ch-TOGp Is Required for Microtubule Aster Formation in a Mammalian Mitotic Extract*

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Microtubules induced to polymerize with taxol in a mammalian mitotic extract organize into aster-like arrays in a centrosome-independent process that is driven by microtubule motors and structural proteins. These microtubule asters accurately reflect the noncentrosomal aspects of mitotic spindle pole formation. We show here that colonic-hepatic tumor-overexpressed gene (ch-TOGp) is an abundant component of these asters. We have prepared ch-TOGp-specific antibodies and show by immunodepletion that ch-TOGp is required for microtubule aster assembly. Microtubule polymerization is severely inhibited in the absence of ch-TOGp, and silver stain analysis of the ch-TOGp immunoprecipitate indicates that it is not present in a preformed complex and is the only protein removed from the extract during immunodepletion. Furthermore, the reduction in microtubule polymerization efficiency in the absence of ch-TOGp is dependent on ATP. These results demonstrate that ch-TOGp is a major constituent of microtubule asters assembled in a mammalian mitotic extract and that it is required for robust microtubule polymerization in an ATP-dependent manner in this system even though taxol is present. These data, coupled with biochemical and genetic data derived from analysis of ch-TOGp-related proteins in other organisms, indicate that ch-TOGp is a key factor regulating microtubule dynamics during mitosis.

Chromosome segregation during both mitosis and meiosis is driven by a complex microtubule-based structure called the spindle (1). Spindles are symmetric, fusiform structures whose constituent microtubules have well defined orientations. Microtubule plus ends either associate with kinetochores on chromosomes, extend to the cell equator where they interdigitate with other microtubules, or extend to the cell cortex (2). Microtubule minus ends converge to form two spindle poles that are clearly identifiable by light microscopy. The spindle poles are functionally defined as the sites to which the sister chromatids move upon segregation at anaphase (3). Thus, proper focusing of microtubule minus ends at spindle poles is important for the overall fidelity of chromosome segregation.

In somatic cells, the number of spindle poles is determined by the number of centrosomes. Centrosomes are the dominant centers of microtubule nucleation, and they duplicate once during the cell cycle. The duplicated centrosomes separate from each other at or just before the onset of mitosis, and the radial array of microtubules emanating from each of the two centrosomes builds the bipolar spindle and establishes the positions of the two spindle poles (4–6). However, centrosomes, and the microtubule arrays nucleated from them, are not sufficient to act as functional spindle poles. Recent experiments have shown that several noncentrosomal proteins are involved in focusing microtubules at spindle poles (3). Among these noncentrosomal proteins are the microtubule motors cytoplasmic dynein (and its associated activating complex dynactin), Eg5, and HSET (also referred to as CHO2, XCTK2, and ncd in hamster, Xenopus, and Drosophila systems, respectively) as well as the structural protein NuMA (7–18). Together, these noncentrosomal proteins appear to act by focusing and anchoring microtubules at spindle poles following their release and/or severing from the centrosomes. Perturbation of the activity of any of these proteins leads to the disorganization of microtubule minus ends at spindle poles despite the presence of centrosomes.

To further explore the molecules and mechanisms of spindle pole formation in somatic cells, we have developed a simple cell-free system for the assembly of microtubule asters (7). The organization of microtubules into asters in this system is cell cycle-dependent, centrosome-independent, and requires the activities of cytoplasmic dynein, dynactin, Eg5, HSET, and NuMA (7–9, 18, 29). Collectively, the data show that these microtubule asters accurately reflect the noncentrosomal aspects of spindle pole formation in somatic cells. To identify additional components of these complex microtubule structures, we have enriched for microtubule asters using density centrifugation. We report here that ch-TOGp is an abundant protein component of these microtubule asters. We have prepared antibodies specific to ch-TOGp and use immunodepletion to show that ch-TOGp is essential for efficient microtubule polymerization during mitotic aster formation in this system. This result is consistent with the prior characterization of homologues of ch-TOGp acting to stimulate microtubule polymerization (19–21) and organize the mitotic spindle (22, 35, 38, 39) and indicates that ch-TOGp plays an important role in regulating microtubule dynamics during spindle assembly in mitosis.

