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High concentrations of STOP protein induce a microtubule super-stable state

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Summary. We have previously shown that mammalian brain crude extracts contained two classes of stable microtubules : "cold stable" and "super-stable" microtubules. We now find that both species are generated by a single protein factor (STOP protein) in a dose dependent manner. These results show that STOP protein action can be extreme, inducing resistance to -80°C or to sonication and that no other factor seems to be required to account for the various subclasses of highly stable microtubules in brain. Finally, the rapid procedure described for the preparation of purified "superstable microtubules" should be useful for the obtention of fractions with high STOP protein activity.

We have studied extensively a neuronal protein factor which induces cold stability in microtubules in vitro. The protein, designated STOP-145 (STOP = stable tubule only polypeptide), has been purified from rat brain (1). STOP-145 has the following properties: it binds tightly to Ca^{2+}-calmodulin (1), its activity is regulated by both calmodulin and ATP (2,3), and it slides diffusionally on its host polymer (4). Most importantly for the present study, STOP-145 protects microtubules to cold induced disassembly at concentrations that are orders of magnitude below that of tubulin in affected polymers (5). The mechanism of this substoichiometric stabilization is probably through the blockage of strictly end-wise disassembly of polymers by only one or a few STOP proteins (5).

In the course of these studies, we have discovered another class of cold-stable microtubule polymer which we have designated "super-stable microtubules" (6). These polymers represent only approximately 2% of all polymers assembled from a brain cytosolic preparation. Properties that distinguish them from cold stable microtubules include their complete resistance to disassembly at -70°C, and their high resistance to salt induced or calmodulin induced polymer destabilization (6).

At the time of the discovery of super-stable microtubules, we were unable to determine whether STOP proteins or another unique factor might be responsible for the induction of stability in this unique microtubule subpopulation. In this report, using new techniques, we demonstrate that super-stable micro-

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tubules may be purified rapidly from brain crude extracts, and that STOP-145 is a prominent protein in these polymers. Further, we have created super-stable microtubules by addback of pure STOP-145 in high concentrations to microtubules in vitro. We therefore conclude that STOP-145, in high concentrations, creates super-stable microtubules. It is possible that STOP-145 subunits interact cooperatively on the polymer surface to create the super-stable state.

Materials and Methods

Materials. The buffer used throughout (designated MME buffer) was composed of 100 mM Mes, 1.0 mM $MgCl_2$, 1.0 mM EGTA, and 0.02% NaN_3 at pH 6.75. DEAE-cellulose (DE-52) was from Whatman and was prewashed according to standard procedures. The calmodulin affinity column matrix was purchased from Bio-Rad.

Protein Isolation. Beef brain microtubule protein was purified by three cycles of temperature dependent assembly and disassembly according to published procedures (7,8) with the following modification. For the third assembly cycle, protein was resuspended from pelleted microtubules and centrifuged in MME buffer for 30 min, 120,000 g (average), 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 hr in a fixed-angle rotor (120,000g average, 30°C). Pellets were stored in liquid nitrogen for later use.

STOP protein was purified to homogeneity for use, according to methods previously published (1). In brief, rat brain cold stable microtubules were assembled and isolated by centrifugation through a sucrose cushion. STOP protein was derived from this material by passage through a DEAE ion exchange column, where it was present in the flow-through fraction; and by calmodulin affinity column chromatography, where it was specifically retained with calcium, and released by EGTA.

Affinity purified rabbit polyclonal STOP specific IgG antibody was produced and isolated as previously described (1).

Other Procedures. Turbidometric measurement of microtubule assembly state was performed at 30°C, and assayed at 350nm in a Varian Cary recording spectrophotometer, as previously described (1). Electron microscopy was performed according to published procedures (7). Protein concentrations were determined by the method of Bradford (9). SDS-polyacrylamide gels electrophoresis was performed as described by Sheir-Neiss et al. (10), and gels were stained with Coomassie blue R. All polyacrylamide gels were 8% acrylamide. Molecular mass markers were supplied by Bio-Rad; sizes are those reported by the supplier. Polyacrylamide gel "blotting" procedure was performed by transfer over to a nitrocellulose sheet from SDS-polyacrylamide gels, and exposure to antibody according to methods of Towbin et al. (11). Bound antibody was visualized with ^{125}I-labeled protein A (purchased from New England Nuclear.

