Complex relationship between TCTP, microtubules and actin microfilaments regulates cell shape in normal and cancer cells

Franck Bazile, Aude Pascal, Isabelle Arnal1, Christophe Le Clainche2, Franck Chesnel and Jacek Z.Kubiak*

CNRS UMR 6061, Institute of Genetics & Development, Mitosis & Metiosis Group, IFR 140 GFAS, Faculty of Medicine, University of Rennes 1, 35043 Rennes cedex, France. 1Equipe Tubulin and Interacting Proteins, UMR 6626 CNRS/University of Rennes 1, 35042 Rennes cedex, France and 2Laboratoire d’Enzymologie et Biochimie Structurales, UPR 3082 CNRS, 91198 Gif-sur-Yvette, France

*To whom correspondence should be addressed: Tel: +33 02 23 46 98 78; Fax: +33 02 23 23 44 78; Email: jacek.kubiak@univ-rennes1.fr

Transitionally controlled tumor-associated protein (TCTP) is a ubiquitous and highly conserved protein implicated in cancers. Here, we demonstrate that interactions of TCTP with microtubules (MTs) are functionally important but indirect, and we reveal novel interaction of TCTP with the actin cytoskeleton. Firstly, immunofluorescence in Xenopus XL2 cells revealed cytoplasmic fibers stained with TCTP but not with tubulin antibodies, as well as MTs free of TCTP. Furthermore, TCTP localized to a subset of actin-rich fibers in migrating cells. Secondly, Xenopus laevis TCTP did not affect in vitro assembly/disassembly of MTs and lacked MT-binding affinity both in pull-down assays and in cell-free extracts. Although TCTP also failed to bind to purified filamentous actin (F-actin), it was associated with microfilaments in cell-free extracts. Thirdly, TCTP concentrated in mitotic spindle did not colocalize with MTs and was easily dissociated from these structures except at the poles. Finally, RNA interference knockdown of TCTP in XL2 and HeLa cells provoked drastic, MT-dependent shape change. These data show that although TCTP interacts with MTs, it does not behave as classic MT-associated protein. Our evidence for an association of TCTP with F-actin structures, and for an involvement in cell shape regulation, implicates this protein in integrating cytoskeletal interactions both in interphase and mitosis providing a new avenue to fully understand the role of TCTP.

Introduction

Transitionally controlled tumor-associated protein (TCTP), also known as p23, IgE-dependent histamine-releasing factor, mortalins or fortillin, is a highly conserved protein, which is upregulated in certain cancers [e.g. prostate cancer cells (1)] and cancerous cell lines [e.g. in colorectal cancer cell line Caco-2 (2)]. Reduced expression of TCTP in cancerous cell lines reverts the transformed phenotype (3,4), whereas overexpression protects from apoptosis (5–7). Its recent identification as a partner of Chfr, the key component of the early mitotic prophase checkpoint, increased again the interest in a potential role of TCTP in cancer etiology (8).

TCTP was initially identified as a growth-related protein on the basis of its transationally dependent regulation of expression in mouse ascetic tumor and erythroleukemic cells (9,10). It is implicated in a broad diversity of intracellular functions as a stimulator of cell proliferation, growth, survival and stress responses (see ref. 11). TCTP shares structural features with Rab-binding proteins from the Mss4/Dss4 family (GFCs), leading to the suggestion that TCTP could be a chaperone (12). Besides certain cancerous cells, TCTP is particularly abundant in highly proliferating cells, e.g. in spermatogonia of fetal rat testis and neonatal as well as in adult human testis (13). In Drosophila melanogaster, TCTP controls cell growth and the rate of proliferation by regulating Drheb GTPase (14). Mouse TCTP gene inactivation is embryonic lethal; however, fibroblasts derived from TCTP−/− embryos apparently proliferate at a wild-type rate (15). This indicated that TCTP is not essential for cell viability (at least in fibroblasts) but may be involved in essential developmental processes in the mouse. TCTP is also a well-known calcium-binding protein (1,16,17).

Mechanisms by which TCTP is implicated in so different intracellular functions remain elusive, except for a recently described role as a transcription factor regulating oct4 and nanog genes expression (18). The activity of TCTP as transcription factor activating the pluripotency genes oct4 and nanog (18) together with abundance of TCTP in highly proliferating cells makes this protein a potential candidate for a regulator of early development and stem cells proliferation. Indeed, a phosphorylated form of TCTP affected the reprogramming of nuclei in bovine nuclear transplant experiments and the rate of successfully cloned calves increased when this form of TCTP was enriched in oocytes (19). This effect of TCTP may depend on its activity as a genetic regulator either as a transcription factor or a regulator of translation as it was reported to interact with elongation factor-1 delta (20).

Given that TCTP also resides in the cytoplasm and is associated with the cytoskeleton, it is probably to have non-genomic, cytoskeleton-mediated cellular functions. Several independent observations have led to a suggestion that TCTP interacts with microtubules (MTs). TCTP has been reported to colocalize with MTs in vivo and could be purified in a complex with tubulin and MTs, with a potential MT-binding domain identified in the N-terminal part of the protein (21). Yeast mutants lacking TCTP are hypersensitive to the MT inhibitor benomyl providing a genetic link between TCTP and MT function (22). Consistent with this, in mouse oocytes and embryos, antibodies raised against TCTP decorate the mitotic spindle (23), while phosphorylation of TCTP by a key cell cycle-regulating kinase Plk1 has been implicated in destabilizing MTs (24). These various observations are suggestive of a close relationship between TCTP and the MT cytoskeleton, which may be important for regulation of cell cycle events, proliferation and therefore also for tumorgenesis.

