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The TOGp protein is a new human microtubule-associated protein homologous to the *Xenopus* XMAP215

Sophie Charrasse[1], Marianne Schroeder[2,5], Cécile Gauthier-Rouviere[3], Fabrice Ango[1], Lynne Cassimeris[4], David. L. Gard[5] and Christian Larroque[1,*]

[1]INSERM Unite 128, 34293 Montpellier, France
[2]Myriad Genetics, Salt Lake City, UT, USA
[3]INSERM, IGM, Montpellier, France
[4]Dept of Biological Sciences, Lehigh University, Bethlehem, PA, USA
[5]Dept of Biology, University of Utah, Salt Lake City, UT, USA
*Author for correspondence (e-mail: larroque@xerxes.crbm.cnrs-mop.fr)

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SUMMARY

We have recently identified a 6,449 bp cDNA, termed colonic, hepatic tumor over-expressed gene (ch-TOG), that is highly expressed in human tumors and brain. Its single open reading frame encodes a putative 218,000 Da polypeptide, TOGp. Antibodies generated against a bacterially expressed TOGp fragment specifically recognize a 218,000 Da polypeptide in two human cell lines and in brain. Immunofluorescence microscopy using affinity-purified TOGp antibodies revealed that the distribution of TOGp was dependent upon the cell cycle. During interphase, TOGp was found concentrated in the perinuclear cytoplasm, where it co-localized with ER markers. In contrast anti-TOGp antibodies stained centrosomes and spindles in mitotic cells. TOGp co-sedimented with taxol-stabilized microtubules in vitro. Moreover, a TOGp enriched fraction promotes microtubule assembly both in solution and from nucleation centers. Finally, sequence comparison and immunologic cross-reaction suggest that TOGp is homologous to XMAP215, a previously described microtubule associated protein (MAP) from *Xenopus* eggs. These results suggest that TOGp is a MAP and that TOGp/XMAP215 may be necessary for microtubules rearrangements and spindle assembly in rapidly dividing cells.

Key words: Microtubule associated protein (MAP), Microtubule dynamics, Mitosis

INTRODUCTION

We have recently reported the discovery of a new human gene, colonic, hepatic tumor over-expressed gene (ch-TOG), and its over-expression in colonic and hepatic tumors versus corresponding healthy tissues (Charrasse et al., 1995). The messenger (6,449 bp) from this gene encodes a putative basic protein (pH_i 8.3) of 1,972 amino acids. In normal human tissues, the ch-TOG messenger was found preferentially expressed in the brain, with lesser amounts in all other tissues examined. Although ubiquitously expressed in brain, ch-TOG messenger was most abundant in the cerebellum, and particularly in the cell bodies of Purkinje cells (Charrasse et al., 1996).

To characterize further the protein product (TOGp) of the ch-TOG gene, and to gain insight into its cellular function, we have raised and used polyclonal antibodies against TOGp to determine its intracellular distribution by immunofluorescence microscopy. Since these studies partially suggest a microtubules (MT)-based function, we have also isolated from pig brain a fraction enriched in TOGp and measured its effects on MT assembly in vitro. In addition, comparison of the amino acid sequence deduced from the ch-TOG cDNA with those of nine peptides fragments of the *Xenopus* XMAP215 microtubule-associated protein strongly suggests that these two proteins are homologous. XMAP215, a previously identified microtubule-associated protein (MAP), was isolated from *Xenopus* eggs (Gard and Kirschner, 1987) and is a potent promoter of plus end microtubule assembly (Gard and Kirschner, 1987; Vasquez et al., 1994). XMAP215 is the only identified assembly-promoting MAP which increases, rather than decreases, microtubule turnover (Vasquez et al., 1994; reviewed by McNally, 1996). The above findings support the hypothesis that both XMAP215 and TOGp define a new class of MAP that may play an important role in regulating MT assembly during spindle morphogenesis. This idea is further supported by the analogies between the N terminus of TOGp and the Stu2p or $p93^{dis1}$, two yeast proteins which also bind MTs MTs (Chen and Huffaker, 1996; Nabeshima et al., 1995).

MATERIALS AND METHODS

Expression and purification of TOGp truncated protein in *Escherichia coli*

The human ch-TOG cDNA fragment (2,556-6,049 bp) was inserted

into the pGEX-2T vector carrying the glutathione S-transferase (GST) gene fusion system (Smith and Jonhson, 1988) (Pharmacia Biotech) in the appropriate reading frame. The plasmid was propagated in *E. coli* strain DH-10 β. The expression of the GST-TOGp (amino acid 844-1,972) fusion protein was induced with 0.4 mM isopropyl-β-thiogalactoside (IPTG) then cells were harvested 4 hours later. Fusion protein was prepared according the method described by Frangioni and Neel (1993). Briefly, bacteria were lyzed in STE buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA), 5 mM DTT in the presence of ionic detergent (1.5% Sarkosyl) and various protease inhibitors. After sonication, the lysate was clarified by centrifugation (30,000 ***g***, 30 minutes, 4°C). The supernatant was transferred to a new tube and 4% Triton X-100 was added. The GST-TOGp (amino acids 844-1,972) fusion protein was purified from supernatant bacterial lysate by affinity chromatography on glutathione Sepharose-4B (Pharmacia Biotech). The TOGp protein was cleaved away from GST by thrombin (Sigma, St Louis, MO). We recovered further fragments of about 40 kDa which were microsequenced to verify the 15 first amino acids of the truncated TOGp protein.

Production and purification of polyclonal antibody

The purified TOGp fragments were diluted in PBS and used as an antigen in 2 rabbits (50 μg per injection) and 2 mice (5 μg per injection). Samples were diluted in an equal volume of Freund's adjuvant and injected subcutaneously in ten different sites on each rabbit. Injection on day 1 was with complete Freund's adjuvant and booster injections on days 14 and 28 were with incomplete adjuvant. Preimmune serum were collected from each animal and test bleeds were obtained (for rabbits) two weeks after each injection. This protocol was performed by Eurogentec (Seraing, Belgium). The resulting rabbit polyclonal antiserum was used as it is or antibodies were affinity purified against the corresponding GST fusion protein bound to CNBr-activated Sepharose 4B (Pharmacia Biotech) following the manufacturer's instructions.

Materials and cell culture

The human adrenergic neuroblastoma cell line SH-SY5Y (Biedler et al., 1973; Ross et al., 1980) was from by DSMZ, GmbH Braunschweig Germany (DSM ACC 209). Cells were grown at 37°C in a 1:1 mixture of Eagle's minimum essential medium (MEM) and of Ham's F12 nutrient mixture, supplemented with 10% fetal calf serum (FCS), penicillin (100 i.u./ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) in a 95% air-5% CO_2 humidified incubator. The human pluripotent embryonal carcinoma cell line NTera 2/cl.D1 (NT2-D1, ATCC CRL-1973) was cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% heat-activated FCS, 2 mM L-glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin. This cell line issues from a primary embryonal carcinoma of testis and is known to differentiate to neurons when exposed to retinoic acid (Pleasure and Lee, 1993).

Nocodazole and brefeldin A were purchased from Sigma Chemical Co (St Louis, MO) and were used at concentrations of 20 μM and 17 μM, respectively, for 2 hours.

Immunoblotting

30 μg of different protein fractions or 50 μg of total proteins were separated on 5% SDS-page and transferred onto nitro-cellulose and probed for the presence of TOGp using various antisera diluted 1:800 in 25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.05% Tween-20 or affinity purified anti-TOGp (0.25 mg/ml) diluted 1:100. Incubation was performed for 1 hour at room temperature. After several washes in the same buffer, horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Amersham) was added for 45 minutes in the same conditions. Detection of the immune signal was done using the ECL western blotting system (Amersham). Western blots of *Xenopus* proteins with anti-TOGp and affinity purified anti-XMAP215 were performed as described by Gard and Kirschner (1987).

Indirect immunofluorescence microscopy

SH-SY5Y cells growing on 35 mm plastic dishes and NT2-D1 cells growing on 12 mm glass coverslips were fixed in 3.7% formalin in PBS for 5 minutes followed by a 30 second extraction in –20°C acetone before rehydratation in PBS containing 1% BSA. Other fixations, including –20°C methanol or combinations of formalin and glutaraldehyde, did not alter TOGp localization. Cells were incubated for 60 minutes at 37°C with anti-TOGp antisera diluted 1:200 in PBS-BSA or with anti-TOGp affinity purified antibody diluted 1:20. After a brief wash in PBS, incubation was carried on with biotinylated anti-rabbit IgG antibody (Amersham) for 45 minutes and with Streptavidin-Texas red (Amersham) for 30 minutes. Stained cells were mounted in 0.25% (w/v) Airvol 205 in PBS and examined with a Leica confocal laser scanning microscope (CLSM).

