Microtubule Nucleation from Stable Tubulin Oligomers*

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Microtubule assembly from purified tubulin preparations involves both microtubule nucleation and elongation. Whereas elongation is well documented, microtubule nucleation remains poorly understood because of difficulties in isolating molecular intermediates between tubulin dimers and microtubules. Based on kinetic studies, we have previously proposed that the basic building blocks of microtubule nuclei are persistent tubulin oligomers, present at the onset of tubulin assembly. Here we have tested this model directly by isolating nucleation–competent cross-linked tubulin oligomers. We show that such oligomers are composed of 10–15 laterally associated tubulin dimers. In the presence of added free tubulin dimers, several oligomers combine to form microtubule nuclei competent for elongation. We provide evidence that these nuclei have heterogeneous structures, indicating unexpected flexibility in nucleation pathways. Our results suggest that microtubule nucleation in purified tubulin solution is mechanistically similar to that templated by γ-tubulin ring complexes with the exception that in the absence of γ-tubulin complexes the production of productive microtubule seeds from tubulin oligomers involves trial and error and a selection process.

Microtubules are fibrous elements in the cytoplasm of eukaryotic cells, where they perform a wide variety of functions. The microtubule building block is the tubulin αβ heterodimer. Within the microtubule, tubulin dimers aggregate through longitudinal interactions into protofilaments. Protofilaments are associated laterally to form a 25-nm-diameter cylindrical structure. Microtubule assembly from purified tubulin involves both microtubule nucleation and microtubule elongation (for a review, see Ref. 1). In fully assembled microtubule solutions, individual polymers exhibit spontaneous length fluctuations involving polymer assembly and disassembly events. Microtubule elongation and disassembly are both well documented at the structural level (2–5). By comparison, microtubule nucleation is still poorly documented, mainly because the molecular species that form during nucleation are small sized and short-lived. The earliest structures observed by time-resolved electron microscopy during microtubule assembly are two-dimensional sheets of protofilaments (reviewed in Ref. 6), which subsequently close to form integral microtubules. Earlier steps of microtubule nucleation have hitherto been deduced from kinetic data (7–9), the interpretation of which is model-dependent.

A salient feature of microtubule nucleation is that its rate depends on the high power of the initial GTP-tubulin concentration. This power currently varies from 6 to 12 (7–12). In classical models, nucleation exponents are supposed to correspond to the number of tubulin molecules that assemble to form a filament from which a tubulin sheet arises. A compelling prediction of these models is that microtubule nucleation should rapidly drop to undetectable levels during tubulin assembly because of the resulting drop in the free GTP-tubulin concentration, and, a fortiori, of the 6th–12th power of this concentration. This prediction has been tested only recently by systematic measurements of microtubule length and number concentration during tubulin assembly (13). Surprisingly, the rate of microtubule nucleation turned out to be constant during tubulin assembly, despite the large drop in the free tubulin concentration. These observations led to a model in which the building blocks of microtubule nuclei are persistent tubulin oligomers present at the onset of tubulin assembly. This model implies that microtubule nuclei contain several of these oligomers to account for the nucleation exponent, whereas in previous models microtubules were supposed to nucleate from a single tubulin oligomer (8–10, 14).

Here we provide direct evidence that microtubules can indeed nucleate from combinations of stable tubulin oligomers. Our data indicate that there is not a unique and fully determined way to integrate oligomers in microtubule nuclei. Instead the nucleation process appears to be flexible with a nucleation exponent that represents an average between alternative pathways.

EXPERIMENTAL PROCEDURES

Preparation of 1/1 GTP-Tubulin Complexes—Tubulin purification from bovine brain was performed as described (13). Purified tubulin (100–150 μM) was incubated in PEM buffer (100 mM Pipes, 1 mM MgCl2 for 10 min at 4 °C) and in the presence of 1 mM GTP. Free nucleotides were removed using Biogel P30 chromatography. The GTP-tubulin concentration was adjusted in PEM buffer, and the aliquots were stored in liquid nitrogen.

Fluorescent Labeling of Tubulin—Tubulin was labeled with carboxytetramethylrhodamine succinimidyl ester as described (15), with one recycling step.