Here, we have examined in greater detail the association of TCTP with the cytoskeleton in Xenopus XL2 and human HeLa cells as well as in Xenopus oocytes‘ and embryos’ cell-free extracts. The main goal was to define the cytoplasmic as opposed to transcriptional roles of this protein. Our data indicate that TCTP association with MTs is qualitatively different from that of conventional MT-associated proteins (MAPs) and is also tightly associated in a MT-independent manner with spindle poles in mitosis. Our major finding is that TCTP associates selectively with certain filamentous actin (F-actin) structures. Functional studies further indicate that TCTP is involved in regulating cell shape both during interphase and mitosis, probably via complex interactions with both the actin and MT cytoskeleton. Our study sheds new light on a plausible cytoskeleton-related role of TCTP in carcinogenesis and especially in tumor reversion (3,4).

Materials and methods

Tissue culture cells

The XL2 cell line was cultured in L-15 medium supplemented with 10% fetal calf serum (full medium) and incubated at 25°C in air. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and incubated at 37°C in 5% CO2. Media were supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml).
Immunocytochemistry of tissue culture cells

HeLa cells seeded on glass coverslips were fixed in 75% methanol, 3.7% formaldehyde, 0.5% phosphate-buffered saline (PBS) or in 3.7% paraformaldehyde in 1× PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min. DNA was visualized using 4’,6-diamidino-2-phenylindole. Polyclonal antibodies against Xenopus laevis TCTP (XITCP) (raised in our laboratory) and against HsTCTP (Santa Cruz Biotechnology, CA) were used at the dilution of 1:1000 and 1:100, respectively, with overnight incubations at 4°C. Anti-β-tubulin (Sigma, Saint Quentin Fallavier, France) and anti-α-tubulin (Euromedex, Souffelweyersheim, France) were diluted 1:200. Purified anti-c-myc antibody (Sigma) was diluted 1:100. Secondary antibodies (fluorescein isothiocyanate and rhodamine) (The Jackson Laboratory, Bar Harbor, ME) were conjugated with biotin, 1:1000 dilution; Molecular Probes were incubated for 1 h at room temperature. F-actin was detected with 2 U/ml of rhodamin-conjugated phallolidin (Molecular Probes, Invitrogen, Cergy Pontoise, France). Coverslips were mounted in Vectashield and examined using a Leica DMRXA2 fluorescence microscope or Leica Confoal SP2 microscope. Photographs were taken using a black and white COOLSNAP ES camera (Roper Scientific, Ely, France) and images were processed using Metamorph software (Universal Imaging, Photometrics, Tucson, AZ).

Cell transfection

For transfection of XL2 cells with plasmids encoding Xenopus Myc-TCTP, 5×10⁵ cells were plated on glass coverslips in a 12-well plate. Cells were transfected with 0.5 µg of plasmid DNA using FuGENE 6 Transfection Reagent (Roche Diagnostics, Meylan, France) following the manufacturer’s instructions.

Cell-free extracts and in vitro spindle assembly

Cytostatic factor (CSF)-arrested extracts were prepared as described (25). For Cell-free extracts and in vitro spindle assembly

DNA was visualized using 4’,6-diamidino-2-phenylindole. Polyclonal antibodies against Xenopus laevis TCTP (XITCP) (raised in our laboratory) and against HsTCTP (Santa Cruz Biotechnology, CA) were used at the dilution of 1:1000 and 1:100, respectively, with overnight incubations at 4°C. Anti-β-tubulin (Sigma, Saint Quentin Fallavier, France) and anti-α-tubulin (Euromedex, Souffelweyersheim, France) were diluted 1:200. Purified anti-c-myc antibody (Sigma) was diluted 1:100. Secondary antibodies (fluorescein isothiocyanate and rhodamine) (The Jackson Laboratory, Bar Harbor, ME) were conjugated with biotin, 1:1000 dilution; Molecular Probes were incubated for 1 h at room temperature. F-actin was detected with 2 U/ml of rhodamin-conjugated phallolidin (Molecular Probes, Invitrogen, Cergy Pontoise, France). Coverslips were mounted in Vectashield and examined using a Leica DMRXA2 fluorescence microscope or Leica Confoal SP2 microscope. Photographs were taken using a black and white COOLSNAP ES camera (Roper Scientific, Ely, France) and images were processed using Metamorph software (Universal Imaging, Photometrics, Tucson, AZ).

For transfection of XL2 cells with plasmids encoding Xenopus Myc-TCTP, 5×10⁵ cells were plated on glass coverslips in a 12-well plate. Cells were transfected with 0.5 µg of plasmid DNA using FuGENE 6 Transfection Reagent (Roche Diagnostics, Meylan, France) following the manufacturer’s instructions.

In vitro MT self-assembly and disassembly

Tubulin was purified from pig brain as described (27). The purity of tubulin was validated by electrophoresis and the absence of endogenous TCTP confirmed by western blotting. Samples of tubulin (50 µM) were incubated for 10 min at 4°C with 1 mM guanosine triphosphate in BRB80 buffer. MT assembly was induced at 37°C and monitored turbidimetrically at 350 nm. After 35 min, MT depolymerization was triggered by lowering the temperature to 4°C. To study the effect of full-length and truncated TCTP (TCTPMAP1B) on MT assembly/disassembly, increasing concentrations of the purified proteins were added to tubulin at 4°C before starting the assembly process. As positive controls (data not shown), we checked that the N-terminal fragment of CLIP-170 named H2 (28,29) and EB1 strongly stimulated MT assembly as reported previously (30,31).

