Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 218-224

BULL SPERM 19S DYNEIN POLYMERIZES BRAIN TUBULIN INTO MICROTUBULES

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Received August 21, 1987

Crude dynein extracted from bull sperm flagella polymerized pure phosphocellulose tubulin isolated from brain tissues into microtubules. This effect was predominantly due to the 19S dynein particle in the extract. ATP stimulated up to five fold the polymerization of brain tubulin by bull sperm dynein. Hydrolysis of ATP was not required since vanadate at a concentration sufficient to block dynein ATPase activity did not interfere with ATP stimulation and because the non hydrolyzable ATP analogue adenylyl (β-γ-methylene) diphosphate (AMPPCP) had effects similar to those of ATP. These results suggest that, in addition to hydrolyzing ATP to generate the driving force necessary for microtubule sliding within the axoneme, dynein may also interact with ATP to polymerize tubulin into microtubules.

Flagellar and ciliary axonemes are generally made of 9 doublet and 2 singlet microtubules. Several structures attached to axonemal microtubules allow them to interact with each other. One of these structures originates from each subfiber A of doublet microtubules and projects towards the subfiber B of adjacent microtubules. The arm-like structure named dynein is a complex molecule made of several peptides and with ATPase activity. The driving force in flagellar or ciliary microtubule sliding is provided by the hydrolysis of ATP by this dynein ATPase (for review see 1).

Whereas the structure and function of ciliary and flagellar axonemes is well established, little is known about their assembly. In mammals, the axonemal microtubules are assembled in a relatively short time at the beginning of spermiogenesis (2). Brain tubulin assembly into microtubules is promoted and regulated by microtubule associated proteins (MAPs) (3-6). In the present study, we

Abbreviations used: MAPs, microtubule associated proteins; AMPPCP, adenylyl (β - γ-methylene) diphosphate.

0006-291X/87 $1.50

investigated whether bull dynein, an axoneme associated protein isolated from spermatozoa, could polymerize bovine brain tubulin into microtubules. We report that bull dynein polymerizes tubulin into microtubules. Furthermore, we show that ATP greatly stimulates the polymerization of tubulin by dynein.

METHODS

Preparation of bull sperm dynein: Dynein was extracted from bull spermatozoa according to Belles-Isles et al (7). This low ionic strength extraction resulted in the solubilization of an average of 7 outer dynein arms per axoneme without significantly affecting other sperm structures (7). These extracts, representing 3-5% of the total proteins of demembranated spermatozoa, were concentrated by ultrafiltration on YM 100 membranes (molecular weight cut-off of 100,000, Amicon Corp) and applied on 5-25% sucrose gradient. After centrifugation at 100,000 x g for 16 h at 4°C, two peaks of ATPase activity sedimenting at 19S and 12S were observed (7). Concentrated crude dynein and pooled fractions from the sucrose gradient were tested for their capacity to polymerize brain tubulin.

Preparation of brain tubulin and MAPs: Microtubule proteins from bovine brain were isolated by the polymerization-depolymerization method of Shelanski et al (8). Tubulin and MAPs were further purified by chromotography on phosphocellulose column (Whatman P11) according to a published procedure (4). The purified tubulin free of MAPs was unable to self assemble into microtubules at a concentration of 3.5 mg/ml.

Assembly of tubulin into microtubules: Polymerization of tubulin was performed in 1 mM $mgCl_2$, 1 mM EGTA, 0.8M glycerol, 0.4 mM GTP and 0.1M 2-(N-morpholino) ethanesulfonic acid, pH 6.8 at 37°C and followed at 350 nm. Polymerized microtubules were visualized by negative staining electron microscopy (8). Protein concentrations were measured by the method of Bradford (9).

RESULTS

Effects of dynein on tubulin assembly into microtubules.

Purified brain tubulin free of MAPs was unable to polymerize into microtubules in the presence of 1mM GTP. However, the addition of crude dynein to the incubation medium triggered a rapid increase in absorbance at 350 nm (Fig. 1A). This increase was caused by a thermolabile factor with a molecular weight above 100,000 since boiling of the crude dynein drastically decreased the enhancement in $A_{350\ nm}$ and that crude dynein ultrafiltrates had only marginal effects. The stimulation of polymerization by the crude dynein was dose dependent until a plateau was reached at 1.5 to 2.0 mg/ml in the presence of 3.5 mg/ml of tubulin (Fig. 1B).

When crude dynein was further purified on sucrose gradient to separate two dynein particles from smaller proteins (7), the highest stimulation in tubulin polymerization (on a constant protein basis) was observed with the 19S dynein peak (fractions 8-13) and with the tail of 19S dynein peak (fractions 1-6) (Fig. 2).

