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An Alternatively Spliced Site in the Proline-Rich Region of Microtubule–Associated Protein 4 Plays an Important Role in Microtubule Stabilization

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Abstract: We previously reported that the microtubule-stabilizing activity of a microtubule-associated protein (MAP) 4 variant, with a deletion in the Pro-rich region (MAP4-SP), was lower than that of a variant with a full length Pro-rich region (MAP4-LP). However, it remained unclear whether the deletion of the specific site in the Pro-rich region is responsible for the reduction of the microtubule-stabilizing activity. To answer this question, we examined the microtubule-stabilizing activities of four different MAP4 variants, MAP4-SP, MAP4-LP, and two additional MAP4-LP variants lacking a part of the Repeat region, and considered the correlation between the activity and the structure. When microtubules assembled in the presence of each of the MAP4 variants were treated with nocodazole for disassembly, the MAP4-SP-induced microtubules were significantly less stable than the other variant-induced microtubules. Another set of experiments, in which the microtubules were allowed to disassemble by dilution, yielded similar results: the MAP4-SP-induced microtubules were significantly less stable than the other variant-induced microtubules. The results clearly indicated that the microtubule-stabilizing activity of MAP4 depends on the specific, alternatively spliced site in the Pro-rich region.

Keywords: microtubule, microtubule-associated protein 4 (MAP4), Pro-rich region, microtubule stability and dynamics, alternatively splicing

Introduction

Microtubule-associated protein (MAP)* 4, which is known as a ubiquitous MAP, plays important roles in the dynamic functions of microtubules *in vivo*[1,2]. MAP4 consists of an amino-terminal projection domain and a carboxyl-terminal micro tubule-binding domain, with the latter further divided into the proline rich (Pro-rich) region, the Repeat region, and the Tail region[1]. We have cloned several MAP4 variants generated by alternative splicing, and examined their functions[3-6]. We recently reported that the microtubule-stabilizing activity of a neural variant of MAP4, with a deletion in the Pro-rich region (MAP4-SP), was lower than that of the full length version (MAP4-LP), both *in vitro* and *in vivo*[5]. Nguyen *et al.* (1997) reported that the over-expression of intact MAP4 stabilized cytoplasmic microtubules and slowed down cell growth,

*Abbreviations: MAP, microtubule-associated protein; Pro-rich, proline rich; SP, short Pro-rich; LP, long Pro-rich; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RB, reassembly buffer; LPA_3T, MAP4 fragment containing the long Pro-rich region, the Repeat region with three AP sequences, and the Tail region; LPA_4T, MAP4 fragment containing the long Pro-rich region, the Repeat region with four AP sequences, and the Tail region; LPA_5T, MAP4 fragment containing the long Pro-rich region, the Repeat region with five AP sequences, and the Tail region; SPA_5T, MAP4 fragment containing the short Pro-rich region, the Repeat region with five AP sequences, and the Tail region.

without inducing mitotic arrest or cell death[7]. The expression of MAP4-SP, which is less competent in stabilizing microtubules, may facilitate the elaborate regulation of microtubule dynamics in a living cell. Since the expression of the MAP4-SP variant is restricted to neural cells[4], we suppose that the variant may contribute to the dynamic behaviors of neural cells in particular[5].

Although it is evident that MAP4-SP has lower microtubule-stabilizing activity, it still remains unclear whether the deletion of a specific site in the Pro-rich region is responsible for the reduction in the activity, since we have compared only limited numbers of MAP4 variants[5]. In addition, the microtubule-binding domain fragment of MAP4-LP used in the experiment bundled microtubules, while that of MAP4-SP did not[4]; therefore, the accurate assessment of the microtubule-stabilizing activity may have been hampered by the formation of microtubule bundles.

In this study, we examined the microtubule-stabilizing activity of four types of MAP4 variants, including MAP4-SP. Since the polypeptide length, the net charge, and the microtubule bundling activity differed among these variants[3,4], we expected that a careful examination of these variants would reveal the factor responsible for the reduction in the microtubule-stabilizing activity. The results showed a strong dependency of the microtubule- stabilizing activity on the alternatively spliced site in the Pro-rich region.

Materials and Methods

Microtubule-binding domain fragments of the MAP4 variants were purified from *Escherichia coli* cells expressing the fragments, as described previously[3,4]. Porcine brain tubulin was prepared by the standard method[8,9]. Protein concentrations were determined by the method of Lowry *et al.*[10], using bovine albumin as the standard. Sodium dodecyl sulfate-polyacrylami-de gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli[11], using 10% gels.

