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DISSERTATION

CALCIUM/CALMODULIN REGULATION OF A NOVEL MICROTUBULE MOTOR PROTEIN FROM ARABIDOPSIS

Submitted by
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In partial fulfillment of the requirements
For the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Spring, 2000

COLORADO STATE UNIVERSITY

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY YU-LIN KAO ENTITLED CALCIUM/CALMODULIN REGULATION OF A NOVEL MICROTUBULE MOTOR PROTEIN FROM ARABIDOPSIS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

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ABSTRACT OF DISSERTATION

CALCIUM/CALMODULIN REGULATION OF A NOVEL MICROTUBULE MOTOR PROTEIN FROM ARABIDOPSIS

Kinesin-like calmodulin-binding protein (KCBP) is a recently identified novel kinesin-like protein that appears to be unique to and ubiquitous in plants. KCBP is distinct from other known kinesins and kinesin-like proteins (KLPs) in having a calmodulin-binding domain (CBD). This CBD, 23 amino-acid long, is located at the C-terminus adjacent to motor domain. The C-terminal motor of the KCBP, with or without CBD, binds to tubulin subunits and MTs and bundles MTs in an ATP-dependent manner. In the presence of Ca^{2+}-CaM, the motor with CBD does not bind to tubulin and MTs and cannot bundle MTs. The KCBP lacking the CBD show no effect of Ca^{2+}-CaM. These results indicate that Ca^{2+}-CaM modulates the interaction of KCBP with MTs and this modulation is due to the CBD in the KCBP. When motor domain and calmodulin-binding domain are in two separate peptides, there is no Ca^{2+}/CaM regulation.

NCD (with a C-terminal motor) and DK (with an N-terminal motor) are KLPs from Drosophila and do not bind CaM. The addition of CBD to these two KLPs confers CaM-binding and similar Ca^{2+}-CaM regulation on binding MTs.

In *Arabidopsis*, there are six CaM-isoforms (AtCaMs). Three AtCaMs and one bovine CaM were used to study Ca^{2+}/calmodulin modulation of KCBP interaction with MTs. All AtCaMs and bovine CaM have inhibited the binding of KCBP to MTs in a calcium-dependent manner. However, AtCaM-2 appears to be more effective in inhibiting KCBP interaction with MTs.

A part of the tail domain in KCBP shows sequence similarity to tail domain of some myosins (MyTH4) and a region in talin (talin-like domain). The tail domain contains a microtubule-binding site and binds unpolymerized and polymerized actin, suggesting that KCBP may connect microtubule and actin cytoskeletal systems. The KCBP is unique in having features of actin-based motors (myosins) and microtubule-

based motors (kinesins). These results of calcium/CaM modulation via CBD suggest regulation of KCBP function *in vivo* by calcium, the first such regulation among all the known kinesins and KLPs. KCBP localizes to MT arrays including the preprophase band, the spindle apparatus and the phragmoplast. The bundling results suggest a role for KCBP in establishing these mitotic MT arrays during different stages of cell division and that Ca^{2+}/CaM is likely regulate the formation of these MT arrays.

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This dissertation will be delicated to my great parents. Your countless love and comfort make me have faith and courage facing everything. Without your support and encouragement, I cannot make this possible. Thanks God for giving me this great opportunity to make things happen and my dream come true.

Table of contents

List of Tables

Pages

List of Figures

CHAPTER 1

Introduction

Cytoskeleton

The cell consists of many different organelles suspended on a network of fibers called the cytoskeleton. The cytoskeleton can function as mechanical support to maintain a cell's shape or to change its shape and to anchor organelles, cytoplasmic proteins, and enzymes. It functions in cellular and intracellular motility, which includes movement of the entire cell or organelles (e.g. mitochondria, chloroplasts and chromosomes) within the cell by specific proteins called motor molecules. The cytoskeleton is constructed from three types of fibers: microtubules, actin filaments (also called microfilaments), and intermediate filaments. The main functions of microtubules are cell motility, chromosome movement, organelle movement, and maintenance of the cell shape. The basic functions of actin filaments are muscle contraction, cytoplasmic streaming, cell motility, cell division, maintenance of the cell shape, and change in cell shape. The intermediate filament's functions are structural support and maintenance of cell shape.

Microtubules

The microtubules (MTs) in plant cells were discovered by Ledbetter and Porter in 1963. These structures play an important role in cellular processes in higher plants. Microtubules and tubulin sheets (α- and β-tubules) are polar structures exhibiting fast growing plus-ends and slower growing minus-ends that often connect via γ-tubulin to the centrosome (Borisy, 1978; Gildersleeve et al., 1992; Oakley, 1992). In interphase cells, microtubules regulate the orientation of cellulose microfibris on cell walls to establish the architecture of cell wall (Gidding and Staehelin, 1991). In prophase, the

preprophase band (PPB) of microtubules establishes the plane of cytokinesis and regulates the arrangement of cells in tissues (Gunning and Wick, 1985; Traas et al., 1995). During mitosis, two kinds of structures are made of microtubules. The first one is the mitotic spindle, which is involved in segregation of duplicated chromosomes, and the second one is the phragmoplast, which is involved in formation of the cell plate between the daughter nuclei to form two daughter cells (Baskin and Cande, 1990; Gunning, 1982; Lambert et al., 1991). The structures often involved in the organization of microtubules are the centrosome in animal cells and spindle pole body (SPB) in fungal cells, both of which are lacking in plant cells. The plant-specific mechanism for organization of microtubules and their functions have not been clarified.

The phragmoplast is formed between the separating chromosomes and involved in the formation of the cell plate (Staehelin and Hepler, 1996). It is composed of two sets of microtubules with their plus ends toward the narrow equatorial zone (Euteneuer and McIntosh, 1980; Hepler and Jackson, 1968). The phragmoplast is implicated in transporting vesicles containing cell plate materials to the forming cell plate (Samuels et al., 1995; Yasuhara et al., 1993). The equatorial region is important in maintenance of the phragmoplast (Inoué, 1963). Exposure of the equatorial region of *Haemanthus* endosperm cells to UV radiation disrupts the phragmoplast microtubules at the irradiation site. Vantard et al. (1990) showed that the equatorial region is not only required for the maintenance of MTs but also the formation of phragmoplast microtubules, since the phragmoplast in plasma membrane-permeabilized endosperm cells of *Haemanthus* incorporate tubulin at the equatorial

region. Asada et al. (1991) showed similar results, and they suggested that ATP- and GTP-dependent machinery translocated microtubules in the minus-end direction and might function at the equatorial region for maintaining the array of phragmoplast microtubules. Later they found two polypeptides which are candidates for motor molecules involved in the organization of the phragmoplast (Asada and Shibaoka, 1994; Asada et al., 1997).

Motor Proteins

There are three kinds of molecular motors: myosins, dyneins, and kinesins (Moore and Endow, 1996; Walker and Sheetz, 1993). These motor proteins convert energy from the hydrolysis of ATP to move along cytoskeletal polymers. Kinesins and dyneins move along microtubules and myosins move on actin filaments.

Myosins were classified as cytoplasmic myosins (myosins I) and filament-forming myosins (myosins II) traditionally. But after the discovery of numerous unconventional myosins, which possess distinct structural or functional properties, myosins have been subdivided into twelve classes (Sellers et al., 1996).

Dynein was first discovered in axonemes involved in movement causing from ciliary and flagellar beating (Gibbons and Rowe, 1965). Dynein bundles microtubules and causes them to slide relative to one another (Shpetner and Vallee, 1989). Dyneins are also involved in transport toward the minus ends of MTs (Paschal and Vallee, 1987; Vallee and Shpetner, 1990).

The conventional kinesins and kinesin-like proteins (KLPs) are members of a kinesin superfamily and are involved in numerous cellular processes including

intracellular transport of organelles and vesicles and cell division, such as mitotic and meiotic spindle formation and elongation, chromosome segregation and cytokinesis (Barton and Goldstein, 1996; Bloom and Endow, 1994; Brady, 1985; Goldstein, 1993; Kozminski et al., 1995; Leopold et al., 1992; Lloyd, 1991; Moore and Endow, 1996; Robb et al., 1996; Sawin and Endow, 1993). The kinesin superfamily contains a conserved motor domain but remarkable structural and functional diversity. There are more than one hundred members of the kinesin superfamily which includes ten structurally distinct sub-families (Endow, 1999; Goodson et al, 1994; Moore and Endow, 1996; http://www.blocks.fhcrc.org/~kinesin/).

Kinesin

Kinesin was first identified and partially characterized during studies of organelle transport in squid giant axons in 1985 (Brady, 1985; Vale et al., 1985b). Using video-enhanced differential interference contrast microscopy, numbers of particles, probably vesicles and other tubulovesicular elements, were observed moving along linear elements, bundles of parallel fibers that correspond to MTs in the anterograde and retrograde directions in dissected samples (Allen et al., 1982; Schnapp et al., 1985) and in extruded axoplasm from squid giant axons (Brady et al., 1982). AMP-PNP (Adenylylimidodiphosphate), a non-hydrolysable analogue of ATP, was shown to block fast axonal transport and the vesicle transport could be reversed by adding excess ATP (Lasek and Brady, 1985). AMP-PNP inhibited movement of vesicles at a concentration of 0.5mM, which is similar to the ATP concentration in the axoplasm. This ATPase involved in fast axonal transport of vesicle movement was isolated and

identified based on enzyme properties and activity (Brady, 1985; Vale et al., 1985c). This ATPase from squid giant axons and optic lobes, which induced this microtubule-based motility, was distinct from myosin and dynein because the inhibition mechanism by AMP-PNP and nucleotide binding to the vesicle-transport ATPase complex are different. AMP-PNP decreased the affinity of dynein to microtubules and myosin to actin, but increased the microtubule affinity of this newly described ATPase (Vale et al., 1985b). NEM (N-ethylmaleimide) inhibited microtubule binding and force generation ability of dynein, but had no effect on ATPase itself (Vale et al., 1985b). This new class of force generating protein was named kinesin (Vale et al., 1985a).

Kinesin is a microtubule-activated ATPase, which can transport cargo (a vesicle, chromosome, microtubule, RNA or protein) along MTs. Kinesin can hydrolyze ATP stimulated by MTs and generate plus-end directed forces along those MTs (Vale et al., 1985a; 1985c). Kinesin contains binding sites for cargoes, ATP and MTs to perform these functions. The structure and domain functions were illuminated via DNA sequence analysis, electron microscopy and monoclonal antibody decoration techniques (Hirokawa et al., 1989; Scholey et al., 1989; Yang et al., 1989). Kinesin is a tetramer containing two heavy chains and two light chains (Bloom et al., 1988; Kuznetsov et al., 1988). The heavy chain has three distinct domains: (1) a globular amino-terminal head or motor domain containing ATP- and microtubule-binding sites; (2) a middle α-helical coiled-coil structure involved in dimerization; and (3) a carboxy-terminal globular tail that interacts with light chains (Hirokawa et al., 1989; Scholey et al., 1989; Vale and Goldstein, 1990; Yang et al., 1988; Yang et al., 1989;

Yang et al., 1990). Although kinesin heavy chains are highly conserved in amino acid sequence and structural features, light chains of kinesin show diversity (Bloom and Endow, 1994). Evidence from quick freeze electron microscopy suggests that the heavy chain binds MTs and the light and heavy chains associate with membrane bounded organelles (Hirokawa et al., 1989). This suggests that each motor protein possesses a conserved force-generating domain joined to a light chain that confers specificity of a kinesin molecule for its particular cargo property.

KLPs

After the first kinesin was discovered, it was thought to be the only member in its class. However, the kinesin-like proteins (KLPs) were found in *Saccharomyces cerevisiae* (Kar3; Meluh and Rose, 1990), *Drosophila melanogaster* (Ncd; Endow et al., 1990) and *Aspergillus nidulans* (BimC; Enos and Morris, 1990). More than one hundred KLPs have been found using different strategies (http:// www.blocks.fhcrc.org/~kinesin/). These proteins contain a region with similar sequence to the motor domain of "conventional" kinesin heavy chain (about 350 amino acids), including ATP- and microtubule-binding sites. Outside of this motor region, including the central coiled-coil domain and tail domain, they are not conserved between "conventional" kinesins and KLPs (Goodson et al., 1994). These KLPs function as organelle- or vesicle-transporters and involve in mitosis or meiosis (Goodson et al., 1994; Moore and Endow, 1996). The "conventional" kinesin is a tetramer consisting of two heavy chains and two light chains and a coiled-coil region to dimerize kinesin heavy chains (KHCs). Those KLPs lacking coiled-coil structure

become monomers and containing more than two heavy chains form heterotetramers, respectively (Cole and Scholey, 1995; Zhang et al., 1990a). Although the "conventional" kinesin contains an N-terminal motor domain, motor domains of some KLPs are located at the N-terminus, C-terminus (such as Kar3 and Ncd), or in the middle (such as MCAK/KIF2) (Bloom and Endow, 1994; Moore and Endow, 1996). The "conventional" kinesin is a plus-end motor protein, but other KLPs move either toward the plus-end or minus-end of microtubules (such as Kar3 and Ncd) (Endow et al., 1994b; Kuriyama et al., 1995; McDonald et al., 1990; Walker et al., 1990).

According to the phylogenetic analysis of the conserved motor domains of kinesin proteins, most KLPs fall into one of ten kinesin subfamilies: BimC (Table.1), Ce1 (Table.2), C-terminal motor (Table.3), chromokinesin/KIF4 (Table.4), KHC (kinesin heavy chain, see Table.5), KIP3 (Table.6), KRP85/95 (Table.6), MCAK/KIF2 (Table.7), MKLP1 (Table.9), and Unc104 (Table.10) (Endow, 1999; Goodson et al., 1994; Moore and Endow, 1996; Reddy et al., 1997; http://www.blocks.fhcrc.org/~kinesin/). However, some KLPs do not fit into these ten subfamilies, so they are likely to represent additional subfamilies (see Table.11). The kinesin members in the same subfamily show higher sequence similarity in motor domain and some sequence similarity even outside the head domain and have similar motility properties and cellular functions. One of these ten subfamilies (KAR3/Ncd, a C-terminal subfamily) possesses a C-terminal motor with minus-end motility and nine (N-terminal and internal motor domain kinesin subfamilies) are plus-end motors (Barton and Goldstein, 1996; Moore and Endow, 1996). Three subfamilies are involved in organelle/vesicle transport (KHC, Unc104 and KRP85/98 subfamilies), four in

spindle function (C-terminal, BimC, Chromokinesin/KIF4 and MKLP1 subfamilies) and one involved in both organelle/vesicle transport and cell division (MCAK/KIF2 subfamily) (Moore and Endow, 1996). The nonconserved globular tail domains confer diversity to interact with specific cargoes and may function in different cellular processes.

C-Terminal KLPs

There are 18 known members of the C-terminal subfamily of KLPs (Endow, 1999; http://www.blocks.fhcrc.org/~kinesin/). Ncd and Kar3 are the most extensively characterized members of this family. The Drosophila C-terminal motor Ncd (non-claret disjunctional) was originally identified from a ca^{nd} mutant because of chromosome misbehavior during female meiosis (Sequeira et al., 1989; Yamamoto et al., 1989). Then this Ncd gene was found to encode a kinesin-like protein with kinesin-like biochemical properties; the sequence is similar to kinesin heavy chain. Ncd protein has microtubule-motor activity, and can cross-link and bundle microtubules (Endow et al., 1990; McDonald and Goldstein, 1990; McDonald et al., 1990). Microtubule gliding assays have shown that Ncd has a minus-end directed polarity (McDonald et al., 1990; Walker et al., 1990) and the direction is not due to the location of the motor domain but is to the domain itself (Stewart et al., 1993). The gliding assays also suggested that this Ncd protein may have a role in chromosome movement along microtubules during prometaphase and/or anaphase (Walker et al., 1990). Using truncated Ncd proteins, Chandra et al (1993) demonstrated that the head domain of Ncd has an ATP-sensitive microtubule binding

site and controls the movement direction; the coiled-coil stalk is involved in dimerization of Ncd; and the tail domain possesses an ATP-insensitive microtubule binding and bundling site.

The Ncd is a minus-end directed molecular motor and "conventional" kinesin is plus-end directed, although there are similarities between these two motors. What determines the directionality of plus- and minus-end kinesins? Crystal structures of Ncd and kinesin have shown that both motor proteins are remarkably similar in their structure (Sablin et al., 1996) and primarily consist of a similar arrow head-shape domain and this structure is similar to the core of the catalytic domain of the actin-based myosin although there is no amino-acid sequence identity (Kull et al., 1996). These studies rule out the possibility that the structural difference in the motor domain determines the directionality difference. Three-dimensional crystal structures indicate that the motor domain binds to the outer crest of microtubules contacting both α and β tubulins (Hoenger and Milligan, 1997). Despite their opposite directionality, studies revealed a similar microtubule binding geometry for both motor domains (Hoenger and Milligan, 1997) and ruled out the directionality models of motors binding in opposite orientation on the microtubules. A model proposed by Taylor (1993) predicted that difference in the timing or strong and weak binding states could determine the opposite directionality of movement. This model was not supported by experimental results (Lockhart and Cross, 1994; Shimizu et al., 1995). A recent report by Song and Endow (1996) ruled out the possibility that motor polarity is determined by differences of binding sites of the motors on microtubules. These results showed that the plus and minus-ended motors bind to partially

overlapping but not identical binding sites on microtubules. The binding sites on microtubules of the same polarity motors (such as Ncd and Kar3) are the same or overlap extensively but the binding sites of motors with opposite directionality (such as KHC and Ncd or KHC and Kar3) partially overlap. It was also proposed that plus- and minus-end-directed motors use different conformational changes to drive different directionality (Arnal et al., 1996). Electron cryo-microscopy and three-dimensional maps of motor-microtubule complexes showed one attached and one unattached motor heads binding to tubulin subunits per dimer (Arnal et al., 1996). These studies exhibited a similar conformation of the attached motor heads and interaction with the same region of the tubulin subunits but distinctly different conformation for the unattached heads, suggesting that differences on binding conformation of free head and dimer could determine directionality (Arnal et al., 1996). Henningsen and Schliwa (1997) discovered that removal of the kinesin motor domain at N-terminus and replacing it with the Ncd C-terminal motor domain result in the movement of the chimeric construct towards the plus-end. They proposed that the position of the motor domain is important for the polarity and is not the only directionality-controlled determinants (Cross, 1997; Henningsen and Schliwa, 1997) but Case et al (1997) proved the "neck" region adjacent to the motor domain is the major directionality-controlled determinant. Case et al. (1997) used a similar idea and created more chimeric constructs to solve this mystery. They used the catalytic domain (the nucleotide and microtubule binding sites in the motor) of Ncd to replace the kinesin's catalytic domain and thus created a chimeric construct that had plus-end-polarity. They proposed that the catalytic domain responds to motor properties but

not directionality; the position of the motor domain does not influence directionality; the minimal motor domain and neck region of kinesin and Ncd are sufficient to generate polarity; and the "neck" serves as a mechanical transducer to determine directionality. They claimed that the neck domain, not visible in the electron density maps, must differ from kinesin to Ncd (Case et al., 1997), and Sablin et al. (1998) confirmed that there is a difference between neck regions of Ncd and kinesin. Endow and Waligora (1998) analyzed the chimeric motor containing the stalk and neck of the Ncd motor and motor domain of kinesin and found that the Ncd stalk and neck reverse kinesin motor polarity. The neck region (defined as a region of 20-40 amino acids between the end of the coiled-coil region and the conserved structural element in motor domain) is very critical for polarity and attachment of the neck to stalk may also be important. Changes in the amino acid residues in the neck region reverse the movement direction (Endow and Waligora, 1998).

