Identification of MINUS, a small polypeptide that functions as a microtubule nucleation suppressor

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In eukaryotic cells, tubulin polymerization must be regulated precisely during cell division and differentiation. To identify new mechanisms involved in cellular microtubule formation, we isolated an activity that suppresses microtubule nucleation *in vitro*. The activity was due to a small acidic polypeptide of 4.7 kDa which we named MINUS (microtubule nucleation suppressor). MINUS inhibited tau- and taxol-mediated microtubule assembly *in vitro* and was inactivated by dephosphorylation. The protein was purified to homogeneity from cultured neural (PC12) cells and bovine brain. Microinjection of MINUS caused a transient loss of dynamic microtubules in Vero cells. The results suggest that MINUS acts with a novel mechanism on tubulin polymerization, thus regulating microtubule formation in living cells.

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Introduction

In eukaryotic cells, microtubules have an essential role in establishing and maintaining the architecture of the cytoplasm. In order to achieve this function, microtubule assembly must be regulated spatially and temporally during cell division and differentiation. It is thought that the polymerization state of microtubules is determined by additional proteins such as microtubule-associated proteins (MAPs) and cellular structures such as the centrosome (for reviews, see Hirokawa, 1994; Joshi, 1998). Most probably, other cellular components also play an important role in directing microtubule nucleation and elongation since microtubule dynamics change dramatically during the cell cycle (for reviews, see McNally, 1996; Hyman and Karsenti, 1998).

In fact, cellular activities that could shift the assembly state of microtubules toward free tubulin have been discovered. A non-proteinaceous activity from juvenile chicken and calf brain which inhibits microtubule assembly

in vitro (SurrIDGE and Burns, 1991), and a nuclear microtubule assembly inhibitor protein (MIP; Kotani *et al.*, 1988) have been reported. More recently, several microtubule-severing proteins have been isolated which may be involved in changing the microtubule array between interphase and mitosis (for a review, see McNally, 1996). In addition, stathmin (also designated Op18, p19, prosolin, metablastin), a ubiquitous cytosolic phosphoprotein, has been found to inhibit microtubule growth by sequestering tubulin (Belmont and Mitchison, 1996; Curmi *et al.*, 1997; Jourdain *et al.*, 1997), and SCG10, a neuron-specific membrane-associated protein with sequence homology to stathmin, has been reported to bind to microtubules, inhibit their assembly and induce disassembly (Riederer *et al.*, 1997). However, the cellular roles of these biomolecules remain unclear. For example, it has been shown that stathmin null mutant mice do not exhibit an overt phenotype regarding development of or predisposition to tumors (Schubert *et al.*, 1996). Moreover, no up-regulation of SCG10, which may functionally compensate for the loss of stathmin in these animals, was observed. To date, the results strongly suggest that key players in microtubule nucleation and assembly still need to be identified.

To search for a biomolecule that controls the formation of microtubules during cell division and differentiation, we designed a strategy to identify and purify negative regulators of microtubule assembly. Since overexpression of the MAP tau in the neural cell line PC12 does not induce an increase in the level of polymerized tubulin (Esmaeli-Azad *et al.*, 1994) or influence generation time (Brandt *et al.*, 1995), PC12 cells could be a good source for the isolation of such factors. Reasoning that negative regulators would interact with tubulin but not with microtubules, a microtubule-depleted fraction from PC12 post-nuclear supernatant was prepared and fractionated further by affinity chromatography on immobilized tubulin. For detection, a sensitive immunofluorescence-based population assay was used specifically to assay an activity that inhibits microtubule assembly.

In this study, we report the purification of a small cytosolic polypeptide with a mol. wt of 4.7 kDa from PC12 cells. This polypeptide functions as a microtubule nucleation suppressor *in vitro* and was thus named MINUS. In contrast to tubulin-sequestering factors such as stathmin or SCG10, MINUS is inactivated after phosphatase treatment. Microinjection studies with the purified protein suggest a role for MINUS in regulating microtubule formation in living cells.

Results

Isolation of a cytosolic activity from PC12 cells that inhibits tubulin polymerization *in vitro*

To search for a putative inhibitor of microtubule assembly, a procedure was designed to isolate a cytosolic activity

Fig. 1. Identification of an activity that inhibits tubulin polymerization *in vitro*. **(A)** Schematic representation of the purification strategy. **(B)** Effect of fractions on microtubule assembly after tubulin affinity chromatography. Mean numbers of microtubules, expressed as a percentage of a control experiment, are indicated. Note the inhibition of microtubule assembly in the 0.25 M KCl eluate from the tubulin column which is absent in the respective myoglobin column. Immunofluorescence micrographs of assembly reactions from a control experiment (buffer) and the respective 0.25 M KCl eluates are shown at the bottom. **(C)** Effect of the inhibitory activity in a microtubule sedimentation assay. A representative anti-tubulin dot-blot and the quantitation of pelleted material are shown. The mean value and standard deviation from three independent experiments are indicated. Affinity chromatography, microtubule assembly and microtubule sedimentation assays were performed as described in Materials and methods. Samples of the non-microtubule fraction after Sephadex G-10 chromatography corresponding to the material from 80 confluent 15 cm TC dishes were divided into two equal portions and loaded onto the myoglobin and tubulin affinity column, respectively. Then 1% (v/v) of each fraction was tested for inhibitory activity in microtubule assembly assays in the presence of 1.5 μ M tau. For the sedimentation assay, assembly was performed as described above, polymerized microtubules were separated by ultracentrifugation, immobilized on PVDF and immunodetected as described in Materials and methods. For quantitation, the amount of polymerized tubulin was normalized to a positive (+tau, -inhibitor, represented as 100%) and a negative (-tau, -inhibitor, represented as 0%) control.

that interacts with tubulin but not with microtubules (Figure 1A). As a sensitive assay, tau-dependent microtubule assembly assays were used to detect inhibitory activity (Fanara and Brandt, 1997). As starting material, post-nuclear supernatant from cultured PC12 cells was prepared and microtubule polymerization was induced with the assembly-promoting drug taxol (Kumar, 1981). Microtubule proteins were separated from the non-microtubule (non-MT) fraction by ultracentrifugation. The pellet (MT fraction) contained ~90% of the total cytosolic tubulin and the neuronal microtubule-associated protein tau. Residual taxol, which was found to interfere with the detection of inhibitory activity, was removed from the non-microtubule fraction by size exclusion chromatography on Sephadex G-10. The protein fraction which eluted in the exclusion volume was essentially free of taxol, as judged

by thin-layer chromatography, and inhibited tau-mediated microtubule assembly *in vitro* (Table I).

To purify further the inhibitory activity based on its interaction with dimeric tubulin, affinity chromatography on immobilized purified tubulin (PC-tubulin) was performed. Inhibitory activity, indicated by a strong decrease in microtubule number in our assay, was present in the 0.25 M KCl eluate from the tubulin column (Figure 1B). No such activity was found in the corresponding eluate from a myoglobin column used as a control for unspecific binding. The presence of the inhibitory activity was confirmed by a microtubule sedimentation assay used as an independent method (Figure 1C); only ~5% of tubulin sedimented in polymeric form in the presence of the inhibitor compared with an assembly reaction in the presence of tau. In addition to inhibitory activity in the

Table I. Purification table

Purification step	Total activity ($\times 10^6$ U)	Total volume (ml)	Volume activity ($\times 10^3$ U/ml)	Volume activity (relative to cytosolic extract)
A. PC12 cells				
Post-nuclear supernatant	0.23	264	0.84	1
Non-MT fraction	0.37	264	1.40	1.7
Affinity chromatography (0.25 M KCl)	2.13	164	13	15.4
Ion-exchange chromatography (MonoQ)	0.87	16	54	64.3
B. Bovine brain				
Cytosolic extract	0.04	50	0.8	1
Non-MT fraction	0.16	50	3.2	4
Affinity chromatography (0.1 M/0.25 M KCl)	3.0	200	15	18.7
Ion-exchange chromatography (MonoQ)	1.24	20	62	77.5

One unit (U) was defined as the amount of the respective fraction that caused a 50% reduction in the number of microtubules as determined from standard microtubule assembly assays. To determine the inhibitory activity during the purification, the respective fractions from individual purifications were pooled. For the unit determination, a standard curve was established using serial dilutions of the inhibitor after the MonoQ column (see Figure 4A). Note that the table does not reflect the total recovery of the inhibitor since partial material of the non-MT fraction (7%) was used for the myoglobin column.

