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Two Kinesins from Arabidopsis, KatB and KatC, Have a Second Microtubule-binding Site in the Tail Domain

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Kinesins, as a kind of microtubule-based motor proteins, have a conserved microtubule-binding site in their motor domain. Here we report that two homologous kinesins in *Arabidopsis thaliana*, KatB and KatC, contain a second microtubule-binding site in their tail domains. The prokaryotic-expressed N-terminal tail domain of the KatC heavy chain can bind to microtubules in an ATP-insensitive manner. To identify the precise region responsible for the binding, a serious of truncated KatC cDNAs encoding KatC N-terminal regions in different lengths, KatC1-128, KatC1-86, KatC1-73 and KatC1-63, fused to Histidine-tags, were expressed in *E. coli* and affinity-purified. Microtubule cosedimentation assays show that the site at amino acid residues 74-86 in KatC is important for microtubule-binding. By similarity, we obtained three different lengths of KatB N-terminal regions, KatB1-384, KatB1-77, and KatB1-63, and analyzed their microtubule-binding ability. Cosedimentation assays indicate that the KatB tail domain can also bind to microtubules at the same site as and in a similar manner to KatC. Fluorescence microscopic observations show that the microtubule-binding site at the tail domain of KatB or KatC can induce microtubules bundling only when the stalk domain is present. Through pull-down assays, we show that KatB1-385 and KatC1-394 are able to interact specifically with themselves and with each other *in vitro*. These findings are significant for identifying a previously uncharacterized microtubule-binding site in the two kinesin proteins, KatB and KatC, and the functional relations between them.

Keywords: Cosedimentation, Kinesin, KatB, KatC, Microtubule-binding site

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Introduction

Members of kinesin superfamily are microtubule-based motor proteins found in all eukaryotic organisms and participate in various intracellular functions, including organelle transport, spindle formation, chromosome segregation, microtubule dynamics, and signal transduction (Hirokawa, 1998; Heald, 2000; Kline-Smith *et al.*, 2002; Nishihama *et al.*, 2002). The conventional kinesin (kinesin-1) is a tetramer with two heavy chains and two light chains (Kuznetsov *et al.*, 1988). The kinesin heavy chains are composed of three distinct domains: a conserved motor domain which contains the ATP-dependent microtubule-binding site that is responsible for force generation and direction of movement (Case *et al.*, 1997; Endow *et al.*, 1998); a long α-helical coiled-coil domain thought to be responsible for dimerization; and a tail domain thought to be involved in cargo or light chain binding and regulation of motor activity (Seiler *et al.*, 2000; Shiroguchi *et al.*, 2003).

Besides its conserved microtubule-binding site in the motor domain, kinesin-1 contains a second microtubule-binding site in the tail domain thought to mediate microtubule-microtubule interactions (Straube *et al.*, 2006). In addition, several kinesin-14 (C-terminal) family members such as Ncd from *Drosophila melanogaster*, Kar3 from *Saccharomyce cerevisiae* and KCBP from *Arabidopsis thaliana* (Meluh and Rose, 1990; Narasimhulu and Reddy, 1998; Karabay and Walker, 1999), also contain a microtubule-binding site in their tail domains, which specifies the ability of respective kinesin to cross-link anti-parallel microtubules. The plus-end directed kinesins of the kinesin-5 (BimC) family also can cross-link and slide apart anti-parallel microtubules by forming tetramers with their motor domains (Kashina *et al.*, 1997; Sharp *et al.*, 1999). It is well established that an antagonistic interaction exists between kinesin-5 proteins and kinesin-14 proteins along anti-parallel microtubules, and this relationship is important for bipolar spindle formation during mitosis (Sharp *et al.*, 2000; Heald, 2000).

KatB and KatC are two highly homologous (84% amino

acid identity) members of sixty-one kinesins in *Arabidopsis thaliana* (Reddy and Day, 2001) and belong to the kinesin-14 family (Lawrence *et al.*, 2004). KatB and KatC share approximately 30% amino acid identity with Ncd, a kinesin-14 family member isolated from *Drosophila melanogaster*. The C-terminal half of both KatB and KatC contains the kinesin motor domain and possesses ATP-sensitive microtubule-binding and microtubule-stimulated ATPase activities (Mitsui *et al.*, 1994) common to kinesin proteins. *In vivo* studies showed that KatB and KatC expression increased during mitosis in synchronized BY-2 cells (Mitsui *et al.*, 1996).

