Monoclonal Antibody to Microtubule-Associated STOP Protein: Affinity Purification of Neuronal STOP Activity and Comparison of Antigen with Activity in Neuronal and Nonneuronal Cell Extracts†

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ABSTRACT: Microtubules, ordinarily cold-labile structures, are made entirely resistant to cold temperature by the presence of substoichiometric amounts of STOP (stable tubule only polypeptide), a microtubule-associated protein. We have produced a monoclonal antibody which specifically recognizes a 145-kDa protein previously implicated in STOP activity in rat brain extracts. An antibody affinity column removes both the 145-kDa protein and STOP activity from solution. A urea eluate from the affinity column contains the 145-kDa protein and exhibits substantial STOP activity. We conclude the 145-kDa protein accounts for all measurable STOP activity in rat neuronal extracts. For this work, we have developed an assay of microtubule cold stability which is generally applicable to the detection of STOP activity in various tissues. Using this assay, we show STOP activity is most abundant in neuronal tissue but is detectable in all tissues tested, with the exception of heart muscle. In all tissues that we have examined, STOP activity elutes as a single peak from heparin affinity columns, and in common with brain STOP, all activity is Ca^{2+}–calmodulin sensitive. The monoclonal antibody recognizes the 145-kDa STOP in rat neuronal extracts but reacts with no protein in active fractions from other tissue. A similar, but not identical, analogue of brain STOP thus appears to be widespread in mammalian tissues.

Microtubules in the cell cytoplasm and the mitotic apparatus for the most part rapidly depolymerize when exposed to cold temperature or to assembly-inhibiting drugs. A substantial subpopulation in cycling cells, however, is indefinitely resistant to these agents (Brinkley & Cartwright, 1975; Krystal et al., 1978; Schulze & Kirschner, 1986). In mammalian brain, cold-stable microtubules are common (Heidemann et al., 1985).

When microtubules are polymerized in vitro from mammalian brain crude extract, cold-stable microtubules appear in abundance (Webb & Wilson, 1980; Margolis & Rauch, 1981). A protein factor, which we have designated STOP[1] (stable tubule only polypeptide), induces stability at concentrations that are highly substoichiometric to tubulin (Job et al., 1982; Margolis et al., 1986b). We have presented evidence that such profound stabilization occurs because STOPs indefinitely block depolymerization past the subunit they come to occupy on the polymer end (Job et al., 1982). In vitro, one can readily demonstrate that STOP activity is strongly modulated by such physiological regulators as calmodulin (Job et al., 1981), to which STOP binds avidly (Margolis et al., 1986b), and ATP (Job et al., 1983).

In addition to its stabilizing capacity, STOP protein exhibits an in vitro sliding behavior characterized by a free diffusional migration of STOP protein on its host polymer (Pabion et al., 1984). Sliding behavior suggests that STOP may have motility as well as stabilizing functions in the cell.

We have previously reported a tentative identification of a 145-kDa protein as a brain-derived STOP, on the basis of its predominance in highly enriched preparations with STOP activity (Margolis et al., 1986b). We report here that we have formed a highly specific monoclonal antibody which recognizes the brain-derived 145-kDa protein previously identified as copurifying with STOP activity. An affinity column formed with the monoclonal antibody removes the intact 145-kDa protein from brain isolates and depletes STOP activity. Further, a single polypeptide of 145 kDa elutes from the affinity column with 6 M urea and exhibits STOP activity by in vitro assay. We thus confirm that brain STOP activity is expressed by a 145-kDa microtubule-associated protein against which we have formed a monoclonal antibody.

Cold-stable microtubules are also present in nonneuronal cells in culture and often coincide with microtubule subpopulations of unique function (Brinkley & Cartwright, 1975; Krystal et al., 1978). It has therefore been of substantial interest to determine if STOP activity is widespread in tissue distribution and if STOP-like stabilizing functions in nonneuronal tissues can be correlated with a protein factor antigenic to the monoclonal antibody.

We find that STOP activity is indeed present in a variety of nonneuronal tissues. The activity that we detect shares some properties in common with brain-derived STOP. As with brain STOP, all activity found in nonneuronal extracts elutes in a moderate salt step from heparin–agarose columns and is entirely labile to Ca^{2+}–calmodulin in the micromolar range. However, no 145-kDa antigen is detectable in fractions with STOP activity purified from lung, kidney, or liver.

We conclude that a STOP protein activity is present in nonneuronal tissues and that STOP activity is associated with a factor similar in behavior to brain-derived STOP, but this homologue lacks the epitope recognized by the brain STOP-specific antibody.

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[1] Abbreviations: MAP, microtubule-associated protein; STOP, stable tubule only polypeptide; MME, buffer composed of 100 mM Mes, 1 mM $MgCl_2$, and 1 mM EGTA, pH 6.75; PBS, phosphate-buffered saline.

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In addition to the monoclonal antibody, we have developed a new and highly sensitive filter assay of microtubule stabilization for this work. Our previous work on STOP protein had relied on turbidity assays, which were adequate to measure the substantial activity present in brain extracts. Such assays were not sensitive enough to detect the small amounts of STOP activity present in other tissue extracts. Since it involves sample dilution prior to assay, the filter assay we have developed has the further advantage of allowing assay of column eluate samples containing salt. As we have shown elsewhere (Job et al., 1985), variants on the filter assay used here can detect microtubule stabilization by low concentrations of other microtubule-associated proteins.

