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Bundling of Microtubules by Motor and Tail Domains of a Kinesin-like Calmodulin-Binding Protein from *Arabidopsis:* Regulation by Ca^{2+}/Calmodulin

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Kinesin-like calmodulin-binding protein (KCBP), a novel kinesin-like protein from plants, is unique among kinesins and kinesin-like proteins in having a calmodulin-binding domain adjacent to its motor domain. KCBP localizes to mitotic microtubule (MT) arrays including the preprophase band, the spindle apparatus, and the phragmoplast, suggesting a role for KCBP in establishing these MT arrays by bundling MTs. To determine if KCBP bundles MTs, we expressed C-terminal motor and N-terminal tail domains of KCBP, and used the purified proteins in MT bundling assays. The 1.5 C protein with the motor and calmodulin-binding domains induced MT bundling. The 1.5 C-induced bundles were dissociated in the presence of Ca^{2+}/calmodulin. Similar results were obtained with a 1.4 C protein, which lacks much of the coiled-coil region present in 1.5 C protein and does not form dimers. The N-terminal tail of KCBP, which contains an ATP-independent MT binding site, is also capable of bundling MTs. These results, together with the KCBP localization data, suggest the involvement of KCBP in establishing mitotic MT arrays during different stages of cell division and that Ca^{2+}/calmodulin regulates the formation of these MT arrays.

MTs, one of the three major components of cytoskeleton, have been shown to play an important role in a variety of cellular functions during cell division, cell growth and cell differentiation in plants. In dividing plant cells, four distinct arrays of MTs appear sequentially in a cell cycle-dependent manner (1). Cell division in plants, although similar to animals in most aspects, differs in not having well-defined centrosomes and in possessing unique MT arrays such as the preprophase band and the phragmoplast (2). How the MTs are organized into various arrays and the molecular mechanisms that regulate spatial organization of MT arrays in plants are poorly understood. The members of the kinesin superfamily which consists of kinesins and a large number of related proteins called kinesin-like proteins (KLPs) are involved in controlling many functions associated with MTs and their dynamics and organization (3). KLPs have a conserved motor domain, which is located either at the N-terminus, C-terminus or in the center (4). Several plus- and minus-end MT motors have been shown to be necessary for spindle formation (5).

In a screen for calmodulin-binding proteins from *Arabidopsis,* we isolated a unique kinesin-like protein, kinesin-like calmodulin-binding protein (KCBP) (6). Although the KCBP contains three distinct regions (a motor domain, a coiled-coil stalk and a tail) that are found in most kinesin-like proteins, it has some domains that are not found in known kinesins or kinesin-like proteins. These include (i) a calmodulin-binding domain adjacent to the motor domain at the C-terminus, and (ii) a myosin tail homology region (MyTH4) in the N-terminal tail which suggests a possible interaction of KCBP with actin cytoskeleton (7). The calmodulin-binding domain allows KCBP to interact with calmodulin in a Ca^{2+}-dependent fashion and the binding of calmodulin to KCBP inhibits its interaction with MTs (8, 9). KCBP, like other C-terminal kinesins, is a minus-end directed motor with a velocity similar to *Drosophila* Ncd (10). A homologue of KCBP has been found in phylogenetically divergent plant species in dicots and monocots (11, 12, 17, Abdal-Ghany and Reddy, unpublished results). A homologue of KCBP has not been found in non-plant systems including *S. cerevisiae* and *C. elegans* whose genomes have been completely sequenced. However, a calmodulin-binding kinesin was identified recently in sea urchin cells (13). In phylogenetic trees, KCBP constitutes a distinct group within the C-terminal family of motors

