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Functional Analysis of Microtubule-binding Domain of Bovine MAP4

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ABSTRACT. **Bovine microtubule-associated protein 4 (MAP4) consists of an amino-terminal projection domain and a carboxyl-terminal microtubule-binding domain. The carboxyl-terminal domain of MAP4 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, which consists of 22 amino acid residues (AP sequence region), and a hydrophobic tail region (Tail region). The subdomain structure of MAP4 microtubule binding domain is similar to those of other MAPs (MAP2 and τ).**

In order to study the function of each subdomain *per se* of bovine MAP4 microtubule-binding domain, we purified a series of truncated fragments of MAP4, expressed in *Escherichia coli*. Binding affinity of the PA_4T fragment (containing the Pro-rich region, the AP sequence region and the Tail region) is only four times higher than that of the A_4T fragment (containing the AP sequence region and the Tail region), while the microtubule nucleating activity of the PA_4T fragment is far greater. We propose that the Pro-rich region promotes the nucleation of microtubule assembly.

The A_4 fragment (corresponding to the AP sequence region) stimulated the assembly of tubulin into cold-stable amorphous aggregates. The AP sequence region of MAP4 failed to promote microtubule assembly. On the other hand, the fragment has an activity to stimulate microtubule elongation.

The function of the MAP4 Tail region is not clear at present. The A_4T fragment (containing the AP sequence region and the Tail region) promote both microtubule nucleation and elongation step, but the A_4 fragment only promotes microtubule elongation, suggesting that the Tail region is indispensable for the nucleation step. However, the fragment containing only the Tail region could not bind to microtubule. Although MAP4 was considered to be long, thin and flexible molecule, never the Tail region may contribute to be the proper folding of MAP4, and/or may interact with other molecules.

We concluded that both the Pro-rich region and the AP sequence region take part in the promotion of tubulin polymerization, and that the former is important for the lateral protofilament-protofilament interaction, and the latter is important for the longitudinal affinity between each tubulin dimer in a protofilament.

Key words: MAP4/microtubule/microtubule assembly

Microtubule is a highly dynamic structure that reorganizes continuously as the cell changes shape, divides, and responds to its environment. In eukaryotic cells, microtubules play a role in diverse cellular functions in all over the cytoplasmic regions such as mitosis, intracellular transport, and determination of cell shape (23). Many microtubule-dependent cellular processes include microtubule depolymerization and repolimerization as main-events (30, 54). As one might expect from the diverse and dynamic functions of microtubules, various kinds of accessory proteins exist, which confer specific role and/or dynamic property on microtubules. The putative roles of accessory protein include linking filaments to one another or to other cell components, regulating the rate and extent of tubulin polymerization, and regulating their stability against disassembly.

Actually, microtubules prepared by repeated cycles

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of assembly-disassembly contain several non-tubulin components, so called microtubule-associated proteins (MAPs). MAPs promote tubulin polymerization and stabilize microtubules *in vitro*, thus MAPs are believed to regulate microtubule dynamics *in vivo* (7, 33, 43, 49). MAPs are classified into two groups, neural MAPs and non-neural MAPs. Three classes of neural MAPs (MAP1, MAP2, and τ) were purified from mammalian brain and characterized well (19, 20, 35, 37, 38). These MAPs are expressed almost exclusively in the brain; τ is present only in axons (10), while MAP2 in dendrites and the cell body (9, 46), suggesting that these MAPs contribute axon- or dendrite-, and cell body-specific function to microtubules (8, 14, 15, 18). On the other hand, many types of mammalian organs or cultured cells contain a non-neural MAP with relative molecular weight of ~200,000 on SDS-PAGE (12, 36, 48, 53, 56). These 200-kDa MAPs were generitically termed MAP4 (36). The distribution of MAP4 is ubiquitous (4, 36), suggesting that this MAP supplies microtubules with microtubule-dependent fundamental cellular processes, and not the cell type specific function: one of the plausible role for MAP4 is to control microtubules dynamics.