Microtubule stability assay

The microtubule-stabilizing activity of the MAP4 variants was analyzed as described previously[5]. Tubulin (15 μM) was polymerized in the presence of the MAP4 variants (2 μM) in reassembly buffer (RB: 100 mM 2-morpholinoethanesulfonic acid, pH 6.8, 0.1 mM ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 0.5 mM $MgCl_2$) containing 0.5 mM GTP, at 37˚C for 30 min. The samples were treated with nocodazole (50 μg/ml) or were diluted 10-fold by adding pre-warmed RB. After a 10 min incubation, the treated samples and the untreated control sample were centrifuged at 16000 x g for 30 min. The pellets were resuspended in the original volume of RB. The supernatant and the pellet solutions were supplemented with a set volume of SDS-PAGE loading buffer, and a 15 μl aliquot of each sample was analyzed by electrophoresis on a 10% gel. For quantitative analysis, representative gels were prepared from three independent experiments. The amounts of tubulin on the polyacrylamide gels were quantified by image analysis (NIH image, NIH, USA).

Results

Preparation of the Proteins

In this study, we used four MAP4 fragments (Fig. 1A), each corresponding to the entire microtubule-binding domain of one of the MAP4 variants[3,4]. Three of the four fragments contain the intact Pro–rich region (LPA_5T, LPA_4T, and LPA_3T), while the other has a deletion in the region (SPA_5T). The fragments were renamed to show the length of the Pro–rich region explicitly. LPA_5T, LPA_4T, LPA_3T, and SPA_5T correspond to the former PA_5T, PA_4T, PA_3T[3], and SP[4], respectively. The MAP4 fragments and the tubulin were purified to homogeneity (Fig. 1B).

Stability of microtubules assembled in the presence of the MAP4 variants

To assess the microtubule-stabilizing activities of the MAP4 variants, we examined the resistance of microtubules against disassembly induced by the addition of nocodazole or dilution, as described previously[5]. Microtubules assembled *in vitro* in the presence of the MAP4 variants were treated with nocodazole or were diluted with pre–warmed RB, and then the samples were centrifuged to recover the microtubules remaining in the pellet fractions. Fig. 2 shows the percentage of the

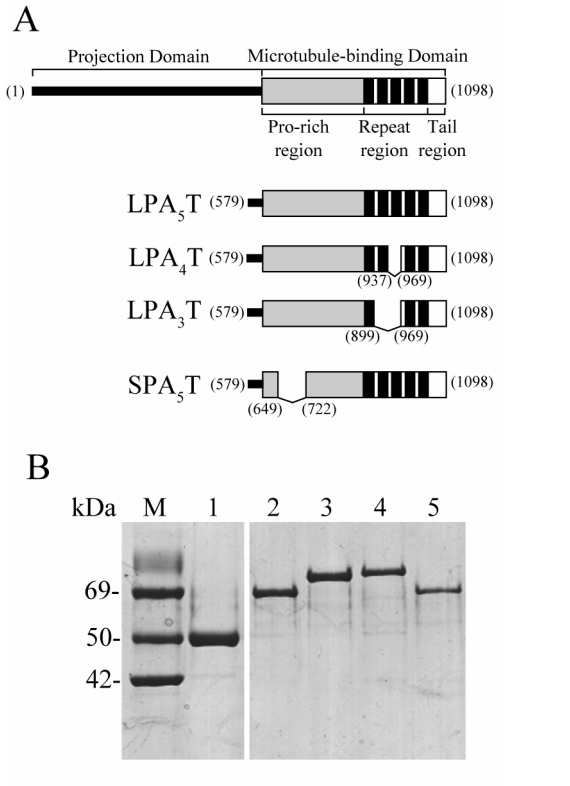

Fig. 1. Schematic structures of the microtubule-binding domain fragments of MAP4 variants, and SDS-PAGE patterns of the proteins used in this study. (A) Schematic structures of intact MAP4, LPA_5T, LPA_4T, LPA_3T, and SPA_5T. The numbers in parentheses indicate the amino acid positions of intact MAP4. (B) SDS-PAGE of the purified proteins. Lane M is the molecular mass markers: bovine serum albumin (69 kDa), porcine tubulin (50 kDa), and rabbit actin (42 kDa). Lane 1, porcine tubulin; lane 2, LPA_3T; lane 3, LPA_4T; lane 4, LPA_5T; lane 5, SPA_5T.

