MICROTUBULE ASSOCIATED PROTEINS STABILIZE THE COLCHICINE BINDING ACTIVITY OF TUBULIN

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1. Introduction

The binding of the alkaloid colchicine to tubulin is highly specific and has therefore become a sensitive tool to localize and quantitate tubulin in subcellular fractions. Since the capacity of soluble tubulin to bind this drug decays relatively fast, though the binding reaction is relatively slow, for reliable quantitative estimates binding is measured at various time points and initial binding capacities are determined by extrapolation [1]. An additional parameter deducible from this type of experiment is the stability of the tubulin molecules assayed, because stability correlates directly to the rate of decay.

We have used this method for estimating the stability of tubulin purified from cultured rat glioma C-6 cells by spontaneous in vitro polymerization [2] and that of tubulin present in crude cell extracts of various Chinese hamster lung cell lines [3]. Since we found differences in the decay rates of tubulins extracted from non-transformed versus SV40-transformed cell lines [3] we initiated a search for cellular factors that directly affect the stability of tubulin. As a first approach we measured the decay rates of hog brain tubulin, in the presence and absence of proteins that copurify with tubulin in repeated cycles of in vitro polymerization [4,5]. We found that these components, collectively referred to as microtubule associated proteins, reduce the decay rate of tubulin.

2. Materials and methods

[^{3}H]Colchicine (7.6 Ci/mmol) was purchased from The Radiochemical Centre, Amersham; bovine serum albumin (A 7511) from Sigma Chemicals Co., St Louis, MO; and urease from Böhringer, Mannheim.

Abbreviations: MAP, microtubule associated proteins; PC-tubulin, phosphocellulose purified tubulin

2.1. *Preparation of microtubule protein, tubulin and MAP*

Microtubule protein was prepared from hog brain shortly after slaughter by in vitro polymerization/depolymerization cycles as in [6]. Twice cycled preparations were stored in 8 M glycerol at −20°C. For binding experiments, samples were polymerized an additional time, microtubule pellets gently resuspended in 25 mM sodium morpholinoethane sulfonate, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM GTP (pH 6.4) (solution A) and then incubated on ice for 45 min. Suspensions were then cleared by centrifugation at 100 000 × *g* for 30 min at 4°C, protein assayed as in [7], and samples immediately used for experiments.

To obtain preparations of tubulin and MAP, microtubule protein in solution A was chromatographed on phosphocellulose columns according to [4]. Tubulin eluting in the flow through (PC-tubulin) was used immediately for binding experiments after determination of protein [7]. MAP were eluted in solution A plus 0.8 M NaCl and 1 mM β-mercaptoethanol, subsequently desalted by passage through a Bio-Gel P-6 column equilibrated with solution A, and stored frozen. Alternatively, MAP were desalted by dialysis against 50 mM ammonium acetate, 0.1 mM $MgCl_2$, 0.2 mM EGTA, 10 mM β-mercaptoethanol (pH 6.4), then concentrated by lyophilization. Both preparations of MAP gave rise to similar results.

2.2. *Assay of colchicine-binding activity*

This assay was performed similarly as in [3]. In brief, assay mixtures (final vol. 150 μl) consisted of 2 parts 10 mM sodium phosphate, 5 mM $MgCl_2$, 0.1

mM GTP (pH 7.0); and 1 part protein sample in solution A. (In some cases as stated in the text, the addition of GTP was omitted.) These mixtures were preincubated for various times (0–4 h) at 37°C. At the end of each preincubation period 5 μl aqueous 0.186 mM [^{3}H]colchicine solution (2.16 Ci/mmol) was added to the samples to be tested for binding activity. Then an additional incubation was done for 1 h at 37°C and samples were analysed by DEAE–Sephadex chromatography. Assays were routinely run in triplicate and time points represent the average value. Decay curves were constructed by linear least square fit to these averaged points; at least 3 time points/experiment were taken.

3. Results

Hog brain microtubule proteins prepared by repeated cycles of in vitro polymerization/depolymerization consisted of ~80% tubulin, the rest were MAP ([8], and data not shown). By chromatography of such preparations on phosphocellulose columns [4] highly purified (MAP-free) tubulin preparations (PC-tubulin) were obtained. As shown in fig.1, the capacities of both preparations to bind colchicine decayed with time following the kinetics of first order reactions [1]. Furthermore, in both cases the rate of decay was dependent on GTP and protein concentration (fig.2). Increasing from ~0.05–0.4 mg protein/ml caused the half-lives of unfractionated microtubule protein (fig.2A) to increase by ~3 h, those of highly purified tubulin preparations (fig.2B) by ~2 h. This effect was observed in the absence (lower curves, fig.2A,B) as well as in the presence (upper curves, fig.2A,B) of GTP. The addition of 0.4 mM GTP at any protein concentration tested slowed down the decay rate of either tubulin preparation.