Ncd is necessary for the formation of bipolar spindle in oocyte meiosis. Ncd has been proposed to focus meiotic spindle microtubules into spindle poles using its minus-end directed motility and microtubules bundling ability (Hatsumi and Endow, 1992). The localization of Ncd on mitotic spindles, including spindle poles and centrosomes, suggests that Ncd keeps centrosome attaching on spindle poles and prevents it from splitting in mitosis (Endow et al., 1994a). Ncd has the ability to bundle microtubules *in vitro* (McDonald et al., 1990). The truncated N-terminal tail of Ncd lacking the C-terminal motor domain has an ATP-insensitive microtubule-binding site and bundling ability (Chandra et al., 1993), supporting the hypothesis that Ncd is involved in organization of spindle poles.

KAR genes were first identified in mutants defective in karyogamy, a process in which two haploid nuclei fuse to form a diploid nucleus during yeast *Saccharomyces cerevisiae* mating (Polaina and Conde, 1982). The KAR1 gene is involved in spindle pole body (SPB) duplication (Rose and Flink, 1987). The KAR2 gene encodes a protein with homology to HSP70 protein in the endoplasmic reticulum (Normington et al., 1989; Rose et al., 1989). Kar3 mutants accumulate many mitotically delayed or arrested cells with a single nucleus and a short mitotic spindle (Meluh and Rose, 1990). Immunolocalization of Kar3 shows microtubule-dependent localization to spindle pole body, cytoplasmic microtubules (Meluh and Rose, 1990) and the mitotic apparatus (Page et al., 1994). Kar3 is a C-terminal minus-end directed motor, showing both structural and functional similarities to Ncd (Endow et al., 1994b). Outside the motor domain, the sequence of Kar3 is different from Ncd. However, both proteins contain a basic and proline-rich region and also a microtubule-association site in the N-terminal tail domain (Meluh and Rose, 1990). In mitosis, Kar3 can move chromosomes by attaching to kinetochores and pulling them polewards (Endow et al., 1994b). The Kar3 is unusual in destabilizing taxol-stabilized microtubules at their minus-ends (Endow et al., 1994b). Using this destabilizing activity, Kar3 functions to limit the number and length of cytoplasmic microtubules from START (the entry to S phase) to anaphase of the cell cycle (Saunders et al., 1997), and this depolymerizing activity for regulating microtubule turnover may be the major function for Kar3. A timing mechanism controlled by spindles is capable of initiating spindle elongation at the "correct" time in anaphase even without chromosomes (Zhang and Nicklas, 1996) and the Kar3 may maintain a

rapid turnover of the cytoplasmic microtubules to ensure proper spindle elongation and position until the right time in anaphase (Saunders et al., 1997). According to the results of double mutant analysis, function of Kar3 to spindle assembly is balanced by opposing forces generated by Cin8 and Kip1 in separating spindle poles and spindle elongation (Sauders and Hoyt, 1992); the spindle length is determined by antagonistic forces exerted by Kar3 and Cin8/Kip1 on the spindle (Sauders et al., 1997); and the cytoplasmic microtubule numbers are controlled by Kar3 and Kip2 (Huyett et al., 1998). Kar3 is important, not essential, in mitosis but essential for meiosis I (Bascom-Slack and Dawson, 1997). In Kar3 mutants, meiotic cells are blocked in prophase I with monopolar microtubule arrays and incomplete synaptonemal complexes, suggesting that Kar3 may participate in meiosis and culminate in chromosome synapsis (Bascom-Slack and Dawson, 1997). The Kar3 mutants show greater sensitivity to NaCl-stress and sucrose-stress but are not sensitive to heat-stress, nitrogen-starvation or to ethanol as a carbon source. This indicates that KAR3 may have a function in osmotic stress in addition to karyogamy, mitosis and meiosis (Schoch et al., 1997).

Plant KLPs

Until recently, intracellular motility in plants was investigated only in a few cases. The cytoplasmic streaming in non-dividing cells caused by an actin-based motor, such as myosin, was identified in lily pollen tubes and in characean algae (Shimmen and Yokota, 1994; Williamson, 1993). In pollen tubes, how microtubule-based motors are involving in the dynamic changes of the cortical microtubules requires

further investigation (Asada and Collings, 1997). Microtubule-based motors involved in cell division have been investigated. The studies result in a better understanding of the roles of molecular motors in cell division. However, the understanding of motors in plants is still in its infancy as compared to yeast and animal systems.

Many cell division processes such as spindle formation, chromosome movement and spindle pole elongation are similar among plant, animal and yeast systems suggesting that some plant KLPs may be similar to motors from animal and yeast systems. The plant spindle, which is formed in the absence of a well-defined centrosome, and several unique microtubule arrays such as the preprophase band (PPB) and the phragmoplast may require unique plant KLPs. The centrosome-free meiotic spindle in *Drosophila* oocytes requires the minus-end-directed kinesin motor, Ncd, to help focus microtubules to form spindle poles (Endow et al., 1990), implying that minus-end-directed microtubule-based motors might be involved in bipolar spindle formation in plants. The rapid turnover of all plant microtubules suggests that the transition between microtubule arrays and changes in orientation may involve depolymerization/repolymerization of microtubules, rather than direct movement (Hepler and Hush, 1996). Although the mechanisms that regulate the formation and dynamic instability of microtubule arrays in plants are not completely clear, microtubule motors may be involved in these processes just like the depolymerizing activity of Kar3, *Xenopus* KCM1 and KIF2 in regulating microtubule turnover (Desai et al., 1999; Endow et al., 1994b; Saunders et al., 1997).

An actin-based myosin is found in *Nicotiana alata* (Tang et al., 1989), which is localized in the cytoplasm of pollen tubes and involved in organelle transport and cell

division. Studies to identify microtubule-based kinesin motors have focused on three strategies: isolating proteins from phragmoplast extracts, using antibodies against kinesin and/or cloning via the polymerase chain reaction (PCR).

Two polypeptides (125 kDa and 120 kDa) found in an extract of isolated phragmoplasts of tobacco BY-2 cells cause minus-end directed microtubule gliding with microtubule-dependent ATPase and GTPase activity (Asada and Shiboaka, 1994; Asada et al., 1991). Microtubules glide by their minus-end in the lead indicating that the proteins are plus-end-directed motors (Asada and Shiboaka, 1994). Analysis of the cDNA for the 125 kDa polypeptide, named TKRP125 (tobacco kinesin-related polypeptide of 125kDa), indicates that it possesses an amino-terminal kinesin-like motor with strong similarity to members of the BimC subfamily (Asada et al., 1997). TKRP125 does not cross-react with antibodies against conventional kinesin (Asada and Shiboaka, 1994). An antibody against motor domain of TKRP125 inhibits GTP- and ATP-dependent translocation of phragmoplast microtubules (Asada et al., 1997). TKRP125 shows a cell-cycle-dependent expression with absence at the G1 phase, but appearance at the S phase, and accumulation in the G2 and M phase. TKRP125 localizes to the cortical microtubules in the S phase, along microtubules of the preprophase band (PPB) and perinuclear microtubules in prophase, the equatorial plane of spindle microtubules in metaphase and anaphase, and the phragmoplast microtubules in telophase and cytokinesis (Asada et al., 1997). These observations suggest that the TKRP125 might be involved in the changes of microtubule arrays, especially in chromosome movement and phragmoplast function (Asada et al., 1997).

A microtubule-interacting kinesin homologue is found in the cytoplasm of *Nicotiana tabacum* pollen tube (Tiezzi et al., 1992). The pollen kinesin homologue (PKH) was identified and showed ATP-dependent microtubule-binding ability and microtubule-stimulated ATPase activity. Using antibodies against conventional kinesin (Cai et al., 1993), the cross-reacting protein colocalizes with microtubules in the apex of pollen tubes, a place rich with Golgi-vesicles (Tiezzi et al., 1992). A kinesin-related polypeptide and homologue to tobacco PKH, was identified in a Golgi vesicle-enriched fraction from *Corylus avellana* pollen with microtubule-activated ATPase activity (Liu et al., 1994). A kat gene family (katA, katB, katC, katD, and katE) encoding kinesin-like proteins in *Arabidopsis thaliana* has been characterized using PCR. These KLPs contain a motor domain and share sequence similarity in ATP-binding and microtubule-binding sites with kinesin-like proteins (Mitsui et al., 1993; Mitsui et al., 1994; Tamura et al., 1999). The C-terminal region of katA protein shows homology with the motor domain of kinesin heavy chain, and its predicted secondary structure similar to kinesin-like proteins (Mitsui et al., 1993). KatA (89 kDa) protein in *Arabidopsis* has been shown to have a minus-end-directed motor, and similar proteins has been found in carrot and tobacco (Liu et al., 1996). KatA is localized near the midzone at metaphase and anaphase and in the phragmoplast in suspension cells of *Arabidopsis* and tobacco BY-2 (*Nicotiana tabacum* cv. Bright Yellow-2), and is detectable but weak in the cytoplasm and nucleus during interphase (Liu et al., 1996). Using antibodies against KatB (82 kDa) and KatC (84 kDa), a kinesin-like polypeptide, KatB/C, is detected in synchronized tobacco BY-2 cells and increases during M phase and decreases after cytokinesis

(Mitsui et al., 1996). Microtubule-based structures, such as the spindle and phragmoplast, may be the action site of the KatB/C protein (Mitsui et al., 1996). The central region of KatD shares sequence similarity to kinesin heavy chain (Tamura et al., 1999). This central region is followed by about 240- amino acid residues on C-terminus and that exhibits no significant similarity to other KLPs (see Figure 1.1) which suggests that KatD may belong to the internal motor kinesin subfamily (MCAK/KIF2) (Tamura et al., 1999). This would make KatD phylogenetically distant from other plant KLPs (Tamura et al., 1999). The predicted secondary structure shows the absence of a α-helical coiled-coil region indicating that KatD is a monomeric motor (Tamura et al., 1999). The amino-terminal region shows homology to calponin homology (CH) domain, a highly conserved region in actin-cross-linking proteins, suggesting that KatD participates in actin-binding (Tamura et al., 1999). Cytoskeleton and cytoskeleton-motor proteins are required for the development and growth of pollen tubes (Cai et al., 1997). KatD is a floral organ-specific motor protein that may contribute to the actin cytoskeleton in pollen (Tamura et al., 1999).

From Arabidopsis, Reddy et al. (1996b) have isolated a kinesin-like calmodulin-binding protein (KCBP) which possesses a C-terminal motor domain with minus-end motility (Song et al., 1997). There have been twenty-four kinesin-like proteins cloned from *Arabidopsis,* and the structural functions of them are shown in Figure 1.1. Homologues of KCBP have been cloned in tobacco, potato and maize (Abdel-Ghany and Reddy, unpublished data; Reddy et al., 1996a; Wang et al., 1996). KCBP will be discussed in detail in next section.

Kinesin-Like Calmodulin-Binding Protein (KCBP)

A unique *Arabidopsis* kinesin-like calmodulin-binding protein (AKCBP) has been isolated by screening an expression library for calmodulin-binding proteins (Reddy et al., 1996b). This KCBP is 1261 amino acid long with an estimated molecular mass of about 143 kD. A stretch of 340 amino acids at the C-terminus shows significant similarity with the kinesin motor domain containing ATP- and microtubule-binding sites. The calmodulin-binding domain (CBD) in the very end of C-terminus is mapped to a stretch of 23 amino acids and located adjacent to the motor domain. Outside the motor domain, there is no sequence similarity between KCBP and other KLPs. There are three major domains existed in KCBP. A motor domain located on C-terminus and a putative coiled-coil domain in the middle link to an N-terminal globular tail domain. In addition to providing a rotational freedom for the kinesin molecule, this coiled-coil stalk has been reported to aid in dimerization (Bloom and Endow, 1994; Chandra et al, 1993), so the KCBP is hypothesized to be a dimer (Reddy et al., 1996b). The region in the amino-terminal part of KCBP shows homology to a region in some myosins, called the myosin tail homology domain (MyTH4) (Chen et al., 1996; Reddy et al., 1996b). The MyTH4 domain is a typical feature of myosin subfamilies IV, VIIa, X and XII. A region of 300 amino acids near the center of the KCBP shows significant homology to talin, which can interact with membranes and the actin filaments (Niggli et al., 1994; Nuckolls et al., 1990). The roles of MyTH4 and talin homology domains in KCBP are not known. The N-terminal tail domain contains a PEST motif rich in proline, glutamate, serine and theronine (Reddy et al., 1996b), and suggests that KCBP is a rapidly turned over

protein. These three domains (calmodulin-binding domain, MyTH4 and talin homology domain) have not been found in any other known members of the KLP superfamily. These predicted functional domains of KCBP are showed in Figure 1.2. Homologs of KCBP have been cloned and characterized from potato (PKCBP) (Reddy et al., 1996a), tobacco (TCK1) (Wang et al., 1996) and maize (Abdel-Ghany and Reddy, unpublished data), suggesting that the KCBP is ubiquitous in plants.

The KCBP motor domain has microtubule-stimulated ATPase activity and ATP sensitive microtubule binding activity (Reddy et al., 1996a). KCBP is a minus-end directed microtubule motor which translocates on MTs with a velocity of 8-10 μm/min (Song et al., 1997) with opposite polarity to "conventional" kinesins. The C-terminal motor domain and polarity of KCBP is the same as Ncd and has been classified as a member of the C-terminal motor kinesin subfamily. AKCBP motor cannot bind to microtubules in the presence of calcium/calmodulin, but calcium alone or calmodulin alone has no effect (Song et al., 1997). There is an inhibition of binding of the motor domain to microtubules in the presence of both calcium and calmodulin, and this negative regulation is dependent on the concentration of calcium/calmodulin (Deavours et al., 1998). Calcium/calmodulin does not influence the basal ATPase activity of KCBP but reduces the microtubule-stimulated ATPase activity. The results from Deavours et al. (1998) suggest that calcium/calmodulin might regulate the activity of KCBP by inhibiting its association with microtubules. In the N-terminal tail domain of KCBP, there is a second microtubule-binding site in a stretch of 270 amino acids which is ATP-independent (Narasimhulu and Reddy; 1998).

The anti-KCBP antibody is raised against the synthetic peptide of calmodulin-binding domain specific in the KCBP motor. In the tobacco BY2 suspension cells, KCBP is detectable in interphase but increased in mitotic cells (Bowser and Reddy, 1997). Using the affinity-purified anti-KCBP antibody, KCBP is localized on the preprophase band (PPB), mitotic spindle, phragmoplast and cell plate in tobacco, *Arabidopsis* and *Haemanthus* endosperm (Bowser and Reddy, 1997; Smirnova et al., 1998). In *Haemanthus* endosperm, KCBP is highly concentrated within the kinetichore fibers and kinetichore microtubules during metaphase then redistributed to spindle polar regions in anaphase (Smirnova et al., 1998).

The *ZWICHEL* (*ZWI*) gene that is involved in the trichome morphogenesis pathway in *Arabidopsis*, has been cloned by T-DNA tagging (Oppenheimer et al., 1997). The *ZWI* gene product is identified as KCBP, suggesting a physiological role for KCBP in trichome development. To date, KCBP is the only member in the kinesin superfamily with a calmodulin-binding domain. KCBP is unique to plants, and its absence in animals implies the possibility that it may be involved in some aspects of cellular development that are specific to plant cells.

Table 1. BimC, the kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
BimC	*A. nidulans*	spindle, poles		SPB separation, spindle bipolarity	Enos and Morris, 1990; O'Connell et al., 1993
Cin8	*S. cerevisiae*	spindle		SPB separation, spindle bipolarity	Hoyt et al., 1992; 1993
Cut7	*S. pombe*	spindle, poles		SPB separation, spindle formation	Hagan and Yanagida,1990;1992
Eg5	*H. sapeins*	spindle, poles	(+)	Centrosome separation	
	X. laevis	spindle, poles	(+)	Spindle bipolarity formation	Blangy et al., 1995; 1998; Koshland et al., 1988; Le Guellec et al., 1991; Rothwell et al., 1993; Sawin et al., 1992; Sawin and Mitchison, 1995; Sawin and Nurse, 1998
KIF8	*M.musculus*				
KIF11	*M.musculus*				
Kip1	*S. cerevisiae*	spindle, poles		Spindle bipolarity, Redundant with Cin8	Hoyt et al., 1992; 1993; Rodionov et al., 1990; Roof et al., 1992
Klp61F (KRP130)	*D. melanogaster*	spindle, poles	(+)	Centrosome separation	Cole et al.,1994; Heck et al.,1993; Wilson and Fuller, 1993
Krp	*D. melanogaster*				
KRP125	*N. tabacum*				

Table 2. The C-terminal kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
CHO2	*C. griseus*	spindle, centrosomes, poles	(-)	centrosome in interphase, spindle in mitosis	Dragas-Granoic et al., 1993; Kuriyama et al., 1995
CTK1	*X. laevis*				
2	*X. laevis*	spindle, poles		Pole formation	Walczak et al., 1996; 1997
FKIF2	*M. saxatilus*			accumulated in fish retina	Bost-Usinger et al., 1997
HSET	*H. sapiens*				Ando et al., 1994
Kar3	*S. cerevisiae*	spindle, poles	(-)	karyogamy (nuclear fusion after mating) opposite to Cin8/Kip1	Endow et al., 1994b; Hoyt et al., 1993; Meluh and Rose, 1990; Middleton and Carbon, 1994; O'Connell et al.,1993
KatA	*A. thaliana*	spindle, phragmoplast			Liu et al., 1996; Mitsui et al., 1993
B	*A. thaliana*			accumulated in mitosis	Mitsui et al., 1996
C	*A. thaliana*				Mitsui et al., 1994
KCBP	*A. thaliana* (AKCBP)	spindle, preprophase band, phragmoplast	(-)	part kinesin, part myosin, trichome development	Reddy and Reddy, 1999; Oppenheimer et al, 1997
	N. tabacum (TCK1)				Wang et al., 1996
	S. tuberosum (PKCBP)				Reddy et al., 1996a
	Z. may				Abdel-Ghany and Reddy, unpublished data
KIFC1	*M. musculus*				Hirokawa, 1996
2	*M. musculus*	neuron, organelles		non-mitotic motor	Hanlon et al., 1997; Saito et al., 1997
3	*M .musculus*				Hirokawa, 1996
Klp2	*S. pombe*				Troxell and McIntosh, 1993
Klp3	*C. elegans*				Khan et al., 1997
KlpA	*A. nidulans*	spindle		opposite force to BimC, complements kar3 mutant	O'Connell et al., 1993
Ncd	*D. melanogaster*	spindle, centrosomes, poles	(-)	spindle formation, spindle integrity	Endow et al., 1990; 1994a; McDonald and Goldstein, 1990; Moore and Endow, 1996
Pkl1	*S. pombe*	spindle, poles			Pidoux and Cande, 1993; Pidoux et al., 1996; Troxell and McIntosh, 1993

