sucrose density gradient set in an SRP28-SA tube (Hitachi, Tokyo, Japan) with 3.5 ml of 60% sucrose (wt/wt), 3.5 ml of 40% sucrose (wt/wt) prepared in 20 mM Pipes, pH 6.8, 0.5 mM MgCl<sub>2</sub>, 1 mM EGTA and 0.1% β-mercaptoethanol, and run at 14,500 rpm for 1 h. Fractions were collected from the bottom and analyzed for microtubule nucleation ability as follows, according to Mitchison and Kirschner (1984).

1 µl of each fraction was incubated with 12.5 µl of tubulin solution (40 mM tubulin of porcine brain, 80 mM Pipes, pH 6.8, 1 mM MgCl<sub>2</sub>, 1 mM EGTA and 1 mM GTP) at 37°C for 10 min. Microtubules were fixed by adding glutaraldehyde, sedimented onto poly-L-lysine-coated coverslips, and then subjected to immunofluorescence. Using Zeiss Axio Photo (Carl Zeiss Inc.), the aster possessing more than 10 microtubules was counted. We counted the total number of the asters formed on a coverslip three times. The mean value and the standard deviation (SD) of the obtained numbers are shown in the text.

### Preparation of Ran and Its Derivatives

*E. coli*-produced wild-type and mutated Ran proteins were prepared as described (Dasso et al., 1994).

### Immunoblotting Analysis

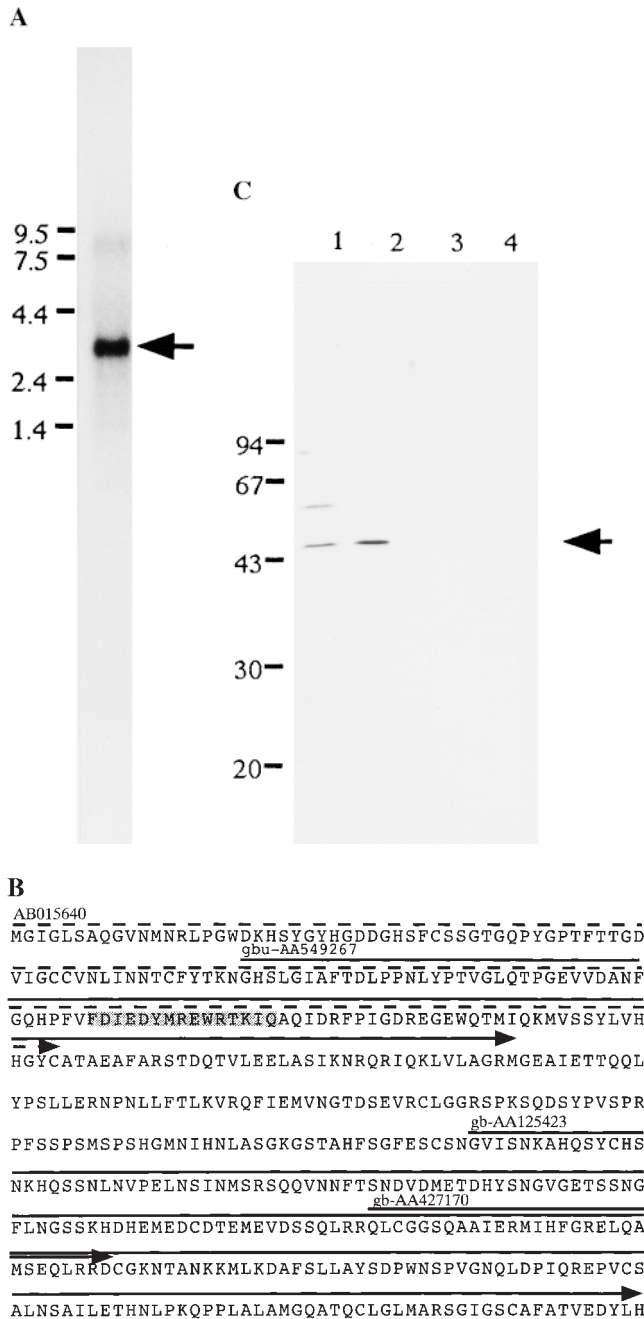
Cells were lysed in buffer containing 62.5 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% (wt/vol) SDS, and 10% glycerol. Cellular proteins were electrophoresed in an SDS-12% polyacrylamide slab gel, transferred to a polyvinylene difluoride (PVDF) membrane and probed with antibodies as described previously (Nakashima et al., 1993).

## Results

### Isolation and Identification of RanBPM

The human cDNA clone *B8* was isolated by the two-hybrid method using human Ran as a bait (Yokoyama et al., 1995). Northern analysis using the *B8* clone as a probe revealed the presence of a 3.1-kb mRNA in human HeLa cells (Fig. 1 A). Subsequently, the human cDNA library was screened using the cDNA clone *B8* as the probe. Finally, the cDNA clone of 2.8 kb, whose nucleotide sequence has been deposited as GenBank/EMBL/DBJ accession number AB008515, was isolated. Since there was no stop codon at the site upstream from the first methionine, we repeatedly carried out 5' RACE using as primers the nucleotides localized either near the 5' end or the middle of the *RanBPM* cDNA. But there was no cDNA clones extended from the 5' end of *RanBPM* cDNA. We concluded the ORF of RanBPM (Fig. 1 B) to consist of 500 amino acid residues, encoding a protein with a calculated molecular mass of 55,079 Da.

