The Plant Cell, Vol. 10, 957–965, June 1998, www.plantcell.org

Characterization of Microtubule Binding Domains in the Arabidopsis Kinesin-like Calmodulin Binding Protein

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The kinesin-like calmodulin binding protein (KCBP) is a new member of the kinesin superfamily that appears to be present only in plants. The KCBP is unique in its ability to interact with calmodulin in a Ca^{2+}-dependent manner. To study the interaction of the KCBP with microtubules, we expressed different regions of the Arabidopsis KCBP and used the purified proteins in cosedimentation assays with microtubules. The motor domain with or without the calmodulin binding domain bound to microtubules. The binding of the motor domain containing the calmodulin binding region to microtubules was inhibited by Ca^{2+}–calmodulin. This Ca^{2+}–calmodulin regulation of motor domain interactions with microtubules was abolished in the presence of antibodies specific to the calmodulin binding region. In addition, the binding of the motor domain lacking the calmodulin binding region to microtubules was not inhibited in the presence of Ca^{2+}–calmodulin, suggesting an essential role for the calmodulin binding region in Ca^{2+}–calmodulin modulation. Results of the cosedimentation assays with the N-terminal tail suggest the presence of a second microtubule binding site on the KCBP. However, the interaction of the N-terminal tail region of the KCBP with microtubules was insensitive to ATP. These data on the interaction of the KCBP with microtubules provide new insights into the functioning of the KCBP in plants.

INTRODUCTION

Kinesins and kinesin-like proteins (KLPs), a family of microtubule motor proteins, are involved in many dynamic cellular processes, such as transport of vesicles and organelles, nuclear fusion, spindle formation, and chromosome movement during cell division (Bloom and Endow, 1994). In recent years, a large number of KLPs have been identified and characterized in non-plant systems (Barton and Goldstein, 1996). Phylogenetic analysis of the motor domains of kinesin proteins has shown that most members of the kinesin superfamily can be placed into one of eight subfamilies (Moore and Endow, 1996). Although most members of the kinesin superfamily translocate toward the plus end of microtubules, members of the C-terminal family, such as Ncd and KAR3, show minus-end–directed translocation (McDonald et al., 1990; Walker et al., 1990; Endow et al., 1994; Kuriyama et al., 1995). However, it has been demonstrated that the direction of Ncd motor movement can be reversed by fusing it to the tail of a plus-end kinesin from Neurospora, suggesting the importance of domain organization and the tail in determining the direction of motor movement (Cross, 1997; Henningsen and Schliwa, 1997). Similar results were reported by Case et al. (1997) using a chimera in which the motor domain in conventional kinesin is replaced with the motor domain of Ncd.

Information on KLPs and their role in various cellular processes in plants is very limited (Asada and Collings, 1997). Kinesin-related proteins were first detected in plants using the antibodies raised to animal kinesin heavy chain (Tiezzi et al., 1992; Cai et al., 1993; Liu et al., 1994). Recently, three cDNAs (KatA, KatB, and KatC) encoding KLPs with C-terminal motor domains have been isolated from Arabidopsis (Mitsui et al., 1993, 1994). The KatA protein, a minus-end motor, was shown to localize to the mitotic spindle and the phragmoplast (Liu et al., 1996). A plus-end microtubule motor protein in tobacco (TKRP125) also localized to the spindle and the phragmoplast (Asada et al., 1997). In a protein–protein interaction-based screening with calmodulin, we isolated a calmodulin binding protein (KCBP) from Arabidopsis that showed strong sequence similarity to kinesins and KLPs (Reddy et al., 1996b). Homologs of KCBP have been isolated from potato and tobacco (Reddy et al., 1996a; Wang et al., 1996). Using blot overlay assays, we have demonstrated that the KCBP can bind tubulin subunits (Narasimhulu et al., 1997). Antibodies specific to the KCBP recognized a protein of the expected size in Arabidopsis, tobacco, and Haemanthus, suggesting the presence of the KCBP in evolutionarily distant species of higher plants (Bowser and Reddy, 1997; Smirnova et al., 1997).