Microtubule assembly consists of nucleation and elongation step (45), with MAPs promoting both. MAPs speed up the nucleation step of tubulin polymerization *in vitro*, and thus, lower the critical concentration of tubulin polymerization. Moreover, MAPs stabilize the microtubules once they have formed (22, 40, 50). It is important to analyze the function of a MAP to promote microtubule assembly *in vitro*. We have purified MAP4 from bovine adrenal cortex (48), and have been characterized it biochemically (1, 3–5, 34, 48, 55). We have also prepared several truncated fragments of MAP4 (5), and have measured their microtubule-binding activity, and nucleation-/, elongation-promoting activity (5, 34, 55). Here, we review the mechanism of MAP4-induced microtubule assembly in term of its domain structure.

Structure of MAP4 and other MAPs (MAP2 and τ)

Electron microscopic observation of microtubules reconstituted from purified tubulin and MAP4 revealed the presence of lateral projections extending from the microtubule surface (48), while microtubules reconstituted from tubulin and the proteolytic fragment of MAP4 had no lateral projections, although the fragment could promoted tubulin polymerization (1). It is considered that MAP4 consists of two domains; a microtubule-binding domain and a projection domain.

A cDNA of bovine MAP4 was cloned, and the complete amino acid sequence of MAP4 was determined (4). MAP4 is a bipolar molecule consisting of an amino-termina acidic domain and a carboxy-terminal basic domain (4, 17, 57). The polar nature of the MAP4 molecule is similar to other MAPs, MAP2 and τ (4, 16, 31). The microtubule-binding proteolytic fragments of the three MAPs, can promote tubulin polymerization, and correspond to a part of the carboxy-terminal basic domain of each MAP (1, 2). Consequently, the fundamental architecture of a MAP molecule can be illustrated as in Figure 1a: a projection domain in their amino-terminal acid domain is followed by a microtubule-binding domain in their carboxy-terminal basic domain.

The projection domain of MAP4 shows no significant sequence homology with those of τ, and MAP2, while, the microtubule-binding domains of the these MAPs contains the homologous region (4). The MAP4 projection domain has a unique sequence without significant homology with those of other proteins ever sequenced and contains a specific structure with $18^{1}/_{2}$ repeats of a unit sequence of 14 residues. Because the amino acid sequences of the projection domains of MAP4, τ, and MAP2 are different from each other, each projection domain may play a specific role, such as the interaction with its own intracellular counterparts, or the regulation of MAP activity though chemical modifications. The microtubule-binding domain is fur-

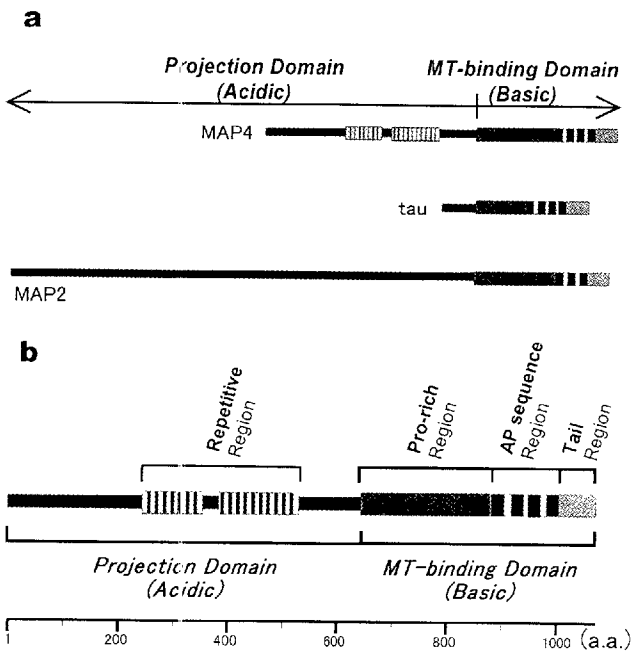

Fig. 1. Schematic diagram of the three MAPs (MAP4, MAP2, and τ). (a) comparison of the structures of MAP4, τ, and MAP2. (b) schematic structures of MAP4. The residue numbers are presented on the *bottom*. Reiterated AP sequences of 18 residues and the spacer sequences in the carboxyl-terminal basic domain are indicated by *filled* and *open boxes*, respectively.

ther divided into three subdomains (4): a region rich in proline and basic residues (Pro-rich region), a region containing four repeats of an assembly-promoting (AP) sequence (3), which consists of 18 amino acid residues (AP sequence region), and a region rich in acidic and hydrophobic residues (Tail region) (see Fig. 1b).