For dual-labeling experiments involving TOGp and dihexyloxacarbocyanine iodide (DiOC6) or (NBD)-ceramide, cells were fixed in 3.7% formalin and permeabilized with 0.05% saponin in 80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$ for 5 minutes. Free aldehyde groups were quenched with 50 mM NH_4Cl in PBS for 10 minutes. After incubation with antibodies as described above (NBD)-ceramide diluted 1:1,000 or DiOC6 diluted 1:10,000 are mixed with streptavidin-Texas red. For double staining of TOGp and the rough endoplasmic reticulum (RER) or the medium compartment in the Golgi apparatus, cells were fixed in 3.7% formalin and permeabilized with 0.1% Triton in PBS for 5 minutes. Antibodies against RER were diluted 1:100 (Louvard et al., 1982) and CTR433 (Jasmin et al., 1989), a hybridoma cell culture supernatant was used undiluted. For MT, centrosome and Golgi staining the second antibody was a fluorescein (FITC)-conjugated goat affinity purified antibody to mouse IgG (50-fold dilution; Cappel). For dual-labelling experiments involving TOGp and RER, we have used the mouse polyclonal antiserum anti-TOGp because antibodies raised against RER were produced in rabbit. For double staining of TOGp with microtubules or centrosomes, cells were fixed in methanol at –20°C for 4 minutes. Anti-β-tubulin antibody (Amersham) was used at a dilution of 1:50 and anti-centrosome antibody CTR453 (Bailly et al., 1989) was used at a dilution of 1:100.

Preparation of human brain MT and MAPs using taxol

The strategy for the purification of MT and MAPs from human brain tissue was carried out as described by Vallee (1986). 3.5 g of human brain obtained from surgically resected brain and collected under the recommendation of the local ethics committee were used for this preparation. Purification was achieved in a single polymerization step with taxol used at 20 μM. In this procedure, a first centrifugation at 30,000 ***g*** for 30 minutes was run to discard the majority of membranes. This pellet was resuspended in PEM buffer and used to prepare the nuclear and mitochondrial fractions as described above. Microsomes were sedimented during the 180,000 ***g*** centrifugation preceding the polymerization of microtubules (see Fig. 4A).

Preparation of the TOGp enriched fraction

Pig brains obtained a maximum of 15 minutes after the animals were killed were kept on ice during transportation (1 hour in our case). Typically, one brain was homogeneized in 100 ml PEMD buffer (Pipes 100 mM, EGTA 1 mM, $MgSO_4$ 1 mM, DTT 0.1 mM, pH 6.9) plus 1 mM Pefabloc (Pentapharm, AG, Basel) as protease inhibitor. Homogenate was spun at 130,000 ***g*** for 30 minutes and the supernatant applied to a phosphocellulose P11 (Whatman) column (5 grams of dry cellulose powder per 100 g of crude brain tissue) equilibrated in the same buffer. Most of the tubulin was eluted from the gel by washing the column with this buffer. After each chromatographic step, fractions were analysed by western blot with the anti-TOGp antibody (see above). Bound proteins were then eluted stepwise with washes of 0.2, 0.35 and 0.5 M NaCl in PEMD buffer plus Pefabloc (0.1 mM). About 70-80% of the bound proteins were eluted with the first 0.2 M NaCl wash. Fractions of the 0.35 M wash

that contained the protein TOGp were pooled, diluted 5 times with PEMD-MS buffer (Pipes 50 mM, EGTA 1 mM, $MgSO_4$ 1 mM, DTT 0.1 mM), Pefabloc 0.1 mM. The pH was adjusted to 6.5 and this fraction was applied to a Mono-S 5/5 FPLC cation exchange column. Bound proteins (about 20% of the applied fraction) were eluted with a linear gradient of 0-0.5 M NaCl and collected in 500 μl fractions. TOGp was eluted from the gel at 0.3 M NaCl in 4 fractions containing increasing amounts of lower molecular mass polypeptides corresponding to TOGp degradation. Only the fraction 25, with a single 218 kDa polypeptide was used in assembly experiments. This fraction, which was estimated to be enriched in TOGp by a 840 factor, also contains additionnal uncharacterized lower molecular mass contaminants which do not react with antibodies to TOGp. At this stage, each additional separating step resulted in further degradation of TOGp and the addition of other proteases inhibitors did not solve this problem. However, this enriched TOGp preparation was free of high molecular mass contaminants and western blots against tau (rabbit polyclonal, Boehringer) and MAP2 (monoclonal antibody, Sigma) were negative. The concentration of TOGp was estimated from both western blots and Coomassie blue stained gels. A fraction containing the identical contaminant bands (fraction 22), but not TOGp, was used in control experiments to confirm that the MT assembly-promoting activity was due to TOGp. Note that neither TOGp nor its degradation products were found in discarded fractions after the various chromatographic steps suggesting that TOGp is present at a low level in pig brain cytosol.

MT assembly assays

Tubulin purification

Tubulin was purified from either bovine or porcine brains. Bovine tubulin was isolated by a cycle of assembly and disassembly followed by phosphocellulose chromatography (Mitchison and Kirschner, 1984). Porcine tubulin was isolated by a similar protocol but an additional step of assembly in Na glutamate was included after the phosphocellulose column (Walker et al., 1988).

Spectrophotometric assays

Tubulin polymerization was monitored turbidimetrically at 350 nm with a Uvikon 321 (Kontron Instrument) spectrophotometer equipped with a thermostatted cuvette holder. The cuvette had a 10 mm path length and was 2 mm wide internally. It was prewarmed at 37°C prior to addition of the reaction mixture. It was verified that the time to reach 37°C for the reaction mixture did not exceed 20 seconds. Experiments were run in PEM buffer (100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$, pH 6.9), with 8 μl of the tested fraction in a total volume of 100 μl. All samples contained 13 μM tubulin (isolated from bovine brain) and 1 mM GTP. The final concentration of TOGp was estimated to be approximately 0.04 μM. To confirm that the assembly-promoting activity was due to TOGp in the fraction, TOGp was immunodepleted from the fraction using rabbit anti-TOGp coupled to Protein A-Sepharose.

Real-time assembly assays

Real-time analysis of individual MT assembly dynamics from axoneme fragments was performed using video-enhanced DIC microscopy as described by Vasquez et al. (1994). These experiments used porcine brain tubulin. Rates of MT growth and shortening, and transitions between these states, were determined as described by Vasquez et al. (1994). Samples contained 10 μM tubulin and an estimated concentration of 0.15 μM TOGp. The TOGp used for these studies was stored at −80°C prior to experimentation.

Assembly assays using centrosomes

HeLa cells plated on coverslips were treated with 33 μM nocadazole for 90 minutes at 37°C, rinsed with PEM buffer (see above) then extracted for 2 minutes in PEM supplemented with 0.1% Triton X-100. After a last wash with PEM, cells were incubated in PEM supplemented with 20% glycerol, 1 mM GTP, 20 μM tubulin (isolated from bovine brain) and the fraction to be assayed (2-4 μl corresponding to 0.04 μM for TOGp). This assay mixture was put on the coverslip and then incubated at 37°C for 12 minutes in a humid chamber. The coverslips were briefly rinsed and cells fixed in PEM buffer plus 0.5% gluteraldehyde for 5 minutes. Then the tubulin detection was performed as described above for NT2 cells.

Microsequencing of XMAP215

XMAP215 protein was isolated as described by Gard and Kirschner (1987). XMAP215 was digested with endo-Lys-C or endo-asp-N and peptides were separated by reverse phase HPLC. Nine peptides were sequenced by gas-phase microsequencing at the University of Utah Peptide Sequencing Facility.

Sequence homologies were analysed using the BLAST program on the NCBI facilities or Bestfit and Pileup from the Genetic Computer Group (Madison, Wisc.).

