INITIATION OF BRAIN TUBULIN ASSEMBLY BY A HIGH MOLECULAR WEIGHT FLAGELLAR PROTEIN FACTOR

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Although much information is available concerning the structure and function of cilia and flagella (26), little is known about the assembly of these organelles and in particular about the control of their assembly. Available experimental evidence suggests that some component or components released into the cytoplasm by resorbing cilia and flagella limit the subsequent regeneration of these structures in the absence of protein synthesis (5, 22, 23). Williams (33) has suggested that in Tetrahymanena there exists a high molecular weight axonemal protein that regulates ciliary assembly.

Protein factors that control both the initiation and extent of assembly of brain tubulin dimers in vitro have been found in preparations of mammalian and avian brain microtubule protein obtained by cycles of in vitro polymerization (12, 14, 20, 32, footnote 1). These observations prompted a search for similar regulatory factors within the flagellar axoneme.

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MATERIALS AND METHODS

Chick and calf brain microtubule protein were obtained by cycles of polymerization/depolymerization in vitro as previously described (6, 27). Twice-cycled microtubule protein was fractionated by chromatography on Bio-Gel A1.5m as previously described (6) in order to obtain a preparation of tubulin dimers.

*Chlamydomonas reinhardtii* flagella were obtained by the sucrose-PH method of Witman et al. (34). Whole flagella, or flagella demembranated by treatment with 1% Nonidet P-40 in 10 mM Tris-HCl at pH 7.8 for 10 min at 4°C, were dialyzed for 48 h at 4°C against 200 vol of a buffer containing 1 mM Tris-HCl, 0.1 mM EDTA, and 0.01% 2-mercaptoethanol at pH 8.0. The preparation was centrifuged at 100,000 g for 30 min at 4°C and the supernate was concentrated by pressure dialysis with Amicon PM 30 filters (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). In some cases, this preparation was fractionated by gel filtration using a 55 x 2.5 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), equilibrated and eluted with dialysis buffer. Cytoplasmic extracts were prepared from deflagellated *Chlamydomonas* by passage through a French pressure cell (American Instrument Co., Silver Spring, Md.) followed by centrifugation for 60 min at 100,000 g.

*Arabia punctulata* sperm flagellar axonemes were obtained by the method of Stephens (28) and extracted for 2-5 min at 24°C with a buffer containing 0.5 M KCl as described by Gibbons and Gibbons (11) and Kincade, Gibbons, and Gibbons (16). The extraction was terminated by centrifugation at 30,000 g for 10 min at 4°C. The supernate was concentrated as described above. All extracts were carefully monitored by negative-stain electron microscopy to ensure the absence of any microtubule pieces, membrane fragments, or other particulate material.

All flagellar and cytoplasmic fractions were transferred into polymerization buffer (0.1 M piperazine-N,N'-bis (2-ethane sulfonic acid) (PIPES), 2 mM EGTA, 1 mM GTP at pH 6.9) by desalting on a 1.5 x 10 cm column of Sephadex G-25 (Pharmacia) before the polymerization experiments. Most of these preparations possessed a magnesium-stimulated ATPase activity of 1-2 μmoles Pi liberated/mg protein/min at 24°C. This ATPase activity is comparable to that reported by others for sea urchin sperm flagellar dynein preparations (10). When increasing amounts of the sea urchin flagellar extract were added to a constant amount of brain tubulin dimers, the maximum rate of turbidity increase was a linear function of the concentration of flagellar material but the maximum change in absorbance was independent of the concentration of flagellar material (Figs. 2 and 3). Significant stimulation of microtubule formation was obtained at ratios of flagellar extract to brain tubulin (wt/wt) as low as 1:300 (Fig. 2, curve labeled 7). When assembled microtubules were sedimented at 100,000 g and processed for electron microscopy, thin sections of the pellets showed little difference between the microtubules formed from tubulin dimers alone and those formed when tubulin di-