0.25 M KCl eluate, strong inhibition was found in the flow-through fraction. The majority (>90%) of this activity could be recovered by reloading this fraction on the tubulin column (data not shown). This suggests that most of the inhibitory activity of the non-microtubule fraction is due to the fraction that can be eluted from the tubulin column.

Identification of the inhibitory activity as a small polypeptide: purification, biochemical and physicochemical characterization

Since it has been reported that DNA inhibits tubulin polymerization (Vater *et al.*, 1978), the inhibitory fraction was analyzed for the presence of nucleic acids. No DNA or RNA was detected by ethidium bromide staining after agarose gel electrophoresis (data not shown). In agreement, treatment with RNase did not affect the inhibitory activity (Table II). In contrast, the inhibitory activity was decreased substantially after digestion with carboxypeptidase Y or papain, indicating that a protein is responsible for the observed inhibition of microtubule assembly. Also, a brief heat treatment (5 min at 100°C) resulted in a complete loss of the inhibitory activity.

Additional purification of the inhibitor was performed using gel chromatography on a Superdex 200 HR operated with an FPLC system. The activity eluted from the Superdex column as a single peak with an M_r of 6.0 kDa (Figure 2A). To determine the homogeneity of the eluted activity, the peak fraction was subjected to anion exchange chromatography on a MonoQ column. The elution profile showed the presence of a single peak eluting at an NaCl concentration of 0.1 M, indicating the presence of a single slightly acidic polypeptide. An overview of the purification of the PC12 inhibitor, as judged from its activity in inhibiting tubulin polymerization, is given in Table I. A >60-fold enrichment of the volume activity was obtained during the different steps. The increase in total activity during the first two isolation steps is probably due to the removal of assembly-promoting proteins or of factors that interfere with the assembly assay.

To test for proteolytic activity of the isolated inhibitor, tubulin and tau were incubated with an excess of the purified inhibitor for 30 min at 25°C. No degradation as

Table II. Biochemical characteristics of the inhibitory activity

Sample	No. of microtubules (% of controls)
Control: + CPY ^a	100 $\pm$ 1.2
+ inhibitor + CPY	98 $\pm$ 1.5
+ inhibitor + PMSF	18 $\pm$ 1.2
+ inhibitor	19 $\pm$ 1.3
Control: + PMSF + CPY ^b	100 $\pm$ 14.7
+ PMSF + CPY + inhibitor	43 $\pm$ 5.6
+ inhibitor	15 $\pm$ 5.7
Control: + papain	100 $\pm$ 1.7
+ inhibitor + papain	77 $\pm$ 1.2
+ inhibitor + leupeptin	16 $\pm$ 1.0
+ inhibitor	19 $\pm$ 1.3
Control: + leupeptin + papain ^b	100 $\pm$ 9.8
+ leupeptin + papain + inhibitor	22 $\pm$ 12.0
+ inhibitor	32 $\pm$ 12.0
Control: + RNase	100 $\pm$ 1.8
+ inhibitor + RNase	15 $\pm$ 1.7
+ inhibitor	12 $\pm$ 1.5
Control: + RS ^c	100 $\pm$ 2.2
+ inhibitor + RS	18 $\pm$ 1.2
+ inhibitor	15 $\pm$ 1.5
Control: + AP ^d	100 $\pm$ 1.7
+ inhibitor + AP	96 $\pm$ 1.9
+ inhibitor	10 $\pm$ 2.0

^aCarboxypeptidase Y.

^bPre-incubation of protease inhibitor with protease.

^cGTP-regenerating system.

^dCalf intestinal alkaline phosphatase.

Protease, RNase and alkaline phosphatase treatments were performed as described in Materials and methods. All microtubule assays were performed in the presence of 1.5 μ M (RNase and RS) or 2.5 μ M (protease and AP) tau. Numbers are normalized to the respective control and represent the mean $\pm$ SD from five randomly chosen microscopic frames.

determined by immunoblot analysis was observed (data not shown), indicating that the inhibition was not due to proteolytic degradation of tau or tubulin. Inhibitory activity could also be due to GTPase activity since the presence of GDP inhibits microtubule nucleation (Johnson and Borisy, 1977; Bayley *et al.*, 1986). To test for this possibility, assembly assays were performed in the presence of a GTP-regenerating system (GTP-RS) as described previously (Mitchison and Kirschner, 1984a). The presence

Fig. 2. Purification of the inhibitory activity. (A) FPLC size exclusion chromatography (Superdex 200 HR) of the inhibitory activity after elution from the tubulin column. Protein elution at 214 nm (top) and inhibitory activity of individual fractions from the eluted peak (bottom) are shown. The mean numbers of microtubules, expressed as a percentage of a control experiment, are indicated. No inhibitory activity was observed in fractions 1–16 and 22–32 (not shown). Note the presence of a single peak with inhibitory activity with an estimated M_r of 6 kDa. Absorbance of the column effluent at 280 nm resulted in a peak at the same elution volume (not shown). (B) Ion exchange chromatography (MonoQ) of the inhibitory activity after elution from the Superdex 200 HR column. Protein elution at 214 nm (top) and inhibitory activity of individual fractions from the eluted main peak (bottom) are shown; no inhibitory activity was observed in fractions 1–10 and 15–50 (not shown). Note that the inhibitor eluted as a main peak at 0.1 M NaCl (arrow). Absorbance of the column effluent at 280 nm resulted in a main peak at the same NaCl concentration (not shown). Chromatography and cell-free assembly assays were performed as described in Materials and methods. Fractions eluting at 0.25 M KCl after tubulin affinity chromatography were loaded onto the Superdex 200 HR, and 1% (v/v) aliquots of the eluted fractions were tested in microtubule assembly assays. Active fractions were pooled, brought to 10 mM Tris-HCl and loaded onto an anion exchange column. Aliquots of 10% (v/v) of the eluted fractions were tested in microtubule assembly assays.

of the GTP-RS did not change the efficacy of the inhibition (Table II), suggesting that a change in the GDP:GTP ratio is not involved in the inhibitory activity. To characterize a potential regulation of the inhibitory activity further, we determined whether the isolated activity was sensitive to dephosphorylation. After treatment with alkaline phosphatase, the inhibitory activity was completely lost (Table II), indicating that the protein requires phosphorylation in order to be active.

Physicochemical characterization of the protein was performed by two-dimensional SDS-PAGE (2D-PAGE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The protein was resolved as a single spot with an M_r of 6.5 kDa and an estimated isoelectric point (pI) of 5.6 (Figure 3A). MALDI-MS analysis yielded a single charged peak with an M_r of 4.7 kDa (Figure 3B), a size which is ~20% lower than the estimated mass from the size exclusion column.

The 4.7 kDa protein functions in vitro as a microtubule nucleation suppressor

To characterize the mechanism by which the 4.7 kDa protein regulates tubulin polymerization, functional studies were performed using cell-free microtubule assembly assays. The protein inhibited tau-mediated microtubule assembly in a dose-dependent manner (Figure 4A). The number of microtubules was decreased similarly when the assembly was promoted with the microtubule-polymeriz-

ing drug taxol (Kumar, 1981; Figure 4B), indicating that the inhibitor acted on tubulin rather than on MAPs.