Two other kinesin-14 family members ATK1 (KatA) and ATK5 (KatE) that have high amino acid sequence identity to KatB and KatC (60% and 57% respectively) are also known to be involved in mitotis and meiotis (Liu *et al.*, 1996; Chen *et al.*, 2002; Marcus *et al.*, 2003; Ambrose *et al.*, 2005). Both ATK1 and ATK5 localize to interzonal microtubules of the anaphase spindle and phragmoplast (Liu *et al.*, 1996; Ambrose *et al.*, 2005). During mitosis, *atk1-1* null mutant cells exhibit an atypical spindle during prophase and metaphase, and after anaphase, bipolar spindles form and chromosomes segregate normally (Marcus *et al.*, 2003). While in meiosis, however, *atk1-1* mutant cells show abnormal spindle poles, resulting in defective chromosome segregation and lower male fertility (Chen *et al.*, 2002). On the other hand, *atk5-5* null mutants exhibit an abnormally broadened mitotic spindles in metaphase and anaphase. (Ambrose *et al.*, 2005). The cellular localization and functions of KatB and KatC in plants have not yet been investigated. To examine the physiological functions of both KatB and KatC, it is important to characterize their biochemical properties. In this study, we expressed and purified N-terminal regions of varying lengths of both KatB and KatC heavy chains of *Arabidopsis thaliana*. These purified polypeptides were used to examine interactions between KatB or KatC and microtubules, and between KatB and KatC *in vitro*. Our results show that both KatB and KatC contain a second microtubule-binding site in the tail domain, and that the peptide YI/VKRLRLCIRWFQ is important for microtubule-binding. These data on the interaction of the KatB and KatC with microtubules provide new clues to the unknown function of KatB and KatC in plants.

Materials and Methods

Construction of recombinant plasmids. The cDNA sequences of *KatB* and *KatC* available in GenBank (accession no. D21137 and D21138 respectively) were referenced to design primers. *KatB* and *KatC* cDNA were amplified by RT-PCR with the following primers:

KatBfwd (5'-GGTACCATGGTTGGGGAAATGACGAAC-3')
KatBtrev (5'-GAGCTCTCATCCAAGGCTCAAGCGATA-3')
KatCfwd (5'-GGTACCATGGTGGGGGCGATGGCAAAC-3')
KatCtrev (5'-GAGCTCTCATCCAAGGCTCAACCGATTTTCC-3')

The two amplified fragments were then inserted into pGEM-Teasy vector (Promega) to yield pT-KatB2235 and pT-KatC2262. The diagrams of constructs used in this study are shown (Fig. 1A, 5A). DNA fragments encoding different fragments of the KatB and KatC tail regions were amplified by PCR from pT-KatB2235 and pT-KatC2262 plasmids, and *EcoR*I-*Sal*I sites were introduced into each fragment and amplified. Amplified DNA fragments were then digested with *EcoR*I and *Sal*I and cloned into pET-30a (Novagen) to produce 6 × His-tagged fusion proteins or into pGEX-4T-1 (Promega) to produce GST fusion proteins. All constructs were sequence-confirmed.

Expression and purification of proteins. All plasmid constructs were transformed into *E.coli* BL21 (DE3) cells for expression. Bacterial cultures were grown at 37°C to an OD_{600} of 0.6, and fusion protein expression was induced by addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 6 h at 22°C, cultures were pelleted, washed, suspended in lysis buffer (0.3 M NaCl, 20 mM imidizole, 1 mM PMSF and 20 mM Tris-HCl, pH 7.5) and sonicated in an iced water bath. Lysates were spun at 12,000 × *g* for 30 min to separate soluble and insoluble protein fractions. The resulting supernatant was loaded onto a column containing 2 ml of Ni-NTA agarose resin (Amersham Pharmacia) for purification according to the manufacturer's protocol. Proteins purified from *E. coli* were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Fig. 1B, 5B).

Tubulin isolation, purification, and microtubule preparation. Isolation and purification of bovine brain tubulin were performed as previously described (Robley *et al.*, 1987). To prepare microtubules, purified tubulin was polymerized at 37°C for 30 min containing 1 mM GTP (Sigma) and stabilized with 20 M taxol (Paclitaxel; Sigma).

Microtubule cosedimentation assay. Microtubule cosedimentation assays were performed in PEM buffer (2 mM $MgCl_2$, 1 mM EGTA, and 100 mM PIPES, pH 6.9) plus 1 mM DTT, and 10 μM taxol. Protein (4 μM in a reaction volume of 100 μL) was mixed with 4 μM taxol-stabilized microtubules in the absence or presence of 5 mM 5'-adenylylimidodiphosphate (AMP-PNP; a non-hydrolyzable ATP analogue; Sigma) and 5 mM MgATP, respectively. Control reactions contained no microtubules. After incubation for 20 min at 22°C, reactions were centrifuged at 100,000 × *g* for 30 min at 22°C in Beckman ultracentrifuge (Beckman). Supernatant and pellet were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. This assay was also used to determine the binding affinity (K_d) of the KatC tail domain (KatC1-128) to microtubules. For this, the taxol-stabilized microtubule concentration was kept constant (5 μM), and KatC1-128 concentration varied (1 μM to 25 μM). The reaction was incubated for 20 min at 22°C, centrifuged and analyzed by SDS-PAGE as described above. After SDS-PAGE analysis, the concentration of KatC1-128 in the supernatant and pellet was quantified by measuring protein band intensities with Graphpad. prism.v4.03 software, and K_d was determined with a simple quadratic equation.