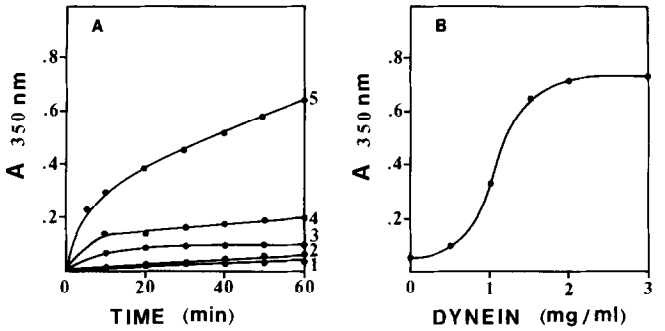

Fig. 1. Effects of dynein on tubulin polymerization.
A) Tubulin (3.5mg/ml) and dynein (1.5mg/ml) were incubated alone or together at 37°C. The polymerization of tubulin into microtubules was followed by measuring the absorbance at 350 nm. Tubulin alone (1); dynein alone (2); Amicon YM 100 (100,000 molecular weight cut-off) ultrafiltrate of crude dynein concentrated on YM 10 membranes (10,000 molecular weight cut-off) and incubated at 1.5 mg/ml with tubulin (3); tubulin + boiled (5 min) dynein (4); and tubulin + dynein (5).
B) Tubulin (3.5mg/ml) was incubated for 60 minutes at 37°C with increasing concentrations of dynein.

Proteins on the top of the gradient had little effect. On a mg protein basis, the 19S dynein was 3 to 4-fold more efficient in increasing A_{350nm} than crude dynein. The increase in absorbance was associated with the appearance of microtubules (Fig. 3) whereas none were observed when dynein or tubulin (data not shown) were incubated separately. The microtubules formed in the presence of dynein had no periodic

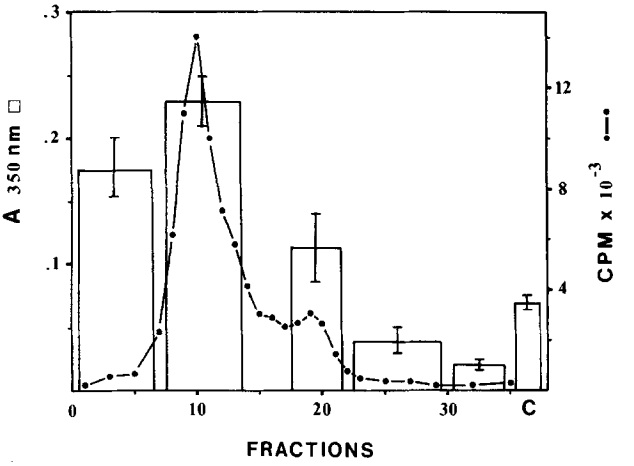

Fig. 2. Polymerizing capacity of dynein purified on sucrose gradient.
Dynein was extracted from bull sperm axonemes as described elsewhere (7) and purified on a 5-25% sucrose gradient. Two peaks of dynein ATPase sedimenting at 19S (tube 10) and 12S (tube 19) were separated. Fractions were combined and tested at a final concentration of 100 ug/ml for their capacity to polymerize tubulin (3.5mg/ml) into microtubules. ATPase activity is expressed in CPM of Pi liberated per 10 min whereas the polymerizing capacity is expressed as absorbance at 350 nm/20 min/100 ug protein. The column on the right indicates the polymerizing capacity of the crude dynein (C) loaded on the gradient tested under the same experimental conditions. Vertical bars represent the mean ± S.E.M. of 4 dynein preparations.