Materials and Methods

Materials. [^{3}H]GTP (25–50 Ci/mmol) was obtained from New England Nuclear; nucleotides and acetate kinase were products of Boehringer-Mannheim; the filtration assay used GF/C glass fiber filters from Whatman. The buffer used for protein purification was 100 mM 2-(*N*-morpholino)ethanesulfonic 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 (Ultragel A4R) was from LKB or (Sepharose; CL-4B) from Pharmacia. Rats (Sprague-Dawley) were from IFFA–CREDO (France) or (W/FU) from a Hutchinson Cancer Center inbred colony. Calmodulin was purified from ram testis by the method of Autric et al. (1980).

Microtubule Protein Isolation. Cold-labile microtubule protein from beef brain was isolated by three cycles of assembly and disassembly, in MME buffer, according to published procedures (Asnes & Wilson, 1979; Job & Margolis, 1984), with the following modification. For the third assembly cycle, protein was resuspended from pelleted microtubules and centrifuged in MME buffer for 30 min, 120000g_{av}, at 4 °C. The supernatant fraction was reassembled in 2 mM GTP at 30 °C for 45 min, layered on 50% sucrose in MME buffer, and centrifuged 2 h in a fixed-angle rotor (120000g_{av}, 30 °C). Pellets (referred to as "MAP-microtubule protein") 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., 1986b) 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 150000g_{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 150000g 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 120000g for 30 min at 4 °C. The supernatant was used for further separatory procedures or for STOP activity measurements.

Preparation of Cytosols. Bovine brain from a local slaughterhouse was processed at 0–4 °C within 1 h after slaughter. Rats were anesthetized with diethyl ether, decapitated, and dissected for organs. For this study, we removed brain, spinal cord, lung, liver, skeletal muscle, heart, and kidney. Approximately 7–15 g of tissue was further washed in MME, minced, and homogenized in MME buffer (1:1, g/mL) containing 5 mM dithiothreitol, 10 μg/mL leupeptin, and 10^4 units/mL aprotinin at 4 °C using a motor-driven Potter–Elvehjem glass homogenizer. The homogenate was centrifuged for 30 min at 4 °C at 150000g and the supernatant ("cytosol") was used for further separatory procedures or for STOP activity assay.

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 & Wilson, 1978) 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 with 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 less than 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 32000 cpm, and disassembled blanks were approximately 1500 cpm. Results, expressed as percent stability, are the ratio of the cpm after 40 min at 0 °C minus blanks versus the total assembly cpm minus blanks.

Turbidimetric Assay. Microtubule protein samples were assembled in microcuvettes (0.3 mL) in MME buffer containing a GTP regeneration system and 0.05 mM GTP (as above in the STOP filter assay). Changes in optical density were followed at 350 nm and 30 °C by using a UVIKON 810 recording spectrophotometer equipped with a constant-temperature chamber and a cell programmer. Assembly was initiated by warming to 30 °C samples which had been previously adjusted to the base line. After 30 min at elevated temperature, the chamber temperature was rapidly decreased to 7 °C using a preequilibrated water bath, and disassembly was monitored for 40 min. (Pabion et al., 1984). Results, expressed as percent stability, were the ratio of ΔOD at 350 nm after 40 min at 7 °C vs ΔOD at 350 nm after total assembly.

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 by using an ELISA assay (Engvall, 1980) and were subcloned 3 times by limiting dilution to ensure monoclonality. Hybridoma cells were then grown in pristane-primed mice to produce high-titer antibody in

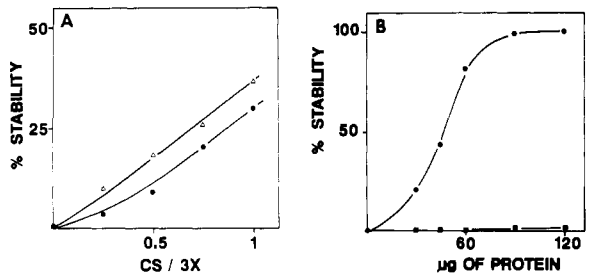

FIGURE 1: Filter assay of microtubule cold stability. (A) Validation of the filter assay for STOP activity. Beef brain three cycle purified MAP-microtubule protein ("3X"; 2 mg/mL), purified as specified under Materials and Methods, and cold-stable rat microtubule protein ("CS"; 3 mg/mL) were mixed in the proportions indicated, diluted to a final concentration chosen to yield equivalent final assembly levels, and assembled at 30 °C. Total assembly and cold-induced disassembly levels were determined by turbidity (△) or filter assay (●). The filter assay is described under Materials and Methods. Results are expressed as percent stability after 40 min at 0 °C. (B) Specificity of the STOP filter assay. After assembly of beef brain recycled MAP-microtubule protein (1.5 mg/mL) for 30 min at 30 °C, with [^{3}H]GTP, 40 μL of microtubules was diluted into 2 mL of 40% sucrose–MME. The indicated aliquots from 3 mg/mL stocks of cold-stable rat microtubule protein (●) or recycled beef brain protein (■) were added (maximum added volume, 40 μL) and assayed for STOP activity as described under Materials and Methods. Stabilization depends on the presence of STOP protein.

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. In this experiment, a mouse anti-tubulin IgG monoclonal antibody ("YL1/2"; generous gift of J. Wehland) was used to generate a control column.

Other Methods. Protein concentrations were determined by the method of Bradford (1976). Polyacrylamide–sodium dodecyl sulfate slab gels (8%) were prepared according to the methods of Sheir-Neiss et al. (1978). Western blots were processed according to the methods of Towbin et al. (1979), using ^{125}I-protein A.

