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Different protofilament-dependence of the microtubule binding between MAP2 and MAP4

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Abstract

To see a molecular basis of the difference in the microtubule binding between MAP2 and MAP4, we compared the binding of them onto microtubule and Zinc-sheet in the presence of various concentrations of NaCl. The Zinc-sheet is the lateral association of protofilaments arranged in an antiparallel fashion with alternatively exposed opposite surfaces, so that binding requiring adjacent protofilaments is restricted. While the salt-dependence of the MAP2 desorption was not altered between these tubulin polymers, MAP4 dissociated from Zinc-sheet at lower concentrations of NaCl than from microtubule. These results suggest that single protofilament is sufficient for microtubule binding of MAP2 as observed by Al-Bassam et al. [J. Cell Biol. 157 (2002) 1187], but MAP4 appeared to interact with adjacent protofilaments during microtubule-binding. Weakened binding on Zinc-sheets was also observed in the projection domain–deletion mutants of MAP4, so that the difference in the protofilament-dependence would lie in the relatively conserved microtubule-binding domain.

Keywords: Microtubule; MAP2; MAP4; Protofilament; Zinc-sheet

Microtubules, the major cytoskeletal components of all eukaryotes, are essential in various cellular processes, including mitosis, organelle transportation, and cellular morphogenesis. Microtubules are hollow polymers with a diameter of 25 nm, composed of 13 protofilaments associated in a parallel fashion. Protofilaments are straight head-to-tail arrangements of αβ-tubulin heterodimers [1,2]. Individual microtubules show an intrinsic behavior known as dynamic instability [3], which is repeated alternations between relatively slow polymerization phases and rapid depolymerization phases both in vivo and in vitro [4–8]. A variety of protein factors are shown to participate in the regulation of the dynamic instability [9]. Among them, the classical heat-stable MAPs (microtubule-associated proteins), including MAP2 (microtubule-associated protein 2), MAP4 (microtubule-associated protein 4), and tau have been intensively studied for long time.

MAP2, MAP4, and tau are shown to associate on the surface of microtubules, and to stimulate tubulin polymerization. They share a common asymmetric molecular structure consisting of the amino-terminal projection (PJ) domain and the carboxyl-terminal microtubule-binding (MTB) domain [10–13], although they are derived from distinct genes. The PJ domain, which protrudes from the microtubule wall and does not bind to microtubules, completely differs among these MAPs in terms of the lengths and amino acid sequences excepting their overall acidic property. In contrast, several common structures are found in the C-terminal MTB domain that contains sequences required for microtubule binding and assembly. The MTB domain is commonly sub-divided into three distinct regions, the proline-rich (Pro-rich) region, the assembly-promoting-repeated sequence (AP) region with three to five 18 amino acid imperfect repeats, and the hydrophobic tail region. Both

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the Pro-rich and AP regions can promote tubulin assembly [14–18].

Direct observations of single microtubule dynamics using various optical microscopies suggested that assembly promotion by these heat-stable MAPs is due to the suppression of the transition from the polymerization state to the depolymerization state (called catastrophe) [20–22]. In addition, the increase in the frequency of the phase transition from depolymerization to polymerization (called rescue) also contributes to the stabilization of microtubules and thereby inclining the dimer–polymer equilibrium toward the polymer state [21,23–25]. To know the molecular basis of the MAP2-dependent rescue, we have correlated the distribution of MAP2 on individual microtubules with the dynamic phase changes of the same microtubules using fluorescent-labeled MAP2. We have found that stop of depolymerization required locally higher density of MAP2 on the microtubule lattice [26]. To stop the microtubule depolymerization, in which individual protofilaments are cooperatively curled off from the depolymerizing ends [27], the curvature of individual protofilaments must be restricted. By this viewpoint, we have proposed two models to explain the MAP2-cluster dependent stop of depolymerization [26]. MAP2 may be a linker for adjacent protofilaments that reduces the *Z*-axis flexibility of individual protofilament so as to decrease their curvature. Alternatively, MAP2 may link tubulin dimers only along the *Z*-axis of a single protofilament to restrict its curvature, acting as a splint. For the MAP2-cluster dependent stop of depolymerization, either model requires an appropriate arrangement and a certain density of MAP2 in a small area on microtubule lattice. Recently, cryo-electron microscopy revealed longitudinal binding of MAP2 along the protofilaments [28], indicating that the latter model is accurate. Since a common structure is shared among heat-stable MAPs especially at the microtubule-binding region, this binding mode may be applicable to MAP4. On the other hand, the characteristics of microtubule binding of MAP2 was reported to differ from that of MAP4 or tau [29]. It may be possible that the binding-geometry is somehow different between MAP2 and MAP4.