In the carboxy-terminal region of MAP4, MAP2, and τ, they share a homologous AP sequence region which contains a trio or quartet of non-identical repeats (4, 25, 28, 32, 39, 42). The synthetic polypeptides containing the AP sequence of MAP4, τ, and MAP2 bind to microtubules and stimulate microtubule elongation *in vitro* (3, 24, 31, 44). Thus, this region is considered to play a leading role in the interaction of MAPs with microtubules. In the amino-terminal side, the AP sequence region is flanked by the Pro-rich region, the amino acid compositions of which are similar among the three MAPs, yet no significant sequence homology was detected (4). This region is mainly composed of five kinds of amino acid residues, Pro, Ala, Lys, Ser, and Thr (4). MAP2 and τ share a homologous Tail region (42), but the primary structure of the Tail region of MAP4 is not similar to those of MAP2 and τ (4).

As discussed above, the primary structures of the three subdomains of microtubule-binding region have distinctive features. Therefore, a possibility is that each subdomain has a role distinguishable from the other subdomains in tubulin polymerization and microtubule stabilization. To address this issue, we have constructed several truncated fragments of MAP4 microtubule-binding domain as shown in Figure 2, and have characterized them. The possible roles for the three subdomains will be discussed in the following sections.

The microtubule-binding domains of the three MAPs resemble each other in subdomain structure on the whole. So these MAPs may interact with microtubules by the same mechanism, which is partly supported by the fact that MAP4 and τ compete with each other for binding to microtubules (3). Therefore, the knowledge of the function of MAP4 microtubule-binding domain will be helpful to clear up the common mechanism of MAPs-microtubule interaction.

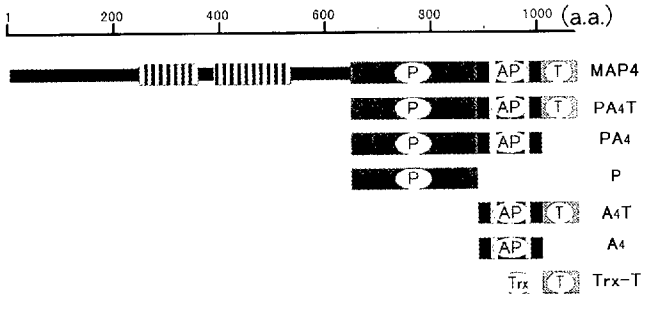

Fig. 2. Schematic diagram of truncated MAP4 fragments expressed in *E. coli*. Schematic structures of MAP4, the PA_4T fragment, the PA_4 fragment, the P fragment, the A_4T fragment, the A_4 fragment, and the Trx-T fragment are presented. The residue numbers are presented on the *top*.

The function of each subdomains of MAP4 MTs-binding domain

The role of the Pro-rich region

Aizawa *et al.* (1) reported that a proteolytic fragment of MAP4, which consist mainly of the Pro-rich region, binds to microtubules. It was considered that the binding of MAP4 to microtubules is stabilized by the basic Pro-rich region which could bind to the acidic tubulin molecule by the electrostatic interaction. To study the function of the Pro-rich region *per se*, the activity of the P fragment, containing the Pro-rich region alone, to bind to microtubules was measured by co-sedimentation analysis. The P fragment co-sedimented with microtubules (5), indicating that the Pro-rich region have microtubule-binding activity. An activity of the P fragment to stimulate microtubule assembly was examined by turbidity change at 350 nm. The turbidity no sooner increased that the P fragment was mixed with tubulin at 0°C, so the initial turbidity change was not observed. When the mixture was incubated at 37°C, the turbidity increased further (Fig. 3a). In case of MAP-dependent tubulin polymerization, the turbidity do not increase till 37°C incubation. After a 10 min incubation at 37°C, the reaction mixtures were incubated at 0°C for 5 min. The turbidity levels remained unchanged, indicating that the products were cold-stable. Electron microscopic observation revealed that the products constructed were amorphous aggregates (Fig. 3b). No microtubules were observed. The result indicated that the P fragment stimulates unfavora-

