Purification and assay of a 145-kDa protein (STOP145) with microtubule-stabilizing and motility behavior

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ABSTRACT The capacity of microtubules to disassemble in vitro is profoundly affected by a protein factor designated STOP (stable tube only polypeptide). Here we report the isolation of STOP protein and confirm that its activity is, as predicted, highly substoichiometric to the tubulin in microtubules. The isolation of the 145-kDa STOP (STOP145) protein has been effected from isolated cold-stable microtubules by two column steps: DEAE ion-exchange and a calmodulin affinity column. To confirm the protein’s activity we have produced an antibody against STOP145 and have used the antibody to specifically remove the protein and the activity using an antibody-linked affinity column. We conclude that the STOP145 protein accounts for the observed in vitro stabilization of microtubules.

The diverse functions that microtubules perform in cells often require the precise control of assembly equilibrium and of polymer stability (1). These properties are invested in the polymer, we believe, by specialized proteins that selectively associate with microtubules for specific purposes. Among these proteins, STOP (stable tube only polypeptide) is one of particular interest, since it displays stabilizing and motility behavior on the polymer.

STOP induces stability in the polymer by preventing the endwise dissociation of subunits. Microtubules in the presence of STOP are indefinitely stable to low temperature, to millimolar calcium, or to assembly-inhibiting drugs. STOP protein is remarkably potent in effect; we have inferred previously that it induced polymer stability at concentrations highly substoichiometric to tubulin (2). Also embodied in the STOP protein is the capacity to slide on microtubules (3). It is not known at this time which activity, stabilization or sliding, predominates in the cell. It is possible that both aspects of its behavior coordinately act to promote intracellular motility. Recent evidence from our laboratory indicates that STOP protein is present among cold-stable microtubules in the spindle of dividing cells (unpublished observations).

We have postulated, in this case, that sliding and stabilization may act coordinately to create the poleward movement of chromosomes in anaphase (4).

In the initial work on microtubule cold stability, it was reported that the activity was not intrinsic to tubulin but was due to a protein factor associated substoichiometrically with microtubules (5). We tentatively identified a protein of 145 kDa and a few smaller polypeptides as candidates for STOP protein based on their unique association with cold-stable microtubules (2).

Here we report on the purification to near homogeneity of a STOP protein of 145 kDa and confirm its substoichiometric activity. Stable microtubules readily respond to simple metabolic signals. They become labile when exposed to ATP (6–8) or to micromolar calmodulin (CaM) (9). We have exploited the CaM binding capacity of the active moiety to effect its purification, using a CaM affinity column.

EXPERIMENTAL PROCEDURES

Materials. All reagents not otherwise identified were from Sigma and were the purest grades available. Podophyllotoxin was a gift from L. Wilson. DEAE-cellulose (DE-52) was from Whatman and was prewashed according to standard procedures. The CaM affinity column matrix was purchased from Bio-Rad. The buffer used throughout (designated MME buffer) was composed of 100 mM Mes, 1.0 mM MgCl2, 1.0 mM EGTA, and 0.025% NaN3 at pH 6.75.

Purification of Microtubule Protein. Beef brain microtubules were purified by three assembly/disassembly cycles according to Margolis and Wilson (10) and Asnes and Wilson (11), except that all procedures were carried out in MME buffer. After the third cycle of polymerization, the preparation was cleaned by centrifugation of microtubules through 50% (wt/vol) sucrose in MME buffer (200,000 × g, 2.0 hr, Beckman 70.1 Ti rotor, 25°C). Cold-stable microtubules were purified from the brains of adult rats (inbred strains W/FU and Sprague–Dawley) according to published procedures (2, 7). After a sucrose cushion sedimentation step, cold-stable microtubules were depolymerized by rapid and repeated passage at 0°C through a syringe fitted with a 25-gauge needle in MME buffer with added 1.0 mM free Ca2+. After 10 min on ice, the depolymerized microtubule protein was centrifuged at 12,000 × g for 30 min at 4°C and the supernatant was used for further separatory procedures.