In this paper, we used Zinc-sheets to examine whether the protofilament-dependence of the microtubule binding of MAP4 is different from that of MAP2 or not. A Zinc-sheet is a polymorphic tubulin polymer composed of side-by-side protofilaments alternating in polarity and in inside-outside orientation, without changing their overall conformation [30]. For example, kinesin movement along a single protofilament was shown using Zinc-sheets [31]. Microtubule binding requiring two adjacent protofilaments is expected to be weakened when using the Zinc-sheet. Here, we examined the salt-dependent desorption from tubulin polymers, since salt-dependency of MAPs for microtubule binding is a good criterion for the microtubule-binding activity [32,33]. Present results suggest that the mode of protofilament-dependence upon microtubule binding differs between MAP2 and MAP4.

Materials and methods

Chemicals and protein preparations. Paclitaxel was purchased from Molecular Probe (OR, USA) and GTP was purchased from Yamasa (Tokyo, Japan). All other chemicals are certified to be reagent grade.

Tubulin and MAP2 were purified from bovine brain microtubule proteins as described previously [33]. Tubulin was purified by DEAE–Sepharose column chromatography using FPLC (Pharmacia, Sweden). MAP2 was purified from the crude microtubule proteins by boiling in the presence of 0.8 M NaCl and 50 mM β-mercaptoethanol followed by the separation using gel filtration with Superdex 200 (Pharmacia, Sweden). Full-length human MAP4 or its carboxyl-terminal half fragment, MTB (amino acid residues 693–1152), was purified by Mono-S column chromatography (Amersham–Pharmacia, Sweden) from a heat stable fraction of the cell lysate of *Escherichia coli* carrying pET-MAP4 or pET-MTB as described previously [34,35]. In the case of the purification of shorter fragment, MTB1 (amino acid residues 800–1152), bacterial lysate was directly applied onto Mono-S column without heat treatment at 100 °C for 5 min due to the lack of heat stability. Purification was not sufficient by the omitting of this process, so that the MTB-1 rich fraction obtained from Mono-S column was further purified with hydroxylapatite column (Bio-Rad, USA) to remove contaminant proteins. Pure MTB1 was eluted at 0.2 M K-Pi (data not shown). All the protein preparations were stored at −80 °C until use. Schematic diagrams of MAP2, MAP4, and MAP4 mutants are shown in Fig. 1.

Preparation of various polymorphic tubulin polymers. Zinc-sheet or C-tubule was prepared according to the methods of Kamimura and Mandelkow [31]. Tubulin stock in PM-buffer (0.1 M Pipes (pH 6.9), 1 mM EGTA, 0.5 mM $MgSO_4$, and 1 mM GTP) was incubated at 37 °C for 30 min to induce microtubule polymerization after adding 4 M glycerol, then microtubules were harvested by ultracentrifugation at 100,000*g* for 60 min at 37 °C. The resulting microtubule pellet was resuspended with cold 0.1 M Mes (pH 6.0), 0.5 mM EGTA, 0.5 mM $MgCl_2$, 1 mM $ZnCl_2$, 0.1 mM DTT, and 3 mM GTP, and stood on ice to allow depolymerization. After adding 20 μM paclitaxel, Zinc-sheets were polymerized by incubation at 30 °C for 20 min. When examining microtubule binding of MAPs at pH 6.0, microtubules were polymerized with PM-buffer containing 8% DMSO for 30 min at 37 °C. Microtubules were sedimented after adding 20 μM paclitaxel, and then the microtubule pellet was re-suspended at 37 °C with 0.1 M Mes (pH 6.0), 0.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM DTT, and 3 mM GTP containing 20 μM paclitaxel. In the case of C-tubule formation, 50 μM tubulin solution in PM-buffer was incubated at 37 °C for 20 min in the

