The *Xenopus* TACC Homologue, Maskin, Functions in Mitotic Spindle Assembly[□]

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Submitted October 26, 2004; Revised March 9, 2005; Accepted March 16, 2005

Monitoring Editor: Trisha Davis

Maskin is the *Xenopus* homolog of the transforming acidic coiled coil (TACC)-family of microtubule and centrosome-interacting proteins. Members of this family share a ~200 amino acid coiled coil motif at their C-termini, but have only limited homology outside of this domain. In all species examined thus far, perturbations of TACC proteins lead to disruptions of cell cycle progression and/or embryonic lethality. In *Drosophila*, *Caenorhabditis elegans*, and humans, these disruptions have been attributed to mitotic spindle assembly defects, and the TACC proteins in these organisms are thought to function as structural components of the spindle. In contrast, cell division failure in early *Xenopus* embryo blastomeres has been attributed to a role of maskin in regulating the translation of, among others, cyclin B1 mRNA. In this study, we show that maskin, like other TACC proteins, plays a direct role in mitotic spindle assembly in *Xenopus* egg extracts and that this role is independent of cyclin B. Maskin immunodepletion and add-back experiments demonstrate that maskin, or a maskin-associated activity, is required for two distinct steps during spindle assembly in *Xenopus* egg extracts that can be distinguished by their response to “rescue” experiments. Defects in the “early” step, manifested by greatly reduced aster size during early time points in maskin-depleted extracts, can be rescued by readdition of purified full-length maskin. Moreover, defects in this step can also be rescued by addition of only the TACC-domain of maskin. In contrast, defects in the “late” step during spindle assembly, manifested by abnormal spindles at later time points, cannot be rescued by readdition of maskin. We show that maskin interacts with a number of proteins in egg extracts, including XMAP215, a known modulator of microtubule dynamics, and CPEB, a protein that is involved in translational regulation of important cell cycle regulators. Maskin depletion from egg extracts results in compromised microtubule asters and spindles and the mislocalization of XMAP215, but CPEB localization is unaffected. Together, these data suggest that in addition to its previously reported role as a translational regulator, maskin is also important for mitotic spindle assembly.

INTRODUCTION

The mitotic spindle is essential for the process of partitioning a complete set of chromosomes to each daughter cell during cell division. It consists of microtubules, microtubule-based motor proteins, and hundreds of other proteins that function together to produce the configurations and movements required for chromosome segregation (reviewed by Gadde and Heald, 2004; Kline-Smith and Walczak, 2004). Not surprisingly, mitotic spindle assembly is

highly regulated and requires the precise spatial and temporal coordination of many processes, including microtubule dynamics, attachment and alignment of chromosomes, and focusing of spindle poles (Compton, 2000). A detailed molecular understanding of the components of the mitotic spindle, their interactions, and their regulation is essential for our understanding of the molecular basis of cell division.

Extracts made from *Xenopus laevis* eggs (Lohka and Masui, 1983) have been used extensively for identifying and characterizing spindle assembly proteins. The extracts readily assemble spindles in vitro when supplemented with sperm chromatin (Lohka and Masui, 1983; Murray *et al.*, 1989; Sawin and Mitchison, 1991) or upon addition of RanGTP or RanGTP mimics (reviewed in Kahana and Cleveland, 1999; Dasso, 2002). Spindle assembly in egg extracts proceeds in at least three distinguishable stages: 1) microtubule asters form initially, which 2) rearrange to form “half-spindles,” and 3) two half-spindles fuse to form a bipolar spindle (Sawin and Mitchison, 1991). Although this pathway differs slightly from the events observed during early embryogenesis (Sawin and Mitchison, 1991), spindles assembled in this manner have been used successfully to assess the functions of various spindle assembly proteins (see reviews by Gadde and Heald, 2004, and Kline-Smith and Walczak, 2004).

Depending on the buffers used during extract preparation, *Xenopus* egg extracts can be made mitotic, interphasic, or made to cycle between these states (Murray, 1991). They can

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-10-0926>) on March 23, 2005.

□ The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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Abbreviations used: CPEB, cytoplasmic polyadenylation element binding protein; GST, glutathione S-transferase; MT, microtubule; TACC, transforming acidic coiled coil; XMAP, *Xenopus* microtubule associated protein.

be arrested in a mitotic state by the addition of a nondegradable truncated cyclin B protein, $\Delta 90$ cyclin, which is missing the first 90 amino acids including the "destruction box" (Murray *et al.*, 1989). This has the advantage that the results of perturbations of key spindle assembly components can be interpreted independently of their potential role(s) in cell cycle progression.

One such protein that has a role in cell cycle progression but that might also be important for mitotic spindle assembly is maskin. Maskin was initially identified in *Xenopus* oocyte extracts as a binding partner of the cytoplasmic polyadenylation element binding protein (CPEB), an important regulator of cytoplasmic polyadenylation of maternal mRNAs (Stebbins-Boaz *et al.*, 1999). Maskin is thought to repress translation by binding to CPEB until phosphorylation of CPEB by Aurora A/Eg2 disrupts their interaction and activates polyadenylation (Hodgman *et al.*, 2001; Mendez *et al.*, 2000).

Two lines of evidence support a role for maskin in cell cycle control. First, disrupting maskin in *Xenopus* early embryos by antibody injection blocks cell division in the injected blastomeres, and this is due at least in part to the failure to translate cyclin B mRNA near the mitotic spindle (Groisman *et al.*, 2000). Consistent with this, both CPEB and maskin are present on the mitotic apparatus of animal pole blastomeres in *Xenopus* embryos (Groisman *et al.*, 2000; reviewed in Richter and Theurkauf, 2001). Second, disruption of maskin by antibody addition, or by antisense RNA, in cycling *Xenopus* egg extracts blocked the extract in mitosis and prevented cycling to the next interphase (Groisman *et al.*, 2002). In these extracts, cyclin B1 concentration and H1 kinase activity remained high when maskin was disrupted. Thus, the failure to proceed to the next interphase could be attributed to the effect of maskin on the translation of cyclin B (Groisman *et al.*, 2002).

Analysis of the domain structure of maskin (Stebbins-Boaz *et al.*, 1999) revealed that it is a member of the highly conserved transforming acidic coiled coil (TACC) family of proteins (reviewed in Gergely, 2002; Still *et al.*, 2004). TACC family members share a ~200 amino acid coiled coil domain located at the C-terminus of the protein but show little homology outside of this domain (reviewed by Gergely, 2002). Members of the TACC family were originally identified in human cells, but TACC proteins are highly evolutionarily conserved (reviewed by Still *et al.*, 2004). The three human TACC homologues map closely to chromosomal translocation breakpoints that are associated with certain cancers (Still *et al.*, 1999a, 1999b; Chen *et al.*, 2000). Maskin, the only known TACC family member in *Xenopus*, is most closely related to human TACC protein 3 (hTACC3), a protein of unknown function that is highly expressed in various transformed cell lines (Pu *et al.*, 2001; Still *et al.*, 1999).