EXPERIMENTAL PROCEDURES

Microtubule Aster Preparation and Enrichment—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mm glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin and synchronized in the cell cycle by double block with 2 mM thymidine followed by treatment with 40 mg/ml nocodazole. Preparation of the mitotic extract from synchronized HeLa cells and assembly of microtubule asters were performed as described previously (7). To enrich for microtubule aster proteins we prepared the mitotic extract with the following modifications. Following mitotic cell shake off and incubation at 37 °C with 20 μg/ml cytochalasin B, the cells were washed

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1 The abbreviations used are: ch-TOGp, colonic hepatic tumor-overexpressed gene; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

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twice with cold phosphate-buffered saline and once with cold KHMBuffer (78 mM KCl, 50 mM HEPES, pH 7.0, 4 mM MgCl$_2$, 2 mM EDTA, 1 mM dithiothreitol) without cytochalasin B, and no cytochalasin B was used in any subsequent steps. The 100,000 × g supernatant was collected as described previously (7), supplemented with 3.0 mM ATP, 50 µM GTPγS, and 100 µM GTP. The extract was then subjected to centrifugation at 100,000 × g for 10 min, and the resulting supernatant fraction was collected and supplemented with 20 µM taxol and 1.5 mM ATP, and microtubule asters were assembled by incubation at 30 °C for 60 min. The extract containing the microtubule asters was then layered on top of KHMBuffer containing 20% (w/v) sucrose, and the asters were collected as a pellet following centrifugation at 150,000 × g for 120 min. Indirect immunofluorescence microscopy of the mitotic asters and immunoblotting of the soluble, insoluble, and immunoprecipitated fraction derived from the mitotic extract were performed as described previously (7).

Immunodepletion from the extract prior to aster assembly were carried out using 100 µg of either the ch-TOGp-specific rabbit polyclonal IgG or a preimmune rabbit IgG. As described previously (7, 8), the antibodies were adsorbed onto 25 µl of protein A-conjugated agarose (Roche Molecular Biochemicals), and depletions were performed in two sequential steps of 60 min each. Following the immunodepletion reactions, the extract was recovered, and microtubule asters were induced as described previously (7, 8). The agarose pellet derived from the supernatant fraction was also recovered and solubilized with SDS-PAGE sample buffer.

Antibody Production—The ch-TOGp-specific antibodies were prepared by immunizing rabbits with recombinant ch-TOGp expressed in bacteria. A 1118-base pair EcoRI/NotI fragment from the ch-TOGp est cDNA clone AA496098 (Genome Systems Inc., St. Louis, MO.) was ligated at the unique EcoRI/NotI site in the multicloning region. This construct results in the fusion of the open reading frames for GST and the C-terminal 301 amino acids of ch-TOGp. The orientation of the ch-TOGp sequence was verified by multiple combinations of restriction digests, and the construct transformed into Escherichia coli BL21 (Stratagene, La Jolla, CA). Expression of the GST-ch-TOGp fusion protein was induced by the addition of 1 mM isopropyl-l-thiogalactopyranoside to a liquid culture. Cells were harvested after 4 h, pelleted by centrifugation at 7000 rpm at 4 °C, resuspended in 10 ml of phosphate-buffered saline containing protease inhibitors (5 µg/ml chymostatin, leupeptin, antipain, pepstatin, and 100 µg/ml phenylmethylsulfonyl fluoride) and sonicated on ice. The lysed cells were then incubated on ice for 30 min with 1% Triton X-100 and the insoluble debris was removed by centrifugation at 11,000 rpm for 15 min at 4 °C. The soluble fraction was collected and passed over a column of packed glutathione-Sepharose-4B (Amersham Pharmacia Biotech). The column was washed twice with phosphate-buffered saline to remove any nonbound protein after which the bound GST-ch-TOGp protein was eluted by three successive washes with 10 ml reduced glutathione in 50 mM Tris-HCl, pH 8.0. This pure GST-ch-TOGp fraction was used to immunize two rabbits (Covance Research Products Inc., Richmond, CA), which produced two similar ch-TOGp-specific antibodies. The IgG fraction was purified from the crude serum by affinity chromatography using protein A-conjugated agarose (Roche Molecular Biochemicals).

The remaining antibodies used in these experiments were as follows: NuMA was detected with the rabbit polyclonal antibody (7); tubulin was detected using the monoclonal antibody DM1a (Sigma); Eg5 was detected using a rabbit polyclonal antibody raised against the central rod domain expressed as clone M4F (23); HSET was detected using a rabbit polyclonal antibody (18); and finally, MAP-4 was detected using a rabbit polyclonal antibody (LHB) courtesy of J.C. Bulinski (Columbia University, NY).