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Like other kinesins and KLPs, the KCBP contains three distinct domains: a highly conserved motor domain with ATP and microtubule binding sites, a coiled-coil stalk region involved in heavy chain dimerization, and a globular tail region. However, the KCBP has several unique features, suggesting that it is a new member of the kinesin superfamily. Unlike all known KLPs, the KCBP contains a calmodulin binding domain adjacent to the motor domain in the C terminus (Reddy et al., 1996b). In addition, the N-terminal tail domain of the KCBP shows significant sequence similarity to a region found in the tail domain of some myosins (Chen et al., 1996; Reddy et al., 1996b). In vitro motility studies have demonstrated that the KCBP is a minus-end microtubule motor (Song et al., 1997). Phylogenetically, all three KCBPs from plants fall into a group with other minus-end C-terminal motors like Kar3 and Ncd, which are implicated in cell division (Moore and Endow, 1996; Reddy et al., 1998). However, all KCBPs fall into a distinctive group within the C-terminal subfamily (Hirokawa, 1998; Reddy et al., 1998). Immunolocalization studies using antibodies specific to the KCBP showed an association of the KCBP with microtubule arrays in dividing cells, including plant-specific microtubule arrays such as the preprophase band and the phragmoplast, suggesting a role for this protein in the formation and/or functioning of these structures (Bowser and Reddy, 1997). In addition, the KCBP plays an essential role in trichome morphogenesis in Arabidopsis (Oppenheimer et al., 1997).

Regulation of directed movements in microtubule-dependent motor processes can occur by modulating the activity of the molecular motors. Although many (close to 70) KLPs have been identified in non-plant systems, little is known about the mechanisms by which the activity/function of these proteins is regulated in the cell. A few studies suggest that phosphorylation is involved in targeting or localization of some KLPs (Liao et al., 1994; Blangy et al., 1995; Sawin and Mitchison, 1995). Calcium-dependent binding of calmodulin to the kinesin light chain was shown to inhibit the ATPase activity of native kinesin but not of cAMP-dependent protein kinase phosphorylated kinesin (Matthies et al., 1993). The KCBP is the only known KLP with a calmodulin binding domain. The presence of a calmodulin binding domain in the KCBP and its interaction with calmodulin in the presence of micromolar calcium raise an interesting possibility that its function is modulated by calcium, a well-recognized messenger molecule in plants (Poovaiah and Reddy, 1993; Gilroy and Trewavas, 1994).

In this study, we have used different regions of the Arabidopsis KCBP in microtubule cosedimentation assays to investigate the interaction of the KCBP with microtubules and the effect of Ca^{2+}–calmodulin on these interactions. Our data show that there are two microtubule binding regions in the KCBP: one in the N-terminal region and a second one in the C-terminal motor domain. In addition, the interaction of the KCBP motor domain with microtubules is regulated by Ca^{2+}–calmodulin, and this regulation is mediated through the calmodulin binding domain.

RESULTS

Expression and Purification of Different Truncated Versions of the KCBP

To characterize the interaction of different domains of the KCBP with microtubules, we expressed different regions of the KCBP protein, including the full-length protein, in a pET expression system, as described in Methods. The full-length clone produced two breakdown products: a 90-kD N-terminal end protein (recognized by an S protein) and a 55-kD C-terminal end protein (recognized by a biotinylated calmodulin probe). A single protein of the expected size of 140 kD was not detected (data not shown). Hence, further efforts were limited to the analysis of the subdomain constructs. Five truncated versions of the KCBP designated as 1.5 N, 1.5 C, 1.0 C, 0.8 N, and 0.4 C were used in this study. The number in each construct indicates the length of the cDNA, and N and C represent N-terminal and C-terminal regions of the KCBP, respectively. All fusion proteins contained a histidine tag, whereas 1.5 C, 1.5 N, and 0.8 N fusion proteins, which are expressed in pET32 vector as thioredoxin fusion proteins, contained an additional S tag, which allowed detection of these proteins by the S protein (Figure 1). The S tag is a 15–amino acid peptide that binds to a 104–amino acid S protein from pancreatic ribonuclease A (McCormick and Mierendorf, 1994). Detection of proteins with the S tag by the S protein is highly specific and sensitive because of the strong interaction between them. Fusion proteins 1.5 C, 1.0 C, and 0.4 C contained amino acids 821 to 1261, 860 to 1210, and 1210 to 1261 of the C-terminal region of the KCBP, respectively (Figure 1). Fusion proteins 1.5 C and 0.4 C, which contained the calmodulin binding domain, were purified to near homogeneity by using calmodulin–Sepharose chromatography. Fusion proteins 1.5 N and 0.8 N contained amino acids 12 to 503 and 12 to 262 of the N-terminal region of the KCBP, respectively (Figure 1). Proteins from 1.0 C, 1.5 N, and 0.8 N were purified by the His-Bind affinity purification system.