The inhibitor may inhibit assembly by sequestering tubulin, by depolymerizing microtubules or by blocking microtubule nucleation. To distinguish between these possibilities, the effect of the inhibitor on the kinetics of tubulin polymerization was analyzed. In the absence of inhibitor, the total amount of polymerized tubulin reached a maximum at 10 min and remained constant during further incubation (up to 30 min analyzed; Figure 4C, left). In the presence of the inhibitor, the amount of assembled polymer was reduced to ~40% after 5 min of the reaction. No effect was detected during the first 2 min, suggesting that the inhibitor did not prolong the nucleation as would be expected from a tubulin-sequestering factor (Jourdain *et al.*, 1997). The reduction in the amount of polymerized tubulin was due to a decreased number of microtubules but not to a change in microtubule lengths (Figure 4C, middle and right). This indicates that the inhibitor blocks microtubule nucleation but does not alter microtubule growth and stability, suggesting a specific function of the 4.7 kDa protein on the nucleation process. As an additional test, microtubules were polymerized in the presence of cross-linked microtubule seeds (EGS-seeds) which provide a nucleating site and circumvent the requirement for *de novo* nucleation (Symmons *et al.*, 1996). Incubation of the seeds with tubulin resulted in an increased mean length of the microtubules both in the

Fig. 3. (A) Two-dimensional PAGE (2D-PAGE) of the purified inhibitor after iodination with [125 I]Na. On the left, the same sample was run in one dimension. Note the presence of a single spot having an M_r of 6.5 kDa and an estimated pI of 5.6. (B) MALDI-MS of the purified inhibitor. The spectrum shows the region of the single charged peak with a molecular mass of 4721.360 Da. Active fractions after the MonoQ column were iodinated and separated by 2D-PAGE as described in Materials and methods. About 10^5 c.p.m. of the iodinated sample was loaded onto the gel for the first dimension. For the second dimension, another 10^5 c.p.m. were loaded directly on the left of the gel. For mass spectrometry, active fractions after Superdex 200 HR were dialyzed against water. Separation was done on an HPLC reversed-phase C18 column (Vydac), and eluted peaks were subjected to MALDI-MS.

absence and in the presence of the inhibitor (Figure 4D), confirming that the inhibitor does not affect microtubule growth under conditions in which it blocks microtubule nucleation.

To test for a potential microtubule-severing activity of the protein, microtubules were assembled in the presence of tau or taxol for 10 min. Then, the inhibitor was added to the pre-formed microtubules and the reaction products were analyzed after different incubation times. No significant change in the mean lengths of the microtubules was observed after 2 and 5 min (Figure 4E), excluding microtubule-severing activity of the factor. To analyze specifically the function of the 4.7 kDa protein during the microtubule nucleation period, the effect of the addition of the inhibitor at different time points was determined

under conditions where the total incubation was continued until 5 min. A decrease in microtubule number was observed when the inhibitor was added during the nucleation phase (0–30 s; Figure 4F, top). In contrast, no effect was observed when the inhibitor was added during the elongation phase (30–600 s), consistent with a selective function of the inhibitor on microtubule nucleation. To resolve better the nucleation phase, the same experiment was performed at 27°C to retard microtubule assembly (Murphy *et al.*, 1977). Under these conditions, the inhibitor completely blocked microtubule assembly when added during the nucleation phase (Figure 4F, bottom), confirming its function as a microtubule nucleation suppressor.

In most cell types, microtubule nucleation appears to be confined largely to specialized organelles called microtubule-organizing centers (MTOCs). To test for an effect of the inhibitor on MTOC-mediated microtubule polymerization, cell-free microtubule assembly assays were performed in the presence of isolated centrosomes as a nucleating agent. As described earlier, centrosome-mediated microtubule nucleation and growth can then be analyzed by pelleting the centrosome-nucleated microtubules through a glycerol cushion followed by anti-tubulin immunofluorescence staining (Mitchison and Kirschner, 1984b; Fanara and Brandt, 1997). Addition of MAPs at concentrations well below the critical concentration to induce microtubule assembly in the absence of MTOCs caused an increase in the number and length of the centrosome-nucleated microtubules (Figure 5B and C). In the presence of the inhibitor, the number of centrosome-nucleated microtubules was >2-fold reduced, both in the absence and presence of MAPs (Figure 5A–C). No significant effect on the mean lengths of the centrosomal microtubules was observed. The data indicate that MINUS acts as a potent inhibitor of microtubule nucleation, both in the presence and absence of MTOCs *in vitro*.

Identification and purification of a similar activity from bovine brain

To investigate whether a similar activity is present also in differentiated neural tissue, bovine brain was used as source for the purification. Fresh brain was homogenized and cytosolic extract was prepared by differential centrifugation. The cytosol was subjected to the same purification steps as described in Figure 1A. After affinity chromatography, an activity that inhibited tau-dependent microtubule assembly was detected in the 0.1–0.25 M eluates (Figure 6A). When loaded on the Superdex column, this fraction eluted as a single peak ($V_e = 18$ ml) (data not shown) having the same M_r of 6.0 kDa as the inhibitor from PC12 cells. However, in contrast to the PC12 inhibitor, the Superdex peak eluted in two major peaks from the MonoQ column (at 0.1 and 0.2 M NaCl, respectively), with only the latter having inhibitory activity (Figure 6B). A summary of the purification is shown in Table I. The effect of the bovine brain inhibitor on the kinetics of tubulin polymerization was almost identical to what was observed with the PC12 protein (Figure 6C, compare with Figure 4C). In addition, the bovine brain inhibitor was sensitive to proteolytic digestion (carboxypeptidase Y, papain) and to dephosphorylation as analyzed by assembly assays (data not shown). The fraction that eluted at 0.2 M NaCl was iodinated and analyzed by

Fig. 4. Effect of the inhibitory protein on *in vitro* tubulin polymerization. (A) Concentration-dependent inhibition of tubulin polymerization by the purified protein. (B) Effect of the inhibitor on tau- and taxol-mediated microtubule assembly. Note that the inhibitor decreases the number of microtubules similarly under both conditions. (C) Dose- and time-dependent inhibition of tau-mediated microtubule assembly. Whereas the number of microtubules is much lower in the presence of the inhibitor, no effect on microtubule length is observed. (D) Effect of the inhibitor on seeded microtubule assembly. EGS-stabilized microtubule seeds were pelleted directly (seeds only) or elongated for 10 min in the presence of 15 μ M tubulin with or without 4 U of the inhibitor. Incubation with tubulin results in an increased length of the seeds, in both the absence and presence of the inhibitor. (E) Effect of the inhibitor on pre-assembled microtubules in the presence of tau (top) and taxol (bottom). Microtubules were assembled in the presence of 1.5 μ M tau or 20 μ M taxol. After 10 min, 6.5 U of the inhibitor were added (time 0). At the times indicated, aliquots were removed and analyzed. The inhibitor induces a transient reduction in microtubule number, but neither permanent depolymerization nor severing of microtubules are observed. (F) Influence of inhibitor addition during the nucleation and elongation process. Assembly was started with 2.5 μ M tau at 37 or 27°C. After the times indicated, 6.5 U of the inhibitor were added and the reaction continued until a total incubation time of 5 min. The number of microtubules decreases only when the inhibitor is added during the nucleation process (0–30 s) but not later during the elongation phase. Microtubule assembly assays and number or length determinations were performed as described in Materials and methods. In (A), microtubules were assembled for 10 min at 37°C in the presence of tau (2 μ M) and various amounts of the inhibitor after the MonoQ column. In (B), assembly was performed using tau (1.5 μ M) or taxol (20 μ M) and 6.5 U of the inhibitor for 10 min at 37°C; in (C), 1.5 μ M tau was used. From the geometry of the microtubule-pelleting device, the absolute amount of polymer can be calculated. For the plateau of assembly in the absence of the inhibitor (C, left), it corresponds to ~50% of the total tubulin in the reaction. For the unit calibration in (A), a logarithmic curve fit of the indicated data was used. Mean and standard deviation are shown.

