The 'assembly-promoting sequence region' of microtubule-associated protein 4 failed to promote microtubule assembly

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Abstract In order to study the function of the bovine MAP4 microtubule-binding domain (the assembly-promoting (AP) sequence region), a fragment corresponding to the AP sequence region was prepared using an *Escherichia coli* expression system. When the fragment was mixed with purified tubulin at 37°C, the fragment caused a time- and dose-dependent turbidity increase, and the fragment bound to tubulin. However, the products were cold-stable, and amorphous aggregates were observed by electron microscopy. Using axonemes as the seeds for microtubule assembly, the microtubule-elongating activity of the fragment was examined. A dose-dependent turbidity increase of the sample was observed, and electron microscopic observation revealed that microtubules were dose-dependently elongated from the axonemes. Consequently, the AP sequence region does not nucleate microtubules, but elongates them.

Key words: Microtubule; Microtubule-associated protein 4; Tau; Microtubule-associated protein 2

1. Introduction

Microtubule-associated proteins (MAPs) are accessory proteins of microtubules. They are associated with microtubules both in vivo and in vitro, and are proposed to play diverse roles in microtubule-dependent cellular functions. Four classes of MAPs (MAP1, MAP2, τ, and MAP4) have been purified and biochemically characterized [1–4]. The ubiquitous distribution of MAP4 suggests that this MAP contributes to microtubule-dependent fundamental cellular processes.

MAP4 is a polar molecule consisting of an amino-terminal domain and a carboxy-terminal domain [5]. The carboxy-terminal domain is further divided into three subdomains: a region rich in proline and basic residues (Pro-rich region), a region containing four repeats of an assembly-promoting (AP) sequence [6], which consists of 22 amino acid residues (AP sequence region), and a hydrophobic tail region (tail region). The polar structure of MAP4 is common to other MAPs (MAP2 and τ) [5,7,8]. The three MAPs share a homologous AP sequence region [5,9,10], which is considered to be requisite for the MAP-microtubule interaction in vitro and in vivo [6,7,11–16]. MAP2 and τ share a homologous tail region [10], but the primary structure of the tail region of MAP4 is not similar to those of MAP2 and τ [5]. It has been suggested that the tail regions of MAP2 and τ participate in microtubule bundling [15], yet the function of the tail region of MAP4 has not been investigated.

The AP sequence region is reported to play a leading role in the promotion of MAP-induced microtubule assembly [5–8,10–13]. We and other investigators have reported that synthetic peptides corresponding to an AP sequence have microtubule-binding and/or assembly-promoting activity [6,7,12]. Studies of the function of recombinant polypeptides consisting mainly of the AP sequence region of τ in vitro [14,17], and MAP4 in vivo [16] have also been published. However, in vitro analyses of a MAP4 fragment containing only the AP sequence region have not yet been performed.

To study the function of the AP sequence region per se of MAP4, we expressed and purified a fragment corresponding to the AP sequence region without the tail region. We tested the new fragment for an activity to nucleate free microtubules, and for an activity to elongate microtubules nucleated by axonemes. This is the first report on the function of the MAP4 AP sequence region in vitro.

2. Materials and methods

2.1. Materials

All materials not specifically mentioned were of reagent grade.

2.2. Protein preparations

Bovine brain tubulin from two-cycled microtubule protein fractions [18] was prepared by the method of Weingarten et al. [19]. MAP4 was purified from bovine adrenal cortex according to the method of Murofushi et al. [4]. Axonemes were prepared from the spermatozoa of *Hemicentrotus pulcherrimus* as described [20].

2.3. Construction of expression plasmid for MAP4 fragment

To construct an expression plasmid pA_4' for the A_4' fragment, we truncated the pA_4 gene [11], encoding the AP sequence region and the tail region of MAP4, with *Apa*I and *Sac*I to remove the 3′-terminal side of the insert, and a synthetic oligonucleotide with a termination codon was inserted into the restriction site. The resultant plasmid encodes amino acid residues 870–1016 of MAP4 with an additional 13 residues (MTMITPSLHACRS) on the amino-terminus. The pA_4 for the A_4 fragment, encoding amino acid residues 870–1072, was constructed as described [11].