RESULTS

Validation of the Filter Assay. In prior publications on microtubule cold stability, we have used principally two methods for quantitation of cold stability levels: measurement of residual polymer turbidity at cold temperature by spectrophotometry [e.g., see Job et al. (1984)] or assay of radioactive cold-resistant polymers trapped on glass fiber filters (Pabion et al., 1984; Job et al., 1985). In the present work, we have developed a variant on the filter assay which is highly sensitive to small amounts of STOP activity, and since it is performed under conditions of high dilution of the constituents, can be used to detect STOP activity in protein fractions originally containing high salt.

The assay involves admixture of possible STOP-containing fractions to highly diluted cold-labile microtubules (stabilized in a sucrose-containing buffer) that have been radiolabeled by [^{3}H]GTP incorporation during assembly (Margolis & Wilson, 1978). After cold exposure of these highly diluted polymers, the only microtubules that survive and yield a filter assay signal are those stabilized by STOP-like activity.

The specificity and sensitivity of this assay for STOP activity are validated in Figure 1, in which we have compared cold-stable levels of brain-derived cold-stable microtubules as measured by turbidity with the levels of cold stability detected for the same sample by filter assay. In this comparison, recycled cold-labile microtubule protein (MAP-microtubules, containing the full complement of MAPs) was mixed in the ratios indicated with recycled cold-stable microtubule protein from rat brain; the samples were then assembled to steady state and subjected to analysis by the two methods. We find that the filter assay closely parallels the turbidity assay in detection of cold stability levels, although it is somewhat nonlinear at low cold-stable levels (Figure 1A). It is generally reliable for cold stability levels which exceed 25%, although one can expect a small but reproducible underestimation of cold stability compared with that obtained by turbidity analysis. The presence of MAPs other than STOP on microtubules fails to stabilize microtubule polymers in this assay (Figure 1B, lower line).

The assay works equally well for addition of samples that contain STOP protein to microtubules preassembled to steady state. To demonstrate the specificity of the assay for the presence of STOP activity, we preassembled recycled cold-labile microtubules to steady state, then diluted aliquots, and added the indicated amounts of unassembled cold-labile or cold-stable microtubule protein (Figure 1B). The result indicates that stabilization of steady-state polymers is detected only when a sample containing STOP protein is added to the assay mixture. Further, the assay yields as much as 100% cold stability and detects STOP protein at quite low concentrations.

Detection of STOP Protein Activity in Heparin Column Fractions. We have applied the filter assay of STOP protein activity to quantitate the presence of STOP protein activity during column purification. For this purpose, either crude brain cytosol or cold-stable microtubule protein derived from such a cytosol preparation was loaded onto a heparin–agarose column, and fractions eluting in a linear salt gradient were assayed for the presence of STOP activity. Because of the high dilution of column fractions during assay, the final concentration of salt contributed by the column fractions to the assay was negligible. We find that STOP protein activity consistently elutes from the column as a single peak at 0.25–0.3 M salt (Figure 2).

Monoclonal Antibody Identification of STOP Activity of the Brain-Derived 145-kDa STOP Antigen. We have previously reported that STOP activity is associated with a highly purified protein fraction from brain extracts, the major component of which was a polypeptide of 145 kDa (Margolis et al., 1986b). We have now raised a monoclonal antibody against the 145-kDa protein, which we had suspected to be the protein with STOP activity.

The antibody recognizes only one polypeptide in heparin column eluates (Figure 3B). To confirm that the antigen recognized by the monoclonal antibody has STOP activity, we have assayed for depletion of STOP by passage through an affinity column containing covalently linked STOP antibody. When heparin column eluate containing STOP activity is passed through a column containing the STOP antibody, the overall protein profile of the eluate is not perceptibly altered, but for the absence of a band at 145 kDa. As determined by Western blot analysis, the STOP antigen is specifically removed from the heparin column eluate (Figure 3). A control affinity column, containing an equal amount of an irrelevant antibody (monoclonal anti-tubulin), retains no STOP protein (Figure 3). The eluates from the two columns were subjected to STOP protein filter assay, as above. The assay clearly demonstrates that the STOP antibody affinity column specifically depletes STOP protein activity (Figure 3). We conclude that the monoclonal antibody specifically recognizes a 145-kDa protein with STOP activity in brain tissue.

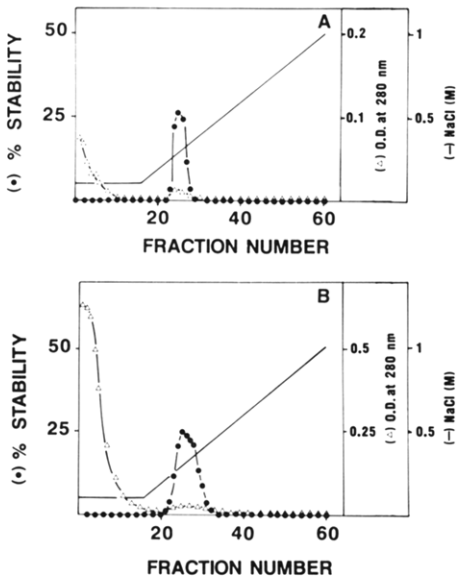

FIGURE 2: Chromatography of STOP protein on heparin–Sepharose columns. (A) A cold-stable microtubule pellet derived from rat brain cytosol (containing 10 mg of protein) as described under Materials and Methods or (B) whole rat brain cytosol (4.5 mL containing 75 mg of protein) was run onto a 0.9-mL heparin–agarose column. After the column was washed with additional 0.1 M NaCl–MME buffer, a 15 column volume linear gradient of 0.1–1.0 M NaCl was applied (—). STOP activity (●) was assayed by filter assay of 10-μL aliquots of cytosol protein (A) or 20-μL aliquots of a cold-stable microtubule preparation (B), and results were expressed as percent stability. Optical density at 280 nm (△) was measured following a 50-fold dilution of samples in MME buffer containing 1 M salt. A single peak of microtubule cold-stabilizing activity eluted at 0.25–0.30 M NaCl.