RESULTS

To identify new protein components of microtubule asters assembled in a mammalian mitotic extract, we separated the asters from the soluble components of the extract by centrifugation through 20% sucrose. Because the extract is prepared as a 100,000 × g supernatant, the microtubule asters represent one of the only structures present in the extract that have sufficient mass to sediment through 20% sucrose under these conditions. The other major structure that assembles under these conditions is actin, and actin and its associated proteins appeared as major contaminants in our initial attempts to enrich for microtubule asters. To reduce this contamination by actin, we modified our protocol for microtubule aster assembly by eliminating cytochalasin B from every step except the initial 30-min incubation which is necessary for efficient cell rupture during homogenization. Next, we supplemented the mitotic extract with ATP and phallolidin, but not taxol, and incubated at 30 °C for 15 min. Phallolidin drives the polymerization of actin under these conditions, and a majority of the actin fibers and bundles were removed from the extract by sedimentation at 100,000 × g (Fig. 1A, lane 1). In the absence of taxol, microtubules do not polymerize in these extracts, and the microtubule-associated proteins involved in microtubule aster formation such as NuMA, Eg5, cytoplasmic dynein, dynactin, and HSET remain soluble (7–9, 18). Microtubule asters were induced in the extract following removal of actin filaments by adding taxol and additional ATP and incubating at 30 °C for 60 min. Microtubule asters assembled under these modified conditions were morphologically indistinguishable from asters assembled under our previous conditions (7–9, 18) and were enriched upon sedimentation through 20% sucrose as judged by the abundant tubulin band on the gel (Fig. 1A, lane 2).

To identify the protein components of these microtubule asters, we separated the proteins by SDS-PAGE, cut selected proteins from the gel, and obtained peptide sequence information from tryptic peptides by mass spectrometry. In addition to tubulin (Fig. 1A, lane 2) and a variety of proteins with minor abundance, two of the most abundant proteins in the enriched microtubule aster fraction are ~120 and 200 kDa (Fig. 1A, lane 2). Fifty-nine internal peptide sequences ranging in size from 7 to 28 amino acids representing 41 unique peptide sequences after elimination of duplicates were obtained from the 120-kDa protein (Table I). All of these peptide sequences were 100% identical to the published sequence of the human Eg5 protein. Eg5 is a kinesin-related protein that is known to be involved in microtubule aster assembly in this (8) and other systems (24–26).

The 200-kDa protein contained two polypeptides, and based on the abundance of the peptides sequenced, one protein was more abundant than the other. Fifty-four internal peptide sequences ranging in size from 7 to 32 amino acids representing 26 unique peptides after elimination of duplicates were obtained from the protein of less abundance (Table I). All of these peptide sequences were identical to the published sequence of human MAP-4. MAP-4 is an abundant microtubule-associated protein in HeLa cells (36). Immunodepletion of >90% of MAP-4 from the mitotic extracts showed no detectable change in microtubule aster morphology (data not shown). This suggests that MAP-4 is not essential to microtubule aster assembly in this system consistent with in vivo results showing that MAP-4 is not essential for mitotic spindle assembly (37). Eighty-five internal peptide sequences ranging in size from 7 to 31 amino acids representing fifty-seven unique peptides after elimination of duplicates were obtained from the more abundant 200-kDa protein (Table I). All of these peptide sequences were identical to the published sequence of human protein ch-TOGp. ch-TOGp is predicted to be 218 kDa and is overexpressed in many human tumors (27–28). It is associated with mitotic spindles in cultured cells (21) and is homologous to XMAP-215, Zyg9, mps, p95astro1, and Stra2 (20, 22, 35, 38, 39). XMAP-215 is a Xenopus protein identified by its ability to promote microtubule polymerization (19, 20). Zyg9 and mps are genes in Caenorhabditis elegans and Drosophila, respectively, which when
Fig. 1. A, enrichment of microtubule asters by density centrifugation. Lane 1, the majority of actin and actin-associated proteins were removed from the extract by preincubation with phalloidin. Lane 2, microtubule asters were assembled in the actin-diminished extract and collected by sedimentation through 20% (w/v) sucrose. The position of tubulin (T) as well as Eg5 and ch-TOGp, both identified by peptide sequencing, are indicated on the right. B, specificity of polyclonal antibodies against ch-TOGp. Total cell protein (~50 mg) from human HeLa cells was immunoblotted with a rabbit polyclonal serum against ch-TOGp. The position of tubulin (T) as well as Eg5 and ch-TOGp, both identified by peptide sequencing, are indicated on the left side of A and B.

