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Specific association of STOP protein with microtubules *in vitro* and with stable microtubules in mitotic spindles of cultured cells

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STOP (Stable Tubule Only Polypeptide) is a neuronal microtubule associated protein of 145 kd that stabilizes microtubules indefinitely to *in vitro* disassembly induced by cold temperature, millimolar calcium or by drugs. We have produced monoclonal antibodies against STOP. Using an antibody affinity column, we have produced a homogeneously pure 145 kd protein which has STOP activity as defined by its ability to induce cold stability and resistance to dilution induced disassembly in microtubules *in vitro*. Western blot analysis, using a specific monoclonal antibody, demonstrates that STOP recycles quantitatively with microtubules through three assembly cycles *in vitro*. Immunofluorescence analysis demonstrates that STOP is specifically associated with microtubules of mitotic spindles in neuronal cells. Further, and most interestingly, STOP at physiological temperature appears to be preferentially distributed on the distinct microtubule subpopulations that display cold stability; kinetochore-to-pole microtubules and telophase midbody microtubules. The observed distribution suggests that STOP induces the observed cold stability of these microtubule subpopulations *in vivo*.

Key words: microtubules/mitotic spindles/STOP protein

Introduction

In general, microtubules are labile structures which depolymerize at cold temperature, and are sensitive to calcium, various drugs, absence of GTP and dilution at physiological temperature (Dustin, 1984). However, many tissues and cells in culture contain subpopulations of disassembly resistant microtubules. In brain tissue, the axonal microtubules of neuronal cells are disassembly resistant (Jones *et al.*, 1980; Black *et al.*, 1984; Heidemann *et al.*, 1985), and can exist without direct attachment to a microtubule organizing center (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). In cultured cells, some stable polymers are present in interphase arrays (Schulze and Kirschner, 1986; Behnke and Forer, 1967). During mitosis, the location of stable polymers is characteristic and particularly well defined. At this stage, the kinetochore-to-pole microtubules of the mitotic spindle (Brinkley and Cartwright, 1975) and midbody microtubules connecting recently divided cells (Mullins and McIntosh, 1982) are indefinitely resistant to drug or cold temperature induced disassembly. Most such microtubule stabilizations are defined by resistance to disassembly rather than kinetic inactivity. Throughout this paper, we refer to stability of microtubules as the resistance to disassembly of polymers which nevertheless may exhibit substantial steady-state kinetic activity.

Microtubule stabilization has functional significance. In neurons the existence of stable, but not necessarily kinetically inactive, polymers is thought to be central to the organization and maintenance of axons. Stable microtubules appear to serve as nucleating centers that maintain microtubule orientation at sites distant from centrosomes (Baas and Heidemann, 1986) and that may recapitulate the proper organization of the axonal cytoskeleton upon recovery from cold temperature (Heidemann *et al.*, 1984).

Microtubule stabilization in mitosis may function to generate motility. We have proposed that interplays between selective microtubule stabilization and the intrinsic dynamic properties of these polymers can, in principle, be used to generate chromosome movement by a mechanism we call 'polymer guided diffusion' (Margolis *et al.*, 1986a; Garel *et al.*, 1987). Koshland *et al.* (1988) have recently provided experimental evidence that an *in vitro* microtubule system is intrinsically capable of generating such movements, and have proposed an equivalent model of chromosome motion based on 'biased diffusion'.

We have isolated a protein from brain tissue which has the ability to induce a high degree of microtubule stability *in vitro*. We have designated this protein STOP, for Stable Tubule Only Polypeptide. STOP protein, as isolated from rat neuronal tissue, is a 145 kd polypeptide (Margolis *et al.*, 1986b). Microtubule stabilizing activity with similar isolation and regulatory properties is also demonstrable in non-neuronal tissues (Pirollet *et al.*, 1989), though the protein responsible for this activity has not yet been identified. At low stoichiometry relative to tubulin *in vitro*, the neuronal STOP protects microtubules against depolymerization induced by cold temperature (Margolis *et al.*, 1986b) and by drugs such as podophyllotoxin (Job *et al.*, 1981) and by millimolar calcium (Job *et al.*, 1981). At higher concentrations relative to tubulin, STOP induces a microtubule 'superstable' state, in which polymers resist freezing and sonication (Job *et al.*, 1987).

A critical step toward understanding the physiological role of STOP protein lies in the characterization of its association with microtubules *in vivo* and *in vitro*. We have previously provided extensive evidence that STOP protein is tightly associated with microtubules *in vitro* (Pabion *et al.*, 1984). This conclusion was based on kinetic studies of the STOP effect on polymers. Here, using specific anti-STOP monoclonal antibodies, we present evidence that this protein behaves as a standard MAP in that it remains quantitatively with polymers on microtubule recycling. We also show that it stabilizes pure tubulin microtubules to dilution induced disassembly *in vitro*. Further, we show that STOP is microtubule associated *in vivo*. Finally, we demonstrate its preferential association, in cultured neuronal

cells at physiological temperature, with the cold stable subset of microtubules in the mitotic spindle and in the midbody. Biochemical analysis has given evidence that STOP protein moves in a diffusional manner on microtubules at physiological temperature *in vitro* (Pabion *et al.*, 1984). This finding predicted that microtubules could provide a substrate for diffusional sliding of microtubule associated proteins, as recently reconfirmed by microscopic observation of the behavior of dynein (Vale *et al.*, 1989). STOP protein thus appears capable of both stabilizing polymers and sliding on them. It therefore appears to have the activity and the position in the mitotic spindle to generate the proposed diffusional poleward movement of chromosomes in anaphase (Garel *et al.*, 1987; Koshland *et al.*, 1988). These results must, however, be tempered by the fact that the monoclonal antibodies thus far derived are specific for neuronal STOP. Therefore, firm conclusions as to the generality of these results must await generation of more cross-reactive probes.