Table 3. Ce1, the kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
C06G3.2	*C. elegans*	ND	ND	ND	http://www.blocks.fhcrc.org/~kinesin/
LC33H5.4	*C. elegans*	ND	ND	ND	http://www.blocks.fhcrc.org/~kinesin/

ND, non-determined

Table 4. Chromokinesin/KIF4, a kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
Chrkin	*G. gallus*	chromosome arms		binds on DNA	Wang and Adler, 1995
KIF4	*H. sapiens*				
	M. musculus	membranous vesicles, mitotic spindle	(+)	vesicle transport	Sekine et al., 1994
Klpl	*X. laevis*	chromosome arms		positioning mitotic chromosomes, stabilizing bipolar spindle	Vernos and Karsenti, 1995; Vernos et al., 1995
Klp3A	*D. melanogaster*	meiotic spindle midbody		midbody	William et al., 1995

Table 5. KHC, a kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
KH3	*M. musculus*				
KHC					
CeKHC(Unc116)	*C. elegans*	vesicles, organelles	(+)	axonal transport of synaptic vesicle, also orients first mitotic spindle	Hall et al., 1991; Patel et al., 1993; Sulston et al., 1992
DmKHC	*D. melanogaster*	cytoplasm, vesicles, organelle	(+)	required for viability and neuromuscular function	Saxton et al., 1988; 1991; Stewart et al., 1993; Yang et al.,1988;1989
HsKHC	*H. sapiens*	uniform in cultured neurons, vesicles, organelle	(+)	ubiquitously expressed	Navone et al., 1992; Niclas et al., 1994
HsnKHC	*H. sapiens*	cell body in cultured neurons	(+)	specific in neural tissue	
LpKHC	*L. pealii*	membranous vesicles	(+)		Kosik et al., 1990; Pfister et al., 1989; Vale et al., 1985a; b; c;
MmKHC(KIF5)	*M. musculus*	microsomes, vesicles, organelles	(+)	specific in brain	Aizawa et al., 1992; Hirokawa et al., 1989; Kondo et al.,1994; Noda et al., 1995
NcKHC	*N. Crassa*		(+)	high velocity of microtubule transport	
SpKHC	*S. purpuratus*	membranous vesicles	(+)		Cohn et al., 1987; Henson et al., 1992; Scholey et al., 1985; Wright et al., 1991
KIF1B	*M. musculus*				
KIN1	*N. haematococca* *S. racemosum*				Wu et al., 1998

Modified from http://www.blocks.fhcrc.org/~kinesin/

Table 6. KIP3, the kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
BC2F12.13	*S. pombe*	ND	ND	ND	http://www.blocks.fhcrc.org/~kinesin/
BC649.01c	*S. pombe*	ND	ND	ND	http://www.blocks.fhcrc.org/~kinesin/
KIP3	*S. cerevisiae*	ND	ND	ND	http://www.blocks.fhcrc.org/~kinesin/

ND, non-determined

Table 7. KRP85/95, a kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
Fla10	*C. reinhardtii*	flagella		required for flagellar assembly/maintenance	Walther et al., 1994
KIF3A	*M. musculus*	microsomes/synaptic vesicles	(+)	fast axonal transport	Aizawa et al., 1992; Kondo et al., 1994
KIF3b	*M. musculus*				Aizawa et al., 1992; Kondo et al., 1994
Kip3	*X. laevis*				
Kip68D	*D. melanogaster*		(+)	possible motor for anterograde axonal transport	Pesavento et al., 1994
KRP85	*S. purpuratus*		(+)	heterotrimer with KRP95	Cole et al., 1992; 1993
KRP95	*S. purpuratus*		(+)	heterotrimer with KRP85	Cole et al., 1993; Rashid et al., 1995
Osm3	*C. elegans*	vesicles		required for cilia, transport of vesicles in sensory apparatus	Perkins et al., 1986; Shakir et al., 1993

Table 8. MCAK/KIF2, a kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
CAK	*H. sapiens*				
DSK1	*C. fusiformis*	spindle			Foss et al., 1993; Wein et al., 1996
HK2	*H. sapiens*				
KCM1	*X. laevis*	centromeres		MT dynamics; destabilizing MT	Desai et al., 1999
KIF2	*M. musculus*	membranous vesicles	(+)	axons, transporting specific organelles	Aizawa et al., 1992; Noda et al., 1995
	X. laevis			destabilizing MT	Desai et al., 1999
Krp2	*R. norvegicus*				Sperry and Brady, 1993; Sperry and Zhao, 1996
MCAK	*C. griseus*	kinetochore		kinetochore MTs	Maney et al., 1998; Wordeman and Mitchison, 1995

Table 9. MKLP1, a kinesin subfamily.

Protein	Organism	Location	Motor polarity	Functions	References
CHO1	*C. griseus*	spindle, midbody		separates poles at anaphase B?	Kuriyama et al., 1994
M03D4.1	*C. elegans*				
MKLP1	*H. sapiens*	spindle, midbody and poles	(+)	separates poles at anaphase B?	http://www.blocks.fhcrc.org/~kinesin/
PAV-KLP	*D. melanogaster*				

Table 10. Unc104, kinesin subfamily.

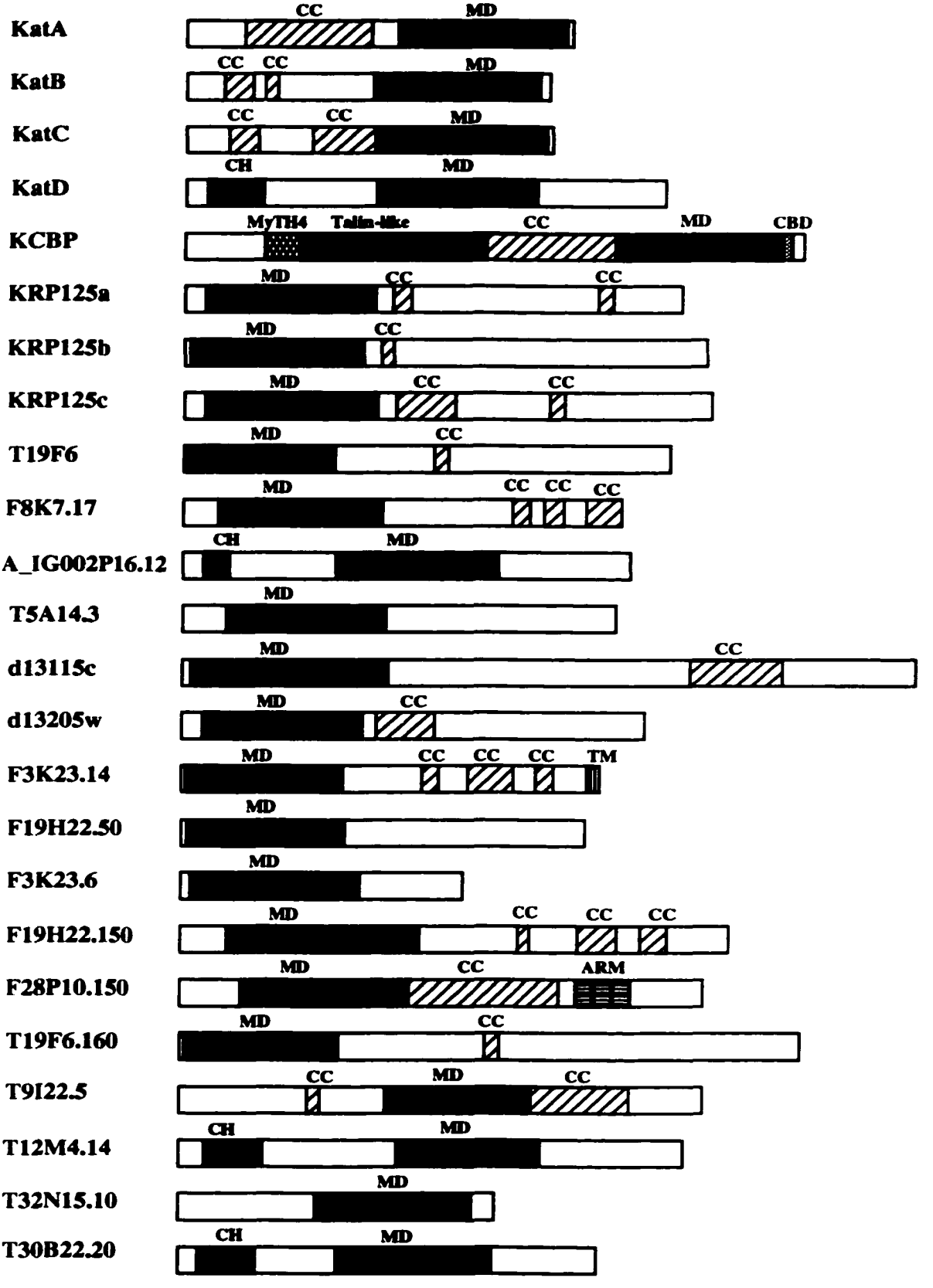
Protein	Organism	Location	Motor polarity	Functions	References
ATSV	*H. sapiens*				
KIF1A	*M. musculus*	axon	(+)	in synaptic vesicles	Okada et al., 1995
KIF1B	*M. musculus* *R. norvegicus*	mitochondria	(+)	transport mitochondria	Nangaku et al., 1994
KIF1D	*R. norvegicus*				
Klp73	*D. melanogaster*				
Unc104	*C. elegans*			synaptic vesicle transport	Hall and Hedgecock, 1991; Otsuka et al., 1991

Table 11. The ungrouped kinesin-like proteins (KLPs).

Protein	Organism	Location	Motor position/polarity	Functions	References
CENP-E	*H. sapiens*	centrosome, kinetochore	N/(+)	kinetochore localization, chromosome movement	Brown et al., 1996; Duesbery et al., 1997; Lombillo et al., 1995a; Thrower et al., 1995; Wood et al., 1997; Yen et al., 1991; 1992; Zecevic et al., 1998
Kid	*H. sapiens*	chromosome, centromere		spindle formation, chromosome segregation	Tokai et al., 1996
Kin	*L. chagasi*				
KIP3	*S. cerevisiae*	spindle fibers, asters midzone		nuclear migration, spindle positioning	
Klp1	*C. rheinhardtii*	central pair MTs	N/ ND	flagellar beating	Bernstein et al., 1994
Klp2	*X. laevis*	spindle, poles		spindle bipolarity, Centrosome separation	
Klp3A	*D. melanogaster*	mitotic spindle, midbody			
Klp67A	*D. melanogaster*				
Nod	*D. melanogaster*	meiotic chromosome	N/ND	binds to DNA	Zhang et al., 1990b; Afshar et al., 1995
Smy1	*S. cerevisiae*		N/ND	secretion vesicle	Lillie and Brown, 1992; 1993; 1994

ND, non-determined

Figure 1.1: Schematic representation of the kinesin-like proteins (KLPs) cloned from *Arabidopsis* described in the text (Reddy, 1999).
CC, α-helical coiled-coil region; MD, motor domain; CH, calponin homology domain; MyTH4, myosin tail homology domain; Talin-like, talin homology domain; CBD, calmodulin-binding domain; TM, transmembrane; ARM, Armadillo domain.



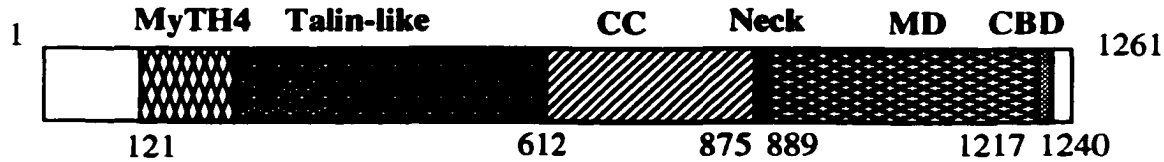


Figure 1.2: Schematic representation of *Arabidopsis* kinesin-like calmodulin-binding protein (AKCBP).
AKCBP: The full-length *Arabidopsis* kinesin-like calmodulin-binding protein showing various domains that are identified based on sequence similarity or functional analysis of *E. coli* expressed protein (amino acids 1-1261) (Chen et al., 1996; Reddy et al., 1996b; Reddy and Reddy, 1999).
Tail domain, a region located on the N-terminal part of AKCBP (amino acids 1-612), including MyTH4 and Talin-like domains.
MyTH4, a domain presents in the tail region of some myosins.
Talin-like, a domain showed homology to talin.
CC, α-helical coiled-coil region located on the middle (amino acids 612-875).
Neck, neck domain located between CC and MD (amino acids 875-889).
MD, motor domain located on the C-terminus of AKCBP (amino acids 889-1217).
CBD, calmodulin-binding domain adjacent to MD (amino acids 1217-1240).

CHAPTER 2

Interaction of *Arabidopsis* kinesin-like calmodulin-binding protein with tubulin subunits: modulation by Ca^{2+}-calmodulin

Part of these results have been published as:
Interaction of *Arabidopsis* kinesin-like calmodulin-binding protein with tubulin subunits: modulation by Ca^{2+}-calmodulin,
S.B. Narasimhulu, Y-L. Kao, and A.S.N. Reddy, Plant J. 12 (1997) 1139-1149.

Abstract

Kinesin-like calmodulin-binding protein (KCBP) is a recently identified novel kinesin-like protein that appears to be unique to and ubiquitous in plants. KCBP is distinct from all other known KLPs in having a calmodulin-binding domain adjacent to its motor domain. Different regions of KCBP have been used to study its interaction with tubulin subunits and the regulation of this interaction by Ca^{2+}-calmodulin. The results show that the carboxy-terminal part of the KCBP, with or without calmodulin-binding domain, binds to tubulin subunits, and this binding is sensitive to nucleotides. In the presence of Ca^{2+}-calmodulin the motor with calmodulin-binding domain does not bind to tubulin. This Ca^{2+}-calmodulin modulation is abolished in the presence of antibodies specific to the calmodulin-binding domain of KCBP. Similar binding studies with the carboxy-terminal part of KCBP lacking the calmodulin-binding domain show no effect of Ca^{2+}-calmodulin. These results indicate that Ca^{2+}-calmodulin modulates the interaction of KCBP with tubulin subunits, and this modulation is due to the calmodulin-binding domain in the KCBP. Calcium-dependent calmodulin modulation of KCBP interaction with tubulin suggests regulation of KCBP function by calcium, the first such regulation of a kinesin heavy chain among all the known kinesin-like proteins.

Introduction

Interaction of motors with microtubules is an important component in translocation of kinesins along microtubules and is likely to be highly regulated. Both plus-end and minus-end kinesins interact with tubulin subunits, and this interaction is modulated by polyglutamylation of tubulin subunits (Larcher et al., 1996; Walker, 1995). There is also evidence for mechanisms such as phosphorylation that modulate the interaction of motors with microtubules (Blangy et al., 1995; Liao et al., 1994; Sawin and Mitchison, 1995). Among motor proteins, the heavy chain of some actin-based myosin motors and the light chain of microtubule-based kinesin motors show calcium and calmodulin-dependent regulation (Matthies et al., 1993; Wolenski, 1995). The presence of a calmodulin-binding domain next to the motor domain in KCBP and its binding to calmodulin at micromolar calcium concentration raises an interesting possibility that calcium, an important messenger molecule in plant and animal systems (Gilroy and Trewavas, 1994; Poovaiah and Reddy, 1993; Roberts and Harmon, 1992), through calmodulin, may regulate the interaction of KCBP with tubulins. In this chapter, the binding of KCBP to tubulin subunits and the possible role of calcium/calmodulin in modulating KCBP interaction with tubulin subunits was investigated.

Materials and methods

Construction of recombinant plasmids

Plasmids expressing different parts of KCBP were prepared as follows. A *SacI/SalI* fragment of *Arabidopsis* KCBP cDNA containing nearly complete coding sequence (amino acid 12-1261) was cloned in-frame into pET 28b expression vector. A 1.6-kb *ScaI/NotI* fragment corresponding to the 3' end of KCBP was deleted from this clone by digesting with respective restriction enzymes. The ends of the digested plasmid were repaired with Klenow (Sambrook et al., 1989) and religated to generate 2.5 N construct (2.5-kb from N-terminal end) that produced a truncated KCBP containing the amino-terminal region (amino acids 12-806). For preparing the 1.5 C (1.5-kb at C-terminus) construct, a 1.5-kb *EcoRI* fragment was excised from a previously constructed pGEX/AKCBP plasmid (Song et al., 1997) and cloned in-frame into *EcoRI* site in pET32b expression vector.

Preparation of 1.4C and 1.0 C constructed was described earlier in Reddy et al., (1996b). This 1.4-kb *SalI/NotI* cDNA fragment containing the motor domain and calmodulin-binding domain (CBD) on the carboxyl-terminal region was isolated from the full-length of AKCBP in pET 28b vector. The construct with 1.0-kb *SalI/PvuI* region (containing motor domain without CBD) was generated by digested 1.4-kb insert of pET28b vector with *NotI* following by partial digestion with *PuvI* to eliminate the 0.4-kb fragment with CBD. The *SalI/PvuI* ends of 1.0-kb cDNA were filled with T4 DNA polymerase and ligated. These constructs were introduced into *E. coli* BL21 (DE3) strain. The fusion protein was grown in LB medium and induced by adding IPTG to the culture. Soluble and insoluble protein fractions from un-

induced and induced cultures were isolated according to the instructions provided by Novagen.

Protein induction and isolation

Bacterial cultures in LB medium were allowed to grow at 37°C to an OD_{600} of 0.6. Expression of fusion protein was induced by adding IPTG to a final concentration of 0.2mM and the culture was allowed to grow further for an additional 8 hours at 20°C. The bacterial cell suspension was spun, washed and resuspended in one-tenth volume of a resuspension buffer. For 1.5 C and 1.4 C fusion protein that were purified by a calmodulin Sepharose affinity column, the induced cell pellet was resuspended in 50mM Tris, pH7.5, 150mM NaCl and 0.1% Triton. In the case of 2.5 N and 1.0 C fusion proteins which were purified by a His-Bind affinity column the cells were resuspended in 20mM Tris, pH 7.9, 500mM NaCl, 5mM imidazole and 0.1% Triton. The re-suspension buffer for all the constructs contained 200µg/mL lysozyme and "Complete" proteinase inhibitor (a commercial mixture of several proteinase inhibitors from Boehringer-Mannheim). Cells in the resuspension buffer were incubated on ice for an hour followed by 10 pulses of sonication (Virsonic 475, Virtis) at power scale 9, each of 10sec duration, in an iced water-bath, with 2 minutes intervals between each pulse. The sonicated extracts were centrifuged at 100,000g to separate the soluble and insoluble protein fractions. To confirm the induction of fusion protein, soluble and insoluble protein fractions of both uninduced and induced cultures were separated by the denaturing SDS electrophoresis. One gel was stained with Coomassie Blue and the other gel was blotted onto a nitrocellulose membrane

using a Bio-Rad mini transfer cell in a buffer containing 25mM Tris base, 192mM glycine, pH 8.3, and 20% methanol at 100V for an hour, then detected with an appropriate probe.