In vitro assays for XITCP binding to MTs and F-actin

Taxol-stabilized MTs were incubated for 15 min at 37°C with TCTP or CLIP-170 (H2) in BRB80 buffer [80 mM 1,4-piperazineidine-sulfonic-acid, 1 mM MgCl₂ and 1 mM ethyleneglycol-bis(aminooxyethyl)-tetraacetic acid]. MTs were sedimented by centrifugation at 80 000g for 30 min at 37°C. F-actin (monomeric actin) from rabbit skeletal muscles in G-buffer (5 mM Tris–HCl, pH 7.8, 0.1 mM CaCl₂, 0.2 mM adenosine triphosphate, 1 mM dithiothreitol, 0.01% NaN₃) was polymerized into F-actin by addition of 100 mM KCl, 1 mM MgCl₂ and 0.2 mM ethyleneglycol-bis(aminooxyethyl)-tetraacetic acid. F-actin was incubated for 15 min at room temperature with TCTP in buffer F (5 mM Tris–HCl, pH 7.8, 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.2 mM adenosine triphosphate, 1 mM dithiothreitol, 0.01% NaN₃). The filaments were collected by centrifugation at 90 000g for 30 min at 20°C. In both assays, proteins in the supernatants and pellets were solubilized in sodium dodecyl sulfate sample buffer and analyzed after sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining or western blotting.

RNA interference

Human and Xenopus TCTP small interfering RNAs (siRNAs) were purchased from Eurogentec (Angers, France). A single Xenopus TCTP siRNA with the following nucleotide sequence 5’-CAAGAGGACCUUCACAAAGA-3’ and three human TCTP siRNAs with sequences A: 5’-UUCGCUCAUGUGG-GAAAU-3’, B: 5’-GCAAGAAGGUGAUCAGUGA-3’ and C: 5’-GGUAGCAGGACACAGUAGU-3’ were used. The ‘universal negative control’ siRNA (OR-00300-neg05) designed by Eurogentec was also used. Cells were transfected with oligonucleotides using HiPerFect Transfection Reagent (Qiagen) following the manufacturer’s instructions (in 12-well plate: Xenopus siRNA at 50 nM final with 9 µl HiPerFect; Human siRNAs at 5 nM final with 3 µl HiPerFect).

Results

TCTP partially colocalizes with the MT cytoskeleton in XL2 cells

TCTP was previously identified as a putative cell cycle-dependent MAP in monkey COS cells on the basis of immunofluorescence localization and by detection of the affinity binding to MTs and tubulin (21). To investigate the cytoskeletal roles of this protein, we first examined the intracellular localization of endogenous and exogenous (Myc-tagged) XITCP in Xenopus XL2 cells. The specificity of the antibody used in this study was confirmed by western blotting (Figure 1A, a and b). Immunofluorescence and transfection experiments revealed equivalent patterns of intracellular TCTP localization (Figure 1A, c–g), confirming to the previous description (21). In interphase cells (methyl–formaldehyde fixation), TCTP formed a fibrous MT-like network, whereas in mitotic cells it accumulated in the spindle as well as in granular or fibrous structures in the cytoplasm (Figure 1A, c–g) that is MT free during mitosis. Similarly, as in monkey COS cells, TCTP was not detected in the midbody (Figure 1A, d; tubulin staining in the inset) (21). Similar pattern of TCTP was also shown recently in XL2 cells (8). Careful examination of cells contained with β-tubulin, however, revealed small but consistent differences between the TCTP network and the MT network in interphase cells (Figure 1B). Many instances of individual fibers stained exclusively with anti-β-tubulin or exclusively with anti-TCTP were decreasing amounts of urea and finally concentrated by centrifugation using a MicroSeq™ device (molecular weight 10 K cutooff; PALL, St Germain en Laye, France). The full-length 6xHis-tagged XITCP was used to raise rabbit polyclonal antibody against Xenopus TCTP (Charles River Laboratories, L’arbresle cedex, France) and both proteins were also used in various assays.

F.Bazile et al.

556
detected, especially in thin and flattened areas at the edges of cells (Figure 1B, a–d). In cells treated with colchicine to cause partial disassembly of the MT network, differences in the distributions of TCTP and β-tubulin became obvious also in the central region of interphase cells (Figure 1B, e and f). Prolonged colchicine treatment resulted in total disassembly of both MT and TCTP networks (data not shown) suggesting that despite local differences, the two networks are related and that the TCTP network is dependent on the integrity of MTs. The pattern of TCTP fibers bears some resemblance to that described for vimentin intermediate filaments. However, double immunofluorescence for TCTP and vimentin showed that these networks are clearly distinct (supplementary Figure 1 is available at Carcinogenesis Online). A strong granular signal of TCTP staining was also present in the cytoplasm (particularly well visible in the cytoplasm of mitotic cells, Figure 1A, c) suggesting that a significant fraction of TCTP is either soluble or associated with a cytoplasmic lattice, consistent with the abundance of the protein apparent in western blots. We conclude that TCTP is present in XL2 cells’ cytoplasm as MT-like ...
fibrous network and cytosolic pool. Although the fibrous TCTP localization is highly reminiscent of the MT network, the colocalization is not strict and TCTP-free MTs as well as tubulin-free TCTP-positive fibers coexist within the cell.