RESULTS

Characterization and specificity of anti-TOGp antibodies

Polyclonal antibodies were raised in rabbits and mice against a protein fragment consisting of amino acids 844-1,972 of human TOGp (see Materials and Methods). Immunoblot analysis of

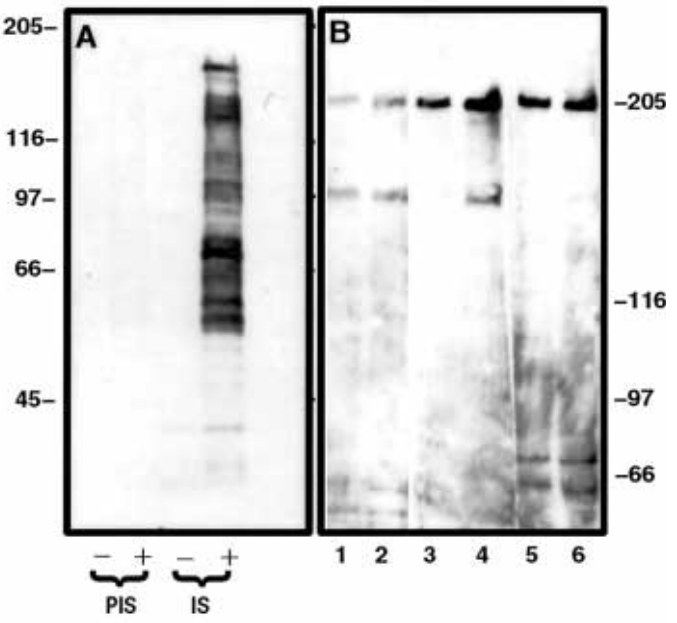


Fig. 1. Characterization of anti-human TOGp antibodies. (A) The specificity of anti-TOGp antibodies was assessed by immunoblotting against bacteria DH-10 β induced or not by IPTG for the production of truncated TOGp fusion protein. Shown are non-induced bacterial extracts (lanes 1 and 3) and extracts induced by IPTG (lanes 2 and 4). Lanes 1 and 2: immunoblot with the pre-immune serum (PIS); lanes 3 and 4: immunoblot with the immune serum anti-TOGp (IS). (B) The specificity of different antibodies raised against TOGp was assessed using cell extracts from: human pluripotent embryonal carcinoma NT2-D1 (lanes 1, 3 and 5) and human neuroblastoma cells SH-SY5Y. Immunoblots were revealed with the affinity purified anti-human TOGp antibodies (lanes 1 and 2), rabbit antisera anti-TOGp (lanes 3 and 4) or with mouse antisera anti-TOGp (lanes 5 and 6). Molecular mass sizes are given in kDa.

bacterial cells producing the GST-TOGp fusion product with the anti-TOGp immune serum revealed a 150 kDa polypeptide corresponding to the GST-TOGp fusion protein, as well as several degradation products with lower molecular masses (Fig. 1A, lane 4). No staining was observed when the immune serum was used to stain blots of protein extracts from uninduced bacteria (Fig. 1A, lane 3). Preimmune serum did not react with any proteins in protein extracts prepared from either induced (Fig. 1A, lane 2) or uninduced bacteria (Fig. 1A, lane 1).

To further insure the specificity of the TOGp antiserum, the immune sera were affinity purified using bacterially-expressed TOGp protein lacking the 844 N-terminal amino acids. To examine the capacity of these different antibodies to recognize the human endogenous TOGp protein, we used these affinity-purified antibodies to probe immunoblots of total protein extracts from two human cell lines: NT2-D1 (Fig. 1B, lanes 1, 3 and 5) and SH-SY5Y (Fig. 1B, lanes 2, 4 and 6). For comparison, similar blots were probed with the affinity-purified antibodies (Fig. 1B, lanes 1 and 2), the unpurified immune serum from rabbits (Fig. 1B, lanes 3 and 4) or mice (Fig. 1B, lanes 5 and 6). All three antibody preparations specifically bound the same polypeptide of approximately 220 kDa in both cell lines (the predicted molecular mass of TOGp is 218 kDa). An additional band of approximately 150 kDa was recognized by both the affinity-purified and the crude rabbit antisera. In different protein preparations, in which the 150 kDa polypeptide was initially absent, its appearance then increase is correlated with a decreasing abundance of the 220 kDa band. Furthermore, the reaction to both 220 kDa and 150 kDa polypeptides was eliminated by preadsorption of the antisera with the TOGp antigen. From these results we conclude that the 150 kDa species is a breakdown product resulting from proteolysis of the 220 kDa TOGp.

Localization of the TOGp protein in human interphasic cells

To further characterize the intracellular distribution of TOGp, we used anti-TOGp antibodies to examine permeabilized NT2-D1 and SH-SY5Y cells by immunofluorescence microscopy. All three antibody preparations (affinity-purified, and mouse or rabbit immune serum) gave identical staining patterns in both NT2-D1 (see Fig. 3A and G) and SH-SY5Y cells (not shown). Staining was concentrated in the perinuclear cytoplasm, in a somewhat punctuate pattern. Neither cell line was appreciably stained with either preimmune sera.

In order to better determine the localization of TOGp, we double stained cells with anti-TOGp antibodies and either NBD-ceramide or DiOC6, which localize preferentially within elements of the *trans*-Golgi (NBD-ceramide) (Pagano et al., 1989) or in vesicle-like organelles, plasma membrane and reticulum (DiOC6) in neurons (Dailey and Bridgman, 1989) and non-neuronal cells (Lipsky and Pagano, 1985; Terasaki et al., 1984). Anti-TOGp staining closely correlated with both DiOC6 or NBD-ceramide staining (Fig. 2).

Because NBD-ceramide and DiOC6 are not restricted to a unique intracellular structures, we double labeled cells with anti-TOGp and antibodies against marker proteins of the rough endoplasmic reticulum (RER) (Louvard et al., 1982) or the Golgi apparatus (CTR433; Jasmin et al., 1989). As shown in Fig. 3, the RER of NT2-D1 cells extends throughout the cytoplasm (green in B), and some colocalization of TOGp (red in A) was observed (areas of overlap appear yellow). However, TOGp was more concentrated in the perinuclear cytoplasm, and TOGp staining did not entirely correspond with that of the RER marker. Only partial colocalization of the perinuclear Golgi apparatus (Fig. 3, green in H) and TOGp (red in G) was observed (yellow in I).

To further examine the cytoplasmic localization of TOGp, we disrupted localization of the RER or Golgi with nocodazole (NZ) or brefeldin A (BFA), respectively, and examined the effects on TOGp distribution by immunofluorescence microscopy. As shown in Fig. 3, treatment with the microtubule depolymerizing drug nocodazole induced the ER to retract towards the cell center (Fig. 3, green in E) (Terasaki et al., 1984), where substantial co-localization of TOGp and the RER was observed (Fig. 3, yellow in F). In contrast, treatment of the

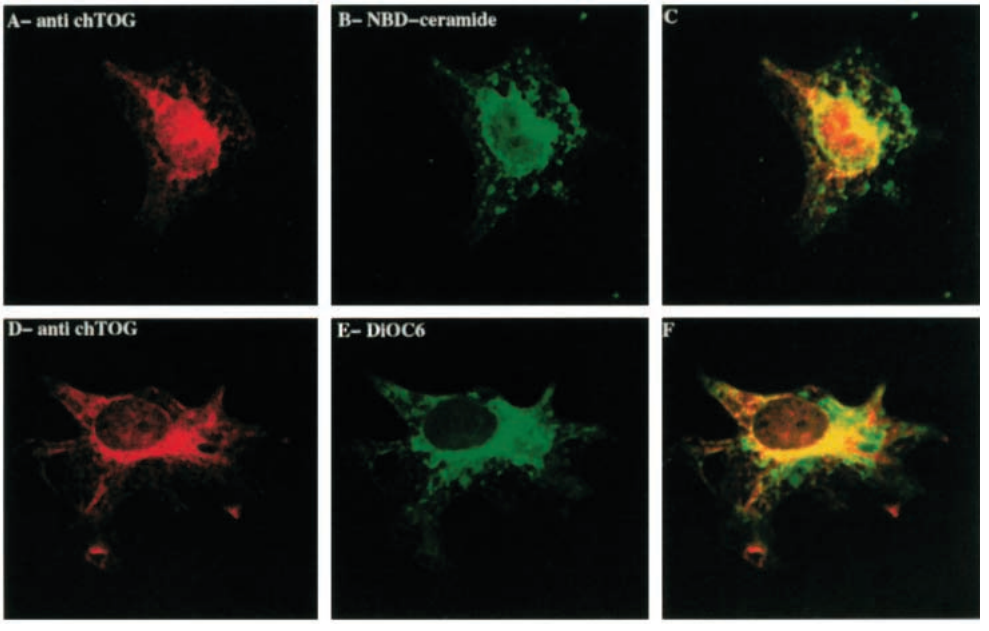


Fig. 2. Human interphasic NT2-D1 cells double stained with subcellular markers. NT2-D1 cells were fixed in formalin and permeabilized with Triton before staining with the mouse anti-TOGp antibodies (A and D, red); the *trans*-Golgi marker NBD ceramide (B, green); or the plasma and reticulum marker DiOC6 (E, green). The colocalization after staining summation appears in yellow (C and E).

NT2-D1 cells with brefeldin A, which causes rapid redistribution of Golgi proteins into the ER (Fujiwara et al., 1988) (Fig. 3, green K), had no significant effect on the cytoplasmic localization of TOGp (Fig. 3, red in J). Only a few regions of co-localization between TOGp and the Golgi were visible (Fig. 3, yellow in L). These results suggest that TOGp protein is associated mainly with the ER of interphase cells, consistent with its fractionation with microsomes from brain tissue (see below).