Detection of induced proteins

To detect the fusion proteins with calmodulin-binding domain using biotinylated calmodulin, the membranes were blocked for 2 hours in a buffer containing 3% gelatin in TBS/Ca/Mg (50mM Tris pH 7.5, 50mM $MgCl_2$, 200mM NaCl and 0.5 mM $CaCl_2$). The blots were washed 3 times in TBS/Ca/Mg, 10 min each, and then incubated for 2 hours in 5μg/mL biotinylated calmodulin (Gibco BRL) made in TBS/Ca/Mg containing 1% gelatin and 0.05% Tween20 (Fordham-Skelton et al., 1994; Reddy et al., 1996b). The blots were washed as above and incubated for 45 min in the presence of Vectastain ABC.HRP complex made in TBS/Ca/Mg containing 0.1% Tween 20. The blots were washed as above and the calmodulin-binding proteins were detected colorimetrically by placing the membrane in a solution containing 100mM Tris, pH 7.5, 0.8mg/mL diaminobenzedine (DAB), 0.4mg/mL $NiCl_2$, and 0.08% hydrogen peroxide.

For detection with anti-KCBP antibodies, the membranes were blocked for 2 hours in 3% gelatin in TBS (20mM Tris, pH 7.5, and 0.5M NaCl), washed 3 times, 10 min each, and incubated in the presence of affinity-purified anti-KCBP antibodies from rabbit (1:5000) made in TBS but supplemented with 1% gelatin for a period of 2 hours. The blots were washed in TBS and incubated in the presence of a secondary antibody, an alkaline phosphatase conjugated anti-rabbit IgG raised in goat.

Immunoreactive protein bands were detected coloremetrically by placing the blots in substrate solution containing 0.34mg/mL nitroblue tetrazolium (NBT) and 0.175mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100mM Tris, pH 9.5, 100mM NaCl and 5mM $MgCl_2$.

To detect 1.0 C, 1.4 C and 2.5 N fusion proteins with T7.tag antibody, the protein blots were blocked for 30 min in 3% gelatin made in TBST (10mM Tris pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and washed for 15 min in TBST. Then the membranes were incubated in the presence of T7.tag monoclonal antibodies from mouse (1:10000 dilution) in TBST for 30 min at 30°C. This was followed by a incubation with an anti-mouse IgG antibody conjugated to alkaline phosphatase. The membrane was washed in TBST as above and immunoreactive protein bands were detected using NBT and BCIP as described above. The 1.5 C KCBP is expressed in pET32 vector as thioredoxin fusion protein and contains an additional S tag which is a 15-amino acid peptide which can bind to S protein from pancreatic ribonuclease A (McCormick and Mierendorf, 1994). Detection of 1.5 C protein based on its S protein is essentially same as that of T7.tag except that we used a covalent complex of purified S protein chemically cross-linked with calf intestinal alkaline phosphatase (Novagen).

Protein purification

Although the fusion proteins were abundantly expressed only a part of the expressed fusion protein (10-40%) was found in the soluble fraction and the rest was present in the inclusion bodies. Only the soluble fraction was used to purify the

protein to ensure that the purified protein is in native form. The high-speed supernatant of the induced bacterial lysate was used for purification of soluble proteins under native conditions.

Fusion proteins from 1.5 C and 1.4 C constructs that contained the calmodulin-binding domain were purified by calmodulin Sepharose column chromatography. Calmodulin Sepharose-4B column was prepared and equilibrated in binding buffer according to the instructions provided by the manufacturer (Pharmacia Biotech Inc). The high speed supernatant was supplemented with 2 mM $CaCl_2$, loaded onto the column and washed with at least 10 times the volume of binding buffer (50mM Tris, pH 7.5, 150mM NaCl and 2mM $CaCl_2$). The calmodulin-binding proteins were eluted in a buffer containing 50mM Tris, pH 7.5, 150mM NaCl and 2mM EGTA. Purified protein was dialyzed against a buffer containing 50mM Tris, pH 7.5 and 150mM NaCl. Fusion proteins from 2.5 N and 1.0 C constructs were purified by taking advantage of His.Tag sequences, a stretch of 6 consecutive histidine residues that is present at the N-terminal end of the fusion proteins. The His.Tag sequence binds to divalent cations immobilized on the His-Bind metal chelation resin (Novagen). The high speed supernatant extract of the induced bacteria was applied onto the His-Bind column and allowed to bind in the presence of binding buffer containing 20mM Tris, pH 7.9, 0.5M NaCl and 5mM imidazole followed by extensive washing in a buffer containing 20mM Tris, pH 7.9, 0.5M NaCl and 20mM imidazole. The bound proteins were eluted in an elution buffer that is similar to that of wash buffer but contained 100mM imidazole in place of 20mM imidazole.

Purified protein was dialyzed against a buffer containing 50mM Tris, pH 7.5, and 150mM NaCl.

Generation and purification of the anti-KCBP antibodies

A synthetic peptide corresponding to a stretch of 23 amino acids in the C-terminal end of *Arabidopsis* KCBP (Reddy et al., 1996b) was synthesized and conjugated to 20mg Keyhole Limpet Hemocyanin (KLH). The conjugate was diluted in part deionized water and one part Freund's complete adjuvant and injected intradermally at multiple spots on New Zealand White rabbits. Booster injections (100-300 μg immunogen in 1:1 ratio of deionized water: Freund's incomplete adjuvant) were performed 14, 28, 42, and 56 days after initial injection. Bleeds were obtained before the initial injection and on each day a booster injection was given. Serum from the terminal bleed (90 days after initial injection) was used to purify antibodies.

Anti-KCBP antibodies were obtained from serum by affinity purification. The peptide was covalently attached to Immobilon-AV membrane as described in Millipore technical protocol TP015. A 2x2 cm strip of immobilon-AV was incubated with 2mL of peptide solution (1mg of peptide dissolved in 2mL of 0.5M potassium phosphate, pH 7.4) for 2 hours. The strip was rinsed in PBS with 0.1% Tween 20 and incubated in 10% monoethanolamine in 1M $NaHCO_3$ for 2 hours. Following two 30-min rinses in PBS containing 0.1% Tween 20, the strip was incubated with 2mL of serum for 3.5 hours. The strip was rinsed and the antibodies were eluted in 0.9 mL of 100mM glycine, pH 2.5 for 10 min. The solution was removed and neutralized with 0.1 mL of 1M Tris, pH 8.0 (Harlow and Lane, 1988).

Blot overlay assay

Bovine brain tubulin was prepared as described previously (Willams and Lee, 1982; Reddy et al., 1996a). The interaction of KCBP with tubulin was analyzed following a blot overlay assay (Boucher et al., 1994; Larcher et al., 1996). About 30 µg tubulin protein was electrophoresed in an 8 or 10% SDS gel with a single continuous well comb. The gel was transblotted onto a nitrocellulose membrane using a transfer cell in a buffer containing 25mM Tris base, 192mM glycine, pH 8.3, and 20% methanol at 100V for an hour. After blotting, the membrane was stained with 0.2% Ponceau S made in 3% trichloroacetic acid for identifying the location of tubulin subunits. Each membrane carrying 30 µg protein was cut into 15 equal strips and marked for orientation. These strips were placed in a sterile 5cm Petri dish and blocked overnight in MES buffer (50mM MES, pH 6.8, 2mM $MgCl_2$, 1mM dithiothreitol (DTT), 0.1% Tween 20, and 0.1% gelatin). This buffer is a modification of the previously reported MES buffers (Boucher et al., 1994) in that we have removed the chelating agent, EGTA. The membranes were incubated with 4 mL of overlaying protein solution containing KCBP at various concentrations (0.1 to 5µg/mL) diluted in MES buffer. After incubating with KCBP for one hour, the overlaying protein fraction was washed away thoroughly, a minimum of 5 washes of 10-min duration each, using MES buffer. A 30-min incubation in 0.5% formaldehyde made in MES buffer followed by another 30-min incubation in 2% glycine (in MES buffer) was used to stabilize the protein-protein interaction (Kremer et al., 1988) for

subsequent detection studies. The blots were equilibrated for a total duration of 45 min with three changes of buffer. The buffers used for equilibration are (i) TBS with 0.1% gelatin for probing with KCBP antibodies, (ii) TBS/Ca/Mg with 0.1% gelatin for probing with biotinylated calmodulin, or (iii) TBST with 0.1% gelatin for probing with T7.tag antibodies or S protein as required. The binding of KCBP to tubulin was detected with an appropriate probe (biotinylated calmodulin, anti-KCBP antibodies, T7.tag antibody or S protein) as describe above.

Results

Expression and Purification of different parts of KCBPs

To study the interaction of different domains of KCBP with tubulin α and β subunits and the possible modulation by calcium and calmodulin, different parts of KCBP (the full-length, amino- and carboxy-terminal parts of KCBP in *E. coli* as fusion proteins) were expressed and used in pelleting assays. To express full-length KCBP, the coding region corresponding to amino acid residues 12-1261 was cloned in-frame in pET 28. This full-length construct is expected to produce a protein of about 140 kD, but two breakdown products were produced instead. One break down product is a 90-kD protein which is located on N-terminal part of KCBP and can be detected by S protein, the other is a 55-kD protein which is located on C-terminal part of KCBP and can be detected by biotinylated calmodulin or KCBP specific antibody. A single protein of full-length (140 kD) KCBP was never observed, even in the presence of high concentration of proteinase inhibitors.

Four different truncated proteins representing different parts of KCBP were expressed as fusion proteins (Figure 2.1). The 2.5 N contained the amino-terminal part of KCBP (amino acids 12-806). The 1.5 C contains the carboxy-terminal fusion protein of KCBP including motor and calmodulin-binding domains and a part of coiled-coil stalk from amino acids 821-1261. The 1.4 C contains the carboxy-terminal fusion protein of KCBP (like 1.5 C) including motor and calmodulin-binding domains and a shorter coiled-coil stalk from amino acids 860-1261. This extended stalk of coiled-coil region had been reported to help in dimerization for providing a rotational freedom for the kinesin molecule (Bloom and Endow, 1994; Chandra et al., 1993). The 1.0 C contains the carboxy-terminal fusion protein of KCBP like 1.4 C including the motor and shorter coiled-coil stalk but without calmodulin-binding domain (amino acids 860-1210).

The fusion proteins were obtained from inducing the bacteria at room temperature in the presence of 0.2mM IPTG (Figure 2.2). Fusion proteins from 2.5 N and 1.0 C construct were purified by using His-Bind affinity column and the proteins from 1.5 C and 1.4 C constructs were purified by a calmodulin-Sepharose affinity column because they contain the calmodulin-binding domain. The purity of the fusion proteins were assessed by Bio-Rad assay, Coomassie Blue staining and probing the blots with appropriate probes. Fusion protein from 2.5 N, 1.4 C, or 1.0 C was detected with T7. tag monoclonal antibody. Biotinylated calmodulin or KCBP antibody was used to detect 1.5 C and 1.4 C fusion proteins because they contain the calmodulin-binding domain. 1.5 C KCBP was detected with S protein also.

Motor domain of KCBP interacts with α and β tubulin subunits

The motor domain of *Arabidopsis* KCBP can bind to microtubules (Deavours et al., 1996; Reddy et al., 1996a), however, whether KCBP binds to unpolymerized α and β subunits of tubulin is not known. Different truncated versions of KCBP protein were used in the blot overlay assay to test whether KCBP binds to unpolymerized tubulin subunits. As showed in Figure 2.3a, 1.5 C, 1.4 C and 1.0 C fusion proteins can bind to tubulin subunits. Therefore, the absence of calmodulin-binding domain in the 1.0 C fusion protein (Figure 2.3a, lane 1) or shorter length of coiled-coil stalk in 1.4 C KCBP (Figure 2.3a, lanes 2 and 3) does not affect its binding to tubulin monomer. The 2.5 N fusion protein containing only the amino-terminal part of KCBP but not the motor domain did not bind to tubulin subunits (Figure 2.3a, lane 6) suggesting that the motor domain only can bind to tubulin subunits. Similar blot overlay assay containing BSA instead of tubulin did not bind to any KCBP (Figure 2.3b), indicating the specificity of the blot overlay assay. The data from Reddy et al. (1996a) showed that the calmodulin used in the binding assay cannot bind to tubulin. These results show that the C-terminal motor domain of KCBP with or without the calmodulin-binding domain can interact with both tubulin α and β subunits.

KCBP interaction with tubulin subunits is concentration dependent

In order to determine the concentration dependence of KCBP binding to tubulin subunits, blots containing a fixed amount of tubulin were incubated with increasing concentration (from 0.1 to 5 μg/mL) of 1.5 C protein. Probing the blots with KCBP

antibodies indicated increased binding of KCBP to tubulin following with increasing concentration of KCBP up to a saturation point (3 μg/mL) (Figure 2.4a). Blots that were probed with biotinylated calmodulin showed similar results (Figure 2.4b). A competition experiment with tubulin indicated that binding of KCBP to immobilized tubulin is strongly inhibited with increasing concentrations of free tubulin in the overlay solution (Figure 2.5). Concentration-dependent interaction of KCBP with tubulin subunits and the competitive inhibition of this binding by tubulin in overlay solution indicate specificity of KCBP binding to tubulin subunits.

GTP and ATP affect binding of KCBP to tubulin subunits

Kinesins use energy derived from hydrolysis of ATP or GTP to translocate on microtubules. According to the microtubule pelleting assay result from Reddy et al. (1996a), the KCBP motor domain cannot bind to microtubules in the presence of ATP. In the blot overlay assay, KCBP did not bind to immobilized tubulin subunits in the presence of 0.5 mM ATP (Figure 2.6). In the presence of GTP, the binding between KCBP and tubulin subunits was reduced only at concentration of 5 mM, several orders higher concentrations than ATP (Figure 2.6). Considering the KLPs preference for ATP as a substrate over GTP, the difference between these two nucleotides is not surprising. These results indicate that tubulin monomers in the blot overlay assay behave like polymerized microtubules.

Ca^{2+}-calmodulin modulates KCBP binding to tubulin subunits

KCBP is unique among all kinesin-like proteins (KLPs) in possessing a calcium dependent calmodulin-binding domain (Reddy et al., 1996a; b; Wang et al., 1996). The presence of a calmodulin-binding domain suggests that KCBP function is modulated via Ca^{2+}-calmodulin. Calcium had no effect on the binding of 1.5 C protein to tubulin subunits (Figure 2.7). The binding of KCBP to tubulin was reduced in the presence of calmodulin alone. However, the KCBP motor domain failed to bind on tubulin subunits in the presence of both calcium and calmodulin (Figure 2.7). The effect is similar with both α and β tubulin subunits. These results are consistent with results obtained in *in vitro* motility assays and microtubule pelleting assays in the presence of calcium and calmodulin (Deavours et al., 1996; Song et al., 1997).

Antibodies to the calmodulin-binding domain of KCBP abolish Ca^{2+}-calmodulin modulation of KCBP interaction with tubulin subunits

KCBP specific antibody was raised using a synthetic peptide of 23 amino acids corresponding to the calmodulin-binding domain. In mobility shift assay, it was shown that the calmodulin binds to this synthetic peptide with high affinity in a calcium-dependent manner (Reddy et al., 1996b). Affinity purified antibody against this synthetic peptide recognizes a protein of expected size in different plant (data not shown) and bacterial cells that express KCBP (Figure 2.2b and c). To determine if the calmodulin-binding domain is responsible for Ca^{2+}-calmodulin modulation of KCBP binding to tubulin, binding studies in the presence of antibodies specific to the

calmodulin-binding domain were performed. Inclusion of antibodies in the overlay solution restricted the use of the anti-KCBP antibodies or biotinylated calmodulin as probes to detect binding. So the advantage of the S protein tag available at the amino end of the fusion protein was taken to detect binding of motor to tubulin subunits. Such an analysis indicated that the motor domain protein containing the calmodulin-binding region is able to bind to tubulin subunits. The calcium and calmodulin-dependent modulation that we observed with 1.5 C alone is no longer evident in the presence of antibodies (Figure 2.8) indicating that the effective blockage of the calmodulin-binding domain by the antibodies.

Motor domain lacking calmodulin-binding region fails to show Ca^{2+}-calmodulin modulation

To address whether deletion of calmodulin-binding domain abolishes Ca^{2+}-calmodulin modulation, binding assays with 1.0 C KCBP protein which lacks the calmodulin binding domain were performed in the presence of calcium alone, calmodulin alone, or in the presence of both calcium and calmodulin. Analysis of binding of 1.0 C protein to tubulin showed no effect of calcium-calmodulin (Figure 2.9). The binding of 1.0 C KCBP to tubulin in the presence of calcium/calmodulin is similar to that of binding in the absence of Ca^{2+}-calmodulin. The results of this experiment demonstrate that the Ca^{2+}-calmodulin dependent modulation of KCBP interaction with tubulin requires the calmodulin-binding region located at the carboxy-terminus.

Discussion

The blot overlay assay was used to study the interaction of KCBP with unpolymerized tubulin subunits. Blot overlay assays do not use chemical cross-linkers which may cross-link proteins that are not binding partners (Bloom and Endow, 1994). Bovine brain tubulin was used in all these blot overlay assays for several reasons. (1) The amino acid sequences of tubulins are highly conserved between plants and animals (Fosket and Morejohn, 1992). (2) Microinjection of animal tubulin into plant cells results in incorporation of animal tubulin into different microtubule arrays (Hepler and Hush, 1996; Zhang et al., 1990a). (3) *In vitro* motility studies have shown translocation of *Arabidopsis* KCBP on animal microtubules (Song et al., 1997). (4) Microtubule pelleting assays showed binding of KCBP to animal microtubules (Reddy et al., 1996a). (5) The binding of KCBP to animal microtubules regulated by bovine calmodulin and *Arabidopsis* calmodulins in a similar manner (see chapter 5 "Regulation of KCBP interaction with CaM Isoforms").

Results presented here demonstrate that bacterial expressed truncated KCBPs are functional and interact with α- and β-subunits of tubulin. Both subunits were found to interact equally with KCBPs (Figure 2.3a). Nitrocellulose strips containing immobilized bovine serum albumin that were incubated with KCBP failed to show any signal when probed with either T7. tag antibody, S protein, KCBP antibody or biotinylated calmodulin (Figure 2.3b). The binding of KCBP to tubulin subunits increased proportionately with increasing concentration of KCBP in overlay solution (Figure 2.4). A competition experiment showed that binding of KCBP to the immobilized tubulin decreased proportionately with increasing concentrations of free

tubulin in the overlay solution (Figure 2.5). These results show that both α- and β-tubulin subunits have intrinsic ability to bind to KCBP.

Analysis of interaction of truncated proteins of KCBP with tubulin subunits indicated the requirement for the motor domain. Three fusion proteins (1.5 C, 1.4 C and 1.0 C) containing motor domain were able to bind to tubulin subunits (Figure 2.3). The motor domain of KCBP contains four (I to IV) highly conserved motifs implicated in microtubule-binding that are typically present in motor domains of kinesins or KLPs (Bloom and Endow, 1994). The interaction of motor domain of KCBP with tubulin subunits is similar to that with microtubule polymer. Analysis of binding between tubulin and 1.5 C or 1.4 C, which are different in stalk length, showed no difference. Extended stalk region can aid in dimerization of two motor heavy chains and confer more efficient transportability of motor along polymer tracks than motor domain alone or motor with a shorter stalk (Bloom and Endow, 1994). Dimerization of kinesin heavy chain enhances the binding of motor to tubulin subunits (Larcher et al., 1996). The amino-terminal part of KCBP (2.5 N fusion protein) containing the globular tail and coiled-coil stalk region but lacking the motor domain did not show clear binding to tubulin subunits (Figure 2.3a, lane 6). Some KLPs, especially minus end motors, have been shown to contain a second microtubule binding sit in the tail region that in insensitive to ATP (Chandra et al., 1993; Endow et al., 1994b; Kuriyama et al., 1995; Liao et al., 1994; McDonald et al., 1990). Although KCBP amino-terminal fusion protein does not appear to interact with tubulin subunits here, the results from microtubule pelleting assays allowed us to

determine that there is an additional microtubule-binding site in the amino-terminal part of KCBP (Narasimhulu and Reddy, 1998).