Microtubule fluorescence observation. In a reaction volume of 10 μL, 10 μM NHS-Rhodamine labeled microtubules (provided by Dr. Ming Yuan's lab in China Agricultural University) were mixed with or without 5 μM protein. After incubation (20 min at 22°C), the reaction was terminated using 1% glutaraldehyde in PEM buffer.

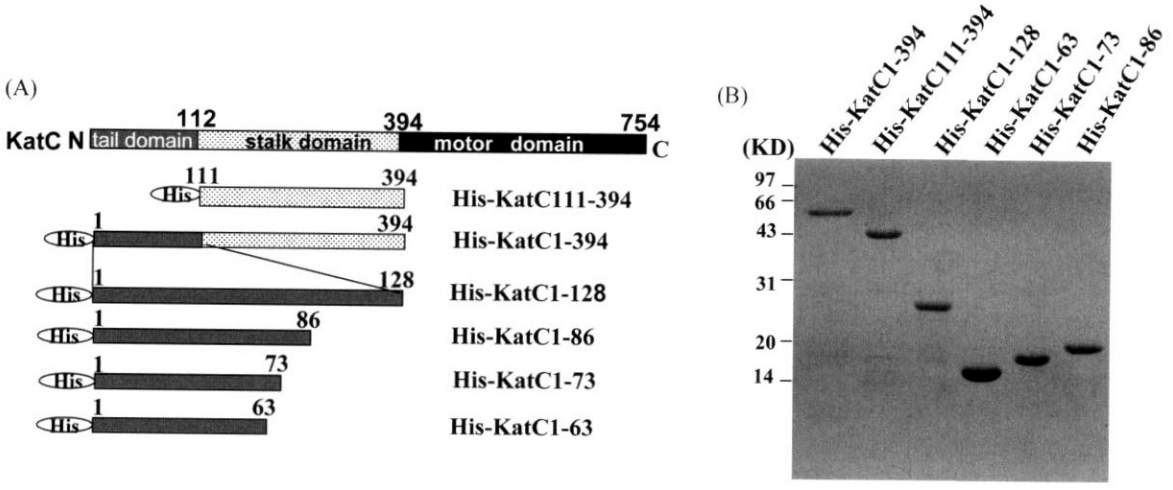

Fig. 1. Constructs and proteins. (A) The domain structures of the Arabidopsis KatC and constructs of different His-tagged fusion proteins. Numbers refer to corresponding amino acids. (B) SDS-PAGE (15% gel) analysis of His-tagged KatC N-terminal polypeptides. Numbers on the left indicate the position of the molecular weight markers.

Samples were diluted 20 times with PEM buffer for observation and examined under an Olympus microscope (Tokyo) equipped with 100 × oil objective (NA 1.3). Images were collected with cooled CCD (CoolSNAP HQ).

The interaction of KatB and KatC *in vitro*. The interaction between KatB and KatC *in vitro* was examined as previously described (Pan *et al.*, 2004). In brief, the bacteria expressing GST fusion proteins (GST control, GST-KatB1-384, GST-KatC1-394) were lysed in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 1 mM ATP, 1 mM DTT, 2 mM $MgCl_2$, 1 mM PMSF and 1% Triton X-100. After sonication, lysates were centrifuged at 12,000 × *g* for 30 min at 4°C. Supernatants (1.5 mL) were incubated with 1 ml immobilized glutathione gel (Amersham Pharmacia) for 30 min at 4°C. After washing, the gel was divided into two equal parts: one was incubated with 6×His-KatB1-384 and the other with 6 × His-KatC1-394 overnight at 4°C. Gel was centrifuged at 70 g for 5 min at 4°C, washed thoroughly, and then GST fusion proteins were eluted with 20 mM reduced glutathione. Collected samples were loaded on SDS-PAGE gels for immunoblotting experiments with either anti-6 × His antibody (R&D) or anti-GST antibody (Sigma).

Results

The N-terminal region of KatC has an ATP-insensitive microtubule-binding site. To determine whether the KatC N-terminal region is microtubule binding, a microtubule cosedimentation assay was performed. The KatC tail and stalk fragment containing residues 1 to 394 (KatC1-394), KatC tail domain containing residues 1 to128 (KatC1-128) and KatC stalk domain containing residues 111 to 394 (KatC111-394) were used for cosedimentation assays. Proteins were incubated with taxol-stabilized microtubules in the absence or presence of either AMP-PNP or ATP and analyzed by SDS-PAGE (Fig. 2A). KatC1-394 and KatC1-128 were found in the pellet fractions regardless of AMP-PNP presence. In the control reaction (no microtubules), little protein was found in the pellet fraction. Addition of ATP (5 mM) had no effect on microtubule-binding ability of KatC1-394 and KatC1-128 (Fig. 2A, top, middle). As expected, KatC111-394 was found not to cosediment with microtubule (Fig. 2A, bottom). These results suggest that, in addition to the motor domain (Mitsui *et al.*, 1994), KatC has another ATP-insensitive microtubule-binding site in the N-terminal region.