Column Separatory Procedures. DEAE-cellulose column. Routinely, the one-cycle purified microtubule protein from eight rat brains was depolymerized in 0.75 ml of MME buffer with 1 mM free Ca2+ and centrifuged as indicated above. The supernatant protein was run into a 4.5-ml DE-52 column. The flow-through fraction was then eluted in a stepwise fashion with successive 0.5-ml Ca2+/MME buffer additions and collected in plastic tubes. In some experiments, 0.1 M NaCl was included during protein preparation and column elution. Peak fractions were combined and used for stability assay after addition of 2 mM EGTA or were used directly for further separatory procedures.

CaM affinity column. The flow-through eluate peak fractions from a DE-52 column were run into a 1.5-ml CaM column (Bio-Rad) in MME buffer plus 1.0 mM free Ca2+ by stepwise addition of 0.5-ml aliquots at 10-min intervals (4°C). After binding, the column was washed with Ca2+/MME buffer and then eluted with 6.0 ml of MME buffer containing 0.3 M KCl and 1.0 mM Ca2+. After another Ca2+/MME buffer wash, the column was eluted with 1.0 ml of MME buffer and 1.0 ml of MME buffer containing 0.3 M CaCl2 and 1.0 mM MgCl2. The flow-through from this step was saved. The pool of CaM affinity column fractions from two DE-52 columns was used for antibody production.

Abbreviations: STOP, stable tube only polypeptide; CaM, calmodulin.
buffer wash, the CaM-specific polypeptides were eluted in MME buffer containing 0.1 M KCl and collected in 0.5-ml aliquots. The peak of activity usually eluted at ~2.5 ml. The addition of a column wash containing 0.1% Triton X-100 in Ca1+ /MME buffer following column loading greatly improved purification, as described in the text.

Agarose column. STOP protein was run at 4°C in a Bio-Gel A-1.5m (Bio-Rad) column in MME buffer containing 0.1% 2-mercaptoethanol. The Stokes radius was determined by comparison with standards: thyroglobin (8.5 nm), catalase (5.2 nm), and aldolase (4.6 nm) (12).

Turbidimetric Measurement. Assays of assembly, disassembly, and cold-stable levels of polymer were performed with 1.0 ml samples in semimicro quartz cuvettes. Changes in optical density [which linearly covary with the microtubule assembly state (13)] were followed at 350 nm and 30°C by using a Varian Cary 219 recording spectrophotometer equipped with a constant-temperature chamber and a "cell programmer" for assaying five samples successively. Assay was initiated by the addition of 1.0 mM GTP and warming to 30°C. Spectrophotometric disassembly assays were performed as described (7, 8).

Isolation and Use of 145-kDa Protein-Specific Antibody. The 145-kDa STOP protein was excised from preparative NaDodSO4/polyacrylamide gels after visualization either by Coomassie blue R stain or by UV light. Antibody was then raised in a rabbit, utilizing an injection schedule with 100-200 µg of the 145-kDa protein per injection. IgG was purified from rabbit serum through standard procedures (14), and the 145-kDa STOP-specific antibody was further purified by adsorption to an Affi-Gel 10 (Bio-Rad) column containing covalently crosslinked 145-kDa protein. The specificity of the antibody was assayed by exposure to a "blot" of whole cold-stable microtubule protein, transferred over to a nitrocellulose sheet from a NaDodSO4/polyacrylamide gel (15). Bound antibody was visualized with 125I-labeled protein A (purchased from New England Nuclear). The slot blotting procedure (Fig. 3) was according to the method of Olnsted (16), except that antibody-bound nitrocellulose sheets were additionally washed with phosphate-buffered saline containing 2 M NaCl and with 2 M urea in 10 mM Tris-HCl (pH 7.3), and the antibody was removed with 5 M NaI and 1 M sodium thiocarbonate. For the purpose of forming an antibody affinity column, the 145-kDa STOP-specific antibody was bound to Affi-Gel 10 at a final protein concentration of 0.35 µg/ml of column, and 5 ml of this material was used to bind and extract protein from the DEAE column flow-through eluate. Column loading was performed identically to the loading procedure for the CaM affinity column, discussed above. The running buffer was MME supplemented with 0.1 M NaCl.