**TCTP associates strongly with mitotic spindle poles**

To examine in detail TCTP localization within the spindle, we used ‘CSF’ cytoplasmic extract (metaphase-arrested oocytes) in which spindles were assembled around sperm nuclei and visualized using rhodamine-labeled tubulin. Immunofluorescence of spindles frozen within thin layer of crude cell-free extract (Figure 2A, top panel) confirmed enrichment of TCTP in the spindles, as seen in XL2 cells (Figure 1A, c and f). However, the fibrous not linked to MTs organization of TCTP staining was restricted to the cytoplasm (Figure 2A, top panel; MT-free cytoplasm around spindles in corners of the Figure 2A, b and d). Moreover, the intense staining of the spindles was

![Figure 2](https://academic.oup.com/carcin/article-abstract/30/4/555/2476733)

**Fig. 2.** TCTP is abundant in the mitotic spindle, but it is not associated with spindle MTs. (A) Mitotic spindles were assembled in the CSF extract following addition of sperm heads and rhodamine tubulin. Samples were frozen in liquid nitrogen, post-fixed in cold methanol (crude spindles) and stained for DNA (a), TCTP (b) and MTs (c; merged image in d). Spindles show high, but not fibrous, TCTP staining and a granular network of TCTP in the adjacent cytoplasm that does not colocalize to MTs (especially visible in corners of b–d). Two examples of MT fibers (black arrows in c) that do not correspond to any TCTP fibers (white arrows in b) are shown. In parallel, spindles assembled in the extract were purified on glycerol cushion, fixed (purified spindles) and processed for DNA staining (e), TCTP immunofluorescence (f) and MTs labeling with rhodamine tubulin (g; merged image in h). The majority of TCTP staining disappeared from purified spindles and discrete fluorescent dots of TCTP remained on the spindle poles (white arrows in f). Merged image of DNA, TCTP and rhodamine tubulin (h) shows clear red staining of spindle MTs with yellow dots on its poles indicating that only in these areas the colocalization of TCTP and tubulin is detected. Inset in h shows details of a merged image of another spindle pole with TCTP remnants in higher magnification. Bars = 20 μm. (B) Either 6xHis-XTCTP or 6xHis-XTCTPD MAP1B does not modify assembly/disassembly of MTs in vitro. Tubulin alone (control) or mixed with two concentrations of recombinant wild-type TCTP (TCTP 2 and 8 μM) or ΔMAP1B deletion mutant (ΔMAP1B 2 and 8 μM) was incubated at 37°C (0–35 min) or 4°C (35–60 min) and optical density of samples was measured at 350 nm light wave to follow assembly (0–35 min) and disassembly (35–60 min) of MTs. All curves are very similar indicating that neither 6xHis-XTCTP nor 6xHis-XTCTPD MAP1B modifies MTs dynamics. (C) MAP1 domain does not influence localization of TCTP. Myc-TCTPΔMAP1B localizes to mitotic spindles (b; here in metaphase) after expression in XL2 cells and forms a network in the cytoplasm of both mitotic (b) and interphasic cells (a). XL2 cells were transfected with an appropriate vector and were fixed and processed for anti-Myc immunofluorescence. Single confocal sections of DNA (blue) and anti-Myc immunofluorescence (red) in an interphase (a) and metaphase (b) cells are shown. Note that peripheral cells that do not express Myc-TCTP are negative, while stained with anti-Myc antibody providing the control of the specificity of anti-Myc detection for the exogenous protein. Bars = 20 μm.
relatively homogenous compared with the tubulin staining (Figure 2A, top panel; black arrows show tubulin-positive fibers, white arrows show the absence of TCTP fibers at the corresponding sites within the spindle). In the spindle, TCTP thus did not show details of features of distribution predicted for a MAP. Unexpectedly, in vitro assembled spindles isolated by centrifugation through a glycerol cushion showed a major loss of TCTP staining, with some signal retained selectively at the spindle poles (Figure 2A, f). The residual TCTP remaining within such spindles was again neither fibrous nor colocalized with MTs. These results confirm that TCTP is enriched in mitotic spindles, but indicate that it is not linked directly to MTs, with a strong association only at the spindle poles.

Neither TCTP nor TCTPMAP1B modify MTs assembly/disassembly in vitro

The results described above suggest that either TCTP is not associated directly with MTs or that this association differs significantly from that of other MAPs. Nevertheless, TCTP possesses a domain resembling the MT-binding domain of MAP1B (21). To clarify the MT-interacting properties of TCTP, we produced a recombinant His-tagged mutant protein, TCTPMAP1B, lacking the MAP1B-like domain (amino acids 82–121) and compared its activity with intact recombinant protein in in vitro MT assembly/disassembly assays. In contrast with the stimulatory effects of an N-terminal fragment of His-tagged mutant protein, TCTPMAP1B, with the MAP1B-like domain, in the in vitro MT assembly/disassembly assays did not significantly affect rates of MT polymerization at 37°C or MT disassembly at 4°C (Figure 2B). We also examined the localization of TCTPMAP1B after expression in XL2 cells and found that this truncated form became distributed very similarly to intact TCTP cytoplasmic staining in interphase and both cytoplasmic and spindle staining in mitosis (Figure 2C). We conclude from these studies that neither intact TCTP nor a form lacking the putative MT-binding domain modifies MT dynamics and that this domain is not required for the MT-like distribution of TCTP in cells.