At >0.1 mg/ml and in the presence of 0.4 mM GTP unfractionated microtubule proteins decayed significantly slower than highly purified tubulin samples (compare the two upper curves in fig.2A,B). At the lowest protein concentration (0.05 mg/ml) tested under these conditions the $t_{1/2}$ measured were about the same. Under conditions where GTP was omitted from incubation mixtures (lower curves, fig.2A,B) a significant difference between the two tubulin preparations was observed only at the highest (0.45 mg/ml) protein concentration tested. Thus it appeared that at least in the range of the higher pro-

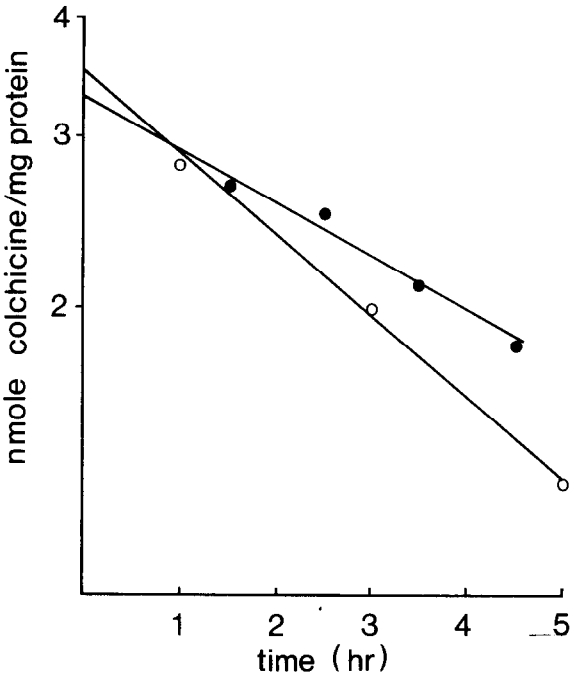

Fig.1. First-order decay of colchicine binding activities. 3 × cycled hog brain microtubule protein and PC-tubulin were prepared and assayed for decay of colchicine binding as in section 2. PC-tubulin was assayed in the absence of GTP. (●–●) 3 × cycled microtubule proteins, 0.15 mg/ml; (○–○) PC-tubulin, 0.4 mg/ml. The initial binding capacities and decay half-lives were 3.3 × 10^{-6} mmol colchicine/mg protein and 5.2 h for microtubule proteins; and 3.5 × 10^{-6} mmol colchicine/mg protein and 3.7 h for PC-tubulin, respectively.

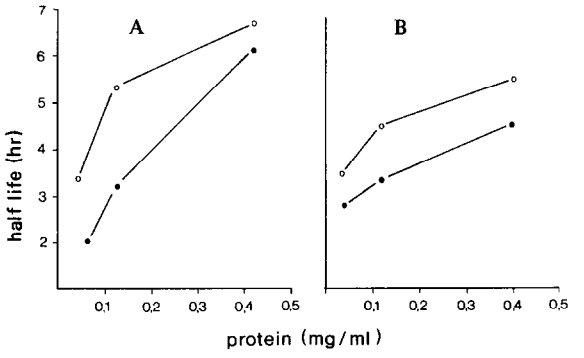

Fig.2. Effects of GTP and protein concentration on decay half-lives. Aliquots of microtubule protein (panel A) and PC-tubulin (panel B) were assayed in the presence (○–○) or absence (●–●) of 0.4 M GTP. Half-lives were determined as in the text.

Table 1
Reduction of decay rate by MAP

Experiment	Half-life (h)[a]
Microtubule protein (0.5 mg/ml)	5.78[b] ± 0.55[c]
PC-tubulin (0.4 mg/ml)	3.92[d] ± 0.45
PC-tubulin (0.4 mg/ml) + MAP (0.1 mg/ml)	5.85[e] ± 0.65

[a] Preincubations and colchicine binding assays were run as described in the text in the absence of GTP; [b] Average of 3 expt; [c] Standard deviation; [d] Average of 7 expt; [e] Average of 3 expt

tein concentrations tested, tubulin was more stable in the presence of MAP.