To identify the protein encoded by the isolated cDNA clone, the antibody was prepared against the peptide of the putative ORF highlighted in Fig. 1 B, shaded box. The obtained serum was purified by the affinity column to the peptide used as the antigen. Total cell extracts were prepared from cultures of human HeLa and MRC5 cell lines,



**Figure 1.** Identification of human RanBPM. (A) Northern analysis. Poly (A)<sup>+</sup> RNA (2  $\mu$ g/lane) extracted from HeLa cells was electrophoresed in 1.4% agarose gel and analyzed, using as a probe the <sup>32</sup>P-labeled *RanBPM* cDNA fragment as described in Materials and Methods. Arrow, 3.1 kb of *RanBPM* mRNA. RNA size markers are given in kilobases (kb). (B) Amino acid sequence. The amino acid sequence of human *RanBPM* was deduced from the nucleotide sequence of human *RanBPM* cDNA deposited under GenBank/EMBL/DDBJ accession number AB008515. Solid black arrows, amino acid sequences deduced from the deposited nucleotide sequences of mouse cDNA fragments, the GenBank/EMBL/DDBJ accession numbers of which are gbu-AA549267, gb-AA125423, and gb-AA427170, respectively. Dotted arrows, amino acid sequence deduced from cloned hamster *RanBPM* cDNA, the nucleotide sequence of which has been deposited under GenBank/EMBL/DDBJ accession number AB015640. Shaded block, position of peptides used to raise the anti-*RanBPM* antibodies. (C) Immunoblotting analysis. Total

and then subjected to immunoblotting analysis using the affinity-purified antibodies. In both cell extracts, a major band of protein with a molecular mass of 55 kD that is consistent with the molecular mass calculated based on the putative ORF, was specifically recognized (Fig. 1 C). The higher bands of 57 kD which were also specifically recognized by the antibodies (Fig. 1 C, compare lanes 1 with 3), could in fact be modified forms of the protein encoded by the putative ORF or other proteins cross-reactive to the antibodies. Although the absence of in-frame termination codons upstream allows for the possibility of a small extension at the NH<sub>2</sub> terminus, we concluded that the putative ORF encodes a predicted protein of 55 kD, designated as *RanBPM*.

### *RanBPM Is Well Conserved through Evolution*

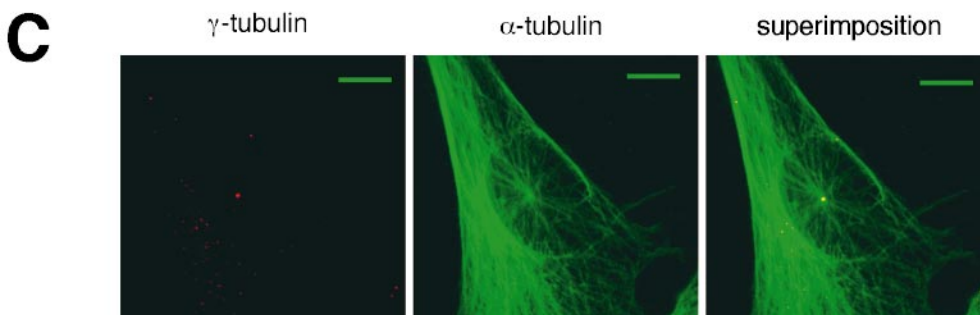
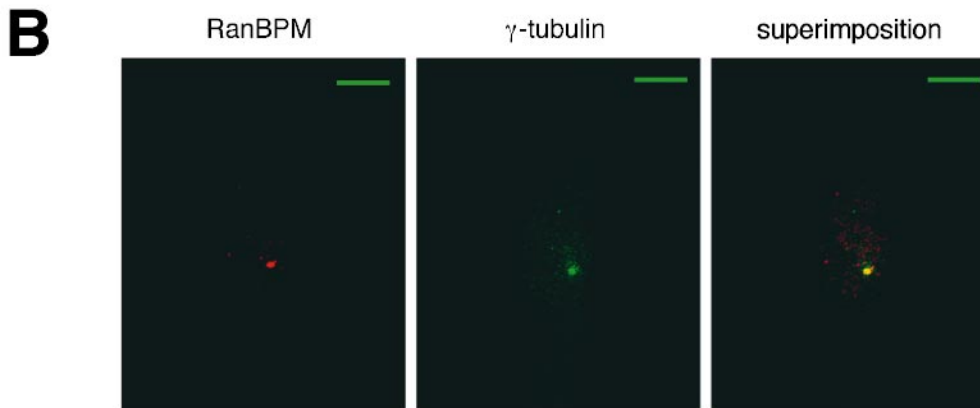
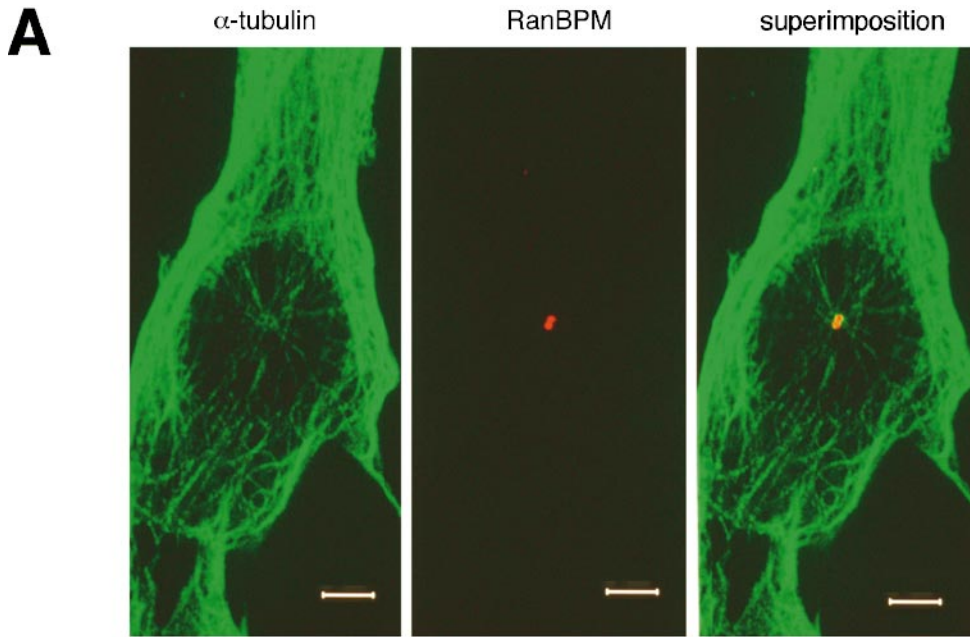
By homology search, several mouse cDNA fragments, the accession numbers of which are shown in Fig. 1 B, were found to encode a section of *RanBPM*. The amino acid sequences deduced from these mouse cDNA fragments are 100% identical to those deduced from human *RanBPM*, indicating that *RanBPM* is well conserved among mammals. To confirm this issue, the library of hamster cDNA was screened for *RanBPM* cDNA. The isolated cDNA fragment encoded a polypeptide that is identical to human *RanBPM* (Fig. 1 B, dotted arrows).