Ca^{2+}–Calmodulin Regulates Binding of the Motor Domain to Microtubules

We have shown previously that the motor domain of the KCBP that contains residues 860 to 1261 is capable of binding to microtubules both in the absence of ATP and in the presence of AMP-PNP (adenosine 5′-[β,γ-imido]triphosphate), an ATP analog (Deavours et al., 1996; Reddy et al., 1996a). We have also shown that the motor domain does not bind microtubules in the presence of ATP. Because KCBPs differ from all other kinesins and KLPs in the presence of a unique calmodulin binding domain at their C terminus, we investigated the role of this domain in KCBP function. To determine the effect of Ca^{2+}–calmodulin, we

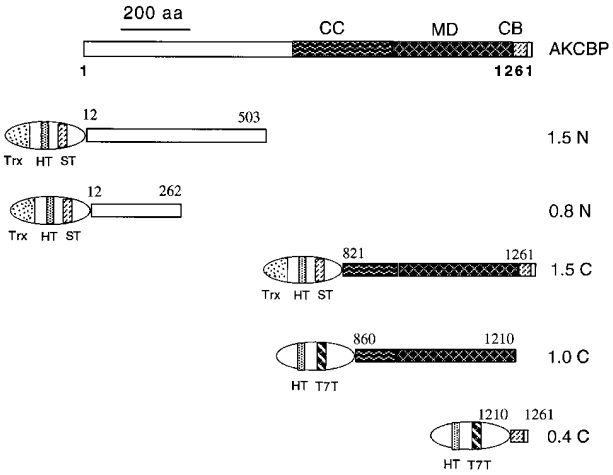

Figure 1. Schematic Diagram of Different Fusion Proteins of the Arabidopsis KCBP.

AKCBP is the full-length protein showing various domains that are identified based on sequence similarity or functional analysis of the *E. coli*–expressed protein (Reddy et al., 1996b). 1.5 N is the N-terminal fusion protein containing amino acids (aa) 12 to 503 of the KCBP. 0.8 N is the N-terminal fusion protein containing amino acids 12 to 262 of the KCBP. 1.5 C is the C-terminal fusion protein (amino acids 821 to 1261) of the KCBP containing the motor and calmodulin binding domains and a limited coiled-coil stalk. 1.0 C is the C-terminal fusion protein (amino acids 860 to 1210) of the KCBP containing a short coiled-coil stalk and the motor domain but without the calmodulin binding domain. 0.4 C is the C-terminal fusion protein (amino acids 1210 to 1261) of the KCBP containing only the calmodulin binding domain. CB, calmodulin binding domain; CC, α-helical coiled-coil region; HT, histidine tag for affinity purification of fusion protein; MD, motor domain; ST, S tag for fusion protein detection; Trx, thioredoxin for increased solubility of fusion protein; and T7T, T7 tag for immunodetection.

used cosedimentation assays to analyze the interaction of the 1.5 C protein with microtubules in the absence of any nucleotide but in the presence of Ca^{2+}, calmodulin, or Ca^{2+}–calmodulin. The results of these experiments indicate that the motor protein cosediments with microtubules in the presence of Ca^{2+} alone or calmodulin alone. However, most of the protein remained in the supernatant when microtubules were supplemented with Ca^{2+}–calmodulin (Figure 2), suggesting that in the presence of calcium/calmodulin, the 1.5 C protein is released from microtubules.

Antibodies Raised to the Calmodulin Binding Domain of the KCBP Abolish Ca^{2+}–Calmodulin Regulation

To test whether the calmodulin binding domain is responsible for Ca^{2+}–calmodulin regulation of the KCBP interaction with microtubules, we performed a similar experiment, as described above, in the presence of antibodies specific to the calmodulin binding domain. We previously mapped the calmodulin binding domain in the 1.5 C protein to a stretch of 23 amino acids (Reddy et al., 1996b). Antibodies raised to this synthetic peptide recognize a protein of ~140 kD in Arabidopsis suspension-cultured cells and seedlings (Bowser and Reddy, 1997). We conducted microtubule binding reactions with the 1.5 C protein, as shown in Figure 2, in the presence of antibodies. Because the 1.5 C protein and the antibody to the calmodulin binding domain have identical mobility on gels, we blotted a duplicate gel and probed it with the S protein, which recognizes the N terminus of the 1.5 C protein. This allowed us to distinguish the motor protein from the antibody. In the presence of the antibody, calcium/calmodulin did not release the KCBP from microtubules (Figures 3A and 3B). This provides indirect evidence that the binding of the antibody to the calmodulin binding domain interferes with the Ca^{2+}–calmodulin—mediated modulation of the kinesin interaction with microtubules.