Recent studies suggest that members of the TACC family of proteins are centrosome- and microtubule-interacting proteins important for mitotic spindle assembly (Gergely *et al.* 2000a, 2000b; Cullen and Ohkura 2001; Lee *et al.*, 2001; Bellanger and Gönczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003). Changes in the structure and/or the expression of TACC proteins and TACC-interacting proteins may have profound consequences for cellular growth and survival (reviewed by Theurkauf, 2001 and Raff, 2002; Gergely *et al.*, 2000a; Bellanger and Gönczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003). TACC proteins in every species examined thus far interact with members of the ch-TOG protein family, and this interaction is important for mitotic spindle assembly (reviewed by Gergely, 2002). The involvement of TOG family members in mitotic spindle assembly is highly conserved

and well documented (Cassimeris and Skibbens, 2003; Gadde and Heald, 2004; Kline-Smith and Walczak, 2004). The founding member of the TOG family, *Xenopus* XMAP215 (Gard and Kirschner, 1987), is a microtubule associated protein that is required for microtubule assembly in *Xenopus* egg extracts (Wilde and Zheng, 1999; Tournebise *et al.*, 2000; Popov *et al.*, 2001, 2002). The human TOG protein was also recently implicated in focusing microtubule minus ends and in maintaining centrosome integrity (Cassimeris and Morabito, 2004). The importance for spindle assembly of the interaction between TACCs and TOGs is becoming clear from studies in *Drosophila* (Cullen and Ohkura, 2001; Lee *et al.*, 2001), *C. elegans* (Bellanger and Gönczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003), *Saccharomyces cerevisiae* (Wang and Huffaker, 1997; Chen *et al.*, 1998; Usui *et al.*, 2003), *S. pombe* (Garcia *et al.*, 2001; Sato *et al.*, 2004), and humans (Lauffart *et al.*, 2002; Conte *et al.*, 2003; Gergely *et al.*, 2003).

In this study, we show that the depletion of maskin from mitotic *Xenopus* egg extracts disrupts microtubule aster and spindle assembly. Maskin, or a maskin-associated activity, is required at two distinct steps during spindle assembly. Maskin interacts with XMAP215 and is required for the proper localization of XMAP215 in egg extracts. Maskin also interacts with CPEB in the extracts, but CPEB localization is unaffected by maskin depletion. Our results suggest that maskin plays a direct role in spindle assembly and that at least part of this role is mediated by its TACC domain.

MATERIALS AND METHODS

Buffers and Reagents

Antibody diluting solution (AbDil) Tris-buffered saline (TBS), 0.1% Triton, 2% bovine serum albumin, 0.1% sodium azide; BRB80: 80 mM K-PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8; Cleavage buffer: 20 mM Tris-HCl, 150 mM NaCl, 1 mM TCEP, pH 7.5; CSF-XB: 10 mM K-HEPES, pH 7.6, 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose, 5 mM EGTA; H100: 50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 100 mM NaCl, pH 7.6; H250: 50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 250 mM NaCl, pH 7.6; H500: 50 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 500 mM NaCl, pH 7.6; protease inhibitors: leupeptin, pepstatin, and chymostatin, 10 μ g/ml each (from a 10 mg/ml stock in dimethyl sulfoxide); TBS: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4; TBS-T: TBS, 0.1% Triton X-100; XB buffer: 10 mM K-HEPES, 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose, pH 7.6.

Construction of Maskin Expression Vectors

Full-length maskin cDNA was a generous gift from M. Sheets (UW-Madison). This cDNA was generated by PCR amplification of an expressed sequence tag (EST; IMAGE clone ID 5048343). Sequencing of this EST revealed five differences to the previously published protein sequence. These are: Y205S, Y300D, S399L, T736A, and R888A. Several independent ESTs in the database agree with our sequencing results, whereas others agree with the previously published sequence, and we speculate that the differences arose from polymorphisms in the population.

To generate a fusion protein with glutathione-S-transferase (GST), full-length maskin was subcloned into the EcoRI-site of the pGEX-4T2 vector (Amersham, Piscataway, NJ) modified to include the recognition site for the Tobacco Envelope Virus (TEV) protease, which allows the removal of the GST portion of the fusion protein following purification on GST-agarose by digestion with the rTEV protease (pGEX-4T2-rTEV). A GST fusion protein with the TACC domain of maskin (amino acids 714–931 of maskin) was generated using primers (5': CGGGATCCTCAACTTCTGATGCCATC; 3': CCGCTC-GAGTCAGATCTTCTCCATCTTTAAAT) that introduced 5' BamHI and 3' XhoI sites (underlined in the primer sequences, respectively) to amplify the desired region of maskin by PCR and subcloned into the BamHI/XhoI sites of the pGEX-6P2 vector. We also generated a "TACC-less" maskin (amino acids 1–713) using a similar approach. However, this construct was poorly expressed and insoluble.

Recombinant Protein Expression and Purification

The following fusion protein were expressed in *Escherichia coli* BL21 and purified by affinity chromatography using standard protocols for the respective tags: 6His-CPEB (a kind gift from M. Wickens), GST-RanL43E (described in Wilde *et al.*, 1999), GST-maskin, and GST-TACC domain (see above). All

purified proteins were concentrated to >2 mg/ml, dialyzed against XB, flash-frozen in liquid nitrogen in small aliquots, and stored at -80°C.

Antibodies

Polyclonal antibodies to maskin were generated in rabbits or chickens using purified, bacterially expressed recombinant full-length maskin fused to GST as antigen. Antibodies raised in either species gave identical results by Western blotting and immunofluorescence. Here, we report the results obtained using affinity-purified antibodies raised in rabbits, which were diluted 1:80,000 for Western blots and 1:50,000 for immunofluorescence. Anti-CPEB antibodies were raised in chickens against full-length 6-his-tagged CPEB (a kind gift from M. Wickens).

Other antibodies were as follows: mouse anti-Eg2 monoclonal antibodies (kind gift from C. Prigent), rabbit or guinea pig anti-CPEB antibodies (kind gift from L. Hake), polyclonal antibodies to XMAP215 (kind gift from Y. Zheng); monoclonal antibodies to α -tubulin (clone DM1 α), γ -tubulin (clone GTU88), FITC-conjugated anti-chicken antibodies, and alkaline phosphatase-linked secondary antibodies (for Western blots) were obtained from Sigma (St. Louis, MO). Alexa-488 and Alexa-594 anti-mouse or anti-rabbit secondary antibodies (for immunofluorescence) were purchased from Molecular Probes (Eugene, OR).