By BLAST search, we found that the COOH-terminal half of the *S. cerevisiae* ORF; YGL227w, is highly homologous to the human *RanBPM*. Percent amino acid identity of *RanBPM* with YGL227w is 29.8% and the probability that such sequence similarity is realized by chance is less than 10<sup>-13</sup>, when calculated as described (Toh et al., 1983). In particular, the amino acid sequence of *RanBPM* (1-78) is homologous to the SPRY domain found in several proteins, although its function remains unknown (Schultz and Bork, 1997).

### *Cellular Localization of RanBPM*

Immunoblotting analysis using the affinity-purified anti-*RanBPM* antibodies showed that whereas several protein bands were recognized in the extracts of HeLa cells, only a single band of 55 kD was recognized in the extracts of MRC5 cells. To determine the cellular localization of *RanBPM*, cultures of MRC5 cells were doubly stained with the affinity-purified anti-*RanBPM* antibodies (*red*) and the mAb to  $\alpha$ -tubulin (*green*) (Fig. 2 A). When both staining patterns overlapped, the microtubule was found to be nucleated from the matrix which was stained with the affinity-purified anti-*RanBPM* antibodies. These results suggested that the protein encoded by the *B8* clone was localized within the centrosome, and thereby *RanBPM* stands for Ran-binding protein in MTOC.

cell extract of HeLa cells (lanes 1 and 3) and MRC5 cells (lanes 2 and 4) was analyzed by 12% SDS-PAGE and then transferred to a PVDF membrane. The membrane was probed with affinity-purified anti-*RanBPM* antibodies in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of the peptide used for the immunization.



**Figure 2.** Cellular localization of RanBPM. (A) Non-synchronized cultures of MRC5 cells were fixed and permeabilized as described in Materials and Methods. Cells were double stained with the mAb to  $\alpha$ -tubulin (green) and with the affinity-purified anti-RanBPM antibodies (red). Red and green staining patterns were superimposed by electronic image processing (superimposition). The representative figures are shown. Bars, 5  $\mu$ m. (B and C) Non-synchronized cultures of MRC5 cells were fixed and doubly stained with affinity-purified anti-RanBPM antibodies (red) and the mAb to  $\gamma$ -tubulin (green) (B), or with affinity-purified anti- $\gamma$ -tubulin antibodies (red) and the mAb to  $\alpha$ -tubulin (green) (C). Red and green staining patterns were superimposed by electronic image processing (superimposition). Cells within the same visual field are demonstrated by antibody staining and phase-contrast. Bars, 10  $\mu$ m.

To confirm that RanBPM was localized in the centrosome, cultures of MRC5 cells were doubly stained by the affinity-purified anti-RanBPM antibodies (red) and by the mAb to  $\gamma$ -tubulin (green) (Fig. 2 B). When superimposed, the color changed to yellow, thereby revealing that RanBPM was colocalized with  $\gamma$ -tubulin, one of the important centrosomal residents (Oakley and Oakley, 1989;

Kalt and Schliwa, 1993; Kellogg et al., 1994). To confirm that the  $\gamma$ -tubulin stained in MRC5 cells was located within the centrosome, cells were double stained by the affinity-purified  $\gamma$ -tubulin antibodies (red) (Masuda et al., 1996) and the mAb to  $\alpha$ -tubulin (green) (Fig. 2 C). When superimposed, the affinity-purified  $\gamma$ -tubulin antibodies stained the central matrix of the microtubule network, as



expected. In mitotic HeLa cells, the area corresponding to the centrosome was also stained by the affinity-purified anti-RanBPM antibodies (data not shown).

Based on these staining results, we concluded that RanBPM was localized within the centrosome throughout the cell cycle, similar to  $\gamma$ -tubulin.

### Overexpression of Human RanBPM cDNA Causes Ectopic Nucleation of Microtubules In Vivo

To determine the biological function of RanBPM, we overexpressed *RanBPM* cDNA in COS cells and examined its effect on the microtubule network. *T7-fused RanBPM* cDNA carried on the pcDEB vector was introduced into COS cells as described in Materials and Methods. 36 h later, transfected cells were fixed and doubly stained by the affinity-purified anti-RanBPM antibodies (*red*) and by the mAb to  $\alpha$ -tubulin (*green*). In contrast to cells transfected with the vector alone (Fig. 3 A, panel *c*), a normal radial network of microtubules was broken in cells transfected with *T7-RanBPM* cDNA (the representative figures are shown in Fig. 3 A, panels *a* and *b*). When transfected cells were doubly stained by the mAb to the T7-tag (*green*) and the affinity purified anti- $\gamma$ -tubulin antibodies (*red*), RanBPM and  $\gamma$ -tubulin were found to be distributed as spots throughout the cytoplasm (Fig. 3 B). Both staining spots were colocalized when superimposed (Fig. 3 B, *superimposition*), indicating that there is some type of interaction between  $\gamma$ -tubulin and RanBPM. We thought that upon overexpression of T7-RanBPM,  $\gamma$ -tubulin was recruited onto RanBPM, resulting in reorganization of the microtubule network similar to the case of overexpressed

$\gamma$ -tubulin (Shu and Joshi, 1995). To confirm this issue, we monitored the recovery of microtubules after complete disassembly induced by lowering the temperature to 0°C as described (Joshi et al., 1992; Shu and Joshi, 1995). *RanBPM* cDNA or, as a control, the vector alone, was transfected into COS cells. To avoid rapid cell death, we transfected a smaller amount of *RanBPM* cDNA per cell (10 ng/dish), compared with the experiment described above (1  $\mu$ g/dish). Under this condition, the average number of RanBPM-staining spots was  $\sim$ 3–5 per cell. 24 h later, transfected cells were placed on ice for 1 h. Cells were then incubated in fresh medium at 30°C for varying time periods ranging from 0 to 5 min, lysed to remove free tubulin and then fixed to visualize the initiation of microtubule assembly sites in the green channel and RanBPM in the red channel by double immunofluorescence microscopy (Fig. 4). In cells transfected by the vector alone, after return to 30°C, short microtubules emerged from a single RanBPM-stained spot and became progressively elongated as previously reported (Shu and Joshi, 1995). In contrast to cells transfected by the vector alone, multiple RanBPM-stained spots appeared in cells transfected with *T7-tagged RanBPM* cDNA (Fig. 4). After incubation at 30°C for 1 min,  $\alpha$ -tubulin gathered around multiple RanBPM-stained spots. Subsequently, short microtubules emerged from the multiple RanBPM-stained spots, although the microtubules were shorter than the microtubules that emerged from the centrosome of the untransfected cells. The number of ectopically nucleated microtubules was the same as the number of RanBPM-stained spots. Thus, we concluded that overexpressed T7-tagged RanBPM caused ectopic nucleation of the microtubule assembly in vivo.