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(14, 15). KCBP localizes to mitotic MT arrays including the preprophase band and the phragmoplast (16, 17). Furthermore, KCBP protein accumulates in a cell cycle-dependent manner to a high level in M phase and declines as the cells enter interphase (16). An essential role of KCBP in *Arabidopsis* trichome morphogenesis has also been reported (18). This study suggests KCBP involvement in cell expansion since the trichomes are unicellular structures whose development involves primarily cell expansion, a process in which MTs play a critical role (19). At least three other kinesin-like proteins have been reported in *Arabidopsis* and several more are found in the partially sequenced *Arabidopsis* genome, suggesting that there is a large family of kinesin-like proteins in *Arabidopsis* (20). A plus-end directed KLP (TKRP125) of the bimC subfamily was isolated from tobacco cells (21). Two C-terminal motors (KCBP and Kat A) and one N-terminal motor (TKRP-125) from plants localize to mitotic MT arrays, suggesting their involvement in cell division (16, 17, 21, 22). Colocalization of KCBP with MT arrays suggests that it may have a role in forming these arrays by bundling MTs. To determine if KCBP has MT bundling activity, purified proteins of KCBP were incubated with Taxol-stabilized MTs and examined by video-enhanced differential interference contrast (VE-DIC) microscopy. The results presented here demonstrate that the KCBP tail and motor domains are directly capable of bundling MTs *in vitro* and the motor domain-induced bundling is sensitive to Ca^{2+}/calmodulin. KCBP is the first kinesin-like protein to cause MT bundling in plants.

MATERIALS AND METHODS

Expression and purification of different regions of KCBP. *Arabidopsis* 1.4 C protein of KCBP was expressed using the pET28 system whereas 1.5 N and 1.5 C proteins were expressed in pET32. Figure 1 shows the domains present in each of the expressed proteins. Construction of plasmids was described previously (6, 8, 23). The 1.4 C and 1.5 C proteins were purified either by calmodulin-Sepharose affinity column or Talon metal affinity column (8, 23). The 1.5 N protein was purified using His-Bind metal chelation resin as described previously (9, 23). Proteins purified with calmodulin-Sepharose were dialyzed against a buffer containing 50 mM Tris, pH 7.5, and 150 mM NaCl. The purity of the proteins was assessed by Coomassie blue staining of gels and by probing the blots with appropriate antibodies or biotinylated calmodulin (23).

Expression and purification of MC1 and MC6. Construction of MC1 and MC6 pET plasmids containing the *Drosophila* motor domain was described by Chandra *et al.* (24). MC1 contains 492 residues corresponding to amino acids 209–700 of Ncd. MC6 encodes amino acids 333–700 of Ncd, which contains a motor domain and a short coiled-coil stalk. Induction and purification of MC1 and MC6 was performed as described earlier (25).

MT bundling assay. Tubulin was purified according to Walker *et al.* (26). Bundling assays were done in AB buffer (20 mM Pipes, pH 6.9, 1 mM Mg SO_4, 1 mM EGTA) containing 50 mM NaCl, 40 μM Taxol, 5 mM Mg AMP-PNP, 1 or 5 μM tubulin as Taxol-stabilized MTs, and purified motor or tail protein. Calcium and/or calmodulin were added to the appropriate reactions. After 30 min of incubation, MTs were observed by video-enhanced differential interference contrast microscopy.

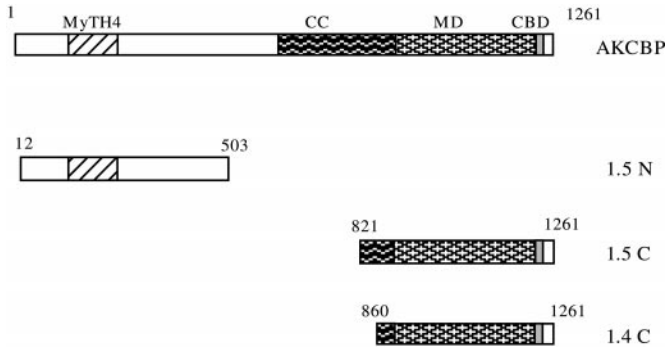

FIG. 1. Diagram showing the domains in bacterially expressed proteins of *Arabidopsis* KCBP. AKCBP: The full-length *Arabidopsis* kinesin-like calmodulin-binding protein showing various domains (6). MyTH4, a myosin tail homology region; CC, coiled-coil region; MD, motor domain; CBD, calmodulin-binding domain. 1.5 N: Fusion protein containing the N-terminal region of KCBP. 1.5 C: The C-terminal fusion protein of KCBP containing the MD, CBD, and a limited coiled-coil stalk. 1.4 C: The C-terminal fusion protein containing a short coiled-coil stalk, MD, and CBD.