mutated, cause defects in spindle assembly in early embryonic cells (22, 35). P93D61 and Stu2 are genes in Schizosaccharomyces pombe and Saccharomyces cerevisiae, respectively, that encode essential proteins of the spindle pole body (38, 39). The identification of three known mitotic spindle-associated proteins as major constituents of the insoluble pellet fraction illustrates the utility of this technique for identifying microtubule aster proteins, and we are currently identifying other proteins within these structures through similar techniques.

To characterize the potential role of ch-TOGp in microtubule aster assembly, we prepared polyclonal antibodies against the C-terminal 301 amino acids of the protein. Immunoblot analysis of total HeLa cell protein with this polyclonal antibody shows specific reactivity to a protein of ~200 kDa in agreement with the molecular weight of ch-TOGp (Fig. 1B). Consistent with published data on ch-TOGp (21), indirect immunofluorescence microscopy of cultured cells showed the protein distributed throughout the cytosol with some enrichment in the perinuclear region during interphase, and throughout the spindle during mitosis (data not shown). Indirect immunofluorescence microscopy also showed ch-TOGp to be distributed throughout the microtubule asters assembled in our cell-free extract (data not shown), and immunoblot analyses of the soluble and insoluble fractions obtained following microtubule aster assembly showed that ch-TOGp is highly enriched in the insoluble, aster-containing pellet fraction (Fig. 2C). The solubility of ch-TOGp in these extracts was not sensitive to the presence or absence of ATP (see Fig. 4) and mirrored that of tubulin in that it only appeared in the insoluble fraction under conditions where microtubule polymerization was induced. Moreover, the efficiency with which ch-TOGp associated with microtubules was not altered if either NuMA, Eg5, cytoplasmic dynein, or dynactin were depleted from the extract prior to microtubule polymerization or if the activity of HSET was perturbed by the addition of HSET-specific antibodies (data not shown). These data indicate that the rabbit polyclonal antibodies are specific to ch-TOGp. Further, in accordance with our identification of ch-TOGp from an enriched microtubule aster preparation, these data show that ch-TOGp is highly enriched on the microtubule asters assembled under these conditions.

To determine if ch-TOGp is involved in microtubule aster assembly, we used the ch-TOGp-specific antibodies to deplete the protein from the extract prior to stimulating aster assembly. In control samples, we depleted the extract with IgG prepared from preimmune rabbit serum. Depletion of the extract with this antibody had no effect on the morphology of the microtubule asters, the concentration of NuMA at the core of the asters, or the efficiency with which the various aster components associated with the insoluble aster-containing fraction (Fig. 2, A and C; Refs. 7 and 8). Depletion of the extract with the ch-TOGp-specific antibodies resulted in the removal of >98% of ch-TOGp from the extract as judged by immunoblotting (Fig. 2C). In the absence of ch-TOGp, microtubule aster formation was severely inhibited (Fig. 2B). Microtubule polymerization was significantly reduced, and only small clumps of microtubule fragments were observable by immunofluorescence microscopy. The reduction in microtubule polymerization efficiency in the absence of ch-TOGp was clearly apparent by immunoblot analysis as the quantity of tubulin present in the insoluble, aster-containing fraction is reduced 4-fold in the sample depleted of ch-TOGp compared with the sample depleted with the control IgG (Fig. 2, C and D). The small microtubule clumps formed in the absence of ch-TOGp stained positively for NuMA (Fig. 2B) and were similar in appearance, albeit somewhat smaller, to the central core of microtubule asters formed under control conditions. This indicates that the
minor amount of microtubule polymerization that occurred in the absence of ch-TOGp was sufficient for the recruitment of other aster components. This conclusion was supported by immunoblots showing either no significant change or only slight diminution in the efficiency with which NuMA, Eg5, HSET (Fig. 2C), cytoplasmic dynein, and dynactin (data not shown) associated with the insoluble pellet fraction in the absence of ch-TOGp. Thus, ch-TOGp or a complex containing ch-TOGp plays an essential role in stimulating robust microtubule polymerization during microtubule aster formation in this system.