Egg Extract Preparation and Immunoprecipitation

CSF-arrested *Xenopus* egg extracts were prepared as described (Murray, 1991) and supplemented with $\Delta 90$ cyclin (1:20) to arrest them in mitosis (Murray *et al.*, 1989). For immunoprecipitations, Affi-prep Protein A beads (Bio-Rad, Richmond, CA) were washed with TBST and incubated with antibodies for 1 h at 4°C with gentle rotation. The beads were then washed with CSF-XB plus protease inhibitors. To immunoprecipitate proteins from CSF-extracts, 100 μ l of egg extracts were incubated with the antibody beads for 1 h at 4°C with gentle rotation. The beads were pelleted and washed, and the immunoprecipitates and depleted egg extract were analyzed by Western blotting. Briefly, proteins were separated on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes, blocked with 5% milk in TBS with 0.1% Tween 20, and probed with alkaline-phosphatase (AP)-linked secondary antibodies for 1 h at 23°C. After three 5-min washes with TBS/Tween-20, blots were developed using an AP substrate kit (Bio-Rad) according to the manufacturer's instructions.

Immunofluorescence Microscopy

Spindles and asters assembled in egg extract were spun onto coverslips, fixed in -20°C methanol, and processed for immunofluorescence as previously described (Wilde and Zheng, 1999). Primary and secondary antibodies diluted in AbDil were applied to the coverslips for 1 h in a humidified chamber at room temperature, as follows: rabbit anti-maskin, 1:50,000; chicken anti-CPEB, 1:50; rabbit anti-XMAP215, 1:1000 (Wilde and Zheng, 1999); anti- α -tubulin (DM1 α), 1:1000; XenC anti- γ -tubulin, 1:1000; anti-acetylated tubulin, 1:1000; all secondary antibodies, 1:1000. To visualize DNA, coverslips were rinsed in water, dipped into a solution of 200 μ g/ml bisbenzimidazole H33342 trihydrochloride fluorophore (Hoechst dye; Calbiochem, La Jolla, CA) in water for 10 s, rinsed again in water, and mounted in mounting medium (Sigma). Spindles and asters were photographed with a Photometrics CoolSnap HQ cooled CCD camera (Roper Scientific, Tucson, AZ) through a 60 $\times$ /1.4 NA plan apo objective mounted on a Nikon Eclipse E800 fluorescence microscope (Melville, NY) equipped with MetaMorph software. Twelve-bit images were obtained using MetaMorph (Universal Imaging, West Chester, PA) and processed using Adobe Photoshop software (San Jose, CA).

Aster and Mitotic Spindle Assembly Assays

Aster and spindle assembly were induced with RanL43E (1 mg/ml) or sperm chromatin (150 sperm/ μ l), depending on the experiment. Rhodamine-labeled tubulin was added to the extract to a final concentration of 0.2 mg/ml. For excess maskin addition, protein was added and quantitated by immunoblotting. Reactions were incubated at room temperature for 15 min (asters) or 90 min (spindles).

For quantification of asters at the 15-min time point, images were captured at the same camera setting, and fluorescence intensity was quantitated using MetaMorph software. For fluorescence intensity measurements, images were taken at 60 $\times$ magnification, the gray values of all pixels in a 100 $\times$ 100-pixel area were integrated, and a background value was determined from an equal-sized area on the same image was subtracted. For quantification of structures at the 90-min time point, images were scored and classified into three categories as asters, normal, or abnormal spindles.

Microtubule-binding Assays

Taxol-stabilized microtubules were mixed with recombinant maskin protein (~1 μ g) in BRB80 with 1 mM GTP, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 20 μ M taxol and incubated for 30 min at room temperature. The reactions were then centrifuged for 25 min at 120,000 $\times$ g at 30°C in a Beckman TLA100.2 rotor (Fullerton, CA), and supernatant and pellet fractions were analyzed separately by SDS-PAGE. The Coomassie-

stained gel was digitally scanned with an Epson (Nagano, Japan) Perfection 2450 Photo Scanner, and the amount of maskin protein in each fraction was measured using the histogram function of the Adobe Photoshop software.

To determine whether maskin bound to the sides or the ends of microtubules, one aliquot of taxol-stabilized microtubules was sheared by repeatedly passing the microtubule solution through a 25-gauge needle. Recombinant maskin was then incubated with either sheared or unsheared microtubules, pelleted, and analyzed as above.

Microtubule Polymerization Assays

Microtubule polymerization assays were performed as previously described (Oegema *et al.*, 1999) with the following modifications: microtubule assembly reactions (5 μ l) containing different concentrations of purified recombinant maskin protein (0, 0.1, 0.2, and 0.8 mg/ml) and 32.5 μ M bovine brain tubulin (supplemented with a small amount of rhodamine-labeled tubulin) were incubated at 30°C for 10 min, fixed with 10 volumes of 1% glutaraldehyde in BRB80 at 23°C for 3 min, and diluted with 250 μ l of 70% glycerol in BRB80. Microtubule assembly reactions were spotted onto microscope slides, covered with a coverslip, sealed with nail polish, and viewed in the microscope. To measure the effect of maskin on nucleation, the number of microtubules in 50 random microscope fields was counted. To analyze the effect of maskin on microtubule assembly, we measured the average length of microtubules in 50 fields.

GST Pull-down Experiments

CSF extracts (200 μ l per sample) were "precleared" with glutathione-agarose beads for 1 h at 4°C with rotation. The precleared extract was then incubated with ~100 μ g of GST or GST-fusion protein at 4°C for 30 min with rotation. After the addition of 100 μ l of glutathione-agarose, the extracts were incubated for an additional 60 min. The beads were collected by centrifugation and washed five times with H100, once with H250 plus 0.1% Triton X-100, and twice with cleavage buffer. The beads were then incubated at 4°C overnight with protease (rTEV for GST-maskin, PreScission protease (Amersham) for GST and GST-TACC, respectively) in 100 μ l of cleavage buffer to cleave the GST portion from the respective fusion protein. Cleaved protein (including binding partners) was then eluted from the beads with 1 bead volume of cleavage buffer and analyzed by SDS-PAGE (Coomassie-stained gel or Western blot).

Maskin Immunodepletions and Add-backs

Immunodepletions for add-back assays were performed by first binding antibody (anti-maskin for depletions or normal rabbit serum for mock depletions) to Protein A Dynabeads (Dyna Beads, Lake Success, NY) for 40 min at room temperature. The beads were washed three times with CSF-XB plus protease inhibitors and were then added to the extract. Extracts were incubated with the beads for 1 h at 4°C with gentle rocking, and the beads were removed using a magnet. For reconstitution experiments, the depleted extracts were supplemented with a small amount of rhodamine-tubulin to visualize microtubules and with the protein to be tested to a final concentration of 20 nM. Control reactions were incubated with protein dilution buffer instead of protein (CSF-XB containing protease inhibitors). After a 20-min incubation on ice, microtubule assembly was initiated by adding sperm chromatin or RanL43E and moving the reaction to room temperature. Asters (15 min) and spindles (90 min) were then processed as described above. For quantification, images were captured at the same camera setting, and threshold levels were quantitated using MetaMorph software. For threshold level measurements, images were taken at 60 $\times$ magnification, and the gray values of all pixels above a set threshold were recorded. The data were normalized against mock-depleted samples.