The most unique character of KCBP is the calmodulin-binding domain, which does not appear in any other known KLPs (Reddy et al., 1996a; b). Calmodulin, a ubiquitous Ca^{2+}-binding protein in eukaryotes, interacts and modulates the activity and function of several proteins (Poovaiah and Reddy, 1993; Roberts and Harmon, 1992). The binding of the motor domain to tubulin is independent of the calmodulin-binding domain (Figure 2.3a). The truncated KCBP containing the motor domain and calmodulin-binding domain cannot bind to tubulin subunits in the presence of calcium and calmodulin (Figure 2.7, lane 4). A similar effect was observed with both α- and β-tubulin subunits. Blocking KCBP with antibodies specific to the calmodulin-binding domain abolished the Ca^{2+}-calmodulin modulation of KCBP binding to tubulin subunits (Figure 2.8). However, with antibody blocking assays, it is difficult to clarify whether the abolition of Ca^{2+}-calmodulin regulation is the direct effect due to blocking of calmodulin-binding domain by the antibody or indirect effect due to the binding of the large IgG molecule to the motor. Hence, a truncated 1.0 C protein lacking the calmodulin-binding domain was used o perform the tubulin-binding study in the presence of Ca^{2+}-calmodulin. There is no Ca^{2+}-calmodulin modulation observed with the KCBP lacking calmodulin-binding domain (Figure 2.9). These results suggest that the interaction of KCBP with tubulin monomers is similar to that with microtubule polymer, although the possibility of subtle variations has not been ruled out (Deavours et al., 1996).

MTs are highly dynamic structures. Individual MTs alternate stochastically between periods of polymerization and depolymerization, leading to rapid exchange between tubulin subunits and MT polymer (Mandelkow and Mandelkow, 1995). Recent studies indicate that some motor proteins may also be important in regulating MT assembly (Desai et al., 1999; Endow et al., 1994; Lombillo et al., 1995a; b; Waters and Salmon, 1996). Kar3, a minus end-directed motor protein, induced depolymerization of the minus-end of taxol-stabilized MTs (Endow et al., 1994). Shortening of microtubule plus-ends by KLPs has also been reported (Lombillo et al., 1995a; b). Two kinesin-like proteins from *Xenopus* (XKCM1 and XKIF2) have been shown to regulate stability of microtubules (Desai et al., 1999; Walczak et al., 1996). Calcium and CaM have been implicated in assembly and disassembly of MTs. Calcium ions exert their effect on MTs by two modes of action, one direct and the other indirect (Lee and Wolff, 1982; Serrano et al., 1986). The direct effect was mediated by interaction of calcium with tubulin, which behaves like calcium-binding protein. The indirect effect was mediated by calmodulin and microtubule-associated proteins (MAPs). In plants, calcium and calmodulin are involved in stabilizing and destabilizing cortical MTs (Cyr, 1991; Fisher et al., 1996). Because CaM does not bind to tubulin (Lee and Wolff, 1984; Reddy et al., 1996a), the effects of CaM on MTs are likely to be mediated by calmodulin-binding microtubule-associated proteins rather than direct interaction between tubulin and CaM (Durso and Cyr, 1994; Kotani et al., 1985; Lee and Wolff, 1984; Margolis et al., 1986; Pirollet et al., 1992). Whether KCBP, a calmodulin-binding microtubule motor protein, has any direct effect on polymerization/depolymerization remains to be seen.

Among motor proteins, light chains of bovine brain kinesin and actin-based unconventional myosin heavy chains are the only ones that bind to calmodulin (Matthies et al., 1993; Wolenski, 1995). The binding of calmodulin to kinesin light chain inhibits kinesin's microtubule stimulated ATPase activity by 50% (Matthies et al., 1993). Phosphorylation of kinesin by protein kinase A reverses the calmodulin inhibition of ATPase activity. Many unconventional myosins bind calmodulin in the absence of calcium which is contrary to most target enzymes including KCBP that bind calmodulin in the presence of calcium (Wolenski, 1995). In the case of brush border myosin I, calcium causes partial dissociation of calmodulin from the motor resulting in complete inhibition of motility (Wolenski et al., 1993). Also, calcium has profound effects on actin-independent and actin-dependent MgATPase activity (Collins et al., 1990; Cozzelman and Mooseker, 1987; Swanljung-Collins and Collins, 1991; Wolenski et al., 1993). The observation that the amino terminal protein sequence of the three KCBPs that we have isolated show homology to myosins (Chen et al., 1996; Reddy et al., 1996b; Reddy and Reddy, 1999) suggests that KCBP has features of both microtubule- and actin-based motors. These results demonstrate that KCBP can interact with unpolymerized tubulin subunits and that the calmodulin-binding domain confers Ca^{2+}-calmodulin modulation of KCBP interaction with tubulins. Calcium-dependent calmodulin modulation of KCBP interaction with tubulin provides a mechanism by which cytosolic calcium, an important messenger molecule can regulate function of KCBP.

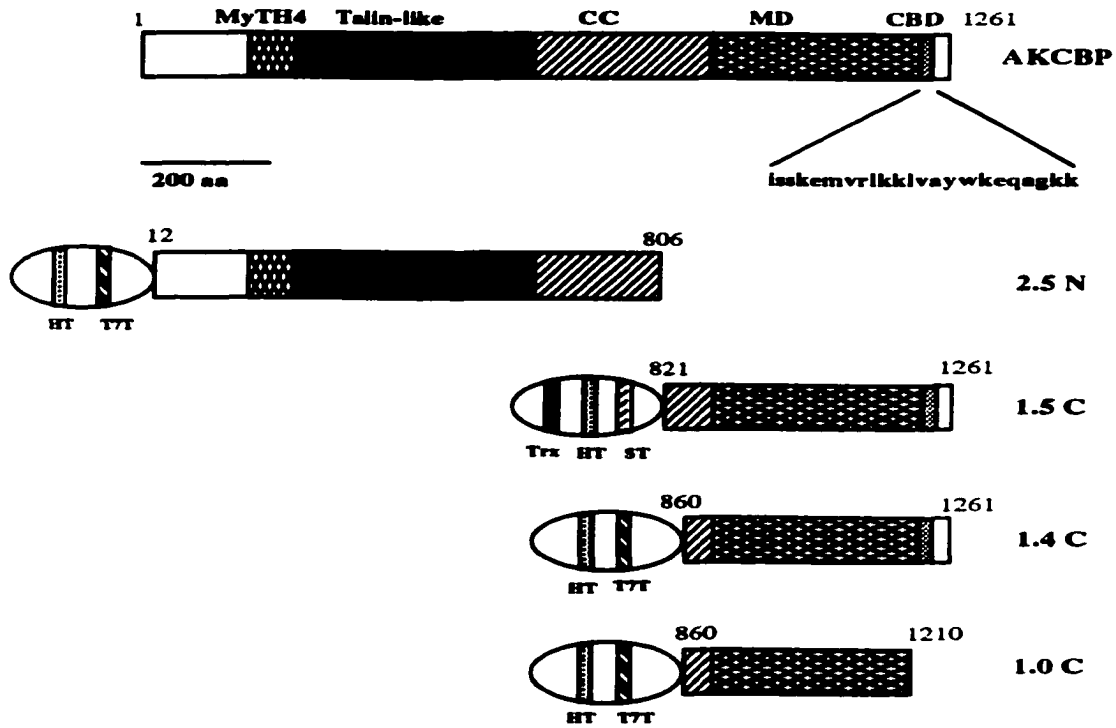


Figure 2.1: Schematic representation of different fusion proteins of *Arabidopsis* kinesin-like calmodulin-binding protein.

AKCBP: The full-length *Arabidopsis* kinesin-like calmodulin-binding protein showing various domains that are identified based on sequence similarity or functional analysis of *E. coli* expressed protein (Chen et al., 1996; Reddy et al., 1996b). MyTH4, a domain present in the tail region of some members of the myosin superfamily (VIIa, IV, X and XII); CC,α-helical coiled-coil region; MD, motor domain; CBD, calmodulin-binding domain. The amino acid sequence of the calmodulin-binding domain used in raising KCBP antibodies is indicated in single letter code.

2.5 N: Fusion protein containing the amino-terminal region (amino acids 12-806) of KCBP.

1.5 C: The carboxy-terminal fusion protein (amino acid 821-1261) of KCBP containing motor and calmodulin-binding domains and a limited coiled-coil stalk.

1.4 C: The carboxy-terminal fusion protein (amino acid 860-1261) containing a short coiled-coil stalk, motor and calmodulin-binding domains.

1.0 C: The carboxy-terminal fusion protein (amino acid 860-1210) containing a short coiled-coil stalk and motor domain but without calmodulin-binding domains.

HT, His.tag for affinity purification of fusion protein; T7T, T7.tag for immunodetection; Trx, thioredoxin for increased solubility of fusion protein; and ST, S tag for fusion protein detection.

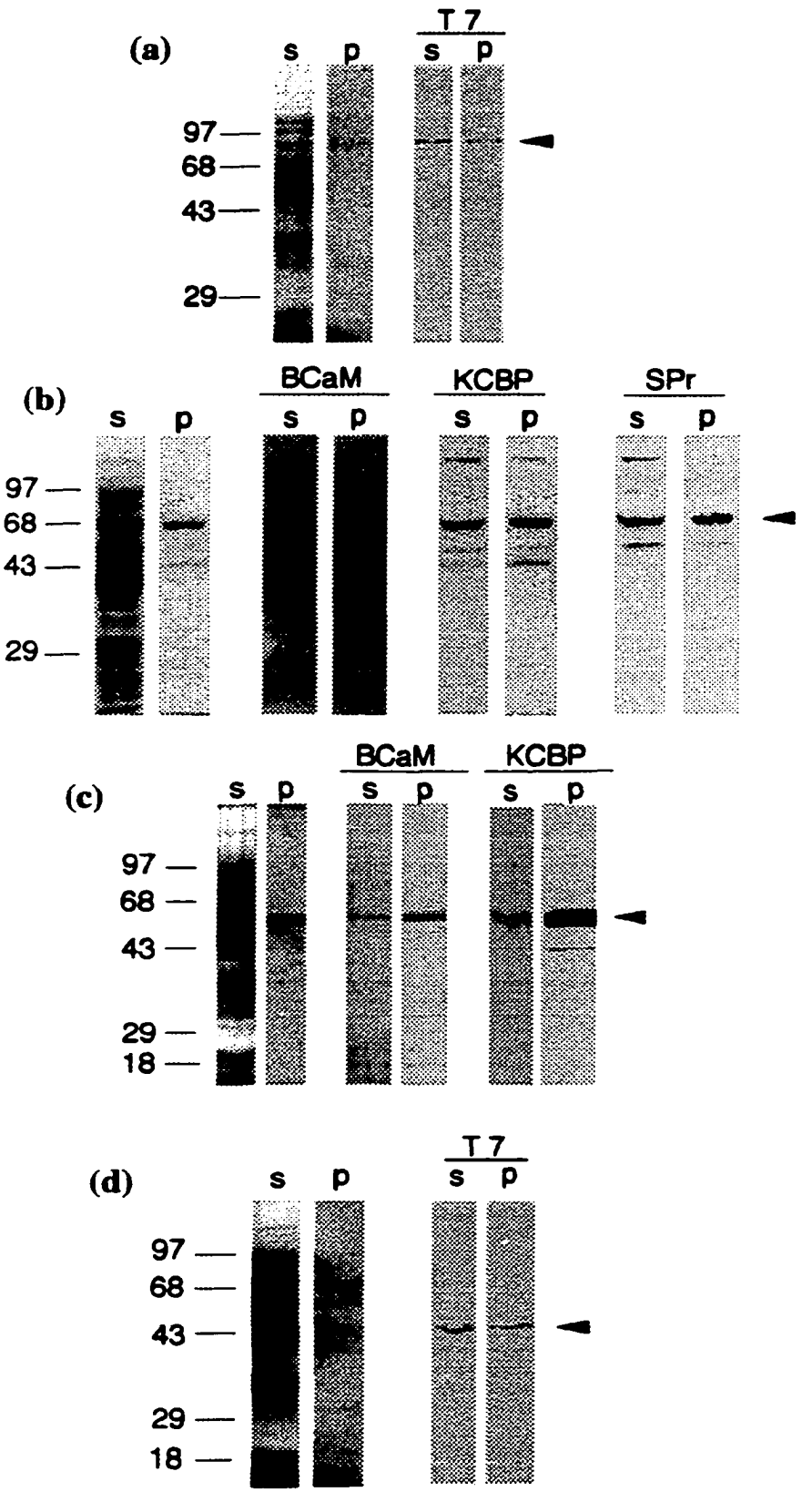
Figure 2.2: Purification and detection of *E. coli* expressed fusion proteins. Soluble proteins from induced culture (s) and purified fusion protein (p) were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (first two lanes in each panel) or blotted and probed to detect fusion protein. T7, blots probed with T7.tag antibodies; BCaM, blots probed with biotinylated calmodulin; KCBP, blots probed with anti-KCBP antibodies, SPr, blots probed with S protein. Molecular mass markers are indicated (in kD) in the left. Location of fusion proteins is indicated by arrowhead.

(a) 2.5 N fusion protein. His-Bind affinity column was used to purify the fusion protein.

(b) 1.5 C fusion protein. Fusion protein was purified using calmodulin Sepharose column.

(c) 1.4 C fusion protein. CaM Sepharose column was used to purify the fusion protein.

(d) 1.0 C fusion protein. Purification of fusion protein was performed with His-Bind affinity column.



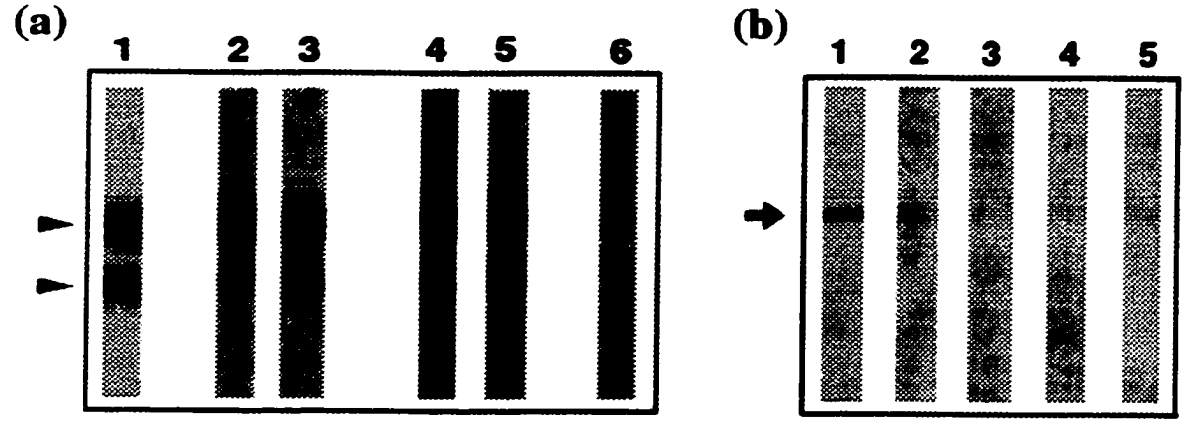


Figure 2.3: Interaction of different regions of KCBP with tubulin subunits (a) and BSA (b).

(a) Nitrocellulose strips containing 2 μg of purified tubulin were incubated with 3 μg/mL fusion proteins in a blot overlay assay. Arrowheads indicate the position of tubulin subunits. The binding of KCBP to tubulin subunits was detected using the following probes.

Lane 1: Blot incubated in the presence of 1.0 C fusion protein was probed with T7.tag monoclonal antibody.

Lane 2 and 3: Blots incubated in the presence of 1.4 C fusion protein were probed with either KCBP antibodies (lane 2) or biotinylated calmodulin (lane 3).

Lane 4 and 5: Blots incubated in the presence of 1.5 C fusion protein were probed with either KCBP antibodies (lane 4) or biotinylated calmodulin (lane 5).

Lane 6: Blot incubated in the presence of 2.5 N fusion protein was probed with T7.tag monoclonal antibody.

(b) Nitrocellulose strips containing 2 μg of BSA were incubated with 3 μg/mL fusion proteins in a blot overlay assay. Position of BSA is indicated with an arrow. The interaction of KCBP with BSA was detected using the following probes.

Lane1: Gel containing BSA was stained with Coomassie blue.

Lane 2: Blot incubated in the presence of 1.0 C fusion protein was probed with T7.tag monoclonal antibody.

Lane3-5: Blots incubated in the presence of 1.5 C fusion protein were probed with KCBP antibodies (lane 3), biotinylated calmodulin (lane 4) or S protein (lane 5).

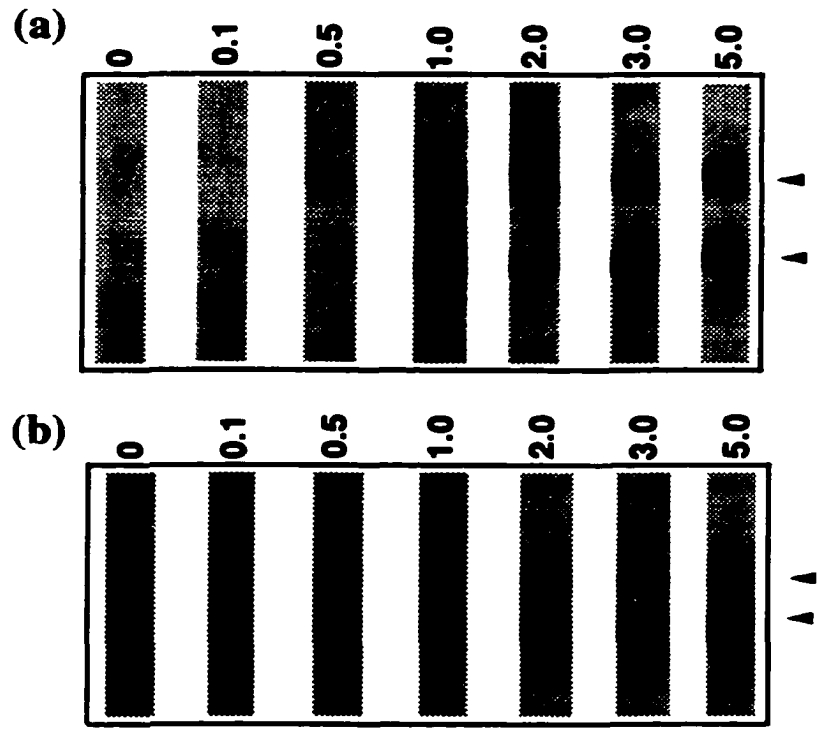


Figure 2.4: Concentration-dependent binding of 1.5 C fusion protein with tubulin subunits.
Tubulin (2 μg) containing nitrocellulose strips were incubated in the presence of increasing concentrations (0-5 μg/mL) of 1.5 C fusion protein. Blots were probed with either KCBP antibody (a) or biotinylated calmodulin (b). Numbers on the top of each lane indicate the concentration of protein in μg/mL. Arrowheads indicated the position of tubulin subunits.