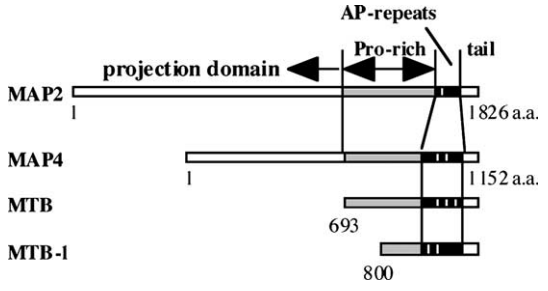

Fig. 1. Diagram of MAP2 and truncated MAP4 fragments used in this work. Truncated constructs were made as described previously [34,35].

presence of 15% DMSO. Formation of either polymer was monitored with electron microscopy (JEM-1200-EXII, JOEL, Japan) after negative staining with 1% uranyl acetate. Morphological difference among these tubulin polymers could also be detected with dark-field microscopy [33].

Assay of tubulin polymer binding activity. The binding ability of MAPs (MAP2, MAP4 MTB or MTB1) to various tubulin polymers was examined by a co-sedimentation experiment as follows. One μM of various MAPs was mixed with 10 μM paclitaxel-stabilized microtubules or 10 μM of other tubulin polymorphic polymers and incubated for 10 min at 37 °C in the presence of various concentrations of NaCl up to 0.3 M and fixed concentration of paclitaxel (20 μM). Polymers were then ultracentrifuged at 100,000*g* for 60 min at 37 °C using TLA 55 rotor for a TL-100 ultracentrifuge (Beckman, USA), and resulting supernatants and pellets were examined by SDS–PAGE with 10% polyacrylamide gel. After protein bands were stained, gel images were incorporated into a Power Macintosh 8500 computer (Apple, USA) as PICT files using an image scanner (Scan Jet 4c/T, Hewlett Packard, USA). Band concentrations were then determined by using the program NIH-image 1.61. The ratios of MAPs in the precipitant and MAPs in the supernatant were then calculated.

Miscellaneous. SDS–PAGE was performed by the methods of Laemmli [36]. Gels were stained with Coomassie Brilliant Blue R (CBB). All protein concentrations were estimated by bicinchoninic acid protein assay reagent (Pierce Chem., USA) or Bio-Rad protein assay reagent (Bio-Rad, USA) using BSA as a standard.

Results

MAPs do not bind to the lumenal face of protofilaments

If MAPs have an ability to bind to the lumenal surface of a protofilament, the use of Zinc-sheets to evaluate the mode of microtubule binding is meaningless. To examine this possibility, we used the C-tubule, an opened tubule in which lumenal faces of individual protofilaments are exposed. If MAPs can bind to the lumenal surface of protofilaments, the amount of MAPs bound to C-tubule would significantly increase from that bound to microtubules. However, when excess amount (2 μM for 1 μM tubulin polymers) of MAP2 or MAP4 was co-sedimented with microtubules or with C-tubules, no significant difference in the unbound proteins was detected between these two different polymers (data not shown). From this result, it is not likely that the lumenal surface of protofilament has an ability to bind either MAP2 or MAP4.