Microtubule Assembly and Length Measurements—Microtubule assembly was carried out in PEM buffer with purified tubulin (12 μM), and various amounts of ethylene glycol bis succinimidyl succinate (EGS) suspension. Assembly was monitored at 350 nm, 35 °C, in a spectrophotometer. 

Alternatively, the microtubules were assembled from [3H]GTP-labeled tubulin. Rat tail fibroblasts were incubated in PEM buffer containing [3H]GTP (1 μM), and tubulin was isolated from the cell lysate. Purified tubulin (1 mM) was incubated in PEM buffer (100 mM Pipes, 1 mM MgCl2) for 10 min at 4 °C in the presence of 1 mM GTP. Free nucleotides were removed using Biogel P30 chromatography. The GTP-tubulin concentration was adjusted in PEM buffer, and the aliquots were stored in liquid nitrogen.

Fluorescent labeling and assembly were performed as described for nonradioactive tubulin. 

Acknowledgments

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1. The abbreviations used are: Pipes, 1,4-piperazinediethanesulfonic acid; GEG, ethylene glycol bis succinimidyl succinate; γ-TuRC, γ-tubulin ring complex; SLS, static light scattering.
beaded tubulin (18 μm). The assembly conditions, procedures designed to measure assembled tubulin concentration, determination of microtubule mean length, and microtubule number concentration are described in Ref. 13.

Procedures for Electron Microscopy—Vitreous ice-embedded samples were prepared as described previously (16). 4 μl of sample (EGS suspension or microtubules assembled at 37 °C from tubulin (20 μM), GTP (1 mM), and EGS suspension (0.1 μM)) were pipetted onto a holey carbon grid, briefly blotted, and plunged quickly into liquid ethan (for microtubules, the grid was maintained in a humid atmosphere at 37 °C before blotting). The specimens were stored in liquid nitrogen and observed in a Philips CM 12. Images were recorded on Kodak S.O 163 film under low dose conditions at 28,000x and 35,000x magnifications and ~ 2.3 μm underfocused. The micrographs were digitized with a UPRES-A 6026 CNRS scanner and with the Machine A Mesurer en Astronomie (M.A.M.A.) microdensitometer (Centre d’Analyse des Images Institut des Sciences de l’Univers/CNRS/Observatoire de Paris). The diameter and length of particles in the EGS suspension were measured using NIH Image software.

Light Scattering—Static light scattering (SLS) and dynamic light scattering (DLS) experiments were performed by means of a spectrometer equipped with an argon ion laser (Spectra Physics model 2020) operating at λ = 488 nm, an ALV-5000 correlator (ALV, Langen-Germany Instruments), a computer-controlled and stepping motor-driven variable angle detection system, and a temperature-controlled sample cell. The scattering spectrum was measured through a band pass filter (488 nm) and a pinhole (200 μm for the static experiments and 100 μm for the dynamic experiments) with a photomultiplier tube (ALV).

In the SLS experiments, the excess of scattered intensity I(q) was measured with respect to the solvent, where the magnitude of the scattering wave vector q is given by the following equation.

\[ q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2} \]  

(1)

where n is the refractive index of the solvent (1.34 for the water at 25 °C), λ is the wavelength of light in the vacuum, and θ is the scattering angle. In our experiments, the scattering angle θ varied between 20° and 150°, which corresponds to scattering wave vectors q in the range from 6 × 10^-4 to 3.2 × 10^-3 Å^-1. The absolute scattering intensities I(q) (i.e. the excess Rayleigh ratio) were deduced using a tolune sample reference for which the Rayleigh ratio is well known. The plots of I(q)/c versus q^2 were extrapolated to q = 0 to give intercepts I(q)/c, where c is the concentration of the scattering objects. If the length scale q^-1 is sufficiently large compared with the radius of gyration R_g of the objects, the form factor obeys Guinier law, and the radius of gyration R_g can be determined from the intercept and the initial slope of these plots using a scattering inverse Lorentzian law of the form (17).

\[ K_c \frac{I(q,c)}{I(0,c)} = \frac{q^2 R_g^2}{3} \]  

(2)

where \( K = 4\pi n^2 \text{dn} \text{d}c/N_c \) is the scattering constant, dn/dc is the refractive index increment, and N_c is Avogadro’s number. The apparent radius of gyration is obtained by a mean square linear fit of the inverse of the scattered intensity versus q^2.