Results

We have previously demonstrated that STOP activity from rat brain copurified with a microtubule associated protein of 145 kd (Margolis *et al.*, 1986b). STOP activity and the 145 kd protein, associate with high affinity with Ca^{2+}–calmodulin, and release from calmodulin with EGTA (Margolis *et al.*, 1986b). The activity also binds quantitatively to an antibody affinity column bearing a monoclonal antibody specific for the 145 kd protein and coelutes with the 145 kd protein on elution of the column bound fraction with 6 M urea (Pirollet *et al.*, 1989).

We have now purified STOP to homogeneity and we demonstrate the concentration dependent stabilization of microtubules on addback of the protein *in vitro* (Figure 1). The purification involves heparin–sepharose column chromatography of the whole brain cytosol and monoclonal antibody affinity chromatography of the heparin column fraction with peak activity. Both procedures have been previously described in detail (Pirollet *et al.*, 1989). The affinity column specific eluate was then subjected to polyacrylamide gel electrophoresis. A horizontal strip, calculated by R_f to contain STOP protein, was sliced out and the protein collected by electroelution.

The electroeluted protein ran as a single band on re-electrophoresis (Figure 1b) and was competent to induce microtubule cold stability, as determined by filter assay of cold resistant polymers (Figure 1a). A comparison of specific activity of the electroeluted STOP with STOP obtained from the monoclonal antibody affinity column shows a consistent 1.6-fold rise in specific activity over the previous purification step (not shown). Since the STOP protein has been exposed to SDS during electrophoresis, it is evident that its activity is not seriously degraded by exposure to chaotropic agents. A direct test of the effect of 30 min exposure of STOP to SDS prior to assay in fact shows no apparent effect of SDS on STOP specific activity (Figure 1a).

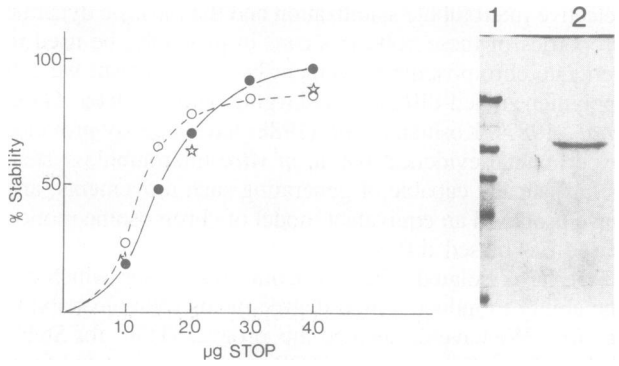

Fig. 1. STOP purification to homogeneity by electroelution. STOP protein was purified from rat brain by published procedures (Pirollet *et al.*, 1989), including heparin column chromatography and retention on a monoclonal antibody affinity column, followed by elution with 6 M urea. The protein was then subjected to polyacrylamide gel electrophoresis. The 0.5 cm of the gel encompassing the 145 kd position was sliced from the gel, electroeluted and assayed for STOP activity by filter assay procedure (see Materials and methods). Activity levels per μg of electroeluted protein are indicated by stars. An aliquot of the same electroeluted STOP protein was rechromatographed by gel electrophoresis, and is shown at right, Coomassie stained (lane 2). In these experiments 60 μg of tubulin (M_r = 100 kd) were used in each assay, thus the molar ratio of STOP (M_r = 145 kd) to tubulin at 50% stabilization is ~1:60. Lane 1 contains mol. wt standards: 205, 116, 97, 66 and 45 kd. Other areas of the gel, similarly sliced and electroeluted, showed no activity above background by filter assay (not shown). To determine the effects of SDS on STOP activity, STOP purified by monoclonal antibody affinity column was incubated at 0°C for 30 min in MME buffer with or without 0.1% SDS. Upon filter assay of microtubule cold stability, we obtained the stabilization values indicated per μg of added STOP. The STOP protein concentration in column eluates was calculated from total protein present and from the percent of protein at 145 kd in gel scans of the eluted protein. The percent stability indicated is the level of residual cold stable polymer (the 100% value is the total polymer assembly level at warm temperature). Results are shown for preincubation with SDS (dashed line, open circles); and for the control, containing no SDS (solid line, closed circles). The minimal dilution of STOP protein for assay of microtubule stabilization after preincubation or after electroelution was at least 50 fold. The filter assay for microtubule cold stability described under Materials and methods was modified to include 1% Triton X-100 in the dilution buffer to compete with the SDS.