Deletion of the Calmodulin Binding Region Abolishes Ca^{2+}–Calmodulin Modulation of the KCBP Interaction with Microtubules

Because the 1.5 C protein contains a motor domain and a calmodulin binding domain, we determined the effect of calmodulin binding domain deletion on the ability of the motor to bind to microtubules. We performed two different experiments using protein from the 1.0 C construct. In the first experiment, pelleting assays were performed with the 1.0 C protein, which lacks the calmodulin binding domain, to test its ability to bind to microtubules in the absence of a nucleotide, in the presence of AMP-PNP, and also in the presence of ATP. The results of this experiment indicate that the 1.0 C protein binds microtubules both in the absence of any

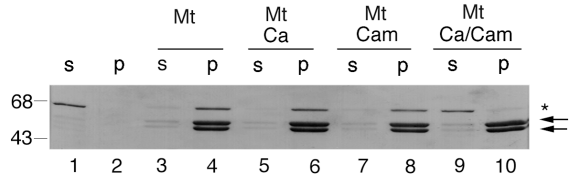

Figure 2. Ca^{2+}–Calmodulin Regulates the Interaction of the 1.5 C Protein with Microtubules.

The purified protein and microtubules were incubated in the absence (Mt) or presence of Ca^{2+} (Mt Ca), calmodulin (Mt Cam), or Ca^{2+}–calmodulin (Mt Ca/Cam) and centrifuged at 100,000*g*. The resulting supernatant (s) and pellet (p) fractions from these assays were separated on an SDS–polyacrylamide gel and stained with Coomassie blue. The supernatant and pellet obtained with the 1.5 C protein alone are shown in lanes 1 and 2. The asterisk indicates the position of 1.5 C protein. Arrows point to the two tubulin subunits. Molecular mass markers are shown at left in kilodaltons.

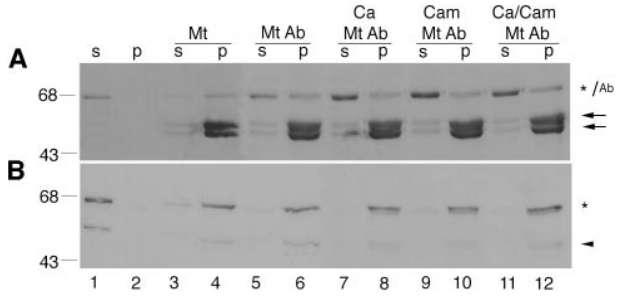

Figure 3. Antibodies Raised against the Calmodulin Binding Domain Abolish Ca^{2+}–Calmodulin Regulation.

(A) The 1.5 C protein and microtubules were incubated in the absence (Mt) or presence of antibodies (Mt Ab), antibodies plus Ca^{2+} (Ca Mt Ab), antibodies plus calmodulin (Cam Mt Ab), and antibodies plus Ca^{2+}–calmodulin (Ca/Cam Mt Ab). Supernatant (s) and pellet (p) fractions from each assay were analyzed as given in the legend to Figure 2. The supernatant and pellet obtained with the 1.5 C protein alone are shown in lanes 1 and 2.

(B) A duplicate gel of **(A)** was blotted and probed with the S protein. Tubulin subunits are indicated by arrows. Asterisks indicate the positions of the 1.5 C protein. Ab indicates the position of the antibody. A breakdown product of 1.5 C protein is indicated by an arrowhead. Molecular mass markers are shown at left in kilodaltons.

nucleotide and in the presence of AMP-PNP (Figure 4). The 1.0 C protein did not bind microtubules in the presence of ATP, suggesting that the 1.0 C protein interacts with microtubules in an ATP-sensitive manner (Figure 4). To determine whether the calmodulin binding domain is necessary for Ca^{2+}–calmodulin regulation, we performed microtubule pelleting assays with the 1.0 C protein in the presence of Ca^{2+}, calmodulin, and Ca^{2+}–calmodulin. A predominant portion of the 1.0 C protein remained in the pellet in all three cases, suggesting that the Ca^{2+}–calmodulin effect observed with the 1.5 C protein is no longer evident with the motor domain protein lacking the calmodulin binding domain (Figure 5).

Calmodulin Binding Domain Alone Does Not Interact with Microtubules

Using protein derived from the 0.4 C construct, which contains the last 52 C-terminal amino acid residues, including the 23–amino acid stretch representing the calmodulin binding domain, we performed microtubule pelleting assays in the absence or presence of a nucleotide (ATP or its analog AMP-PNP). In the absence of any nucleotide or in the presence of ATP or AMP-PNP, the 0.4 C protein remained in the supernatant, suggesting that the 0.4C protein does not bind to microtubules (Figure 6). Pelleting assays with the 0.4 C protein in the presence of Ca^{2+}, calmodulin, or Ca^{2+}–calmodulin were also conducted to test their effect on the 0.4 C interaction with microtubules. In these assays, the 0.4 C protein remained in the supernatant (data not shown), suggesting that Ca^{2+}, calmodulin, or Ca^{2+}–calmodulin had no effect.