Figure 2.5: Binding of 1.5 C fusion protein to tubulin subunits in the presence of increasing concentrations of tubulin.
Blots containing tubulin (2μg) were incubated in the presence of KCBP (3 μg/mL) and varying concentrations (0-100 μg/mL), indicated on the top of each lane) of tubulin in the incubation buffer. All the blots were probed with biotinylated calmodulin. Arrowheads indicate the position of tubulin subunits.

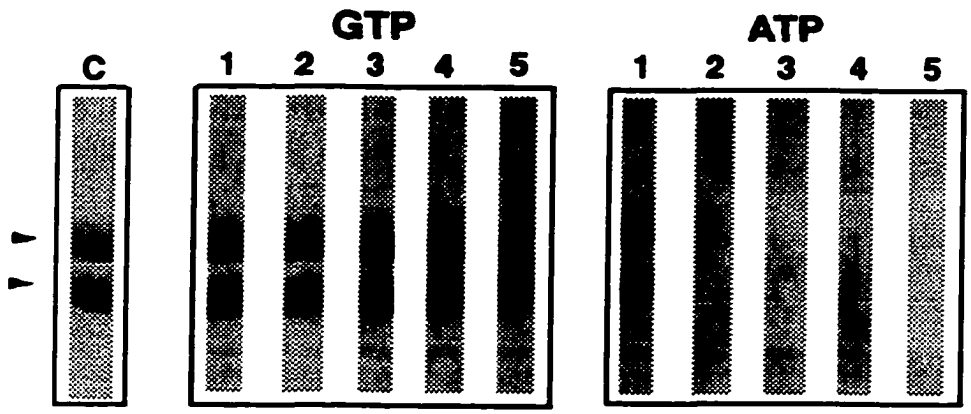


Figure 2.6: Binding of 1.5 C fusion protein to tubulin subunits in the presence of varying concentration (0-5 mM) of GTP or ATP.
Nitrocellulose strips containing tubulin (2 μg) were incubated in the presence of 3 μg/mL 1.5 C Fusion protein containing increasing concentration (1-5 mM) of either GTP or ATP. The blots were probed with biotinylated calmodulin. Lane (1) 0.1mM; lane (2) 0.5mM; lane (3) 1mM; lane (4) 2mM; lane (5) 5mM. Control blot that was incubated in the absence of any nucleotide is presented in lane C. Arrowheads indicate the position of tubulin subunits.

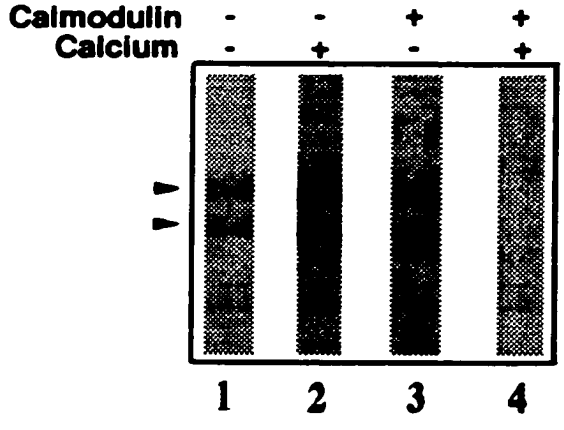


Figure 2.7: Modulation of binding of 1.5 C fusion protein to tubulin subunits by calcium/calmodulin.
Nitrocellulose strips with bound tubulin were incubated with 1.5 C fusion protein in the presence (+) or absence (-) of calcium (0.5 mM) and/or calmodulin (10 μg/mL). The binding of fusion protein to tubulin subunits was detected with S protein.

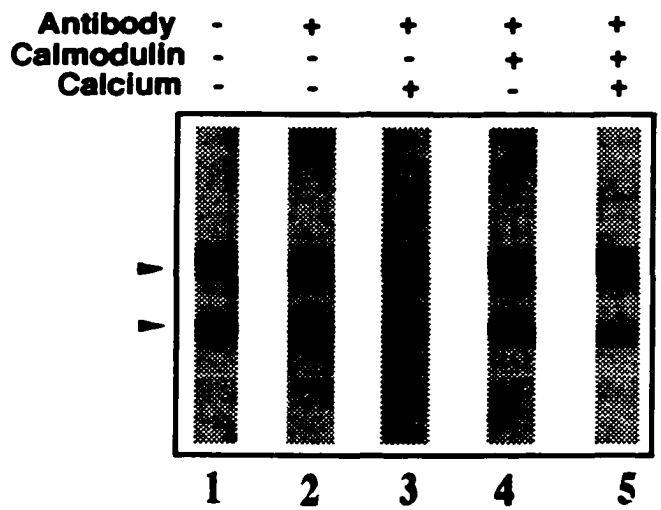


Figure 2.8: Binding of 1.5 C fusion protein to tubulin subunits in the presence of calcium/calmodulin and KCBP antibodies.
Tubulin containing nitrocellulose strips were incubated with 1.5 C fusion protein in the presence (+) or absence (-) of KCBP antibodies, calcium (0.5 mM) and/or calmodulin (10 μg/mL). The binding of the fusion protein to tubulin was detected with S protein.

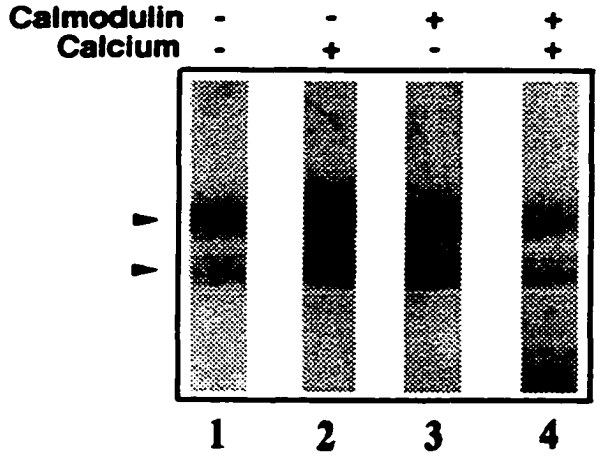


Figure 2.9: Binding of 1.0 C fusion protein to tubulin subunits in the presence of calcium and calmodulin.
Nitrocellulose strips with bound tubulin were incubated with 1.0 C fusion protein (3 μg/mL) in the presence (+) or absence (-) of calcium (0.5mM) and/or calmodulin (10 μg/mL). The blots were probed with T7.tag antibodies.

CHAPTER 3

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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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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Abstract

Kinesin-like calmodulin-binding protein (KCBP), a recently discovered 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 the C-terminal motor (with and without the calmodulin-binding region) 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. A 1.0 C protein lacking the calmodulin-binding domain also caused MTs to bundle. However, Ca^{2+}/calmodulin did not cause dissociation of these bundles. 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 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.

Introduction

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 (Lloyd, 1991). In dividing plant cells, four distinct arrays of MTs appear sequentially in a cell cycle-dependent manner (Goddard et al., 1994). In interphase cells, MTs are arranged at the cell cortex and control the deposition of cellulose microfibrils. These cortical MTs are replaced, in late G2, with a narrow band of MTs called the preprophase band (PPB). The site of the preprophase band determines the location of future cell wall. By metaphase, MTs are organized into a spindle apparatus, which ensures proper segregation of chromosomes to opposite poles. In early telophase, MTs are arranged into a phragmoplast between the segregated chromosomes. The phragmoplast consists of two sets of MTs, whose plus ends interdigitate at the equatorial plane. The phragmoplast functions in transporting Golgi-derived vesicles to the equatorial plane where they fuse to form the cell plate (Staehelin and Hepler, 1996). Mitosis 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 (Baskin and Cande, 1990; Fosket and Morejohn, 1992; Lloyd, 1994). In elongating cells, MTs are arranged perpendicular to the direction of expansion. Furthermore, hormonal and environmental signals that affect the direction of cell expansion alter the orientation of cortical MTs (Lloyd, 1994; Shibaoka, 1991), suggesting a tight regulation of spatial organization of MTs in plant cells. 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 activities associated with MTs (Barton and Goldstein, 1996; Bloom and Endow, 1994). Native kinesin is a tetramer consisting of two heavy chains and two

light chains. The heavy chain has an N-terminal head or motor domain of about 340 amino acids with ATP and microtubule binding sites, a central α-helical coiled-coil stalk involved in dimerization, and a C-terminal tail to which light chains bind (Bloom and Endow, 1994; Gauger and Goldstein, 1993). KLPs have a conserved motor domain, which is located either at the N-terminus, C-terminus or in the center (Moore and Endow, 1996). Although most KLPs contain a stalk and tail, the amino acid sequence in these regions is not conserved among different KLPs. Several plus- and minus-end MT motors have been shown to be necessary for spindle formation (Walczak and Mitchison, 1996; Walczak et al., 1998).

In a screen for calmodulin-binding proteins from *Arabidopsis*, a unique kinesin-like protein, kinesin-like calmodulin-binding protein (KCBP) was isolated (Reddy et al., 1996b). 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. There are instances where interactions between actin cytoskeleton and MTs are mediated by MT-associated proteins (Pedrotti et al., 1994a; b). 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 (Deavours et al., 1998; Narasimhulu and Reddy, 1998). Several lines of evidence suggest the involvement of KCBP in cell division. High level KCBP expression was found in tissues with meristematic cells and in suspension cultures (Reddy et al., 1996a; b). KCBP localizes to mitotic MT arrays including the preprophase band and the phragmoplast (Bowser and Reddy, 1997; Smirnova et al., 1998). Furthermore, KCBP protein accumulates in a cell cycle-dependent manner to a high level in M phase and declines as the cells enter interphase (Bowser and Reddy,

1997). An essential role of KCBP in *Arabidopsis* trichome morphogenesis has also been reported (Oppenheimer et al., 1997). 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 (Lloyd, 1994). Two C-terminal motors (KCBP and Kat A) and one N-terminal motor (tobacco kinesin-related polypeptide of 125kDa; TKRP-125) from plants localize to mitotic MT arrays, suggesting their involvement in cell division (Asada et al., 1997; Bowser and Reddy, 1997; Liu et al., 1996; Smirnova et al., 1998). 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 either by video-enhanced differential interference contrast (VE-DIC) microscopy or electron 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 and 1.0 C proteins of KCBP were expressed using the pET28 system whereas 1.5 N and 1.5 C proteins were expressed in pET32. Figure 3.1 shows the domains present in each of the expressed proteins. Construction of plasmids was described previously (Deavours et al., 1998; Narasimhulu et al., 1997; Reddy et al., 1996b). *E. coli* BL21(DE3)pLYS cells containing a recombinant pET plasmid were grown at 37°C to an OD_{600} of 0.6 and fusion protein expression was induced by isopropyl β-D-thiogalactopyranoside (IPTG). The cultures were grown overnight at room temperature, spun, washed, and processed for purification. The 1.4 C and 1.5 C proteins were purified either by calmodulin-Sepharose affinity column or Talon metal

affinity column (Deavours et al., 1998; Narasimhulu et al., 1997). The 1.0 C and 1.5 N proteins were purified using His-Bind metal chelation resin as described previously (Narasimhulu et al., 1997; Narasimhulu and Reddy, 1998). Proteins purified with calmodulin-Sepharose were dialyzed against a buffer containing 50mM Tris, pH 7.5, and 150mM 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 (Narasimhulu et al., 1997).

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. (1993). 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 (Walker, 1995).

MT bundling assay

VE-DIC microscopy:

Tubulin was purified according to Walker et al. (1988). Bundling assays were done in AB buffer (20mM Pipes, pH6.9, 1mM $MgSO_4$, 1mM EGTA) containing 40μM taxol, 5mM 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 (VE-DIC) microscopy.

Negative-stain electron microscopy:

Bovine brain tubulin was isolated and purified as described earlier (Williams and Lee, 1982). To prepare MTs, tubulin was polymerized in the presence of 1mM GTP,

4M glycerol, and 20μM taxol for 1 hr at 37°C. The MT bundling reaction was performed in a 50 or 100 μL reaction volume containing buffer (50mM Pipes, pH 6.9, 0.5mM MgSO4, 1mM DTT and 40μM taxol), 1μM tubulin as taxol-stabilized MTs, and purified KCBP constructed protein. Calcium and/or calmodulin were added to the appropriate reactions and incubated for 30 min at room temperature. The samples were then processed for negative-stain electron microscopy. Five μL of sample was placed on acetone cleaned 200-mesh copper grids that were coated with Formvar (0.4% in ethylene dichloride) and carbon, and allowed to adsorb to the grid surface. Grids were then dried with a filter paper wick, washed with 5 μL of water and stained with 5 μL of 2% uranyl acetate. MTs on grids were observed using a JEOL 2000 EXII electron microscope at 100 kV. Micrographs were recorded at 30,000 x magnification.

Sucrose gradient

Preparation of the sucrose gradient:

The 5-35% sucrose gradient containing 50mM Tris and 150mM NaCl was prepared by freezing and thawing sucrose solution as described by Davis and Pearson (1978). One mL of solution of the Tris, NaCl and 35% sucrose solution was added into the centrifuge tube, frozen at -20°C, and then 30% sucrose with Tris/NaCl was added onto the top layer, frozen at -20°C and then 25%, 20%, 15%, 10% and 5% solution was added sequentially. The seven milliliters of this solution was frozen in -20°C, and then thawed at 4°C as one-cycle gradients. The two-cycle gradients were cycled one more time at -20°C and 4°C again. The freeze-thaw cycle takes 3 to 4 hours and makes 5% to 35% continuous sucrose gradients. The three-cycle sucrose gradients can be stored at -20°C and just before layering the KCBP samples, the three-cycle sucrose gradients thawed in 4°C. BSA (molecular weight: 66kDa) is used as molecular-weight marker in this assays.

Sucrose gradient centrifugation:

Sample of 650 µL containing KCBP and BSA in buffer (50mM Tris and 150mM NaCl) was layered onto the top of 5% - 35% linear sucrose gradients. This sucrose gradient was centrifuged at 47,000 rpm in a vertical rotor, VT150, for 3 hours at 4°C using Beckman L7-55 ultracentrifuge. After centrifugation, fractions of 250 µL were collected from the top to the bottom of the sucrose gradient immediately. Each fraction was collected individually and analyzed on the SDS-PAGE gel and western blot to identify the protein and its quantity.

Results

Different parts of KCBP in a bacterial system were expressed and the purified proteins were used in a MT bundling assay. Four truncated proteins of KCBP (1.5 N, 1.5 C, 1.4 C and 1.0 C) were used in the present study. The 1.5 N protein has the amino-terminal region (amino acids 12 to 503) of KCBP with myosin tail homology (MyTH4) region. Proteins 1.5 C, 1.4 C and 1.0 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 Figure 3.1). The 1.4 C (amino acids 860-1261) protein is similar to 1.5 C except that it has a shorter coiled-coil stalk. Protein 1.0 C (amino acids 860-1210) has the motor domain and stalk as in 1.4 C protein but lacks the calmodulin-binding domain. Purified protein from each of these constructs was obtained as described previously (Deavours et al., 1998; Narasimhulu et al., 1997; Narasimhulu and Reddy, 1998). Expression of a full-length KCBP cDNA using pET 28 expression system resulted in degradation of full-length protein into two smaller products (Narasimhulu et al., 1997). Hence, the bundling assays with the full-length KCBP could not be performed.

The 1.5 C protein with motor and calmodulin-binding domains of KCBP bundles MTs

The 1.5 C protein contains an ATP-sensitive MT binding site (Deavours et al., 1998; Narasimhulu and Reddy, 1998). 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 (VE-DIC) microscopy and electron microscopy. In the absence of protein, MTs were randomly distributed and remained as single microtubules (Figures 3.2A and 3.3A). Addition of 1.5 C protein to taxol-stabilized MTs caused extensive bundling of MTs where MTs appeared as thick cables due to clustering of MTs (Figures 3.2B and 3.3B). Lateral association of MTs was clearly visible under the EM (Figure 3.3B). Several MTs were present in each bundle (see Fig. 3.3B). The bundling was observed best at equimolar concentrations of tubulin and motor protein. The bundles were long and several times the diameter of a single MT. The 1.5 C protein did not cause bundling in the presence of ATP (Figure 3.2F). MTs incubated under the same conditions with BSA have not shown any bundling activity (data not shown).

Ca^{2+}/calmodulin dissociate 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 (Deavours et al., 1998; Narasimhulu and Reddy, 1998; Song et al., 1997). Furthermore, Ca^{2+}/calmodulin can disassociate preformed KCBP-MT rigor complexes formed in the presence of AMP-PNP (Deavours et al., 1998). 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 (Figures 3.2E and 3.3C). Calcium alone or calmodulin alone in the reaction did not dissociate KCBP-induced MT bundles (Figures 3.2C and D). These results suggest that

Ca^{2+}/calmodulin regulate the MT bundling activity of KCBP. These results are consistent with the previous studies on the effect of Ca^{2+} and calmodulin on KCBP interaction with MTs (Deavours et al., 1998; Narasimhulu and Reddy, 1998).

1.4 C protein exists as monomer

The sucrose gradient method has been used widely to determine the oligomeric nature of proteins (Blangy et al., 1998). In this method, proteins are separated by differences in their sedimentation rate. This rate is determined by the molecule size, shape, and mass as well as the properties of the centrifugation medium (density and viscosity) and the applied centrifuged force (Davis and Pearson, 1978). In order to determine the native form of 1.4 C KCBP, sucrose gradient centrifugation was used. As shown in Figures 3.4A and B, the 1.4 C KCBP appeared mostly in fractions #1 (top layer of sucrose gradient, containing proteins with smaller molecular mass) and #2, then the quantity decreased, but BSA started to be present from fraction #3 and keep its presence until the bottom of centrifuge tube. This result suggested that the molecular mass of native form of 1.4 C KCBP protein is less than BSA (66KDa), indicating that the 1.4 C exists as a monomer.

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 seventy amino acids and this region allows it to form dimer (Deavours et al., 1998). 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, a shorter version (1.4 C protein) of KCBP was used in bundling assays. The 1.4 C protein has a very short stalk (about 30 amino acids) and exists as a monomer (Figure 3.4). Motor domains of other proteins that contain short stalk regions as in 1.4 C protein were found to be monomers (Blangy et al., 1998; Chandra et al., 1993; Moyer et al., 1996). As shown in

Figure 3.5A, the 1.4 C protein is also capable of bundling MTs. Addition of Ca^{2+} alone or calmodulin alone had no effect on bundling (Figures 3.5B and C), whereas Ca^{2+}/calmodulin together "unbundled" 1.4 C-induced bundles (Figure 3.5D). These results suggest that dimerization of motor protein is not required for bundling activity. Inclusion of 5mM ATP also disrupted MT bundling (data not shown). However, Ca^{2+}/calmodulin was more effective than ATP in dissociating MT bundles into single MTs.