Subcellular localization of the TOGp protein during mitosis

Immunofluorescence microscopy revealed significant changes in the cytoplasmic distribution of TOGp as cells progressed from interphase into M-phase. To more carefully correlate the distribution of TOGp with cell cycle changes in cytoplasmic organization, NT2-D1 cells were double stained using anti-TOGp (red staining), and anti-tubulin antibodies (Fig. 4A, green staining) or CTR453 anti-centrosomes antibodies (Fig. 4B, green staining). During interphase, TOGp does not co-localize with either MTs or centrosomes. In contrast, anti-TOGp antibodies clearly stained the centrosomes of prophase cells (prophase Fig. 4A,B), without binding to the substantial MT network present in these cells. During prometaphase and metaphase (Fig. 4A), anti-TOGp staining was apparent throughout the mitotic spindle, while at anaphase, TOGp

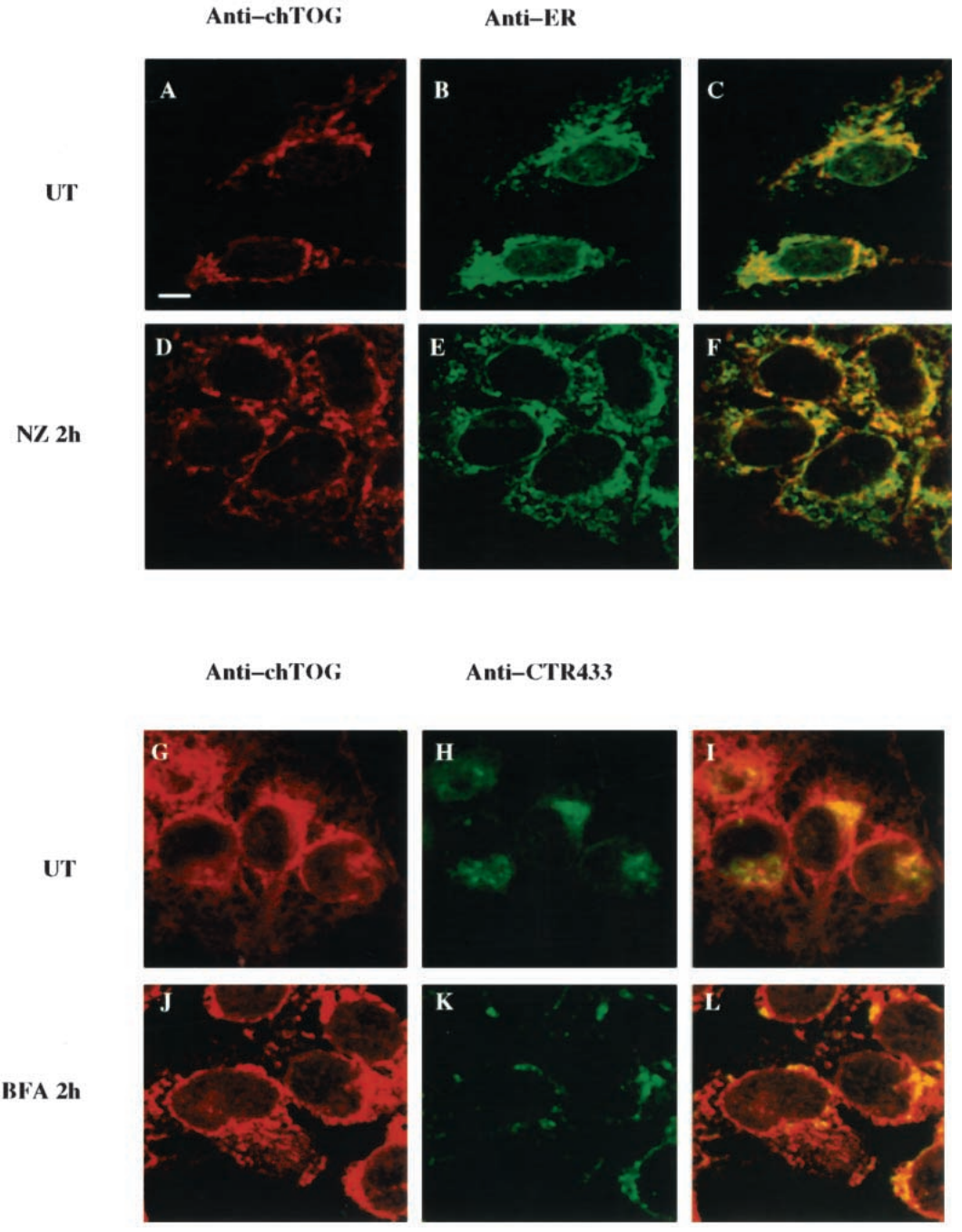
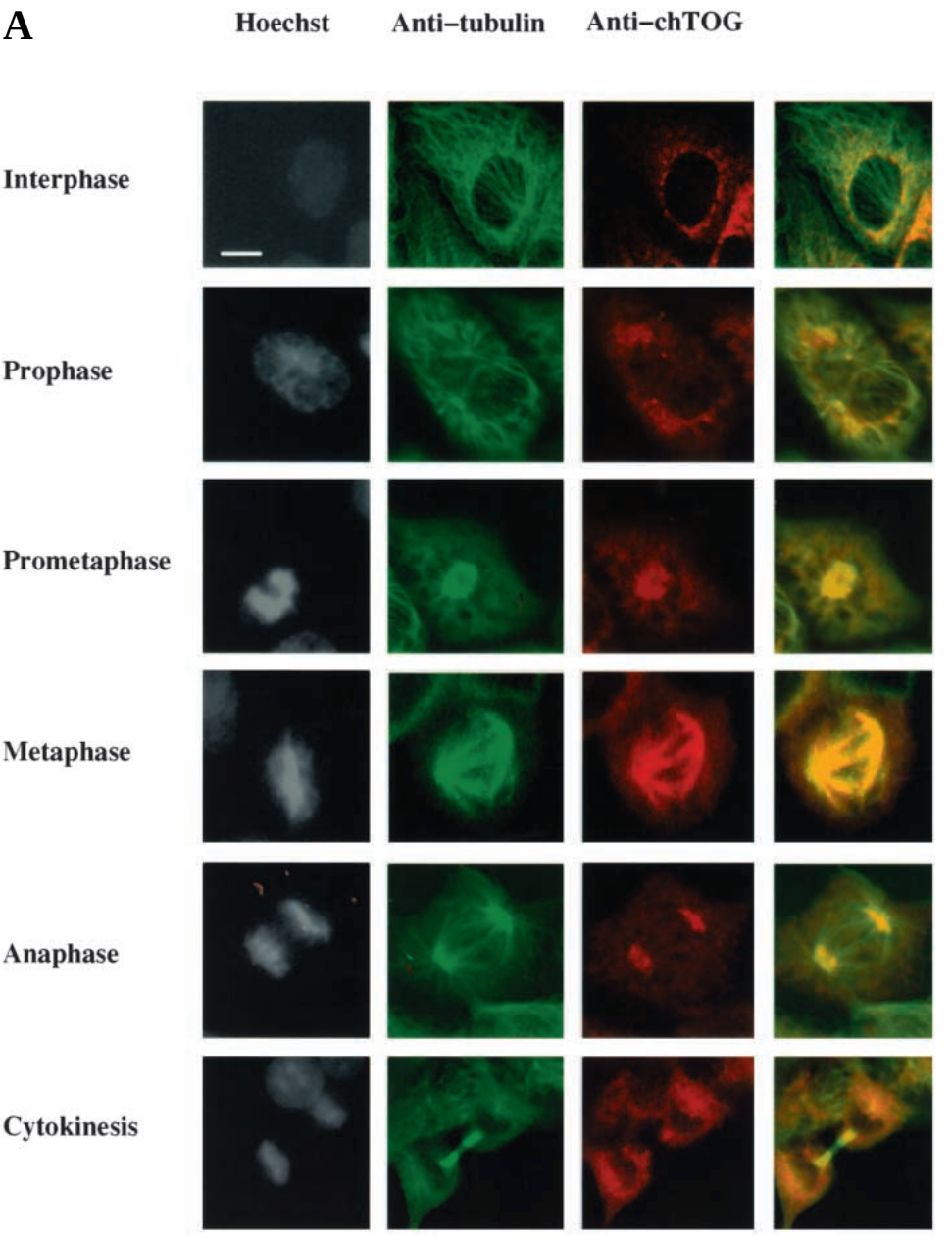


Fig. 3. Subcellular localization of TOGp protein in interphasic human cells and effects of nocodazole and brefeldin A treatment. NT2-D1 cells were fixed in formalin and permeabilized with Triton before staining with the mouse anti-TOGp antisera (A and D), affinity-purified TOGp antibodies (G and J, red), anti-RER (B and E), and anti-Golgi (H and K, green). The colocalization after staining summation appears in yellow (C,F,I,L). UT, untreated cells (A,B,C,G,H,I); NZ, nocodazole treatment (D,E,F), BFA, brefeldin A treatment (J,K,L). Bar, 10 μm.



appeared to be concentrated at the spindle poles. Finally, during cytokinesis, TOGp protein was observed concentrated between the nuclei and centrosome of the two daughter cells, whereas most of the microtubules become concentrated at the cleavage furrow (Fig. 4A).