RESULTS

We expressed different parts of KCBP in a bacterial system and used the purified proteins in a MT bundling assay. Three truncated proteins of KCBP (1.5 N, 1.5 C and 1.4 C) were used in the present study. The 1.5 N protein has the amino-terminal region (amino acids 12 to 503) of KCBP. Proteins 1.5 C and 1.4 C contain the C-terminal regions of KCBP. The 1.5 C protein with amino acids 821–1261 contained the motor domain with ATP- and MT-binding sites, a 23-amino acid calmodulin-binding domain, and ~70 amino acids from the predicted coiled-coil stalk (see Fig. 1). The 1.4 C (amino acids 860–1261) protein is similar to 1.5 C except that it has a shorter coiled-coil stalk. Purified protein from each of these constructs was obtained as described previously (8, 9, 23). Expression of a full-length KCBP cDNA using pET 28 expression system resulted in degradation of full-length protein into two smaller products (23). Hence, we could not perform bundling assays with the full-length KCBP.

The 1.5 C protein with motor and calmodulin-binding domains of KCBP bundles MTs. To investigate if the C-terminal motor of KCBP is capable of bundling MTs, Taxol-stabilized MTs were incubated in the presence or absence of 1.5 C protein, and then examined by video-enhanced differential interference contrast microscopy (VE-DIC). In the absence of protein, MTs were randomly distributed and remained as single microtubules (Fig. 2A). Addition of 1.5 C protein to Taxol-stabilized MTs caused extensive bundling of MTs where MTs appeared as thick cables due to clus-