Maskin Immunodepletion and Cycloheximide Addition

Immunodepletions were performed as described above using Affi-prep Protein A beads (Bio-Rad). Depleted extracts were supplemented with 50 μ g/ml cycloheximide (from a 10 mg/ml stock in water) and were incubated on ice for 20 min before sperm chromatin was added. The reactions were then incubated at room temperature and processed as described above. To assess the effect of cycloheximide on spindle assembly, the samples were viewed in the microscope and the first 50 structures encountered were classified as asters, normal spindles, or abnormal spindles. To assess the effect of cycloheximide on aster assembly, microtubules in at least 50 asters were quantitated using threshold level measurements as described above. The results reported are mean values $\pm$ SD, measured using at least three independent experiments.

Sucrose Gradient Sedimentation

Sucrose gradients (5–40%) were poured as step gradients in H100 (low salt gradients) or H500 (high salt gradients) as described (Martin *et al.*, 1998; Oegema *et al.*, 1999). Clarified egg extract (20 μ l), or a mixture of protein standards, were fractionated on parallel 2-ml sucrose gradients. Fractions (16 $\times$ 130 μ l) were separated by SDS-PAGE on 10% gels and proteins (as indicated in Supplementary Figures S1–S3 legends) were detected by Western

Figure 1. Maskin localizes to microtubule structures in egg extracts. (A) Western blot to characterize our maskin antibody. rec, recombinant maskin; ext, extract. Molecular-weight markers are indicated on the left. (B) Western blot to titrate the amount of maskin present in egg extract. The amount of egg extract and recombinant maskin of known concentration loaded in each lane is indicated. The concentration of maskin depends on the egg extract and ranges between 10 and 20 nM (4 independent measurements using 4 different extracts); maskin concentration is ~14 nM in the extract shown (total protein concentration in this extract is ~50 mg/ml). (C) Maskin (green) localizes along microtubules (red) in asters and spindles induced by addition to the egg extract of GST-RanL43E (top row) or sperm chromatin (bottom rows; DNA is blue in the overlays). Scale bar, 20 μ m.

blotting. Sucrose gradient standards: ovalbumin (3.5 S), rabbit muscle aldolase (7.35 S), bovine liver catalase (11.3 S), and ferritin (17.6 S). Images of spindles were acquired digitally and a line was drawn on the digital images through and slightly extending beyond the poles of the spindle to be analyzed. Linescan analysis was performed along this line 1-pixel wide using MetaMorph software. The resulting graphs were aligned at one pole and rendered using KaleidaGraph Software (Synergy Software, Reading, PA). Fluorescence intensity is in arbitrary units.

RESULTS

To examine the role of maskin in microtubule assembly in *Xenopus*, we first raised polyclonal antibodies in rabbits against bacterially expressed GST-maskin (see *Materials and Methods*). These antibodies recognize a distinct protein of ~150 kDa in mitotic egg extract that comigrates with bacterially-purified maskin (Figure 1A). Comparing the amount of maskin in egg extracts with purified maskin by quantitative Western blotting using our affinity purified antibodies, we estimate that the concentration of maskin in extracts is generally between ~10 and 20 nM (Figure 1B).

Maskin Localizes along Microtubules

If maskin plays a direct role in spindle assembly in the egg extract, it is expected to localize to microtubules. Microtubule assembly in *Xenopus* egg extracts requires sperm chromatin, but can also be induced by addition of an allele of the small GTPase, Ran, that mimics its GTP-bound state (e.g., RanL43E; Wilde and Zheng, 1999). Spindle assembly (which requires 60–90 min of incubation time) proceeds via several intermediate structures, the earliest of which (10–15 min of incubation) are microtubule asters that are organized

around the centrosome associated with the sperm chromatin. RanGTP induces similar structures, microtubule asters and spindles, when added to *Xenopus* egg extracts; however, these structures assemble in the absence of centrosomes or chromosomes (reviewed in Kahana and Cleveland, 1999; Sazer and Dasso, 2000; Karsenti and Vernos, 2001; Dasso, 2002). In this study, we examined the role of maskin in the assembly of asters and spindles induced by sperm chromatin or by RanL43E (a RanGTP mimic).

To examine whether maskin associates with microtubules in the egg extract, we induced microtubule assembly by the addition of sperm chromatin or RanL43E. Immunofluorescence with anti-maskin antibodies revealed that maskin localized along the microtubules of asters and spindles assembled in egg extracts in vitro (Figure 1C). Maskin antibodies decorated the length of the microtubules and the label did not appear to preferentially accumulate at the center of microtubule asters or at spindle poles (Figure 1C). Maskin staining of microtubule structures formed in the egg extracts is consistent with a previous report that maskin localizes to mitotic spindles in early *Xenopus* blastomeres (Groisman *et al.*, 2000). We concluded that maskin associates with microtubules in *Xenopus* egg extracts.

Maskin Is Involved in Mitotic Spindle Assembly in *Xenopus* Egg extracts

To examine whether maskin may play a role in spindle assembly and to begin to characterize this role, we used our affinity-purified anti-maskin antibodies and recombinant maskin for depletion and add-back experiments. We routinely removed >80% of maskin from the egg extract by immunodepletion (Figure 2).

Similar to control mock-depleted reactions, maskin-depleted egg extracts assembled microtubule asters around exogenously added sperm centrosomes within 15 min of incubation. Furthermore, maskin-depleted extracts also supported spindle assembly (Figure 2). However, morphological defects were apparent at both time points: the asters assembled in maskin-depleted extracts were significantly smaller than asters assembled in control, mock-depleted extracts (Figure 2, A and B), and the spindles assembled in maskin-depleted extracts were mostly (60%) misshapen and often less robust, disorganized, and had poorly aligned chromosomes (Figure 2, C and D; see also below). Readdition of 20 nM purified bacterially expressed full-length maskin to maskin-depleted extracts was able to restore the asters to control levels (Figure 2, A and B). Interestingly, aster assembly could also be rescued by readdition of 20 nM bacterially expressed purified TACC domain of maskin (amino acids 714–931) to maskin-depleted extracts, suggesting that residues outside of the TACC domain of maskin are not required for aster assembly in the egg extracts. In contrast, the defects in spindle assembly caused by maskin-depletion could not be rescued by readdition of maskin (Figure 2D). This strongly suggests that maskin depletion resulted in the removal of a factor that is required for spindle assembly but that is dispensable for aster assembly. This also suggests that maskin may be required for more than one step during spindle assembly.

Maskin Depletion Does Not Affect γ -tubulin Recruitment

We reasoned that the small aster phenotype caused by maskin depletion may be the result of disrupting one (or more) of three activities required for microtubule formation: 1) recruitment of microtubule nucleators to the centrosome, 2) centrosome function, or 3) microtubule stability.