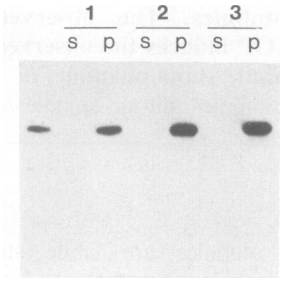

Fig. 2. MAP-like behavior of STOP. To demonstrate the recycling behavior of STOP protein with microtubules *in vitro*, we collected one cycle purified rat brain cold stable microtubules (Job *et al.*, 1982) as a sucrose cushion pellet by centrifugation (Margolis and Wilson, 1978). Microtubule cold stability was assayed in the next assembly cycle (not shown), and STOP protein abundance was followed through three subsequent assembly cycles. Dissociation of sucrose pellet microtubules and intervening disassembly steps, were performed by shearing polymers at cold temperature in the presence of 2 mM calcium, then centrifuging at 0°C for 25 min after chelation of calcium with 1 mM excess EGTA. Samples were then brought to 2.5 mM GTP and assembled for 30 min at 30°C. Microtubules were collected by centrifugation and the process reiterated through three assembly cycles. The various experimental conditions were maintained for three assembly cycles, and supernatant and pellet fractions were subjected to Western blot analysis using anti-STOP monoclonal antibody. Gel lanes were loaded with equal protein. STOP becomes relatively greater in abundance due to its more quantitative retention in the polymer fraction compared with tubulin. The lane on the left is the one cycle purified starting material. Cycles are indicated by numbers; S, supernatant; P, pellet.

Properties of STOP protein on microtubules in vitro

A stringent criterion for the identification of a protein as a true microtubule associated protein (MAP) is its quantitative association with microtubules upon recycling of polymers (Murphy *et al.*, 1977). Such a demonstration of the MAP-like behavior of STOP protein cannot be achieved by direct visualization of the protein because of its relatively low abundance as compared with other MAPs. It can however, be demonstrated using a specific monoclonal antibody probe for STOP protein, and Western blot analysis of microtubule protein during successive assembly cycles.

We have recycled all microtubules from a brain crude extract through three *in vitro* cycles of assembly. Cold stable microtubules are recovered at each cycle by disassembly induced by shearing and millimolar calcium at cold temperature (Margolis *et al.*, 1986b). STOP protein is quantitatively retained on polymers in each cycle and not detectably present in supernatant fractions (Figure 2). Because it is quantitatively retained, but some tubulin is lost in each cycle, its relative abundance increases with each cycle. No STOP protein pellets during centrifugation in the absence of microtubules, as demonstrated by its retention in the supernatant fraction during microtubule disassembly centrifugation steps (see legend to Figure 2).

To assay for the ability of STOP to protect microtubules against disassembly at elevated temperature, pure STOP protein was mixed with microtubules assembled from purified tubulin, and the resulting polymers were assayed by microscopy for resistance to dilution induced disassembly. Mixed at a molar ratio to tubulin that induced 100% cold stability by filter assay, STOP protected polymers against dilution induced disassembly at elevated temperature (Figure 3), and the stabilized polymers characteristically associated into loose bundles (Figure 3b).

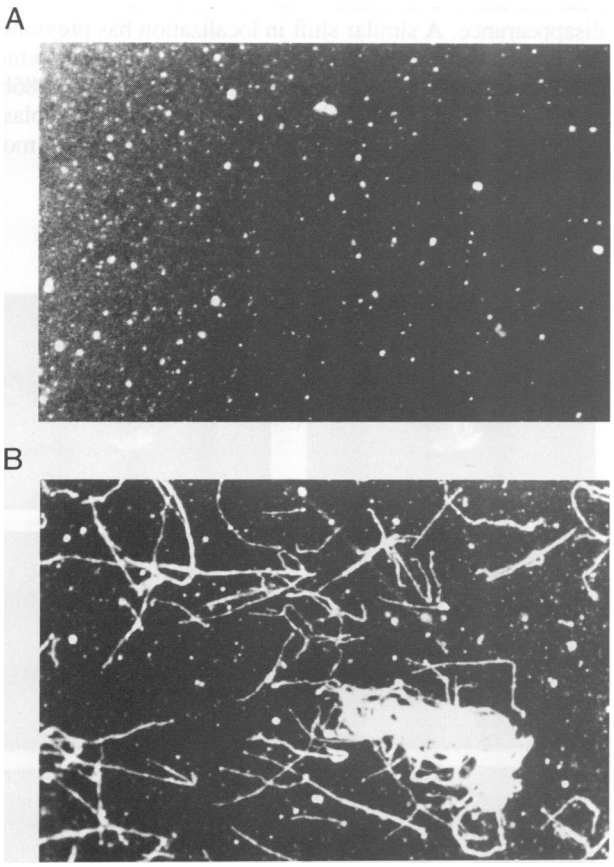

Fig. 3. Immunofluorescence assay of microtubule stabilization to dilution by STOP. Pure tubulin microtubules were assembled for 50 min at 30°C at 1.5 mg/ml, then challenged for stability to dilution either in the absence (**A**), or presence (**B**), of a 1:10 ratio to tubulin of purified STOP protein. The experimental conditions and the immunofluorescence analysis are as detailed in Materials and methods. (A) Microtubules, in the absence of STOP protein, were diluted by a factor of 200 under conditions destabilizing to pure tubulin microtubules prior to fixation and centrifugation onto coverslips. (B) Same conditions as in A, except the dilution buffer contained 3.4 μg STOP protein.

Association of STOP protein with cold stable microtubule subpopulations

We have used anti-STOP monoclonal antibody to determine the distribution of STOP protein in rat dorsal root ganglion cells in primary culture. STOP protein is present in these cells and reacts with the monoclonal antibody on Western blot analysis of a crude cytosolic preparation (Figure 3). Immunofluorescence microscopy shows the antigen localizes to the region of kinetochore microtubules in the spindle at physiological temperature (Figure 4). The presence of STOP is dependent on microtubules. When microtubule assembly is prevented by incubation of cells with nocodazole, STOP is not detectable by immunofluorescence in mitotic cells (Figure 4).