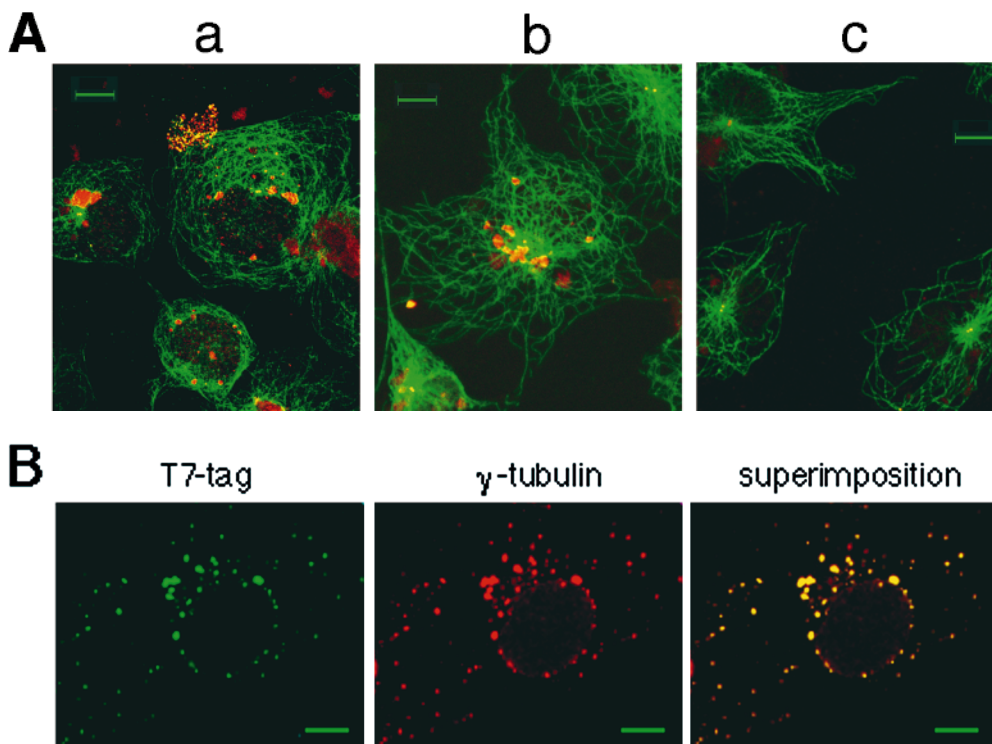
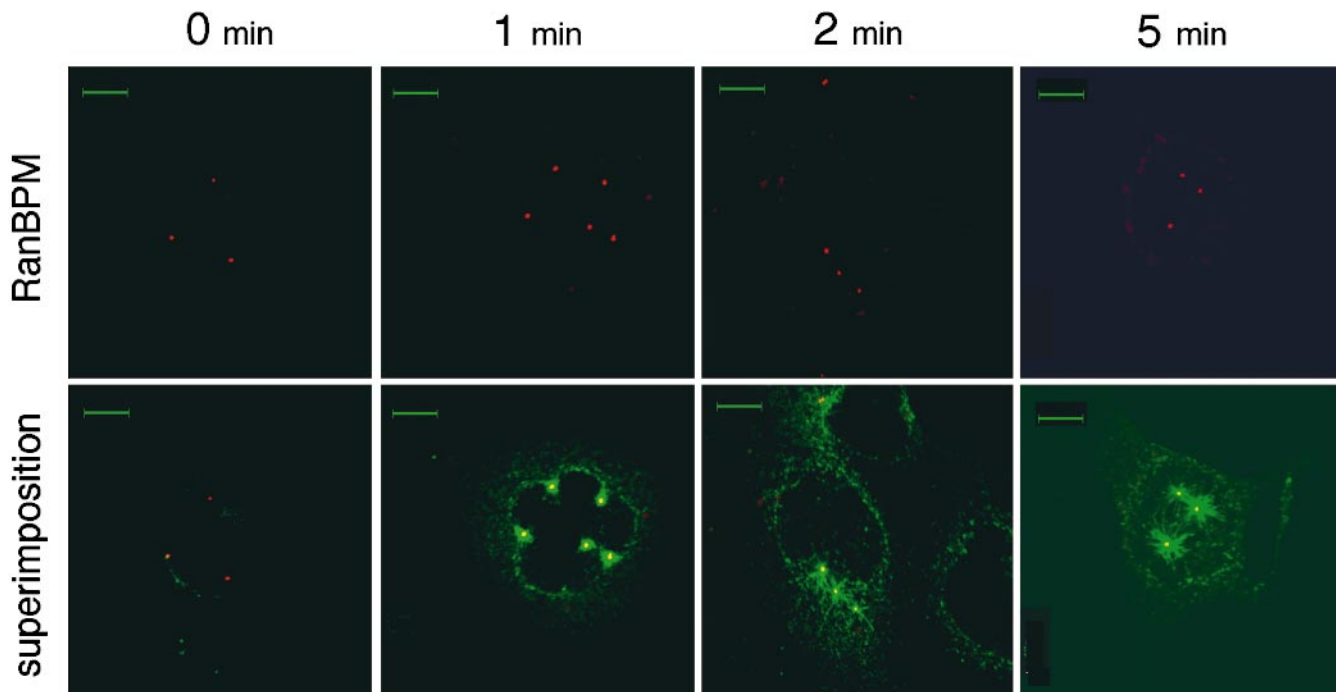


Figure 3. Reorganization of microtubule network in COS cells transfected with *RanBPM* cDNA. (A) Cultures of COS cells were transfected with 1  $\mu$ g of either pcDEB-T7-RanBPM (panels *a* and *b*) or pcDEB vector alone (panel *c*) per dish (35-mm-diam). 36 h later, cells were fixed and double stained with the mAb to  $\alpha$ -tubulin (*green*) and affinity-purified anti-RanBPM antibodies (*red*). The representative figures of cells which were superimposed are shown. (B) COS cells transfected with pcDEB-T7-RanBPM were doubly stained with the mAb to T7 (*green*) and affinity-purified anti- $\gamma$ -tubulin antibodies (*red*). Both staining patterns were superimposed by electronic image processing (*superimposition*). Bars, 10  $\mu$ m.