In DLS experiments, the normalized time autocorrelation function g(0)(q,t) of the scattered intensity is measured (16). The latter can be expressed in terms of the field autocorrelation function or equivalently in terms of the autocorrelation function of the concentration fluctuations g(1)(q,t) through the following equation.

\[ g(0)(q,t) = \frac{(I(q,0)I(q,t))}{(I(q,0))^2} = A + \beta g(1)(q,t)^2 \]  

(3)

where A is the base line, and \( \beta \) is the coherence factor, which in our experiments is equal to 0.7–0.9. The normalized dynamical correlation function g(1)(q,t) of concentration fluctuations is defined as follows.

\[ g(1)(q,t) = \frac{\left< \delta c(q,0)\delta c(q,t) \right>}{\left< \delta c(q,0)^2 \right>} \]  

(4)

where \( \delta c(q,0) \) and \( \delta c(q,0) \) represent fluctuations of polymer concentration at time t and time 0, respectively.

In our experiments, inspection of the angular dependence shows that the relaxations are diffusive with characteristic time \( \tau \) inversely proportioned to \( q^2 \). It is then possible to determine diffusion constant D. The latter is related to the average hydrodynamic radius \( R_H \) of the objects through the following equation.

\[ D = \frac{kT}{6\pi \eta R_H} \left( \frac{1}{q^2} \right) \]  

(5)

where \( k \) is the Boltzmann constant, \( \eta \) is the solvent viscosity, and \( T \) is the absolute temperature.

To determine the average relaxation time \( \tau \), we used the Contin method based on the inverse Laplace transform of \( g(1)(q,t) \) (19). If the spectral profile of the scattered light can be described by a multi Lorentzian curve, then \( g(1)(q,t) \) can be written as follows.

\[ g(1)(q,t) = \int G(\Gamma) \text{exp}(\Gamma \tau) d\Gamma \]  

(6)

where \( G(\Gamma) \) is the normalized decay constant distribution. This method is appropriate for solutions characterized by several relaxation mechanisms.

Assay of Nucleotide Exchange—Various amounts of EGS suspension were incubated with GTP and [γ-32P]GTP for 15 min at 4 °C. The mix was filtered by Biogel P30 chromatography. The protein concentration and radioactivity of the eluate fraction were measured. The eluted radioactivity corresponded to the fraction of the nucleotide associated with EGS oligomers.

Quantitative Analysis of Rhodamine Spot Fluorescence—Contours of spots were determined with a detection threshold higher than the mean background value measured around the spot. The threshold was adjusted near the sharp rise of the spot region, to reject the flat outer wings of diffused light. This strategy slightly underestimated the integrated intensity of the spot, but it minimized uncertainties caused by background fluctuations. The fluorescence intensities of spots were calculated as the sums of pixel values contained inside the selected contours after subtracting the corresponding background.

To determine the number of dimers contained in individual spots, we estimated the mean fluorescence intensity value corresponding to one dimer. For this, rhodamine-labeled microtubules were assembled with the same batch of rhodamine tubulin as the one used for preparation of EGS oligomers. Measurements of fluorescence intensity per microtubule length unit yielded an estimation of the mean fluorescence intensity per tubulin dimer, assuming 1625 dimers/μm of microtubule length (20).

The number of tubulin dimers in a spot was determined by dividing the integrated fluorescence intensity inside the contour of the spot by the fluorescence intensity of one tubulin dimer. The number of oligomers/spot was finally deduced from the number of tubulin dimer in one EGS oligomer.

RESULTS

Preparation of Stable Tubulin Oligomers—It is well known that microtubule disassembly produces a profusion of oligomers of various sizes and forms (21, 22). To stabilize, isolate, and characterize tubulin oligomers with microtubule nucleation capacity, we developed procedures in which microtubules were cross-linked and then disassembled. Cross-linked oligomers were recovered from disassembly products.