There is a distinct non-uniform distribution of cold stability among microtubules in cycling mammalian cells. In the mitotic apparatus, only the kinetochore associated microtubules are cold stable. In telophase, the midbody microtubule bundle is cold stable, while neighboring interphase microtubules are largely cold labile.

Double label analysis, comparing the distribution of STOP with that of tubulin, shows STOP is abundant in only the kinetochore-to-pole subset of microtubules at metaphase (Figure 5a). Astral microtubules and interpolar microtubules,

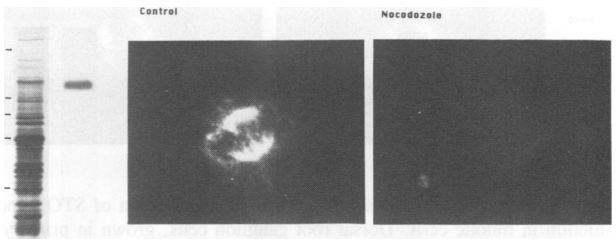

Fig. 4. Immunofluorescence of STOP in the mitotic spindle. A monoclonal antibody to rat brain STOP recognizes only one polypeptide, of 145 kd, in Western blots of newborn rat dorsal root ganglion extract. (**left**) The left lane is a Coomassie stained gel lane of whole cytosolic extract, and the right lane is a duplicate gel lane transferred to nitrocellulose for Western blot analysis. Mol. wt standards ran as indicated. (**right**) STOP protein has localized to microtubules in cycling cells by immunofluorescence, using the same monoclonal antibody '296' as on the left. For this purpose, dorsal root ganglion cells were explanted from newborn rats and maintained in primary culture. Cells on coverslips were prepared for immunofluorescence according to the methods of Osborn and Weber (1982). Monoclonal antibody was applied at 1:1000 dilution of ascites fluid. The secondary antibody (TAGO goat anti-mouse; 1:200) contained a rhodamine fluor. The immunofluorescence image on the left is of a mitotic spindle fixed at physiological temperature, and visualized with anti-STOP antibody. On the right, a mitotic cell (located with DAPI stain) blocked with 0.06 μg/ml nocodazole of 4 h prior to fixation and exposed identically to antibodies.

easily visible with anti-tubulin antibody, appear to lack STOP protein. STOP is readily visible only in the spindle region lying between the kinetochores and poles. In telophase, midbody microtubules contain STOP, but the antigen is not highly evident on interphase microtubule arrays in these same cells (Figure 5b). When cells are exposed to cold temperature, the cold stable microtubules remain and in this case the distribution of STOP is coincident with the residual microtubules of the mitotic spindle (Figure 5c). It is difficult to assess the presence of STOP protein on interphase microtubules, due to the relatively weak signal on these polymers. If present, it is evidently less abundant than it is on midbody microtubules in the same cells (Figure 5b).

Perhaps the clearest images of preferential distribution of STOP protein to microtubule subsets in the mitotic spindle are evident in anaphase, when interpolar microtubules that span between the chromatid sets may easily be distinguished from kinetochore-to-pole microtubules. A gallery of double labeled anaphase spindles (Figure 6) shows that the greatest abundance of STOP is clearly on the kinetochore-to-pole microtubules.

In control experiments, we have observed no signal cross-over when only one primary and both secondary antibodies are present (not shown). Additionally, images of STOP distribution are as described above when cells have been exposed to anti-STOP antibody alone. In these experiments, only the kinetochore-to-pole microtubules of the spindle appear to contain abundant antigen when visualized with single label at physiological temperature (Figure 4) and at cold temperature the kinetochore microtubules, which resist disassembly, contain STOP both in metaphase (Figure 7a) and in anaphase (Figure 7b). In telophase, the midbody microtubules resist cold temperature disassembly, and contain STOP (Figure 7c).

To summarize the pattern of STOP distribution, interphase cells display little specific STOP staining on microtubules. On entry into mitosis the spindle has little or no STOP staining until it appears that chromosomes are engaged in the prometaphase spindle. Staining is abundant on kinetochore microtubules and weak or absent on astral and interpolar microtubules in metaphase and anaphase. As cells enter telophase the STOP stain 'jumps' to the midbody microtubules, coincident with kinetochore microtubule disappearance. A similar shift in localization has previously been noted for calmodulin (Welsh *et al.*, 1978), with which STOP associates strongly *in vitro* (Margolis *et al.*, 1986b). In G_1, where midbody microtubules coexist in cytoplasm with interphase microtubules, STOP remains much more abundant on midbody microtubules.