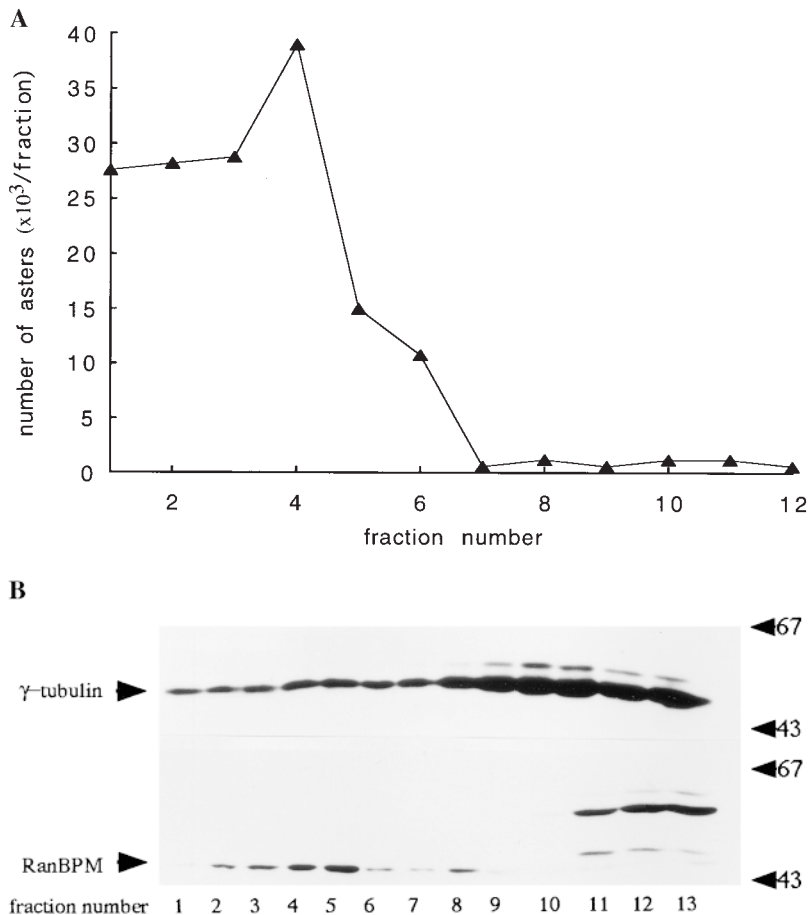
**Figure 4.** Ectopic microtubule nucleation in COS cells transfected with *RanBPM* cDNA. Cultures of COS cells growing on the coverslip were transfected with 10 ng of pcDEB-T7–*RanBPM* per dishes (35-mm-diam). 24 h later, transfected cells were incubated on ice for 1 h and then either processed immediately for immunofluorescence (0 time point of recovery), or else the cold medium was replaced with warm (30°C) medium. Coverslips were harvested for immunofluorescence at 1, 2, or 5 min after medium change as indicated. Cells were fixed and stained with the affinity-purified anti-*RanBPM* antibodies (*red*) and the mAb to  $\alpha$ -tubulin (*green*). Both staining patterns were superimposed by electronic image processing (*superimposition*). Bars, 10  $\mu$ m.

### *RanBPM* Is Localized within the Central Part of the Microtubule Asters

We then determined the relationship between *RanBPM* and microtubule nucleation, using the isolated centrosome. To achieve this, the centrosome extracts were prepared from HeLa cells by sucrose-density gradient centrifugation as described (Mitchison and Kirschner, 1984; Bornes et al., 1987). Fractions containing the centrosomes were determined by microtubule nucleation ability (Fig. 5 A). Immunoblotting analysis revealed that both *RanBPM* and  $\gamma$ -tubulin were cosedimented with the centrosome fractions (Fig. 5 B). The protein bands higher than 55 kD which were fractionated into the top fractions were recognized by the affinity-purified anti-*RanBPM* antibodies. The major band of these corresponds to the band of 57 kD recognized in the total extract of HeLa cells (Fig. 1 C). We do not know whether they are modified forms of *RanBPM* or proteins cross-reactive to the affinity-purified anti-*RanBPM* antibodies as mentioned above. These proteins were concentrated into the top fractions, suggesting that they were localized in the cytoplasm. Similarly, a majority of  $\gamma$ -tubulin was fractionated into the top fraction, being consistent with the previous report that the majority of  $\gamma$ -tubulin is localized in the cytoplasm (Stearns and Kirschner, 1994).

To determine the localization of *RanBPM* in the microtubule asters assembled *in vitro*, the asters were doubly

stained by the mAb to  $\alpha$ -tubulin (*green*) and by the affinity-purified anti-*RanBPM* antibodies (*red*). As expected from the *in vivo* results, *RanBPM* was localized at the central part of the microtubule asters (Fig. 6) where  $\gamma$ -tubulin was also localized (see Fig. 9). These results are consistent with the notion that *RanBPM* is one of the centrosomal components which is involved in microtubule nucleation. To address this issue, the centrosome fractions were preincubated with the affinity-purified anti-*RanBPM* antibodies, and then assayed for aster formation. Although the preimmune IgG had no effect on aster formation, the number of microtubules nucleated by the centrosome was greatly reduced by addition of anti-*RanBPM* antibodies (Fig. 7). In a control reaction mixture containing the preimmune IgG or the buffer alone, the total number of asters formed on a coverslip was 1442 (SD = 27.0) and 1489 (SD = 51.1), respectively. In contrast, it was 151 (SD = 10.1) in the presence of anti-*RanBPM* antibodies. Thus, the aster-forming ability of the centrosome fractions was reduced to about 10% of the buffer alone by the addition of the affinity-purified anti-*RanBPM* antibodies. Under the same conditions, the polymerization of microtubules without centrosome fractions was not inhibited by the affinity-purified anti-*RanBPM* antibodies (data not shown), this being consistent with the result showing that the length of the microtubules was not reduced by the addition of anti-*RanBPM* antibodies (Fig. 7).