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\[2\] Although little assembly occurred in the brain tubulin
FIGURE 1 SDS-polyacrylamide gel electrophoresis on 8% slab gels of: (a) Calf brain tubulin dimers purified by chromatography on Bio-Gel A1.5 m. (b) 0.5 M KCl extract of sea urchin sperm flagellar axonemes. (c) *Chlamydomonas* flagellar extract obtained by dialysis of axonemes against Tris-EDTA-mercaptoethanol. (d) Fraction of the *Chlamydomonas* flagellar extract that elutes with the void volume of a Sephadex G200 column. The positions of dynein (D), the tubulins (Tα and Tβ) and a 125,000 mol wt protein (125K) are indicated on the gels.

stimulation of tubulin dimer assembly by *Chlamydomonas* flagellar extracts

Extracts of *Chlamydomonas* flagellar axonemes obtained by low ionic strength dialysis in the presence of EDTA and mercaptoethanol contained primarily dynein and tubulin, although other components were also observed by SDS-PAGE (Fig. 1c). These preparations uniformly contained a magnesium-stimulated ATPase activity of 0.5 μmol Pi liberated/mg protein/min at 24°C. This is comparable to the values obtained by others for *Chlamydomonas* dynein preparations (29–31).

When increasing amounts of the *Chlamydomonas* flagellar extract were added to a constant amount of brain tubulin dimers, the maximum rate of turbidity increase was a linear function of the flagellar extract concentration while the total change in absorbance was independent of the concentration of flagellar material (Figs. 4 and 5). By contrast, when the flagellar extract concentration was maintained constant, both the maximum rate of turbidity increase and the total turbidity change were found to be a linear function of the brain tubulin dimer concentration (Figs. 6 and 7). In order to obtain equivalent maximum rates of microtubule assembly (with the same brain tubulin concentration), it was necessary to use a 10-fold higher concentration of *Chlamydomonas* than of sea urchin flagellar extract (compare Figs. 2 and 4). When the *Chlamydomonas* flagellar extract was fractionated by Sephadex G200 chromatography, all of the magnesium-stimulated ATPase activity and all of the brain tubulin assembly-stimulating activity eluted with the void volume of the column (Fig. 8). SDS-PAGE (Fig. 1d) showed that the void volume contained primarily dynein and a 125,000 mol wt component although some tubulin was still present.

Other characteristics of the stimulatory activity

In all cases, greater than 90% of the turbidity increases observed at 37°C in the brain tubulin-flagellar extract mixtures were reversed by incubation at 4°C. Preincubation of either of the flagellar extracts at 80°C for 5 min or with 10 μg/ml of pancreatic trypsin for 10 min at 24°C (followed by addition of a 10-fold excess of soybean trypsin
FIGURES 2 and 3  Kinetics of assembly of a constant concentration of calf brain tubulin dimers in the presence of varying concentrations of sea urchin sperm flagellar extract.

FIGURE 2  Plot of $A_{350}$ vs. time for 2.2 mg/ml of calf brain tubulin in the presence of 0, 7, 14, 28, 56, and 82 $\mu$g/ml of flagellar extract.

Variability occurred from one brain tubulin dimer preparation to another in the maximum levels of turbidity achieved for a given dimer concentration, and hence valid comparisons of the total amount of assembly obtained could only be made within a particular experiment. The variability was assumed to result from different percentages of the tubulin dimers being assembly-competent in different preparations.

A microtubule assembly-stimulating activity similar to that present within the flagellar extracts was searched for within the cytoplasm of *Chlamydomonas*. When high-speed supernates of cytoplasmic extracts from deflagellated *Chlamydo-
Figures 4 and 5  Kinetics of assembly of a constant concentration of calf brain tubulin dimers in the presence of varying concentrations of *Chlamydomonas* flagellar extract.

**Figure 4**  Plot of $A_{350}$ vs. time for 3.8 mg/ml of calf brain tubulin in the presence of 0, 95, 190, 380, and 760 $\mu$g/ml of flagellar extract.

