GDP-TUBULIN INCORPORATION INTO GROWING MICROTUBULES MODULATES POLYMER STABILITY

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Supplemental Results

Absence of GDP conversion to GTP during microtubule assembly- Tubulin preparation can contain contaminating NDP kinase activity which then induces GDP conversion to GTP using either GTP itself or another triphospho-nucleotide such as ATP as a phosphor donor (35). To test for the presence of such contaminating activity in our preparations, GDP-tub (75 µM) was incubated with ATP (0.5 mM) at 35°C and assayed for microtubule assembly by turbidimetry changes at 350 nm. In the presence of significant amounts of NDP kinase, GDP should be converted to GTP using ATP as a phosphate donor, with resulting tubulin assembly. In fact, no detectable tubulin assembly was observed (Figure S1 A). We also tested whether GTP had nevertheless been generated during incubation, although at sub-critical concentrations. GDP-tub (75 µM, 40 µl) was incubated in the presence of [γ–^{32}P]-ATP (10 µCi/µM) and ATP (0.5 mM) at 35°C during different times. Reactions were stopped by sample immersion in liquid nitrogen. Then samples were thawed at 4°C and diluted with 80 µl of cold PEM buffer. To test whether samples contained [γ–^{32}P]-GTP bound tubulin, free nucleotide was removed using Biogel P30 chromatography. ^{32}P radioactivity was counted and converted in tubulin concentration using labelled nucleotide specific activity. Three independent experiments were performed. The resulting level of labelled GTP-tub was about 2 µM (Figure S1, B). As a positive control of maximal nucleotide binding, GDP-tub (75 µM, 40 µl) was incubated in the presence of [γ–^{32}P]-GTP (10 µCi/µM) and GTP (0.5 mM) during different times, at 11°C to avoid microtubule assembly. Under these conditions, the originally tubulin bound nucleotide GDP was replaced by labelled GTP. Samples were processed as above for ^{32}P radioactivity measurements. The labelled GTP-tub concentration was about 68 µM (Figure S1, B). These results indicate absence of detectable conversion of GDP to GTP during incubation.

Quantitative analysis of microtubule nucleation and mean length of microtubules assembled from GTP-tub/GDP-tub mixtures- To determine microtubule mean length, GTP-tub/GDP-tub mix were assembled as described in Experimental Procedures section (*Microtubule assembly conditions*) and assembly reaction was stopped by adding to samples 1 ml of 100 mM Mes (pH 6.7) containing 1 mM EGTA, 1 mM $MgCl_2$, 0.75% glutaraldehyde and 50% (w/v) sucrose. Then, microtubules were diluted in PEM buffer containing 10% glycerol, centrifuged on coverslips at 63,000g during 30 min and fixed in methanol at -20° for 6 min. Microtubules were processed for indirect immunofluorescence analysis using tubulin antibody TUB2.1 (Sigma-Aldrich). The microtubule length was measured with help of image analysis controlled by MetaMorph software (Universal Imaging Corp.). At least 200 microtubules were analyzed on each coverslip. To determine the microtubule number concentration, we derived the number of tubulin dimers in polymers per unit of volume from the molar concentration of polymeric tubulin and the Avogadro number. The microtubule number concentration was calculated as the ratio of the number of tubulin molecules per unit of volume divided by the average number of tubulin molecules per microtubule. This average number was calculated from the microtubule mean length, assuming 1625 tubulin dimers per µm of microtubule length. In our hands, microtubule length determination becomes unreliable when the length of the longest microtubules exceeds 100 µm (22), due to hardly avoidable breakage of longer microtubules during experimental manipulations. When microtubules are assembled from GTP-tub alone, microtubule elongation is independent of the initial GTP-tub concentration and constant during most of the assembly phase, with a rate of variation of the microtubule mean length of about 8 µm/min, corresponding to a predicted rate of individual microtubule elongation of 2x8=16 µm/min (22). Therefore, reliable microtubule length determinations should not be performed after 100/16=6 min.

Results indicated that the microtubule mean elongation rate was constant (8 µm/min) in the absence of GDP-tub (Figure S2, A), as previously observed (22). No detectable modification of the microtubule elongation rate was observed in the presence of added GDP-tub (Figure S2, A). The

corresponding microtubule nucleation rates remained in the same range at all tested GDP-tub concentrations (Figure S2, B).

Turbidimetry measurements- Microtubule assembly was followed by turbidimetry measurements in a spectrophotometer. For this, aliquots (100 µl) of different tubulin suspensions were prepared at 4°C in PEM buffer were transferred to thermostated cuvettes (35°C) and the change in absorbance at 350 nm was measured (Fig. S3).