TCTP has no affinity for taxol-stabilized MTs in vitro

We next investigated the affinity of TCTP for in vitro assembled taxol-stabilized MTs. When recombinant 6xHis-tagged XlTCTP was incubated alone at 37°C in the MT stabilization buffer and then spun down, approximately half of it was pelleted (Figure 3A), probably reflecting the capacity of TCTP to oligomerize (32). Surprisingly, even high amounts of taxol-stabilized MTs did not significantly increase the quantity of TCTP in the pellet (Figure 3B, TCTP in the middle panel). Under the same conditions, the H2 N-terminal fragment of CLIP-170 clearly interacted with MTs (Figure 3B, H2 in the bottom panel). Thus, the soluble fraction of 6xHis-tagged XlTCTP lacks detectable affinity for MTs in the cosedimentation assay. Being aware that the recombinant protein could be misfolded and thus could not reflect the properties of the native protein, we carefully analyzed the affinity of MTs with a TCTP protein expressed in conditions assuring its functionality. Rigorously equivalent results were obtained in a cosedimentation assay with untagged as well as Myc-tagged XlTCTP translated in rabbit reticulocyte lysates. (Figure 3C). This shows that the absence of detectable TCTP/MT interactions was neither related to the bacterial origin nor to the purification procedure of the recombinant protein shown in Figure 3B but that the properly folded TCTP protein indeed has no detectable affinity for MTs in our assay.

To test for interactions between native TCTP and MTs in the presence of endogenous cytosplasmic proteins, we applied a cosedimentation assay using Xenopus egg cytoplasmic extracts (Figure 3D). Taxol-stabilized MTs were mixed with either interphase or M-phase (CSF) extracts diluted in appropriate buffer, incubated and spun down. The ratio of distribution of endogenous TCTP in the supernatant and pellets was compared. Under these conditions, the kinesin-related Eg5 protein, known to interact with MTs (33), was shown to become enriched in the MT pellet as expected (Figure 3D, bottom); pelleted MTs appear saturated with endogenous Eg5 and moderate increase is observed. The amount of endogenous TCTP found within the fraction along with MTs did not, however, increase in either type of cell-free extract (Figure 3D). We conclude that neither bacterially expressed nor native TCTP behave as MT-binding proteins under these assay conditions.

Finally, to modulate the quantity of MTs and eventual proportions of TCTP in supernatant and pellet fractions, we added into interphase and M-phase extracts either a low dose of nocodazole or taxol to, respectively, decrease or increase the volume (number and length) of MTs in extracts and the volume of the pellet. As expected, the amount of MTs in the insoluble fraction (P) diminished slightly in the presence of nocodazole and clearly increased when taxol was added (Figure 3E, right). However, the quantities of TCTP in the pellet fraction rigorously did not change at all (Figure 3E, left). Thus, the endogenous TCTP present in X.laevis interphase and M-phase-arrested cell-free extracts lacks an affinity for MTs that could be detected in cosedimentation assay.

TCTP associates with actin-rich structures in XL2 cells

Careful examination of TCTP immunofluorescence and Myc-tagged exogenous TCTP in paraformaldehyde-fixed XL2 cells revealed a distinctive discrete pattern at the cell border in spreading cells (Figure 4A). Double staining of TCTP and F-actin (using rhodamine phalloidin) in these cells revealed a significant colocalization between the two proteins in these cellular regions (Figure 4B). TCTP strongly colocalized with F-actin at the leading edge of peripheral ruffles. In addition, TCTP colocalized with very actin-rich fibers proximal to the lamellipodia and those resembling dorsal ruffles (Figure 4B). In contrast, stress fibers, the most solid and abundant F-actin structures, were not stained with TCTP antibody (data not shown). Actin-rich peripheral and dorsal ruffles were visible also in XL2 cells transfected with Myc-TCTP and in spreading HeLa cells (data not shown). Cytochalasin D treatment depolymerizes F-actin and provokes, as a side effect, formation of actin-rich residual foci within cells. TCTP clearly colocalized with these cytochalasin-induced actin foci (Figure 4B, bottom), indicating that TCTP can accompany actin-rich structures upon their rearrangement. These data suggest that TCTP associates with F-actin structures at the periphery of spreading cells and with actin foci upon cytochalasin D treatment. The absence of TCTP in stress fibers suggested that this association is under complex regulation and/or may be indirect.

TCTP associates with F-actin in cell-free extracts

As TCTP colocalizes with certain actin structures in vivo, we tested potential interactions between F-actin and TCTP using an in vitro cosedimentation assay (34). The sedimentation of recombinant 6xHis-tagged XlTCTP did not increase in the presence of actin filaments (Figure 4C). Similar results were obtained when F-actin was incubated with a reticulocyte lysate containing in vitro translated XlTCTP or Myc-XlTCTP (data not shown). However, when actin filaments were incubated in egg extracts, a significant cosedimentation of endogenous TCTP with F-actin was observed (Figure 4D). The proportion of TCTP found in the pellet was comparable with that of tropomyosin, a well-characterized F-actin-binding protein (Figure 4D, bottom). The exclusion from the actin pellet of proteins detected non-specifically by the anti-tropomyosin antibody additionally confirmed the specificity of the assay for F-actin-binding proteins. The cosedimentation of endogenous TCTP with F-actin in egg extracts but not of recombinant or reticulocyte-translated TCTP with purified F-actin indicates that this interaction is indirect and/or requires some post-translational modifications of either TCTP or actin.