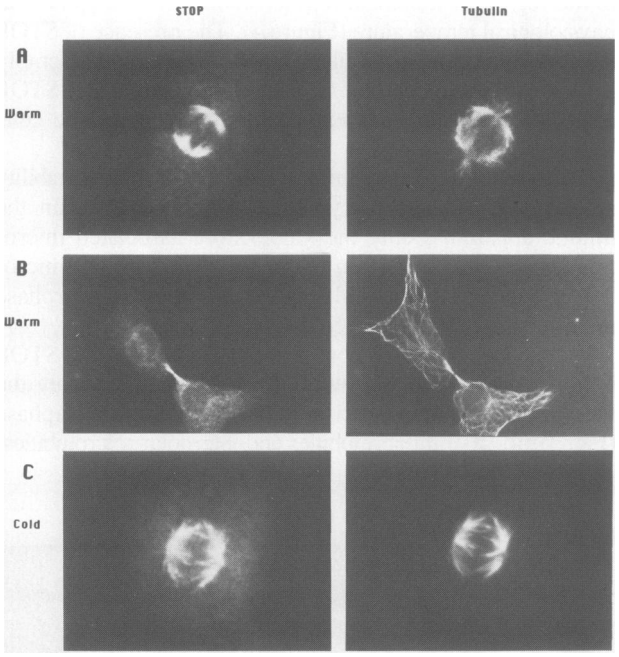

Fig. 5. Double label analysis of the relative distribution of STOP and tubulin in mitotic cells. Dorsal root ganglion cells, grown in primary culture, were prepared for immunofluorescence analysis with both rabbit anti α- and β-tubulin (Chemicon, AB935, used at 1:60) and mouse anti-STOP antibodies. The distribution of the two antibodies was determined using secondary antibodies at 1:200 with different fluors (fluorescein, STOP, rhodamine, tubulin). The secondary antibodies (Chemicon) were purchased 'pre-cleared' to avoid cross-species reactivity. Controls using both secondaries but only one primary were free of non-specific fluorescence. The top two sets of images show cells fixed at physiological temperature, the bottom set has been fixed after 20 min exposure to 0°C. (**A**) A metaphase cell is shown. At physiological temperature, only a subset of spindle microtubules contains STOP. Astral microtubules and interpolar microtubules, clearly apparent with anti-tubulin, do not appear to contain STOP antigen. (**B**) Cells connected by a midbody bridge of microtubules show the presence of STOP in the midbody at physiological temperature, but show no organized presence of STOP on the interphase array of microtubules seen with tubulin antibody. (**C**) At cold temperature, the kinetochore-to-pole microtubules resist disassembly (anti-tubulin image is at right), and this resistant population is coincident with the distribution of STOP antigen in these cells (left).

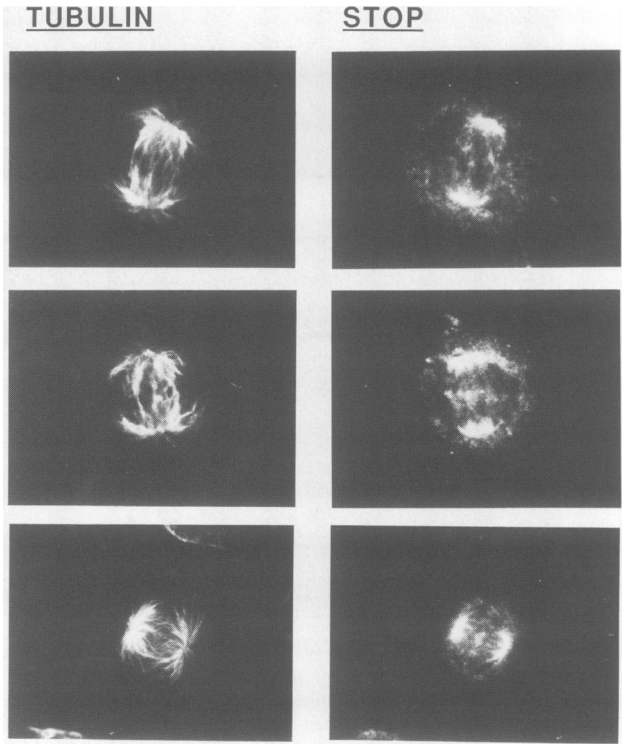

Fig. 6. Double label analysis of the distribution of STOP and tubulin in a gallery of anaphase cells. Cells and conditions of culture and preparation for microscopy are as in Figure 5. All cells were fixed at physiological temperature. The horizontal pairs represent single anaphase cells labeled with both anti-tubulin (left) and anti-STOP (right) antibodies.

We conclude that STOP protein distribution is preferentially associated with mitotic microtubule subsets at physiological temperature, and that the distribution of microtubules which display cold stability is apparently coincident with the intracellular distribution of STOP protein prior to cold temperature exposure.

Discussion

When exposed to cold temperature, certain microtubule subpopulations remain stable. In neuronal cells such microtubules are abundant (Jones *et al.*, 1980). In mitotic cells, cold stable microtubules are represented by specific subpopulations; the kinetochore-to-pole microtubules (Brinkley and Cartwright, 1975) and midbodies (Mullins and McIntosh, 1982). The results presented here demonstrate the non-uniform distribution in the mitotic spindle and in midbodies of STOP, a microtubule associated protein with well characterized *in vitro* properties. STOP protein, purified from brain tissue, is sufficient for microtubule cold stability *in vitro*. We have purified the protein to homogeneity and have raised monoclonal antibodies that are monospecific for this protein. At physiological temperature STOP appears substantially localized by immunofluorescence to those microtubule subpopulations of cycling cells that exhibit cold stability. At cold temperature, STOP is coincident in distribution with these cold resistant polymers.