Other Procedures. Protein concentrations were determined by the method of Bradford (17). NaDodSO4/polyacrylamide gel electrophoresis was performed by using the procedures of Schier-Neiss et al. (18), and gels were stained either with Coomassie blue R or by using a silver staining procedure (19). All polyacrylamide gels were 8% acrylamide unless noted otherwise. Molecular mass markers were supplied by Bio-Rad; sizes shown are those reported by the supplier. Isoelectric focusing was performed on 15-cm (length) slab gels, reading the pH every 6 mm. LKB Ampholines (pH range, 3.5-10) were used. Densitometry of gels was performed with an LKB 2202 laser densitometer equipped with a recording integrator.

The crosslinker analysis used dimethyl suberimidate, dimethyl suberate, and disuccinimidyl tartarate (all from Pierce) according to the procedures of Smith et al. (20), except that in all cases STOP protein was in MME buffer with 0.1% 2-mercaptoethanol. Glycerol gradients were run according to published procedures (21). Amino acid analysis was performed according to the method of Cohen et al. (22).

RESULTS

Assay of Microtubule Cold Stability. The capacity of STOP protein to induce microtubule cold stability may be conveniently assayed and quantitated through use of a turbidimetric assay. As we have reported previously (2, 7), the extent of cold stability of a microtubule preparation may be demonstrated by chilling the assembled product for 10 min at 0°C and measuring the residual turbidity of the solution. In the absence of STOP protein, the turbidity always returns to the base value. Any turbidity above baseline therefore is indicative of cold-stable microtubules (see Fig. 3B).

Data collected over a STOP protein (DEAE column eluate) concentration gradient, while maintaining a constant microtubule protein concentration, show (Fig. 1) that the degree of cold stability varies as an apparently linear function of STOP protein concentration. This result is independent of whether DEAE column-eluate protein is added prior to or subsequent to microtubule assembly, a finding in accord with our previous determination that STOP protein binding occurs randomly on the polymer (2, 3). Since variance in cold stability is a nearly linear function of STOP protein concen-

![Figure 1](attachment:image.png)

**FIG. 1.** Titration of STOP protein cold-stabilizing activity. Protein derived from cold-stable microtubules as the flow-through eluate from DE-52 columns was added in varying amounts to purified cold-labile microtubule protein (2.4 mg/ml) in order to assay the induction of cold stability. DE-52 column eluates were diluted in buffer to proper concentrations prior to mixture with microtubule protein so that final admixture volume ratios were always 1:1. Addition of the cold-stabilizing protein fraction was performed either prior to or after the assembly to steady state of the microtubule protein. For all assays, assembly was initiated by warming the protein solutions to 30°C in MME buffer containing 0.5 mM GTP and was monitored by turbidity change. In steady-state addition assays, controls were always included in which microtubules were diluted without any eluate protein and the values derived were used as background. Additions prior to assembly (◆) were assayed at steady state, after addition of 25 µM podophytoxin, by chilling to 0°C for 10 min and then measuring turbidity after rewarming (refs. 2 and 7; see also Fig. 3B). Additions at steady state (●) were diluted at steady state with prewarmed DE-52 eluate and buffer as indicated above. These samples were subsequently allowed to incubate for an additional 5 min, 25 µM podophytoxin was added, and samples were chilled to 0°C for 10 min and then rewarmed for assay. "% cold stable" refers to the percent of polymer mass that is cold stable in each sample.
tation, this assay can be used to calculate STOP protein specific activity during stages of purification. We define specific activity as the percent cold stability generated, multiplied by the total assembly state (\( \Delta OD_{350} \)) and divided by the mg of protein with STOP activity added to the assay.

**Purification of STOP Protein.** Cold-stable microtubules are purified by assembly from the rat brain crude extract and subsequent centrifugation of the polymeric product through a sucrose cushion. The microtubule pellet (Fig. 2) is disassembled by exposure to millimolar calcium and 0°C for 10 min and used as a source material for the further purification of an active stabilizing fraction.