Results

The major criterion by which we have defined super-stable microtubules is their resistance to disassembly on exposure to -70°C (6). Our strategy to purify superstable microtubules in abundance was therefore to assemble rat brain crude extract microtubules, freeze the resulting polymers to -70°C, thaw the sample on ice in the cold room and sediment the residual polymers through a sucrose step gradient. We have shown previously that sucrose step gradients effectively purify microtubule polymers away from contaminant proteins (7).

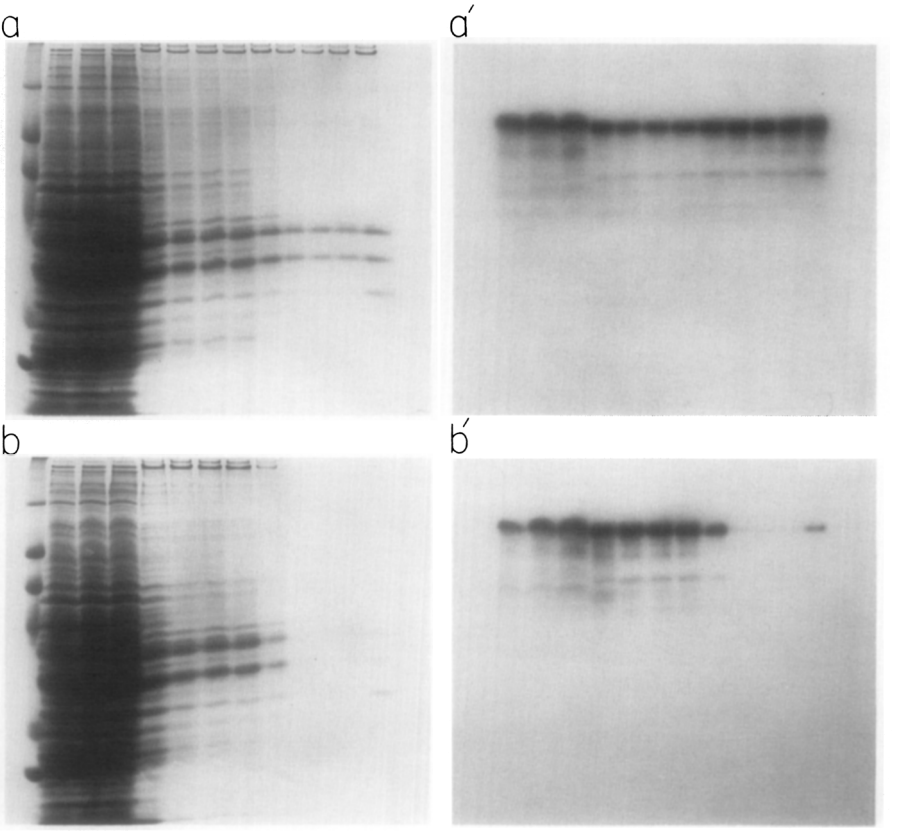

Figure 1. Sucrose gradient analysis of the protein content of superstable microtubules. Rat brain crude extract microtubules were assembled in MME buffer at 30°C for 45 min, then frozen to -70°C for at least 0.5 hr. The preparation was thawed at 0°C in the cold room, and then loaded onto discontinuous sucrose gradients. The gradients, 12 ml total, contained 3 ml of loaded sample, 4.5 ml of 20% sucrose (with or without NaCl), and 4.5 ml of 50% sucrose. Gradients were then centrifuged in a Beckman SW41 rotor for 90 min at 35,000 rpm (4°C). We then collected 12 one ml fractions, using the last fraction to resuspend the small pellet. Protein profiles obtained by this procedure are shown; (a), no salt, (b) 0.15 M salt. The top of the gradient is to the left. Immunoblots of similar gels are shown on the right; the primary antibody being anti-STOP rabbit polyclonal IgG_1 used at a dilution of 1:500. Visualization of the bound antibody was with ^{125}I-protein A. The protein markers at left are myosin (205 kDa); beta-galactosidase (116 kDa); phosphorylase b (97 kDa); bovine albumin (66 kDa); egg albumin (45 kDa, and carbonic anhydrase (29 kDa).

A polyacrylamide gel of the result of such a gradient run shows that the bulk of non-microtubule proteins remain above the 20% sucrose step (figure 1a), and that all proteins not tightly associated with microtubules remain above the 20%-50% sucrose interface. The only proteins, other than tubulin, identifiable in the 50% cushion are high molecular weight MAPs and a protein of 145 kDa, which we suspected to be STOP-145, the microtubule stabilizing protein previously described.