The N-Terminal Region of the KCBP Has an Additional Microtubule Binding Site

Two different N-terminal constructs of the KCBP protein representing amino acid residues 12 to 503 (1.5 N) and 12 to 262 (0.8 N) were expressed. Proteins from these two constructs tended to aggregate and pellet, even in the absence of microtubules. However, we solved this problem by supplementing the binding reactions with 0.2 M NaCl. The 1.5 N and 0.8 N proteins failed to bind biotinylated calmodulin, indicating that there is no calmodulin binding domain in this region (data not shown). Cosedimentation assays with microtubules indicated that both proteins have the ability to bind to microtubules (Figures 7A and 7B). This is evident from the observation that a large part of the protein remained with the pellet in the case of the 1.5 N construct. However, some of the protein remained in the supernatant with the 0.8 N construct in the presence of microtubules. The protein that remained in the supernatant may have been inactivated and lost its microtubule binding activity during purification. The other possibility is that the protein from the 0.8 N construct may have less affinity to microtubules. Unlike the microtubule domain in the C terminus, the N-terminal proteins bound microtubules both in the presence and absence of ATP, indicating that the microtubule binding domain in the N terminus of the KCBP is insensitive to ATP (Figure 7).

DISCUSSION

Analysis of the interaction of various truncated versions of the KCBP with microtubules indicates that there are two mi-

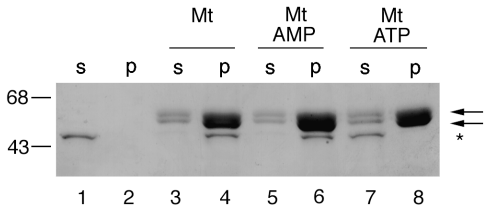

Figure 4. C-Terminal Protein (1.0 C) Lacking the Calmodulin Binding Domain Shows ATP-Sensitive Binding.

The purified protein was incubated with microtubules in the absence (Mt) or presence of either AMP-PNP (Mt AMP) or ATP (Mt ATP) and centrifuged at 100,000*g*. Supernatant (s) and pellet (p) fractions were analyzed as described in the legend to Figure 2. The asterisk indicates the position of 1.0 C. Arrows indicate tubulin subunits. Molecular mass markers are shown at left in kilodaltons.

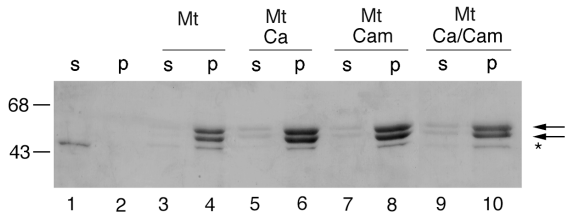

Figure 5. Effect of Ca^{2+}–Calmodulin on the Binding of the 1.0 C Protein to Microtubules.

Cosedimentation assays with 1.0 C and microtubules (Mt) in the presence of Ca^{2+} (Mt Ca), calmodulin (Mt Cam), or Ca^{2+}–calmodulin (Mt Ca/Cam) were performed and analyzed essentially as described in the legend to Figure 2. Supernatant (s) and pellet (p) fractions obtained with the 1.0 C protein alone are shown in lanes 1 and 2. The asterisk indicates the position of 1.0 C. Arrows indicate tubulin subunits. Molecular mass markers are shown at left in kilodaltons.

crotubule binding domains, one located at the C terminus and the second one located at the N terminus. The microtubule binding site located at the C terminus has conserved amino acid stretches typical of all kinesins (Reddy et al., 1996b). The presence of ATP-independent microtubule binding regions in the nonmotor domains of KAR3, Ncd, and the kinesin heavy chain has been reported (Meluh and Rose, 1990; Navone et al., 1992; Chandra et al., 1993). However, there is no sequence similarity between the N-terminal tail region of the KCBP and known KLPs. The N- and C-terminal binding domains differ in their sensitivity to ATP. The C-terminal region spanning the motor domain does not bind microtubules in the presence of ATP, whereas the N terminus of the protein is capable of binding to microtubules in the presence of ATP (Figure 7). The specificity of the binding response is also evident from the fact that under the same conditions, the 52–amino acid C-terminal region representing the calmodulin binding domain does not bind microtubules (Figure 6).

The conclusion that the calmodulin binding domain in the KCBP confers Ca^{2+}–calmodulin regulation of the KCBP interaction with microtubules is supported by the results obtained from three independent sets of experiments. The motor protein containing the calmodulin binding domain did not bind microtubules in the presence of Ca^{2+}–calmodulin (Figure 2). This is consistent with the data on the effect of Ca^{2+}–calmodulin on microtubule gliding (Song et al., 1997). The binding response of the motor in the presence of Ca^{2+} or calmodulin alone was similar to its response in the absence of Ca^{2+} or calmodulin, suggesting that the activated calmodulin is responsible for these differences. Supplementing the binding reaction with antibodies specific to the calmodulin binding domain abolished the Ca^{2+}–calmodulin modulation of the KCBP interaction with microtubules (Figure 3). More direct evidence, however, was obtained by using the C-terminal motor protein without the calmodulin binding domain. This protein binds to microtubules, suggesting that residues 860 to 1210 are necessary and sufficient for microtubule binding (Figure 4). The binding of this protein to microtubules in the presence of Ca^{2+}, calmodulin, or Ca^{2+}–calmodulin remained essentially similar to the binding in the absence of these components (Figure 5), indicating that the missing calmodulin binding domain is critical for Ca^{2+}–calmodulin—modulated interaction of the KCBP with microtubules. Protein with the calmodulin binding domain alone (capable of binding to biotinylated calmodulin) failed to bind microtubules (Figure 6).