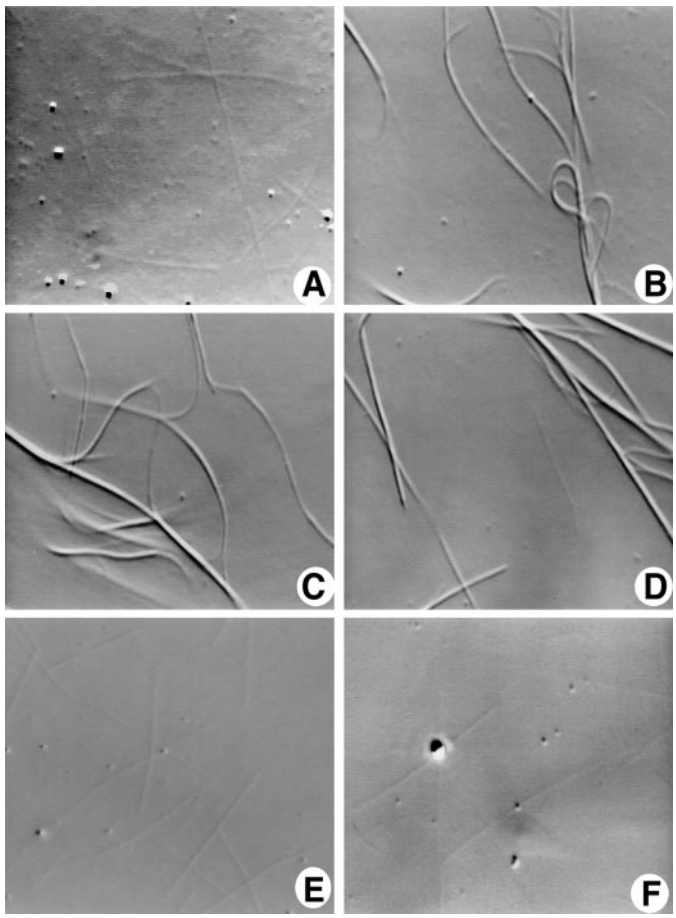

FIG. 2. 1.5 C protein induces MT bundling. Taxol-stabilized MTs (5 μM) were incubated with 1.5 C protein (5 μM) in the presence or absence of Ca^{2+} (20 μM) and/or calmodulin (15 μM) for 30 min and observed by VE-DIC microscopy. (A) MTs, (B) MTs plus 1.5 C protein, (C) MTs plus 1.5 C protein and Ca^{2+}, (D) MTs plus 1.5 C protein and calmodulin, (E) MTs plus 1.5 C protein and Ca^{2+}/calmodulin, (F) MTs plus 1.5 C protein and 5 mM ATP. The width of these and all other VE-DIC micrographs is 26 μm.

tering of MTs (Fig. 2B). The bundling was observed at equimolar concentrations of tubulin and motor protein. The bundles were long and several times the diameter of single MT. The 1.5 C protein did not cause bundling in the presence of ATP (Fig. 2F). MTs incubated under the same conditions with BSA have not shown bundling activity (data not shown).

Ca^{2+}/calmodulin dissociated 1.5 C-induced MT bundles. Previous studies with the 1.5 C protein in motility and MT pelleting assays have shown that Ca^{2+}/calmodulin together inhibit the binding of KCBP to MTs (8–10). Furthermore, Ca^{2+}/calmodulin can disassociate preformed KCBP-MT rigor complexes formed in the presence of AMP-PNP (8). Based on these data we hypothesized that Ca^{2+}/calmodulin would affect the KCBP-induced MT bundling. Addition of Ca^{2+}/calmodulin together to the bundling assay resulted in dissociation of MTs (Fig. 2E). Calcium alone or calmodulin alone in the reaction did not dissociate KCBP-induced MT bundles (Figs. 2C and 2D). These results suggest that Ca^{2+}/calmodulin regulates the MT bundling activity of KCBP.

1.4 C protein has MT bundling properties similar to 1.5 C protein. The 1.5 C protein has a coiled-coil stalk region of about 70 amino acids and this region allows it to form dimer (8). It is likely that in dimeric form each head interacts with a different MT resulting in bundling. To determine if the bundling caused by 1.5 C protein requires dimerization, we used a shorter version (1.4 C protein) of KCBP in bundling assays. The 1.4 C protein has a very short stalk (about 30 amino acids) and elutes as a monomer on gel-filtration column (data not shown). Motor domains of other proteins that contain short stalk regions as in 1.4 C protein were found to be monomers (24, 27, 28). As shown in Fig. 3A, the 1.4 C protein is also capable of bundling MTs. Addition of Ca^{2+} alone or calmodulin alone had no effect on bundling (Figs. 3B and 3C), whereas Ca^{2+}/calmodulin together "unbundled" 1.4 C-induced bundles (Fig. 3D). These results suggest that dimerization of motor domain is not required for bundling activity. Inclusion of 5 mM ATP also disrupted MT bundling (data not shown). However, Ca^{2+}/calmodulin was more effective than ATP in dissociating MT bundles into single MTs.