To determine the state of the fusion protein-bound microtubules, both KatC1-394 and KatC1-128 were mixed with NHS-Rhodomine labeled microtubules respectively in the absence or presence of ATP, and observed under a fluorescence microscope. KatC1-394 induced large bundles of microtubules in the buffer regardless of the presence of ATP (Fig. 2B, c, d), while KatC1-128 could not under identical conditions. Instead, microtubules appeared as single filaments (Fig. 2B, b) similar to our control (Fig. 2B, a). These results demonstrate that the presence of the microtubule-binding site and stalk region neighboring the motor domain could make KatC bundle microtubules *in vitro*, suggesting that KatC may function as dimers *in vivo* during the interactions among microtubules.

To quantify the binding of the KatC tail domain to microtubules, various concentrations (1-25 μM final) of KatC1-128 proteins were mixed with microtubules (5 μM final) (The KatC1-394 construct was not used for these experiments since it contains the predicted coiled-coil region at the stalk domain which may encourage dimer formation). Supernatants and pellets were separated by SDS-PAGE after centrifugation (Fig. 2C). KatC1-128 in supernatant fractions increased in proportion to total protein concentration (Fig. 2C, top). In comparison, KatC1-128 increased in the pellet fractions as total protein increased to 10 μM, but at a higher

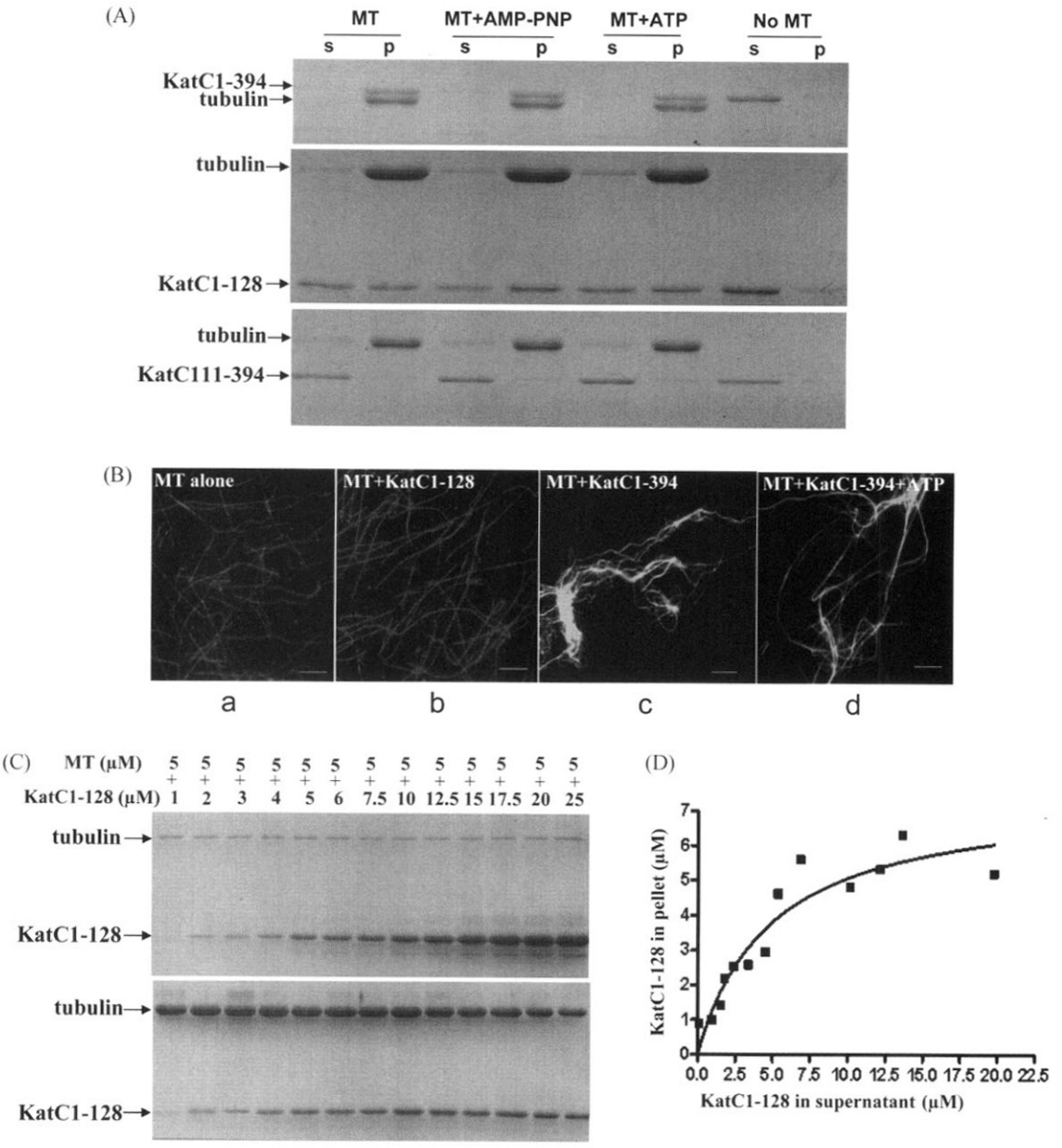

Fig. 2. The N-terminal region of the KatC can interact with microtubule in an ATP-insensitive manner. (A) Cosedimention assays of KatC1-394 (top), KatC1-128 (middle) and KatC111-394 (bottom) with microtubules. Cosedimention experiments were performed with 4 M fusion proteins in the absence or presence of either AMP-PNP or ATP. Resulting proteins in the supernatant (s) and pellet (p) are shown. (B) Images observed by fluorescence