*Chlamydomonas* were added to brain tubulin dimers, no assembly-stimulating activity was observed; in fact, the normal background assembly of microtubules observed in the brain tubulin dimer preparations alone was abolished. This observation was consistent with the observation of Farrell and Burns (7) that *Chlamydomonas* cytoplasm inhibits microtubule assembly. Since it has been suggested by Bryan et al. (3) that cytoplasmic RNAs are inhibitors of microtubule assembly, the *Chlamydomonas* cytoplasmic extracts were incubated with RNase A (120 $\mu$g/ml) before assaying for assembly-stimulating activity, but still no activity was observed.

**Figure 5**  Plot of maximum rate ($\Delta A_{350}/5$ min) (○—○) and maximum $A_{350}$ (Δ—Δ) vs. concentration of flagellar extract for the data shown in Fig. 4.
FIGURES 6 and 7 Kinetics of assembly of varying concentrations of calf brain tubulin dimers in the presence of a constant concentration of Chlamydomonas flagellar extract.

FIGURE 6 Plot of $A_{350}$ vs. time for 0.4 mg/ml of flagellar extract in the presence of 3.2, 3.8, 4.5, and 5.1 mg/ml of brain tubulin.

FIGURE 7 Plot of maximum rate ($\Delta A_{350}/5$ min) (O—O) and maximum $\Delta A_{350}$ (Δ—Δ) vs. concentration of brain tubulin for the data shown in Fig. 6.

DISCUSSION

Flagellar extracts from Chlamydomonas and sea urchin sperm stimulate the assembly of tubulin dimers isolated from calf and chick brain. The stimulatory activity is proteinaceous, heat labile and of high native molecular weight, as judged by gel filtration on Sephadex G200. All flagellar extracts that stimulate brain tubulin assembly contain one or both of the dynein bands on SDS-PAGE in addition to magnesium-stimulated ATPase activity.

As the concentration of flagellar extract material is increased in the presence of a constant amount of brain tubulin dimers, the maximum rate of microtubule assembly increases while the total mass of microtubules assembled, as judged by turbidity, remains constant (Figs. 2–5). These kinetics are consistent with the conclusion that the flagellar extract material is exerting an effect only on the initiation of microtubules. If this conclusion is correct, increasing concentrations of flagellar material added to the same concentrations of tubulin dimers should result in increasing numbers of microtubules while the average length of these microtubules at equilibrium should decrease. Preliminary data (not presented) suggest that this is the case. Since the flagellar extracts affect the maximum rate of microtubule assembly but not the final amount of assembled material, the apparent equilibrium amount of microtubule assembly should depend only on the brain tubulin concentration; this is indeed the case (Figs. 6 and 7).

The data presented in this paper show that some high molecular weight protein or proteins within the flagellum are capable of stimulating the initiation of brain tubulin assembly in vitro.

A number of fractions obtained from in vitro cycled brain microtubule protein preparations by gel filtration or ion exchange chromatography have the ability to stimulate the assembly of purified brain tubulin dimers into microtubules (12, 14, 20, 32, footnote 1). There are several important differences between these brain-derived fractions and the flagellar fractions described in the present report. All of the brain fractions have the property that they affect both the maximum rate of microtubule assembly and the final equilibrium amount of assembly (12, 14, 32, footnote 1). The microtubules stimulated to assemble in the presence of the brain fractions have filamentous material associated with their surfaces (6, 20); in contrast, the microtubules stimulated to assemble by the flagellar fractions are smooth and without any obvious surface decoration (Fig. 9). All of the brain fractions already contain or have the ability to stimulate brain tubulin dimers to form presumptive nucleation structures in the form of rings, disks, or similar structures (12, 20, 32, footnote 1). There are no obvious candidates for
Fractionation of *Chlamydomonas* flagellar extract on Sephadex G-200. The dashed line is the plot of $A_{280}$ vs. fraction number while the solid line is the plot of magnesium-stimulated ATPase activity ($\mu$mol Pi liberated/50 $\mu$l/min) vs. column fraction number (3-ml fractions). The first adsorbance peak contains protein, all of the magnesium-stimulated ATPase activity, all of the brain tubulin assembly-stimulating activity, and elutes with the void volume. The second adsorbance peak contains protein; the third adsorbance peak contains no protein and elutes with the included volume of the column.

nucleation structures in the flagellar extracts or in mixtures of brain tubulin and flagellar material incubated under a variety of conditions.