2D-PAGE. A single spot with an M_r of 6.5 kDa and a pI of 5.1 was visualized (Figure 6D, left). Therefore, the protein from bovine brain showed the same relative mobility, indicating an identical molecular weight as that in PC12 inhibitor cells, but was slightly more acidic. Since the activity of the inhibitor was sensitive to dephosphorylation, the bovine brain inhibitor was treated with alkaline phosphatase and separated by 2D-PAGE. A shift in the M_r (to 6.0 kDa) and the pI (to 6.3) consistent

with dephosphorylation of the protein was observed (Figure 6D, right). Taken together, the data suggest that both inhibitors are biochemically and functionally closely related and represent MINUSes.

Microinjection of MINUS causes a transient disassembly of dynamic microtubules in Vero cells

To analyze the activity of MINUS in living cells, microinjection experiments in African green monkey kidney

Fig. 5. Effect of the inhibitor on centrosome-mediated microtubule assembly. (A) Fluorescence micrograph of centrosome-nucleated microtubules that have been assembled in the absence or presence of the inhibitor. (B and C) Effect of the inhibitor on the number distribution (left) and mean microtubule lengths (right) of centrosome-nucleated microtubules that have been assembled in the absence (B) or presence (C) of tau protein. Under both conditions, the mean number of microtubules per centrosome decreased as a result of the presence of the inhibitor. In contrast, no major effect on the mean lengths of the centrosome-nucleated microtubules is observed. Centrosome-mediated microtubule assembly assays and number or length determinations were performed as described in Materials and methods. Assembly was with or without tau (0.15 μM) or purified inhibitor (4 U) for 10 min at 37°C. Microtubule staining employed monoclonal anti-tubulin antibody (DM1A) and Cy3-coupled secondary antibody. For the microtubule lengths, mean and standard deviation are shown.

(Vero) cells were performed. Vero cells were chosen as a model since they contain highly dynamic microtubules which turn over very fast (Bré *et al.*, 1987; Kreis, 1987). To determine the organization of cytoskeletal filaments, cells were processed for anti-tubulin immunofluorescence after a combined fixation-extraction protocol. Shortly after injection of the inhibitor, a diffuse tubulin staining but no intact microtubules were observed (Figure 7A–C). In contrast, a typical filamentous microtubule pattern was observed in control experiments [fluorescein isothiocyanate (FITC)-dextran injection; Figure 7G and H]. At 2 h after injection of the inhibitor, most cells had recovered as judged by the presence of an intact microtubule array (Figure 7D–F). Quantitation revealed that >80% of the cells had lost their microtubule network 15 min after microinjection of MINUS (Table III). More than 95% of the cells had re-established their microtubule array after 2 h. To determine more closely the time course of the recovery of the microtubule network, cells were analyzed at different time points after microinjection. Whereas no intact microtubules were observed in the majority of cells (89%) 30 min after injection, most cells (90%) had reestablished their microtubule network 60 min after injection, indicating that the microtubule array disas-

sembles and reforms in <1 h. To confirm that the loss of microtubules is due to the activity of MINUS, the inhibitory activity was boiled for 10 min prior to injection to inactivate the protein. Consistent with the *in vitro* results, injection of the heat-denatured inhibitor did not induce a loss of the microtubule network in the majority of cells (>80%) (Figure 8C and D).

Since the data from the cell-free assembly assays indicate that MINUS specifically blocks centrosomal and non-centrosomal microtubule nucleation, MINUS should only be active in the cell when dynamic microtubules reassemble. If true, conditions which decrease microtubule dynamics in the cell should inhibit the activity of MINUS. For this purpose, cells were incubated with low concentrations of the drug taxol which is known to decrease cellular microtubule dynamics. As a measure for reduced microtubule turnover, the ratio of acetylated to total tubulin was determined as described previously (Ilschner and Brandt, 1996). Treatment with 10^{-9} M taxol for 2 h resulted in no apparent change in the morphology of the cells but caused an almost 10-fold increase in the ratio of acetylated:total α -tubulin, indicating an efficient reduction of microtubule turnover. In the presence of taxol, no loss of microtubules was observed after injection of MINUS (Figure 8A and B), indicating that the activity of MINUS requires dynamic microtubules. The microinjection data confirm a function of the 4.7 kDa protein as a microtubule nucleation suppressor and suggest a role for MINUS as a regulator of microtubule assembly in cells.

Discussion

In this study, we identified an activity in cultured cells and bovine brain that inhibits microtubule assembly *in vitro* and in cells. The inhibition appeared to be due to a novel mechanism by which microtubule nucleation was suppressed and which we named MINUS for microtubule nucleation suppressor. Interestingly, MINUS did not affect the length distribution of microtubules in our assays, suggesting a specific function of MINUS in regulating microtubule nucleation versus microtubule growth and stability in the cell. It should, however, be noted that the assays used in this study allow the analysis of the behavior of microtubule populations rather than of individual microtubule ends, and other assays will have to be explored for a detailed mechanistic analysis of MINUS. In contrast to tubulin-sequestering proteins such as stathmin that prolong the nucleation phase and slow down microtubule growth (Belmont and Mitchison, 1996; Jourdain *et al.*, 1997), the isolated activity blocked microtubule assembly early during the nucleation period without affecting the elongation phase. Moreover, compared with a microtubule-destabilizing protein such as katanin (McNally and Vale, 1993), the inhibitor had no microtubule-severing activity.

We demonstrated that the activity was due to a small acidic polypeptide of 4.7 kDa which could be purified to homogeneity from cultured neural (PC12) cells. It is conceivable that MINUS escaped earlier efforts aimed at purifying negative regulators of microtubule assembly for three reasons. First, purification of a small polypeptide such as MINUS required a Tricine gel system since it would not separate during standard SDS-PAGE. Second, MINUS could not be visualized using in-gel staining

Fig. 6. Isolation of the inhibitory protein from bovine brain. **(A)** Effect of fractions eluted from tubulin and myoglobin columns on microtubule assembly. Mean numbers of microtubules, expressed as a percentage of a control experiment, are indicated. Note the inhibition of microtubule assembly in the 0.1 and 0.25 M KCl eluates from the tubulin column which is absent in the respective myoglobin column. **(B)** Ion exchange chromatography (MonoQ) of the inhibitory protein after elution from the Superdex 200 HR column. Protein elution at 214 nm (top) and inhibitory activity of individual fractions from the eluted main peak (bottom) are shown; no inhibitory activity was observed in fractions 1–20 and 25–50 (not shown). Note that the inhibitor eluted as a small peak at 0.2 M NaCl (arrow). **(C)** Time- and dose-dependent inhibition of tubulin polymerization by the purified protein from bovine brain cytosol. Note that the bovine brain inhibitor caused a drastic decrease in the number of microtubules but only a minor change in microtubule length similar to what was also observed for the purified PC12 inhibitor (compare with Figure 4C). **(D)** 2D-PAGE of the purified inhibitor after iodination with [¹²⁵I]Na without (–AP) and with (+AP) prior treatment with alkaline phosphatase. Note the presence of a single spot having an M_r of 6.5 kDa and an estimated pI of 5.1. After alkaline phosphatase treatment, a shift in the M_r (to 6.0 kDa) and the pI (to 6.3) is observed. Preparation of bovine brain cytosol, chromatographies, *in vitro* assembly assays and 2D-PAGE were performed as described in Materials and methods. A non-microtubule fraction from 50 ml of bovine brain cytosol (corresponding to the material from 160 g of bovine brain) was prepared, and taxol excluded by size exclusion chromatography. Equal portions of the protein eluates were dialyzed against BRB80 and loaded on the myoglobin and tubulin affinity column, respectively. Eluates were tested in standard microtubule assembly assays as described in the legend of Figure 1. Fractions eluting at 0.1 and 0.25 M KCl after tubulin affinity chromatography were combined and loaded onto the Superdex 200 HR and the eluted fractions tested in assembly assays as described in Figure 1. Active fractions were pooled, brought to 10 mM Tris–HCl and loaded on the MonoQ column. Samples of the eluates were tested for activity as described in Figure 1. In (C), microtubules were assembled in the presence of tau (2 μM) and various amounts of the inhibitor after the MonoQ column as described in the legend of Figure 4. Active fractions were iodinated and separated by 2D-PAGE as described in Materials and methods. A total of 4 × 10⁴ c.p.m. of the iodinated sample was either directly loaded onto the gel for the first dimension or treated with phosphatase prior to electrophoresis.

procedures such as Coomassie or silver staining, probably because it eluted during the fixation or washing steps as a result of its small size. Third, it appears that the design of our assay, which was aimed at detecting microtubule assembly inhibitors sensitively, was critical for the identification of MINUS. MINUS would not show up in assays using pre-assembled microtubules such as those used to detect microtubule-destabilizing activities (McNally and Vale, 1993; Belmont and Mitchison, 1996).