2.4. Purification of MAP4 fragments expressed in bacterial cells

Unless otherwise mentioned, preparations were done at 0–4°C. The A_4' fragment was expressed in *Escherichia coli* MM294 cells carrying the recombinant plasmids. Bacterial cells were disrupted in a buffer containing 10 mM Tris-HCl (pH 7.5) and 2 mM EDTA supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and the cellular debris was removed by centrifugation at 20 000×*g* for 30 min. The supernatants were collected, heat-treated at 95°C for 10 min, and

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Abbreviations: MAP, microtubule-associated protein; AP, assembly-promoting; PMSF, phenylmethylsulfonyl fluoride; MES, 2-morpholinoethanesulfonic acid; RB, reassembly buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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centrifuged at 20000×*g* for 30 min. The A_4' fragment was purified from the heat-stable supernatant by phosphocellulose column chromatography and gel filtration chromatography (Sephadex G-100, Pharmacia Biotech, Uppsala, Sweden). The A_4 fragment was purified as described [11].

2.5. Assay of microtubule assembly

Tubulin and the MAP4 fragment were mixed at 0°C in reassembly buffer (RB; 100 mM MES (pH 6.8), 0.1 mM EGTA, and 0.5 mM $MgSO_4$) containing 0.5 mM GTP. Microtubule assembly was initiated by raising the temperature from 0°C to 37°C. Polymerization was monitored by the change in absorbance at 350 nm using a Hitachi U-2000A spectrophotometer, as described [6]. The axonemes used as the seeds for microtubule assembly were sheared by 20 passes through a 1.9-cm 27-gauge needle just before use.

2.6. Electron microscopy

For thin section observations, the samples were centrifuged at 16000×*g* for 30 min at 37°C, the pellets were fixed with 2.5% glutaraldehyde, 3% formaldehyde, and 1% tannic acid in 100 mM cacodylate buffer (pH 7.4) for 1 h at 37°C, and then postfixed with 1% OsO_4 dissolved in the same buffer for 1 h. After dehydration with a graded ethanol series, the pellets were embedded in Poly/Bed 812 mixtures (Polysciences, Inc., Pennsylvania, USA). Ultra-thin sections were cut on a Leica Super Nova ultra-microtome and were stained with uranyl acetate and lead citrate. For negative staining, samples were mounted on Formvar- and carbon-coated grids, fixed by 2.5% glutaraldehyde in RB prewarmed at 37°C, and then negatively stained with 2% uranyl acetate. Observations were performed with a Jeol JEM-1200EX electron microscope operating at 80 kV.

2.7. Other methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [21] using 15% polyacrylamide gels. Protein concentrations were determined by the method of Lowry et al. [22] using bovine serum albumin as the standard.

3. Results

3.1. Purification of the A_4' fragment expressed in E. coli

We constructed a recombinant plasmid (pA_4') encoding the A_4' fragment as described in Section 2.3. The primary structures of the intact MAP4, the A_4 fragment, and the A_4' fragment are illustrated in Fig. 1a.

Heat treatment allowed the removal of more than 80% of the proteins in the *E. coli* crude extract (Fig. 1b, lane 1). When the heat-stable fraction was chromatographed on a phosphocellulose column, the A_4' fragment was eluted with ~0.5 M NaCl (Fig. 1b, lane 2). The phosphocellulose fraction was subjected to gel filtration chromatography, and the A_4' fragment was purified almost to homogeneity (Fig. 1b, lane 3, arrowhead). The major band below the A_4' fragment band (Fig. 1b, lane 3, dot) is a proteolytic fragment, since the amount of the band increased with the passage of time. PMSF could not completely stop the degradation.

3.2. Assembly-promoting activity of the A_4' fragment

When the A_4' fragment was mixed with tubulin at 37°C, the turbidity at 350 nm increased in a time-dependent manner (Fig. 2a). Increasing the A_4' fragment concentration led to a linear increase in the turbidity (Fig. 2b). The minimal concentration of the A_4' fragment required for the polymerization of 15 μM tubulin was 9 μM (Fig. 2b). On the other hand, an increase in turbidity was induced by no more than 6.3 μM of intact MAP4 in the presence of 15 μM tubulin (Fig. 2a, broken line), which is consistent with our previous report which showed that normal tubulin polymerization was promoted by intact MAP4 with a concentration higher than 0.5 μM [11].