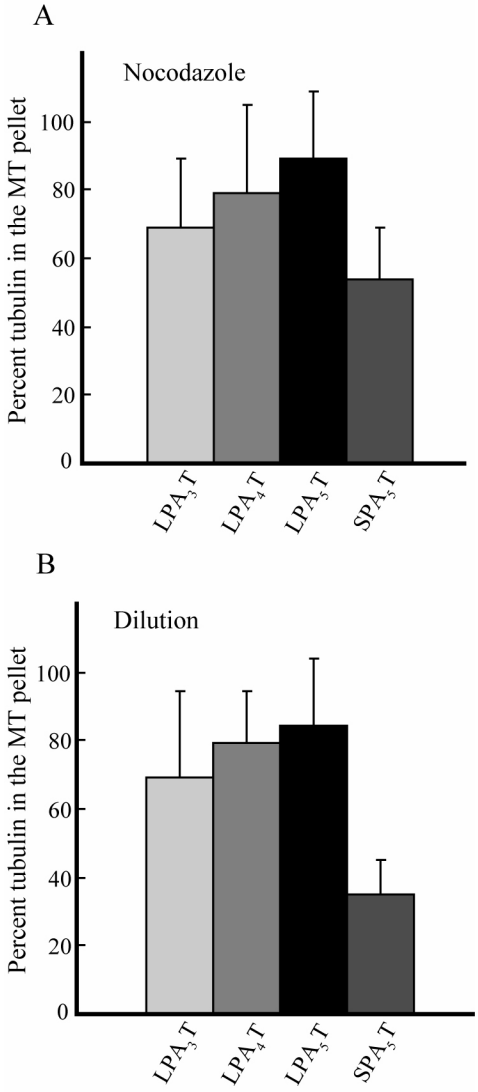

Fig. 2. Stability of microtubules assembled in the presence of the MAP4 variants. Tubulin (15 μM) was polymerized in the presence of each MAP4 variant (2 μM). The samples were supplemented with 50 μg/ml of nocodazole (A) or were diluted 10-fold by the addition of pre-warmed RB (B). After a 10 min incubation, the treated samples were centrifuged. The amount of pelleted tubulin in each sample was quantified, as described in the Materials and Methods, and the results are presented as the percentages of the untreated sample (100%). Data are averaged from three independent experiments. Error bars denote S.D.s.

amount of sedimented tubulin in each sample relative to that of each untreated sample (100%). The SPA_5T-induced microtubules were clearly less resistant to nocodazole treatment than the LPA_5T-induced microtubules (Fig. 2A). Although the LPA_3T- and LPA_4T-induced microtubules were also more susceptible than the LPA_5T-induced microtubules, the effect was not as pronounced as that on the SPA_5T-induced microtubules. The same is true of the dilution experiment (Fig. 2B). The SPA_5T-induced microtubules were much less stable than any of the MAP4-LP variant (LPA_3T, LPA_4T, and LAP_5T)-induced microtubules. The results clearly indicated that the SPA_5T-induced microtubules are less stable, and in other words, more dynamic. The dilution was more effective than the nocodazole treatment in depolymerizing the SPA_5T-induced microtubules: The percentage of microtubules remaining was 35% in the diluted sample, while it was 54% in the nocodazole-treated sample. Meanwhile, the two treatments showed similar effects on the depolymerization of the other three fragment-induced microtubules.

Discussion

In this study, we revealed that the microtubule-stabilizing activity of MAP4 strongly depends on the specific site in the Pro-rich region. Although

a deletion within the Repeat region also affected the stability of the microtubules against nocodazole treatment and dilution, the effect was only marginal, as compared with the deletion within the Pro-rich region.

Protein-protein interactions are generally affected by the polypeptide length and the charge[12]. Although we previously reported the modulation of the microtubule-stabilizing activity of MAP4 by a deletion in the Pro-rich region[4], it still remained unclear whether the primary factor in the reduction of the microtubule-stabilizing activity was the deletion of the specific sequence in the Pro-rich region or simply the length and/or the charge of the polypeptide. To address this issue, we prepared four MAP4 variants with different polypeptide lengths and net charges in this study. Table 1 summarizes the parameters of the MAP4 microtubule-binding domain fragments used in this study. The data in the table show that all three of the parameters (length, net charge, and bundling activity) of SPA_5T, in addition to the deletion in the Pro-rich region, are different from those of LPA_5T. Apparently, it is difficult to specify which factor is dominant in lowering the activity of SPA_5T. In fact, the microtubule-stabilizing activities of LPA_5T, LPA_4T and LPA_3T decreased in that order, which correlates well with the order of the three parameters, raising the possibility that the low microtubule-stabilizing activity of SPA_5T is associated with a general length and/or charge effect, and is not specific to the alternative splicing in the Pro-rich region. However, the activity of SPA_5T is much less than those of the others. Especially, the difference in the activity between SPA_5T and LPA_3T should be noticed. The polypeptide length of SPA_5T (448- amino acid residues) is similar to that of LPA_3T (451-amino acid residues), and their net charges are the same (+41). In addition, both fragments lack microtubule bundling activity[3,4]. Despite these similarities, the microtubule-stabilizing activity of SPA_5T was significantly lower than that of LPA_3T, indicating that the difference in the microtubule-stabilizing activity does not depend on the polypeptide length, net charge, or microtubule bundling activity. In other words, MAP4 regulates its microtubule-stabilizing activity by alternatively splicing a specific site in the Pro-rich region.