In most cell types, microtubules are attached to special-

ized organelles called MTOCs, such as the centrosome. However, the capacity of the cytoplasm to nucleate microtubules independently of an MTOC has been demonstrated using cell fragments and cytoplasts (Karsenti *et al.*, 1984; McNiven and Porter, 1988). Assembly of non-centrosomal microtubules which appear spontaneously in the cytoplasm has also been shown directly in cultured PtK1 cells (Vorobjev *et al.*, 1997). In these cells, free microtubules are stable at their minus ends and persist in the cytoplasm, suggesting that a ‘minus-end pathway’ for the formation

Fig. 7. Effect of microinjection of the inhibitory protein into living cells. Vero cells were injected with ~ 0.5 – 1 mU of purified inhibitor together with FITC–dextran (A–F) or FITC–dextran only (G and H) and analyzed after 15 min (A–C, G and H) or 2 h (D–F) incubation for an intact microtubule network using a combined fixation–extraction protocol. Phase contrast micrograph of a field with injected cells (A and D), FITC–dextran fluorescence to reveal injected cells (B, E and G), and anti-tubulin immunofluorescence to visualize the microtubule network (C, F and H) are shown. A diffuse tubulin staining but no intact microtubules were observed in cells 15 min after injection of the inhibitor (C). Control cells (H) and inhibitor-injected cells after 2 h of incubation (F) show an intact microtubule network. Cell culture, microinjection and immunofluorescence were performed as described in Materials and methods. Microtubule staining employed monoclonal anti-tubulin antibody (DM1A) and Cy3-coupled secondary antibody. Scale bar, $10\ \mu\text{m}$.

Table III. Effect of microinjection of the inhibitory protein on the microtubule network in Vero cells

Sample injected	Time after microinjection (min)	Total No. of injected cells analyzed	% of injected cells with loss of microtubules ^a
Control ^b	15	117	2.2 ± 2.2
Control ^b	120	120	2.8 ± 2.4
Inhibitor ^c	15	91	83.6 ± 4
Inhibitor ^c	120	115	4.1 ± 1.3
Taxol + inhibitor	15	126	1.5 ± 1.3

^aMean $\pm$ SD ($n = 3$).

^bFITC–dextran.

^cInhibitor with FITC–dextran.

Cell culture, microinjection and immunocytochemistry were performed as described in Materials and methods. The ratios of injected (FITC-positive) cells with no intact microtubule network was determined by visual inspection after anti-tubulin staining. For taxol treatment, cells were pre-incubated for 2 h in 10^{-9} M taxol in the culture medium. No taxol was present after the injection. For each condition, three independent experiments were performed. The loss of microtubules that occurred 15 min after injection of the inhibitor in the absence of taxol was statistically significantly different ($P < 0.005$) from the control or the taxol experiment.

of cytosolic microtubules exists. The function of MINUS *in vitro* and in cells presents it as a candidate molecule for the regulation of centrosomal and non-centrosomal microtubule nucleation in the cytoplasm. It will be interes-

ting to determine whether MINUS can affect the centrosomal and the minus-end pathway in the cell differentially, thus potentially regulating the relative proportion of microtubule formation through both pathways. Our functional

Fig. 8. Effect of microinjection of the inhibitory protein into taxol-pre-incubated living cells and effect of heat-inactivated inhibitor. (A and B) Vero cells were pre-incubated with 10^{-9} M taxol in culture medium for 2 h and injected with ~ 0.5 –1 mU of purified inhibitor containing FITC-dextran. (C and D) Vero cells were injected with FITC-dextran and ~ 0.5 –1 mU of purified inhibitor which had been boiled for 10 min to inactivate the protein. Cells were analyzed after 15 min incubation for an intact microtubule network using a combined fixation-extraction protocol. FITC-dextran fluorescence was used to reveal injected cells (A and C) and anti-tubulin immunofluorescence to visualize the microtubule network (B and D). An intact microtubule network is observed in taxol-pre-incubated cells 15 min after injection of the inhibitor and in untreated cells after injection of heat-inactivated inhibitor. Cell culture, microinjection and immunofluorescence were performed as described in Materials and methods. Microtubule staining employed monoclonal anti-tubulin antibody (DM1A) and Cy3-coupled secondary antibody. Scale bar, 10 μ m.

and physicochemical data provide evidence that the activity of MINUS is regulated through its phosphorylation-dephosphorylation state. Interestingly, dephosphorylation renders MINUS inactive, which is opposite to what has been reported for the tubulin-sequestering factor stathmin (Horwitz *et al.*, 1997). In addition, we have shown that MINUS activity is present in brain tissue, which may suggest a role for MINUS in establishing the characteristic neuronal microtubule organization. It is known that axonal and dendritic microtubules freely exist in the neuronal cytoplasm without an apparent connection to an MTOC (Bray and Bunge, 1981; Baas and Joshi, 1992) and it is possible that MINUS is involved in the temporal and spatial regulation of the assembly of these microtubules.

It is generally assumed that microtubule self-assembly proceeds through distinct nucleation and elongation steps (Voter and Erickson, 1984). The precise composition of the nucleation complex is unknown; however, a recent theoretical study suggests the presence of a four stage nucleation process which consists of intermediate and reversible steps where tubulin aggregates with increasing mass are formed (Flyvbjerg *et al.*, 1996). Based on turbidity measurements of assembly reactions, a critical size of 15 monomers for the stable nucleus has been concluded. For centrosomal nucleation, a ring-shaped structure containing γ -tubulin appears to act as the microtubule-nucleating unit (Moritz *et al.*, 1995; Zheng *et al.*,

1995). From our data, we propose that MINUS suppresses microtubule assembly either by blocking the formation of a stable nucleation complex or by disintegrating intermediate nucleation products. The low stoichiometry of the MINUS:total tubulin ratio (1:80 as estimated from the absorbance at 214 nm) required to induce a 50% reduction of microtubule number is consistent with a mechanism where MINUS interacts with small tubulin aggregates rather than with dimeric tubulin.

Given the function of MINUS as a microtubule nucleation suppressor, it appears surprising that microinjection of this protein causes a complete loss of the microtubule network in Vero cells. However, it is known that microtubules are highly dynamic in these cells and thus continuously disassemble and assemble (Bré *et al.*, 1987; Kreis, 1987). An activity which blocks nucleation would inhibit the reassembly of the depolymerizing microtubules thus resulting in a quick destruction of the microtubule array. Such a scenario is consistent with our finding that under conditions which decrease cellular microtubule dynamics (presence of low concentrations of taxol), the microtubule network is not affected by microinjection of MINUS. The fact that MINUS-injected cells re-establish an intact microtubule array could indicate that regulatory mechanisms exist that effectively control the function of MINUS *in vivo*. Since our *in vitro* data showed that MINUS requires phosphorylation to be active, a candidate would be regulation of the phosphorylation state of MINUS

by kinases/phosphatases. Alternatively, microinjected MINUS could have been degraded proteolytically in the cells after some time, thus rendering it inactive.

Given the novel mechanism of its function and the difference in its regulation compared with known microtubule-destabilizing proteins, it is likely that MINUS belongs to a new class of microtubule assembly regulators. Our purification strategy should allow the design of a method to isolate sufficient amounts of MINUS from tissue to perform additional functional assays, to obtain sequence information and to develop antibodies that will be useful to examine its biological roles in a definitive manner.