Figure 2. Maskin depletion disrupts microtubule aster and spindle assembly in *Xenopus* egg extracts. (A) Fluorescence micrographs of microtubule asters (formed within 15 min of incubation) assembled in mock-depleted, maskin-depleted, or maskin-depleted plus bacterially expressed purified recombinant protein (as indicated). Amino acids 714–931 of maskin constitute the TACC domain of maskin (see Figure 7 for schematic). Recombinant proteins were expressed as GST fusion proteins, but GST was cleaved off following purification. Top row, tubulin staining; microtubules are red and DNA is blue in the overlays (bottom row). Scale bar, 25 μ m. (B) Quantitation of microtubule fluorescence intensity of asters assembled in depleted extracts. The graph represents the average of three independent experiments; the error bars represent the SD. (C) Examples of normal and abnormal spindles (formed after 90 min of incubation) in maskin-depleted extracts, as indicated. Microtubules are red and DNA is blue in the overlays. Scale bar, 25 μ m. (D) Quantitation of the ratio of normal and abnormal spindles in mock- and maskin-depleted extracts, and in maskin-depleted extracts reconstituted with recombinant full-length maskin, as indicated. The graph represents the average of three independent experiments.

The idea that maskin could be involved in the recruitment of nucleators is based in part on the observation that the *S. cerevisiae* TACC homolog, Spc72p (Tien *et al.*, 2004), tethers γ -tubulin to the cytoplasmic side of the spindle pole body (the fungal equivalent of the centrosome; Knop and Schiebel, 1998). γ -Tubulin is an essential component of the centrosome that is critical for microtubule nucleation in *Xenopus* egg extracts (Felix *et al.*, 1994; Zheng *et al.*, 1995). Along with other components of the centrosome, γ -tubulin needs to be recruited to the sperm tip from the egg extract, and this recruitment of γ -tubulin is essential for sperm aster formation (Felix *et al.*, 1994). To test whether maskin is involved in the recruitment of γ -tubulin to centrosomes, we incubated sperm chromatin in mock-depleted or maskin-depleted egg extracts. These experiments were done in the presence of the microtubule-destabilizing drug, nocodazole, to suppress microtubule formation in the extracts and thus facilitate visualizing (Figure 3A) and quantitating (Figure 3B) the amount of γ -tubulin recruited to the sperm tip by immunofluorescence (Felix *et al.*, 1994). Quantitation of the γ -tubulin immunofluorescence associated with the sperm centrosome revealed that maskin depletion had little or no effect on the amount of γ -tubulin recruited to sperm tips. This suggests that the reduction in aster size in maskin-depleted extracts could not be explained by a failure to assemble γ -tubulin onto the centrosome. These results indicate that maskin depletion may affect microtubule stability rather than centrosome assembly, although they do not rule

out that maskin may also be required for centrosome function.

Maskin Is Involved in Centrosome-independent Microtubule Formation

Next, we wanted to examine whether maskin depletion had an effect on microtubule stability. For these experiments, we took advantage of the ability of RanL43E to induce microtubule formation in the egg extract independent of centrosomes. Because Ran-induced microtubule structures are devoid of centrosomes, any defects in aster formation are likely to be due to defects in microtubule stability.

Consistent with a role of maskin in microtubule stability, depleting maskin from the egg extract reduced both the number and the length of the microtubules in Ran asters (Figure 4). As was the case for sperm asters, addition of 20 nM purified maskin was able to rescue the aster defects. Moreover, increasing the maskin concentration by addition of 20 nM maskin to Ran-treated but undepleted extracts resulted in the formation of asters with microtubules that were both more numerous and longer (Figure 4). These results support the notion that maskin is involved in microtubule stability in the egg extracts.

There are three possibilities for how maskin could influence microtubule stability: 1) it could directly bind to microtubules and influence their dynamics, or it could indirectly affect microtubule stability by 2) repressing the synthesis of a microtubule destabilizing protein (via its role in transla-

Figure 3. Maskin depletion has little effect on γ -tubulin recruitment to centrosomes. (A) Fluorescence micrographs of sperm chromatin incubated in mock-depleted (top rows) or maskin-depleted (bottom rows) egg extract supplemented with nocodazole to prevent microtubule formation. Acetylated tubulin (a marker for centrosomes) is red, γ -tubulin is green, and DNA is blue in the overlays (right panels). Scale bar, 5 μ m. (B) Quantitation of the amount of γ -tubulin recruited to sperm centrosomes. To quantitate the amount of γ -tubulin recruited, the γ -tubulin fluorescence intensity associated with the sperm centrosomes was measured using MetaMorph software. The graph represents the average of 11 independent experiments $\pm$ SEM.

tion—depletion of maskin would then be expected to make microtubules less stable), or 3) it could be required for the function of one or more microtubule stabilizing proteins.

Maskin Interacts Directly with Microtubules . . .

To distinguish these possibilities, we first tested whether maskin interacts directly with microtubules. We generated microtubules by polymerizing highly purified bovine brain tubulin in the presence of paclitaxel (taxol). We then incu-

Figure 4. Maskin is involved in centrosome-independent microtubule assembly in *Xenopus* egg extracts. (A) Ran-aster formation in mock- and maskin-depleted extracts. Fluorescence micrographs of microtubule structures assembled in mock-depleted (top left-hand panel), maskin-depleted (top right-hand panel), maskin-depleted plus bacterially expressed recombinant maskin (bottom left-hand panel), or mock-depleted plus an extra bolus of bacterially purified recombinant maskin (bottom right-hand panel). Scale bar, 20 μ m. (B) Quantitation of Ran-induced structures assembled in depleted extracts. Graph on the left, average fluorescence intensity; graph on the right, average microtubule length; mock, mock-depleted; depl, maskin-depleted; recon, maskin-depleted extract plus 20 nM bacterially purified recombinant maskin; extra, mock-depleted extract plus 20 nM bacterially purified recombinant maskin. (C) Western blot to quantitate the levels of maskin depletion and add-back, as indicated.

bated bacterially expressed maskin with the stabilized microtubules in vitro, and the mixture was centrifuged to separate microtubule-bound maskin from unbound protein. Depending on the microtubule concentration, most of the maskin pelleted with the microtubules (Figure 5A). These data indicate that maskin interacts directly with microtubules. This is in contrast to what has been reported for the *Drosophila* and human TACC proteins, which appear to require at least one additional protein for stable interaction with microtubules (Gergely *et al.*, 2000b; Lee *et al.*, 2001), but

“sheared” sample compared with the “unsheared” sample, as measured by fluorescence microscopy. The amount of maskin copelleting under each condition is indicated below the gel. (D) Maskin does not have an effect on microtubule polymerization. Representative fields from microtubule polymerization reactions containing 32.5 μM tubulin and increasing amounts of recombinant maskin protein (as indicated in the micrographs). Under these conditions, microtubules form spontaneously even in the absence of stabilizing proteins (0 μM sample). Bar, 20 μm .