We tested the identity of the 145 kDa protein by blotting an identical gel to nitrocellulose and probing with antibody specific for STOP protein

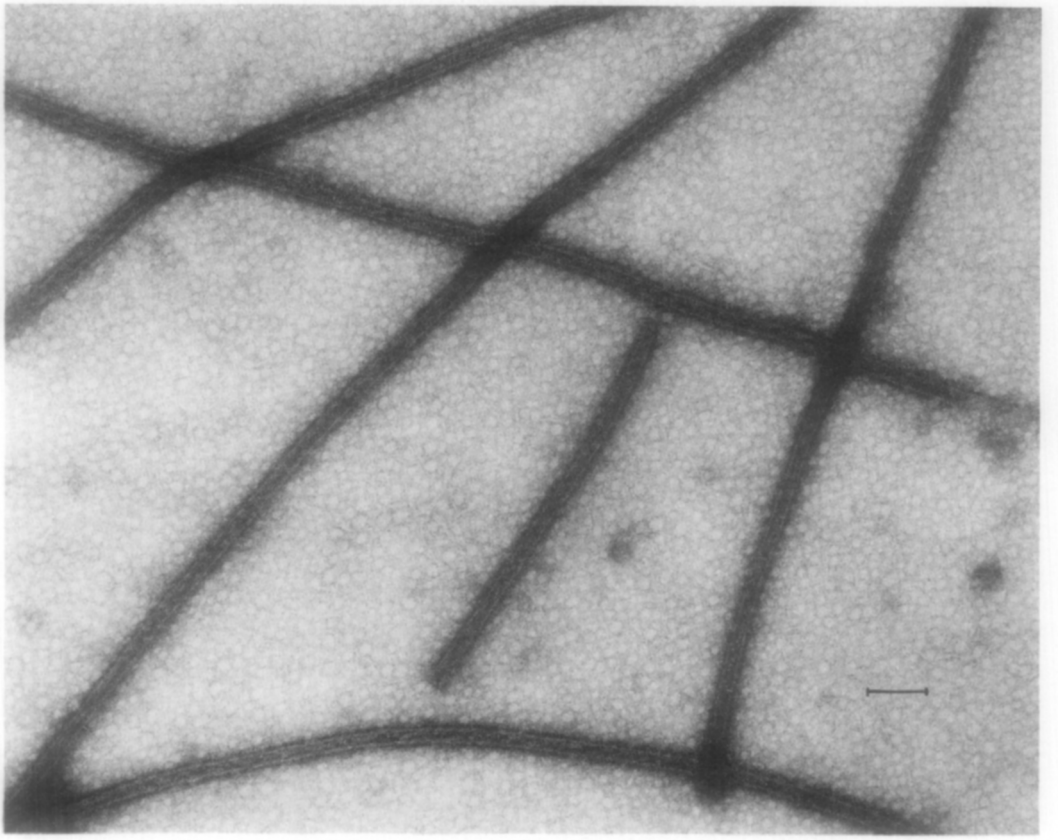

Figure 2. Electron micrograph of superstable microtubules. One ml of purified superstable microtubules from the 50% sucrose cushion step was rediluted in 12 ml of MME buffer, frozen at -70°C for 30 min, thawed and respun in the SW41 rotor at 15,000 rpm for 90 min. By this procedure, all the protein forms a pellet which contains microtubules, as shown. Bar = 100 nm.

(1). We find (figure 1a') that STOP-145 is present throughout the gradient and remains abundant in the 50% cushion, although the quantity of microtubule polymer is diminished in this region.

Previously we showed that super-stable microtubules were slowly dissociated by relatively low concentrations of salt. Inclusion of salt (0.15 M NaCl) in the gradient would therefore be expected to cause disassembly of superstable microtubules in the gradient. The predicted result was obtained (figure 1b), and essentially no polymer entered the 50% cushion after exposure to salt. No other proteins are present in the 50% cushion, including STOP (figure 1b'), suggesting the dependency of these proteins on superstable microtubules for entry into the 50% cushion.

A sample of the material present in the 50% cushion was examined by electron microscopy and was found to consist of morphologically normal microtubules, with little visible contaminant (figure 2).