TOGp co-fractionates with microsomes and MTs

The immunofluorescence localization suggested that TOGp was capable of binding both endoplasmic reticulum membranes (during interphase) and MTs (during mitosis). To confirm these localizations, we fractionated human brain subcellular structures into a microsome fraction and a MT/MAP fraction. The latter fraction was prepared by taxol-induced MT assembly (see Materials and Methods and Fig. 5A). As shown in Fig. 5B, TOGp is present in both MT (Fig. 5B, lane 2) and microsomal fractions (Fig. 5B, lane 4) from brain, while most of the tubulin was found in the microtubule fraction (data not shown). These results are consistent with those obtained by immunofluorescence microscopy. Most of the 150 kDa protein fragment identified with anti-TOGp antibodies (Fig. 1) copurified with microtubules. The TOGp observed in the nuclear fraction (Fig. 5B, lane 5) probably results from contamination of the nuclear fraction with microsomes or other cytoplasmic membranes.

Protein fractions enriched in TOGp promote MT assembly

The above results indicated that TOGp was capable of binding taxol MTs. We therefore tested whether TOGp modulates MT assembly in vitro. A protein fraction enriched in TOGp was prepared from pig brains by ion exchange chromatography (see Materials and Methods) (Fig. 6A). Two major contaminants

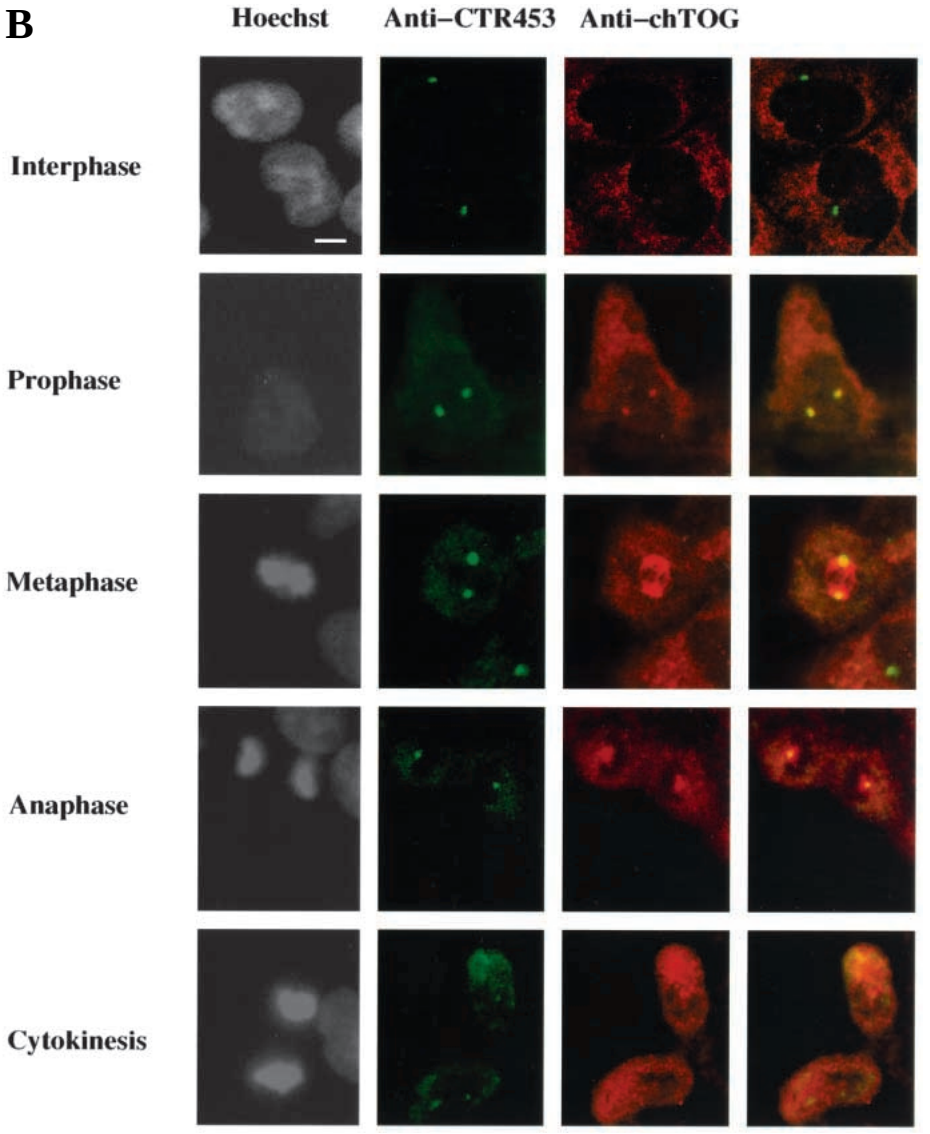


Fig. 4. Subcellular localization of TOGp in mitotic human cells. Confocal indirect immunofluorescence comparison of the subcellular localization of TOGp (red in A and B) with that of MTs (green in A) or centrosomes (green in B) during mitosis. A co-localization of TOGp with a reference structure appears in yellow (fourth column on both figures). Pictures were obtained after methanol (–20°C) fixation by the use of anti-TOGp antibodies, anti-tubulin antibodies and anti-CTR453 antibodies (see Materials and Methods). The first column of each figure represents the Hoechst staining of DNA. Bars, 10 μm.

(molecular mass 66 and 48 kDa) were found to co-purify with TOGp. The effects of this TOGp enriched fraction on the tubulin polymerization was first examined by light scattering (Fig. 6B). The initial velocity of tubulin polymerization in the presence of TOGp (0.011±0.002 ∂A.minutes^{-1}) is enhanced 5- to 6-fold versus control experiments (0.002±0.001 ∂A.minutes^{-1}) (Fig. 6B) that include addition of the fraction lacking TOGp and the fraction immunodepleted in TOGp with anti-TOGp antiserum (see Materials and Methods). Turbidimetry readings demonstrate an increase in the tubulin polymer concentration. To complete this finding, we repeated the assembly experiment in the presence of microtubule organizing centers (i.e. centrosomes from HeLa cells). As seen in Fig. 7, the initial nocadazole treatment of living cells resulted in complete depolymerization of MTs (A). The resulting lysed cells were then used to nucleate MT assembly. Addition of tubulin alone (B) or tubulin plus the fraction lacking TOGp (C) resulted in the formation of few short MTs. In contrast, in the presence of the TOGp rich fraction, the tubulin polymers formed and assembled as asters around specific structures (Fig. 7C,D,F). This phenomenon is abolished by immunodepletion of TOGp with anti-TOGp antiserum (E) but not with the preimmune serum (F).

Since MTs continually assemble and disassemble by a process termed dynamic instability (Mitchison and Kirschner, 1984; Cassimeris, 1993), we then examine individual MT assembly from axoneme fragments using video enhanced DIC microcopy in the presence or absence of TOGp. As shown in Fig. 8A and B, TOGp promoted faster MT elongation rates at both plus and minus ends by approximately twofold (P<0.01). Similar results were obtained with two tubulin preparations. These tubulin

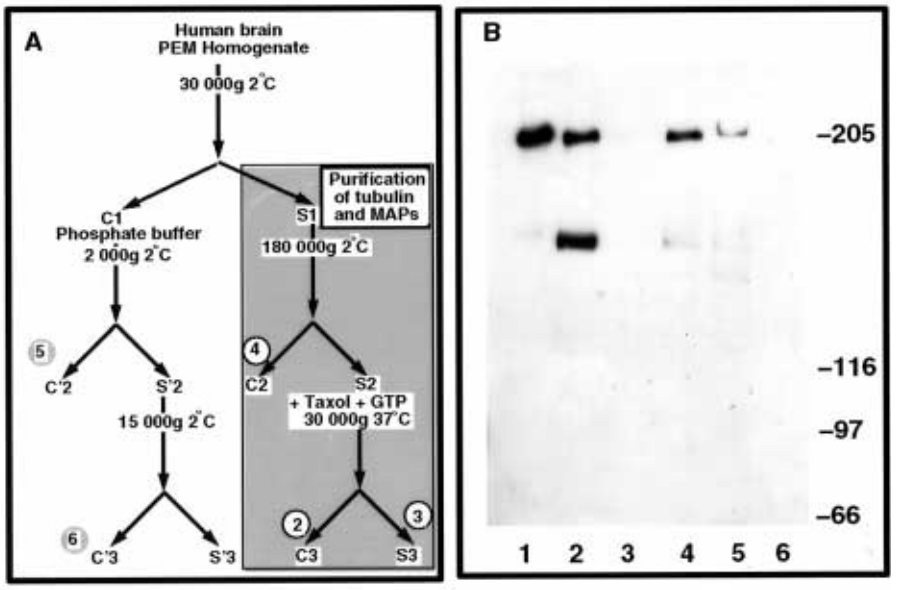


Fig. 5. Co-purification of TOGp with MTs isolated from human brain. MTs from human brain and associated proteins were purified using taxol as described in Materials and Methods. (A) Schematic protocol of the different purification steps. The numeral associated with each fraction refers to the lane number in B of the figure. (B) SDS-PAGE and immunoblot analysis of TOGp protein: lane 1: microsomal fraction of NT2-D1 cells used as control; lane 2: MAP-containing MTs (30,000 ***g*** pellet after taxol addition); lane 3, 30,000 ***g*** supernatant; lane 4: corresponding microsomal pellet; lane 5: nuclear fraction; lane 6: mitochondrial fraction.

preparations had a low rate of catastrophe (the switch from growth to shortening) so limited information is available on the effects of TOGp on catastrophe, shortening and rescue. However, these experiments suggest that TOGp inhibits catastrophes but does not inhibit shortening velocity.