Among motor proteins, myosin heavy chains and light chains of kinesin are the only ones that bind to calmodulin. Kinesin heavy chains other than the KCBP are not known to bind to calmodulin (Matthies et al., 1993; Reddy et al., 1996b). The binding of calmodulin to the kinesin light chain reduces kinesin's microtubule-stimulated ATPase activity by 50%. Phosphorylation of kinesin by protein kinase A reverses the calmodulin inhibition of ATPase activity. The light chains, however, are not required for kinesin to exhibit motor activity (Yang et al., 1990).

Ca^{2+} and calmodulin have been to shown to regulate the enzymatic and mechanochemical properties of brush border myosin, an actin-based motor. Brush border myosin, which is found in the microvilli at the apical surface of intestinal brush border cells, has a calmodulin binding domain in its heavy chain (Coluccio and Bretscher, 1988; Wolenski, 1995). This protein binds tightly to calmodulin in the absence of Ca^{2+}. Ca^{2+} inhibits motility by inducing dissociation of calmodulin from the heavy chain (Collins et al., 1990; Wolenski, 1995). A second isoform that contains an additional calmodulin binding site introduced by an alternative splicing event (Halsall and Hammer, 1990) was also reported. In addition, Ca^{2+} and calmodulin bind several

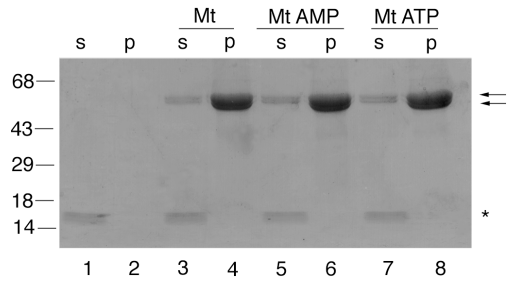

Figure 6. The Calmodulin Binding Domain Does Not Bind to Microtubules.

Protein (0.4 C) containing the calmodulin binding domain was incubated with microtubules in the absence (Mt) or presence of either AMP-PNP (Mt AMP) or ATP (Mt ATP) and centrifuged at 100,000*g*. Supernatant (s) and pellet (p) fractions were as described in the legend to Figure 2. Lanes 1 and 2 show supernatant and pellet from an assay with the 0.4 C protein only. The asterisk indicates the position of the 0.4 C protein. The position of tubulin subunits is indicated by arrows. Molecular mass markers are shown at left in kilodaltons.

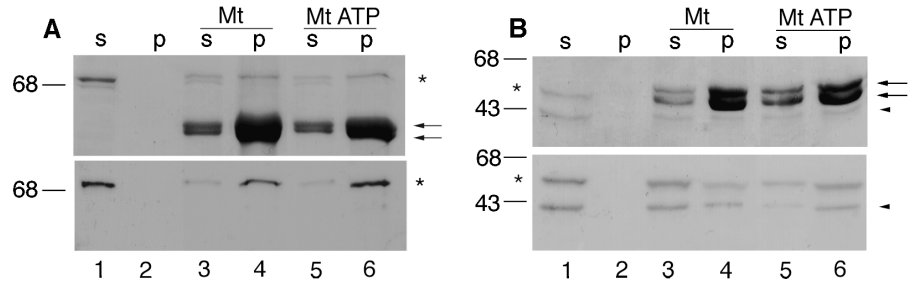

Figure 7. The N-Terminal Region of the KCBP Contains a Second Microtubule Binding Domain.

(A) Purified 1.5 N.

(B) Purified 0.8 N.