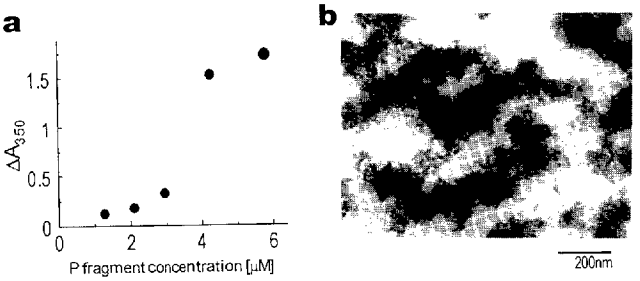

Fig. 3. Assembly-promoting activity of the P fragment. (a) tubulin (15 μM) was mixed with various concentration of the P fragment. The turbidities at 10 min are plotted against the concentrations of the P fragment. (b) electron microscopy of the products reconstituted from tubulin (15 μM) and the P fragment (3.6 μM). The samples were mixed in 100 mM MES buffer (pH 6.8) containing 0.5 mM GTP, and incubated at 37°C for 30 min. The sample was fixed, embedded, and sectioned, and those in d and e were negatively stained.

ble assembly of tubulin at 0 μM, and the products were cold-stable amorphous aggregates. The Pro-rich region alone would not stimulate microtubule nucleation. Using axonemes as the seeds for microtubule assembly, the microtubule-elongating activity of the fragment was examined. Electron microscopic observation revealed that microtubule did not elongate from the axonemes. The Pro-rich region would not stimulate microtubule elongation, either.

Next, to analyze the function the Pro-rich region fused with the other subdomain(s), microtubule assembly-promoting activity of the PA_4T fragment (containing the Pro-rich region, AP sequence region, and Tail region) was compared to those of the A_4T fragment (containing the AP sequence region and the Tail region).

The assembly promoting activity of the PA_4T fragment and the A_4T fragment were examined. The products constructed from tubulin and the PA_4T fragment or the A_4T fragment were cold-labile, and electron microscopic observation revealed that the products were normal microtubules (5), indicating that both fragments can stimulate microtubule assembly. However, the critical concentration of the PA_4T fragment for the promotion of microtubule assembly was 20 times lower than that of the A_4T fragment (5, 55). The presence of the Pro-rich region enhanced the assembly-promoting activity of the PA_4T fragment. The ability of the Pro-rich region to lower the critical concentration may be ascribed to the enhancement of the binding affinity, but the binding affinities determined by the quantitative analyses of the binding of the fragments to taxol-stabilized microtubules do not support this idea (55). Regardless the PA_4T fragment is above 20-fold more potent than the A_4T fragment as an inducer of microtubule assembly, binding affinity of the PA_4T fragment is only four times higher than that of the A_4T fragment (55), which is not as large as we expected. The high microtubule assembly-promoting activity of the Pro-rich region can not be explained simply by the enhancement of the binding affinity. The Pro-rich region should have a distinct role in microtubule nucleation. Microtubule nucleating activity of the Pro-rich region was assesses by counting the number concentration of microtubules in the early stage of assembly (55). Actually, the maximum microtubule number of the PA_4T fragment samples in the early stage of assembly was greater than that of the A_4T fragment samples (55). The presence of the Pro-rich region possibly affects the nucleating activity rather than the binding affinity of the MAP, and thereby augments the assembly promoting activity. One possible model of the Pro-rich region induced microtubule nucleation is presented elsewhere (55).

The role of the AP sequence region

We showed that MAP4 and τ shared a common sequence of 18 residues in their microtubule-binding proteolytic fragments, and also demonstrated that the synthetic polypeptide corresponding to the sequence bind to microtubules and stimulates microtubule elongation (3). We considered that the sequence is essential for MAP activity, and named it "AP (assembly-promoting) sequence" (3). Bovine MAP4 contains four imperfect repeats of the AP sequence. The presence of the repeats of the AP sequence within a single polypeptide chain maybe necessary to make nuclei for microtubule assembly (4).