To determine if ch-TOGp is precipitated from these extracts as part of a multiprotein complex, we analyzed the immunoprecipitate by SDS-PAGE followed by silver staining (Fig. 3). On both 8.5% (Fig. 3) and 12.5% (data not shown) SDS-PAGE, we were unable to detect any proteins that co-immunoprecipitated with ch-TOGp. This indicates that ch-TOGp is not part of a multiprotein complex in extracts prepared under these conditions, although we cannot rule out the possibility of an associated protein of ±50 and ±20 kDa because of the antibody molecules.

Finally, we investigated whether the reduction in the efficiency of microtubule polymerization in the absence of ch-TOGp was dependent on ATP added exogenously to the extract. For this experiment we depleted the extract with either the control antibody or the ch-TOGp-specific antibody.

### Table I

| Peptide sequences derived from the 120- and 200-kDa microtubule aster-associated proteins |
|---|
| **120 kDa** | **200 kDa (a)** | **200 kDa (b)** |
| QHNIFLDQMTIEKLIQNLELNETIKSTEISSAET | AALATVNAAFKGEQKMDLEGEDLSEELK | IVFKDLVLSSIEEVAQANDII |
| TSIATISPASINLNEETLSLEYAHKRK | HSTSGDEGEDGEPDGDNGDVLPLLPR | DFIATLAEAFDDVGFETVG |
| VSSLLEYNELPDNLNSPDSVQRSIFAPQDNPLK | LNDNKLIVLQLTINNQHAVAMGPNIK | MYYDDLSLVPFSATADT |
| GLEITVHNDEKQIEK | NAALNTIVTVNHGQDQVF | VALSSETIVLVAR |
| NNLQNPNNMDLLIK | PAVPTVASTDMHLHSG | VGSNDVHLPAGVAGV |
| EYITSALESTEEKLHDAASK | GLEALYTVYENAHVAGK | HYYFVGQVQQNK |
| AVOHQQNAEQQDFGKTEK | TASQVVLGLDVKDQGVK | DMQQSMEDMALVDLMEL |
| TVYFSEQWSSSLERLK | MPEFEDRRQLSQIDAEEFK | EIIHAEEEREPQALEIMMG |
| TTAATLMTLINAYSSR | EAEMGSHIQDPLATPMQLR | NWLPTETEFAFQDVLKL |
| LNLVLDAGSENNGR | EFQDIDQERIENDGFTVR | KPMGLASGLVFPAPFK |
| EAGNINQSSLTLKR | DTVNMLVAAK | TDDMPSKETEMALAK |
| EKGNVYIIEENFR | TSSLQEDKSDPFIHPPNVGK | TDIPIEDVDEK |
| LTVGEEQIVELEIK | NSQETLNLWNSAIAK | TAPFISAAQK |
| LIQANLQNEITIK | DNLAPPFQHNINSVQLK | LATNTSAPDLK |
| HSVSEVFVTIHK | LTQITFPVLIPEFK | KFPNSAVTK |
| TSLTVADKIDQHK | IDLEDQGQVIP | TDILMSPTETEMALAK |
| VTELIFMDNK | FBTDMSAVQQLK | ATFTMPRSSPSTFIDK |
| NVYVISENLF | TAASAGGADGTGK | SFVAPSLSRPK |
| LLNTVEETTKK | AQDALLFIPPMHMLQYK | DMFPFPQHK |
| LMLNLETRE | FPQNNIGEPTALPK | KTEAAATTP |
| LTNDGERSVK | VNDFLAIEFKK | TFKPLAQQAFK |
| VITALVRE | DAAFEALGTALK | HFKPGGGDVK |
| LTHPIEFER | DLHMLGTLMLDSL | DMALATK |
| LQDSIQLGR | MQQSFQFPAPTR | ETEMALAK |
| ISEQTEOR | GEQAVQELIK | DVATPTVFK |
| TQLETTQIK | LIGNSEKDMSELER | QTQVAK |
| NIQVVR | HINSAPEVR | |
| SYLYPSSTLVR | TIGVEGSGSVS | |
| ETITIDGEELVK | GKFAPPAGGANTGTR | |
| LQMDTPOPI | EIAYHIHGDINV | |
| EQSQAESSK | EGALEYEY | |
| EHLDLQIK | ALAVMVOHLESEK | |
| EYTEIEER | SVNLLVVK | |
| DVSGLHISK | VMFQFAFPPTK | |
| NPQOSGTK | WNFTPSGEYIELQK | |
| AMLEVK | GLDKNKPK | |
| LVEESVK | LEAGDYADLVK | |
| TQIHPYVR | FSELYVK | |
| SDLQNK | LIAVEYR | |
| MNFHISO | DEYETEQK | |
| TPHVPIR | RPAAPIK | |
| PMGSSAP | PMGSSAPK | |
| KGPAEDMSSK | KGPAEDMSSK | |
| LSGYEEALK | LSGYEEALK | |
| ILLSHFIFMK | ISQETEOR | |
| SGPIFIVNVPGK | VELLGSK | |
| ITSELVK | VELLGSK | |
| SQLELAK | SQLELAK | |
| OVEQFOR | OVEQFOR | |
| ATSPPGMSSAP | ATSPPGMSSAP | |
| DQVLAMEK | DQVLAMEK | |
| DISAPKFGELK | DISAPKFGELK | |
| ALSFEGSK | ALSFEGSK | |
| QELLGHLAEK | QELLGHLAEK | |
| VIEMER | VIEMER | |
| EASTGVLK | EASTGVLK | |
| AIPISNVK | AIPISNVK | |
divided the depleted extracts into two parts. One part was untreated, the other part was supplemented with ATP, and microtubule polymerization was induced in both parts by the addition of taxol and incubation at 30 °C for 60 min. The morphology of the microtubule structures formed under each condition was determined by immunofluorescence microscopy, and the efficiency of microtubule polymerization was assessed by immunoblot analysis of the soluble and insoluble fractions (Fig. 4).