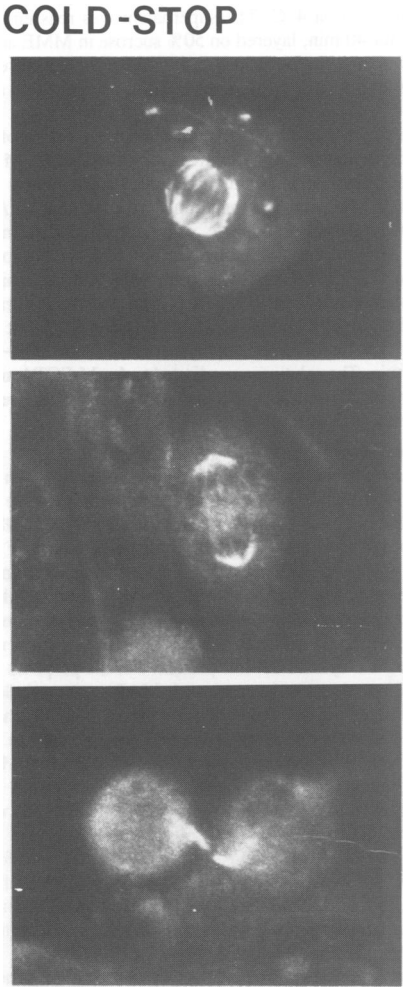

Fig. 7. Single label analysis of STOP distribution in mitotic cells exposed to cold temperature. Dorsal root ganglion cells in primary culture were exposed to cold temperature for 20 min prior to fixation, and were analyzed for STOP distribution with anti-STOP monoclonal antibody, as in Figure 3. A metaphase cell (**A**); an anaphase cell (**B**); and a telophase cell (**C**); are shown.

The presence of STOP protein on such polymer subpopulations correlates with and probably causes, their observed cold temperature stability. We have found that STOP protein can induce, under some conditions, a state of extreme stability in microtubules *in vitro* (Job *et al.*, 1987). Midbody microtubules appear to be similarly superstable in that they resist not only cold temperature, but also dilution and other destabilizing conditions during purification (Mullins and McIntosh, 1982). Midbodies are probably composed of microtubules that are stable at physiological temperature and STOP is now a reasonable candidate to explain the induction of the stable state in this microtubule subpopulation.

In contrast to midbody microtubules, microtubules of the mitotic spindle are evidently dynamic structures at physiological temperature. The recent elegant experiments of Mitchison have given evidence of a robust poleward flux of kinetochore microtubules at metaphase (Mitchison, 1989). Such apparent treadmilling behavior must entail rapid subunit uptake and loss from these polymers at physiological temperature.

Indeed, direct evidence for the free equilibrium with subunits of the kinetochore associated microtubule end has been convincingly demonstrated *in vitro* (Koshland *et al.*, 1988). This suggests a mechanism in which polymers associate with kinetochores and centrosomes by lateral linkages that allow tubulin subunits to freely enter or leave the polymer ends. Such a mechanism requires a microtubule associated protein that will slide or walk on the polymer surface. This protein might be associated with the microtubule itself or with the kinetochores or centrosomes.

We have previously shown that STOP protein has the apparent capacity to slide on its host polymer at physiological temperature (Pabion *et al.*, 1984). In accord with this finding, we have determined that the protein does not measurably interfere with microtubule treadmilling *in vitro* (Margolis *et al.*, 1986a). The principle that the microtubule polymer may act as a substrate for diffusional sliding of proteins has recently been reconfirmed by microscopic observation of dynein behavior *in vitro* (Vale *et al.*, 1989). Thus, although STOP stabilizes microtubules against rapid disassembly induced by cold temperature, the protein nevertheless appears to permit the dynamic behavior of microtubule ends by sliding on the polymer at elevated temperature. We believe these properties of STOP may interplay to create chromosome motility in anaphase spindles (Margolis *et al.*, 1986a; Garel *et al.*, 1987).

Our present observations are tempered by the fact that the available monoclonal antibodies are specific to neuronal STOP. We have, in fact, raised polyclonal antibodies which give identical immunofluorescent patterns on spindle and midbody microtubules in a variety of cell types. These antibodies probably recognize a non-neuronal STOP, but definitive analysis awaits the demonstration that the antigens recognized have STOP activity *in vivo* or belong to a STOP gene family. We are presently sequencing the cDNA for neuronal STOP and should soon be able to probe for non-neuronal homologues, and to generate specific antibodies against them.

Two previous publications on microtubule associated proteins in the mitotic spindle are relevant to the present report. Zieve and Solomon reported the selective presence of a 150 kd protein on microtubules in the mitotic spindle and its absence from interphase microtubules of NIL8 cells (Zieve and Solomon, 1982). STOP protein, as isolated from rat brain, has an apparent mass of 145 kd, and our immunofluorescence images show it is abundant only on mitotic microtubules. A definitive correlation with the previously identified protein cannot be drawn until we obtain STOP antibodies with a broader range of specificity.

Recently, a mitotic apparatus associated protein was reported to undergo Ca^{2+}−calmodulin dependent phosphorylation in sea urchin spindles as the microtubules become labile (Dinsmore and Sloboda, 1988). Microtubule cold stability *in vitro* due to STOP protein, as we have previously demonstrated (Job *et al.*, 1983), is labile to Ca^{2+}−calmodulin dependent phosphorylation. It is possible that the sea urchin spindle substrate is an echinoderm analogue of STOP protein.

The mechanism by which STOP preferentially binds to a subset of microtubules is unknown. It might relate to covalent modifications of the tubulin present in stable polymers *in vivo* such as detyrosinolation and acetylation (Gundersen *et al.*, 1987; Piperno *et al.*, 1987; Wehland and Weber, 1987). However, we have recently shown that the tyrosinolation status of tubulin does not interfere with STOP activity (Paturle *et al.*, 1989). Furthermore, detyrosinolation appears to follow rather than precede microtubule stabilization *in vivo* (Wehland and Weber, 1987; Gundersen *et al.*, 1987). On the other hand, tubulin acetylation occurs rapidly after polymer assembly in cells (Schatten *et al.*, 1988). The apparent limited distribution of acetylated tubulin to stable kinetochore polymers (Schatten *et al.*, 1988; Wilson and Forer, 1989) may therefore suggest a role for acetylation in STOP binding.