Table 1. Parameters of the microtubule-binding domain fragments of MAP4 variants.

Variant names	LPA_3T	LPA_4T	LPA_5T	SPA_5T
Length [a. a.][a]	451	489	520	448
Net charge[b]	+41	+46	+52	+41
Bundling[c]	–	+	++	–
Nocodazole [%][d]	69 ± 20	79 ± 25	89 ± 19	54 ± 15
Dilution [%][e]	70 ± 25	79 ± 15	84 ± 19	35 ± 9

[a,b]Length and Net charge of MAP4 fragments, calculated from amino acid sequence data.
[c]Bundling activities reported by Tokuraku *et al.*[3] and Matsushima *et al.*[4].
[d,e]Microtubule–stabilizing activities from Figure 2.

Why is the microtubule-stabilizing activity of MAP4 reduced by the absence of a part of the Pro-rich region? Intact MAP4 enhances the rescue frequency without decreasing the catastrophe frequency, and reduces the shortening lengths[13,14]. Mandelkow *et al.*[15] found that the tips of shrinking microtubules have the protofilaments coiled inside out, and concluded that the protofilaments of shrinking microtubules peel outward, and consequently, the microtubules rapidly depolymerize (Fig. 3A). According to our hypothesis[16], the Pro-rich region of MAP4 facilitates the lateral association of protofilaments by bridging them. If so, then the shrinking of a microtubule will stop at the MAP4-bound region (Fig. 3B), and it subsequently will be rescued. The microtubule- stabilizing activity of the MAP4-SP fragment (SPA_5T) was

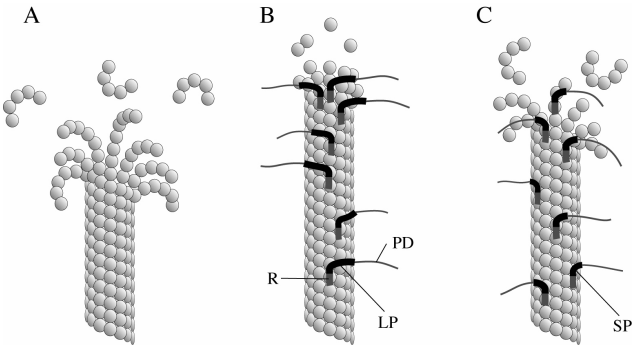

Fig. 3. Schematic illustration of disassembling microtubules in the presence or absence of MAP4 variants. (A) The first step in microtubule disassembly is the coiling of the protofilament tips. Consequently, the lateral bonds between them are disrupted; after that, they peel off, and subsequently break apart. (B) MAP4-LP enhances the lateral association of protofilaments by bridging them; therefore, the disassembly does not proceed over the MAP4-bound region because the peeling is blocked by the bridging. (C) The short Pro-rich region cannot bridge the adjacent protofilaments efficiently enough to stop the disassembly. PD, R, LP, and SP indicate projection domain, Repeat region, long Pro-rich region, and short Pro-rich region, respectively.

significantly lower than those of the other MAP4-LP fragments (LPA_3T, LPA_4T, LPA_5T), suggesting that the region essential for the protofilament bridging activity is missing in the MAP4-SP variant (Fig. 3C). The mechanism by which the missing sequence contributes to the bridging will be clarified by further experiments.

MAP4 variants are derived from a single gene by alternative RNA splicing[1]. The expression of these variants depends on the tissue types and the developmental stages[4,17]. As reported by Matsushima *et al.*[4], the expression of the MAP4-SP variant, which lacks part of the Pro-rich region, was restricted to neural cells[4]. Since we demonstrated that the structure of the Pro-rich region dictates the microtubule-stabilizing activity of MAP4 in this study, it is quite possible that this variant contributes to the dynamic behaviors of neural cells, as speculated previously[5].

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