Compared with intact MAP4 (Fig. 2a, broken line), an abnormal 5-fold increase in turbidity was observed in the presence of the A_4' fragment (Fig. 2a). After a 10 min incubation at 37°C, the reaction mixtures were incubated at 0°C for 5 min. However, the turbidity levels were unchanged, indicating that the products were cold-stable (data not shown). Electron microscopic observation revealed that the products constructed from tubulin and the A_4' fragment were amorphous aggregates (Fig. 2c). No microtubules were observed. On the other hand, the products induced by intact MAP4 (Fig. 2d) and by the A_4 fragment (Fig. 2e) were normal microtubules. And they were cold-labile (data not shown). The results indicate that the A_4' fragment stimulates the assembly of tubulin into cold-stable amorphous aggregates.

3.3. Microtubule elongation activity of the A_4' fragment

Next, the axoneme-mediated microtubule elongation assay was done to assess the effect of the A_4' fragment on the

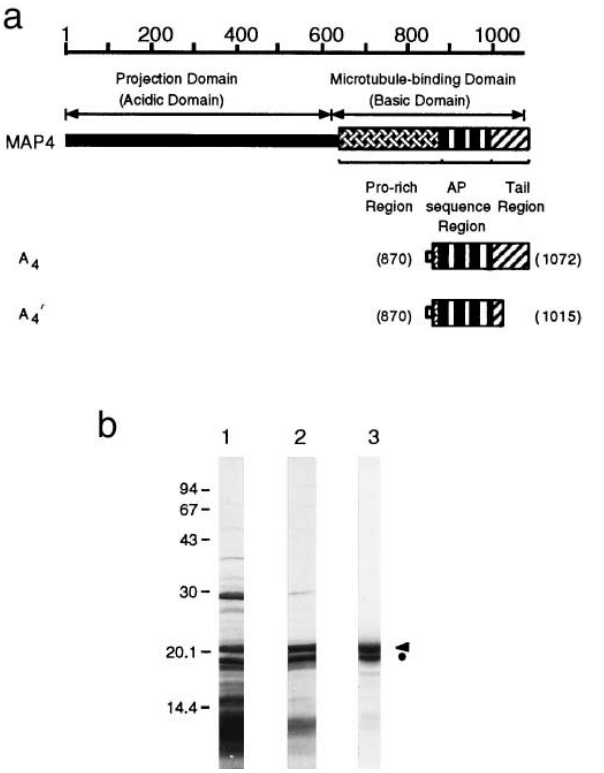

Fig. 1. Schematic diagram of truncated MAP4 fragments expressed in *E. coli* and SDS-PAGE of the A_4' fragment. a: Schematic structures of MAP4 (residues 1–1072), the A_4 fragment (residues 870–1072), and the A_4' fragment (residues 870–1015) are presented. Additional sequences derived from the *lac'*Z gene and the polylinker site of pUC are indicated by open thin boxes. The residue numbers are presented at the top. b: Purification of the A_4' fragment expressed in *E. coli*. Lane 1, heat-stable fraction prepared from bacterial cells; lane 2, phosphocellulose column fractions; lane 3, gel filtration column fraction. Arrowheads indicate the A_4' fragment, and the dot indicates the putative proteolytic product of the A_4' fragment. The relative molecular masses of the markers are indicated in kDa on the left side of the figure.

elongation of microtubules. When purified tubulin (10 μM) and axonemes were incubated in the absence of the A_4' fragment, only a slight increase in turbidity was observed (Fig. 3a, curve 1), and the microtubules were slightly elongated from the axonemes, as observed by electron microscopy (Fig. 3b). As the concentration of the A_4' fragment was increased, a larger increase in turbidity was observed (Fig. 3a). Electron microscopic observation revealed that longer microtubules were elongated from the axonemes (Fig. 3c) as compared with those prepared without the A_4' fragment, indicating that the A_4' fragment has an activity to stimulate microtubule elongation.