Table I: Assay of STOP Activity in Affinity Column Urea Eluate[a]

μL of eluate added	μg of pure STOP	% cold stability
8	1	17
10	1.25	36
15	1.8	57
20	2.5	66

[a] A heparin column eluate containing STOP activity was loaded onto an affinity column containing covalently bound monoclonal antibody "296", as described in Figure 3. The specific column-bound fraction was obtained by washing the column with 1 M salt, then with 0.5% Triton X-100 in 0.1 M salt, and then with 0.1 M salt again (all washes in MME buffer). The specifically bound protein was then eluted with 6 M urea. The protein, initially 44 μg/mL, was concentrated 6-fold by using a Centricon centrifuge concentrator, and the product was used for filter assay of STOP activity. In the assay, the minimum dilution of STOP (and of urea) was 100-fold.

Table II: Purification of STOP Activity from Brain Extract[a]

fraction	sp act.	total act.
cytosol	1.0	248
heparin–0.4 M salt	28.6	241
antibody affinity column, urea eluate	625.0	63

[a] Conditions of assays were as described under Materials and Methods. "Cytosol" protein represents the amount loaded onto a heparin column; "heparin–0.4 M salt" represents the fraction eluted from a heparin column in 0.4 M salt; "antibody affinity column, urea eluate" represents the heparin eluate that specifically bound to a monoclonal antibody affinity column containing covalently bound "296" antibody, and which was specifically eluted with 6 M urea and concentrated for assay as described for Table I. Specific activity and total activity determinations: A dose–effect curve was constructed for STOP activity at each stage of purification. We define STOP specific activity as liters per milligram of protein yielding 50% stability in the filter assay. Total activity was the ratio of total protein in the fraction (milligrams) × specific activity per gram of original tissue.

Purification of the 145-kDa STOP. The 145-kDa protein specifically bound to the monoclonal antibody affinity column can be eluted from the column with 6 M urea (Figure 3). We have assayed the eluted polypeptide by the filter method and find the eluted 145-kDa protein retains STOP activity (Table I). The two-step combination of the heparin column and the antibody affinity column thus yields an apparently pure 145-kDa STOP protein which retains activity. This protocol therefore represents a rapid two-step purification procedure for brain-derived STOP protein (Table II).

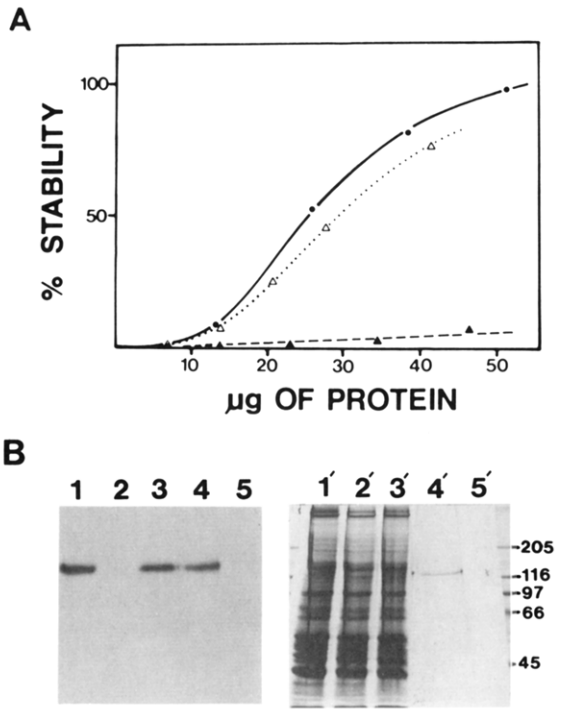

FIGURE 3: Depletion of STOP activity by monoclonal antibody affinity column. Rat brain cytosol was run into a heparin–Sepharose column, and the salt fraction with STOP activity was collected by step gradient elution (see Figure 4). The salt eluate was diluted 2-fold, and a 1.2-mL aliquot was loaded onto a 400-μL Sepharose CL-4B affinity column preequilibrated with 0.2 M NaCl in MME buffer. The columns contained covalently linked antibody, either purified anti-STOP antibody (mouse ascites monoclonal IgG, designated "296") or anti-tubulin mouse monoclonal IgG antibody, the generous gift of J. Wehland (designated YL1/2). Antibodies had been bound to the columns at 5 mg/mL, after CNBr activation of the column matrices. (A) Assay of STOP activity in column eluates. STOP activity was assayed before and after the column runs by the filter assay described under Materials and Methods. Data are expressed as percent stability induced by the indicated micrograms of protein assayed. Heparin column eluate (●); flow-through eluate from the anti-tubulin column (△); flow-through eluate from the anti-STOP column (▲). (B, left) Western blot analysis of column eluates, using anti-STOP antibody as the probe: lane 1, heparin column eluate; lane 2, anti-STOP column flow-through fraction; lane 3, anti-tubulin column flow-through fraction; lane 4, urea eluate of bound material from anti-STOP column; lane 5, urea eluate of bound material from anti-tubulin column. (B; right) Corresponding SDS–polyacrylamide gel of materials analyzed on left. Urea washes were obtained by washing the columns with 4 volumes of 0.2 M NaCl in MME buffer, and finally eluting the columns with MME containing 6 M urea. The various samples were run into 8% SDS–polyacrylamide gels and either stained with Coomassie blue R or transferred onto nitrocellulose sheets and immunoblotted as described under Materials and Methods. The urea eluate (lane 4) was used for assay of STOP activity, as described in Table I.