As noted previously (5, 7, 8), stabilizing activity may be recovered from a DEAE ion-exchange column flow-through eluate, whereas tubulin and the more abundant microtubule-associated proteins (MAPs and 7) remain bound to the column (Fig. 2). When mixed with pure tubulin, this flow-through eluate fraction has no measurable capacity to induce microtubule assembly (data not shown), thus distinguishing STOP activity from other MAPs and 7 (1).

Previously, we characterized the microtubule cold-stabilizing factor as CaM labile (9). The STOP protein factor apparently binds directly to CaM (2). It is therefore possible to remove the cold-stabilizing activity from the DEAE flow-through eluate by passage of this material through a CaM affinity column in the presence of calcium. One may then retrieve the active species from the CaM affinity column with a calcium-free buffer wash (MME buffer plus 0.1 M KCl). This elution is performed after a moderate salt wash of the column (0.3 M KCl) in the presence of calcium. We found the majority of proteins present either in the flow-through column eluate in calcium-containing buffer or in the moderate salt wash. Only a small fraction of the total protein was specifically retained and subsequently released with EGTA.

Assay of equal amounts of each of the fractions revealed that all of the assayed microtubule-stabilizing activity was in the CaM column specific fraction. At this point, a 145-kDa polypeptide was the predominant protein species and was \( \approx 85\% \) pure. A dramatic improvement in purity could be effected in the CaM column procedure by inclusion, after loading, of a buffer wash containing 0.1% Triton X-100 in Ca\(^{2+}\)/MME buffer. When the protein is subjected to two successive column runs performed in this manner, the final specific eluate consists of 93–96% 145-kDa polypeptide (Fig. 2). The extent of purity is based on densitometric scans of electrophoretic gels performed after each of several purification procedures. The purification scheme for STOP protein, with yields for the different purification steps, is presented in Table 1. We now designate the polypeptide with the apparent activity "STOP145."

**Antibody to STOP145.** We have produced a rabbit polyclonal antibody to STOP145. Once extracted, the antibody was further purified from a serum IgG fraction by affinity chromatography on a column with covalently linked STOP145. By immunoblot analysis the antibody recognized the STOP145 strongly and weakly reacted with a couple of polypeptide bands of lower molecular mass, as exemplified here in the reaction with the DEAE column flow-through eluate protein (Fig. 3A). The same polypeptides were recognized by antibody at each stage of purification (data not shown), and the weakly reacting bands appeared to copurify with STOP145. In fact, at the state of highest purity obtained for STOP145, they remain as the only visible contaminants (see Fig. 2).

In the multiple binding reactions of the antibody preparation, we assayed for antibody specificity by extracting bound antibody from the 145-kDa polypeptide band on an immunoblot and reapplying the antibody to another protein blot containing a DEAE column flow-through eluate fraction. The result is clear (Fig. 3A); the 145-kDa specific antibody recognizes the same pattern of polypeptides. The most reasonable interpretation of this result is that the major contaminants of the purified STOP145 are proteolytic fragments of this protein. Other observations lend support to this conclusion: (i) they are the same polypeptides identified, along with the 145-kDa protein, as STOPs on one-cycle purified cold-stable microtubules (2); (ii) they cannot be totally removed from the 145-kDa protein by any of a few additional column procedures attempted (data not shown). Inclusion of proteolysis inhibitors (leupeptin, phenylmethylsulfonyl fluoride, aprotinin) during purification has not thus far eliminated the contaminant bands. It is possible the proteolyzed fragments are present in the cell prior to extraction.