Materials and methods

Materials

All chemicals were purchased from Sigma (Deisenhofen, Germany), and cell culture materials were obtained from Life Technologies (Gaithersburg, MD), unless stated otherwise.

Preparation of tau, tubulin and EGS-seeds

Tau was purified from pET-3d human fetal tau-transformed and induced *Escherichia coli* BL21(DE3)pLysS cells as described previously (Léger *et al.*, 1997). Tubulin was isolated from bovine brain by two assembly-disassembly cycles and phosphocellulose chromatography as described previously (Brandt and Lee, 1993). The purified tubulin was free from MAPs as judged by Coomassie brilliant blue staining of heavily overloaded gels. Covalently cross-linked microtubule seeds (EGS-seeds) were prepared by a modification of the method of Koshland *et al.* (1988). Purified tubulin (30 μ M) in a total volume of 400 μ l of 80 mM K-PIPES pH 6.8, 1 mM EGTA, 50% (v/v) glycerol, 5 mM MgCl₂ and 1 mM GTP was polymerized for 20 min at 37°C, passed five times through a syringe with a 24-gauge needle and incubated further for 5 min. A 40 μ l aliquot of 37.5 mM ethylene glycol-bis-succinimidylsuccinate (EGS; Calbiochem, La Jolla, CA) in dimethylsulfoxide (DMSO) was added and the incubation continued for 15 min. The mixture was diluted into 2 ml of BRB80 (80 mM K-PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8) containing 50% sucrose, 10 mM K-glutamate and 1 mM GTP, passed four times through a syringe with a 24-gauge needle, and incubated for 1 h at room temperature. Microtubules were pelleted for 30 min at 100 000 g and dissolved in 100 μ l of BRB80 containing 1 mM GTP, quick-frozen in liquid nitrogen and stored in small aliquots at -80°C. The EGS-seeds were counted and the mean length determined after collecting them onto coverslips, followed by anti-tubulin immunofluorescence as described below. The procedure yielded 5×10^7 EGS-seeds/ μ l with a mean length of $\sim 2 \mu$ m.

Microtubule assembly and sedimentation assays

Microtubule assembly assays were performed as described previously (Fanara and Brandt, 1997) in the presence of 15 μ M tubulin, 1 mM GTP and various tau or taxol concentrations adjusted to a final volume of 25 μ l in BRB80. After glutaraldehyde fixation, 0.1% (v/v) aliquots were collected by centrifugation onto polylysine-treated coverslips. Seeded microtubule assembly was performed in the presence of 15 μ M tubulin, 1 mM GTP and 10^8 EGS-seeds in a total volume of 25 μ l of BRB80. Centrosome-mediated assembly assays were performed in the presence of 15 μ M tubulin, 1 mM GTP, 4×10^5 isolated centrosomes and with or without 0.15 μ M tau adjusted to a final volume of 50 μ l in BRB80. At this tau concentration, centrosome-mediated nucleation and microtubule growth were promoted, but no assembly of non-centrosomal microtubules was observed. After fixation, samples were loaded onto a glycerol cushion and collected by centrifugation onto polylysine-treated coverslips. After post-fixation, the samples were prepared for anti-tubulin immunofluorescence and number and length determinations were performed as described previously (Fanara and Brandt, 1997). For sedimentation assays, microtubule assembly assays were performed as described above. After 10 min incubation, reaction products were pelleted for 30 min at 100 000 g at 37°C and dissolved in 100 μ l of cold BRB80. Then 0.5 μ l of the solution were diluted with 500 μ l of dot-blot buffer [10 mM Tris-HCl, 0.9% (w/v) NaCl, 30% (v/v) methanol, 0.5% (w/v) deoxycholate, pH 7.4] and immobilized on pre-equilibrated Immobilon-P (Millipore, Bedford, MA) using a dot-blot hybridization chamber (Loxo, Dossenheim, Germany). Immunodetection with monoclonal anti-tubulin

(DM1A) and horseradish peroxidase-coupled goat anti-mouse (Dianova, Hamburg, Germany) antibodies and development using enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK) were performed as described previously (Ilschner and Brandt, 1996). Quantitation employed an image capture computer (Cybertech) and the program NIH Image 1.59.

Purification of MINUS

PC12 cell post-nuclear supernatant (PNS) was prepared as described previously (Brandt *et al.*, 1995), quick-frozen in liquid nitrogen and stored at -80°C. Bovine brain cytosolic extract was prepared from one fresh brain (320 g) which had been cleared of meninges, white matter and the cerebellum. The remaining tissue was homogenized in 2 vols of buffer A [38 mM K-aspartate, 38 mM K-gluconate, 38 mM K-glutamate, 10 mM HEPES, 2 mM Mg-acetate, 5 mM Na-carbonate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.4] using a Dounce homogenizer with eight up and down movements at 1000 r.p.m. The homogenate was centrifuged at 10 000 g at 4°C for 15 min, and the supernatant collected and centrifuged at 200 000 g at 4°C for 1 h. The supernatant (cytosolic fraction) was quick-frozen and stored at -80°C.

To prepare a non-MT fraction, aliquots of the PC12 cell PNS or the bovine brain cytosolic fraction were thawed on ice, and GTP and taxol were added to a final concentration of 2 mM and 20 μ M, respectively. After incubation for 30 min at 37°C, assembled microtubules were pelleted at 54 000 g for 35 min at 20°C. The supernatant (non-MT fraction) was carefully removed and loaded onto a Sephadex G-10 column (Pharmacia, bed volume 200 ml, column size 2.5 $\times$ 50 cm) equilibrated in 10-times diluted BRB80. Chromatography was performed using an automated econo system Model ES-1 (Bio-Rad) at 2.5 ml/min, and the elution profile at 280 nm was recorded. Fractions of 1 ml were collected, and the eluted 280 nm peak fractions were combined. The absence of taxol was confirmed by thin-layer chromatography (TLC) of the eluted protein on TLC aluminium sheets 20 $\times$ 20 cm silica gel 60 F 254 (Merck) as described previously (Parness and Horwitz, 1981).

To prepare a tubulin affinity column, 5 g of an active oxirane-Sepharose (Sepharose 4-B, Fluka) gel with a 12 atom spacer arm was synthesized according to Sundberg and Porath (1974). Coupling was performed with 20 mg of tubulin in 3 ml of 0.5 M Na₂CO₃, pH 9.5 for 24 h at 25°C with an average efficacy of 95%. After sequential washing with 0.5 M Na₂CO₃ pH 9.5 containing 1 M NaCl, double-distilled water, 0.1 M glycine pH 3.0, 50 mM Tris-HCl pH 7.8 containing 0.5 M NaCl, the remaining reactive groups were blocked with 2 M glycine, pH 8.5 at 23°C for 24 h. The resin was washed extensively with BRB80 containing 5% ethanol and 0.05 M thimerosal, and packed in a 1.0 $\times$ 20 cm chromatography column (bed volume 10 ml). Before use, the column was equilibrated overnight in BRB80. The sample was pre-cleared by centrifugation at 24 000 g for 5 min, diluted 20-fold with BRB80 containing 0.5 mM GTP, and loaded overnight in a cycle at a flow rate of 1 ml/min at 4°C. The column was washed sequentially with 20 ml of BRB80 containing 0.5 mM GTP, 20 ml of BRB80 containing 0.5 mM GTP and 4 mM EDTA, and eluted with 10 ml each of 0.1, 0.25, 0.5, 0.75 and 1 M KCl in BRB80. The myoglobin column to control for unspecific binding was prepared identically, except that coupling was with 20 mg myoglobin.