As shown below, although other mammalian tissues contain assayable STOP activity, no antigen is detectable with the monoclonal antibody used here (see Figure 6). Until we obtain an antibody that recognizes nonneuronal STOP proteins, the purification procedure will remain specific for neuronal extracts.

Search for STOP Activity in Various Tissues. Having

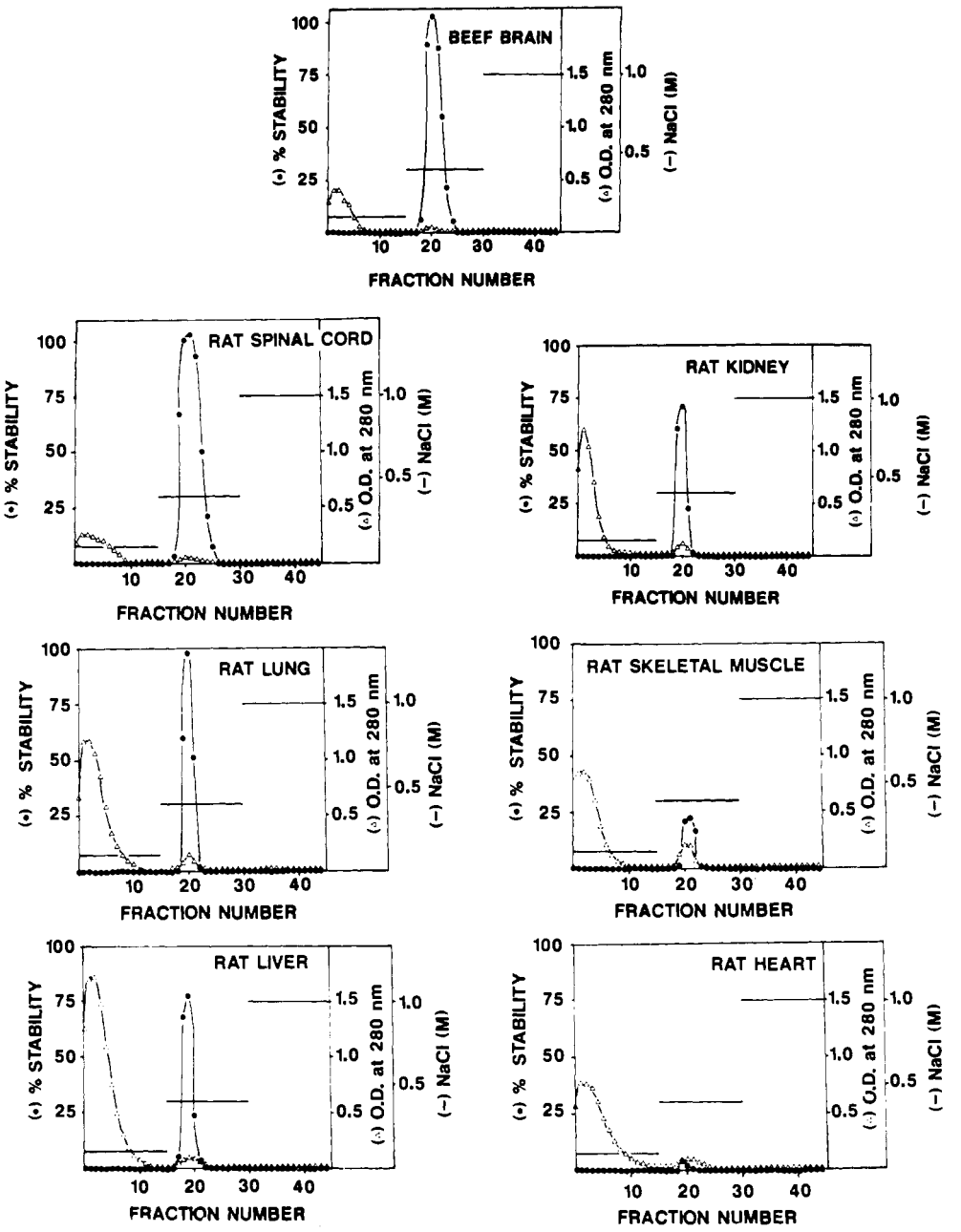

FIGURE 4: Heparin–Sepharose chromatography of STOP activity in various tissues. The cytosol fractions of various tissues were prepared as described under Materials and Methods, and 4.5 mL of supernatant was subjected to chromatography on 0.9-mL heparin–Sepharose columns. Stepwise salt elution in MME buffer was performed (as presented by horizontal bars), and protein concentration [as OD at 280 nm after 50-fold dilution of samples in MME buffer containing 1 M salt (△)] was recorded for each 300-μL column fraction. Fractions were then used for filter assay of microtubule cold stability (see Materials and Methods). For the assay, column fractions were diluted 50-fold (40 μL into 2 mL) at 30 °C into MME buffer containing 40% sucrose and 40 μM GTP (see Materials and Methods). Microtubule stability, expressed as percent of total possible stability, is shown for each column fraction (●).

established that STOP activity derived from brain tissue could readily be detected in heparin column fractions, we proceeded to use the filter assay to search for STOP activity present in heparin column fractions derived from the cytosol of various tissues. In these assays, heparin columns were eluted with step gradients of NaCl. In every case, STOP activity was detectable and was always quantitatively present in the 0.4 M salt step off the column (Figure 4). Rat spinal cord, lung, liver, and kidney all contained appreciable amounts of STOP activity; whereas the two muscle tissues assayed, skeletal muscle and heart, contained only minor amounts of activity. STOP activity could also be readily detected in protein derived from brain tissue of another species, beef (Figure 4).