TCTP knockdown changes the shape of XL2 and HeLa cells

To analyze the role of TCTP in vivo, we used siRNA to reduce levels of this protein in XL2 cells. In cells treated with TCTP siRNA oligonucleotides, TCTP levels were reduced by ~50% (Figure 5A). Similar difficulty to knockdown TCTP in XL2 cells was reported recently (3).
**Fig. 3.** XlTCTP does not associate with taxol-stabilized MTs in cosedimentation assay. (A) Recombinant 6xHis-XlTCTP precipitates alone in MT-stabilizing buffer in concentration-dependent manner (0.5 and 2.5 mM). Proportions of TCTP in supernatants and pellets are similar in the two concentrations studied. (B) Taxol-stabilized MTs alone precipitate in pellet (P) and lower supernatant (LS), whereas only traces of tubulin are detected by western blotting in the upper supernatant (US; top row, right). The amount of TCTP is very similar in MT-devoid (-; left) and MT-containing pellet (+; right) (middle row) despite high accumulation of MTs in the pellet (P) and LS. Proportions of control H2 fragment of CLIP-170 recombinant protein (known to bind to MTs; bottom row) in the supernatant and pellet change in relation with the presence of MTs. H2 is mostly accumulated in the LS, whereas the lowest proportion was found in the pellet (P) in the absence of MTs (-). It is enriched in the pellet and diminishes in the US and LS in the presence of MTs (+) accordingly with the abundance of tubulin signal in each fraction (the very bottom of the western blot). (C) XlTCTP and Myc-XlTCTP expressed in reticulocyte lysate are not enriched in the MT-containing pellet. XlTCTP (upper panel) and Myc-XlTCTP (lower panel) were expressed *in vitro* in the lysate of rabbit reticulocytes. Taxol-stabilized MTs were added (+) or not (-) to the lysates expressing either of the two forms of XlTCTP. Tubulin western blots show that MTs accumulate specifically in the pellets (P; right in upper rows of both panels). TCTP western blots show a very similar pattern of distribution of XlTCTP and Myc-XlTCTP, respectively, in the supernatants (S) and in the pellets (P) in the presence and absence of MTs (bottom rows in each panel, respectively). The plasmid containing untagged XlTCTP allows expression of TCTP (arrow) as well as an additional non-identified protein visualized as a slower migrating band present exclusively in the supernatants (S) and in the pellets (P) in the absence of MTs. Myc-XlTCTP migrates as a single major band (arrow in the bottom row of the lower panel), which is predominantly found in the supernatants (S) and only as traces in the two type of pellets (P) regardless of presence or absence of MTs. Note that proportion of Myc-XlTCTP in the pellet (with or without MTs) is much less important than in the case of untagged TCTP. This suggests that Myc tag may limit oligomerization of TCTP. (D) Endogenous XlTCTP from CSF and interphase extracts is not enriched in MTs pellet indicating that it does not associate with MTs. Endogenous XlTCTP from CSF and interphase extracts is not enriched in MTs pellet indicating that it does not associate with MTs. CSF and interphase extract were supplemented (+) or not (-) with taxol-stabilized MTs, incubated at 37°C, centrifuged and collected as US, LS and pellet (P). No difference between CSF and interphase extract was observed. In the presence of taxol-stabilized MTs, the pellet is enriched in MTs as indicated by the high signal on anti-tubulin western blot (upper row, rightmost). Tubulin signal in LS and P in the absence of taxol-stabilized MTs comes from endogenous MTs and tubulin (-; left). The pattern of distribution of endogenous TCTP in US, LS and P fractions is rigorously the same in the absence (-; left) and presence (+; right) of taxol-stabilized MTs. Control endogenous protein, kinesin Eg5 (known to associate with MTs), is enriched in the pellet (P) in the presence of taxol-stabilized MTs (rightmost; bottom row) in parallel with enrichment in MTs. (E) Modulation of the quantity of MTs in pellets of CSF and interphase extracts does not modify the amount of endogenous pelleted XlTCTP. Endogenous TCTP is found rigorously the same proportions (left) between US, LS and pellet (P) in control interphase and CSF extracts (+ dimethyl sulfoxide; upper row) in the presence either of taxol (middle row) or nocodazole (bottom row). Proportions of endogenous tubulin signal on western blots corresponding to the same fractions (right) vary considerably. It is shifted in favor of the pellet in the presence of taxol (middle row) and has a tendency to diminish in the presence of nocodazole (bottom row).
The most striking effect of TCTP knockdown was a drastic change in the shape of the cells, which became elongated with characteristic perpendicular protrusions, losing the flatness of the cell body such that nuclei could not be distinguished in phase contrast images as they easily could be in flattened control cells (Figure 5A, right). Moreover, the cells seem to change their contact properties. Whereas control XL2 cells grow in ‘islands’ of well-attached flattened cells, those in which TCTP was downregulated showed only a limited area of cell–cell contact and despite that they did not tend to form islands (Figure 5A, right). Since XL2 cells are resistant to transfection and their RNA interference (RNAi) is quite inefficient (8), we performed highly more efficient RNAi experiments in HeLa cells, using three different siRNA oligonucleotides directed toward human TCTP. All three siRNAs provoked significant reductions in TCTP levels as shown by western blotting (Figure 5B). Besides well-known effects of TCTP knockdown as slowed down proliferation and increased apoptosis, the most dramatic phenotypic effect was again a drastic change of cell shape that became elongated with long protrusions (Figure 5A, right).
The same elongated phenotype was equally visible in dispersed as in closely apposed cells (compare Figure 5B with Figure 6B–D), showing that the change in cells shape is not due to isolation following slowed down proliferation and increased apoptosis. HeLa cells do not adhere closely one to another as XL2 cells do, and there was no obvious change in their cell–cell contact properties following RNAi (compare dispersed cells in Figure 5B and more closely apposed ones in Figure 6B). In highly elongated HeLa cells with decreased TCTP levels, the MT cytoskeleton was disorganized and MTs formed dense cables (Figure 5B). Spreading cells disappeared upon TCTP knockdown conditions and lamellipodia in which TCTP colocalized with actin was absent (Figure 6B). The cortical accumulation of actin and numerous fibers perpendicular to the cells axis were present in such highly elongated cells indicating reorganization of actin cytoskeleton (Figure 6B). Interestingly, addition of nocodazole restored the cell shape abnormalities induced by siRNA in interphase cells (Figure 6C), demonstrating that the change is MT dependent. The strong TCTP staining in the spindles of control HeLa cells disappeared (Figure 6A, second and third row) or diminished dramatically (Figure 6A, forth and bottom row) following RNAi. Long cytoplasmic protrusions were occasionally found also during mitosis in siRNA-treated cells (Figure 6A and D) in clear contrast with perfectly round-up control cells (Figure 6A, control). These protrusions were always positioned at a single or two ends of the long axis of elongated cells. Thus, TCTP depletion has dramatic consequences on cell shape both during interphase and mitosis, exerting effects on both actin and MT cytoskeletons. Mitotic...
Discussion
In this paper, we have examined in detail the relationship between TCTP and the cytoskeleton. Our results have demonstrated for the first time that TCTP associates with certain F-actin structures and that this protein is involved in shaping of Xenopus XL2 and human HeLa cells. We have also shown that despite striking similarities between TCTP and MT networks, they are not completely superimposable and that TCTP has no affinity for taxol-stabilized MTs. Gachet et al. (21) were the first to implicate TCTP with MTs by showing that TCTP can be immunoprecipitated with anti-tubulin antibodies and that in COS cells TCTP coaligns with MTs. Rimmerthaler et al. (22) suggested that spindles in cells with knocked down TCTP were indistinguishable from controls when observed with tubulin immunofluorescence (Figure 5B, oligo A, MTs).