MAP2 binding

Since Zinc-sheets are unstable at neutral pH, perhaps due to the reduced solubility of $ZnCl_2$, all the experiments were done at pH 6.0 [31]. In the absence of salt, 100% of added MAP2 was co-sedimented with microtubules and no unbound MAPs were recovered in the supernatant under conditions where the 10 times molar excess of microtubules was mixed with MAP2. Fig. 2A shows a photograph of the representative experiment at pH 6.9 and the calculated data from the densitometry of such a gel image are summarized in Fig. 2B. At low NaCl concentrations, no significant change in salt desorption was observed between pH 6.9 and 6.0. Lowering pH from 6.9 to 6.0 slightly decreased the salt sensitivity of MAP2-binding to microtubule only at higher (0.15 M or more) NaCl concentrations. When the tubulin polymers were changed from microtubules to Zinc-sheets, no significant difference of the salt desorption profile was obtained (Fig. 2B). With either tubulin polymers, 80% of MAP2 remained bound in 0.1 M NaCl and 50% of MAP2 in 0.2 M NaCl. Although a significant amount of MAP2 remained bound on tubulin polymers even in the presence of 0.3 M NaCl in both cases, complete dissociation was observed in 0.5 M NaCl

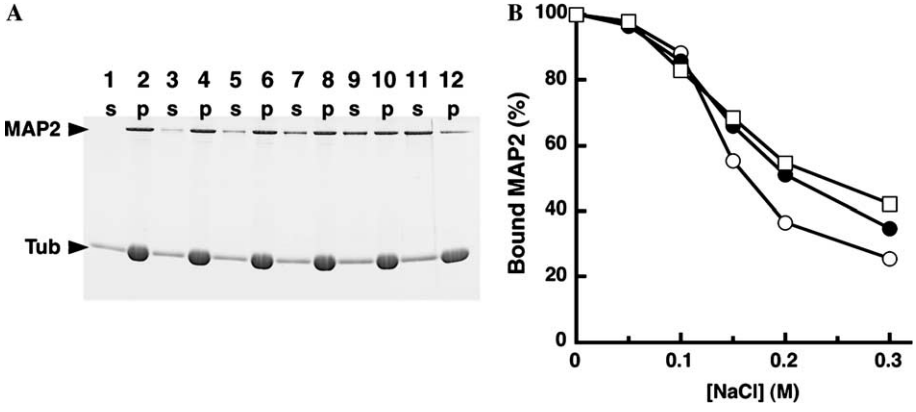

Fig. 2. Salt desorption of MAP2 from tubulin polymers. (A) An SDS–PAGE showing the binding of MAP2 to microtubules at pH 6.9. One μM of MAP2 was mixed with 10 μM of paclitaxel-stabilized microtubules then incubated for 10 min at 37 °C in the presence of various concentrations of NaCl (lanes 1 and 2, 0 M NaCl; lanes 3 and 4, 0.05 M NaCl; lanes 5 and 6, 0.1 M NaCl; lanes 7 and 8, 0.15 M NaCl; lanes 9 and 10, 0.2 M NaCl; lanes 11 and 12, 0.3 M NaCl). They were sedimented by ultracentrifuge and resulting supernatants (lanes 1, 3, 5, 7, 9, and 11) and pellets (lanes 2, 4, 6, 8, 10, and 12) were examined. (B) The amount of MAP2 bound to microtubules at pH 6.9 (open circles), microtubules at pH 6.0 (closed circles) or Zinc-sheets (open squares) was estimated by measuring the density of the band in gels by NIH image after staining with CBB. Each data value is a mean of two independent experiments. Since the SD value for each mean is quite small, the error bar is hidden by the symbol.

(data not shown). This means that binding of MAP2 onto protofilament is not affected even if an adjacent protofilament is not available for binding.

