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Heat-stable microtubule protein MAP-1 binds to microtubules and induces microtubule assembly

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The microtubule-associated proteins, MAP-1, MAP-2 and tau, have been purified from brain tissue via a new approach using a heating step directly on the homogenate, followed by selective adsorption on calmodulin-Sepharose affinity columns and gel-filtration chromatography. Our results indicate that these MAPs share common biochemical properties, including heat stability, calmodulin binding and promotion of tubulin assembly into microtubules.

Microtubule-associated protein; Heat stability; Affinity purification; Microtubule assembly

1. INTRODUCTION

Microtubule-associated proteins (MAPs) appear to play major roles in inducing tubulin assembly and the stabilization of microtubule polymers [1–4]. MAPs have also been implicated in bridging microtubules to other cytoskeleton components, i.e. microfilaments and intermediate filaments [5,6]. Despite several studies pointing to the modulatory function of these proteins in the cell [7] very little is known about their structural characteristics.

MAPs isolated from mammalian brains contain high molecular mass components, namely MAP-1 (~300 kDa), MAP-2 (~270 kDa) and MAP-4 (215 kDa), and intermediate molecular mass proteins, the tau factor (60–66 kDa) [1]. MAP-1 and -2 [8] as well as MAP-4 [9] contain several isoforms as analyzed by high-resolution polyacrylamide gel electrophoresis. Methods for the purification of brain MAPs have some disadvantages in that they are relatively slow and depend on the co-polymerization of MAPs with tubulin [10] and the heat resistance capacity of MAP-2 and tau, but no MAP-1 [11] under specific boiling conditions. For most tissues and cellular systems from non-neuronal origins, attempts to purify MAPs were largely unsuccessful due to the small proportion of MAPs with respect to total proteins and because assembly of cytoplasmic microtubules does not proceed to a significant extent. However, MAPs have been identified in a wide variety of cell types [12]. Furthermore, there is increasing evidence that MAPs are tissue specific and therefore, different MAP components may interact selectively with tubulin subunits depending on their cell origin [4,12].

Here, we report a novel purification procedure based on a heating step of the cellular homogenates under conditions that confer heat stability to MAP-1 in addition to other MAP components, and chromatography on a calmodulin-Sepharose affinity matrix. Purified MAP-1, MAP-2 and tau exhibited the capacity to promote in vitro microtubule assembly.

2. MATERIAL AND METHODS

Tubulin from cow brains was prepared by in vitro cycles of assembly-disassembly followed by phosphocellulose chroma-

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tography to remove MAPs [13]. Total MAPs were obtained by a modification of the boiling procedure [10], in which brains were homogenized (2 ml/g tissue) in 0.05 M Mes buffer, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA in the presence of the protease inhibitors, aprotinin, leupeptin and soybean trypsin inhibitor at a final concentration of 10 μg/ml and 0.2 mM phenylmethylsulfonyl fluoride (buffer I) containing 1 M NaCl. The homogenate was boiled for 5 min in the presence of 10 mM dithiothreitol, and the supernatant obtained by centrifugation at 75000 × g was subjected to 50% ammonium sulfate precipitation. After exhaustive dialysis against 3 × 1 l buffer I without EGTA (buffer II), the MAP-containing sample was adjusted to 0.5 mM $CaCl_2$ and applied to a calmodulin-Sepharose affinity column (1.5 × 6 cm) equilibrated in buffer II containing 0.5 mM $CaCl_2$. The column was washed with 10 vols equilibrium buffer and the retained protein was eluted with 5 mM EGTA. Separation of purified MAP-1, MAP-2 and tau was attained by chromatography in a Sepharose CL-4B column (1.5 × 95 cm) in buffer I containing 0.5 M NaCl. Fractions containing each of the individual MAP components were pooled and concentrated in the Amicon system, using YM-30 filters. Microtubular proteins and MAP concentrations were determined turbidimetrically after 20% trichloroacetic precipitation, using appropriate standard curves. Tubulin concentration was determined spectrophotometrically using $A_{280}^{0.1\%} = 1.15$, tau concentration using $A_{275}^{0.1\%} = 0.28$ and MAP-2 by $A_{275}^{0.1\%} = 0.33$ [14].

MAP-induced tubulin assembly into microtubules was assayed by either the turbidimetric or sedimentation assay. The turbidimetric assay with appropriate blanks was carried out in a Beckman model 25 spectrophotometer. Tubulin in assembly buffer was admixed with either MAPs or purified MAP-1, MAP-2 or tau and allowed to assemble after the addition of 1 mM GTP. Samples were obtained during the assembly, fixed with glutaraldehyde, stained with 1% uranyl acetate and analyzed under the electron microscope. The sedimentation assay was carried out by centrifugation of the assembled polymer at 140000 × g in an air-driven ultracentrifuge [2]. SDS gel electrophoresis was performed on polyacrylamide slab gels using acrylamide gradients of 5–20%.