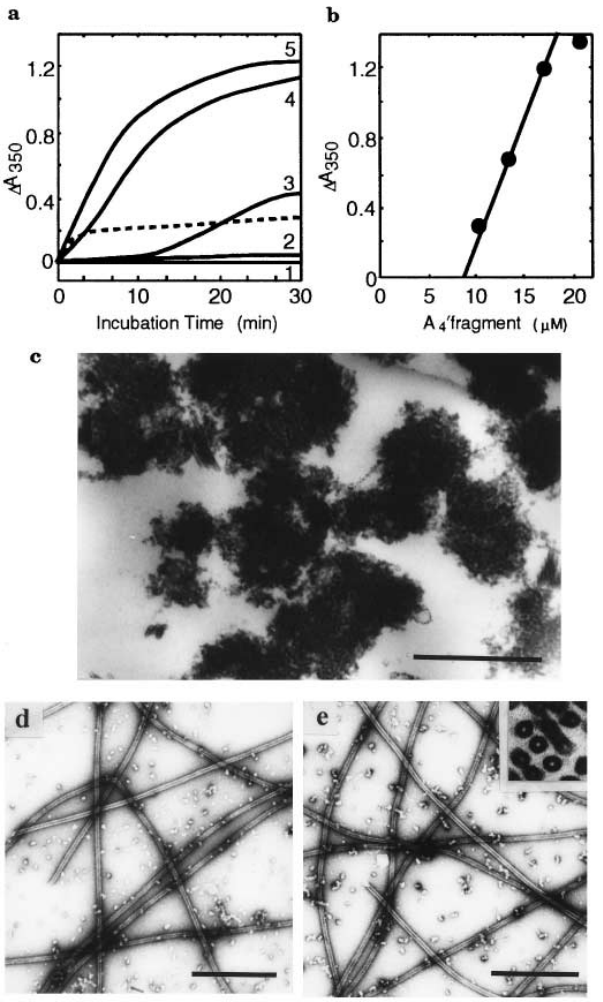

Fig. 2. Assembly-promoting activity of the A_4' fragment. a: Tubulin (15 μM) was mixed with 6.3 μM of intact MAP4 (broken line) or the A_4' fragment; 0 μM (curve 1), 10.1 μM (curve 2), 13.5 μM (curve 3), 16.9 μM (curve 4), 20.3 μM (curve 5). b: The turbidities at 50 min are plotted against the concentrations of the A_4' fragment. c: Electron microscopy of the products reconstituted from tubulin (15.0 μM) and the A_4' fragment (16.9 μM). d: Electron microscopy of the products reconstituted from tubulin (15.0 μM) and intact MAP4 (3.72 μM). e: Electron microscopy of the products reconstituted from tubulin (15.0 μM) and the A_4 fragment (20 μM). The samples were mixed in RB containing 0.5 mM GTP, and incubated at 37°C for 30 min. The samples in c and e (inset) were fixed, embedded, and sectioned, and those in d and e were negatively stained. The bar indicates 500 nm.

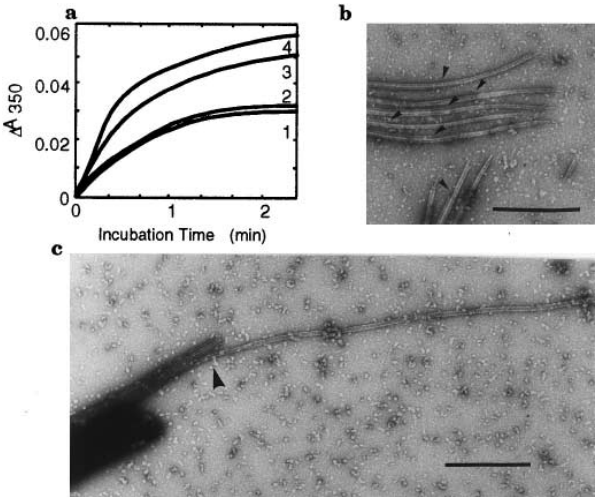

Fig. 3. Elongation-promoting activity of the A_4' fragment. a: Tubulin (10 μM) was mixed with axonemes, and then the A_4' fragment [0 μM (curve 1), 3.4 μM (curve 2), 6.8 μM (curve 3), 10.1 μM (curve 4)] was added to the tubulin-axoneme solution. b, c: Electron microscopy of microtubules elongated from axonemes (negative staining). Tubulin and axonemes were mixed with the A_4' fragment (b, 0 μM; c, 10.1 μM), and then incubated in RB containing 0.5 mM GTP at 37°C for 10 min. Arrowheads indicate the junctional positions of axonemal (nucleus) and elongated microtubules. Bars indicate 500 nm.