Drosophila motor domain (MC1 or MC6) bundles MTs. MT bundling by KCBP motor domain suggests that either the KCBP motor is unique in inducing MT bundling or it is a property of the motor domain of other KLPs also. However, it is not known whether the motor domain of other C-terminal motors is also capable of bundling MTs. To investigate if the motor domain of other C-terminal motors is capable of bundling MTs, we used two proteins, MC1 and MC6, of *Drosophila* Ncd (24). The MC1 protein with amino acids 209–700 contains the motor domain and much of the α-helical coiled-coil region, whereas the MC6 protein (amino acids 333–700) contains the motor domain and a short (~23 amino acids) stalk. Bacterially expressed MC1 and MC6 proteins were shown to exist as dimers and monomers, respectively (24). Both MC1 and MC6 proteins induced MT bundling (data not shown). These results suggest that the Ncd motor domain in dimeric as well as monomeric form is capable of MT bundling. In the presence of 5 mM ATP, MC1 or MC6 did not bundle MTs. Furthermore, Ca^{2+}/calmodulin had no effect on MT bundling caused by MC1/MC6 (data not shown). This is expected since Ncd, unlike KCBP, does not bind calmodulin (Reddy and Endow, unpublished data). Recently, a monomeric form of *Drosophilia* kinesin motor domain (K366), a plus-end motor, was also found to cause extensive bundling of MTs (28). Taken together, these results strongly suggest that the motor