**Figure 5.** Cosedimentation of RanBPM with the centrosomes. The centrosome extracts prepared from HeLa cells ( $2.5 \times 10^7$  cells) which had been incubated in the presence of nocodazole ( $10 \mu\text{g/ml}$ ) and cytochalasin B ( $5 \mu\text{g/ml}$ ) for 2 h, as described in Materials and Methods, were set into a tube for SRP28-SA rotor (Hitachi) with 3.5 ml of 60% and 3.5 ml of 40% sucrose to be fractionated by discontinuous sucrose-density gradient centrifugation at 14,500 rpm for 1 h. Fractions collected from the bottom upwards were analyzed for the ability to form microtubule asters (A), and for the presence of RanBPM and  $\gamma$ -tubulin by immunoblotting using the antibodies to RanBPM and the mAb to  $\gamma$ -tubulin (B). On the horizontal axis, the fraction numbers from the bottom to the top are shown.

### Nonhydrolyzable GTP-Ran Inhibits Microtubule Nucleation

Human *RanBPM* cDNA was isolated by the two-hybrid method using human Ran as a bait (Yokoyama et al., 1995). To confirm whether or not RanBPM interacts specifically with GTP-Ran, mutant forms of Ran, G19V-Ran, and T24N-Ran were prepared as described (Dasso et al., 1994; Kornbluth et al., 1994). The amino acid residues 19 and 24 of Ran were conserved between *ras* and Ran. By analogy of *ras*, G19V-Ran is thought to be locked in an activated state through inability to hydrolyze GTP (McGrath et al., 1984) and T24N-Ran is thought to remain predominantly in a GDP-bound form since the corresponding mutation in *ras*<sup>H</sup> have a profoundly decreased affinity for GTP in vitro (Feig and Cooper, 1988). Indeed, G19V-Ran and T24N-Ran preferentially binds to GTP and GDP, respectively (Kornbluth et al., 1994; Lounsbury et al., 1996).

The cDNA of G19V-Ran and T24N-Ran, both of which were fused in frame with the GAL4-activation domain of pACT (Durfee et al., 1993), were introduced into cultures of the strain Y190 [pAS404-RanBPM]. Transformants were selected in synthetic medium lacking leucine and tryptophan, and plated on synthetic medium either lacking histidine, tryptophan, and leucine but containing 10 mM of 3-aminotriazole (+) or lacking tryptophan, leucine and

3-aminotriazole (-). In the presence of 3-aminotriazole, the strain Y190 [pAS404-RanBPM, pACT-G19VRan] papillated, whereas the strain Y190 [pAS404-RanBPM, pACT-T24NRan] did not (Fig. 8). This result indicates that RanBPM specifically interacts with GTP-Ran.

Based on the above results, we then determined the effects of Ran on microtubule aster formation. The centrosome fractions were preincubated either with GTP-, GTP $\gamma$ S-, or GDP-bound Ran, or with G19V-Ran, and were then assayed for aster formation by the addition of tubulin. The total number of asters formed on a coverslip was 702 (SD = 69.2) without the Ran preparation. By addition of either GTP $\gamma$ S-Ran or G19V, it was reduced to 53 (SD = 7.5) and 56 (SD = 5.2), respectively. On the other hand, in the presence of GTP-Ran or GDP-Ran, the total number of asters was 650 (SD = 26.0) and 592 (SD = 3.7), respectively. Thus, the ability of the centrosome to nucleate microtubules was significantly reduced by addition of GTP $\gamma$ S-bound Ran and G19V-Ran as shown in Fig. 9. By immunoblotting analysis, we found that the centrosome fractions contained a considerable amount of RanGAP1, but no RCC1 (data not shown). Therefore, GTP $\gamma$ S-bound Ran, which can not be hydrolyzed, should remain stable in the reaction mixture.

In the presence of GTP $\gamma$ S-Ran, the central part of the microtubule asters which was stained by both the affinity-



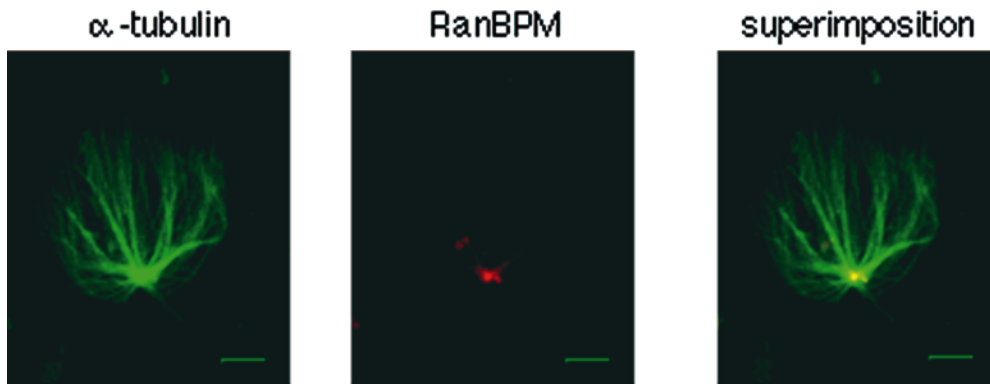


Figure 6. Localization of RanBPM in the microtubule asters. In vitro nucleated microtubules were spun down, fixed, and then double stained with the mAb to  $\alpha$ -tubulin (green) and the antibodies to RanBPM (red) as indicated before being superimposed. Bars, 5  $\mu$ m.

purified anti-RanBPM antibodies and the mAb to  $\gamma$ -tubulin significantly faded, compared with the case of buffer alone (Fig. 9). However, the length of the microtubules was not reduced, and the nonhydrolyzable GTP-Ran seemed to inhibit the nucleation of microtubule assembly, similar to the anti-RanBPM antibodies. Indeed, the microtubule elongation was not inhibited by GTP $\gamma$ S-Ran or G19V-Ran in the absence of the centrosome fractions (data not shown). It is notable that the central part of the microtubule asters did not fade when the anti-RanBPM antibodies were added (Fig. 7). The mechanism for inhibiting microtubule-aster formation, therefore, seemed to be dif-

ferent between the anti-RanBPM antibodies and the non-hydrolyzable GTP-Ran.