As we have previously demonstrated (Job et al., 1981), brain-derived STOP activity is labile to micromolar concentrations of Ca^{2+}–calmodulin. We have assayed the rat brain-associated activity detected off of heparin columns for its lability to Ca^{2+}–calmodulin and have found that calmodulin in the micromolar range effectively destabilizes the induction of cold stability (Figure 5B) at concentrations of calcium that in themselves minimally affect stability in the assay (Figure 5A).

We have also assayed the calmodulin sensitivity of STOP activity derived from various other tissues. The assay was essentially like that performed in Figure 4. All eluates from the heparin column retained full activity in the presence of 0.7 mM total calcium, but all were 100% labile to the combined presence of 0.7 mM calcium and 10 μM calmodulin. For this assay, we used, respectively, 30, 48, 240, 70, and 120 μg of heparin column 0.4 M salt eluate from beef brain, rat

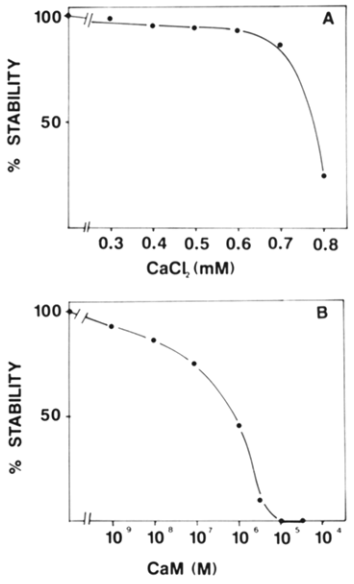

FIGURE 5: Effect of calcium and calmodulin on STOP activity. Recycled microtubule protein from beef brain was assembled in the presence of [^{3}H]GTP as described under Materials and Methods, and 40-μL aliquots were mixed with 1 mL of 40% sucrose–MME at 30 °C. To these microtubules, we then added 27 μg of 0.4 M NaCl–heparin eluate from rat brain cytosol (the fraction with STOP activity; see Figure 4). (A) Effect of calcium concentration. After 15 min at 30 °C, we added 15 μL of $CaCl_2$ from different stock solutions to obtain the indicated total calcium concentration. After an additional 30 min at 30 °C, samples were cooled for 40 min at 0 °C and filter assayed. In this assay, the final EGTA concentration (after dilution of buffer in sucrose) was 0.8 mM. (B) Effect of calmodulin concentration. The assay was performed as above, but all samples contained 0.7 mM calcium and 34 μL of calmodulin added to the final molarities indicated. Controls for STOP activity (100% stability) were samples without calcium (A) or with 0.7 mM calcium but no calmodulin (B). Results, expressed as percent stability, were the ratio of the percent of stability remaining after exposure to calcium or to calmodulin vs the controls.

spinal cord, lung, liver, and kidney. Because of negligible control activity, muscle tissue was not assayed.

Nonneuronal STOP Activity Is Not Antigenic to the Monoclonal Antibody against Brain-Derived STOP. Having confirmed that the STOP activity is associated with a protein of 145 kDa in rat brain extracts, we have probed with the monoclonal antibody raised against the brain-derived 145-kDa STOP to determine if the antigen is present in fractions with STOP activity derived from nonneuronal tissue. Western blots demonstrate that the monoclonal antibody recognizes only one polypeptide, of 145 kDa, in the salt fraction from heparin columns that contains STOP activity in rat brain and spinal cord preparations (Figure 6). Analysis by Western blot of heparin column fractions with STOP activity from liver, lung, and kidney shows there is no demonstrable antigen recognized by the monoclonal antibody in these tissues (Figure 6), despite the fact all fractions represented on the blot were loaded on the polyacrylamide gel on the basis of equal STOP activity.

Despite the fact STOP activity does not correlate with antigenicity when probed with a brain STOP-specific antibody, there are many aspects of STOP behavior in common in neuronal and nonneuronal extracts. We believe the STOP function in nonneuronal cells is associated with a protein quite similar to brain STOP. Although we have not as yet identified the STOP protein in nonneuronal tissue extracts, we can rule out tubulin variants as prospective stabilization candidates. Salt step eluates of various tissue extracts on heparin columns contain all the measurable STOP activity but contain no tubulin as determined by Western blot analysis (data not shown).