Calcium is known to play a central role in the progression of anaphase (Tombes and Borisy, 1989; Poenie *et al.*, 1986). Calmodulin, a calcium dependent regulatory protein, appears to associate specifically with the cold stable kinetochore microtubules of the mitotic spindle (Welsh *et al.*, 1979; Deery *et al.*, 1984; Sweet *et al.*, 1988). In telophase, calmodulin associates with microtubules of the midbody (Welsh *et al.*, 1978). In recent experiments where calmodulin has been specifically down-regulated by expression of antisense RNA, cells arrest in mitosis (Rasmussen and Means, 1989). STOP is the most clearly Ca^{2+}−calmodulin dependent of the known MAPs and the results of our present immunofluorescence studies suggest it may be present on the same microtubule subpopulations where calmodulin has been shown to localize during mitosis.

It is possible that STOP may have a function in both metaphase and anaphase, as proposed in our polymer guided diffusion model (Margolis *et al.*, 1986a; Garel *et al.*, 1987). The known interaction of STOP with calmodulin, and the above observations raise the possibility that STOP may indeed colocalize and interact specifically with calmodulin in the mitotic spindle to create a microtubule based metaphase and anaphase motility machinery. A definitive analysis of STOP distribution and function on microtubules in a variety of tissues now awaits our present efforts to generate specific molecular probes.

Materials and methods

Materials

The buffer used for microtubule protein purification and for microtubule recycling was 100 mM 2-(*N*-morpholino)ethane-sulfonic acid (Mes) (Sigma), 1.0 mM $MgCl_2$ and 1.0 mM ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) (Sigma), pH 6.75 (designated MME). Heparin−agarose (ultrogel A4R) was from Pierce. Rats (Sprague-Dawley) were from IFFA-CREDO (France) or (W/FU) from a Hutchinson Cancer Center inbred colony. [^{3}H]GTP (25−50 Ci/mmol) was obtained from New England Nuclear; nucleotides and acetate kinase were from Boehringer-Mannheim; the filtration assay used GF/C glass fiber filters from Whatman.

Microtubule protein isolation

Cold-labile microtubule protein from beef brain was isolated by three cycles of assembly and disassembly, in MME buffer, by modification of published procedures (Asnes and Wilson, 1979; Job and Margolis, 1984) as follows: Brain tissue was homogenized in 0.75 ml/g in MME buffer at 0°C, then centrifuged 40 min, 20 000 *g* at 4°C. The resulting supernatant was centrifuged 30 min at 150 000 *g* (average). Glycerol was added to the supernatant to 25% (v/v) final concentration, 2 mM GTP added and the solution incubated 45 min at 30°C. Following microtubule assembly, the material was centrifuged 45 min, 150 000 *g* (30°C). The supernatant was discarded, and the pellet resuspended to 10% of the previous volume in MME buffer at 0°C and homogenized. After 20 min at 0°C, the solution was brought to 25% glycerol and centrifuged 45 min at 4°C (150 000 *g*). The supernatant was then brought to 2 mM GTP, then assembled and centrifuged as above. The pellet was again suspended in MME at 15% of the previous volume, homogenized, left at 0°C for 20 min, then stored at −80°C in aliquots. For the third assembly cycle, aliquots were thawed and centrifuged as above at 4°C. The supernatant was reassembled in 2 mM GTP at 30°C for 40 min, layered on 50% sucrose in MME and centrifuged 2½ h in a fixed angle rotor (150 000 *g*, 30°C). Pellets were resuspended in MME, left 20 min at 0°C, then the solutions were stored at −80°C for later use.

Cold-stable microtubules were purified from the brains of 2 month old rats, according to published procedures (Job *et al.*, 1982; Margolis *et al.*, 1986a,b) with minor modifications. Briefly, eight brains (12 g) were homogenized in MME buffer containing 1.5 mM $CaCl_2$. After addition of 3 mM EGTA, the homogenate was centrifuged 30 min at 4°C at 150 000 g_{av}. Microtubules were then allowed to assemble for 1 h at 30°C with 0.05 mM GTP. The extract was cooled to 0°C for 10 min and layered onto a 50% sucrose cushion in MME buffer, and the assembled material was collected by centrifugation at 150 000 *g* for 1.25 h at 25°C. Cold-stable pellets were dissolved by depolymerization in MME−2 mM $CaCl_2$ at 0°C for 30 min. The solution was adjusted to 4 mM EGTA and centrifuged at 120 000 *g* for 30 min at 4°C. The supernatant was used for further assembly cycles.

STOP protein filter assay

The filter assay is essentially as previously reported (Pabion *et al.*, 1984; Job *et al.*, 1985). For the filter assay, microtubules were labeled with [^{3}H]GTP (Margolis and Wilson, 1978; Pirollet *et al.*, 1989) in MME buffer. The assay essentially relies on specific retention of microtubules on glass fiber filters, while unincorporated label and unassembled material pass freely through the filter. Variants on the basic methodology can be used to determine the stabilization of polymers by any microtubule binding protein. Here we have developed a method to assay polymer stabilization to cold temperature by a STOP-like protein. For the purposes of this paper, the following procedures were employed. MAP−microtubule protein was assembled at 1.5 mg/ml in MME buffer wth 0.05 mM [^{3}H]GTP (20 μCi/ml), 10 mM acetyl phosphate and 0.5 μg/ml acetate kinase. After 30 min at 30°C, 40 μl aliquots were mixed with 2 ml of MME at 30°C containing 40% sucrose and 40 μM GTP. The inclusion of GTP was important to reduce background noise in the assay. Samples of proteins to be assayed for STOP activity were mixed at this step and incubated an additional 30 min at 30°C. The assay is thus generally performed with a dilution factor of 50-fold (40 μl in 2 ml). This dilution factor can be diminished to 25-fold or increased at will without modification of the result. Dilution should be large enough so that the final salt concentration is < 15 mM after addition of column fractions.