### Discussion

RanBPM was most frequently isolated by two-hybrid screening of the human cDNA library using human Ran as a bait (Yokoyama et al., 1995). 38 out of a total of 80 clones isolated encode RanBPM. In the same screening, several fragments of *RanBP2* cDNA which encode one of four Ran-binding domains of RanBP2 were isolated, revealing that our screening of Ran-binding proteins was functional. In this context, it is highly likely that RanBPM is yet another Ran-binding protein, although RanBPM has no Ran-binding domain similar to either that of RanBP1 (Coutavas et al., 1993) or that of importin  $\beta$  (Görllich et al., 1997).

The colocalization of RanBPM with  $\gamma$ -tubulin indicates that RanBPM is localized within the centrosome. The  $\gamma$ -TuRC purified from *Xenopus* egg extracts contains at least seven different proteins (Zheng et al., 1995). The proteins homologous to *S. cerevisiae* SPB components Spc97p and Spc98p have been identified in the  $\gamma$ -TuRC and are localized in the centrosome (Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998). Recently, Wigge et al. (1998) prepared a highly enriched spindle pole prep-

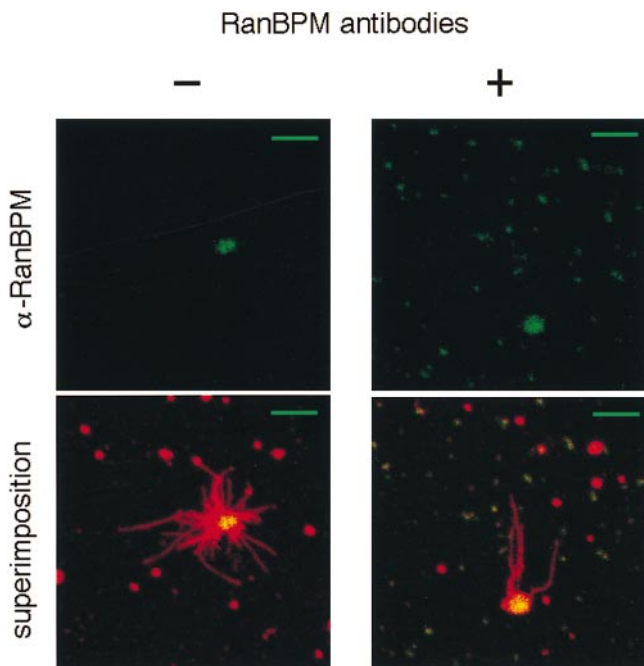


Figure 7. Inhibition of microtubule nucleation by the anti-RanBPM antibodies. 0.6  $\mu$ l of the centrosome fraction were preincubated with 2.1  $\mu$ g of the affinity-purified anti-RanBPM antibodies (+) or buffer alone (-) for 15 min at 37°C, and then tubulin was added to start the microtubule nucleation. The microtubule asters were spun down, fixed, and then double stained with affinity-purified anti-RanBPM antibodies (green) and the mAb to  $\alpha$ -tubulin (red). Both staining patterns were superimposed by electronic image processing (superimposition). Bars, 5  $\mu$ m.

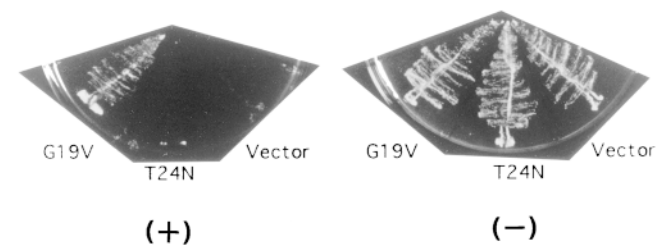
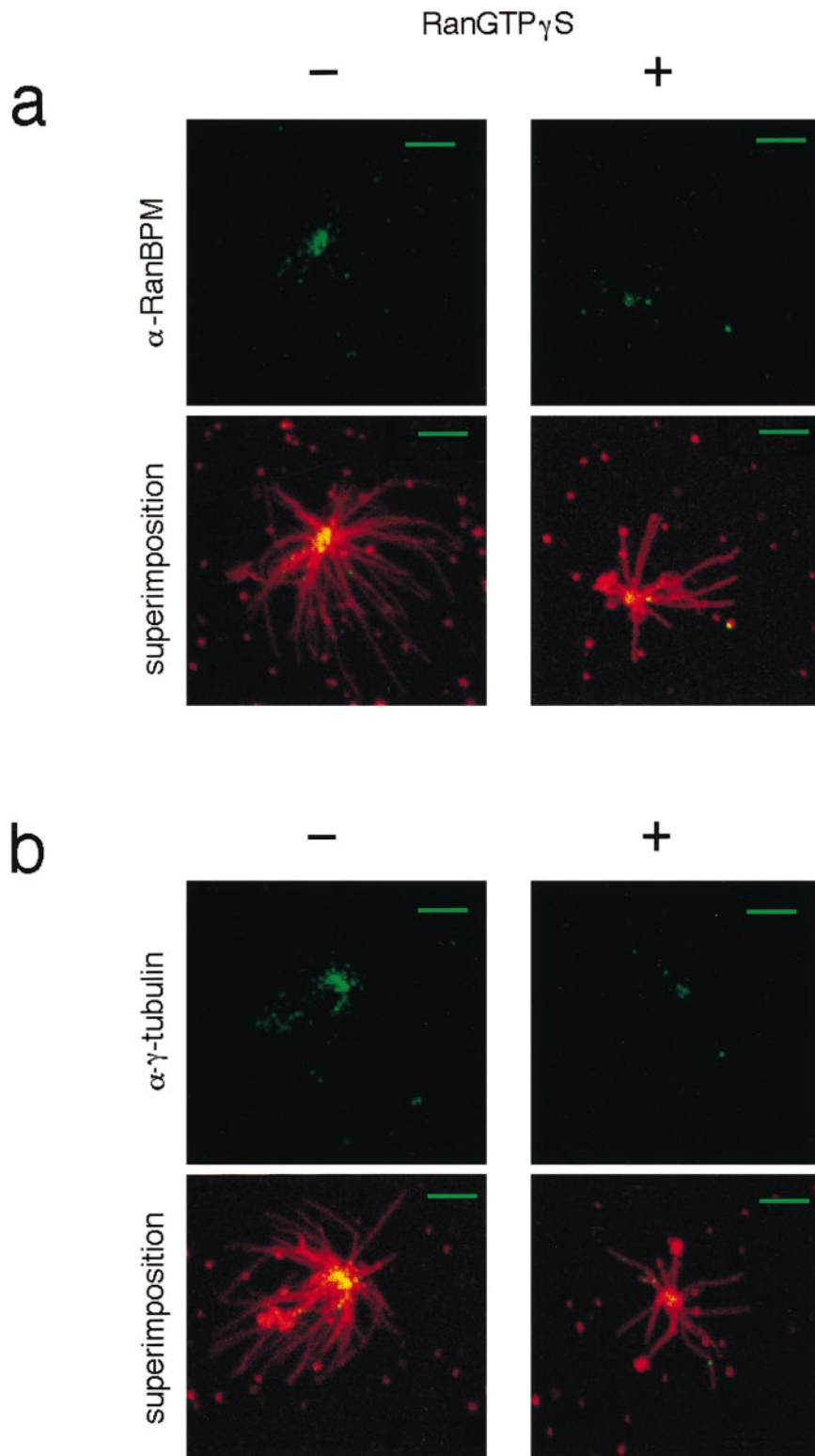