Fig. 6. Phenotype of HeLa cells upon TCTP knockdown. (A) Upon RNAi of TCTP, the staining of TCTP disappears from the mitotic spindle and long protrusions formed during interphase may persist upon mitosis (DNA, TCTP, merge—from left to right). Top row—control. Second, third and bottom row—rounded mitotic cells treated with oligos A, B and C, respectively, in which spindle TCTP staining disappeared or diminished significantly (bottom). Fourth row—metaphase cell treated with oligo B with long protrusion oriented along the spindle axis. (B) Actin localization in control and TCTP siRNA-treated cells. Flattened cells with abundant cortical actin and stress fibers (control) transform into highly elongated ones (RNAi TCTP). Actin staining remains cortical with numerous fibers oriented perpendicularly to the cell axis. Bars = 20 μm. (C) Elongation of HeLa cells upon TCTP knockdown is MT dependent. Control —: control cells. Control +: nocodazole treated for 24 h. Nocodazole increases the number of mitotic cells (rounded up) in comparison with the untreated control (−). Nocodazole treatment does not influence the shape of interphase cells that remain flattened (control +). In oligo B-treated cells (RNAi TCTP +), highly elongated interphase cells are present in the absence of nocodazole (−). The drug treatment (RNAi TCTP +) induces massive flattening of interphase cells, which become morphologically undistinguishable from the controls; compare interphase cells in control (−) with RNAi TCTP (+). Bar = 200 μm. (D) Cell protrusions induced by TCTP RNAi participate in unequal division of daughter cells during cytokinesis. The long protrusions (arrows) formed during interphase remain oriented along the spindle axis during mitosis. They participate in unequal mitotic division (compare the size of daughter cells upon cytokinesis of two dividing cells at 90 min time point). Arrows at 150 min time point show daughter cells that contain the protrusion. Photographs of time-lapse video microscopy of two dividing cells filmed during 150 min. Bars = 200 μm.
TCTP interacts with MTs based on the hypersensitivity of TCTP knockout cells to benomyl. However, in our in vitro experiments, we could neither confirm the affinity of both recombinate and native TCTP to MTs nor detect any influence of TCTP on MT dynamics. Furthermore, in an organism extremely rich in MTs, the protist *Tetrahymena thermophila* green fluorescent protein-tagged TCTP does not localize to MTs and a genetic knockout of *Tt*TCTP gene does not influence the MT cytoskeleton (D.Wloga and J.Gaertig, personal communication). Finally, the abundant TCTP in mitotic spindles does not colocalize with discrete MT fibers but is rather associated with non-fibrous spindle elements and also strongly associates with the spindle poles (this paper). Taken together, these data enable us to refute earlier claims of a direct association of TCTP with MTs (21). Nevertheless, in line with the conclusions of previous research (21,22,24), we did find evidences for a relationship between TCTP and MTs, the changes in the shape of siRNA-treated and TCTP knocked down HeLa cells being found to be MT-dependent (this paper). Given our lack of evidence for direct binding, the effect of TCTP on the MT cytoskeleton is probably indirect.

The MT-like organization of TCTP in a form of network is intriguing. The absence of TCTP on certain MTs and the absence of tubulin in certain TCTP-stained fibers, as well as the increase of tubulin-negative TCTP fibers upon colchicine treatment, suggest that TCTP may associate with MTs following assembly and remain transiently in the place of individual MTs following their disassembly. Such behavior has been reported previously for the 7/13 antigen in mouse oocytes (35). The protein reacting with 7/13 antibody is a component of calcium transport system and it is organized in fibers that trace disassembled MTs and guide newly forming ones (35). Interestingly, TCTP is also a calcium-binding protein (1,16,17) and could thus also be involved in MT guiding.

