Presence of a new microtubule cold-stabilizing factor in bull sperm dynein preparations

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Brain tubulin polymerized with dynein isolated from bull spermatozoa forms cold-stable microtubules, in contrast with microtubules made of brain tubulin polymerized by brain microtubule-associated proteins (MAPs). The level of cold-stable microtubules depends on the concentration of dynein used. Addition of dynein to cold-unstable microtubules renders these microtubules stable to cold. Although ATP and a non-hydrolysable ATP analogue increase the formation of microtubules made of tubulin and dynein, these nucleotides have no effect on dynein cold-stabilizing properties. The data suggests that a new factor, not involving the dynein ATPase active site and present in bull sperm dynein preparations, confers cold-stability to microtubules.

INTRODUCTION

Functions performed by microtubules are dependent on the precise control of their assembly–disassembly [1–3]. Variations in isoforms of tubulin in cold-stable and cold-unstable microtubules have been suggested as the cause for their difference in cold-stability [4,5]. However, the discovery of cold-stabilizing factors in the brain [6–8], coupled to the observation that tubulin isolated from cold-stable flagellar microtubules can polymerize and depolymerize like brain cold-labile microtubules [9,10], suggest that microtubule stability could also be caused by cytoskeletal components other than tubulin. Here we report that microtubules made of brain tubulin polymerized in the presence of bull sperm dynein are stable to 4 °C, in contrast with those polymerized by brain microtubule-associated proteins (MAPs), suggesting that a new ‘microtubule cold-stabilizing factor’ co-purified with mammalian dynein.

METHODS

Microtubule proteins from bovine brain were isolated by three cycles of polymerization–depolymerization according to the method of Shelanski *et al.* [11]. Tubulin and MAPs (0.35 M-NaCl fraction) were further purified from freshly prepared microtubule proteins by passage on a phosphocellulose column as described by Weingarten *et al.* [12].

Dynein was extracted from bull sperm flagella by low-salt buffer and concentrated by ultrafiltration on a 100 000-*M_r* cut-off membrane (Amicon Corp.) as described in detail elsewhere [13,14]. In some experiments the concentrated dynein preparations were further purified on a 5–25% (w/v)-sucrose gradient as previously described [13,14].

Polymerization of tubulin was performed in 1 mM-MgCl₂/1 mM-EGTA/0.8 M-glycerol/1.0 mM-GTP/0.1 M-Mes, pH 6.8 at 37 °C, and monitored by absorbance at 350 nm.

Protein concentrations were measured by the method of Bradford [9].

RESULTS

The incubation of brain tubulin and MAPs at 37 °C in the presence of 1 mM-MgCl₂ stimulated the formation of microtubules, as evidenced by an increase of the turbidity measured at 350 nm (Fig. 1) and the appearance of microtubules revealed by electron microscopy (results not shown). A lowering of the temperature to 4 °C produced a decrease in turbidity (Fig. 1) and the disappearance of microtubules (results not shown). On the other hand, when bull sperm dynein was used to polymerize brain tubulin into microtubules [15], the majority of microtubules formed did not disintegrate in the cold, even after being exposed

Fig. 1. Effects of a 4 °C incubation on microtubules made of tubulin polymerized by MAPs and bull sperm dynein

Tubulin (phosphocellulose-purified, 2.5 mg/ml) was polymerized at 37 °C into microtubules by MAPs (0.35 M-NaCl fraction; 0.8 mg/ml) or bull sperm dynein (mixture of 12 S and 19 S dynein; 1.5 mg/ml) in the presence of 1 mM-GTP and 1 mM-MgCl₂ as described elsewhere [13]. After 1 h of polymerization, incubation mixtures were cooled down to 4 °C for 1 h and rewarmed at 37 °C. Polymerization was monitored by measuring *A*₃₅₀ as a function of time. Curve A, dynein + tubulin; curve B, MAPs + tubulin; curve C, tubulin; curve D, dynein.

Abbreviations used: MAPs, microtubule-associated proteins; p[CH₂]₃ppA, adenosine 5'-[βγ-methylene]triphosphate; STOP, stable tubule only polypeptide.

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Fig. 2. Effects of 4 °C incubation on microtubules made of tubulin polymerized by the 19 S dynein particle

Tubulin (2.5 mg/ml) was incubated at 37 °C with 19 S dynein (0.2 mg/ml) as described in Fig. 1. The incubation mixture was then placed at 4 °C for 1 h and rewarmed at 37 °C. Polymerization was monitored by measuring A_{350} .