To test whether the presence of STOP-145 in abundance created microtubule super-stability, we purified STOP-145 to homogeneity (1), and added it back to

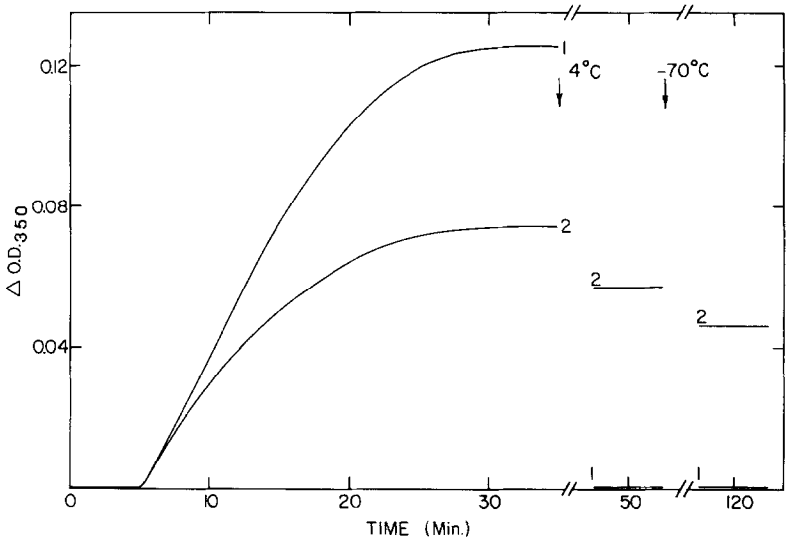

Figure 3. Turbidometric assay of super stable microtubule formation. STOP protein (0.25 ml at 0.2 mg/ml) was added to 0.25 mls of three cycle purified bovine brain microtubule protein (1.7 mg/ml) and allowed to assemble at 30°C for 30 min with the addition of 0.1 mM GTP (final concentration). Samples were then placed in ice slurry for 10 min and then rewarmed in the presence of 25 μm podophyllotoxin to inhibit any further polymerization. The samples were then measured for polymer content turbidometrically. The same samples were then frozen at -70°C and slowly thawed before measuring super stable polymer content. Polymer plateaus have differing full assembly levels due to salt present in the STOP protein sample, which somewhat inhibits assembly. Line 1 represents the control; line 2, the STOP containing sample.

cold labile microtubule protein during assembly. The presence of STOP at these levels created cold stability as expected, but also induced resistance to -70°C for a substantial subfraction of the stable polymers (figure 3). We conclude pure STOP protein can induce microtubule super-stability in vitro.

Discussion

We have demonstrated that microtubule super-stability is created by high concentrations of the same protein, STOP-145, that induces the polymer cold stable state in vitro. The cold-stable state only requires a few STOP proteins per polymer to block end-wise disassembly in the cold (5) whereas we have assumed that resistance to -70°C would require a high density of a stabilizing molecule that could prevent disassembly induced by shearing in the frozen state. Superstable polymers contain an abundance of STOP protein, although it is by no means in molar equivalence to tubulin on these microtubules (figure 1). The stabilizing effect of each STOP protein must therefore extend over many tubulins in the polymer.

By purifying microtubule stabilizing activity, we were able to show that there is only one identifiable cause of microtubule cold stability; STOP-145

(1). It is thus reassuring to determine that an alternate stability state in the polymer is caused by the same factor, but at a higher density.

The fact that STOP could act at extremely low relative concentrations to induce stability suggested its function in the cell could be maximal at quite low concentrations on polymers. Recently, on examining cells by immunofluorescence with anti-STOP antibody, we have determined that certain polymers contain an abundance of STOP protein, notably those connecting kinetochores to poles in mitotic spindles, and those present in midbodies between divided cells (12, and manuscript in preparation).

Similarly, we have recently found high local concentrations of STOP protein on some select microtubule polymers in neuronal cells in culture. The results presented here suggest that high abundance of STOPs can have a unique function on polymers, and may induce a state of extreme stability, both in vivo and in vitro. It is possible that STOPs interact with each other on the polymer surface and therefore may cluster on some polymers or polymer regions.

A secondary outfall of the results described here is that we present a method for the rapid purification of polymers from the brain crude extract that contain high concentrations of STOP protein. This procedure should enable rapid purification of STOP protein from sources that heretofore have shown cold stable activity but no identifiable STOP protein. Using this procedure we have recently found an abundant 145 kDa protein associated with super-stable microtubules from beef brain (F. Pirollet, D. Job and R.L. Margolis, unpublished results). Previously, we had found that STOP purification procedures successful with rat brain did not succeed when scaled up for beef brain. Hopefully, one will now be able to obtain large quantities of pure STOP protein for biochemical analysis.

Acknowledgement

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