MAP4 binding

When MAP4 was co-sedimented with microtubules at pH 6.9, slightly higher salt sensitivity of binding was observed as shown previously [37]. Significant amount of MAP4 tended to desorb from microtubules even in 0.05 M NaCl and almost all MAP4 was removed at 0.15 M NaCl (Fig. 3). The higher sensitivity of binding of MAP4 is a distinct property from that observed in MAP2. At lower pH, MAP4 tended to bind more strongly even at higher concentrations of NaCl. For example, 80% of MAP4 stayed bound at 0.1 M NaCl in pH 6.0, while only 20% bound at same salt concentration in pH 6.9. This suggests that binding of MAP4 is somehow different from that of MAP2 in terms of pH-dependence. When the polymers were changed from microtubules to Zinc-sheets, the salt sensitivity of binding is increased (compare closed circles and open squares). For example, 40% of MAP4 is found to bind to Zinc-sheets at 0.1 M NaCl, and the overall profile of the salt concentration-dependence is rather similar to that at pH 6.9. These results suggest that binding of MAP4 on Zinc-sheets was weakened. Since all of the MAP4 bound onto Zinc-sheets in the absence of NaCl, the basic binding mechanism of MAP4 would be along the protofilament as MAP2. The increasing salt-sensitivity in Zinc-sheets suggests that, however, the absence of an adjacent protofilament with the same orientation weakens the binding of MAP4 onto the microtubule-lattice.

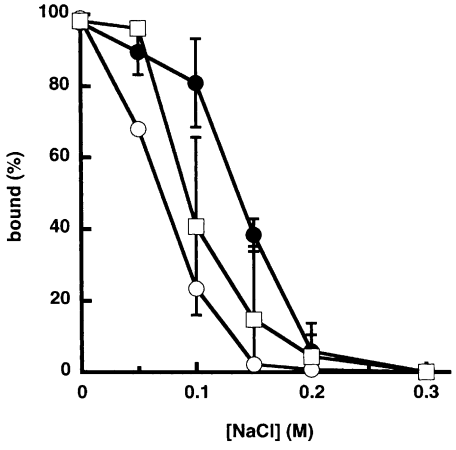

Fig. 3. Salt desorption of MAP4 from microtubules at pH 6.9 (open circles), microtubules at pH 6.0 (closed circles) or Zinc-sheets (open squares). One μM of various MAP4 was mixed with 10 μM paclitaxel-stabilized microtubules or Zinc-sheets and incubated for 10 min at 37 °C in the presence of various concentrations of NaCl up to 0.3 M. Polymers were then sedimented at 100,000*g* for 60 min at 37 °C, and supernatants and pellets were examined by SDS–PAGE. Each data value is a mean of three independent experiments. Error bars indicate SD. Data symbols lacking error bars are SD value smaller than symbol size.

Effects of the removal of projection domain

The prominent difference between MAP2 and MAP4 is in their projection domains. To see the role of projection domain on the binding, we examined the effect of removal of the projection region on the salt sensitivity using MTB, which lacked almost all of the projection region (Fig. 4). Although MTB showed a similar salt sensitivity to MAP4 at pH 6.9 (open circles), the decreased salt sensitivity was observed at pH 6.0 (closed circles). Almost all MTB bound to microtubules even in the presence of 0.15 M NaCl, indicating that the removal of projection region increased the microtubule binding. The salt-resistance of MTB at pH 6.0 somewhat resembles the desorption profile of MAP2. Although the projection region of MAP4 protrudes from the microtubule wall and does not directly bind to the microtubule, it may affect the binding of the MTB domain to the microtubule by an unknown mechanism. Substitution of the polymers from microtubules to Zinc-sheets again decreased the salt-resistance. Approximately 50% of MTB dissociated from Zinc-sheets in the presence of 0.15 M NaCl, a salt concentration in which all MTB bound onto microtubules (open squares). These results suggest that the difference in the protofilament-dependency of MAP4 and MAP2 resides in the MTB region itself.

Truncation of Pro-rich region

The Pro-rich region, the N-terminal half of MTB, has been suggested to be involved in the bridging of adjacent

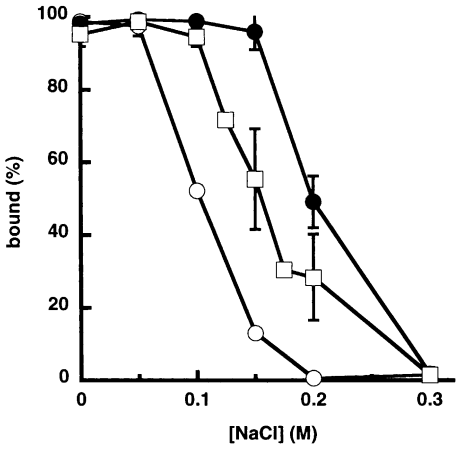

Fig. 4. Salt desorption of MTB from microtubules at pH 6.9 (open circles), microtubules at pH 6.0 (closed circles) or Zinc-sheets (open squares). Details of the data is same as in the legend for Fig. 3.