To study the function of the AP sequence region, the A_4 fragment (containing the AP sequence region) was tested for an activity to nucleate free microtubules, and for an activity to elongate microtubules (34). When mixed with purified tubulin, the A_4 fragment caused a time-dependent and dose-dependent turbidity increase. However, the electron microscopic observation demonstrated that the products were entirely amorphous aggregates (34). The aggregates consist of tubulin and the A_4 fragment, as revealed by co-sedimentation analysis (unpublished result). Using axonemes as the seeds for microtubule assembly, the microtubule-elongating activity of the fragment was examined. A does dependent turbidity increase of the sample was observed, and electron microscopic observation revealed that microtubules were does-dependently elongated from the axonemes (34). Consequently, the AP sequence region does not nucleate microtubules, but elongates them.

The AP sequence region failed to promote microtubule assembly, 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: the multiplicity of the AP sequence do not have a significant role in nucleation of microtubule. Consequently, our model, in which the multiplicity of the AP sequence functions in the nucleation of microtubules, was false. Although, the AP sequence region has been considered to play a leading role in the promotion of MAP-induced microtubule assembly (3–5, 13, 24, 28, 31, 42), the importance of the repeat itself is questioned recently. Coffey *et al.* (21) revealed that, of the three MAP2 AP sequences, only the second AP sequence played a dominant part. In τ, Goode and Feinstein (26) proves that the inter-repeat region (between the first and second AP sequences) binds to microtubules. This is consistent with our quantitative analyses of the binding of the AP sequence region to taxol-stabilized microtubules (55). The A_4T fragment binding stoichiometry was 2.5–1.9 mol of the A_4T fragments$\cdot$mol^{-1} tubulin dimer, and, that of the PA_4T fragment 1.3–0.9 mol of the PA_4T fragments$\cdot$mol^{-1} tubulin dimer (55). Namely, two A_4T

fragments bind to one tubulin dimer, and the PA_4T fragment bind 1 to 1 the tubulin dimer. Only one of the for AP sequence repeat could bind one tubulin dimer at most.

The AP sequence region can promote microtubule elongation (34). Thus, this region can possibly increase a longitudinal affinity between each tubulin dimer, and promote protofilament formation. On the other hand, that the AP sequence region can not promote microtubule nucleation (34), can be explained by supposing that the nucleating step can not be induced by longitudinal binding of tubulin dimers. Since the AP sequence region of MAP4 is similar those of MAP2 and τ, it's considered that this region interact with microtubule by the common mechanism. Actually, the PA_4T fragment of MAP4 and intact τ, or intact MAP2 competed with each other for binding to microtubules, while MAP1a did not compete (Tokuraku *et al.*, manuscript in preparation). Therefore this theory may also be applicable to MAP2-induced and τ-induced assembly.