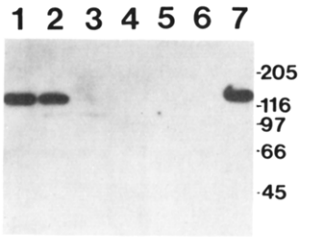

FIGURE 6: Tissue distribution of STOP antigen detected by STOP-145-specific monoclonal antibody. Tissue extracts from various organs were subjected to heparin–Sepharose column chromatography, as described in Figure 4. The column eluates at 0.4 M salt were filter assayed for STOP activity. Each gel lane was loaded with identical STOP activity, based on the assay. Each sample contains STOP activity sufficient to yield 25% cold stability by the filter assay. Samples were subjected to electrophoresis in a sodium dodecyl sulfate–8% polyacrylamide gel. Separated proteins were then transferred to nitrocellulose (1 h, 60 V). The nitrocellulose sheet was then blocked with 0.05% Tween 20 in PBS for 1 h and then exposed to mouse monoclonal anti-STOP-145 ("296") overnight at a 1:1000 dilution in 0.05% Tween 20/PBS. ^{125}I-Protein A (New England Nuclear) was then applied at 0.5 μCi/mL for 30 min in 0.05% Tween 20/PBS. Molecular weight standards ($\times 10^{-3}$) are shown at the right. Tissues of origin are (lane 1) brain extract, (lane 2) spinal cord, (lane 3) lung, (lane 4) liver, (lane 5) kidney, (lane 6) skeletal muscle, and (lane 7) brain (same as lane 1).

Discussion

In previous publications, we have extensively characterized a rat brain-derived microtubule-associated protein, STOP, with potent microtubule-stabilizing properties. When present in low titer relative to tubulin, it indefinitely blocks disassembly induced by cold temperature (Job et al., 1982). Its stabilizing property is regulated in vitro by ATP (Job et al., 1983) and by calmodulin (Job et al., 1981). It has the further intriguing property of sliding on its host polymer at elevated temperatures (Pabion et al., 1984), a result suggesting possible motility functions in the cell, as elaborated in recently published models (Margolis et al., 1986a; Garel et al., 1987). When the activity was extensively purified from brain extracts, it correlated with the presence of a protein of 145 kDa (Margolis et al., 1986b).

For the present report, we have raised a monoclonal antibody against the brain-derived 145-kDa microtubule binding protein that was previously implicated in STOP activity. The antibody specifically recognizes a 145-kDa protein derived from brain tissue and almost totally depletes STOP activity from brain extracts. Subsequent urea eluates from the affinity column contain the 145-kDa protein and exhibit substantial STOP activity. We conclude the 145-kDa protein is responsible for STOP activity in brain extracts.

Cold-stable microtubules are abundant in neuronal tissue (Heidemann et al., 1985). As demonstrated here, the brain-derived STOP antigen accounts substantially for the assayable stabilization of microtubule polymers. It is reasonable to suggest that it may account largely for the observed stabilization of a microtubule subpopulation in vivo. Since STOP protein has an intrinsic capacity to slide on microtubules, STOPs may also function by linking microtubules to other subcellular elements so that the polymers may diffuse past fixed points, or other elements may diffuse passively upon microtubules (Margolis et al., 1986a; Garel et al., 1987).

Stable subpopulations of microtubules are also present in nonneuronal tissue but in relatively reduced numbers compared to those in brain. These microtubules may exist side by side with highly labile polymers (Schulze & Kirschner, 1986). Sometimes, extraordinary stability characterizes specific microtubule subpopulations: microtubules attaching the kinetochore to the pole in the mitotic spindle (Brinkley &

Cartwright, 1975) and microtubules in the telophase midbody (Krystal et al., 1978). An important question, therefore, has been whether STOP protein activity can be detected and attributed to a particular MAP in nonneuronal tissue. On the basis of the evidence presented here, we conclude that STOP protein activity is widespread in tissue distribution.

The 145-kDa antigen derived from brain accounts substantially for brain STOP activity; however, the antibody fails to detect any protein in nonneuronal cell extracts which contain STOP activity. It is therefore clear that neuronal and nonneuronal STOP proteins are divergent at least with respect to a single epitope site. The degree of divergence and its functional meaning remain to be determined.

Filter Assay of Microtubule Stabilization. We have developed a very sensitive microtubule stabilization assay with which we could detect the low levels of STOP activity likely to exist in nonneuronal tissue and have reported that STOP activity is indeed detectable in several tissues, with notable activity present in liver, lung, and kidney. The activity from all tissue sources shares with brain STOP the same elution pattern from heparin–Sepharose columns, and inactivation by micromolar Ca^{2+}–calmodulin. We believe our finding represents the first demonstration of microtubule stabilization by a nonneuronal microtubule-associated protein.

The filter assay we have used is a variant on an assay that we had employed to detect stabilization of microtubule regions by MAP2 and τ (Job et al., 1985). The rationale for these assays is our observation that MAP2 and τ rapidly and quantitatively adsorb onto microtubules, with no detectable dissociation once bound (Job et al., 1985), and that STOP binds rapidly and quantitatively to polymers and does not measurably dissociate once bound (Pabion et al., 1984). Polymers therefore represent a very special protein binding substrate, totally absorbing MAPs regardless of their dilution. Filter assay has clear advantages for detection and purification of the various microtubule-associated proteins likely to be active in nonneuronal cells, or in lower organisms, on the basis of their capacity to stabilize polymers against dissociation. It should, further, be quite useful for the purification of nonneuronal STOP.

The fact that STOP retains activity after exposure to a denaturant (urea) is not exceptional for a cytosekeletal protein. Other MAPs retain microtubule-stabilizing function after exposure to high temperature, intermediate filament proteins retain the capacity to self-assemble after exposure to urea, and actin remains assembly competent after prolonged exposure to acetone.