microscopy when KatC1-128 and KatC1-394 were mixed with NHS-Rhodomine labeled microtubules. a. Microtubules polymerized in the absence of fusion proteins. b. Microtubules still appeared in single filaments in the presence of KatC1-128. c. Microtubule bundles induced by KatC1-394. d. Microtubule bundles induced by KatC1-394 were insensitive to ATP. Bar = 10 μm. (C) Microtubule binding assays in various concentrations of KatC1-128. KatC1-128 (1-25 μM final) were mixed with Taxol-stabilized microtubules (5 μM final) and centrifuged after 20 min incubation at 22°C. Supernatant (top) and pellet (bottom) fractions were then separated on 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue. (D) Values from (C) were plotted and fit with the simple quadratic equation showing $K_d = 5.01 \pm 0.78$ μM. The experiments were repeated three times. MT: microtubule.

level of total protein, the amount of KatC1-128 remained relatively constant (Fig. 2C, bottom). A plot of Kat1-C128 in pellet versus supernatant and subsequent fitting of the data with a simple quadratic equation generated a K_d of 5.01 ± 0.78 μM (Fig. 2D).

The peptide of ^{74}YIKRLRLCIRWFQ^{86} in KatC tail domain is responsible for microtubule binding. Microtubule cosedimentation assays show that KatC has an ATP-insensitive microtubule-binding site in the N-terminal region. To further identify the precise region responsible for this

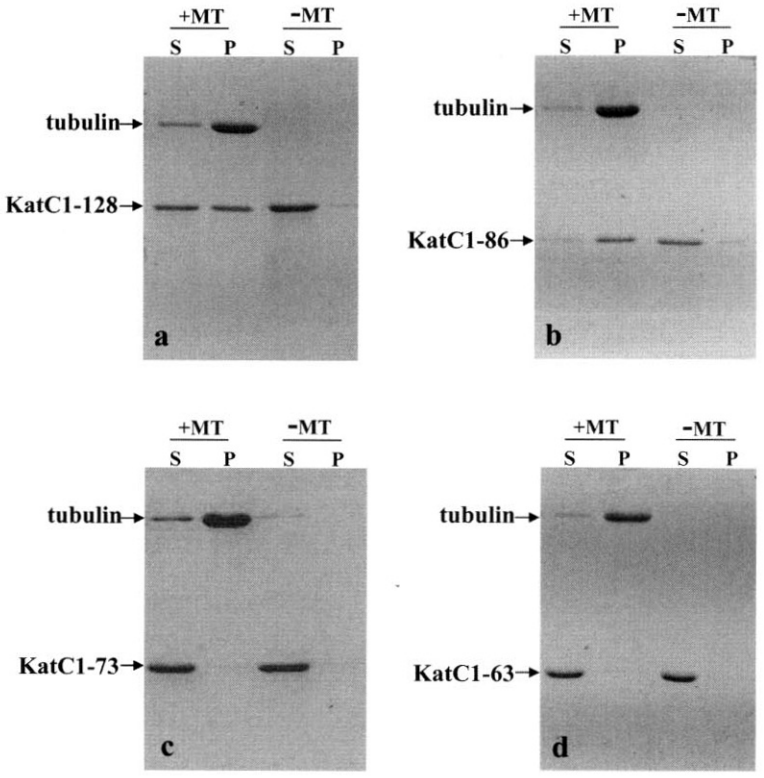

Fig. 3. Microtubule cosedimentation assays of different KatC N-terminal polypeptides. The molar ratio of tubulin:protein (a: KatC1-128, b: KatC1-86, c: KatC1-73, d: KatC1-63) is 1 : 1. Supernatant (s) and pellet (p) fractions in the presence (+MT) and absence (-MT) of microtubules were separated on 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue, indicating that only KatC1-128 and KatC1-86 appeared in the pellet. MT: microtubule.