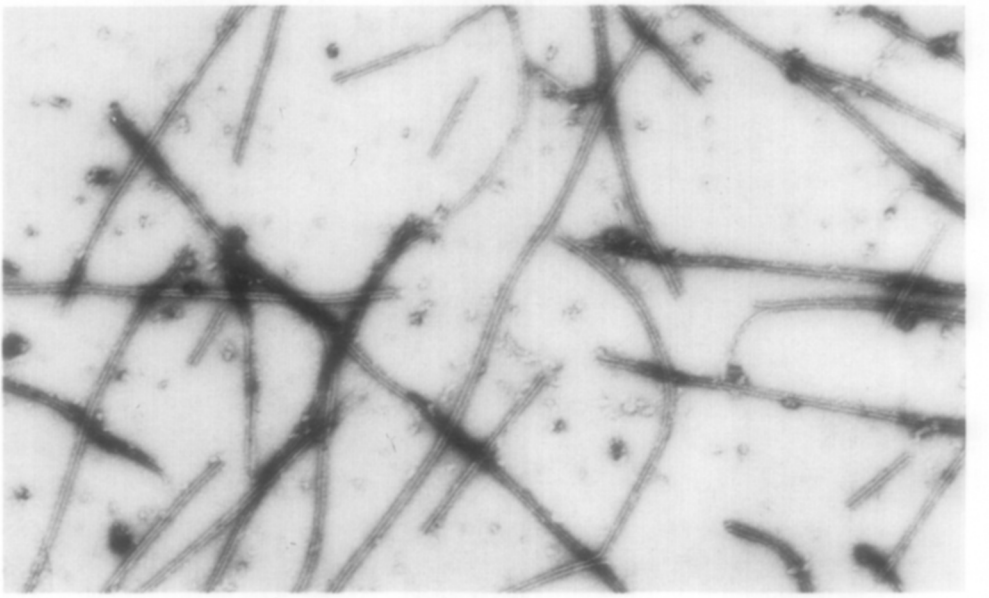

Fig. 3. Electron micrographs of tubulin polymerized by dynein.
Tubulin (3.5mg/ml) was incubated alone or with 1.5mg/ml of dynein at 37°C. After 60 min of incubation, aliquotes were layered on Formwar coated grids and allowed to sit for 60 seconds. Grids were then washed with H_2O, negatively stained with 1% uranyl acetate during 30 seconds and dried.

decoration by dynein molecules (Fig. 4A). However, the addition of 5 mM MgSO4 to the incubation medium induced the binding of dynein to microtubules (see arrows, Fig. 4B).

Effects of ATP on dynein polymerization of tubulin into microtubules.

Since dynein has ATPase activity (1), we investigated the effects of ATP on tubulin polymerization by dynein under the standard polymerizing conditions (e.g. with 1mM GTP). Tubulin polymerization by dynein was greatly stimulated by this nucleotide. Its effect was concentration dependent between 0 and 1 mM where a plateau was reached (Fig. 5B). The non hydrolyzable analogue of ATP adenylyl (β-γ - methylene) diphosphate (AMPPCP) generated the same type of stimulation (data not shown). Moreover, the dynein ATPase inhibitor vanadate (10) had no effect on tubulin polymerization either in the presence or the absence of ATP (data not shown). Microtubules polymerized in the presence of dynein and ATP had smooth surfaces (Fig. 4C) similar to those polymerized by dynein alone (Fig. 4A).

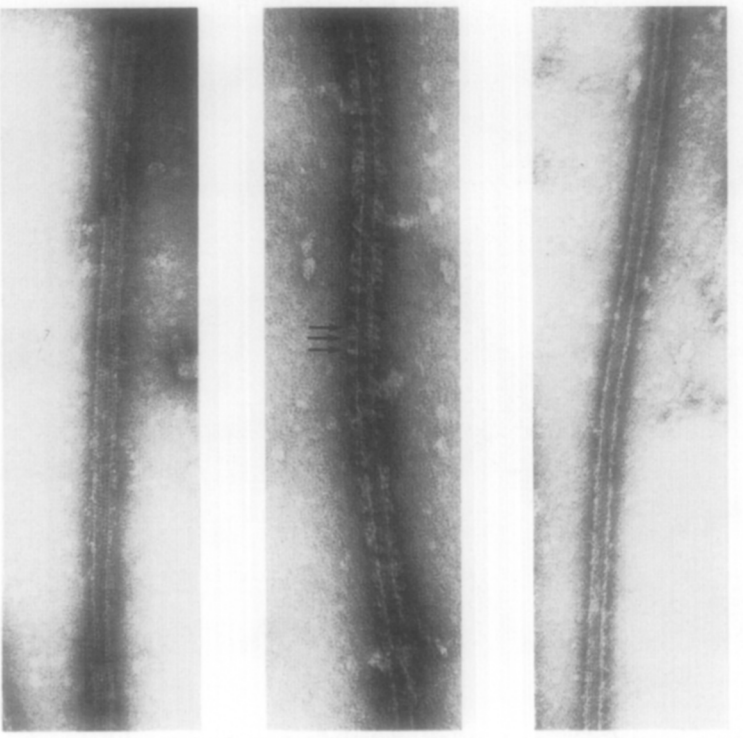

Fig. 4. Electron micrographs of negatively stained microtubules polymerized in the presence or absence of ATP and magnesium.
A) Tubulin (3.5mg/ml) + dynein (1.5mg/ml); B) Tubulin + dynein + 5mM $MgSO_4$; C) Tubulin + dynein + 1mM ATP. Note the decoration of microtubules in B as indicated by arrows and its absence in A and C.