Figure 8. Interaction of RanBPM with GTP-Ran. pACTII carrying *G19V*- or *T24N*-Ran and a vacant vector were introduced into the yeast Y190 strain (*MAT $\alpha$  gal4 gal80 ade2 his3 trp1 leu2 URA3::GAL1-lacZ LYS2::GAL1-HIS3*) (Harper et al., 1993) bearing pAS404-RanBPM. Transformants were selected on synthetic medium lacking tryptophan and leucine, and plated on synthetic medium either lacking histidine, tryptophan and leucine, but in the presence of 3-aminotriazole (10 mM) (+), or lacking tryptophan, leucine and 3-aminotriazole (-).



*Figure 9.* Inhibition of microtubule nucleation by nonhydrolyzable GTP-Ran. 0.6  $\mu$ l of the centrosome fraction were preincubated with 480 pmol of GTP $\gamma$ S-Ran (+) or buffer alone (-) for 15 min at 37°C and then tubulin was added to start the microtubule nucleation. The microtubule asters were spun down, fixed, and then double stained with affinity-purified anti-RanBPM antibodies (*green*) and the mAb to  $\alpha$ -tubulin (*red*) (*a*) or affinity-purified  $\gamma$ -tubulin (*green*) and the mAb to  $\alpha$ -tubulin (*red*) (*b*). Both staining patterns were superimposed by electronic image processing (*superimposition*). Bars, 5  $\mu$ m.

aration and identified a total of 12 known and 11 novel components of the SPB. Among these, there was found no protein homologous to RanBPM, although we found a possible RanBPM homologue of *S. cerevisiae* ORF; YGL227w by Blast search. It remains to be investigated whether RanBPM directly interacts with  $\gamma$ -tubulin. How-

ever, as we discuss below, we demonstrated a functional interaction between RanBPM and  $\gamma$ -tubulin.

Overexpression of the cloned *RanBPM* cDNA causes both a reorganization of the microtubule network and an ectopic microtubule nucleation. These results indicate that the cloned RanBPM cDNA has the biological activity of

nucleating the microtubule assembly *in vivo*. The finding that overexpressed *RanBPM* cDNA causes ectopic microtubule nucleation is quite similar to the case of  $\gamma$ -tubulin reported previously (Shu and Joshi, 1995). Of great interest is that newly formed *RanBPM* spots were costained with the mAb to  $\gamma$ -tubulin. Although the mechanism for the ectopic microtubule nucleation has not been clarified, it may be possible that the overexpression of *RanBPM* activates  $\gamma$ -TuRC or recruits  $\gamma$ -tubulin to the centrosome scaffolds, resulting in microtubule nucleation.

The *in vitro* microtubule nucleation is inhibited not only by the addition of anti-*RanBPM* antibodies, but also by the addition of nonhydrolyzable GTP-Ran. The central part of the microtubule asters stained either by the mAb to  $\gamma$ -tubulin or by anti-*RanBPM* antibodies fades in the presence of nonhydrolyzable GTP $\gamma$ S-Ran or a dominant GTP-bound mutant of Ran, G19V-Ran (Kornbluth et al., 1994; Lounsbury et al., 1996). This is in contrast to the finding that upon addition of the anti-*RanBPM* antibodies, the central part of microtubule asters does not change in size. The anti-*RanBPM* antibodies may structurally hinder the microtubule assembly at the nucleation site. In contrast, it is unclear how the nonhydrolyzable GTP-Ran inhibits the nucleation of microtubule assembly. Recently, it has been reported that the higher-order organization of microtubule-nucleating sites is represented by a centrosomal lattice containing pericentrin and  $\gamma$ -tubulin (Dichtenberg et al., 1998). Therefore, one of possibilities is that the dynamic stability of the centrosomal lattice is regulated by Ran through *RanBPM*. The tight binding of nonhydrolyzable GTP-Ran to *RanBPM* may make the centrosomal lattice unstable, resulting in a shrinking of the central part. However, it could be also possible that GTP $\gamma$ S-Ran inhibits the aster formation independently from *RanBPM*. It is a very interesting question how Ran regulates the centrosome function.

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