We have used an affinity column with covalently linked antibody to STOP145 to assay for the removal of STOP activity from solution. A DEAE column flow-through eluate was passed through this column, and the eluate was tested for cold-stabilizing activity. We find (Fig. 3B) the column totally removes cold-stabilizing activity from solution. A compari-

![Fig. 2. Gel electrophoretic analysis of STOP protein purification: NaDodSO4/polyacrylamide gel analysis of protein aliquots. Lane 1, one-cycle purified cold-stable microtubule protein; lane 2, DEAE column flow-through eluate fraction; lane 3, CaM affinity column specific fraction, following two tandem column runs with Triton X-100 washes. The lanes contain 11 µg, 11 µg, and 2.7 µg of protein, respectively. The gels have been silver stained. Lane 1 has a light appearance due to yellow staining of the overloaded major protein bands. The arrow indicates the 145-kDa STOP protein.](image)

Table 1. Purification of STOP protein

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein, mg</th>
<th>Specific activity</th>
<th>Purification, fold</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>First supernatant</td>
<td>114.0</td>
<td>0.30</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>First cycle</td>
<td>16.2</td>
<td>2.65</td>
<td>8.8</td>
<td>125</td>
</tr>
<tr>
<td>DEAE column</td>
<td>0.61</td>
<td>54.2</td>
<td>181.0</td>
<td>97</td>
</tr>
<tr>
<td>CaM column</td>
<td>0.11</td>
<td>163.0</td>
<td>544.0</td>
<td>53</td>
</tr>
<tr>
<td>+ Triton X-100</td>
<td>0.017</td>
<td>503.0</td>
<td>1680.0</td>
<td>25</td>
</tr>
</tbody>
</table>

First supernatant, high-speed supernatant from brain homogenate; first cycle, cold-stable microtubule protein after one assembly cycle and centrifugation through sucrose; DEAE column, the flow-through eluate peak fractions; CaM column, the protein fraction specific for CaM on a CaM affinity column. Results are shown for the CaM column run in the absence and in the presence of Triton X-100 detergent. The specific activity is determined from turbidometric assays of microtubule cold stability. Specific activity = % cold stability \( \times \) total \( \Delta OD_{350} \) + mg of protein assayed from the isolated fraction. Fold purification = specific activity \( \div \) original specific activity. Yield = mg in fraction \( \div \) original mg \( \times \) fold purification \( \times 100 \) for expression as %. Determination of specific activity rests on the assumption that the % cold stability varies linearly with the concentration of STOP protein in this assay. We verify elsewhere (2), and in Fig. 1, that this is the case.
son of gel profiles of the DEAE eluate protein before and after passage through the column (Fig. 3C) shows the STOP145 and its apparent proteolytic fragments specifically retained on the column. A couple of other minor polypeptides, which possibly interact specifically with STOP145, are also reduced in abundance in the column eluate.

Attempts to use the antibody to block STOP protein activity in solution have been unsuccessful. We believe the antibodies must be recognizing epitopes that do not interfere with STOP binding to the polymer.

**Physical Properties of STOP145.** A preliminary analysis of the STOP145 protein has revealed the following properties. Purified STOP145 stabilizes pure tubulin microtubules to disassembly. For this experiment, phosphocellulose column-derived pure tubulin (1.15 mg/ml) was assembled at 30°C in MME buffer containing 1 mM GTP, 10% glycerol, and 5% dimethyl sulfoxide. To one sample we added CaM column-derived STOP protein at 0.075 mg/ml. At steady state the protein fractions were chilled to 4°C for 10 min and assayed for stability. Compared to a control, the STOP-containing sample was 18% cold stable in a first-assembly cycle and 30% stable in a second cycle. STOP activity therefore requires no MAP mediation. The STOP145 has an isoelectric point of 6.4 and a Stokes radius of 7.8 nm (based on column chromatography results), indicating an elongate geometry. Based on sedimentation in glycerol gradients and on studies with three different bifunctional crosslinkers (see Experimental Procedures), the STOP145 is apparently monomeric. An amino acid analysis of the purified protein (not shown) reveals an unusual abundance of proline in the protein: 19.1% of the total amino acid versus 4.6% for an average protein (23). There are approximately eight cysteines present per monomer, an unusually low value [0.6% of the total versus a 2.8% average occurrence (23)]. We have found, however, that the protein tends to aggregate in the absence of reducing reagents and have therefore recently begun to use 2-mercaptoethanol in buffers during the assay of the protein’s properties.