When the extract is depleted with the control antibody, microtubule asters form normally when the extract is supplemented with ATP (Fig. 4B), but only loose, nonastral microtubule aggregates form in the absence of exogenously added ATP (Fig. 4A). We have previously shown that the lack of aster organization in the absence of ATP is because of the lack of microtubule motor activity and that microtubule asters form equally well if the extract is supplemented with bulk ATP or an ATP regenerating system (7). Despite these ATP-dependent differences in microtubule organization, there was no significant difference in the efficiency with which microtubules polymerized with or without ATP under control depletion conditions (Fig. 4, E and F). Furthermore, there was no detectable difference in the efficiency with which ch-TOGp associates with microtubules in the presence or absence of ATP (Fig. 4E). In the absence of both ch-TOGp and ATP, microtubules were arranged in loose aggregates indistinguishable from those formed under control conditions in the absence of ATP (Fig. 4A, B, and C). The efficiency of microtubule polymerization in the absence of both ch-TOGp and ATP was not significantly different from the control depletion conditions (Fig. 4E and F). In contrast, when the extract depleted of ch-TOGp was supplemented with ATP the microtubules formed small clumps (Fig. 4D) as de-
FIG. 4. Lack of microtubule polymerization in the absence of ch-TOGp depends on ATP. The cell-free mitotic extract was processed for indirect immunofluorescence using antibodies specific for tubulin and NuMA (as indicated) following depletion with either a preimmune antibody (A and B) or the ch-TOGp-specific antibody (C and D). Samples in A and C were untreated, whereas samples B and D were supplemented with ATP prior to inducing microtubule polymerization. E, the immunoprecipitate from each depletion was recovered (P_ab), and the remainder of the extracts were separated into soluble (S) and insoluble (P) fractions by centrifugation at 10,000 × g. These fractions were subjected to immunoblot analysis using antibodies specific for ch-TOGp and tubulin. F, the percentage of tubulin in the insoluble fraction was quantified by densitometry from three independent depletion experiments. The percentage of tubulin in the insoluble fraction in the sample lacking ch-TOGp but containing ATP is significantly reduced compared with the other three samples (t test, p < 0.01). Bar in A—D, 10 μm.