1.0 C protein lacking the calmodulin-binding domain also causes MT bundling

To determine if i) the motor domain alone is capable of inducing MT bundling, and ii) the calmodulin-binding domain is necessary for Ca^{2+}/calmodulin effect, bundling assays were performed in the presence of 1.0 C protein. This protein has the same length stalk as in 1.4 C but lacks the calmodulin-binding domain. The 1.0 C protein like 1.5 C and 1.4 C proteins induced bundling as determined by EM (Figure 3.6B). However, bundling is not as extensive as in the case of 1.5 C or 1.4 C protein. The 1.0C was purified on His-Bind column, which is not as efficient as calmodulin-Sepharose that was used to purify 1.5 C or 1.4 C proteins. The purified protein preparation of 1.0 C, although highly enriched, contained some contaminating bands (Narasimhulu et al., 1997) and also unable to be obtained in a concentrated form. Poor bundling with 1.0 C as compared to other C-terminal proteins is likely due to lower concentration of protein in the assay. Addition of Ca^{2+}/calmodulin did not result in dissociation of bundles even in the presence of high amount of Ca^{2+} (data not shown). These data suggest that the motor domain alone is capable of bundling MTs and the calmodulin-binding domain is necessary for Ca^{2+}-calmodulin regulation of bundling.

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, two proteins, MC1 and MC6, of *Drosophila* Ncd (Chandra et al., 1993) were used. 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 (Chandra et al., 1993). Both MC1 and MC6 proteins induced MT bundling (Figures 3.7A and C). 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 (Figures 3.7B and D). 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 *Drosophila* kinesin motor domain (K366), a plus-end motor, was also found to cause extensive bundling of MTs (Moyer et al., 1996). Taken together, these results strongly suggest that the motor 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 shown binding of this protein to MTs. ATP had no effect on binding of 1.5 N protein to MTs, suggesting the presence of an ATP-independent MT binding site in the N-terminal tail (Narasimhulu and Reddy, 1998). The N-terminal tail of Ncd contains an ATP-independent MT binding site and causes bundling of MTs (Chandra et al., 1993;

Karabay and Walker, 1999). Similar MT binding sites in the tail domain of Kar3 and kinesin heavy chain have been reported (Chandra et al., 1993; Meluh and Rose, 1990; Navone et al., 1992). 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. Taxol-stabilized MTs were incubated in the presence or absence of 1.5 N protein. As shown in Figure 3.8A, 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 (Figure 3.8B). Microtubules appeared as cables due to clustering of MTs. However, the bundling is not as extensive as the bundling observed with motor domain constructs (compare Figure 3.8B with Figures 3.2B and 3.5A). 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 (Narasimhulu and Reddy, 1998), the bundling assays at higher concentrations of motor were unable to be performed. MTs bound to a coverslip coated with 1.5 N protein (data not shown), confirming the 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 (Figure 3.8C). As mentioned above, the N-terminal tail of Ncd exhibits the ability to bundle MTs (Chandra et al., 1993; Karabay and Walker, 1999). However, it is worth noting that the tail domain in KCBP is much longer (about 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 with (1.5 C and 1.4 C) or without calmodulin-binding domain (1.0 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 studies of MT bundling and Ca^{2+}/calmodulin effects on MT bundles using the full-length KCBP. However, I was unable to obtain the full-length protein using a bacterial expression system due to cleavage of the expressed protein into two smaller proteins (Narasimhulu et al., 1997). The MT bundling caused by the 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. Experiments with 1.0 C have shown that calmodulin-binding domain is necessary for Ca^{2+}/calmodulin regulation. 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 (Moyer et al., 1996) indicate that the motor domain of KLPs is capable of MT bundling in monomeric as well as dimeric states.

In animal cells, several MT-associated proteins (MAPs) have been shown to regulate MT assembly, dynamics, bundling and spatial organization (Hirokawa, 1994). MAPs such as MAP2, MAP4 and tau promote MT bundling and enhance the stability of MTs (Barlow et al., 1994; Lee and Rook, 1992; Lewis and Cowan, 1990; Scott et al., 1992; Yoshida et al., 1996). 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 (McDonald et al., 1990). More recently, it was shown that the N-terminal tail of Ncd, which contains an ATP-independent MT binding site, causes MT bundling (Chandra et al., 1993; Karabay and Walker, 1999). Kar 3, also a C-terminal motor from *Saccharmyces cerevisiae*, destabilizes MTs preferentially at their minus ends (Endow et al., 1994b). Kinesin has been shown to cause MT bundling and individual kinesin molecules can form stable crossbridges between MTs (Andrews et al., 1993; Urrutia et al., 1991). Recently, multi-headed kinesin was shown to crossbridge MTs and organize

MTs into dynamic asters (Nédélec et al., 1997). Kinesin, in addition to a MT binding site in the motor domain, contains a second MT-binding site in the N-terminal tail (Navone et al., 1992). It is proposed that two MT-binding sites on a single molecule could crossbridge MTs to cause bundling or sliding of MTs (Andrew et al., 1993; Navone et al., 1992). However, the motor domain alone in monomeric form has been shown to bundle MTs, indicating the existence of other mechanisms by which kinesin promotes bundling (Moyer et al., 1996). A rigor mutant of the human Eg5 motor in dimeric form and the motor domain of mitosis-specific KLP were also found to induce MT bundling (Blangy et al., 1998; Kuriyama et al., 1994).

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 (Tang et al., 1997). 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 (Blangy et al., 1998). 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 of charge on the surface 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 (Nogales et al., 1998; Sackett and Wolff, 1986). 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 (Nogales et al., 1998; Woehlke et al., 1997). Other studies have also shown that the C-

terminal sequences of tubulin play an important role in kinesin binding (Tucker and Goldstein, 1997). The binding of KCBP motor domain, which is basic (pI=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 pI of 9.0 (Narasimhulu and Reddy, 1998), 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 (Smirnova et al., 1998; Song et al., 1997).

Recently, several MAPs have been isolated from plants (Bokros et al., 1995; Chan et al., 1996; Chang-Jie and Sonobe, 1993; Durso and Cyr, 1994; Jablonsky et al., 1993; Marc et al., 1996; Nick et al., 1995; Rutten et al., 1997; Schellenbaum et al., 1993; Vantard et al., 1991). Some of these MAPS were shown to induce MT bundling, and assembly and stabilization of MTs *in vitro*. A calmodulin-binding MAP (elongation factor-1α isoform) was reported from carrot suspension cultures (Durso and Cyr, 1994). This MAP bundled MTs *in vitro*, which are dissociated in the presence of Ca^{2+}/calmodulin. However, so far none of the reported plant MT motor proteins is known to induce MT bundling.

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 (Poovaiah and Reddy, 1993; Trewavas and Malho, 1997). 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 (1987) 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 (Fisher et al., 1996; Hepler, 1992; Zhang et al., 1990a; Zhang et al., 1992). Calmodulin is implicated in stabilizing cortical MTs in carrot at low Ca^{2+} concentration and destabilize them at higher Ca^{2+} concentrations (Cyr, 1991; Fisher et al., 1996). Immunocytochemical studies showed calmodulin localization to cortical MTs and mitotic MT arrays in plant cells (Cyr and Palevitz, 1989; Fisher and Cyr, 1993, Vantard et al., 1985; Wick et al., 1985). However, a recent study raised questions about calmodulin localization to mitotic MT arrays (Vos and Hepler, 1998). Because calmodulin does not bind to tubulin (Lee and Wolff, 1984; Reddy et al., 1996a), 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 via the calmodulin-binding domain in KCBP. 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.

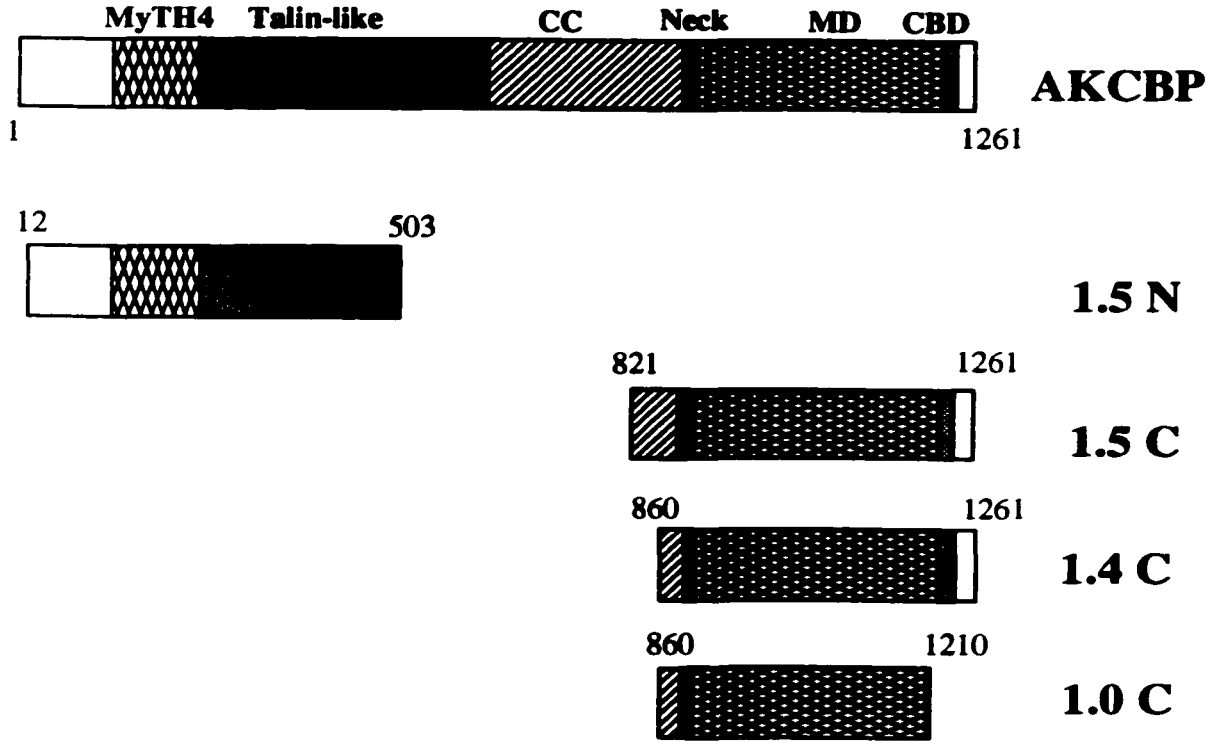


Figure 3.1: Diagram showing the domains in bacterially expressed proteins of *Arabidopsis* KCBP.
AKCBP: The full-length *Arabidopsis* kinesin-like calmodulin-binding protein showing various domains (Reddy et al., 1996b). MyTH4, a myosin tail homology region; CC, coiled-coil region; MD, motor domain; CB, 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 motor and calmodulin-binding domains and a limited coiled-coil stalk.
1.4 C: The C-terminal fusion protein containing a short coiled-coil stalk, motor and calmodulin-binding domains.
1.0 C: The C-terminal fusion protein containing a short coiled-coil stalk and motor domain but without calmodulin-binding domain.

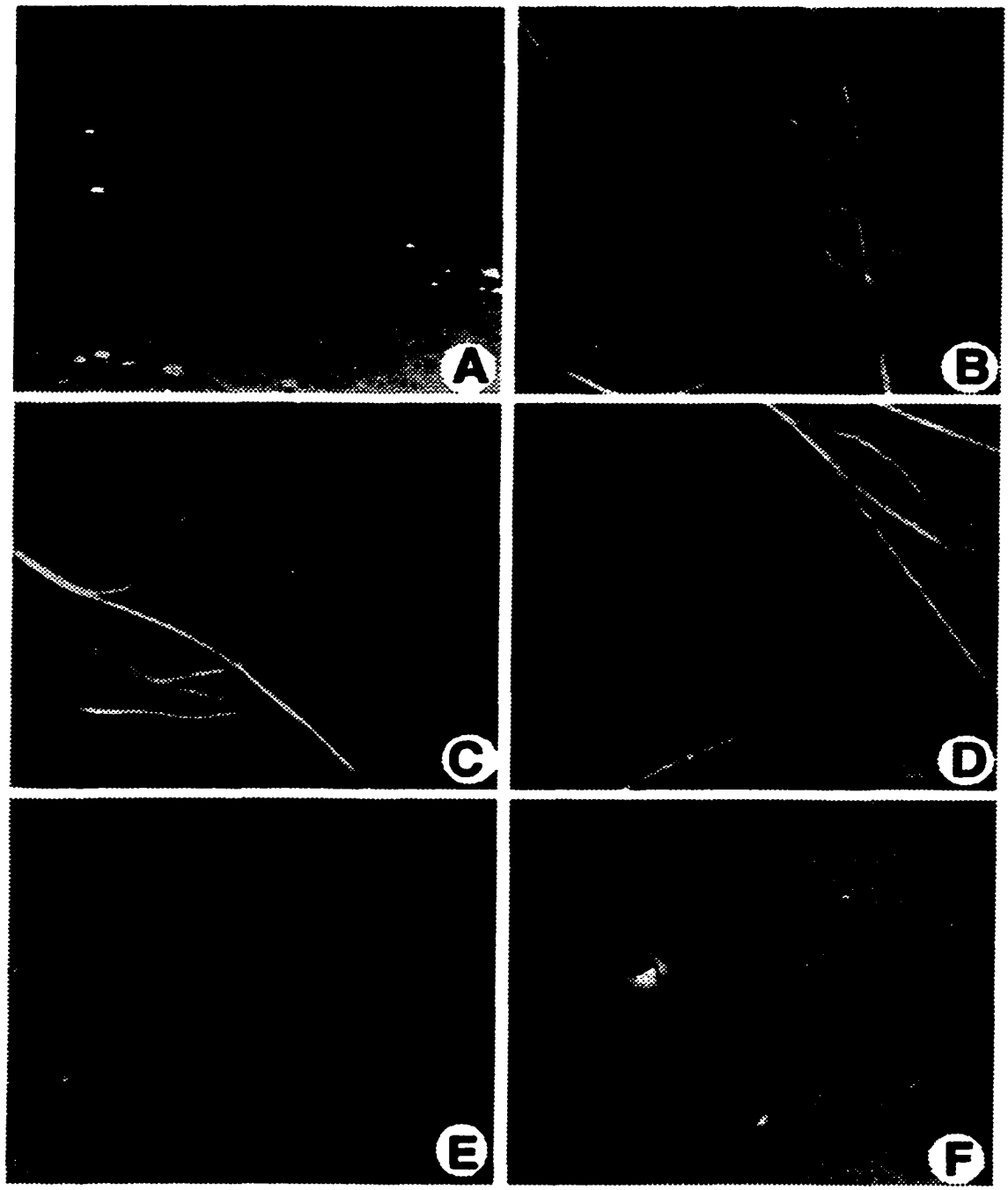


Figure 3.2: 1.5 C protein induces MT bundling.
Taxol-stabilized MTs (5 μM) were incubated with 1.5 C protein (5 μM) in the presence or absence of Ca^{2+} (20 μM) and/or calmodulin (15 μM) for 30 min. and observed by VE-DIC microscopy. A) MTs, B) MTs plus 1.5 C protein, C) MTs plus 1.5 C protein and Ca^{2+}, 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 5mM ATP. The width of these and all other VE-DIC micrographs is 26 μm.

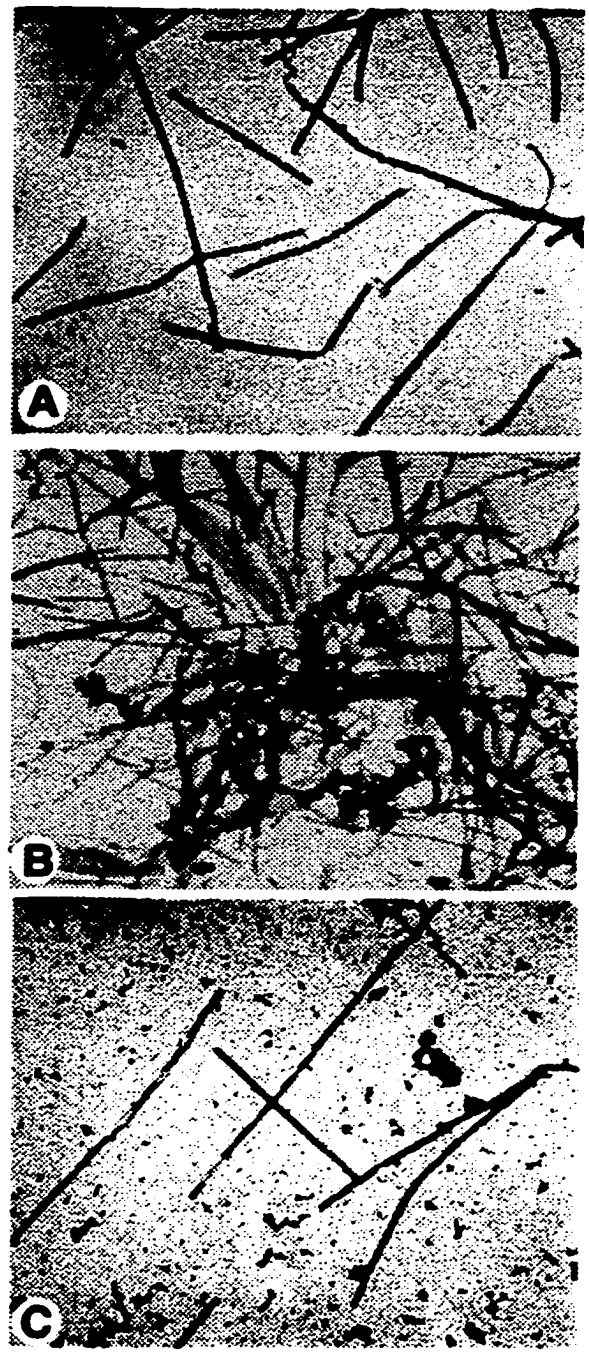


Figure 3.3: MT bundling by 1.5 C visualized by negative-stain electron microscopy. Taxol-stabilized MTs (1 μM) were incubated with 1.5 C protein (1 μM) in the presence or absence of Ca^{2+} (50 μM)/calmodulin (1 μM) for 30 min. and observed by electron microscopy. A) MTs, B) MTs plus 1.5 C protein, C) MTs plus Ca^{2+}/calmodulin. Micrographs are shown at x 30,000 magnification.