**DISCUSSION**

We have purified to near homogeneity a polypeptide responsible for stabilizing microtubules to cold temperature in vitro. The purified protein has an apparent molecular mass of 145 kDa on gel electrophoresis and sometimes appears as a doublet band. Several minor polypeptides copurify with STOP145 but are apparently proteolytic fragments of STOP since they bind to a STOP145-specific antibody. The purified protein is apparently monomeric but migrates on column chromatography at the position expected of a globular protein of 350 kDa, suggesting it has an elongated geometry. The elongated geometry of this MAP is reminiscent of another MAP, τ, which migrates with an apparent molecular mass of 300 kDa on column chromatography but has a true mass in the range of 55–68 kDa (24).

The purification of a specific protein with STOP activity affirms the original conclusion of Webb and Wilson (5) that tubulin itself is not the source of cold stability and affirms our previous assumption that a single protein factor, highly substoichiometric to tubulin, is responsible for the observed activity (2). In contrast, it has been reported recently that a unique tubulin derives from cold-stable microtubules in the brain (25). However, it was not demonstrated that cold-stable microtubules can be created in vitro by using this unique tubulin. In our studies, we have never seen evidence for tubulin itself creating a cold-stable state in vitro.
The STOP we have isolated here is highly substoichiometric in effect. As predicted (2), no more than a few STOPs, randomly bound per polymer, block disassembly by 50%. It is evident that disassembly of microtubules on exposure to calcium, cold temperature, or assembly-inhibiting drugs proceeds through loss of subunits from the polymer ends and that this endwise dissociation may be effectively blocked by highly substoichiometric STOP concentrations.

Cold-stable microtubules are labile to Ca\(^2+\)/CaM (9) and to ATP (6–8). CaM-dependent stability is in accord with our finding here that the 145-kDa STOP (STOP\(_{145}\)) is specifically retained in a CaM affinity column. The ATP lability of cold-stable microtubules is presumably due to a protein kinase activity. It will therefore be of interest to determine if the STOP\(_{145}\) is a substrate for Ca\(^2+\)/CaM-dependent protein kinase in a system containing the purified proteins.

STOP\(_{145}\) apparently is capable of binding to tubulin, since purified tubulin competes with microtubules for STOP protein binding (3), and microtubules composed of pure tubulin are stabilized by the purified STOP\(_{145}\). STOP\(_{145}\) also obviously interacts with CaM in a calcium-specific manner. Other polypeptides that bind to or interact with the STOP protein, perhaps altering its activity, can now be studied in detail by add-back experiments. Hopefully, in this way a complete regulatory system can be reconstructed.

Recently, we have reported that STOP protein has another extraordinary behavior. It slides along the microtubule, successively stabilizing different regions of the polymer (3). This sliding behavior is not attributable to equilibrium exchange of STOP\(_{145}\) between microtubules, since STOP protein activity binds irreversibly to its polymer (3). The irreversible binding of STOPs to microtubules, the sliding behavior, and the interaction of STOP with CaM and ATP all suggest that STOP\(_{145}\) may be intimately associated with microtubule motility mechanisms.

Cold-stable microtubules are abundant in the neuronal tissue from which we have isolated the STOP protein (26). In the axonal system, the role of STOPs in microtubule stabilization and motility has yet to be assessed. One possibility is to serve in assembly and disassembly control. Axonal microtubules presumably are capable of free treadmillling, since they are discontinuous along the axon's length (27–29), and few are therefore attached to a cell center. Since almost all axonal microtubules are oriented with net assembly ends distal to the cell center (30), treadmillling alone may account for slow axonal flow. In this situation, stabilizing blocks may serve to regulate the rate of treadmillling and to maintain a movable population of assembly seeds. In this way, assembly could be favored on preexisting microtubules and the uniform distal polarity of microtubule net assembly ends would be maintained.

There is also a coincident localization of cold-stable microtubules and CaM to kinetochore-to-pole fibers of the mitotic apparatus (31). Preliminary evidence (unpublished observations) indicates the STOP\(_{145}\) antibody binds selectively to the cold-stable kinetochore-to-pole microtubules of cultured cells. If so, it would be reasonable to imagine that STOP protein may serve in a CaM-regulated motility system in the spindle.

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