For microtubule cross-linking, we followed previously published methods (23–25). Purified tubulin (100 μM) was assembled for 20 min at 37 °C in a buffer containing 80 mM Pipes (pH 6.7), 1 mM EGTA, 50% (v/v) glycerol, 5 mM MgCl2, and 1 mM GTP. EGS was then added at 3.4 mM final concentration for 15 min. To quench the EGS in excess, the mixture was diluted into 9 volumes of a buffer containing 80 mM Pipes (pH 6.7), 1 mM EGTA, 50% sucrose, 10 mM glutamate, and 1 mM MgCl2 and incubated for 1 h at room temperature. The solution was then centrifuged at 200,000 × g for 30 min. The pellet containing cross-linked microtubules was resuspended in PEM buffer. In a standard experiment, the microtubule pellet obtained from 400 μl of purified tubulin (100 μM) was resuspended in 180 μl of PEM at a final tubulin concentration of 150 μM. The resuspended microtubules were then subjected to a freezing-thawing cycle (freezing at −80 °C
Fig. 1. Electron micrographs of EGS oligomers. EGS suspension was examined before (A) or following (B) filtration on a 100-nm Millipore filter. Curved filamentary structures of various sizes were detected (arrows). Bar, 30 nm.

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Microtubules had disassembled, and the suspension mainly contained curved filamentary structures of various size, the longest ones looking like open circles (Fig. 1A). Suspensions also contained large protein aggregates (not shown) and, occasionally, residual microtubule fragments. To eliminate such fragments and the large protein aggregates, the suspension (diluted 1:5 in PEM) was subsequently filtered on a 0.1-μm Millipore filter to eliminate residual microtubule fragments. Approximately 5% of the protein was recovered in the filtrate, called “EGS suspension” hereafter, and could be stored at −80 °C for several weeks prior to further processing. Prior to use, the EGS suspension was centrifuged at 200,000 × g for 30 min, and the pellet was recovered in 390 μl of PEM buffer. The EGS suspension mainly contained tubulin oligomers that appeared as curved filaments of 20–60 nm in size, whereas the longest circular tubulin assemblies were largely eliminated (Fig. 1B).

EGS Cross-linked Tubulin Oligomers Nucleate Microtubules—For assay of microtubule nucleation activity, aliquots of the EGS suspension were mixed with 12 μM tubulin in the presence of 1 mM GTP. Tubulin assembly was monitored by optical density measurements (Fig. 2A). To directly verify microtubule production, 20 μl of the suspensions were centrifuged on coverslips and labeled by indirect immunofluorescence with anti-tubulin antibody. In the absence of EGS suspension, no microtubule assembly occurred. Significant microtubule nucleation was observed at a 1:120 oligomeric tubulin/tot al tubulin ratio (Fig. 2A, curve observed at 0.1 μM oligomer concentration). The structure of the microtubules nucleated from the cross-linked oligomers was assessed using cryoelectron microscopy (Fig. 2B). Most of the microtubules were composed of 13 or 14 protofilaments with a small proportion (~5%) composed of 12 or 15 protofilaments. These proportions corresponded to those previously observed in microtubule suspensions assembled in standard conditions (26).

Alternatively, EGS suspension was prepared as previously described, except that the experiment was performed at 4 °C. In this case, the tubulin suspension did not assemble in microtubules, and the resulting EGS suspensions did not display any microtubule nucleation activity (data not shown). Thus, active oligomers could only be obtained from disassembly of cross-linked microtubules.

Light Scattering Analysis of EGS Suspension—Electron microscopy experiments showed that EGS suspension contained oligomers in the form of small linear filaments. Quantitative information on the size of the components of EGS suspensions and on their relative concentrations was obtained using both DLS and SLS experiments.

Fig. 3A shows the results obtained from the DLS experiment. After application of the Contin method (see “Experimental Procedures”), the measured time autocorrelation function of the scattered intensity could be described by a sum of two relaxation times widely separated in time, suggesting that the EGS suspensions contained two main molecular species. The fast relaxation time corresponded to a hydrodynamic radius of 8 ± 1 nm, which is the size of the tubulin dimer. Thus, this relaxation time could be ascribed to the diffusive mode of tubulin dimers. The slow relaxation time corresponded to a hydrodynamic radius of 60 ± 6 nm. This value was in good agreement with the size of the tubulin oligomers seen in electron microscopy (20–60 nm).

The radius of gyration measured using SLS experiments was found to be equal to 50 ± 10 nm. Within the error bars, this value was in good agreement with the value of hydrodynamic radius found for EGS oligomers.