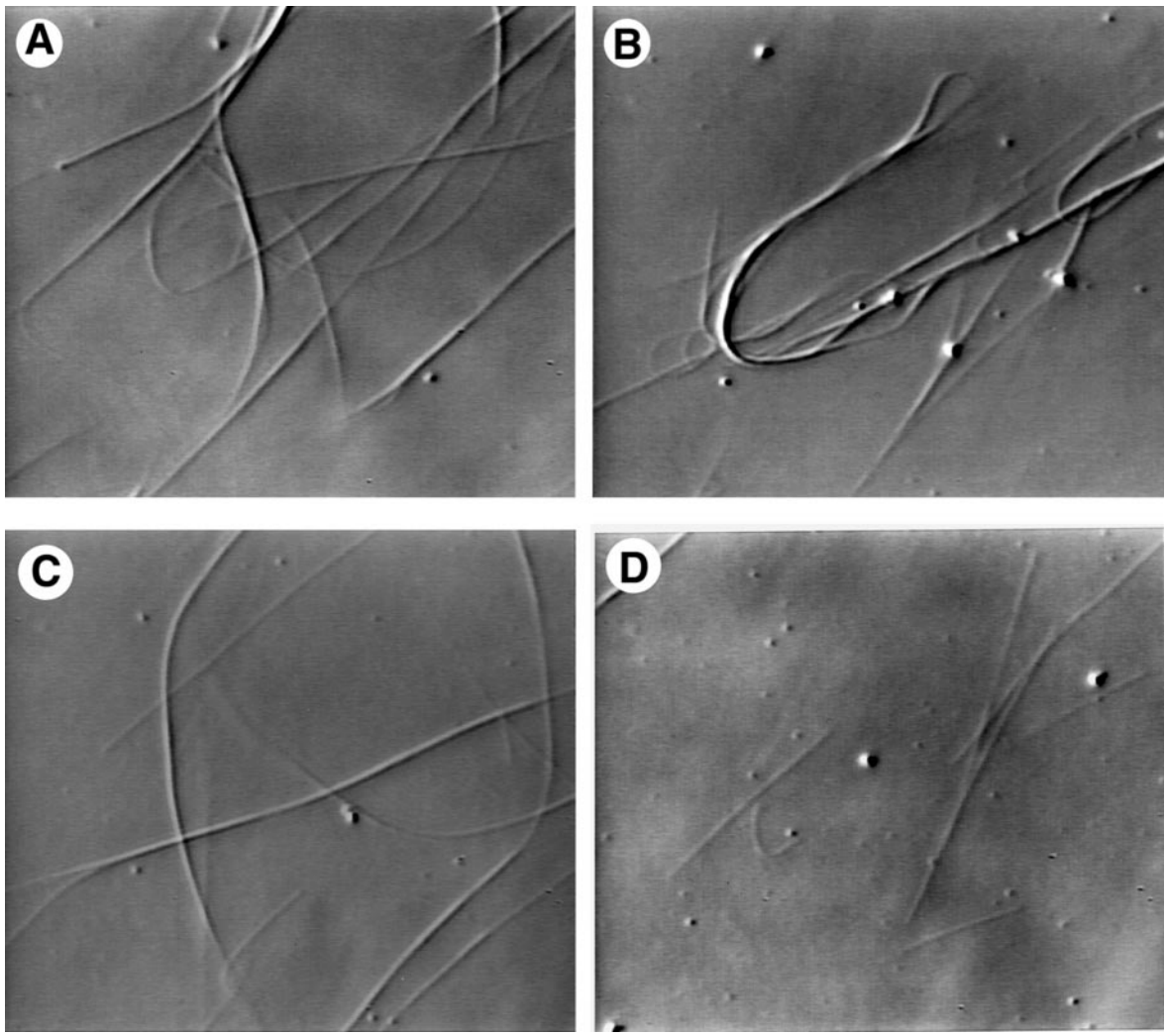

FIG. 3. MT bundling by 1.4 C protein. Taxol-stabilized MTs (5 μM) were incubated with 1.4 C protein (5 μM) in the presence or absence of Ca^{2+} (20 μM) and/or calmodulin (15 μM) for 30 min and observed by VE-DIC microscopy. (A) MTs plus 1.4 C protein, (B) MTs plus 1.4 C protein and Ca^{2+} (20 μM), (C) MTs plus 1.4 C protein and calmodulin (15 μM), (D) MTs plus 1.4 C protein and Ca^{2+}/calmodulin (20 μM/15 μM).

domains of most kinesins and KLPs are likely to exhibit MT bundling activity.

N-terminal region (1.5 N) of KCBP is capable of MT bundling. A previous study with 1.5 N protein in MT pelleting assays has indicated the presence of an ATP-independent MT binding site in the N-terminal tail (9). The N-terminal tail of Ncd contains an ATP-independent MT binding site and causes bundling of MTs (24). Similar MT binding sites in the tail domain of Kar3 and kinesin heavy chain have been reported (24, 29, 30). Our observation that KCBP contains an ATP-insensitive MT binding site in the tail prompted us to investigate if it is capable of bundling MTs. As shown in Fig. 4A, Taxol-stabilized MTs did not cluster or bundle, and remained as single microtubules. However, addition of the 1.5N KCBP to the assay mixture caused bundling of MTs (Fig. 4B). Microtubles appeared as cables due to clustering of MTs. However, the bundling is not as extensive as the bundling observed with motor domain constructs (compare Fig. 4 with Figs. 2 and 3). This is likely due to low amount of 1.5 N protein in the bundling assay. Because of the low yield of 1.5 N protein and its tendency to aggregate (9) we were not able to perform the bundling assays at higher concentrations of motor. MTs bound to a coverslip coated with 1.5 N protein (data not shown), confirming our earlier observation that the tail has a microtubule-binding site. The knobs/bumps on the micrographs are due to some precipitates in the protein preparation. Addition of Ca^{2+} and calmodulin had no effect on 1.5 N bundling activity (Fig. 4C). As mentioned above, the N-terminal tail of Ncd exhibits the ability to bundle MTs (24). However, it is worth noting that the tail domain in KCBP is much longer (about