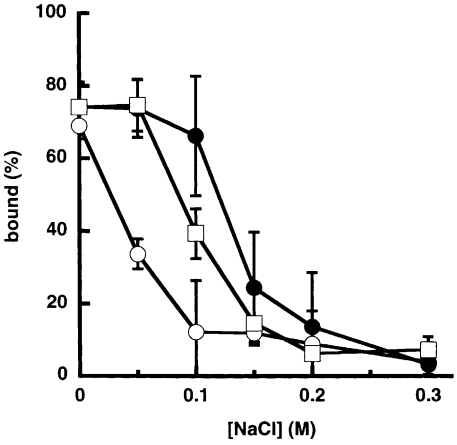

Fig. 5. Salt desorption of MTB-1 from microtubules at pH 6.9 (open circles), microtubules at pH 6.0 (closed circles) or Zinc-sheets (open squares). Details of the data is same as in the legend for Fig. 3.

protofilaments by Tokuraku et al. [19]. We examined the contribution of the Pro-rich region on the difference in the salt-sensitivity between microtubules and Zinc-sheets. We employed MTB1 construct, a deletion mutant of MAP4, which lacks only about half of Pro-rich region in addition to whole projection region, since further deletion of the Pro-rich region was predicted to severely decrease microtubule-binding ability as described below. As shown in Fig. 5, only 70% of added MTB1 co-sedimented with microtubules even in the absence of salt at pH 6.9. This observation is comparable to the results by Tokuraku et al. [19], in which dissociation constant of MTB fragment of bovine MAP4 (PA_4T fragment in their paper) was shown to increase fourfold by the removal of the Pro-rich region. MTB1 also dissociated from microtubules by increasing the NaCl concentrations as in the intact MAP4 or MTB case. Decreasing pH to 6.0 increased the amount of MTB1 bound to microtubules. Substitution of tubulin polymers from microtubules to Zinc-sheets decreased the salt-resistance of the binding especially in 0.1 M NaCl, and a desorption profile (open squares) similar to that of MAP4 and MTB was obtained. These results suggest that the region that interacts with the adjacent protofilament is present in MTB1 despite the decrease in microtubule-binding ability.

Discussion

The present data suggest that bridging across the protofilaments is somehow involved in the binding of MAP4 onto the microtubule wall. Since MAP4 tended to dissociate from Zinc-sheets in lower NaCl concentrations than from microtubules at pH 6.0, it is possible that binding of MAP4 requires the presence of an available adjacent protofilament.

Zinc-sheets of tubulin are a good tool to evaluate the mechanism of the protofilament-dependence of microtubule-binding proteins, since this polymorphic tubulin polymer is a lateral association of protofilaments alternating in polarity and in inside–outside orientation without changing the overall conformation of individual protofilaments [30]. If the microtubule binding of certain protein requires two adjacent protofilaments, the binding ability would be weakened in Zinc-sheets when compared to microtubules. Indeed, our present results for MAP2 binding showed good consistency with the cryo-electron microscopic observation that showed that a MAP2 molecule binds longitudinally along a single protofilament [30]. A microtubule-stabilizer TOGp or a microtubule-destabilizer XKCM1 was predicted to bind along on single protofilaments by the binding assay using the Zinc-sheets [38,39]. Since our results of Zinc-sheets experiment for MAP2 binding are consistent to the structural analysis, the predictions for the above two microtubule-regulators become highly possible.