These experiments indicated that EGS suspensions were composed exclusively of tubulin dimers and EGS cross-linked...
Light scattering experiments. A, distribution function of decay time $A(t)$ obtained using the Contin method for an EGS suspension at 7.5 μM, $\theta = 90^\circ$, and at 35 °C. B, scattered intensity during microtubule assembly in the presence of tubulin (18 μM), EGS suspension (5 μM, expressed in molarity of tubulin dimers), and GTP (1 mM). C, distribution function $A(t)$ at $\theta = 90^\circ$ and at 25 °C for time 0, 540, and 1600 s.

The relative concentrations of free tubulin dimers ($c_{\text{dimers}}$) and tubulin dimers in the form of EGS oligomers ($c_{\text{EGS dimers}}$) in the solution could be deduced from the following equation (27).

$$P = \frac{M_{\text{EGS dimers}}}{M_{\text{dimers}}} = \left( \frac{A_{\text{EGS dimers}}}{A_{\text{dimers}}} \right) \frac{c_{\text{dimers}}}{c_{\text{EGS dimers}}}$$

(Eq. 7)

where $p$ is the number of tubulin dimers in EGS oligomers. Assuming that the tubulin dimer was 8 nm in length and 4 nm in width, for oligomers of 60 nm $p$ is equal to 7.5 for longitudinally associated tubulin dimers and to 14 for laterally associated tubulin dimers.

$M_{\text{EGS dimers}}$ and $M_{\text{dimers}}$ were the weight-average molecular masses of EGS oligomers and dimers, respectively. $A_{\text{dimers}}(q)$ and $A_{\text{EGS dimers}}(q)$ were the relative amplitudes of the relaxations associated with tubulin dimers and EGS oligomers, respectively (Fig. 3A). Their corresponding ratio in Equation 7 extrapolated at $q = 0$ was equal to 100.

The relative concentrations of free tubulin dimers ($c_{\text{dimers}}$) versus tubulin dimers in the form of EGS oligomers ($c_{\text{EGS dimers}}$) could be calculated for two different values of $p$ corresponding to the possibility that the oligomers consisted of laterally or longitudinally associated tubulin dimers, respectively. In the case of EGS oligomers composed of longitudinally associated tubulin dimers, 7% of total tubulin would be in the form of free tubulin dimers, and 93% would be in the form of EGS cross-linked tubulin dimers. For EGS oligomers composed of laterally associated tubulin dimers, 13% of tubulin would be in the form of free tubulin dimers, and 87% would be in the form of EGS cross-linked tubulin dimers.

**EGS Oligomers Can Exchange Nucleotides**—We used a nucleotide exchange assay to determine whether tubulin oligomers in EGS suspensions consisted of laterally or longitudinally associated tubulin dimers. It is known from both biochemical and structural data that GTP at the exchangeable site in β-tubulin is not accessible in longitudinally associated tubulin dimers (28–30). In contrast the same exchangeable sites should be fully accessible in laterally associated oligomers. For nucleotide exchange assay, filtered EGS suspension was prepared with nonradioactive GTP and then incubated in the presence of radioactive [3H]GTP. Free GTP was removed by filtration on Biogel P30 chromatography, and the stoichiometry of GTP binding to tubulin was determined. The results of three independent experiments yielded stoichiometry estimates varying from 0.91 to 0.99 (Table I), suggesting that the exchangeable GTP-binding sites were exposed in the vast majority of tubulin dimers, including those associated in oligomers. This is a strong indication that the nucleating tubulin oligomers consist of laterally associated tubulin dimers.

| EGS oligomers | GTP | Linked GTP | Dimers having exchanged GTP |
|---------------|-----|------------|----------------------------|
| %             | %   | %          |                            |
| 1.2           | 0.25| 1.23 ± 0.12 (n = 4) | 98.4                       |
| 2.5           | 0.25| 2.39 ± 0.14 (n = 4) | 95.6                       |
| 2.5           | 2.75| 2.28 ± 0.09 (n = 3) | 91.2                       |

* Concentration expressed in μM tubulin dimers.
(~200 nm), it was not possible to determine their size using light scattering experiments. These observations mainly showed that only a proportion of the EGS oligomers were incorporated in microtubules during assembly. They did not allow detection of intermediates between oligomers and microtubules, possibly because such intermediates would yield a relaxation overlapping with the microtubule relaxation.