Figure 5. Maskin is a microtubule-binding protein. (A) Coomassie-stained gel of the supernatant (S) and pellets (P) of a representative microtubule copelleting assay using a constant amount of maskin and increasing amounts of taxol-stabilized microtubules, as indicated below the gel. (B) Plot of the percentage of maskin associated with microtubules versus the tubulin concentration. The data shown are the averages ($\pm$ SEM) of three independent experiments. The apparent dissociation constant (K_d), defined as the amount of polymerized tubulin required to pellet half of the added maskin, is 1–2 μM . The graph was generated using the PRISM program (GraphPad software). (C) Maskin does not have a preference for microtubule ends. A Coomassie-stained gel is shown of a microtubule copelleting assay comparing the binding of maskin to 2 μM microtubule samples containing varying numbers of ends. Samples containing shorter microtubules (i.e., more ends) were generated by shearing taxol microtubules through a needle. There were at least fourfold more microtubule ends in the

our findings are consistent with a previous report that maskin copellets with microtubules *in vitro* (Groisman *et al.*, 2000). Plotting of bound maskin versus microtubule concentration and direct hyperbolic curve-fitting (Figure 5B) yielded an equilibrium dissociation constant (K_D) for the interaction between maskin and microtubules of ~ 1 –2 μM , which suggests a moderately high-affinity. This value is well within the range of affinities for other microtubule-interacting proteins, including NuSAP ($K_D = \sim 1$ μM ; Raemakers *et al.*, 2003) and ch-TOG ($K_D = 2$ –5 μM ; Spittle *et al.*, 2000).

Maskin could bind to microtubules in one of two ways: by binding along the side of the polymer, or by preferentially binding to either plus or minus ends, or both. To test whether maskin preferentially binds to microtubule ends, we incubated maskin with a solution of microtubules that had been passed through a fine-gauge needle to generate broken microtubules (in our case, there was a ~ 4 –5-fold increase in the number of microtubule ends as assayed by fluorescence microscopy) and compared the amount of bound maskin to that bound to unbroken microtubules. In both cases, the overall microtubule polymer remained constant (i.e., there is the same amount of microtubule wall). Figure 5C shows that similar amounts of maskin pelleted under both conditions, suggesting that maskin does not have a preference for microtubule ends but instead binds along the wall of the microtubule polymer. This finding is consistent with our observation that maskin seems to bind along the length of microtubules in the egg extracts.

... But Has No Effect on Microtubule Polymerization *In Vitro*

Next, to test whether maskin directly affects microtubule polymerization, we added purified maskin to *in vitro* microtubule polymerization reactions (Figure 5D). If maskin stabilizes microtubules directly, we expect either the number of microtubules, their length, or both, to increase in a dose-dependent manner. Addition of maskin had no effect

on the number of microtubules formed (Figure 5), which remained unchanged when compared with microtubules assembled in the absence of maskin. Similarly, maskin addition to microtubule polymerization reactions had no effect on microtubule length. We concluded that maskin does not stabilize microtubules directly, and that the effect of maskin on microtubule stability in egg extracts is likely to be mediated by other proteins.

Microtubule Assembly Defects Caused by Maskin Depletion Are Not Rescued by Cycloheximide Treatment

We next examined whether the defects in microtubule assembly caused by maskin depletion may be related to maskin's role in repressing translation. In this scenario, it is possible that rather than maskin having a stabilizing effect on microtubules, maskin is required to repress the synthesis of one or more proteins that destabilize microtubules. We therefore tested the effect of the inhibitor of translation, cycloheximide, on asters and spindles formed in maskin-depleted extracts. If maskin depletion induces translation of (a) destabilizing factor(s), we expect cycloheximide to mitigate the defects caused by maskin depletion. We found that addition of 50 $\mu\text{g}/\text{ml}$ cycloheximide had no effect on aster (Figure 6A) or spindle (Figure 6B) assembly in maskin-depleted egg extracts, thus ruling out that maskin depletion increases microtubule-destabilizing activities. Unexpectedly, cycloheximide addition to mock-depleted extracts had an effect on both aster size and spindle assembly: asters in cycloheximide-treated samples were slightly smaller than control asters at the early (15 min) time point, and the number of normal spindles at the later (90 min) time point was consistently reduced (Figure 6). It is not yet clear whether this reflects a requirement of efficient spindle assembly for protein translation or whether this effect is due to some other activity of cycloheximide in the egg extract.

Figure 6. Cycloheximide addition does not rescue the defects caused by maskin depletion. Microtubule structures were induced by addition of sperm chromatin to mock- or maskin-depleted extracts treated with 50 μ g/ml cycloheximide (+) or buffer (–) and asters (A) or spindles (B) were scored as described for Figure 2. (A) Aster size was quantitated in three independent experiments using tubulin fluorescence and is expressed as percent of maximum fluorescence $\pm$ SD. (B) Microtubule structures in 50 randomly chosen microscope fields in spindle assembly reactions (90-min time point) were categorized as asters, normal spindles, or abnormal spindles in maskin- or mock-depleted extracts in the presence or absence of 50 μ g/ml cycloheximide. The graph shows the average of three independent experiments $\pm$ SD. Cycloheximide treatment reduces both the total number of spindles and the ratio of normal to abnormal spindles in mock-depleted extracts but has no effect on maskin-depleted extracts (where the assembly of normal spindles is already compromised).

Maskin Interacts with Several Proteins in Mitotic Egg Extracts

Together, the experiments described above indicated that maskin is likely to exert its effects on microtubule assembly in the egg extracts via its interaction with other proteins. This is also supported by our (unpublished results) observation that addition of maskin to extracts in the absence of sperm or RanGTP had no effect on the extracts. To identify potential maskin binding partners, we expressed fusion proteins of GST with full-length maskin or the C-terminal TACC-domain of maskin (amino acids 714–931) and incubated the purified fusion proteins in egg extracts. Control reactions were incubated with GST alone or buffer. We then reisolated the GST-constructs on GST beads and analyzed copurifying proteins by Coomassie-staining and Western blot (Figure 7, A and B). As an alternative approach, we also immunoprecipitated maskin from egg extracts and analyzed copurifying proteins by Western blot (Figure 7C). These experiments showed that full-length maskin copurified with a number of proteins in the egg extracts that ranged in size from <30 to ~250 kDa. Because TACC proteins in other species are known to interact with members of the TOG protein family, we wondered if the band that migrated near 200 kDa could be the *Xenopus* TOG, XMAP215. Western blot analysis with antibodies to XMAP215 (Wilde and Zheng, 1999) showed that XMAP215 indeed copurified with full-length maskin by immunoprecipitation and with both full-length maskin and maskin's TACC-domain by GST-pull-downs (Figure 7). Interestingly, maskin's binding partner for its role in translational control during oocyte maturation, CPEB, also copurified with full-length maskin in mitotic egg extracts, as did a small amount of the *Xenopus* Aurora A, Eg2 (Figure 7). Neither CPEB nor Eg2 copurified with the TACC-domain. Importantly, the TACC-domain (but not the GST or buffer controls) also pulled down full-length maskin (Figure 7B), suggesting that the coiled-coil domain of maskin is also involved in homodimerization.