Microtubule sedimentation assay- This assay was performed to estimate total polymeric tubulin concentration. GTP-tub or GTP-tub/GDP-tub mix were prepared in PEM buffer at 4°C, aliquoted and assembled as above. Assembly reactions were stopped by adding to samples 1 ml of PEM supplemented with 60% sucrose at 35°C (25)**.** Then samples were centrifuged at 100,000g during 1 hr at 35°C. Microtubule pellets were dissociated at 4°C in PEM and tubulin concentrations were determined by measuring absorbance at 280 nm, the extinction coefficient of tubulin being ε=115,000 M^{-1} cm^{-1} (Fig. S4).

Nucleotide exchange among tubulin variants- We assayed nucleotide exchange among tubulin molecules in either GTP-Detyr-tub and GDP-Tyr-tub mixtures or GTP-Tyr-tub and GDP-Detyr-tub mixtures. To do this, GTP-Detyr-tub (50 µM) was co-assembled with GDP-Tyr-tub (75 µM) and in parallel GTP-Tyr-tub (75 µM) was co-assembled with GDP-Detyr-tub (50 µM) during 15 min at 35°C. At the time of maximum assembly, microtubules were centrifuged (65,000g, 20 min, 35°C) on PEM cushions supplemented with 60% glycerol. Tubulin pellets were diluted in PEM, and then analyzed by SDS-PAGE and quantitative immunoblotting with primary anti-Detyr-tub or anti-tubulin antibodies followed by HRP-conjugated secondary antibody (Jackson Immunoresearch) and ECL (Pierce), or by ^{125}I-labeled secondary antibody (GE Healthcare) and phosphorimager detection. Blot analysis of the Detyr-tub content of microtubules showed that microtubules contained circa 60% Detyr-tub when assembled with initially GTP-Detyr-tub, compared to circa 25% Detyr-tub when assembled with initially GDP-Detyr-tub (Fig. 2A). These proportions are in good agreement with the ratio of GDP-tub/GTP-tub incorporation estimated from radioactivity measurements (Figure 1E). Note that if rapid nucleotide exchange occurred, the proportion of Detyr-tub in microtubules would amount to 50/(50+75) = 40%, whether Detyr-tub was initially carrying GTP or GDP. Thus, our data indicate that, within the time course of our experiments, nucleotide exchange among tubulin molecules is minimal.

Supplemental Figures

Fig. S1. NDP kinase activity. (A) GDP-tub (75 µM) was incubated at 35°C in PEM buffer in the presence of ATP (0.5 mM) (▬). As a positive control of microtubule assembly, GDP-tub (75 µM) was incubated with 1 mM GTP at 35°C (▬). Tubulin assembly blank level was assessed by incubating GDP-tub (75 µM) with 1 mM GTP in the presence of 10 µM nocodazole (●), a drug inhibiting microtubule assembly. Incubations were followed by turbidimetry at 350 nm. (B) GDP-tub (75 µM, 40 µl) was incubated with [γ–^{32}P]-ATP (10 µCi/µM) and ATP (0.5 mM) at 35°C (■) or with [γ–^{32}P]-GTP (10 µCi/µM) and GTP (0.5 mM) at 11°C (■), and the formation of [γ–^{32}P]-GTP-tub was measured at the indicated time points.

Fig. S2. Quantitative analysis of microtubule nucleation and elongation of microtubules assembled from GTP-tub/GDP-tub mixtures. GTP-tub (75 µM) was assembled in the absence (■) or in the presence of 37.5 µM (■), 75 µM (■), 120 µM (□) GDP-tub. (A) Plots of microtubule mean length versus time during assembly. (B) Plots of microtubule number concentration versus time: microtubule number concentrations were estimated from the total polymerized tubulin mass (Fig. 1A-D) and from the microtubule mean lengths (this figure, panel A) at the different time points during assembly phase. Insert, Microtubule nucleation rates versus (initial GDP-tub/initial GTP-tub) ratio: microtubule nucleation rates are given by the slopes of regression lines obtained from microtubule concentrations for each condition.

Fig. S3. Turbidity measurements. Different tubulin preparations were incubated at 35°C and suspension turbidity was followed at 350 nm: GDP-tub (120 µM) alone (▪); GTP-tub (75 µM) alone (▬) or in the presence of GDP-tub (37.5 µM (▬), 75 µM (▬), 120 µM (▬)).

Fig. S4. Microtubule sedimentation assay. GTP-tub (75 µM) was assembled in the absence (■) or in the presence of 37.5 µM (■), 75 µM (■), 120 µM (▫) GDP-tub concentrations. Microtubules were sedimented and polymerized tubulin concentrations in pellets were determined.