**Kinetic Analysis of Nucleation from Stable Tubulin Oligomers**—Each EGS oligomer might be able to nucleate a microtubule (first order reaction), or several oligomers might have to be incorporated in microtubule nuclei ($n^{th}$ order reaction). To distinguish between these possibilities and estimate the $n$ value, we determined the relationship between the oligomer concentration and the initial rate of microtubule nucleation in the presence of GTP-tubulin complexes without GTP in excess (Fig. 4A). The procedures for determination of microtubule concentration from tubulin assembly data and microtubule mean lengths were as in Ref. 13.

Slopes of microtubule concentration plots during the initial fast increase phase were plotted against the oligomer concentration on a log-log plot (Fig. 4B). The slope of the regression line is an estimate of $n$ and was equal to 4.1. This result strongly indicates that several oligomers are incorporated in a single microtubule nucleus.

**Microtubule Nucleation from Fluorescent Tubulin Oligomers**—If several EGS oligomers associated to nucleate a microtubule, we reasoned that it could be possible to detect directly fluorescent EGS oligomers in microtubules by light microscopy and to quantify corresponding fluorescent intensities. EGS oligomers were produced from microtubules obtained using rhodamine-labeled tubulin. Rhodamine-labeled EGS oligomers (1 μM tubulin concentration) were incubated with unlabeled tubulin (18 μM) for 30 min at 37 °C in the presence of 1 mM GTP. The suspension was then centrifuged on coverslips and labeled by indirect immunofluorescence using anti-tubulin primary antibody and fluorescein-labeled secondary antibody. Remarkably, rhodamine-labeled oligomers formed red or yellow (by superposition of green and red colors) spots located either within or at one end of microtubules (Fig. 5A, arrows), strongly indicating that microtubules had indeed polymerized on a seed formed by combined oligomers. The precise location of some spots at one end suggested that the stable seed inhibited microtubule disassembly from the minus end. Some red spots were unconnected with microtubules. In control experiments in which rhodamine-EGS oligomers (1 μM) were incubated for 30 min at 37 °C in PEM buffer with GTP (1 mM) without added tubulin, such orphan spots did not form (not shown). This result suggests that in fitting with the light diffusion experiments, no oligomer complexes form in the absence of free tubulin and that orphan spots represent unproductive microtubule seeds.

The apparent size of the spots showed conspicuous variability both for spots incorporated in microtubules and for orphan spots. A quantitative analysis of spot fluorescence was carried out on spots associated with microtubules (see "Experimental Procedures"). The threshold selected for spot detection was ~10% higher than the mean background value and spot con-
tours determined areas in the range of 1–17 pixels. The mean fluorescence intensity/spot was estimated, assuming 15 tubulin dimers/oligomer. The results indicated that the number of oligomers/spot was variable, ranging from 3 to 40 with a mean value of 14 (Fig. 5B), much higher than the nucleation exponent estimated from kinetic data. This apparent discrepancy may in part result from technical limitations. The fluorescence intensity of pixels in small size spots (lower than 3 pixels) was close to the detection threshold. The number of such spots was therefore probably underestimated and a proportion of these spots, containing five oligomers or less, might have been neglected in our analysis. However, this difference may also result from the fact that large aggregates do not need to form in one step, by simultaneous interaction of dozens of oligomers. Rather, oligomer aggregates might grow in successive steps, and this would account for the apparent discrepancy between fluorescence and kinetic data.

Taken together these data support the view that cross-linked oligomers aggregated in distinct microtubule nuclei in the presence of free tubulin molecules. They also reveal an unexpected variability in the size of such nuclei.

**DISCUSSION**

Although suitable conditions for microtubule assembly *in vitro* have been discovered decades ago (31), the pathway of microtubule nucleation in purified tubulin solutions has remained poorly understood because of the absence of suitable procedures to isolate molecular intermediates between tubulin dimers and tubulin sheets. This report describes the isolation and characterization of stabilized linear tubulin oligomers that represent such molecular intermediates.