There have been some recent indications that flagellar magnesium-stimulated ATPase activity can be associated with in vitro assembled brain microtubules (4, 25, footnote 3). The present work strongly suggests that there is some sort of interaction occurring between flagellar proteins and brain tubulin. Since all of the flagellar extracts contain a number of proteins as judged by SDS-PAGE, it is not possible to assign the assembly-stimulating activity to a particular gel band or enzyme activity. However, it must be noted that all flagellar extracts that possessed the ability to stimulate brain tubulin dimers to assemble into microtubules in vitro also possessed substantial magnesium-stimulated ATPase activity (0.5–2.0 $\mu$mol Pi liberated/mg protein/min at 24°C) and one or both of the gel bands corresponding to flagellar dynein. Further, the extract with the higher relative assembly-stimulating activity is also the extract with the higher magnesium-stimulated ATPase activity (that from sea urchin sperm flagella). It should be possible to follow both the magnesium-stimulated ATPase activity and the brain tubulin assembly-stimulating activity of the flagellar extracts as they are fractionated by a variety of procedures and, by this means, determine whether the two activities co-purify or are separable. Brain microtubules assembled in the presence of sea urchin flagellar extracts and sedimented through 25% sucrose have a magnesium-stimulated ATPase activity which is absent in microtubules assembled in the absence of flagellar material (Bloodgood and Rosenbaum, unpublished results). It cannot be ruled out that this ATPase activity represents protein in the flagellar extract that nonspecifically adsorbed to the formed microtubules.

What is the possible significance of a high molecular weight flagellar protein factor that is capable of initiating the assembly of brain tubulin into microtubules? The in vivo initiation of cilia and flagella always occurs in association with basal bodies. However, the ability of basal bodies to nucleate the assembly of these microtubular organelles may itself be under another level of cellular control because many cell types, particularly among the protozoa, contain basal bodies that nucleate the assembly of cilia or flagella only at
FIGURE 9  Electron micrographs of thin sections of pellets of calf brain microtubules assembled in the absence \( (a) \) and presence \( (b) \) of the sea urchin sperm flagellar extract. Bar equals 0.2 \( \mu \)m. \( \times \) 95,000.
certain times in the cell cycle or upon differentiation. Two types of observations in the literature suggest that the presence of adequate tubulin pools and assembly-competent basal bodies may not be sufficient for microtubule assembly. Heidemann and Kirschner (15) report that injection of preparations of purified Chlamydomonas or Tetrahymena basal bodies into eggs of Xenopus laevis induced the formation of asters. Injection of the same preparations into fully grown oocytes elicited no response even though the oocytes and eggs contained tubulin pools of equal size. In order to explain the difference between the two cases, it is necessary to postulate that the tubulin is modified to make it polymerization competent or that another factor, perhaps modifying the basal body, is necessary for initiation to occur. Gutman and Gorovsky (13) have shown that regeneration of cilia in starved Tetrahymena is accompanied by the rapid synthesis of an 80,000 mol wt protein fraction, although this newly synthesized fraction is not found in any appreciable amount within the regenerated cilium. One interpretation of their data is that a protein must be synthesized which modifies the basal body before the initiation of assembly of new cilia can occur.

**SUMMARY**

A protein factor found within the flagella of Chlamydomonas and sea urchin sperm is capable of stimulating the initiation of calf and chick brain tubulin dimer assembly in vitro.

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