TOGp is homologous to the *Xenopus* microtubule-associated protein XMAP215

Comparison of the TOGp amino acid sequence (deduced from that of its cDNA) with the Swiss Prot data base revealed no extended regions of homology with previously identified vertebrate MAPs. However, peptide sequences from XMAP215, a microtubule-associated protein isolated from *Xenopus* eggs (Gard and Kirschner, 1987), revealed significant sequence similarity with TOGp (see Table 1). Nine XMAP215 peptides exhibitted sequence identity to the predicted sequence of TOGp ranging from 54% (XMAP-7) to 90% (XMAP-1 and 10). When conservative amino acid substitutions are considered, the amino acid sequence similarity between the XMAP peptides and corresponding regions of TOGp ranges from 64% (XMAP-3) to 100% (XMAP-5). As shown in Table 1, the nine sequenced XMAP215 peptides are located throughout the C-terminal three quarters of the TOGp protein, suggesting that the homology between XMAP215 and TOGp extends througout their entire sequences.

To further confirm the structural homologies, cytoplasmic extracts from *Xenopus* eggs, and fractions containing XMAP215 were analyzed by SDS-PAGE and western blotting using polyclonal antibodies specific for TOGp. As shown in Fig. 9, anti-TOGp antibodies detected the same high-molecular weight protein in egg cytoplasm and in all XMAP215 containing fractions. The protein recognized by anti-TOGp antibodies (Fig. 9C) co-migrated with the 215 kDa XMAP215 protein revealed either by Coomassie blue staining (Fig. 9A)

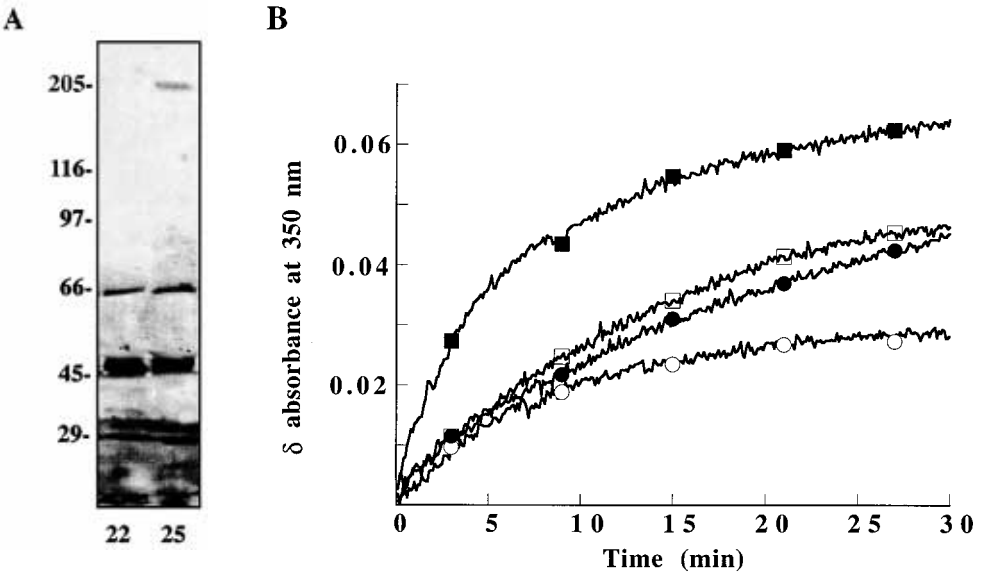
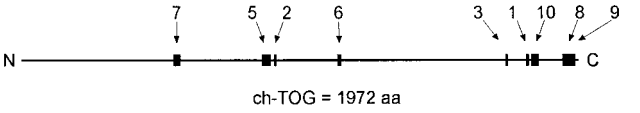


Fig. 6. TOGp promotes MT assembly. (A) Coomassie stained SDS-PAGE gel of a TOGp enriched fraction isolated from pig brain (labeled 25) and a fraction containing identical contaminants but lacking TOGp used in control experiments (labeled 22). (B) Spectrophotometric measurements of MT assembly from tubulin alone (filled circles), tubulin plus a TOGp enriched fraction (filled squares), tubulin plus a fraction (fraction 22) containing identical contaminants (open circles) and fraction 25 pretreated with anti-TOGp antibodies (open squares). The symbols do not represent individual readings but were overlayed on the plots to simplify identification. This experiment was performed three times with similar results.

Table 1. TOGp exhibits sequence similarity to XMAP215

Peptide	Amino acid sequence (one letter code)	% I (% S)
XMAP-1	GGLAE LYEYK	90 (90)
	:::: :::::	
1787-1796	EGLAE LYEYK	
XMAP-2	FIxPx IGHLP RA	67 (75)
	:: : ::.:: :	
893-904	FIQPN IGELP TA	
XMAP-3	ITDGQ VMIEN K	55 (64)
	: : ::.: :	
1714-1724	ILDHL TMIDN K	
XMAP-5	DLLPR TDISD KITSD LVTKI AEKNW KIRKE GLDEV	86 (100)
	::::: :.:::: ::::. ::.:: ..::: ::::: :::::	
850-884	DLLPR TEISD KITSE LVSKI GDKNW KIRKE GLDEV	
XMAP-6	DYKPD PKKTK PGAPA SKAKT AS	55 (82)
	. ::: ::::.: . . . ::::. :	
1119-1140	EPKPD PKKAK APGLS SKAKS AQ	
XMAP-7	GKAAA APKKA PAAKP GGPVK KAKAP A	54 (85)
	. .. . ::: ::::. ::: : :.:.. :	
537-562	APKPG PLKKA PAAKA GGPPK KGKPA A	
XMAP-8	DMLHS KLSQL RESTE QFQxV DLD	83 (83)
	::::: ::::: ::: : : : :::	
1915-1937	DMLHS KLSQL RESRE QHQHS DLD	
XMAP-9	DSNQT RPSTT TSSSA SSTNI DDLKK	68 (92)
	::::: ..: : .::. :..:: :::::	
1937-1961	DSNQT HSSGT VTSSS STANI DDLKK	
XMAP-10	DIKPF LKNSS QFFQS YVERG LRLIE MExEG	90 (93)
	:: :: ::::: ::::: ::::: ::.:: :: ::	
1803-1832	DIEPF LKNSS QFFQS YVERG LRVIE MEREG	

Total identity: 73% (142/194) Total similarity: 88% (170/194)



ch-TOG = 1972 aa

The amino acid sequences of nine proteolytic fragments of XMAP215 (XMAP-1 through -3 and -5 through -10) and the corresponding regions of TOGp are shown. Positions with identical amino acids (:) or conservative substitutions (.) are indicated. Amino acid sequence identity (% I) ranged from 54-90%. Amino acid sequence similarity (% S) ranged from 64-100%. The locations of the nine peptide sequences in the entire TOGp sequence are shown in the accompanying map. XMAP215 was purified according to the method of Gard and Kirschner (1987). Peptides 1-3 were generated by endo-Lys-C digests. Peptides 5-10 were generated by digestion with endo-Asp-N. Peptides were separated by reverse phase HPLC and microsequenced. x designates an unknown amino acid. cDNAs encoding peptides 5 and 10 were subsequently cloned from a *Xenopus* cDNA library using degenerate PCR primers, allowing assignment of unknown amino acids in those peptides (W25 and R28 in XMAP-5 and R22 in XMAP-10) (cloning data from B. Becker, S. Charrasse, C. Larroque and D. L. Gard, unpublished observations).

or detection with affinity purified XMAP215 antibodies (Fig. 9B).

DISCUSSION

TOGp is a MT-associated protein and the human homolog of the *Xenopus* XMAP215

The ch-TOG gene was initially identified as a cDNA over-expressed in human colonic and hepatic tumors (Charrasse et al., 1995). For this report, we have raised polyclonal antibodies against a bacterially expressed C-terminal region of the corresponding protein product (TOGp) and used them to characterize the subcellular localization and functional activities of TOGp. These antibodies specifically recognize an ≈220 kDa protein in human tissues, *Xenopus* eggs and in two human neuroblastoma cell lines, consistent with the predicted size (218 kDa) of the ch-TOG gene product.