After mixture, the tubes were maintained for 40 min on ice to disassemble all but cold-stable microtubules and then mixed with 80 μl of 25% glutaraldehyde. Total assembly level and blanks were determined by diluting MAP−microtubules in sucrose−MME before and after cold exposure. The 30°C values (total assembly levels) were routinely in the range of 32 000 c.p.m., and disassembled blanks were ~1500 c.p.m. Results, expressed

as percent stability, are the ratio of the c.p.m. after 40 min at 0°C minus blanks versus the total assembly c.p.m. minus blanks.

Immunofluorescence assay of microtubule stabilization to dilution by STOP

Recycled bovine brain tubulin was purified by phosphocellulose column chromatography (Weingarten *et al.*, 1975), and pure tubulin was assembled at 1.5 mg/ml for 50 min at 30°C in MME buffer, also containing 10% glycerol (vol/vol), 5% dimethyl sulfoxide (vol/vol), 16 mM $MgCl_2$, 100 μM GTP, 20 mM acetyl phosphate and 0.2 μg/ml acetate kinase (Job *et al.*, 1985). Microtubule assembly was confirmed by both turbidity measurement and by immunofluorescence analysis of microtubules centrifuged onto a coverslip (data not shown). Microtubule aliquots were then diluted 200-fold into MME buffer containing 30% sucrose and incubated a further 20 min at 30°C. The dilution buffer induces the complete disassembly of pure tubulin microtubules, but causes minimal disassembly of MAP containing microtubules (Job *et al.*, 1985). For one sample, the dilution buffer contained 3.4 μg of STOP protein, purified as in Figure 1 (yielding an estimated final ratio to tubulin of 1:10). At the end point, each sample received 0.75% glutaraldehyde. For immunofluorescence, each sample was then further diluted by a factor of 40 into 2 mM of MME buffer (at 30°C) also containing 0.75% glutaraldehyde, and centrifuged onto coverslips in a swinging bucket rotor (10 400 *g*, 60 min, 4°C). Coverslips were treated for 5 min with 10.0 mg/ml $NaBH_4$, washed with PBS + 0.05% Tween-20, then exposed to antibodies as follows: (i) incubated 60 min with 1:100 dilution of anti α- and β-tubulin antibodies (Amersham #356 and 357); (ii) washed 3× with PBS-Tween for 5 min each, and incubated 30 min with 1:100 goat anti-mouse FITC conjugated IgG (Tago #4350); and, (iii) Washed again and incubated 30 min with swine anti-goat FITC conjugated IgG (Tago #6201). Following a final wash, the coverslips were mounted using Dabco mounting medium for microscopy.

Monoclonal antibody

A murine hybridoma producing monoclonal antibody against brain-derived STOP protein, purified according to Margolis *et al.* (1986b), was developed by standard *in vivo* presentation techniques (Goding, 1983). Using the protocol of Gefter *et al.* (1977) to fuse spleen cells from BALB/c mice, inoculated with STOP protein, to NS-1 myeloma cells. Hybridomas producing antibodies against STOP protein were detected using an ELISA assay (Engvall, 1980) and were subcloned three times by limiting dilution to ensure monoclonality. Hybridoma cells were then grown in pristane-primed mice to produce high-titer antibody in ascites fluid. The anti-STOP monoclonal antibody is an IgG and binds protein A. A STOP antibody affinity column was generated by covalent cross-linking of STOP antibody to a Sepharose CL-4B (Pharmacia) column matrix, as detailed elsewhere (Pirollet *et al.*, 1989).

Dorsal root ganglion cell culture

Dorsal root ganglia were explanted from newborn rats under a dissecting microscope, using sterile conditions. The cells were exposed to 0.25% trypsin in Hank's medium for 30 min at 37°C, then dispersed with a Pasteur pipette and plated out for culture. Culture medium was Dulbecco's modified Eagle's, plus 10% fetal calf serum and 5% horse serum. The medium also contained 0.02% glucose and glutamine, and also penicillin/streptomycin and 100 ng/ml nerve growth factor (Sigma). For microscopy, cells were plated onto coverslips, which were pretreated by acid washing and coated with polylysine (0.1 mg/ml), then air dried.

Other methods

Protein concentrations were determined by the BCA procedure (Pierce). Polyacrylamide–SDS slab gels (8%) were prepared according to the methods of Sheir-Neiss *et al.* (1979). For Western blots, slab gels were electro-transferred to nitrocellulose sheets and probed with anti-STOP monoclonal antibody and [^{125}I] protein A, according to Towbin *et al.* (1979). Immuno-fluorescence procedures were according to the methods of Osborn and Weber (1982), fixing with 2.0% paraformaldehyde in PBS at 37°C for 20 min, followed by washes in 0.2% Triton X-100/PBS for 3 min, and then antibody application and wash protocols as above under 'Immunofluorescence assay'. Details of antibody concentrations and fluors used are given in figure legends. All microscopy was performed on a Leitz Diavert epifluorescence microscope at 630× under oil immersion.

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