Together, these findings suggest that maskin forms complexes that contain additional maskin molecules, XMAP215, CPEB, and Eg2 (among other, not yet identified proteins). These proteins could assemble into one complex or form alternative complexes with maskin. To distinguish these possibilities, we immunoprecipitated XMAP215 from egg extracts. Western blots of the immunoprecipitates with antibodies to CPEB (Figure 7D) showed that CPEB coprecipitated

tated with XMAP215, suggesting that XMAP215 and CPEB are in the same complex.

To investigate this question further, we fractionated egg extracts on 5–40% sucrose gradients and probed Western blots of the gradients with antibodies to XMAP215, Eg2, CPEB, maskin, and TPX2, a spindle assembly protein (Wittmann *et al.*, 2000; Supplementary Figure S1). These experiments showed that the majority of maskin migrated as a relative small species with a peak around 8S. This analysis also showed that although the distributions of XMAP215, Eg2, CPEB, and maskin overlapped in several fractions, these proteins migrated differently on sucrose gradients, suggesting that each may form more than one type of complex. This is underscored by the fact that XMAP215 and Eg2, but not maskin and CPEB, migrate as smaller species under high salt (500 mM NaCl) conditions (Supplementary Figure S1). Consistent with a previous report that the interaction between maskin and CPEB is independent of RNA (Stebbins-Boaz *et al.*, 1999), treatment of the extract with RNase had no effect on the sedimentation of maskin or CPEB on sucrose gradients (our unpublished results).

Maskin Depletion Results in Mislocalization of XMAP215 But Does Not Affect CPEB

Disruption of TACC proteins in flies, worms, or humans results in disruption of TOG protein localization (Cullen and Ohkura, 2001; Lee *et al.*, 2001; Bellanger and Gönczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003) and overall protein levels (Bellanger and Gönczy, 2003). Depletion of maskin (>80%) reduced XMAP215 levels by ~25%, presumably by codepletion. To investigate whether maskin depletion had an effect on the localization of XMAP215, we assembled sperm asters or spindles in *Xenopus* egg extracts depleted of maskin or mock-depleted and performed immunofluorescence with antibodies specific for XMAP215 (Figure 8). Consistent with previous reports, XMAP215 localized along microtubules and accumulated at spindle poles in mock-depleted extracts (Tournebise *et al.*, 2000; Popov *et al.*, 2001). In contrast, XMAP215 localization was disrupted in maskin-depleted extracts (Figure 8) at both time points examined. XMAP215 staining along aster microtubules was reduced and/or XMAP215 seemed to aggregate in the vicinity of the microtubules (Figure 8A), depending on the extract used and the extent of maskin depletion. At later time points, XMAP215 staining along spindle microtubules appeared

Figure 7. Maskin has multiple binding partners in *Xenopus* egg extracts. (A and B) Coomassie-stain (A) and Western blot (B) of GST-maskin pull-downs from egg extracts. GST (lane 2), GST-maskin (lane 3), or GST-maskin-TACC domain (amino acids 714–931; lane 4) were incubated with *Xenopus* egg extract (see *Materials and Methods*), retrieved, and separated on a 10% SDS-PAGE gel. Proteins that copurify specifically with full-length maskin (GST-maskin) are indicated by asterisks on the right. GST-TACC, proteins that copurify with the TACC-domain of maskin. GST, proteins that copurify with GST (control); buffer, proteins that copurify with the beads used to retrieve the GST constructs (control). Positions of molecular weight standards are indicated on the right. Full-length maskin migrates near the 150-kDa marker, CPEB migrates around 60 kDa, and Eg2 migrates just below the 50 kDa marker. (B) Western blot of a duplicate gel similar to the one shown in A. After transfer, the blot was cut horizontally into strips using the molecular-weight markers as guides, and the strips probed with antibodies to (from top to bottom) XMAP215, maskin, CPEB, or Eg2, as indicated. The blot shows that GST-maskin pulls down XMAP215 and CPEB, and small amounts of Eg2 (marked with asterisks in the lower panel), whereas the TACC domain pulls down XMAP215 and endogenous maskin, but not CPEB or Eg2. (C and D) Results from immunoprecipitation experiments. Western blots of maskin (C) or XMAP215 (D) immunoprecipitations were probed for XMAP215, maskin, and CPEB, as indicated. Lane 1, mitotic extract (control); lane 2, maskin (C), or XMAP215 (D) immunoprecipitations; lane 3, normal rabbit serum control immunoprecipitations. (E) Schematic of the maskin constructs used for A and B.

mostly normal. However, XMAP215 failed to accumulate at the spindle poles in maskin-depleted extracts (Figure 8 and Supplementary Figure S3). This suggests that XMAP215 localization to microtubules is largely independent of maskin, but its stable association with spindle poles is maskin-dependent. In contrast, CPEB staining along the length of microtubules in asters or spindles was unaffected by maskin depletion (Figure 8B and Supplementary Figure S3), sug-

gesting that maskin is not required for the localization of CPEB. These findings are consistent with reports that in *Drosophila* embryos, D-TACC and Msps (the *Drosophila* TOG) seem to depend on each other for stable association with the centrosome (Cullen and Ohkura, 2001; Lee *et al.*, 2001), whereas CPEB binds to microtubules in vitro independently of maskin (Groisman *et al.*, 2000).

To assess whether localization of other spindle assembly proteins is also disrupted in maskin-depleted extracts, we stained spindles with antibodies to γ -tubulin, TPX2 (Supplementary Figure S2), or pericentrin (unpublished data). Although the immunofluorescence staining was sometimes difficult to assess in severely disrupted spindles, there was little if any effect of maskin depletion on the localization of TPX2 (Supplementary Figures S2 and S3) or pericentrin (unpublished data) to spindle poles.

Consistent with previous reports (Wilde and Zheng, 1999), γ -tubulin localized on spindle poles and along spindle microtubules in mock-depleted extracts (Supplementary Figure S2). γ -Tubulin staining in maskin-depleted spindles was variable, and we often found that one pole of a given spindle stained heavily with γ -tubulin antibodies, whereas the other pole of the same spindle showed reduced or no staining (Supplementary Figure S2). Increased staining often correlated with disorganized microtubules, whereas the more normal-appearing pole seemed to have suppressed γ -tubulin levels. However, we also found examples of disrupted poles with γ -tubulin staining that was indistinguishable from controls and increased γ -tubulin staining at properly organized poles (unpublished data). Moreover, we found similar variability in spindles assembled in mock-depleted extracts, although fewer spindles were affected. Thus, it is not clear whether maskin depletion has a stabilizing or destabilizing effect on γ -tubulin localization to centrosomes, or both. On the other hand, γ -tubulin staining along spindle microtubules appeared to be suppressed in maskin depleted extracts (Supplementary Figure S2).