Figure S1

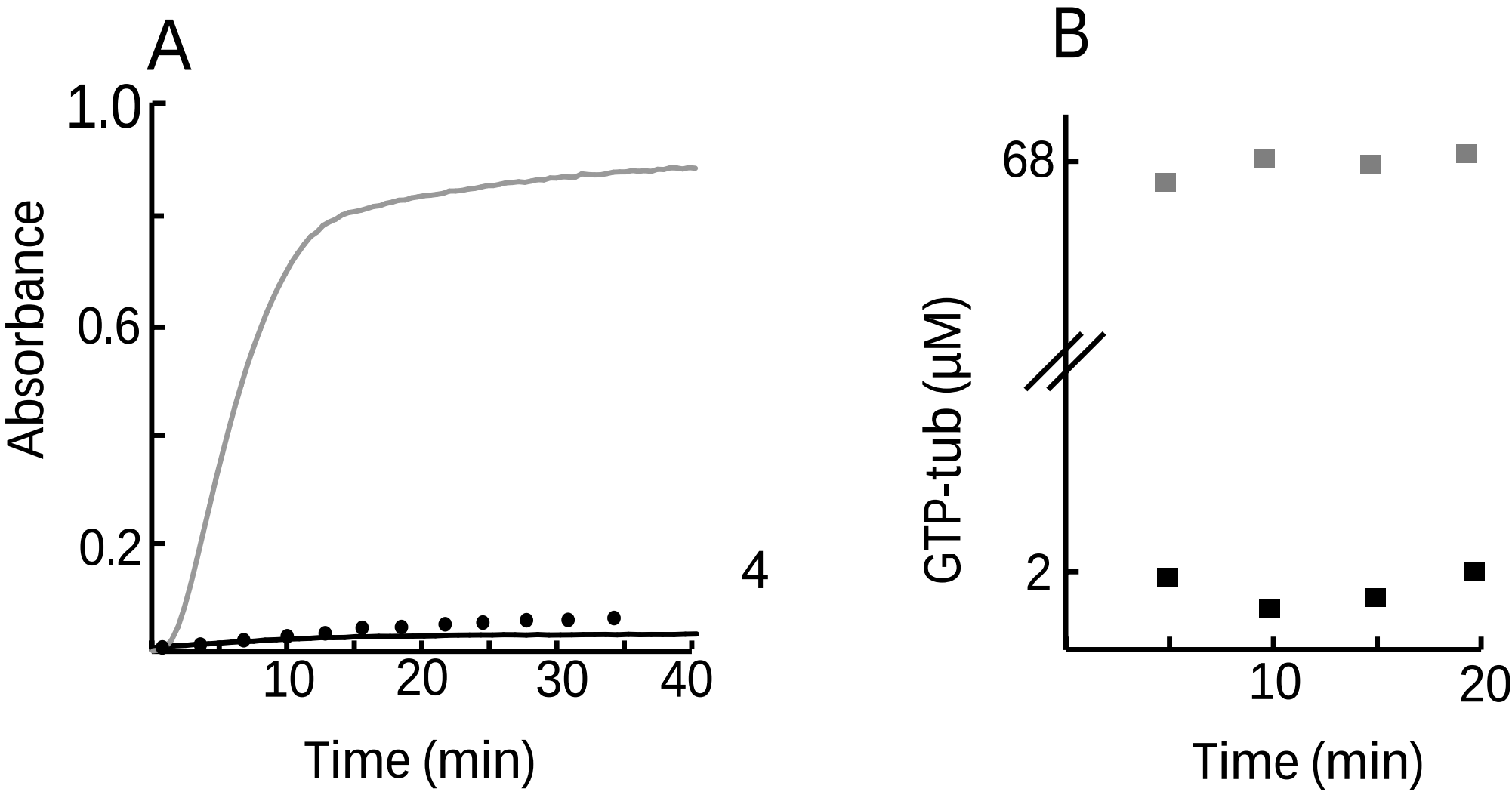
A
1.0
0.6
0.2
Absorbance
4
10
20
30
40
Time (min)
B
68
2
GTP-tub (μM)
10
20
Time (min)