Fig. 4. Sequence alignment of tail domains of KatB and KatC. The identical amino acid residues for both sequences are highlighted with black background and the conserved amino acid residues are shaded.

interaction, 4 cDNAs encoding various lengths of the KatC tail domain were cloned, expressed, and purified. His-tagged fusion proteins with these different deletions were assessed for their microtubule-binding capabilities using a cosedimentation assay. As shown, KatC1-128 (residues 1-128), and KatC1-86 (residues 1 to 86), but not KatC1-73 (residues 1 to 73) or KatC1-63 (residues 1 to 63), have interactions with taxol-stabilized microtubules (Fig. 3). In the absence of microtubules, little fusion protein was found in the pellet fraction. These data suggest that the peptide corresponding to residues 74-86 (^{74}YIKRLRLCIRWFQ^{86}) of the KatC tail domain is responsible for microtubule binding.

KatB tail domain interacts with microtubules in a KatC-like manner. KatB and KatC are highly homologous kinesin-related proteins. Besides an 89.5% amino acid identity in the motor domains (Mitsui *et al.*, 1994), the tail domains show 83% amino acid identity (Fig. 4). The region responsible for their interaction with microtubules (YIKRLRLCIRWFQ) is even more conserved. Therefore, we assessed whether KatB tail domain could interact with microtubule in a similar manner as KatC. We constructed, expressed and purified three different length polypeptides of KatB (Fig 5A, 5B). Microtubule cosedimentation assays show that KatB1-385 (residues 1 to 385) and KatB1-77 (residues 1 to 77), but not KatB1-64 (residues 1 to 64) cosedimented with taxol-stabilized microtubules in the absence or presence of either AMP-PNP or ATP (Fig. 5C), indicating that KatB, as well as KatC, has an ATP-insensitive microtubule-binding site in the N-terminal region. The microtubule-binding site is at amino acid residues 65 to 77 (^{65}YVKRLRLCIRWFQ^{77}). When KatB1-385 was mixed with NHS-Rhodomine labeled microtubules in the absence or