DISCUSSION

In this study, we provide evidence for a novel role for the translational regulator, maskin, in microtubule aster and spindle formation. Furthermore, we show that maskin, or an activity associated with maskin, is required at two separate steps during spindle assembly. The first activity of maskin, which is required for an early stage of spindle assembly (i.e., aster assembly), depends only on the TACC domain of maskin. Our experiments did not allow us to distinguish whether this activity affected centrosome assembly or function, but our finding that the assembly of RanGTP-induced microtubule asters is affected by maskin depletion supports the hypothesis that microtubule stability is affected by maskin depletion. This is further supported by the evidence that maskin depletion had no effect on the amount of γ -tubulin recruited to sperm-associated centrosomes, although we cannot rule out that the amount of maskin left in the extracts (~10–25%, depending on the experiment) is sufficient for γ -tubulin recruitment to centrosomes.

The second activity of maskin is required during later stages of spindle assembly and cannot be rescued by addition of bacterially expressed purified maskin. There are two possible explanations to account for the lack of rescue: 1) the purified protein is misfolded, lacks necessary secondary modifications, or is nonfunctional in some other way, or 2) depletion of maskin resulted in codepletion of one or more component(s) critical for spindle assembly. It is plausible

A. XMAP215 is mislocalized in maskin-depleted extracts

B. CPEB distribution is unaffected by maskin depletion

Figure 8. XMAP215 mislocalizes in maskin-depleted *Xenopus* egg extracts, but CPEB staining is unaffected. (A) Asters (left panel; 15-min time point) or spindles (right panel; 90-min time point) induced by the addition of sperm chromatin to mock-depleted (top row) or maskin-depleted (bottom rows) extract were stained with antibodies to α -tubulin (left column) and XMAP215 (middle column), as indicated on the micrographs. Right column, overlay: green, XMAP215; red, α -tubulin; blue, DNA. (B) CPEB staining of asters (left panel) or spindles (right panel) induced by addition of sperm chromatin to mock-depleted (top row) or maskin-depleted (bottom rows) extracts. Left panels, tubulin; middle panels, CPEB, right panels, overlay; green, CPEB; red, tubulin; blue, DNA. Scale bars, 25 μ m.

that the necessary component could be a protein required for spindle assembly. This hypothesis is supported by our observation that maskin interacts with a number of proteins in the egg extracts, including proteins previously implicated in spindle assembly (e.g., XMAP215). On the other hand, the observation that maskin also interacts with at least one RNA-binding protein, CPEB, leads us to speculate that the critical component codepleted with maskin might also be an mRNA. A role for translation in spindle assembly has not been established, but our observation that treatment with cycloheximide decreases the ratio of spindles (both normal and abnormal) in egg extracts compared with controls (Figure 6) supports the idea that efficient spindle assembly might indeed require the synthesis of one or more spindle assembly factor(s).

Our results that maskin is involved in spindle assembly and interacts with XMAP215 via its TACC domain adds to

the evidence that in addition to sequence homology, TACC proteins from various species share the ability to interact with XMAP215/TOG proteins and influence microtubule stability. We therefore support the notion, originally proposed by Raff and coworkers (Gergely *et al.*, 2000b), that the TACC domain is an evolutionarily conserved specialized coiled coil domain that confers interaction with XMAP215/TOG and/or a structural role in mitotic spindle assembly. The finding that the *C. elegans* TAC-1 protein consists almost entirely of a TACC domain and that TAC-1 interacts with Zyg-9 (the *C. elegans* homolog of XMAP215/TOG) emphasizes the importance of the TACC domain in spindle assembly (Bellanger and Gönczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003). Whether other invertebrate and mammalian TACC proteins also play roles in translational regulation, or in other important cellular processes, remains to be determined.

Although our findings firmly establish maskin as a functional *Xenopus* TACC homolog, they also highlight that the details of the function of TACC proteins in different organisms appear to be distinct. For example, disruption of D-TACC in flies or TAC-1 in worms results in phenotypes that phenocopy disruptions in the TOG family members, Msps and Zyg-9, respectively (Cullen and Ohkura, 2001; Lee *et al.*, 2001; Bellanger and Gönczy, 2003; Le Bot *et al.*, 2003; Srayko *et al.*, 2003), and D-TACC requires Msps for its interaction with microtubules (Lee *et al.*, 2001). This is in contrast to the interactions of maskin with XMAP215 and microtubules: *Xenopus* XMAP215 appears to require maskin for its association with spindle poles, but its association with the microtubules of the mitotic spindle (presumably at least in part via their plus ends) does not seem to be affected by maskin depletion (Figure 8). This calls into question a prevailing model for the function of the interaction between TACCs and TOGs that postulates that TACCs recruit TOGs to centrosomes, thus ensuring that the TOG can bind to and stabilize microtubule plus ends as they grow (Lee *et al.*, 2001).

Another difference between different TACC family members is that the *Drosophila* TACC protein requires an adaptor to interact with microtubules *in vitro* (Gergely *et al.*, 2000b), and Msps (the *Drosophila* TOG family member) might serve as this adaptor (Cullen and Ohkura, 2001; Lee *et al.*, 2001), whereas maskin binds to microtubules with relatively high affinity *in vitro* ($\sim 1\mu\text{M}$), and this interaction does not depend on XMAP215 (Figure 5). Furthermore, D-TACC and Msps are proposed to preferentially bind to microtubule ends (Lee *et al.*, 2001), but maskin has no such preference.

It is clear that maskin depletion results in mislocalization of XMAP215, but not all of the defects in spindle assembly induced by maskin depletion can be accounted for by a sole function of maskin in XMAP215 positioning. In addition to the evidence mentioned above, XMAP215 depletion from *Xenopus* egg extracts affects microtubule length and greatly reduces spindle length (Tournebise *et al.*, 2000), whereas spindles assembled in maskin depleted extracts appear disorganized but microtubule length does not appear to be affected. We therefore speculate that the function of maskin in spindle assembly only partially overlaps with the function of XMAP215. The role of maskin in spindle assembly is likely to require interactions of maskin with additional factors. One of our future challenges will be to identify the remaining maskin-interacting proteins and examine their function in maskin-mediated spindle assembly.

ACKNOWLEDGMENTS

We thank Mike Sheets, Maddie DeBeer, Holly Goodson, and Judy Yanowitz for critically reading the manuscript; John Newman for generating the TACC-domain construct; and Laura Vanderploeg for help with figure preparation. We gratefully acknowledge the gifts of antibodies or clones from J. Richter, L. Hake, Y. Zheng, M. Sheets, M. Wickens, S. Bednarek, and D. Rancour. This work was supported in part by National Science Foundation grant MCB-0344723 (C.W.), a Kimmel Scholar Award from the Sidney Kimmel Foundation for Cancer Research (C.W.), and start-up funds from the UW-Madison Graduate School, the College of Agricultural and Life Sciences, and the Department of Biochemistry.

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