Active fractions from the tubulin column were dialyzed against 5 mM ammonium acetate pH 6.8, lyophilized and redissolved to give a final concentration of 50 mM ammonium acetate. The sample was loaded onto a size exclusion column (Superdex 200 HR, 1.0 $\times$ 30 cm, bed volume 24 ml, Pharmacia), connected to an FPLC system. Chromatography was performed with 50 mM ammonium acetate pH 6.8 at 0.3 ml/min. The elution profile at 214 nm was recorded, and 0.5 ml fractions were collected. For size calibration, the elution pattern of a protein mixture containing 350 μ g of ovalbumin, 150 μ g of carbonic anhydrase, 150 μ g of myoglobin and 100 μ g of aprotinin was determined. Active fractions were pooled, dialyzed against 1 mM Tris-HCl pH 8.0, lyophilized, redissolved to give a final concentration of 10 mM Tris-HCl, and loaded onto a MonoQ column (0.5 $\times$ 5 cm, bed volume 1 ml, Pharmacia). The column was washed with 5 ml of 10 mM Tris-HCl pH 8.0 and eluted with a 20 ml gradient of 0–0.25 M NaCl in Tris-HCl pH 8.0 followed by a steep gradient to 1 M NaCl at a flow rate of 1 ml/min. The elution profile at 214 nm was recorded and fractions of 1 ml were collected. Active fractions were pooled, quick-frozen and stored at -20°C.

Biochemical characterization

For protease treatments, 210 U of purified inhibitor in 25 μ l of BRB80 were incubated with 0.25 μ g of carboxypeptidase or papain for 1 h at 25°C. PMSF or leupeptin was added to a final concentration of 0.1 mM

and 60 μM , respectively, and incubation was continued for another hour on ice. The digestion mixture was diluted to a total of 150 μl with BRB80. Assembly assays were performed using 15 μl of this mixture. In control experiments, carboxypeptidase Y or papain were pre-incubated with PMSF or leupeptin for 1 h on ice, followed by addition of the inhibitor, and further incubated for 1 h at 25°C. For RNase treatment, 32 U of inhibitory protein in 50 μl of BRB80 were incubated with 2.5 μg of RNase A for 15 min at 35°C. Assembly assays were performed using 10 μl of this mixture. To test for the effect of a GTP-regenerating system, microtubule assays were performed with 6.4 U of inhibitor with or without 10 mM Na-acetyl phosphate and 1 U/ml acetate kinase as described previously (Mitchison and Kirschner, 1984a). For alkaline phosphatase (AP) treatment, 6.4 U of inhibitory protein in 10 μl of 50 mM Tris-HCl pH 8.0 were incubated for 10 min at 30°C, 5 μl of calf intestine alkaline phosphatase (10 U) were added and the incubation was continued for another 30 min. The pH was adjusted to 6.8 and the mixture was assayed for inhibitory activity.

Iodination of MINUS

Iodination of MINUS was performed using Iodogen-mediated ^{125}I -labeling according to Markwell and Fox (1978). A 70 μl aliquot of 0.5 mg/ml Iodogen (Pierce) in methylene chloride was evaporated onto the wall of a polypropylene Eppendorf tube in a nitrogen stream. Then 10 μl of 200 mM Na-borate buffer pH 8.5 and purified inhibitor after the MonoQ column in 40 μl of BRB80 were added, followed by 5 μl of [^{125}I]Na (1 mCi/10 μl ; Amersham), and the mixture was incubated for 20 min at room temperature. A 500 μl aliquot of 10% acetonitrile (AcCN), 0.1% trifluoroacetic acid (TFA) was added and the mixture was loaded onto a reverse-phase column (RP C-18 cartridge, bed volume 1 ml, Millipore) which was pre-equilibrated with 10% AcCN, 0.1% TFA. The column was washed with 10 ml of 10% AcCN, 0.1% TFA and eluted stepwise with an increasing concentration of AcCN up to 100% in 0.1% TFA. Fractions with the highest c.p.m. were pooled.

Physicochemical characterization

Two-dimensional PAGE was modified from O'Farrell (1975) in that urea was excluded from the first dimension and the second dimension was performed in a T = 16% Tricine SDS-PAGE according to Schagger and Von Jagow (1987) to resolve small polypeptides. As ampholytes, Servalyt 3–10 Iso-Dalt (Serva) was used. After electrophoresis, the gel was transferred to an exposure cassette containing a Hyperfilm (Amersham) and an intensifying screen and developed at -80°C. The isoelectric point was determined using the relative mobility of the protein in the first dimension and the calibration plot of the pH gradient formed by the ampholytes. For phosphatase treatment prior to SDS-PAGE, 50 μl of the iodinated sample were diluted with 50 μl of 50 mM Tris-HCl pH 8.0 and incubated for 10 min at 30°C. Calf intestine alkaline phosphatase (32 U) was added and incubated for another 30 min at 30°C. The sample was boiled for 5 min and loaded onto the first dimension.

For MALDI-MS, the purified inhibitor was separated by reversed-phase HPLC using a C18 column (Vydac, CA) as described previously (Houthaeve *et al.*, 1995). Eluted peaks were submitted to MALDI-MS as described earlier (Jensen *et al.*, 1996).

Cell culture, microinjection and immunocytochemistry

PC12 cells were cultured as described previously (Brandt *et al.*, 1995). African green monkey kidney (Vero) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) at 37°C and 5% CO_2 . For microinjection, the inhibitor was dialyzed against water, mixed 10:1 with 10% (w/w) FITC-dextran (mol. wt 150 000) used as a co-injection marker, and spun for at least 1 h at 10 000 g. Vero cells were plated onto polylysine-coated glass coverslips (Eppendorf CELLocate, 175 μm^2) in 5 cm TC dishes and injected at 80–90% confluency. Before microinjection, the medium was exchanged against 10 ml of fresh medium supplemented with 10 mM HEPES pH 7.5. Microinjection was carried out for 15 min at room temperature using a motorized micromanipulator (Eppendorf 5171) connected to a pneumatic transjector (Eppendorf 5246). Between 50 and 60 cells per coverslip were injected with ~0.5–1 pl/cell. After microinjection, dishes were washed once with phosphate-buffered saline (PBS), 5 ml of fresh medium were added, and incubation continued for the times indicated.

After incubation, living injected cells were counted and evaluated using immunofluorescence and phase contrast microscopy. Cells were not affected by the microinjection procedure as judged from their morphology and Trypan blue exclusion. For immunocytochemistry, the

coverslips were washed with PBS at room temperature and processed using a combined fixation-extraction protocol, as described previously (Lee and Rook, 1992), to visualize the microtubule network. Immunostaining was performed using monoclonal anti-tubulin (DM1A) and Cy3-coupled donkey anti-mouse antibody (Dianova, Germany). Coverslips were mounted in 1 mg/ml *p*-phenylenediamine in 90% (v/v) glycerol and 10% (v/v) PBS. Cells were photographed with 40 $\times$ Neofluar and 63 $\times$ PL APO lenses on a Zeiss Axiophot. Images were composed using Adobe Photoshop 4.0.

Other methods

SDS-PAGE, Coomassie staining of gels and immunoblots were performed as described previously (Brandt and Lee, 1993).