Our data indicate a lateral association of tubulin molecules in the EGS oligomers. Apparently, whereas in the absence of cross-linker microtubule breakdown in longitudinal filaments, EGS treatment preferentially stabilizes lateral dimer-dimer interactions, and microtubule disassembly by freezing-thawing procedures yields laterally associated tubulin filaments. It is possible that other types of tubulin oligomers could also nucleate microtubule assembly. We have tried a number of other procedures to derive assembly competent tubulin oligomers either from tubulin solutions kept in the cold, which contain abundant linear and circular oligomers (data not shown), or from the disassembly products of microtubules generated during complete microtubule oscillations (22), without success. We cannot exclude the existence of nucleation centers whose structure is altered by cross-linking. It is also possible that natural non-cross-linked oligomers similar to those that we used in the present study would combine somewhat differently to form microtubule seeds. In any case, our results yield new information regarding the possibility and pathways of microtubule nucleation from tubulin oligomers.

We have used the EGS oligomers for a direct test of the pertinence of a model of microtubule nucleation from stable tubulin oligomers that we have previously proposed (13). In classical nucleation models, the slow step is the aggregation of several tubulin molecules into a filament. The rapid step consists in the formation of microtubule from this filament. There is essentially no filament free in solution, and a single filament is incorporated in each polymer (14). In contrast, our model postulated that nucleation-competent tubulin filaments formed very rapidly at the onset of tubulin assembly to yield an excess pool of nucleation competent oligomers that subsequently combined slowly to form microtubule nuclei (13). This study shows that the model that we proposed is sound. Light scattering experiments as well as microscopy studies showed that several EGS oligomers were needed to form a microtubule nucleus and that microtubule nucleation from these oligomers did not proceed in one step, with a pool of oligomers remaining free in solution during the assembly process. However, our study also revealed surprising features of the nucleation process.

The first unexpected observation was that microtubule nuclei do not form from the oligomers themselves but from complexes of these oligomers with free tubulin dimers. Both microscopy and light scattering data provided evidence that the oligomers themselves had no tendency to combine into larger structure, in the absence of tubulin dimers. Upon the addition of tubulin dimers, the same oligomers coalesced into large nucleation complexes visible in light microscopy. It may be that the basic building blocks combining to form productive nucleation complexes are small tubulin sheets arising from one oligomer.

The second surprise was the apparently conflicting flexibility and reliability of the nucleation process. Because *in vitro* tubulin assembly produces *bona fide* microtubules, it is generally thought that microtubule seeds have a rather strictly defined organization and that the nucleation exponent in the nucleation reaction has a clear structural meaning. Our data strongly support a different view. Apparently, the EGS oligomer-tubulin nucleation building blocks form all kinds of complexes, in a presumably flexible succession of association events. Some complexes are unproductive and are visible in microscopy as orphan spots. Other complexes expose a suitable shape to sustain microtubule growth, and this can happen with only a few building blocks or can need the association of a large number of building blocks. The nucleation exponent does not correspond to a strictly defined structure but is an average between many alternative association pathways. We believe that the ultimate reliability of the nucleation process producing *bona fide* microtubules from heterogeneous nuclei requires an efficient selection process. Probably, many tubulin assembly attempts occur on imperfect microtubule nuclei, but the structures formed are eliminated because of their instability. Thus, the general dynamic instability of microtubule assemblies may be as central for the reliability of microtubule assembly as for microtubule function.

The lateral tubulin oligomers that nucleate microtubule assembly in our study bear obvious similarity with the tubulin assemblies that form on γ-TuRCs and can seed microtubule assembly both *in vitro* and *in vivo*. γ-TuRCs template the assembly of a laterally associated tubulin oligomer on which microtubule subsequently elongate (32–34). However, whereas several EGS oligomers are needed for nucleation, a single oligomer is sufficient when templated on a γ-TuRC. A likely explanation is that γ-tubulin complexes specify precisely both the subunit number and the curvature of the tubulin oligomer used for microtubule seeding. With EGS oligomers, exposing laterally associated tubulin dimers with the proper arrangement in space at the surface of microtubule nuclei is apparently achieved through the association and overlap of several tubulin-oligomer complexes. Thus, ultimately, microtubule nucleation in purified tubulin solutions and from γ-TuRCs could be very similar processes, with the exception that γ-tubulin complexes render deterministic and efficient a process that involves trial and error in purified tubulin solutions.

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