Proteins were incubated with microtubules in the absence (Mt) or presence of ATP (Mt ATP) and centrifuged at 100,000*g*. Supernatant (s) and pellet (p) fractions were separated on two gels, as described in the legend to Figure 2. One gel was stained with Coomassie blue (top), and the other gel was blotted and probed with S protein (bottom). Lanes 1 and 2 represent supernatant and pellet from an assay with protein alone. Asterisks indicate the position of the KCBP. Arrows point to tubulin subunits. A breakdown product of 0.8 N is shown with arrowheads. Molecular mass markers are indicated at left in kilodaltons.

unconventional myosins and regulate motility and enzymatic properties of these myosins (Wolenski, 1995). Myosins with calmodulin binding IQ motifs have been reported in plants (Asada and Collings, 1997). However, interaction of these myosins with calmodulin has not been demonstrated. It is noteworthy that the N-terminal protein sequence of the three KCBPs that have been characterized thus far shows sequence similarity to a region in the tail of several myosins (Chen et al., 1996; Reddy et al., 1996a). However, the significance of this conserved region is not known.

The presence of two microtubule binding domains in the KCBP has several important implications in the function of this protein. The formation of mitotic microtubule arrays, such as the preprophase band, phragmoplast, and bipolar spindle (Gunning, 1982; Baskin and Cande, 1990), may involve the KCBP. The majority of higher plants and some animal cells lack well-defined centrosomes, which are known to play a crucial role in bipolar spindle formation in animal cells. The assembly of the acentriolar spindle in plants may involve convergence of microtubule minus ends, leading to the formation of spindle poles. Thus, in these cells, microtubule motors are likely to play a role in spindle formation. Recent reports demonstrating the formation of astral or convergent arrays from microtubules in the presence of motor proteins support this notion (Merdes and Cleveland, 1997; Nedelec et al., 1997). Some members of the Kar3/Ncd family, to which the KCBP belongs, also contain a second N-terminal microtubule binding domain and bundle microtubules (McDonald et al., 1990; Chandra et al., 1993; Endow et al., 1994; Kuriyama et al., 1994). Two microtubule binding sites on the KCBP should allow it to cross-link adjacent microtubules, thereby facilitating the bundling of microtubules. Our localization of the KCBP to the preprophase band and phragmoplasts is consistent with such a possibility (Bowser and Reddy, 1997). In addition, by virtue of its minus-end movement and cross-linking activity, the KCBP may converge microtubules at their minus ends to generate a bipolar spindle in the absence of a centrosome.

Localization of the KCBP to spindle poles in Haemanthus cells at the time of transformation of a barrel-shaped spindle into a convergent bipolar spindle supports this notion (Smirnova et al., 1997). Moreover, it has been shown that Ncd, a C-terminal motor from Drosophila, is involved in forming and stabilizing bipolar meiotic spindles in oocytes that lack microtubule organizing centers (Matthies et al., 1996). Because the interaction of the KCBP with microtubules is modulated by calcium–calmodulin, any changes in the level of cytosolic calcium, a well-recognized messenger in plants (Poovaiah and Reddy, 1993; Trewavas and Malhó, 1997), would affect the interaction of the KCBP with microtubules. In addition to its role in cell division, the KCBP is also involved in trichome development (Bowser and Reddy, 1997; Oppenheimer et al., 1997). Disruption of the KCBP gene has been shown to cause defects in trichome morphogenesis. These defects include a short stalk and fewer branches, suggesting a KCBP requirement for cell expansion and branching in trichomes (Oppenheimer et al., 1997). The orientation of cellulose microfibrils, which dictates the direction of cell expansion, is controlled by cortical microtubules (Shibaoka, 1991). Hence, it is likely that the KCBP may influence cell expansion by regulating cortical microtubule arrays.

METHODS

Construction of Recombinant Plasmids

To prepare 1.5 N and 0.8 N constructs, a 4.0-kb SacI-NotI fragment of the *Arabidopsis thaliana* kinesin-like calmodulin binding protein

(KCBP) cDNA containing the coding region from amino acids 12 to 1261 was cloned into a pET32b expression vector. A 1.0-kb XhoI fragment from the 3′ end of this clone was then dropped and religated to generate a 3.0-kb SacI-XhoI clone. This SacI-XhoI clone was digested with BsiWI-XhoI to delete a 1.5-kb fragment. The cut ends of the plasmid were repaired with the Klenow fragment of DNA polymerase I and religated to generate a 1.5 N clone and produce a truncated KCBP containing the N-terminal region (amino acids 12 to 503). To prepare the 0.8 N construct (amino acids 12 to 262), we deleted a 2.2-kb AvrII-XhoI fragment from the 3′ end of the SacI-XhoI clone. The ends were repaired and ligated as described above, following standard procedures (Sambrook et al., 1989). Construct 1.5 C was made by cloning a 1.5-kb EcoRI fragment from a previously constructed 1.5-kb pGEX/KCBP clone (Song et al., 1997) as a thioredoxin fusion in the pET32b expression vector. Construction of 1.0 C and 0.4 C plasmid was described previously (Reddy et al., 1996b). The plasmid constructs were moved into *Escherichia coli* BL21(DE3)pLYS cells for expression of the fusion protein.