Fig. 3. Effects of dynein concentration on the stability of microtubules made of tubulin polymerized by bull sperm dynein

Tubulin (2.5 mg/ml) was incubated with different concentrations of dynein at 37 °C as described in Fig. 1. After 60 min of polymerization, the incubation mixtures were placed at 4 °C for 60 min. Microtubule stability was calculated as described in Table 1.

to 4 °C for 1–3 h. When dynein preparations were further purified on sucrose gradients, as previously described [13–15], to isolate the predominant 19 S dynein particle, a similar cold-stabilizing activity was observed (Fig. 2). The degree of microtubule stability to cold conferred by dynein varied as a function of the dynein concentration in the incubation mixture up to 1.5 mg/ml. At higher concentrations of dynein, no further increase in the degree of cold-stability could be observed (Fig. 3), suggesting that the binding sites on microtubules for the cold-stabilizing factor are saturable.

To investigate further the molecular mechanism by which dynein stabilizes microtubules, we added dynein to tubulin previously polymerized by brain MAPs (0.35 M-NaCl fraction). This addition conferred cold-stability to the MAPs-polymerized microtubules (Fig. 4). Moreover, the simultaneous addition of MAPs and dynein to tubulin rendered the microtubules formed stable to cold (Fig. 4). In both cases, the degree of stability was not significantly altered by the presence of MAPs. Under these low-Mg²⁺ polymerizing conditions, no obvious decoration of arm-like structures were detected along the microtubule wall. However, regularly spaced arms were observed when the Mg²⁺ concentration was increased to 5 mM, as previously reported [15]. These results demonstrate that the cold-stabilizing factor does

Fig. 4. Effects of the addition of dynein before or after the formation of microtubules on the stability of microtubules to cold

Tubulin (T) (2.0 mg/ml) was incubated at 37 °C as described in Fig. 1 with dynein (Dyn) (1.0 mg/ml), MAPs (0.8 mg/ml) or a combination of both for 20 min. All incubation mixtures were then placed at 4 °C for 30 min, except one incubation mixture containing MAPs and tubulin, which was further incubated for 20 min at 37 °C, in the presence of newly added dynein, which is labelled in this Figure as 'Dyn*' to differentiate it from the Dyn added together with tubulin at the beginning of microtubule polymerization. Polymerization and depolymerization of microtubules were monitored by A_{350} .

Fig. 5. Effects of ATP and p[CH₂]ppA on the polymerization of tubulin by dynein and on the stability to cold of the microtubules formed

Tubulin (3.2 mg/ml) was incubated at 37 °C as described in Fig. 1 with dynein (1.0 mg/ml) in the presence or absence of 1 mM-ATP or 1 mM-p[CH₂]ppA. After a polymerization period of 60 min, the temperature was decreased to 4 °C for 1 h. Incubation mixtures were then heated up to 37 °C for a further 60 min. The formation of microtubules was monitored by A_{350} . A, Tubulin + dynein; B, tubulin + dynein + p[CH₂]ppA; C, tubulin + dynein + ATP.

not need to be incorporated into microtubules during assembly to confer stability.

It has been shown that a cold-stabilizing factor isolated from brain tissues lost its activity in the presence of ATP [7], suggesting some regulation by an ATP-dependent process. Since dynein is an ATPase, the stabilizing properties of dynein in the presence and absence of ATP, a non-hydrolysable ATP analogue and ATPase inhibitor were compared. As previously reported [15], a strong activation of brain tubulin polymerization by dynein was observed with millimolar concentrations of ATP and adenosine 5'-[β -methylene]triphosphate (p[CH₂]ppA). In the presence of these two nucleotides, the microtubules formed had the same degree of stability to cold as those polymerized in the absence of nucleotides (Fig. 5; Table 1). Moreover, when dynein-

Table 1. Effects of ATP, p[CH₂]ppA and vanadate on microtubule cold-stability

Tubulin (2.0 mg/ml) was polymerized into microtubules at 37 °C by MAPs (0.8 mg/ml) or dynein (1.5 mg/ml) in the presence or absence of 1 mM-ATP, 1 mM-p[CH₂]ppA or 0.1 mM-vanadate. After 60 min of polymerization, the temperature was decreased to 4 °C. The percentage of stable microtubules was defined as follows:

$$\text{Percentage of stable microtubules} = \frac{A_{350} \text{ after cooling for 1 h}}{A_{350} \text{ before cooling}} \times 100$$

Conditions	Microtubules stable (%)
Tubulin + MAPs	0
Tubulin + dynein	67
Tubulin + dynein + ATP	70
Tubulin + dynein + p[CH ₂]ppA	75
Tubulin + dynein + vanadate	63
Tubulin + dynein + ATP + vanadate	50

polymerized microtubules (formed in the absence of ATP) were incubated at 4 °C with ATP, no decrease in turbidity was observed (results not shown). Similarly, the presence of a dynein ATPase inhibitor such as vanadate [19], at a concentration (0.1 mM) sufficient to block the enzymic hydrolysis of ATP, did not interfere with the ability of dynein to form cold-stable microtubules in the presence or absence of ATP (Table 1). Taken together, these data show that neither the binding of ATP nor its hydrolysis by dynein ATPase are involved in the stabilization of microtubules to cold.