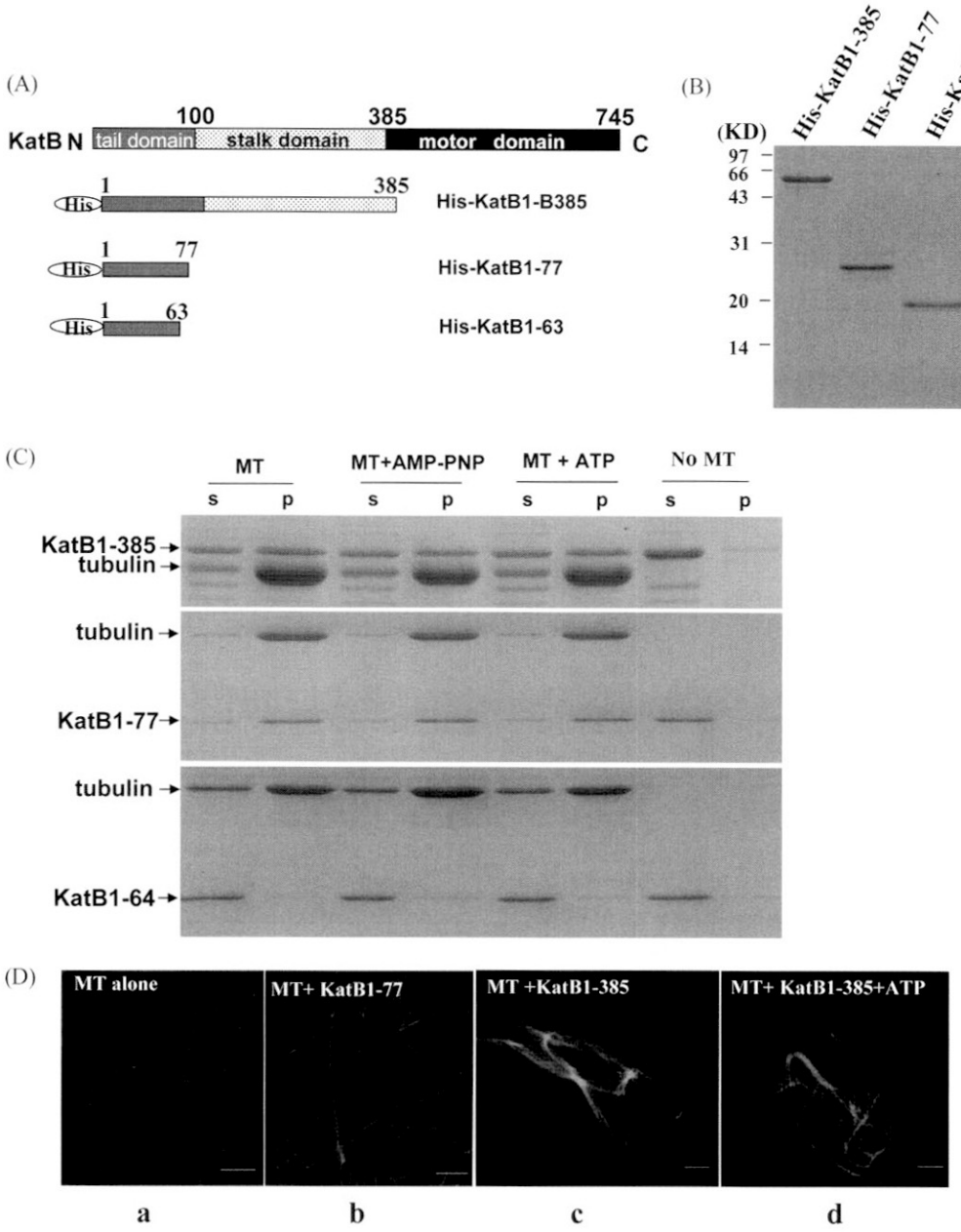

Fig. 5. KatB has an ATP-insensitive microtubule-binding site in the tail domain similar to that of KatC. (A) The domain structures of the Arabidopsis KatB and constructs of three different His-tagged fusion proteins. Numbers refer to corresponding amino acids. (B) SDS-PAGE (15% gel) analysis of truncated His-tagged KatB N-terminal polypeptides. Numbers on the left indicate the position of the molecular weight markers. (C) cosedimentation assays of KatB1-385 (top), KatB1-77 (middle) and KatB1-66 (bottom) with microtubules, respectively. Purified fusion proteins were incubated with microtubules in the absence or presence of either AMP-PNP or ATP. Resulting proteins in supernatant(s) and pellet (p) are shown. (D) Images observed by fluorescence microscopy when KatB1-77 and KatB1-385 were mixed with NHS-Rhodomine labeled microtubules. a. Microtubules polymerized in the absence of fusion proteins. b. Microtubules were still kept in single filaments in the presence of KatB1-77. c. Microtubule bundles were induced by KatB1-385. d. Microtubule bundles induced by KatB1-385 were insensitive to ATP. MT: microtubule; Bar = 10 μm.

presence ATP, bundles of microtubules were also observed (Fig. 5D, c d), reminiscent of those induced by KatC1-C394. KatB1-77, however, could not bundle microtubules. Rather, all microtubules appeared as single individuals similar to those induced by KatC1-128 (Fig. 5D, b).

***In vitro* interaction between KatB and KatC.** Dimerization is commonly observed in kinesins. Because both KatB and KatC have obvious predicted coiled-coils in their stalk domains, it is hypothesized that they may dimerize. In addition, our results showing that microtubule bundles are induced by

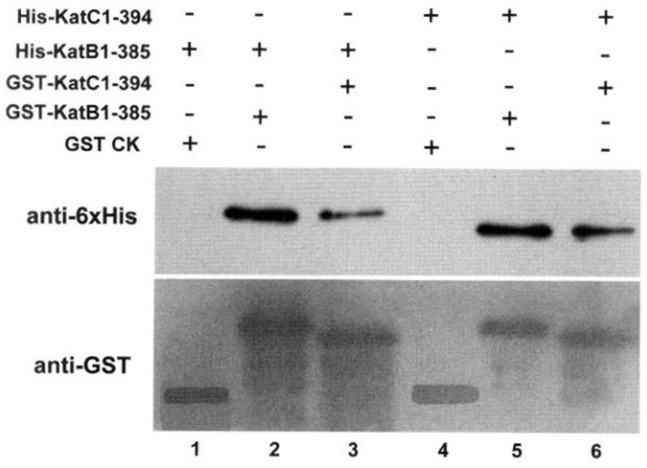

Fig. 6. The interaction between KatB and KatC *in vitro*. Baits: GST-KatB1-385 (lane 2, 5) and GST-KatC1-394 (lane 3, 6). Preys: His-KatB1-385 (lane 1, 2, 3) and His-KatC1-394 (lane 4, 5, 6). GST was used as the control bait (lane 1, 4). Duplicate membranes were probed with anti-GST antibody (top) and anti-6×His (bottom).

KatB1-385 and KatC1-394, but not KatB1-77 and KatC1-128, suggest a possible dimerization *in vitro*. To determine whether the polypeptides with coiled-coil stalk region could interact, GST- and His-tagged fusion proteins were expressed in and purified from *E. coli*. When GST-KatB1-385 (bait) was precipitated with glutathione, 6×His-KatB1-385 (prey) and 6 × His-KatC1-394 (prey) both cosedimented (Fig. 6, lanes 2, 5). A similar phenomenon was found when GST-KatC1-394 was used as bait (Fig. 6, lanes 3, 6). Neither the prey 6 × His-KatB1-385 nor 6 × His-KatC1-394 cosedimented with glutathione in the presence of GST control bait (Fig. 6, lanes 1, 4). These results clearly indicate that KatB and KatC interact specifically with themselves and with each other *in vitro*.