The weakened binding to Zinc-sheets also persisted in MTB and MTB1, which lacks the projection domain and the projection domain/N-terminal half of Pro-rich region, respectively, indicating that the region requiring the presence of adjacent protofilament is present in the microtubule-binding region after 800th amino acid. Tokuraku et al. [19] proposed that the Pro-rich region bridges between two adjacent protofilaments. Although our results do not exclude this, the region in the MAP4 molecule interacting with a neighboring protofilament, we can at least argue that the N-terminal half of Pro-rich region is not required for coordinated binding to two protofilaments. Tau is also thought to use two adjacent protofilaments for microtubule binding. Both of the N-terminal and C-terminal flanking regions of AP-repeat, the Pro-rich region (151–244) and the pseudo-fifth repeat R′ region (369–400), are assumed to be involved in the lateral inter-protofilament interaction according to the "jaws" model [17,40]. With various deletion mutants of tau, Guske et al. [17] postulated that these two flanking regions are more important to microtubule binding than the AP-repeat region, and AP-repeat region is rather thought to contribute to stabilize microtubules. Our present result suggests that microtubule binding of MAP4 is rather similar to tau, and perhaps, the C-terminal half of Pro-rich region and/or the tail region may be involved in the lateral protofilament requirement.

The salt-dependence of MAP4 in desorption from tubulin polymers is different from that observed in MAP2. No significant difference in the binding of MAP2 at increased NaCl concentrations was detected between regular microtubules and Zinc-sheets. This indicates that a single MAP2 molecule does not require an available adjacent protofilament upon microtubule binding. This is a compatible result with the cryo-elec-

tron microscopic observation by Al-Bassam et al. [28]. In addition, distinct modes of microtubule binding between MAP2 and MAP4 were also suggested in terms of the pH-dependent changes in the salt-resistance. Compared to the small change in the salt-desorption profile of MAP2 between pH 6.9 and 6.0, a significant increase in the salt-resistance of microtubule binding of MAP4 was observed at decreased pH. Perhaps, a pH-dependent change in the proton dissociation from the four histidine residues, which are well preserved in the AP-repeats, may participate in this process. According to Tokuraku et al. [29], the microtubule-binding property of MAP2 differs from that of MAP4 or tau. They showed that significant population of MAP2 (50% of 2 μM) bound to microtubules (composed of 15 μM tubulin) even in the presence of excess amount (20 μM) of PA_4T fragment (a bovine MAP4 mutant equivalent to our MTB), whereas MAP4 or tau completely released from microtubules by addition of excess PA_4T fragment. This may be due to the presence of an additional binding site on the tubulin that is only available for MAP2. Different modes of binding between MAP2 and MAP4, as implied by the distinct protofilament-dependence observed here, would be an alternative molecular basis. It is likely that MAP2 is an unique heat-stable MAP that does not require adjacent protofilament upon microtubule-binding, since tau and MAP4 behave similarly, at least, in competition with PA_4T fragment [29].

Recently, electron microscopic observations showed that a part of AP-repeat of tau penetrates into the "paclitaxel-pocket" that is located in the inside of β-tubulin in the absence of paclitaxel [41]. They showed that the microtubule-affinity of tau is much higher in the absence of paclitaxel than in its presence. It is not clear whether MAP2 or MAP4 similarly bind into the paclitaxel-pocket as tau does. Even if so, however, present results are not due to the difference in the way of utilizing the pocket upon binding, since the concentration of paclitaxel is high enough to occupy almost all the pocket in microtubules. Rather, it is conceivable that the difference in the protofilament-dependence observed here would be ascribed to the other structural elements of tubulin at the microtubule surface.

In the case of tau, the microtubule-binding region is proposed to take distinct structures that are specific to 3-repeat or 4-repeat tau isoforms [42]. The number of AP-repeats in human MAP4 is five [43], while that in MAP2 is three. Different numbers of AP-repeats would be the determinant of the mode of microtubule binding. In addition, both the Pro-rich region and the tail region are not highly identical between them. Taken together, it is likely that the MTB-region of either MAP takes a distinct conformation upon microtubule binding as was suggested in tau [42], and thereby different requirements for an adjacent protofilament occur between MAP2 and MAP4.

Acknowledgments

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