Figure S2

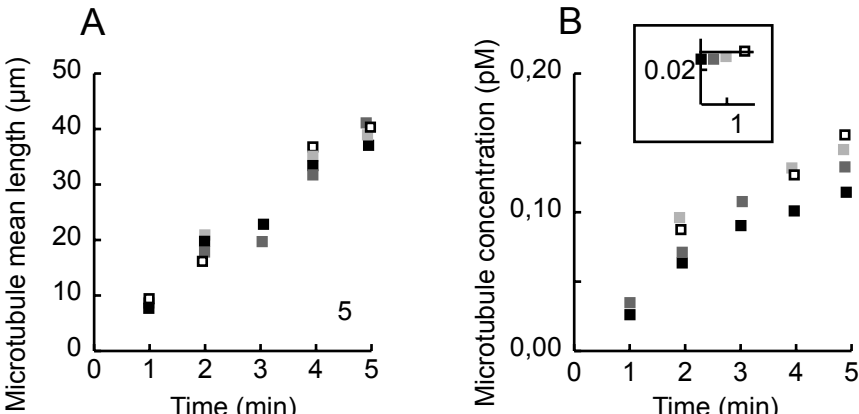
A
Microtubule mean length (µm)
50
40
30
20
10
0
0
1
2
3
4
5
5
Time (min)
B
Microtubule concentration (pM)
0,20
0,10
0,00
0.02
1
0
1
2
3
4
5
Time (min)

Figure S3

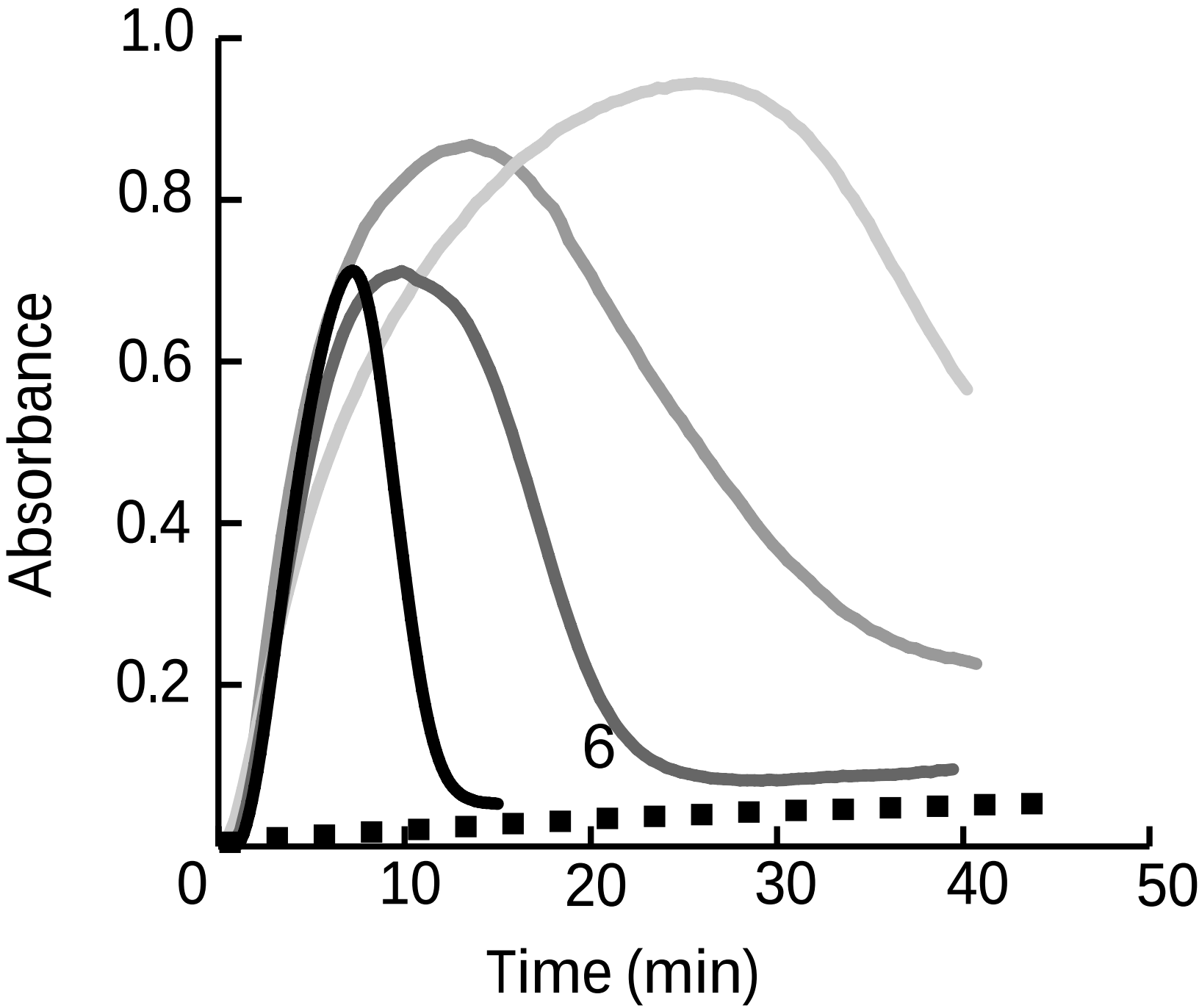

Figure S4