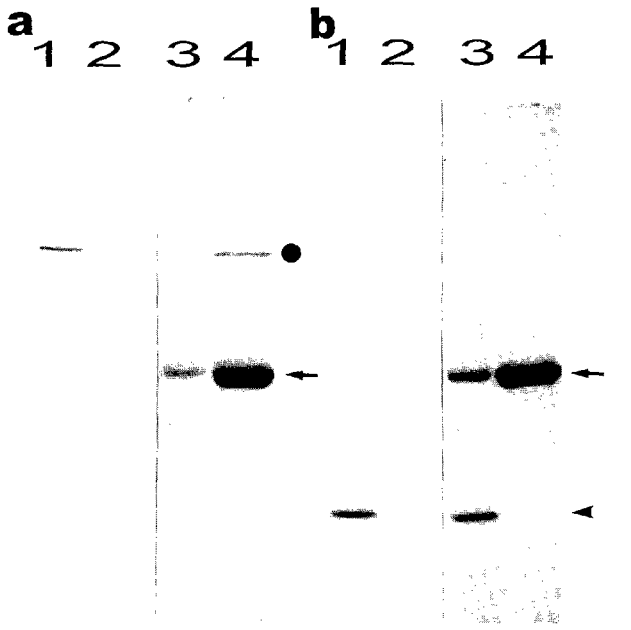

Fig. 4. Co-sedimentation analysis of the Trx-T fragment and the Trx-PA_4T fragment. (a) Co-sedimentation analysis of the Trx-PA_4T fragment. The Trx-PA_4T fragment (2.88 μM) was mixed with; Tubulin 0 μM (*lane 1, 2*), 15 μM (*lane 3, 4*) in the presence of taxol at 37°C for 30 min. (b) Co-sedimentation analysis of the Trx-T fragment. The Trx-T fragment (8.0 μM) was mixed with; Tubulin 0 μM (*lane 1, 2*), 15 μM (*lane 3, 4*) in the presence of taxol at 37°C 30 min. The mixtures were centrifuged at 16,000 × g for 30 min at 30°C. Supernatants and pellets were electrophoresed. *Lanes 1, 3* show the supernatant; *lanes 2, 4*, pellets. *Arrowhead* indicate the Trx-T fragments, and *dot* indicate the Trx-PA_4T fragment, and *arrow* indicate tubulin.

The role of the Tail region

Microtubule assembly consists of nucleation and elongation steps, with MAPs promoting both. The A_4T fragment promotes both steps (5), but the A_4 fragment only promotes microtubule elongation (34), suggesting that the Tail region is indispensable for the nucleation step. To study the function of the Tail region *per se* of MAP4, we tried expressing a fragment consisting mainly of the Tail region. Since the expressed fragment was small in quantity for experiment, we fused the Tail region with Thioredoxin to express.

In Figure 4a, we examined whether Thioredoxin influence the MAP-microtubule interaction, using the Trx-PA_4T fragment (a whole microtubule-binding domain fragment fused with Thioredoxin). When the Trx-PA_4T fragment was incubated with taxol-stabilized microtubule at 37°C, most of the Trx-PA_4T fragment was recovered in the pellets (Fig. 4a, *lane 4, dot*), indicating that the Trx-PA_4T fragment have an activity of microtubule binding. Thioredoxin did not inhibit MAP-microtubule interaction. Then, the Trx-T fragment (containing the Tail region) was tested for an activity to bind microtubules. When the Trx-T fragment was incubated with taxol-stabilized tubulin at 37°C, most of the Trx-T fragment was remained in the supernatant (Fig. 4b, *lane 3, arrowhead*), indicating that the Trx-T fragment do not have an activity to bind to microtubules. It is clear that the Tail region alone do not have microtubule binding ability. Since the Tail region can not bind to microtubules, we considered that this fragment can nether nucleate nor elongate microtubules.

The Tail region alone can not bind to microtubule, or promote microtubule assembly. Next, the function of the PA_4T and PA_4 fragments are compared. When the Tail region is removed from the PA_4T fragment, stoichiometry of the binding between the PA_4 fragment and the tubulin dimers were approximately the same level as those of the PA_4T fragment (55). Microtubule nucleating activity is also the same as that of the PA_4T fragment (55). On the other hand, the binding affinity was up to 10 fold, indicate that the presence of the Tail region inhibit the binding (55). In any case, we consider that the Tail region do not directly interact with microtubule. We suppose that the Tail region contribute to intra- or inter-molecular interaction, for example proper folding or cross-bridge, *in vivo*.

Without the Tail region, the AP sequence region failed to promote microtubule assembly (34). However, in the case of the PA_4 fragment, removal of the Tail region had no effect on the nucleating activity (55). In addition, the nucleation activity of the A_4T fragment is weak (55): critical concentration of the PA_4T fragment for microtubule assembly was 20 times lower

than that of the A_4T fragment (5, 55). Therefore it is the Pro-rich region that mainly promotes nucleation of microtubule assembly in the native MAP molecule, and the contribution of the Tail region to the nucleating activity is negative. The Tail region may substitute the microtubule-nucleating activity of the Pro-rich region, when the Pro-rich region is down-regulated by chemical modifications.