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References

- Baas,P.W. and Joshi,H.C. (1992) Gamma-tubulin distribution in the neuron: implications for the origins of neuritic microtubules. *J. Cell Biol.*, **119**, 171–178.
- Bayley,P.M., Butler,F.M. and Manser,E.J. (1986) Control of nucleation in microtubule self-assembly. *FEBS Lett.*, **205**, 230–234.
- Belmont,L.D. and Mitchison,T.J. (1996) Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell*, **84**, 623–631.
- Brandt,R. and Lee,G. (1993) Functional organization of microtubule-associated protein tau. Identification of regions which affect microtubule growth, nucleation and bundle formation *in vitro*. *J. Biol. Chem.*, **268**, 3414–3419.
- Brandt,R., Léger,J. and Lee,G. (1995) Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain. *J. Cell Biol.*, **131**, 1327–1340.
- Bray,D. and Bunge,M.B. (1981) Serial analysis of microtubules in cultured rat sensory axons. *J. Neurocytol.*, **10**, 589–605.
- Bré,M., Kreis,T.E. and Karsenti,E. (1987) Control of microtubule nucleation and stability in Madin-Darby canine kidney cells: the occurrence of noncentrosomal, stable detyrosinated microtubules. *J. Cell Biol.*, **105**, 1283–1296.
- Curmi,P.A., Andersen,S.S.L., Lachkar,S., Gavet,O., Karsenti,E., Knossow,M. and Sobel,A. (1997) The stathmin/tubulin interaction *in vitro*. *J. Biol. Chem.*, **272**, 25029–25036.
- Esmaeli-Azad,B., McCarty,J.H. and Feinstein,S.C. (1994) Sense and antisense transfection analysis of tau function—tau influences net microtubule assembly, neurite outgrowth and neuritic stability. *J. Cell Sci.*, **107**, 869–879.
- Fanara,P. and Brandt,R. (1997) Immunofluorescence population assays for studying MAP function. In Avila,J., Brandt,R. and Kosik,K. (eds), *Brain Microtubule Associated Proteins: Modifications in Disease*. Harwood, Amsterdam, pp. 259–273.
- Flyvbjerg,H., Jobs,E. and Leibler,S. (1996) Kinetics of self-assembling microtubules: an 'inverse problem' in biochemistry. *Proc. Natl Acad. Sci. USA*, **93**, 5975–5979.
- Hirokawa,N. (1994) Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr. Opin. Cell Biol.*, **6**, 74–81.
- Horwitz,S.B., Shen,H.-J., He,L., Dittmar,P., Neef,R., Chen,J. and Schubart,U.K. (1997) The microtubule-destabilizing activity of metablastin (p19) is controlled by phosphorylation. *J. Biol. Chem.*, **272**, 8129–8132.
- Houthaeve,T., Gausepohl,H., Mann,M. and Ashman,K. (1995) Automation of micro-preparation and enzymatic cleavage of gel electrophoretically separated proteins. *FEBS Lett.*, **376**, 91–94.
- Hyman,A. and Karsenti,E. (1998) The role of nucleation in patterning microtubule networks. *J. Cell Sci.*, **111**, 2077–2083.
- Ilschner,S. and Brandt,R. (1996) The transition of microglia to a ramified phenotype is associated with the formation of stable acetylated and detyrosinated microtubules. *Glia*, **18**, 129–140.

- Jensen, O.N., Podtelejnikov, A. and Mann, M. (1996) Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. *Rapid Commun. Mass Spectrosc.*, **10**, 1371–1378.
- Johnson, K.A. and Borisy, G.G. (1977) Kinetic analysis of microtubule self-assembly *in vitro*. *J. Mol. Biol.*, **117**, 1–31.
- Joshi, H.C. (1998) Microtubule dynamics in living cells. *Curr. Opin. Cell Biol.*, **10**, 35–44.
- Jourdain, L., Curmi, P., Sobel, A., Pantaloni, D. and Carlier, M.F. (1997) Stathmin: a tubulin-sequestering protein which forms a ternary T2S complex with two tubulin molecules. *Biochemistry*, **36**, 10817–10821.
- Karsenti, E., Kobayashi, S., Mitchison, T. and Kirschner, M.W. (1984) The role of centrosome in organizing of microtubule array: properties of cytoplasts containing or lacking centrosomes. *J. Cell Biol.*, **98**, 1763–1776.
- Koshland, D.E., Mitchison, T.J. and Kirschner, M.W. (1988) Polewards chromosome movement driven by microtubule depolymerization *in vitro*. *Nature*, **331**, 499–504.
- Kotani, S., Ikai, A., Kawai, G., Maekawa, S., Yokoyama, S. and Sakai, H. (1988) Microtubule-assembly inhibitor protein: its distribution, localization and physicochemical properties. *Eur. J. Biochem.*, **176**, 573–580.
- Kreis, T.E. (1987) Microtubules containing detyrosinated tubulin are less dynamic. *EMBO J.*, **6**, 2597–2606.
- Kumar, N. (1981) Taxol-induced polymerization of purified tubulin. Mechanism of action. *J. Biol. Chem.*, **256**, 10435–10442.
- Lee, G. and Rook, S.L. (1992) Expression of tau protein in non-neuronal cells: microtubule binding and stabilization. *J. Cell Sci.*, **102**, 227–237.
- Léger, J., Kempf, M., Lee, G. and Brandt, R. (1997) Conversion of serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau. *J. Biol. Chem.*, **272**, 8441–8446.
- Markwell, M.A.K. and Fox, C.F. (1978) Surface-specific iodination of membrane proteins of viruses and eukaryotic cells using 1,3,4,6-tetrachloro-3 β -glycouril. *Biochemistry*, **17**, 278–281.
- McNally, F.J. (1996) Modulation of microtubule dynamics during the cell cycle. *Curr. Opin. Cell Biol.*, **8**, 23–29.
- McNally, F.J. and Vale, R.D. (1993) Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell*, **75**, 419–429.
- McNiven, M.A. and Porter, K. (1988) Organization of microtubules in centrosome-free cytoplasm. *J. Cell Biol.*, **106**, 1593–1605.
- Mitchison, T. and Kirschner, M. (1984a) Dynamic instability of microtubule growth. *Nature*, **312**, 237–242.
- Mitchison, T. and Kirschner, M. (1984b) Microtubule assembly nucleated by isolated centrosomes. *Nature*, **312**, 232–237.
- Moritz, M., Braunfeld, M.B., Sedat, J.W., Alberts, B. and Agard, D.A. (1995) Microtubule nucleation by gamma-tubulin-containing rings in the centrosome. *Nature*, **378**, 638–640.
- Murphy, D.B., Johnson, K.A. and Borisy, G.G. (1977) Role of tubulin-associated proteins in microtubule nucleation and elongation. *J. Mol. Biol.*, **117**, 33–52.
- O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, **250**, 4007–4021.
- Parness, J. and Horwitz, S.B. (1981) Taxol binds to polymerized tubulin *in vitro*. *J. Cell Biol.*, **91**, 479–487.
- Riederer, B.M., Pellier, V., Antonsson, B., Di Paolo, G., Stimpson, S.A., Lütjens, R., Catsicas, S. and Grenningloh, G. (1997) Regulation of microtubule dynamics by the neuronal growth-associated protein SCG10. *Proc. Natl Acad. Sci. USA*, **94**, 741–745.
- Schägger, H. and Von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, **166**, 368–379.
- Schubert, U.K., Yu, J., Amat, J.A., Wang, Z., Hoffmann, M.K. and Edelmann, W. (1996) Normal development of mice lacking metablastin (P19), a phosphoprotein implicated in cell cycle regulation. *J. Biol. Chem.*, **271**, 14062–14066.
- Sundberg, L. and Porath, J. (1974) Preparation of adsorbents for biospecific affinity chromatography I. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. *J. Chromatogr.*, **90**, 87–98.
- Surridge, C.D. and Burns, R.G. (1991) Identification of an inhibitor of microtubule assembly present in juvenile brain which displays a novel mechanism of action involving suppression of self-nucleation. *Biochemistry*, **30**, 10813–10817.
- Symmons, M.F., Martin, S.R. and Bayley, P.M. (1996) Dynamic properties of nucleated microtubules: GTP utilisation in the subcritical concentration regime. *J. Cell Sci.*, **109**, 2755–2766.
- Vater, W., Muller, H. and Unger, E. (1978) Inhibition of microtubule formation by DNA. *Biochem. Biophys. Res. Commun.*, **84**, 721–726.
- Vorobjev, I.A., Svitkina, T.M. and Borisy, G.G. (1997) Cytoplasmic assembly of microtubules in cultured cells. *J. Cell Sci.*, **110**, 2635–2645.
- Voter, W.A. and Erickson, H.P. (1984) The kinetics of microtubule assembly. Evidence for a two-stage nucleation mechanism. *J. Biol. Chem.*, **259**, 10430–10438.
- Zheng, Y., Wong, M.L., Alberts, B. and Mitchison, T. (1995) Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature*, **378**, 578–583.

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