To establish more clearly the role of MAP as stabilizers of tubulin, MAP fractions were recovered from phosphocellulose columns, added back to PC-tubulin and the decay rates of these reconstituted mixtures assayed. The experiments were run under conditions where maximum differences in $t_{1/2}$ (~2 h) between unfractionated and highly purified tubulin preparations had been observed, i.e., at 4 mg protein/ml, and in the absence of GTP (fig.2). Furthermore, the MAP/tubulin ratio (1:5) was kept close to that of unfractionated microtubule protein preparations. As the results compiled from several experiments show (table 1), the addition of MAP to PC-tubulin fully restored the lower decay rate of unfractionated tubulin preparations. This stabilizing effect was apparently specific for MAP, because non-microtubule proteins, such as bovine serum albumin or urease did not cause an elevation of the decay rates (table 2, exp. 1,2). Interestingly, PC-tubulin was able to mimic the effect of MAPs (exp. 3), however only at a considerably higher concentration. The self-stabilization effect of PC-tubulin and the stabilizing effects of MAPs were totally suppressed by high salt (table 2, exp. 4).

Table 2
Specificity of MAP

Experiment[a]		Half-life (h)
1.	PC-tubulin[b]	4.2
	PC-tubulin + Bovine serum albumin (0.6)	4.0
2.	PC-tubulin	3.6
	PC-tubulin + Urease (0.6)	3.8
3.	PC-tubulin + MAPs (0.1)	5.5
	PC-tubulin + PC-tubulin (1.0)	5.6
4.	PC-tubulin	4.4
	PC-tubulin + 0.6 M NaCl	3.5
	PC-tubulin + MAPs (0.1)	5.5
	PC-tubulin + MAPs (0.1) + 0.6 M NaCl	4.1

[a] Decay of colchicine binding was assayed in all cases in the absence of GTP

[b] PC-tubulin was 0.4 mg/ml in all experiments except in exp. 3, line 2, where it totaled 1.4 mg/ml; the concentrations (mg/ml) of other protein components are given in parenthesis

4. Discussion

These data show that protein factors which copurify with tubulin in repeated cycles of in vitro polymerization, MAP, stabilize the tubulin molecule to some extent against decay of its colchicine binding capacity. Stabilization of colchicine binding by increasing concentration of tubulin itself was observed in some [1,9] although not in all cases [3,10] studied. However, this is the first report that polypeptides other than tubulin affect stability. Since the colchicine binding capacity is considered to be a correlate to the native state of the tubulin molecule our data support the conclusion that MAP decrease the denaturation rate of tubulin.

In principle we see two distinct possibilities of how MAP could exert their stabilizing effects.

(i) They interact directly with the colchicine binding sites of tubulin molecules and thereby protect them from decay.

(ii) MAP, by facilitating tubulin–tubulin interaction, induce the aggregation of tubulin dimers to oligomers/polymers whose colchicine binding sites are more resistant towards decay than those of free tubulin dimers.

The observation [11] that MAP and colchicine compete for the same binding site on tubulin would endorse the first possibility. Nevertheless we believe the latter possibility is the more likely one, because the observation of stabilization of phosphocellulose purified (MAP-free) tubulin at increasing concentrations of protein suggests that tubulin–tubulin interactions stabilize the colchicine binding capacity. Furthermore, MAP have been shown [12] to drastically reduce the (critical) concentration of tubulin necessary for polymerization, and it is assumed that they do so by favoring the tubulin–tubulin interactions. Consequently, the increased stability of MAP

containing tubulin preparations might be due to the favored formation of small tubulin aggregates during preincubation. The formation of larger, regularly formed polymers, such as rings, sheets or tubules in this period however, was not observed in the electron microscope (G. W., unpublished). Presumably, the lack of such structures was due to the buffer conditions which were suboptimal for tubulin polymerization. Assuming aggregation of tubulin to be responsible for stabilization, the observation of nearly identical initial binding capacities for MAP-containing and MAP-deficient tubulin preparations (fig.1), is in agreement with [10] where colchicine did not discriminate between different forms (dimers vs bigger aggregates) of tubulin. Likewise, colchicine bound to tubulin in ring form [13]. However, being non-exclusive, the two mechanisms proposed for stabilization of colchicine binding by MAP both might apply.

As reported [3], the colchicine binding activity of tubulin is more stable in extracts of SV40-transformed cells than in those of their non-transformed counterparts. On the basis of the above results one might speculate that these differences are due at least in part to different nature and/or quantities of MAP in the various cell extracts.

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