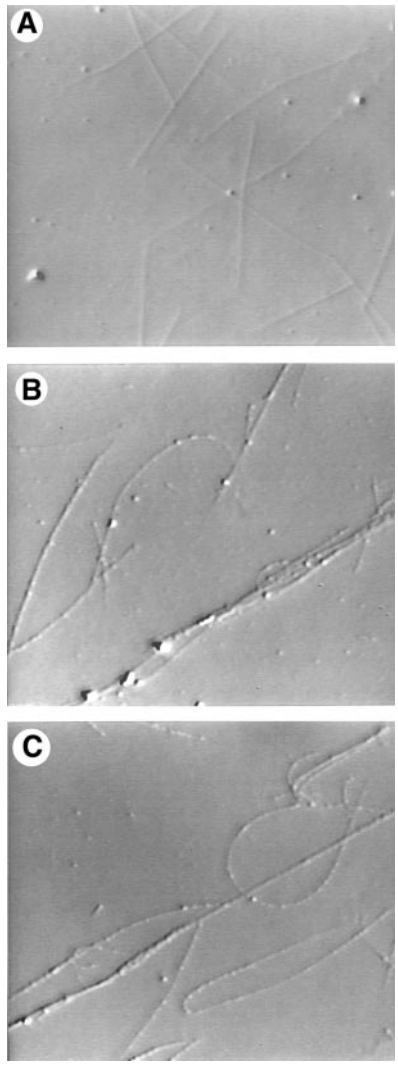

FIG. 4. The N-terminal (1.5 N) tail of KCBP is capable of MT bundling. Taxol-stabilized MTs (1 μM) were incubated in the presence or absence of 1.5 N protein (0.1 μM) for 30 min and observed by VE-DIC microscopy. (A) MTs, (B) MTs plus 1.5 N protein, (C) MTs plus 1.5 N protein and Ca^{2+}/calmodulin (1.25 mM/5 μM).

400 amino acids longer than Ncd tail) and there is no amino acid sequence similarity between the tail domains of KCBP and Ncd or other KLPs.

DISCUSSION

The C-terminal motor (1.5 C and 1.4 C) and N-terminal tail (1.5 N) proteins of KCBP promote redistribution of microtubules from a random pattern into parallel arrays, indicating that there are two regions within KCBP that are directly capable of MT bundling. It would be interesting to perform MT bundling studies and Ca^{2+}/calmodulin effect on MT bundles using the full-length KCBP. However, we were not successful in obtaining the full-length protein using bacterial expression system due to cleavage of the expressed protein into two smaller proteins (23). The MT bundling caused by 1.5 N protein is not sensitive to Ca^{2+}/calmodulin whereas 1.5 C- and 1.4 C-induced MT bundling is sensitive to Ca^{2+}/calmodulin as these bundles are dissociated in the presence of Ca^{2+}/calmodulin. Two proteins representing the monomeric and dimeric forms of Ncd, another minus-end motor, also caused MT bundling. These studies with KCBP and Ncd, and previous observations that the head of kinesin, in monomeric form, can also bundle microtubules (28) indicate that the motor domain of KLPs is capable of MT bundling in monomeric as well as dimeric states.

Some kinesin-like proteins in non-plant systems are also involved in MT dynamics and bundling. *Drosophila* Ncd has been shown to have MT bundling activity (31). More recently, it was shown that the N-terminal tail of Ncd, which contains an ATP-independent MT binding site, causes MT bundling (24). Kar 3, also a C-terminal motor from *Saccharmyces cerevisiae,* destabilizes MTs preferentially at their minus ends (32). Kinesin has been shown to cause MT bundling and individual kinesin molecules can form stable crossbridges between MTs (33, 34). Recently, multi-headed kinesin was shown to crossbridge MTs and organize MTs into dynamic asters (35). Kinesin, in addition to an MT binding site in the motor domain, contains a second MT-binding site in the N-terminal tail (30). It is proposed that two MT-binding sites on a single molecule could crossbridge MTs to cause bundling or sliding of MTs (30, 33). However, recently motor domain alone, in monomeric form, has been shown to bundle MTs, indicating the existence of other mechanisms by which kinesin promotes bundling (28). A rigor mutant of human Eg5 motor in dimeric form and the motor domain of mitosis-specific KLP were also found to induce MT bundling (27, 36).