We show in the current paper that TCTP is a mitotic spindle protein without detectable affinity for MTs. The pattern of non-fibrous staining and the disappearance of TCTP from isolated spindles suggest that the majority of TCTP is either a spindle matrix protein or associates with spindle structures other than MTs. The role of the spindle matrix is still a matter of debate. However, the recent finding that the nuclear intermediate filament protein lamin B contributes to the spindle matrix and plays an important role in spindle morphogenesis strengthened speculation that such matrix could be more important for appropriate cell division than previously thought (36,37). As TCTP is particularly concentrated in the spindle, forms lattice in the cytoplasm (as shown in *Xenopus* cell-free extract; Figure 2A in this paper) and is capable of oligomerization (32); it is a good candidate to participate in a mitotic spindle scaffold. We cannot exclude, however, that TCTP is associated with actin present within spindle or with other spindle components. Persistence of TCTP on the spindle poles after spindle isolation on a glycerol cushion (this paper) could suggest that either it is particularly tightly packed on the spindle poles or forms an integral part of centrosomes. These aspects need further analysis.

We show that TCTP colocalizes with actin structures at the leading edge of lamellipodia-like cells and in actin bundles of peripheral and dorsal ruffles at the rear of the lamellipodia (e.g. 38–40). In agreement with our observations, endogenous TCTP associates with actin filaments in a cosedimentation assay in the presence of cell-free *Xenopus* egg extracts. To our knowledge, this is the first indication of actin-binding capacity for TCTP, although this binding may require a post-translational modification and/or additional adapters to bind since no interaction was observed in the absence of egg extracts. A potential adapter is the light chain of myosin, a well-known actin filament-binding protein that has been described as a TCTP-binding protein (41). Association of TCTP with actin-containing structures, perhaps via myosin, could explain why RNAi depletion of TCTP dramatically affected both the shape and the cytoskeleton organization of tissue culture cells. The actin cytoskeleton plays a critical role in maintaining cell shape, so it is tempting to propose that the ability of TCTP to bind actin filaments is related to its role in cell shaping.

Our results showing that the change of shape of HeLa cells under siRNA treatment is MT dependent do not contradict active interactions between TCTP and actin structures. Moreover, TCTP overexpression also induces changes in the MT cytoskeleton, with the formation of MT cable attributed to the supposed action of the protein as a MAP (21). Such MT reorganizations could, however, result indirectly from perturbations in other cytoskeletal systems, including F-actin structures positioned perpendicularly to the axis of elongated cells with reduced TCTP (this paper, Figure 6B). Even *bona fide* MAPs, like MAP1B, have the capacity to bind both MTs and actin (42) (see ref. 43). Partial colocalization of TCTP with MTs and actin resembles localization of MT- and actin-binding protein Mip-90 in human fibroblasts (44), with notable exception of stress fibers stained with anti-Mip-90 antibody, but not with anti-TCTP. Cell shaping is a complex phenomenon in which all cytoskeletal elements are implicated and interact one with another (see refs 45,46). Thus, F-actin binding of TCTP remains fully compatible with the role of this protein in MT-dependent shaping of tissue culture cells. This conclusion is strengthened by our observation that upon TCTP knockdown changes in cell shape occur also during mitosis. Indeed, the long protrusions in TCTP knockdown HeLa cells may persist in mitosis, resulting in unequally sized daughter cells. Since the cytoplasmic network of MTs disassembles at the beginning of the M-phase in control and RNAi TCTP HeLa cells (Figure 5B; MTs), the protrusions are also devoid of MTs. Interestingly, the protrusions observed in mitotic HeLa cells generally were oriented along the axis of cell division (see Figure 6A and D). This may coincide with the persistence of the strongest retraction fibers during mitosis (47). The occasional character of such phenotype suggests that it concerns only extremely strong retraction fibers. Apparently, in cells with low TCTP level, both actin and MT cytoskeleton escapes from normal control. Cytoskeleton-linked functions of TCTP could play important roles particularly in malignancy as cells flexibility and motility changes in malignant cells. TCTP is a good candidate to regulate the two cytoskeletal networks by integrating their organization and coordinating these two cytoskeletal elements during cell shaping.

In summary, this study provides the first evidence that: (i) TCTP interacts with F-actin; (ii) its relationship with MTs is less direct than thought so far and (iii) this protein regulates cell shape in a cytoskeleton-dependent manner. Further analysis of TCTP interactions both with MTs and F-actin should give us more insights into the role of TCTP in normal and cancer cells including tumor reversion (see Note added in proof).

**Note added in proof**

The most recent review on the role of TCTP in tumor reversion was published online when the current article was under correction: Telerman,A. et al. (2009) The molecular programme of tumour reversion: the steps beyond malignant transformation. *Nat. Rev. Cancer*, in press.

**Supplementary material**

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

**Funding**

Association pour la Recherche contre le Cancer (4900): Ligue Contre le Cancer (Comité d’Ille-et-Vilaine et de Vendée) to J.Z.K.

**Acknowledgements**

We thank Dorota Wloga and Jacek Gaertig (University of Georgia, Athens) who shared with us unpublished observations concerning *Tetrahymena thermophila* TCTP knockout, exchanged information, discussed and helped us to prepare this manuscript including English correction. We wish to thank Stephanie Dutertre (IFR 140 GFAS) for her technical assistance with microscopy work. We are grateful to Evelyn Houliston (Observatoire de Villefranche sur Mer) and William Forrester (Novartis Institutes for...
Biomedical Research) for fruitful discussions, reading the manuscript and valuable English corrections. We thank Robert Amson (ENS, Cachan) for critical reading the manuscript and comments.

Conflict of Interest Statement: None declared.

References


Received September 3, 2008; revised December 24, 2008; accepted January 11, 2009.