Several lines of evidence indicate that TOGp may be a new human MAP. First, TOGp localizes to microtubules of the mitotic spindle and spindle poles in several cell lines. Second, it co-pellets with taxol stabilized MTs from human brain. Third, TOGp promotes rapid MT assembly in vitro. Finally the predicted TOGp amino acid sequence shows substantial sequence similarity to XMAP215, a MAP isolated from *Xenopus* eggs. Indeed, the sequence indentity between these proteins ranges from 55 to 90%, the similarity scores ranging from 64 to 100%. The XMAP215 peptides corresponded to sequences spread throughout the C-terminal three-quarters of TOGp suggesting that homology was not limited to a small protein domain. This conclusion is further supported by preliminary sequencing of cDNA encoding XMAP215 (B. Becker, S. Charrasse, C. Larroque and D. L. Gard, unpublished observations).

Although TOGp is likely a MT-binding protein, sequence comparison revealed that TOGp has no extensive homology with several well characterized MAPs. However, a small motif similar to the typical microtubule binding sequence repeats of MAP2, MAP4 or tau is present in TOGp (amino acids 1,773 to 1,787, see Fig. 10). This sequence is organized around a K(I/V)GS cluster known to regulate the affinity of tau for microtubules by phosphorylation of the serine residue (Biernat et al., 1993). The existence of the KIGS sequence in TOGp suggests that it also may be phosphorylated by the p110 kinase which recognizes the KIGS motif (Drewes et al., 1995). Although this motif forms part of the microtubule binding repeat in the other MAPs (which contain three to five copies of the conserved repeat; Chapin and Bulinski, 1992), it is present only once in TOGp.

The sequence of TOGp also contains a PASAPA motif and a serine and proline residues rich region (S,P domain) (residues 1,000-1,200) in which serine and proline residues are separated by one or two amino acids (mainly SXP or SXXP). This proline-rich region (that included the PASAPA sequence) is also found in MAP4 isoforms (West et al., 1991). Finally, the TOGp sequence does not contain the typical KDM repeat of MAP4 or the KKEE, KK(I/V) repeats of MAP1 (Noble et al., 1989). Thus, the structure of TOGp appears different from the previously described vertebrate MAPs.

Proteins associated with MT and sharing similarities with TOGp have been characterized in yeast. These proteins are smaller than TOGp and the similarities are restricted to the N terminus of TOGp. $p93^{dis1}$ identified in *Schizosaccharomyces pombe* (Nabeshima et al., 1995) encodes an 882 amino acid protein which shows 24% identity and 49% similarity to TOGp. The Stu2p protein of *Saccharomyces cerevisiae* is also likely to be a MT binding protein (Wang et al., 1995). Its 882 amino acid sequence is 23% identical and 46% similar to the N-terminal protein sequence of TOGp. These similarities in sequence and MT-binding suggest that TOGp is the human version of an evolutionarily ancient family of MAPs.

TOGp promotes tubulin assembly

When combined with purified tubulin, a TOGp fraction isolated from pig brain promotes MT assembly both in solution

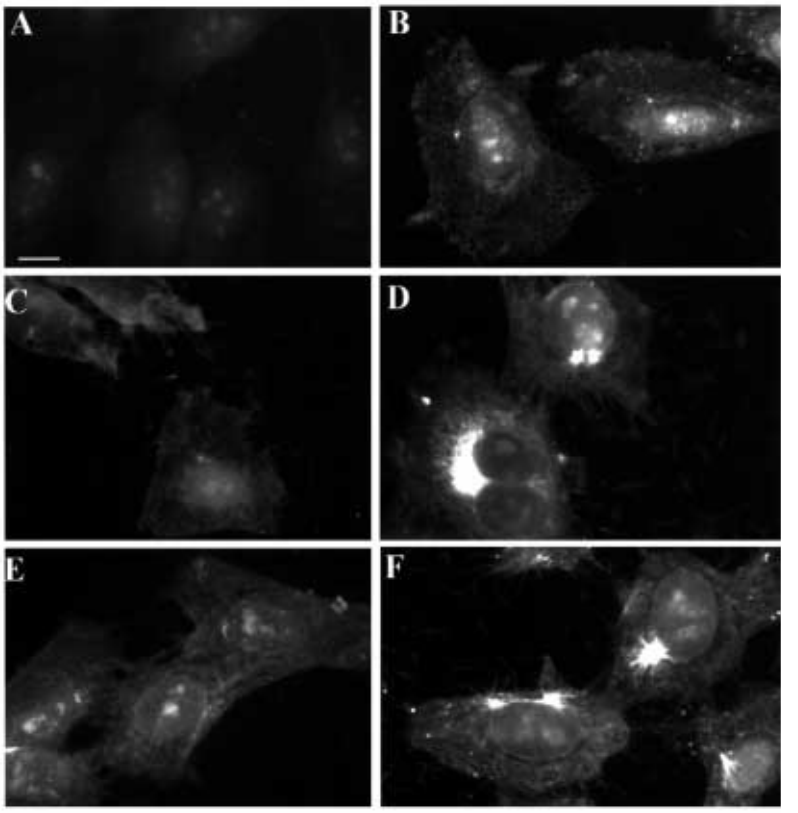


Fig. 7. Re-growth of microtubules in HeLa cells. (A) Endogenous tubulin staining after nocodazole treatment; (B) re-growth of microtubules with 20 μM tubulin alone; (C) 20 μM tubulin in presence of the fraction 22 lacking TOGp (used as a control); (D) 20 μM tubulin in presence of fraction 25 enrichied in TOGp; (E) fraction 25 immunodepleted in TOGp with anti-TOGp antibodies; (F) fraction 25 immunodepleted with preimmune antiserum.

and in the presence of MT organizing centers. While TOGp promotes MT assembly, it differs in activity from that of XMAP215. Unlike other well characterized MAPs, XMAP215 promotes MT assembly in an end dependent manner: it increases plus end assembly rate ~7-fold while only increasing minus end assembly by <2-fold (Gard and Kirschner, 1987; Vasquez et al., 1994). This end specificity was not observed with TOGp. The source of the differences in activity between TOGp and XMAP215 remains unknown. However, preliminary results suggest that several isoforms of TOGp are expressed in different tissues (Charrasse et al., 1996; and L. Cassimeris, unpublished) and it is possible that the brain isoform studied here is less active in promoting MT assembly. TOGp effects MT assembly in a manner similar to MAPs 1, 2 and tau (Pryer et al., 1992; Drechsel et al., 1992; Kowalski and Williams, 1993; Trinczek et al., 1995; Vandecandelaere et al., 1996). In contrast, TOGp does not appear to slow MT shortening rate, suggesting that TOGp's interaction with the MT lattice differs from the neuronal MAPs since these MAPs slow the rate of shortening (Pryer et al., 1992; Drechsel et al., 1992; Kowalski and Williams, 1993; Trinczek et al., 1995; Vandecandelaere et al., 1996).

The concentration of TOGp necessary to see detectable effects on MT assembly was approximately 4-fold higher in the video assays compared to that in the spectroscopic assays. The source of the different efficacies of TOGp in the two assays is unknown. It is possible that some of the TOGp was inactivated in the video assay by binding to glass surfaces of slide and coverslip as it has been noted previously for porcine brain MAPs (Pryer et al., 1992) and XMAP215 (Vasquez et al., 1994). Alternatively, TOGp may have lost some assembly-promoting activity after freezing and thawing since partial loss of assembly-promoting activity has been observed previously with XMAP215 (Gard and Kirschner, 1987).

What is the role of TOGp?

The subcellular fractionation as well as the immunofluorescence microscopy suggest that TOGp is associated with the ER during interphase and with centrosomes and spindles MT during mitosis. Although localization with the ER has not been observed with other characterized MAPs from brain, cell-cycle dependent binding of protein to MTs is not without precedent. For example, NuMA protein binds spindle MTs during mitosis but is present in the nucleus in interphase cells (Compton et al., 1992). Interestingly, the TOGp homologs $p93^{dis1}$, Stu2p and XMAP215 localize to mitotic spindle and/or spindle pole (Nabeshima et al., 1995; Chen and Huffaker, 1996; Gard et al., 1995). While $p93^{dis1}$ also localizes to interphase MTs, the interphase localizations of Stu2p and XMAP215 remain unclear.