Microtubule Asters Require ch-TOGp

We describe an enrichment procedure for microtubule asters formed in a cell-free extract prepared from synchronized cultured cells. Further, we find that ch-TOGp is an abundant component of these microtubule asters, and through immunodepletion, we demonstrate that ch-TOGp is required for microtubule aster assembly in this system. Specifically, microtubule polymerization was significantly reduced in the absence of ch-TOGp, indicating that ch-TOGp plays an essential role in stimulating robust microtubule polymerization in this system. This finding is consistent with the properties of homologues of ch-TOGp, which have been described in other organisms. For example, the Xenopus homologue of ch-TOGp, XMAP-215 (21), was biochemically isolated from frog egg extracts based on its ability to promote microtubule polymerization in vitro (19, 20). XMAP-215 was shown to promote longer, more dynamic microtubules by increasing plus end elongation rates 7–10-fold, in-creasing plus end shortening velocity ~3-fold, and nearly eliminating rescue (20). In line with these in vitro studies, mutation of the zyg-9 gene in C. elegans or the msps gene in Drosophila leads to disorganized, incorrectly oriented mitotic and meiotic spindles that, in the case of zyg-9, have unusually short microtubules (22, 35). Also, the pds4ΔΔ and Stu2 genes encode spindle pole body components in S. pombe and S. cerevisiae, respectively, that are essential for viability, presumably through their role in microtubule organization (38, 39). Thus, evidence generated through in vitro biochemistry, genetics, and mitotic cell extracts all point to the conclusion that ch-TOGp (and its homologues) plays a primary role in modulating microtubule dynamics by promoting microtubule assembly.

One surprising aspect of the results presented here is that the efficiency of microtubule polymerization is reduced in the absence of ch-TOGp despite the fact that taxol is present in these extracts. This might indicate that taxol requires ch-TOGp to stimulate microtubule polymerization in these extracts. However, the fact that taxol is known to stimulate microtubule polymerization without ch-TOGp, that some microtubule polymerization is induced with taxol in these extracts in the absence of ch-TOGp, and that the effect of ch-TOGp on microtubule polymerization is sensitive to ATP suggests an alternative view, namely that multiple factors are present in these extracts and that microtubule polymerization (stabilization) and depolymerization (destabilization) are modulated by these factors in addition to the role that taxol plays in stimulating microtubule polymerization. In this view, ch-TOGp may promote microtubule assembly in this system by counteracting the activity of an ATP-dependent microtubule depolymerizing (destabilizing) factor. This idea is supported by the facts that ch-TOGp does not require ATP to stimulate microtubule polymerization (19, 20), that the reduction in microtubule polymerization efficiency in the absence of ch-TOGp is ATP-dependent, and that nocodazole-induced depolymerization of microtubules in this system requires ATP (29). A candidate for
such an ATP-dependent microtubule depolymerizing (destabilizing) factor is MCAK, a member of the kinI family of kinesin-related proteins (30, 31). Members of the kinI family of kinesin-related proteins display ATP-dependent catastrophe-promoting activities on both native and taxol-stabilized microtubules (32) and play a role in the increase in microtubule catastrophe rate, which has been documented during mitosis (33, 34). We are currently developing antibodies specific to the human kinI kinesin protein to test this hypothesis directly. Thus, whereas taxol is necessary to stimulate microtubule polymerization during spindle assembly in vitro, it is very likely that ch-TOGp is involved in promoting microtubule polymerization during mitotic spindle assembly in vivo. Unfortunately, we have been unable to directly confirm this supposition because microinjection of our antibodies into cultured cells did not alter microtubule organization during interphase or mitosis and did not block the progression of mitosis. Nevertheless, the results presented here, coupled with the defects in spindle assembly observed in both worms and flies carrying mutations in the zyg-9 (22) and msps (35) genes, respectively, strongly supports the conclusion that ch-TOGp (and its homologues) plays an important role in regulating microtubule dynamics during spindle assembly in vivo.