The mechanism by which the motor domain causes MT bundling is not known. The possible mechanisms include (i) crossbridging of MTs by two ATP-sensitive MT binding sites (one in each head) present in the dimer, (ii) presence of two MT binding sites in each head that can crossbridge MTs, and (iii) a change in the MT surface charge by motor binding thereby permitting the interaction between MTs (37). Since both dimeric (1.5 C) and monomeric (1.4 C) proteins are capable of inducing MT bundling, it is likely that dimerization of KCBP motor domain is not required for MT bundling activity. This is in contrast to HsEg5 motor which bundles MTs only in dimeric state (27). There is no evidence currently for the presence of two MT binding sites in the motor domain. Hence, it is likely that the KCBP-induced MT bundling is mediated by a change in the surface charge of MTs leading to an increase in the lateral interactions between MTs. The C-terminal region of tubulin subunits is acidic, highly negatively charged and exposed on the surface

of the MTs (38). Recently, it has been shown that positively charged amino acids in the motor domain are critical for binding of motor to MTs, suggesting electrostatic interaction between positively charged residues in the motor domain and negatively charged residues at the C terminus of tubulin molecule (39). Other studies have also shown that the C-terminal sequences of tubulin play an important role in kinesin binding (40). The binding of KCBP motor domain, which is basic (p*I* 8.7) and therefore positively charged at physiological pH, to tubulin could affect the surface charge of MTs thereby masking tubulin domains (negative charges on the MT surface) that otherwise inhibit microtubule annealing. The 1.5 N protein is likely to exist as a monomer as it does not contain the stalk region. This implies that the 1.5 N-induced bundling may not involve crossbridging of MTs. The binding of 1.5N to MTs may also change the surface charge on tubulin and promote bundling. The smallest N-terminal protein of KCBP that can bind to microtubules in MT pelleting assay is basic with a p*I* of 9.0 (9), suggesting that it interacts with exposed acidic regions of tubulin on MT interface. In addition, two MT binding sites in KCBP, one in the tail and the second one in the motor domain, should also allow it to cross-link two MTs. This cross-linking activity together with its minus-end motor activity are likely to be important for *in vivo* function of KCBP in establishing bipolar spindles (10, 17).

The mechanisms that regulate the spatial organization of MTs in plant cells are not well understood. The ability of KCBP motor domain to bundle MTs is likely to be important to its *in vivo* function. Based on our results we propose that KCBP induces MT bundling (array) in plants by promoting lateral association between MTs. We further propose that the establishment of MT arrays in plant cells is regulated by Ca^{2+}, a universal messenger in eukaryotes, through calmodulin (41, 42). The levels of free Ca^{2+} during different stages of the plant cell cycle are not known to correlate with the establishment of various MT arrays. However, Hepler and Callaham (43) measured free Ca^{2+} in *Tradescantia* stamen hair cells from mid metaphase through cytokinesis. They detected an increase in cytosolic Ca^{2+} for 10–15 min from the onset of anaphase and a decline in Ca^{2+} concentration prior to phragmoplast formation. It is likely that this decline in Ca^{2+} level, prior to phragmoplast formation, could permit KCBP mediated bundling of MTs to form the phragmoplast. Calcium and calmodulin have been implicated in several aspects of cytoskeletal organization and cell division in plants (44–47). Calmodulin is implicated in stabilizing cortical MTs in carrot at low Ca^{2+} concentration and destabilize them at higher Ca^{2+} concentrations (47, 48). Immunocytochemical studies showed calmodulin localization to cortical MTs and mitotic MT arrays in plant cells (49–52). However, a recent study raised questions about calmodulin localization to mitotic MT arrays (53). Because calmodulin does not bind to tubulin (11, 54), localization of calmodulin to MTs is most likely due to interaction with MT associated calmodulin-binding proteins such as KCBP or other MAPs.

In summary, we have demonstrated MT-bundling activity of the KCBP tail and motor domains and regulation of motor domain-induced bundling by Ca^{2+}/calmodulin. KCBP is the first plant MT motor known to induce MT bundling. Furthermore, our finding that Ca^{2+}, through calmodulin, regulates this process has important implications in signal-induced changes in spatial organization of MTs as many hormonal and environmental signals induce changes in cytosolic Ca^{2+}. The data presented here provide evidence for the hypothesis that the organization of microtubules in plant cells involves microtubule motor proteins. Overexpression of motor and tail domains of KCBP in cultured cells should permit *in vivo* analysis of their function in MT organization.

ACKNOWLEDGMENTS

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