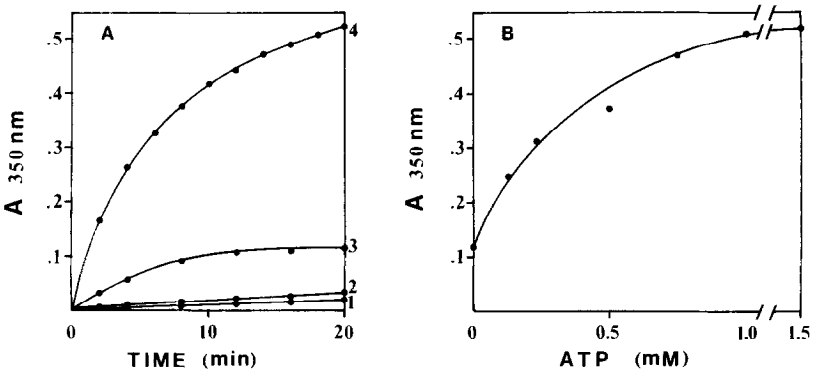

Fig. 5. Effects of ATP on the polymerization of tubulin by dynein.
A) Tubulin 3.5mg/ml and dynein (0.5mg/ml) were incubated alone or together with 1mM GTP in the presence or absence of 1mM ATP. Tubulin + ATP (1); dynein + ATP (2); tubulin + dynein (3); tubulin + dynein + ATP (4).
B) Tubulin 3.5 mg/ml and dynein at 0.5 mg/ml were incubated for 20 min. with 1mM GTP in the presence or absence of various concentrations of ATP.

DISCUSSION

Results reported here clearly indicate that a high molecular weight factor present in low ionic strength extracts from bull sperm axonemes can polymerize phosphocellulose-purified tubulin into microtubules. By contrast to the brain proteins tau and MAP2 which are thermostable (11, 12), the bull sperm factor is unstable to heat. A high molecular weight factor also capable of polymerizing tubulin into microtubules has been reported in high and low salt extracts from sea urchin and Chlamydomonas axonemes, respectively (13). Our results strongly suggest that 19S dynein was the high molecular weight factor predominantly responsible for the stimulation of tubulin polymerization by extracts from bull sperm axonemes.

Dyneins of axonemal origin are known to bind to preformed brain microtubules (14, 15). However, no decoration of dynein on microtubules was observed under our experimental conditions when dynein and tubulin were incubated with or without ATP. Decoration-free microtubules were also obtained when brain tubulin was incubated with high salt extracts from sea urchin axonemes and low salt extracts from Chlamydomonas axonemes (13). This lack of microtubule decoration was not due to an artifact in sample processing for electron microscopy since addition of 5mM Mg^{++} to the dynein and tubulin mixture during polymerization allowed decoration of microtubules by dynein. Thus, decoration by dynein molecules along microtubules does not seem to be essential for dynein effects on tubulin polymerization.

Addition of ATP to a mixture of dynein and tubulin caused a dramatic increase in microtubule formation. This increase by ATP was concentration dependent. Whether ATP acts by binding to dynein, a molecule known to have ATP binding sites (16) and ATPase activity (1), under our experimental conditions is not yet known. The fact that vanadate, an inhibitor of dynein ATPase (10) did not prevent the stimulatory effect of ATP suggests that the ATPase activity of dynein is not essential for ATP stimulated polymerization. Furthermore, the observation that the non-hydrolyzable ATP analogue AMPPCP had effects similar to those of ATP further indicates that only ATP binding rather than ATP hydrolysis is essential for ATP-stimulated polymerization of tubulin by dynein.

Recently, we have shown that 19S dynein isolated from bull sperm axoneme was composed of several peptides including two peptides with molecular weights above 300,000, one peptide at 90,000 and several smaller peptides (7). Whether one or more of these peptides are responsible for the polymerizing effects of 19S dynein remains to be investigated.

In addition to its role in the sliding of axonemal microtubules, the present results suggest that dynein may also play a role in the polymerization of sperm tubulin into microtubules.

ACKNOWLEDGMENTS: The authors are grateful to l'Abattoir Les Cedres, Les Cedres and l'Abattoir Zenon Billette, St.Louis de Gonzague, Quebec, Canada for their generous gifts of bovine testes and brains, respectively. We are also grateful to Drs. M.F. Lally and Y. Clermont from the Department of Anatomy, McGill University, for allowing us to use their electron microscopy facilities and to Dr. J.-F. Leterrier for stimulating discussions. This research was supported by grants from the Mecical Research Council of Canada to C.G.

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