The dual localization of TOGp to the ER and spindle MTs and the paradoxical abundance of its mRNA in both non-dividing tissues, i.e. Purkinje cells (Charrasse et al., 1996) and tumors (Charrasse et al., 1995) is suggestive of different functions for this protein that are dependent on the cell cycle. The relatively high expression of TOGp in brain tissues (Charrasse et al., 1995) suggests that TOGp plays a specific role in neuronal cells. Purkinje cells have an extensive and branched dendritic tree (Junqueira et al., 1992) which is capable of continual reorganization (e.g. Kim and Thompson, 1997). In cultured neuronal cells, dendritic MTs are also dynamic (Baas et al., 1991; Bass and Ahmad, 1992) suggesting

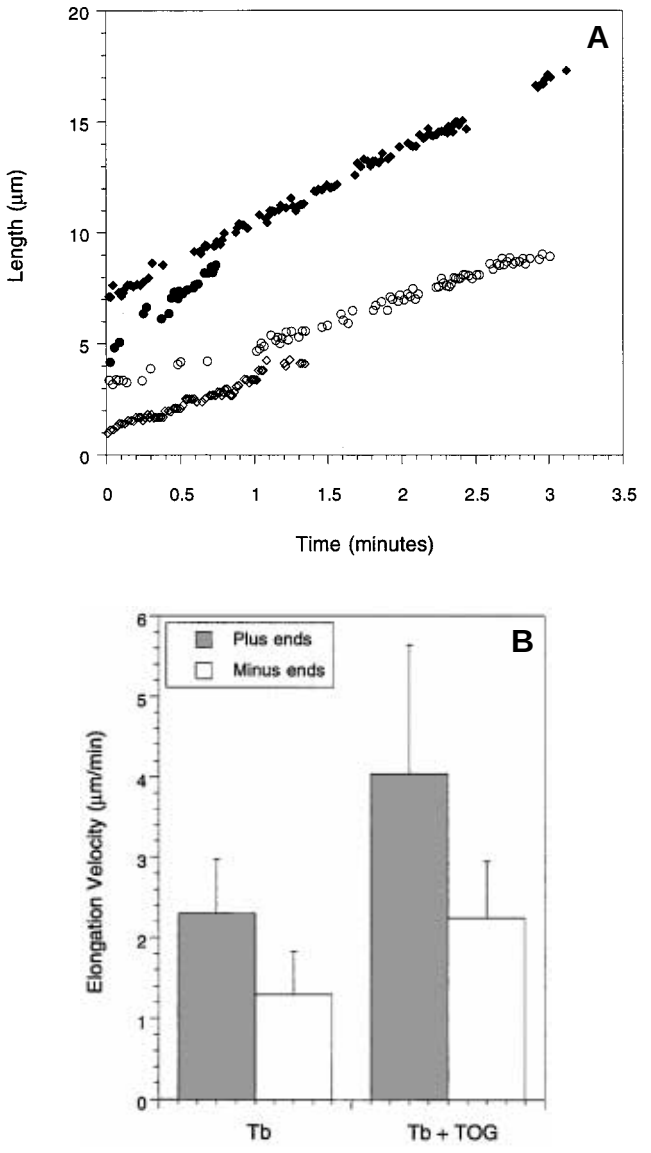


Fig. 8. Individual MT assembly examined using video enhanced DIC microscopy. (A) Examples of individual MT assembly from the plus ends of axoneme fragments observed by video-enhanced DIC microscopy. MTs were assembled from 10 μM tubulin alone (open symbols) or 10 μM tubulin plus the TOG enriched fraction (estimated TOGp concentration of 0.15 μM; closed symbols). (B) Summary of MT elongation rates at plus and minus ends. Addition of the TOGp enriched fraction (~0.15 μM TOGp) to 10 μM tubulin resulted increased elongation velocity ~ 2-fold at both MT plus and minus ends (n = 25 and 15 for plus and minus ends assembled from tubulin alone and n = 27 and 24 for assembly in the presence of TOGp at plus and minus ends).

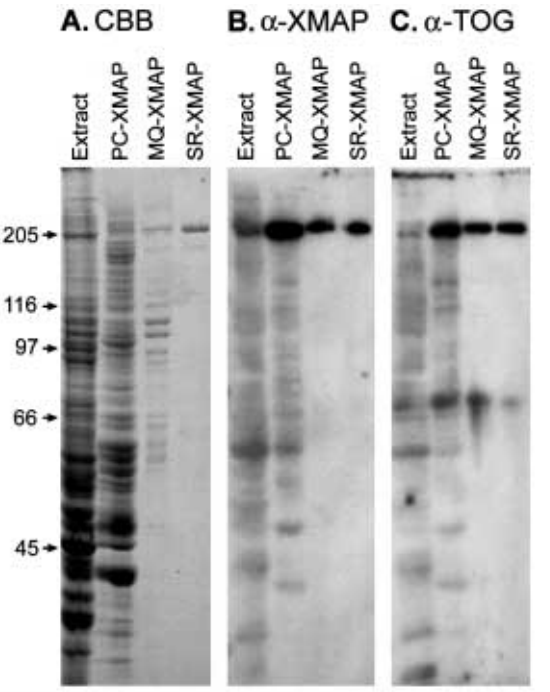


Fig. 9. Cross-reactivity between the XMAP215 protein and TOGp antibodies. Coomassie blue-stained gel showing the purification of XMAP from *Xenopus* eggs (A) and immunoblot identification of XMAP215 with either XMAP215 antibodies (B) or TOGp antibodies (C). Extract: total egg extract; PC-XMAP: phosphocellulose 150 mM-300 mM fraction; MQ-XMAP: peak fractions from mono-Q chromatography; SR-XMAP: peak fractions from Superose 6 chromatography.

that the high concentration of TOGp in Purkinje cells could promote MT assembly and/or contribute to the organization of ER along MTs in these cells.

The number of known proteins required for completion of mitotic events and their temporary recruitment to the centrosome and mitotic spindle is rapidly increasing (King et al., 1995; Chen et al., 1997). The localization of TOGp to the spindle MT suggests that it is likely to play a role during mitosis and that it may also function in spindle organization through its prophase localization to spindle poles. Furthermore, the similarity between TOGp and $p93^{dis1}$ together with the role of $p93^{dis1}$ in sister chromatide separation (Nabeshima et al., 1995) strengthen the idea of a function for TOGp during mitosis.

The over-expression of TOGp in tumors also suggests it has a role in cell division and proliferation. TOGp is not the only MT assembly regulator over-expressed in tumor cells. Belmont and Mitchison (1996) recently identified a MT catastrophe promoter and found that this protein is Op18/stathmin, a protein over-expressed in acute leukemia (Hanash et al., 1988), several solid tumors and in proliferating cell types (Rowlands et al., 1995). While TOGp and Op18 are over-expressed in tumors of different origins, it is possible that expression of these proteins is necessary for rapid remodeling of the MT cytoskeleton or spindle assembly in rapidly dividing tumor cells or early embryos.

While the MT cytoskeleton is clearly required for cell division, mutations in cytoskeletal proteins are unlikely to be causal agents in tumorgenesis (Johnston et al., 1992). Instead, high levels of TOGp and Op18/stathmin may result from a micro-evolutionary process (see Vogelstein and Kinzler, 1993; Nowell, 1993) which would favor the survival of cells over-expressing TOGp and Op18/stathmin by allowing the rapid MT rearrangements necessary for mitosis and completion of rapid cell cycles. The over-expression of ch-TOG mRNA in tumor

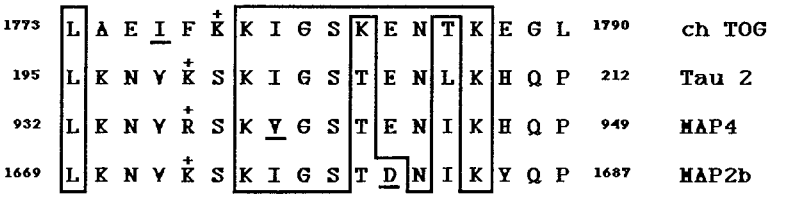


Fig. 10. MT binding motif in TOGp. Comparison between the amino acid sequence of TOGp and the first of the tandem repeats in the tubulin binding domain of human tau 2 (SwissProt access P10636, gb X14474), MAP4 (gb M64571) and MAP2 (gb U01828). Numbers at the left and the right of the sequence indicate the position of the motif in full sequences. Regions of identity are boxed while conservative substitutions are underlined.

cells (Charrasse et al., 1995) and the corresponding higher expression of XMAP215 during early embryogenesis compared to adult tissues (Gard and Kirschner, 1987) may also reflect a de-differentiation of the tumor cells to up-regulate genes normally highly expressed in development, although it is not yet clear whether tumors arise by dedifferentiation (see Sell, 1993). Future studies of mechanisms regulating the expression levels of TOGp and Op18/stathmin may provide important clues to changes occurring in gene expression during tumorogenesis.

In summary, we have presented evidence that the protein product of the human TOG gene functions as an MT-associated protein: it is localized to mitotic spindles in vivo, binds to and promotes MT assembly in vitro, and is structurally homologous to MAPs present in *Xenopus* and yeast. Given the homologies between $p93^{dis1}$, Stu2p, XMAP215 and TOGp, we suggest that these proteins constitute an evolutionarily-conserved family of MAPs, with important functions in mitosis. The lack of substantial sequence similarity to other MAPs whose sequences have been determined also suggests that this family of MAPs contain a unique MT binding domain, supporting a previous proposal by Vasquez et al. (1994) that was based on the novel effects of XMAP215 on MT assembly.

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