STOP and Microtubule Function in Vivo. Recently, there has been substantial interest in the stable subpopulation of microtubules in cultured mammalian cells. These microtubules characteristically are more detyrosinated at the α-subunit carboxyl terminus (Wehland & Weber, 1977; Gundersen et al., 1987) and are more heavily alkylated (Kirschner & Mitchison, 1986) than their unstable neighbors. It is probable that such modifications follow stabilization of the polymers [see Wehland and Weber (1987)]. Such indefinite stabilization is quite possibly the result of a specific and irreversible association of a stabilizing MAP with the stable polymer subpopulation, and the STOP protein is an obvious candidate for such a function. The simplest mechanism by which such unequal MAP distribution can occur is through binding of a limited number of MAPs to the first formed microtubules during reassembly.

STOP is the most potent microtubule-stabilizing protein thus far described. It is reasonable to suggest that it functions in the intracellular environment to stabilize microtubule polymers. In addition, STOP slides in an apparent diffusive manner on microtubules. This sliding behavior suggests the protein embodies a passive motility function in addition to its stabilizing function. As we have suggested previously (Margolis et al., 1986a; Garel et al., 1987), both stabilization and passive motility can act coordinately to create an ordered diffusional movement either of microtubules or of their attached organelles.

In mitosis, such diffusional motility can conceivably cause poleward movement of chromosomes in anaphase, through a mechanism we have designated "polymer guided diffusion" (Garel et al., 1987). The possibility that anaphase motility of chromosomes is diffusion based is made more likely by recent observations that microtubules may displace in vitro relative to the centromere, in the absence of specific energy input (Spurck & Pickett-Heaps, 1987; Koshland et al., 1988). Our demonstration in this report that STOP activity is widespread in tissue distribution raises the possibility that homologues of brain-derived STOP-145 function in nonneuronal microtubule-dependent processes. STOP proteins may, for instance, be directly involved in the recently proposed diffusion-based movements in anaphase, a prospect of obvious interest.

Preliminary evidence obtained by immunofluorescent analysis of STOP antigen distribution in neuronal cells in primary culture shows the STOP antigen is indeed localized to the microtubule cytoskeleton in axons, in midbodies, and in the mitotic apparatus (unpublished observations). An analysis of the precise distribution of STOP-145 in cultured neuronal cells is presently under way.

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Reversible Unfolding of the Gelatin-Binding Domain of Fibronectin: Structural Stability in Relation to Function†

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ABSTRACT: Fibronectin, a large multidomain glycoprotein, binds denatured collagen (gelatin) and mediates cell attachment and spreading on collagen-coated surfaces. Despite the high affinity, binding to gelatin is disrupted by relatively mild conditions. We have examined the effects of denaturants on the structure and function of a 42-kDa gelatin-binding fragment (GBF) isolated from chymotryptic and thermolytic digests of the parent protein. Application of linear gradients to GBF-loaded gelatin–agarose columns resulted in peak elution of the fragment at pH 5.2 or 10.2, at 0.4 M dimethylformamide, 0.9 M GdmCl, or 2.0 M urea, conditions far short of those required to induce structural changes detectable by fluorescence or circular dichroism. Solvent perturbation, fluorescence quenching, and chemical modification experiments indicate that about half of the 8 tryptophans, one-third of the 21 tyrosines, and all of the 9 lysine residues are solvent-exposed in the native protein and that 1 or more of the latter are directly involved in binding to gelatin, most likely through a hydrogen-bonding mechanism. Titration with GdmCl produced a single unfolding transition centered near 2.5 M GdmCl as monitored by changes in fluorescence and circular dichroism. This transition was fully reversible with complete recovery of structural parameters and gelatin binding. Treatment with disulfide reducing agents caused rapid irreversible changes in structure similar to those produced by GdmCl with concomitant loss of gelatin binding. Thus, tertiary and secondary structures are important for binding, but binding can be disrupted without perturbing those structures.

Fibronectin (Fn),[1] a large multidomain glycoprotein, is found in plasma and other body fluids, in the extracellular matrix, and on the surfaces of numerous types of cells (Furcht, 1983; Hynes, 1985; Yamada, 1983; Akiyama & Yamada, 1987). It functions primarily as a cell adhesion protein, mediating the attachment and spreading of cells on various surfaces. Fn binds to several types of denatured collagen (gelatin) and mediates cell attachment to and spreading on collagen-coated surfaces (Kleinman et al., 1976, 1978). The binding site for gelatin has been localized to an ~42-kDa fragment near the N-terminus of each of the two similar but nonidentical polypeptide chains (Hahn & Yamada, 1979). This fragment contains six disulfide-bonded homologous repeat structures, each encoded by a single exon (Patel et al., 1987). Four of these are of the "type I" variety also found in other parts of the molecule, and two are of the "type II" variety unique to the gelatin-binding domain (Skorstengaard et al., 1986). The importance of both types of units for gelatin binding was recently demonstrated by means of fusion proteins expressed in *Escherichia coli* (Owens & Baralle, 1986). Structures having varying degress of homology with the type II units have been identified in bovine seminal fluid protein PDC-109 (Esch et al., 1983), blood coagulation factor XII (Cool et al., 1985), the kringle domain found in several proteins of the blood coagulation/fibrinolytic system (Patthy et al., 1984), the insulin-like growth factor/mannose-6-PO_4 receptor (Lobel et al., 1987), and type IV collagenase (Collier et al., 1988). A three-dimensional structure for the Fn type II unit was recently proposed on the basis of modeling after the X-ray crystal structure of the kringle I domain of bovine prothrombin (Holland et al., 1987).

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[1] Abbreviations: DMF, dimethylformamide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; Fn, fibronectin; GBF, 42-kDa gelatin-binding fragment of Fn; GdmCl, guanidinium chloride; TBS, Tris-buffered saline; TNBS, trinitrobenzenesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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