3. RESULTS AND DISCUSSION

The use of a heating step immediately after homogenization of brain tissue allowed us to obtain a supernatant containing heat-stable MAP-1, MAP-2 and tau, which conserved their activities to promote tubulin assembly into microtubules. The inclusion of the mixture of protease inhibitors in the homogenization step was critical in order to obtain high molecular mass MAPs essentially free of proteolytic products. The supernatant freed of heat-precipitable protein was applied to a calmodulin-Sepharose column equilibrated in buffer I in the presence of 0.5 mM Ca^{2+} to allow the interaction of MAPs with calmodulin. After elution of a major peak of unretained protein, a small peak of MAPs specifically bound to the affinity matrix was eluted with EGTA (fig.1). The protein removed from the calmodulin-affinity resin by EGTA contained 80% MAPs and 20% bound contaminants, including a 30 kDa protein which, in contrast to MAPs, did not bind to microtubules (fig.1). The use of this affinity method avoids the time-consuming cycling procedure to prepare MAPs [10]. In contrast to cycled MAPs, which are apparently non-phosphorylated in their 35 kDa binding domain [15], affinity-purified MAPs appear to contain both phosphorylated and dephosphorylated forms. These forms can be subjected to further separation on the basis of the decreased assembling capacity of the modified MAPs. In vivo phosphorylation of MAPs occurs mainly in the projection domains, which does not affect MAP-2 assembly-promoting activity [15]. A striking characteristic of MAP-2 and tau is their binding to calmodulin [1,4]. This affinity chromatography experiment shows that MAP-1 obtained after the heating step has capacity to bind to calmodulin.

The affinity-purified MAP preparation was

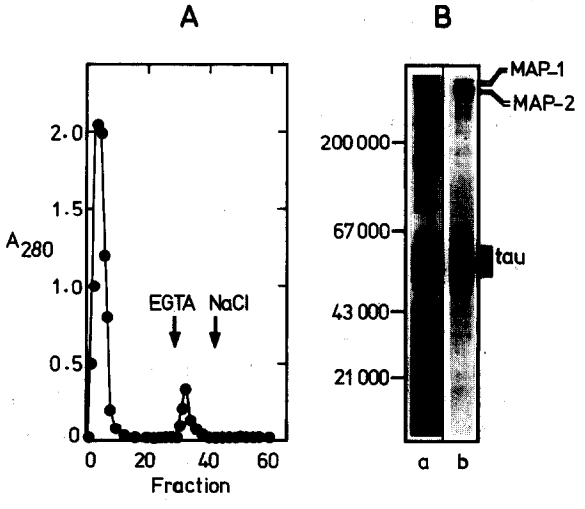

Fig.1. (A) Affinity chromatography. The supernatant obtained after centrifugation of the heated brain homogenate was applied to the affinity column and aliquots of 0.5 ml containing the unretained protein were collected. Bound protein was eluted with 5 mM EGTA in buffer I. (B) Electrophoretic analysis of the input (a) and (b) the pool of fractions eluted with EGTA. Gels were stained with Coomassie blue [21]. Arrows indicate the positions of MAP-1, MAP-2 and tau. The migration of molecular mass markers is also indicated.

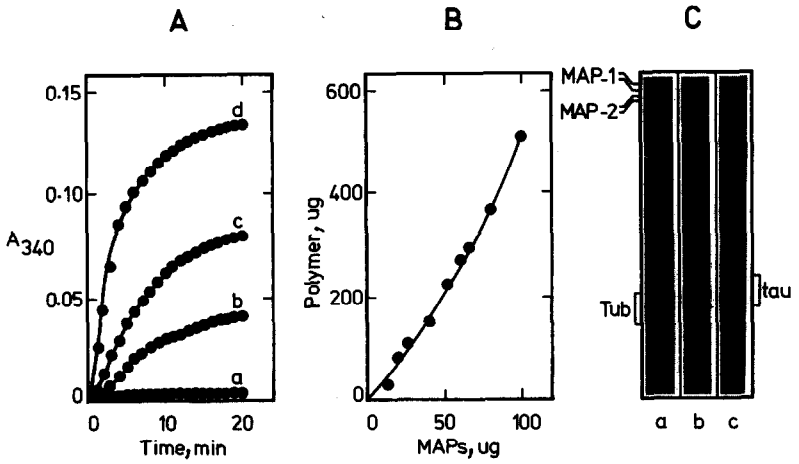

Fig.2. (A) Turbidimetric assay of tubulin (1 mg/ml) assembly induced by affinity-purified MAPs at a final concentration of 50 μg/ml (b), 100 μg/ml (c) and 150 μg/ml (d). Control of tubulin assayed without MAPs is shown in (a). (B) Sedimentation assay of tubulin assembly induced by increasing concentrations of affinity-purified MAPs. (C) Electrophoretic analysis of the supernatant (a) and the pellet of microtubule polymer (b) after assembly. A different microtubular pellet was resuspended in buffer 0.1 M Mes, pH 6.8, brought to 2.5% perchloric acid, and the supernatant containing tau, analyzed by electrophoresis (c).