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REFERENCES
1. McIntosh, J. R. & Koomey, M. P. (1989) Science 246, 622–628
2. Inoue, S. & Salmon, E. D. (1995) Mol. Biol. Cell 6, 1619–1640
3. Compton, D. A. (1998) J. Cell Sci. 111, 1477–1481
4. Mazia, D. (1984) Exp. Cell Res. 153, 1–15
5. McIntosh, J. R. (1983) Mod. Cell Biol. 2, 115–142
6. Sluder, G. & Rieder, C. L. (1985) J. Cell Biol. 100, 897–903
7. Gaglio, T., Saredi, A. & Compton, D. A. (1995) J. Cell Biol. 131, 693–708
8. Gaglio, T., Saredi, A., Bingham, J. B., Hashani, M. J., Gill, S. R., Schreyer, T. A. & Compton, D. A. (1996) J. Cell Biol. 135, 399–414
9. Gaglio T., Dionne, M. A. & Compton, D. A. (1997) J. Cell Biol. 138, 1055–1066
10. Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Beker, P., Hyman, A. & Karsenti, E. (1996) Nature 382, 420–425
11. Heald, R., Tournebize, R., Habermann, A., Karsenti, E. & Hyman, A. (1997) J. Cell Biol. 138, 615–628
12. Matthias, H. J. G., McDonald, H. B., Goldstein, L. S. B. & Theurkauf, W. E. (1996) J. Cell Biol. 134, 455–464
13. Palazzo, R. E., Vaisberg, E. A., Weiss, D. G., Kuznetsov, S. A. & Steffen, W. (1999) J. Cell Sci. 112, 1291–1302
14. Verde, F., Berrez, J.-M., Antony, C. & Karsenti, E. (1991) J. Cell Biol. 112, 1177–1187
15. Walczak, C. E., Verma, S. & Mitchison, T. J. (1997) J. Cell Biol. 136, 859–870
16. Merdes, A., Ramyar, K., Vechio, J. D. & Cleveland, D. W. (1996) Cell 87, 447–458
17. Endo, S. A. & Komma, D. J. (1997) J. Cell Biol. 137, 1321–1336
18. Mountain, V., Simerly, C., Howard, L., Ando, A., Schatten, G. & Compton, D. A. (1999) J. Cell Biol. 147, 351–365
19. Gard, D. L. & Kirschner, M. W. (1987) J. Cell Biol. 105, 2203–2215
20. Vasquez, R. J., Gard, D. L. & Cassimeris, L. (1994) J. Cell Biol. 127, 985–993
21. Charrasse, S., Schroeder, M., Gauthier-Rouviere, C., Ango, F., Cassimeris, L., Gard, D. L. & Larroque, C. (1998) J. Cell Biol. 141, 1159–1168
22. Whitehead, C. M. & Rattner, J. B. (1998) J. Cell Sci. 111, 2551–2561
23. Sawin, K. E., LeFuellec, K., Philippe, M. & Mitchison, T. J. (1992) Nature 359, 540–543
24. Heck, M. S., Periera, A., Pesavento, P., Yannoni, Y., Spradling, A. C. & Goldstein, L. S. B. (1993) J. Cell Biol. 123, 665–679
25. Blangy, A., Lane, H. A., d’Herin, P., Harper, M., Kress, M. & Nigg, E. A. (1995) Cell 84, 49–59
26. Blangy, A., Lane, H. A., d’Herin, P., Harper, M., Kress, M. & Nigg, E. A. (1995) Neurosci. Lett. 119, 119–122
27. Charrasse, S., Obou, C., Arranzuela, C. & Larroque, C. (1996) Neurones Lett. 212, 189–2023
28. Sluder, G. & Mitchison, T. J. (1995) J. Cell Biol. 128, 55–104
29. Walczak, C. E., Mitchison, T. J. & Desai, A. (1996) Cell 84, 57–107
30. Desai, A., Verma, S., Mitchison, T. J. & Walczak, C. E. (1999) Cell 96, 69–78
31. Walczak, C. E., Mitchison, T. J. & Desai, A. (1999) J. Cell Biol. 138, 1055–1066
32. McNally, F. (1996) Curr. Opin. Cell Biol. 8, 23–29
33. Cassimeris, L. (1999) Curr. Opin. Cell Biol. 11, 134–141
34. Cullen, C. F., Deak, P., Glover, D. M. & Ohkura, H. (1999) J. Cell Biol. 146, 1055–1018
35. Olmstead, J. B. (1986) Ann. Rev. Cell Biol. 2, 421–457
36. Wang, X. M., Pelopquin, J. M., Zhai, Y., Bulinski, J. C. & Borisy, G. G. (1996) J. Cell Biol. 132, 345–357
37. Nasahima, K., Kurooka, H., Takeuchi, M., Kinoshita, K., Nakaseko, Y. & Yanagida M. (1995) Genes Dev. 9, 1572–1585
38. Wang, P. J. & Huffaker, T. C. (1997) J. Cell Biol. 139, 1271–1290

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