DISCUSSION

In the present study we clearly demonstrated that dynein preparations obtained from bull sperm contained a microtubule cold-stabilizing factor. Under our experimental conditions, up to 75 % of the microtubules formed during the first cycle of polymerization with 1.0–1.5 mg of dynein/ml became stable to cold. These results show that, like the microtubule stabilizing factor characterized from brain tissues [7,8], the sites of interaction between microtubules and the dynein factor are saturable, possibly reflecting a specific fixation of the factor to particular isoforms of tubulin. The observation that the addition of dynein to cold-unstable microtubules (tubulin polymerized with brain MAPs) rendered the latter stable to cold, clearly indicates that the dynein stabilizing factor does not need to be co-polymerized with microtubules to confer stability. The fact that the presence of MAPs in the incubation mixture before or after tubulin polymerization did not influence the cold-stabilizing properties of dynein clearly suggests that the MAPs-binding sites differ from those of the dynein stabilizing factor and that no synergetic nor competitive interaction exists between these two types of molecules. The brain-derived cold-stabilizing factor (also named 'STOP' for 'Stable Tubule Only Polypeptide') has been reported to have similar properties and act through a similar mechanism [7,8,16–18].

Margolis & Rauch [7] reported that the addition of ATP induced a rapid and total depolymerization of the cold-stable microtubules formed with brain crude tubulin preparations. As suggested by these authors, the effect of ATP on STOP activity could be a reflection of protein kinase activity (possibly Ca²⁺/calmodulin-dependent). The addition of ATP under our experimental conditions resulted, as previously reported [15; Fig.

4], in an increased polymerization of tubulin by dynein. However, after the addition of this nucleotide as well as that of the non-hydrolysable ATP analogue p[CH₂]ppA or the dynein ATPase inhibitor vanadate to incubation mixtures containing dynein and tubulin, the microtubules formed retained the same degree of stability to cold as those polymerized in the absence of nucleotides and ATPase inhibitor. These results suggest that the ATPase site on dynein, or any other ATP-hydrolysing enzyme such as protein kinase, are not involved in the cold-stabilizing properties of dynein preparations. Whether the lack of a depolymerizing action by ATP might be due to the absence of a Ca²⁺/calmodulin-dependent protein kinase, as hypothesized in the case of the brain STOP, remains to be investigated.

Further purification of dynein preparations on sucrose gradient to separate the 19 S dynein particle from the 12 S dynein particle and the soluble proteins as previously described [13–15] indicated that the 19 S particle by itself was able to confer cold-stability to microtubules (Fig. 2) to the same maximal degree as that reported above for dynein preparations. On the other hand, the observation that microtubule stabilization to cold occurred under low-Mg²⁺ concentrations, where no visible decoration of microtubules by dynein particles is detectable [15], may suggest that the intermediate or small peptides rather than the high-molecular-mass components (> 300 kDa; which are detectable by electron microscopy) of the 19 S dynein molecules are involved in the cold-stabilizing properties observed in dynein preparations. However, we cannot yet rule out the possibility that other molecules present in the dynein preparations may contribute to the cold-stabilizing properties.

Recently, microtubule cold-stabilizing activities have also been detected in non-neuronal tissues, including liver, kidney, lung and muscle [20]. All these activities bound to heparin columns and were inhibited by Ca²⁺/calmodulin. The formation of precipitates in the dynein preparation in the presence of low Ca²⁺ concentrations prevented us from testing whether Ca²⁺/calmodulin could inhibit dynein cold-stabilizing properties. Although the properties of non-neuronal STOPS are similar to the better-characterized brain STOP, neuronal and non-neuronal STOPS are different, since monoclonal antibodies to the 145 kDa brain STOP do not cross-react with any of the non-neuronal STOPS.

In summary, dynein preparations from bull sperm flagella have cold-stabilizing properties. This factor can confer cold-stability to pure phosphocellulose–tubulin by interacting with microtubules during assembly and/or once formed. The observations that ATP and non-hydrolysable ATP analogues do not affect dynein stabilizing properties, that neuronal and non-neuronal STOPS are immunologically different [20] and that the 19 S dynein particle has cold-stabilizing properties are consistent with the hypothesis that the present factor is different from the more extensively studied 145 kDa brain STOP [6–8, 16–18].

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