The Tail regions of MAP2 and τ construct cross-bridges, which linked microtubules together *in vivo* (43), while no cross-bridges were detected in microtubules reconstituted from tubulin and the A_4T fragment (containing the AP sequence region and the Tail region) of MAP4 (5). MAP2 and τ share a homologous Tail region (42), but the primary structure and the amino acid composition of the Tail region of MAP4 are different from those of MAP2 and τ (4). 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%). MAP4 Tail region may protrude from acidic microtubules surface, which may have different function from those of τ and MAP2.

Conclusion

In this study, we have made it clear that the three subdomains in MAP4 microtubule-binding domain have distinctive functions, using truncated fragments (Fig. 5). The Pro-rich region promoted microtubule nucleation. The AP sequence region elongates microtubules. The Tail region can not directly interact with microtubules. Since the AP sequences of MAP2, MAP4 and τ are homologous, and the whole AP sequence region and the Pro-rich region of the three MAPs are similar, these regions possibly interact with microtubule by the common mechanism. On the other hand, the Tail region of MAP4 is not similar those of MAP2 and τ. This region may have a MAP4 specific role.

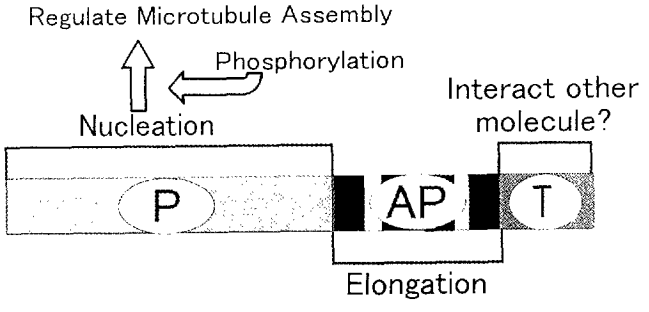

Fig. 5. Subdomains of MAP4 with probable functional assignment. The Pro-rich region promoted microtubule nucleation. The AP sequence region can promote microtubule elongation. The Tail region can not directly interact with microtubules. Phosphorylation of the Pro-rich region may regulate microtubule assembly *in vivo*.

The AP sequence region can only elongate microtubules (34). The function of this region is to increase longitudinal affinity between each tubulin dimer, and to promote protofilament formation. Supposing that the longitudinal binding of tubulin dimers is not sufficient to nucleate microtubules, it is not surprising that the AP sequence region can not promote microtubule nucleation (34). Conversely, it is the lateral protofilament-protofilament interaction that is important for the nucleating step. Since the Pro-rich region promoted microtubule nucleation (55), we consider that the function of the Pro-rich region is to confer nucleating activity on a MAP, possibly. Although several reports have emphasized the importance of the Pro-rich region for the microtubule nucleating activity (6, 11, 27, 41, 47), the underlying mechanism has not been presented so far. Ookata *et al.* (51, 52) reported that Cdc2 kinase phosphorylated MAP4 in the Pro-rich region both *in vivo* and *in vitro*. Phosphorylation did not prevent the binding of MAP4 to microtubules, but abolished its microtubule stabilizing activity *in vitro* (51). This agrees well to our results: Removal of the Pro-rich region from the PA_4T fragment caused its assembly promoting activity to decrease to a twentieth, while its the binding affinity to decrease to only a fourth.

The Tail region may contribute to the proper folding of MAP4 microtubule binding domain, or to the interaction with other molecules. The latter possibility is more likely, since the AP sequence region has an essential activity to interact with tubulin. In the absence of the Tail region, the AP sequence region still can promote microtubule elongation (34), so the AP sequence region also possesses the ability to interact with microtubule or tubulin dimer. The tertiary folding of a MAP molecule has not been taken to account, because a MAP is considered to be a long, flexible molecule with no specific tertiary foldings (48, 58, 29). Why does the A_4T fragment, which lacks the Pro-rich region, promote microtubule assembly? Since the Tail region is rich in hydrophobic residues (5), its hydrophobicity may inhibit amorphous aggregate formation cased by the AP sequence region alone.

Although the subdomains in the microtubule-binding domain have been characterized, some of the points remain unsolved as follows. Should the subdomain be connected to other subdomains to display it's full ability? What is the function of the projection domain? Why does the AP sequence repeats? Definitive answers to these questions will require further investigations.

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