Protein Induction and Purification

Bacterial cells were grown at 37°C to an OD_{600} of 0.6, and fusion protein expression was induced by isopropyl β-D-thiogalactopyranoside (final concentration of 0.2 mM). The cultures were grown for another period of 8 hr at 22°C, spun, washed, and resuspended in a one-tenth volume of lysis buffer.

In the case of the 1.5 C and 0.4 C constructs, the cells were resuspended in buffer A (50 mM Tris, pH 7.5, and 150 mM NaCl) supplemented with 200 μg/mL lysozyme and incubated on ice for 1 hr, followed by five to six pulses of sonication in an iced water bath. The sonicated extracts were spun at 100,000*g* to separate soluble and insoluble protein fractions. The resulting supernatant was supplemented with 2 mM $CaCl_2$ and loaded onto a calmodulin–Sepharose 4B column that was prepared and equilibrated according to the manufacturer's instructions (Pharmacia Biotechnology). The column was washed with at least 10 volumes of binding buffer (buffer A plus 2 mM $CaCl_2$). Bound protein was eluted with buffer A containing 2 mM EGTA.

Proteins expressed from the rest of the constructs (1.5 N, 0.8 N, and 1.0 C) were extracted as given above except that buffer B (20 mM Tris, pH 7.9, 0.5 M NaCl, and 5 mM imidazole) supplemented with 200 μg/mL lysozyme was used to lyse the cells. Fusion proteins from clones 1.0 C, 1.5 N, and 0.8 N contained a histidine-tagged sequence at their N terminus, permitting purification of these proteins on a His-Bind affinity column. Briefly, the high-speed supernatant was allowed to bind to His-Bind resin (Novagen, Madison, WI) in the presence of buffer B and washed extensively with the same buffer containing an additional 15 mM imidazole. The bound proteins were eluted in buffer B supplemented with 100 mM imidazole.

Proteins from 1.5 C, 1.0 C, and 0.4 C, which are relatively more soluble, were dialyzed in a buffer containing 50 mM Tris, pH 7.5, and 150 mM NaCl. The two N-terminal proteins (1.5 N and 0.8 N), which tend to aggregate, were dialyzed in a buffer containing 50 mM Tris, pH 7.5, and 200 mM NaCl.

Tubulin Isolation, Purification, and Microtubule Preparation

Isolation and purification of bovine brain tubulin were performed essentially as described earlier (Reddy et al., 1996a). To prepare microtubules, we thawed frozen tubulin, supplemented it with 4 M glycerol, 1 mM GTP, and 20 μM Taxol (Paclitaxel; Sigma), and incubated it at 35°C for 1 hr. The unpolymerized tubulin protein was separated by sedimenting the microtubules.

Microtubule Cosedimentation Assay

The binding reactions were performed in a 100-μL reaction volume containing 50 μg/mL motor protein with or without 0.5 mg/mL microtubules. The reaction buffer contained 20 mM Pipes, pH 6.9, 2 mM $MgCl_2$, 1 mM DTT, 20 μM Taxol, and 50 mM NaCl. In assays with the 1.5 N and 0.8 N protein, the concentration of NaCl in the reaction buffer was increased from 50 to 200 mM to prevent protein aggregation. ATP (5 mM), AMP-PNP (5 mM), $CaCl_2$ (0.5 mM), or calmodulin (1 μM) was added to the appropriate reactions. In binding reactions with antibodies, we used a twofold excess concentration of antibodies compared with the motor protein. After a 20-min incubation at 22°C, the samples were centrifuged in a TLA 100.3 rotor (Beckman Instruments Inc., Palo Alto, CA) for 20 min at 25°C. The supernatant and pellet fractions were mixed with SDS sample buffer and analyzed by SDS-PAGE. Tubulin subunits and the fusion protein were visualized by staining the gels with Coomassie Brilliant Blue R 250. In cases in which the fusion protein comigrated with either tubulin or the antibody, a duplicate gel was run, blotted, and probed with the S protein. The blot was blocked for 30 min in 3% gelatin prepared in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and washed for 15 min in TBST, followed by a 30-min incubation in the presence of a purified S protein conjugated to alkaline phosphatase. Protein bands reacting with the S protein were detected colorimetrically by incubating the blot in a substrate solution (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.34 mg/mL nitro blue tetrazolium, and 0.175 mg/mL 5-bromo-4-chloro-3-indolyl phosphate).

ACKNOWLEDGMENTS

We thank Dr. Irene Day, Dr. Safadi-Chamberlain, and Jonathan Bowser for their comments on the manuscript. This work was supported by a grant from the National Science Foundation (No. MCB-9630782) to A.S.N.R.

Received January 16, 1998; accepted March 23, 1998.

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