assessed with respect to its activity to promote tubulin reassembly. Both the rate and extent of assembly increased as total MAP concentration in the assay increased (fig.2A). Cold reversibility and electron microscopy showed that the assembly products were normal microtubules (not shown). The MAP dependence of tubulin assembly into microtubules was further quantitated over a broader range of MAP concentrations using the sedimentation procedure (fig.2B). MAP-1 and MAP-2 were incorporated into pelleted microtubules as analyzed by polyacrylamide gel electrophoresis (fig.2C, lane b), whereas only a small fraction of MAP-1 and MAP-2, and non-assem-

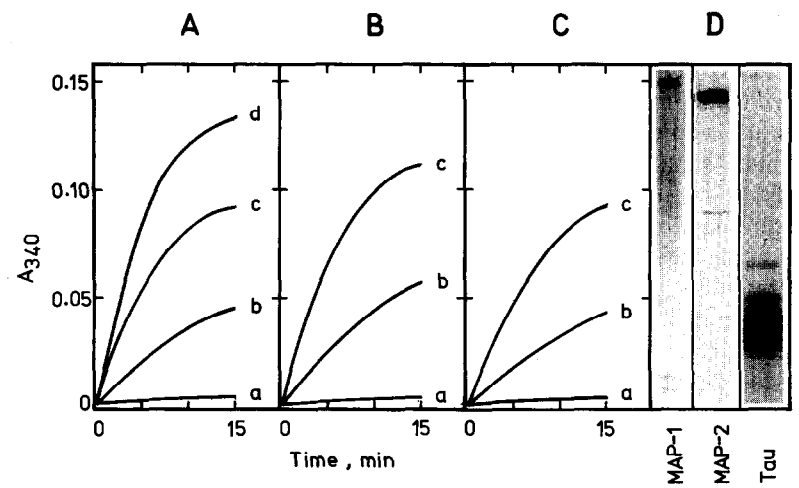

Fig.3. Time course of microtubule polymerization induced by purified tau (A), purified MAP-2 (B) and purified MAP-1 (C). Tubulin concentration was 1 mg/ml. (A) Tau concentrations (mg/ml): (a) 0, (b) 0.06, (c) 0.15, (d) 0.30. (B) MAP-2 concentrations (mg/ml): (a), 0, (b) 0.12, (c) 0.24. (C) MAP-1 concentrations (mg/ml): (a) 0, (b) 0.10, (c) 0.20. (D) Electrophoretic gels of the separated MAP components used in the experiment.

bled tubulin, remained in the supernatant (fig.2C, lane a). Perchloric acid precipitation of the microtubule pellet, resuspended in buffer I, released tau to the supernatant, indicating that tau was incorporated originally into the microtubule pellet (fig.2C, lane c).

In order to assess the assembly-promoting activity of the separate MAP components, we subjected the affinity-purified MAPs to further purification in a Sepharose CL-4B column. Pooled fractions containing either purified MAP-1, MAP-2 or tau were concentrated and assayed in the assembly system (fig.3). All three MAP components induced tubulin assembly in a cold-reversible fashion, with formation of microtubules. This purification approach has proven to be very useful for isolating MAPs from non-neural sources including liver and lung tissue and teratocarcinoma cells [16].

4. CONCLUSIONS

(i) The present purification protocol allowed us to obtain MAP-1, which binds to calmodulin, and with activity to promote tubulin assembly into microtubules. Most interestingly, MAP-1 appears to be a heat-stable protein. A previous classification of MAP-1 as a heat-labile protein [1] was based largely on the purification conditions and, probably, the use of the cycling procedure to obtain MAPs [10]. Our studies agree with those indicating that heat stability of MAP-1 depends on the purification procedure [17].

(ii) MAP-2 obtained by affinity chromatography directly after the heating step of the homogenates also maintained its capacity to promote microtubule assembly, in contrast to observations indicating that a cycling step was required before heating, in order to conserve MAP-2 capacity to induce microtubule assembly [18].

(iii) The data indicate that these MAPs share common biochemical properties such as heat stability, binding to calmodulin, and induction of microtubule assembly. In addition, studies on tubulin-MAP interactions [19] and our findings using synthetic tubulin peptides [20] strongly indicate that MAP-1, MAP-2 and tau have a common binding domain on tubulin. Research on mapping the binding sites of these MAP components should provide further insights on the structural similarities of tubulin binding domains in these polypeptides and their interaction with microtubules.

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