Discussion

This paper reports a previously uncharacterized microtubule-binding site in the tail domains of both KatB and KatC of *Arabidopsis thaliana*. Our results suggest that this binding is specific, although all proteins used in microtubule interactions were modified with a His-tag. Our conclusion, however, is based on our clear findings that His-KatC1-394, His-KatB1-385, His-KatC1-128, and His-KatB1-77, cosediment with microtubules, but under identical conditions, His-KatC111-394, His-KatC1-73, His-KatC1-63 and His-KatB1-64 do not. This binding capacity is also evidenced from the quantitative measure of His-KatC1-128 binding to microtubules, in which KatC1-128 (pellet) remains relatively constant while total protein increases from 10 μM to 25 μM (Fig. 2C, 2D).

Unlike the kinesin motor domain, the interaction of the tail domain of KatB and KatC with microtubules is ATP-insensitive (Fig. 2A, 2B, 5C, 5D). ATP-insensitive microtubule-binding sites in the tail domain have been detected in kinesin-1 (conventional kinesin), Kid, Ncd and Kar3 (Meluh and Rose, 1990; Karabay and Walker, 1999; Shiroguchi *et al.*, 2003; Straube *et al.*, 2006). There is no sequence similarity among the tail domains of these kinesins, although the microtubule-binding sites are highly conserved in their motor domain of these proteins. It has been reported that a high percentage of basic amino acids are found in the tail domains of Kid, Ncd and Kar3, and hypothesized that these regions may play important roles in microtubule-binding (Meluh and Rose, 1990; Karabay and Walker, 1999; Shiroguchi *et al.*, 2003). In fact, many microtubule-associated proteins such as MAP2 and tau also have abundant basic amino acids in their microtubule-binding domains (Lewis *et al.*, 1988; Goode *et al.*, 1997). Consistent with this, there is a high percentage of basic amino acids in the tail domain of *Arabidopsis* KatB and KatC (17% and 16% respectively). We expressed 4 different polypeptides of the tail domain in *E.coli* to identify precise region of the KatC tail domain involved in ATP-insensitive binding to microtubules. Microtubule cosedimentation assays indicated that the residues Tyr74 to Gln86 (^{74}YIKRLRLCIRWFQ^{86}) are important for microtubule-binding (Fig. 3). Compared with most known kinesins, the microtubule binding affinity of KatC tail domain is much lower. The K_d values calculated for the microtubule binding of His-KatC1-128 ($K_d = 5.01 \pm 0.78$ μM) are much higher than that of the Ncd ($K_d = 0.3 \pm 0.05$ μM) and Kar3 ($K_d = 0.2 \pm 0.05$ μM) motor domain (Mackey *et al.*, 2004), and that of the Ncd tail domain with two microtubule-binding sites ($K_d = 0.13 \pm 0.05$ μM), but 1.5 times lower than that of the second binding site of the Kid tail domain ($K_d = 12 \pm 4$ μM) (Shiroguchi *et al.*, 2003). The weaker binding site of KatC tail domain may enhance the affinity of the proteins to microtubules in an easily controlled level for the easier moving motor domain along microtubules between them. It has previously been reported that prolines adjacent to basic amino acid can enhance microtubule-binding (Lewis *et al.*, 1988; Goode *et al.*, 1997; Karabay and Walker, 1999). The KatC polypeptide YIKRLRLCIRWFQ has 4 basic amino acids, but no adjacent proline-rich region, which perhaps explains the weak binding affinity of the KatC tail domain to microtubules. The polypeptide YIKRLRLCIRWFQ is highly conserved between the tail domains of KatB and KatC, so we assessed whether KatB interacted with microtubules at the same site. Our results support this hypothesis (Fig. 5C). The fact that KatB and KatC interact with microtubules at the same site *in vitro* suggests their cooperative and/or redundant roles *in vivo*.

Many proteins with microtubule-binding sites can induce microtubule bundling (Andrews *et al.*, 1993; Chan *et al.*, 1999; Karabay and Walker, 1999). Generally speaking, microtubule bundles are induced by at least two microtubule-binding sites. In our study, the polypeptides of KatC1-128 and KatB1-77, each containing the tail domain alone, can not induce microtubule bundling, but KatC1-394 and KatB1-385

containing the stalk domain responsible for dimerization, do induce microtubule bundling (Fig. 2B, 5D), indicating that the bundles we observed may be induced by dimerization of KatC1-394 and KatB1-385. This was confirmed by pull-down experiments (Fig. 6). In addition, our results show that KatB and KatC can interact with each other *in vitro* (Fig. 6), providing further evidence of a cooperative or redundant role for KatB and KatC *in vivo*. It has been reported that some kinesin-14 members containing microtubule-binding sites in their tail domains, such as Ncd, KAR3 and ATK5, cross-link antiparallel microtubules *in vivo* through a mechanism in which the motor domain interacts with one microtubule, and the tail domain interacts with a second microtubule (Meluh and Rose, 1990; Karabay and Walker, 1999; Ambrose *et al.*, 2005). As two members of kinesin-14 family, KatB and KatC may function in a similar mechanism during mitosis. Future research on the subcellular localization and phenotypes of single or double mutants will further reveal the functions of these two kinesins with a second microtubule-binding site in tail domain.

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