4. Discussion

4.1. Activity of the A_4' fragment

The A_4' fragment caused a time-dependent and dose-dependent turbidity increase, which was also observed in the case of intact MAP4 (Fig. 2a) [11]. However, a 5-fold increase in turbidity was observed, and the products were cold-stable, suggesting that the products were morphologically and functionally distinct from microtubules. This is consistent with the electron microscopic observation, which demonstrated that the products were entirely amorphous aggregates. The aggregates consist of tubulin and the A_4' fragment, as revealed by co-sedimentation analysis (data not shown). In the presence of axonemes, the A_4' fragment promoted elongation from the axonemes without making aggregates. Regardless of the presence of multiple AP sequence repeats, the function of the A_4' fragment is qualitatively similar to that of the tricosapeptide containing only one AP sequence [6]: the A_4' fragment and the peptide can elongate but cannot nucleate microtubules [6].

4.2. Tentative role for the tail region

The tail regions of MAP2 and τ construct cross-bridges, which link microtubules together in vivo [15], while no cross-bridges were detected in microtubules reconstituted from tubulin and the A_4 fragment (containing the AP sequence region and the tail region) of MAP4 [11]. The function of the MAP4 tail region is not clear at present. MAP2 and τ share a homologous tail region [10], but the primary structure and the amino acid composition of the tail region of MAP4 are different from those of MAP2 and τ [5] (Table 1). Basic residues account for 4.5% of MAP4 tail region residues, which is only a third of those percentages for MAP2 and τ (14.5%, Table 1). The MAP4 tail region may protrude from the surface of acidic microtubules.

Table 1
Amino acid composition of the tail region

Amino acid	MAP4[a] (1004–1072)	MAP2[b] (1759–1828)	τ[c] (379–448)
Basic residues			
Lys	1.5	4.3	7.1
His	1.5	2.9	4.3
Arg	1.5	7.1	2.9
Subtotal	4.5	14.3	14.3
Acidic residues			
Asp	2.9	4.3	7.1
Glu	11.6	5.7	5.7
Subtotal	14.5	10.0	12.8
Nonpolar residues			
Gly	17.4	4.3	5.7
Ala	17.4	12.9	10.0
Val	0.0	5.7	8.6
Met	0.0	0.0	1.4
Ile	2.9	4.3	2.9
Leu	8.7	11.4	8.6
Phe	0.0	1.4	1.4
Trp	1.5	0.0	0.0
Cys	1.5	0.0	0.0
Pro	11.6	4.3	4.3
Subtotal	61.0	44.3	42.9
Uncharged polar residues			
Asn	0.0	4.3	2.9
Thr	5.8	4.3	8.6
Ser	8.7	17.1	14.3
Gln	5.8	5.7	2.9
Tyr	0.0	0.0	1.4
Subtotal	20.3	31.4	30.1

[a]Calculated from the MAP4 sequence [5].
[b]Calculated from the MAP2 sequence [10].
[c]Calculated from the τ sequence [8].

We can deduce the function of the tail region of MAP4 from the function of the A_4' fragment. Microtubule assembly consists of nucleation and elongation steps, with MAPs promoting both. The A_4 fragment promotes both steps [11], but the A_4' fragment only promotes microtubule elongation, suggesting that the tail region is indispensable for the nucleation step. It might either contribute to the binding specificity of the AP sequence region, or be required for the proper folding of the peptide. Since MAPs are known to be extended molecules that lack tight folding [23], the former possibility is more likely.

4.3. Conclusion

We constructed a fragment consisting mainly of the AP sequence region (the A_4' fragment), and analyzed the function of the A_4' fragment. The size of the A_4' fragment is small enough for further physicochemical analyses of this region. Moreover, our data provide the first suggestion that the tail region has an essential function in MAPs to nucleate microtubules.

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