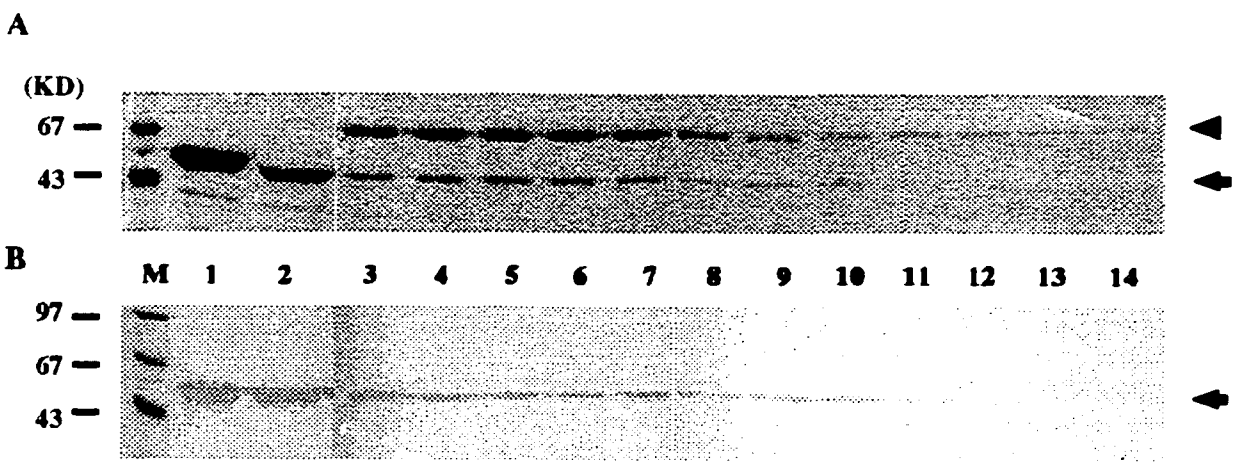


Figure 3.4: The oligomeric form of 1.4 C KCBP.
Using sucrose gradient, mixture of 1.4 C KCBP and BSA was separated into different fractions (marked 1-14 from top to bottom of centrifuge tube and showed from lower to higher molecular weight). Different fractions were run by SDS-PAGE and stained with either A) Coomassie Brilliant Blue, B) blotted and probed with biotinylated calmodulin. The position of BSA is indicated with arrowhead and KCBP as arrow. Molecular weight markers are indicated on the left.

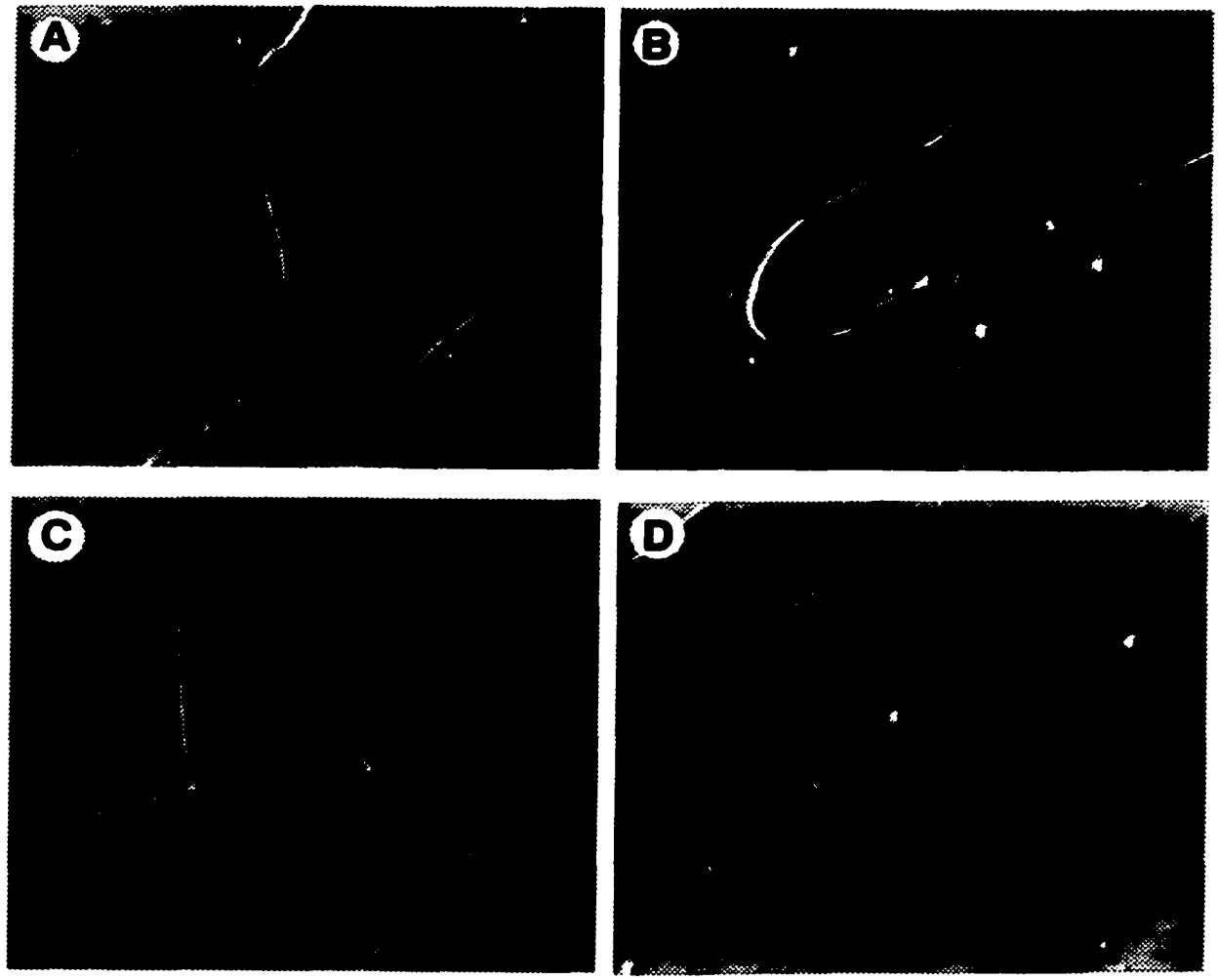


Figure 3.5: MT bundling by 1.4 C protein.
Taxol-stabilized MTs (5 μM) were incubated with 1.4 C protein (5 μM) in the presence or absence of Ca^{2+} (20 μM) and/or calmodulin (15 μM) for 30 min. and observed by VE-DIC microscopy. A) MTs plus 1.4 C protein, B) MTs plus 1.4 C protein and Ca^{2+} (20 μM), 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).

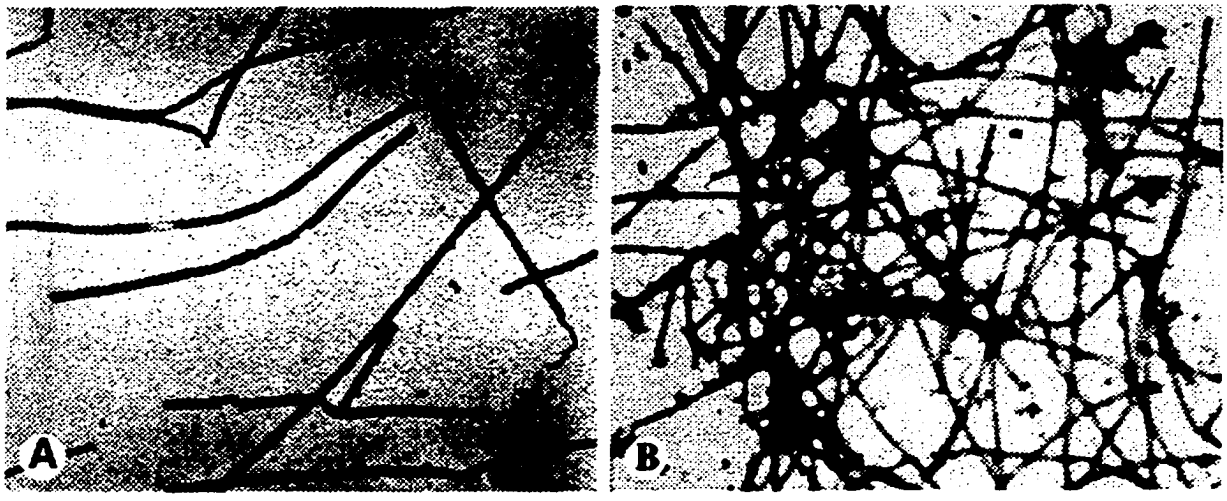


Figure 3.6: MT bundling by 1.0 C.
Taxol-stabilized MTs (1 μM) were incubated with 1.0 C protein for 30 min. and observed by electron microscopy. A) MTs, B) MTs plus 1.0 C protein (0.2 μM). Electron micrographs are shown at x 30,000 magnification.

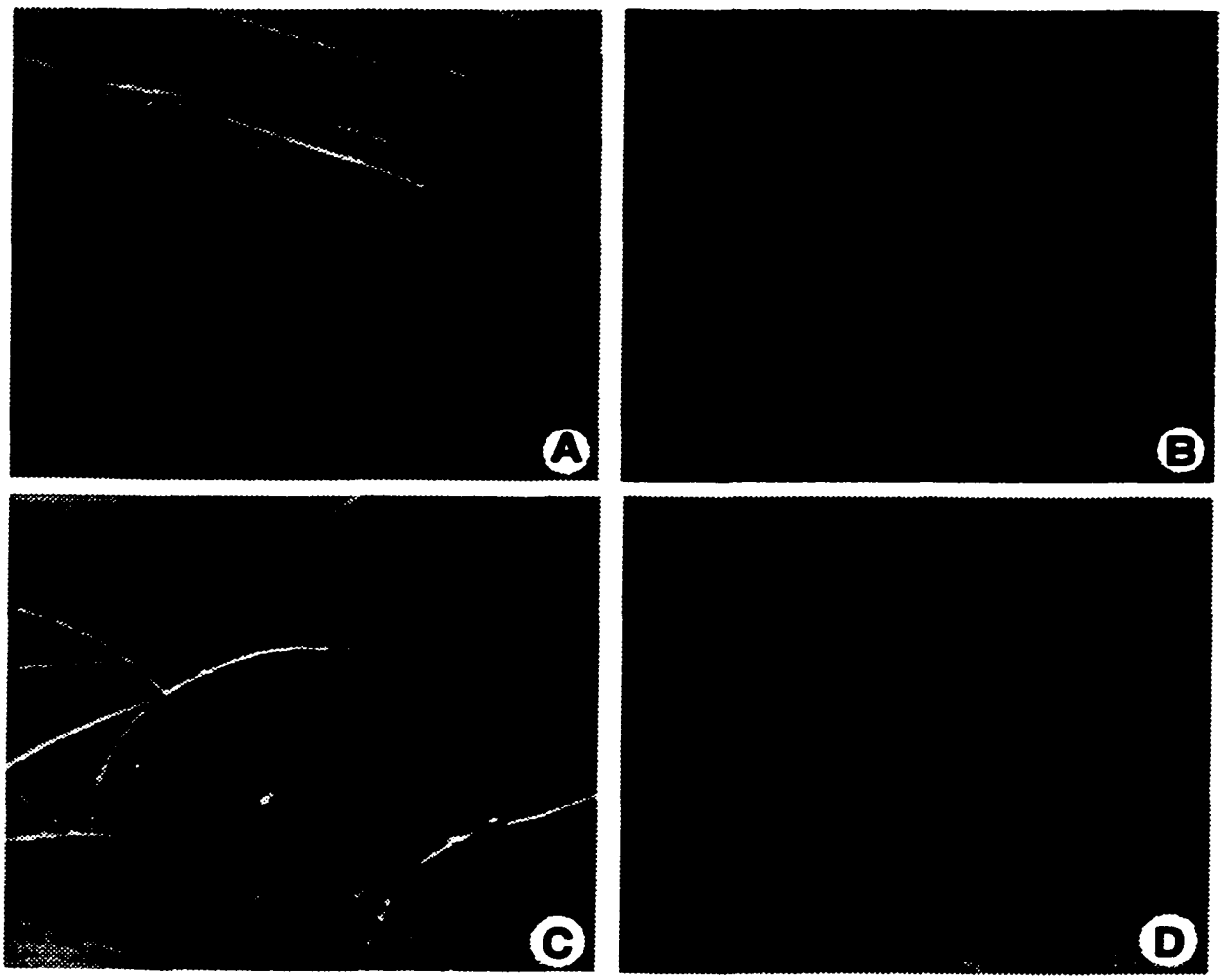


Figure 3.7: *Drosophila* Ncd motor domain (MC1 and MC6) is capable of MT bundling.
Taxol-stabilized MTs (1 μM) were incubated with purified motor protein for 30 min and observed by VE-DIC microscopy. A) MTs plus MC1 protein (2 μM), B) MTs plus 5mM ATP and MC1 protein (1 μM), C) MTs plus MC6 protein (6 μM), D) MTs plus 5mM ATP and MC6 protein (1 μM).

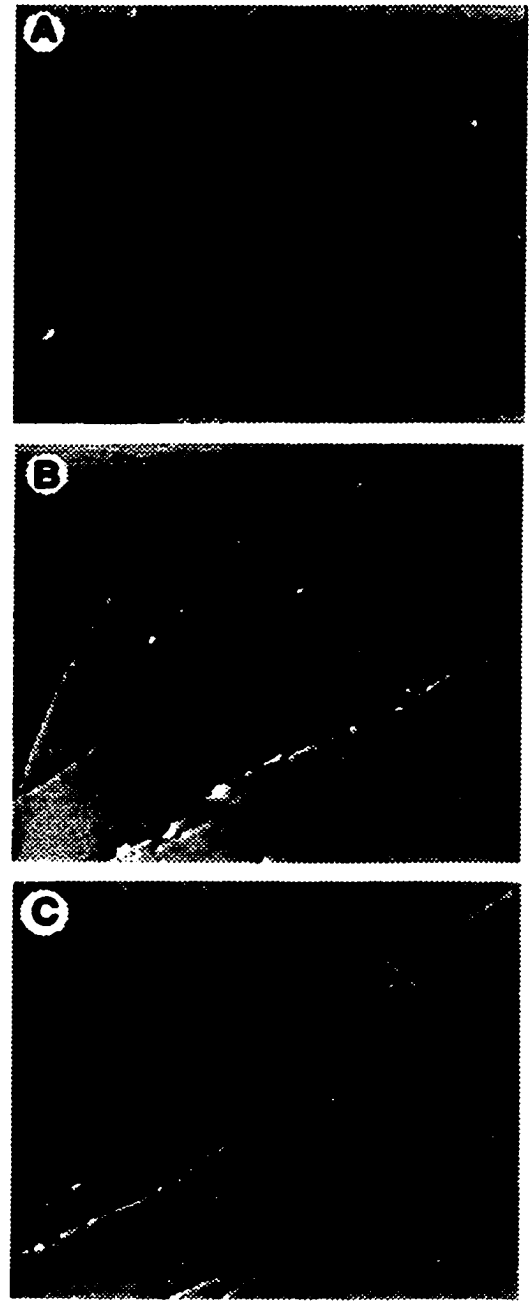


Figure 3.8: The N-terminal (1.5 N) tail of KCBP is capable of MT bundling. Taxol-stabilized MTs (1 μM) were incubated in the presence or absence of 1.5 N protein (0.1 μM) for 30 min. and observed by VE-DIC microscopy. A) MTs, B) MTs plus 1.5 N protein, C) MTs plus 1.5 C protein and Ca^{2+}/calmodulin (1.25 mM/5 μM).

CHAPTER 4

Calcium/calmodulin regulation of NCD-CBD and DK-CBD fusion proteins

Abstract

Kinesin-like calmodulin-binding protein (KCBP) is a novel member of the kinesin superfamily that appears to be unique in plants because it has a calmodulin-binding domain (CBD). This calmodulin-binding domain, 23 amino acid long, is located at the carboxy-terminus adjacent to motor domain. The presence of a CBD in KCBP allows calcium/calmodulin regulation in the interaction of KCBP with microtubules (MTs). To determine if the addition of calmodulin-binding domain to other kinesins, which do not possess CBD, confers the Ca^{2+}/calmodulin regulation, the calmodulin-binding domain from KCBP was fused to two kinesins. The motor domain (MD) of C-terminal motor (NCD) or N-terminal motor (DK) from *Drosophila* kinesin-like proteins was fused to the N-terminus of calmodulin-binding domain of KCBP. The bacterially expressed proteins were tested for their ability to bind calmodulin (CaM) and the effect of Ca^{2+}/CaM on the interaction of motors to MTs. The data indicate that the addition of calmodulin-binding domain of KCBP to these kinesins confers CaM-binding and Ca^{2+}/CaM regulation. Two truncated proteins of KCBP, 1.0 C and 0.4 C were used in pelleting assay to test if they confer Ca^{2+}/CaM regulation when they are in two separate peptides. The data indicate that motor domain and calmodulin-binding domain need to be on the same peptide for Ca^{2+}/CaM regulation.

Introduction

Calcium regulates various cellular and physiological processes through its interaction with calcium-dependent protein kinase (Roberts and Harmon, 1992) and calcium-binding proteins such as calmodulin and calmodulin-like proteins (Poovaiah and Reddy, 1987; Poovaiah and Reddy, 1993) to regulate the activation of the downstream target proteins. These calcium-binding proteins contain EF-hand motifs and undergo conformational change to activate other proteins. Calmodulin, a highly conserved acidic protein with four calcium-binding motifs, has been found in all eukaryotes. The biochemical and physical properties of plant calmodulins are very similar to animal calmodulins (Roberts et al., 1986; Snedden and Fromm, 1998; Zielinski, 1998). Calmodulin contains 148 amino acids arranged in two globular domains (with two EF-hand motifs in each arm) that are connected to each other with a helix domain. The four calcium-binding motifs of calmodulin do not bind calcium simultaneously, but sequentially, due to the unique arrangement of the EF-hand motifs which are classified into high-affinity sites (two) and low-affinity sites (two) (Klee, 1988). Target proteins can also influence the calcium-binding capability of calmodulin. For example, once calmodulin binds to calcium, its affinity for phosphodiesterase increases (Gregori et al., 1985). A feedback inhibition occurs in some cases (Cimler et al., 1985; Fisher et al., 1996), however, enhancement occurs in other cases in which the bound calmodulin has higher calcium-binding affinity and allow the tighter formation of calmodulin-target protein complex (Klee, 1988).

The 12-30 amino acid long calmodulin-binding sites confer binding activity to target proteins that share little other similarity in their amino acid sequence (James et al., 1995). Calcium-calmodulin complex binds to its target protein by hydrophobic interactions in the binding site. However, some calmodulins bind to target proteins in the absence of calcium (Geiser et al., 1993) or have higher affinity to target proteins

than in the presence of calcium (e.g. neuromodulin) or form a stable complex of calmodulin and target protein in low concentration of calcium (e.g. phosphorylase kinase) (Snedden and Fromm, 1998).

Calcium and calmodulin in plants mediate various abiotic (light, cold, heat, drought or gravity) and biotic signals (phytohormones or pathogens) (Snedden and Fromm, 1998). Calmodulin is involved in many cellular and physiological processes in plants such as cell division, microtubule organization (Vantard et al., 1985; Wick et al., 1985), gravitropism (Lu et al., 1996), phytochrome-phototransduction and chloroplast development (Bowler et al., 1994; Neuhaus et al., 1993). Calmodulin interacts with many target enzymes and structural proteins called calmodulin-binding proteins (CBPs), which play important roles in cellular processes in plants (Bown and Shelp, 1997; Durso and Cyr, 1994; Harding et al., 1997; Kinkema and Schiefelbein, 1994; Lu et al., 1996; Malmstrom et al., 1997; Narasimhulu et al., 1997; Oppenheimer et al., 1997; Szymanski et al., 1996).

Calcium and calmodulin have been implicated in cytoskeletal organization and cell division (Durso and Cyr, 1994; Hepler, 1992; Kinkema and Schiefelbein, 1994; Oppenheimer et al., 1997). In animals, calcium and calmodulin together regulate the depolymerization of microtubules (Keith et al., 1983). In plants, calcium/calmodulin affects the stabilization of cortical microtubules (Cyr, 1991; Fisher et al., 1996). Calmodulin is involved in cortical microtubule organization in two different ways depending on calcium concentrations: low calcium concentrations help stabilize cortical microtubules whereas higher concentrations destabilize them (Fisher et al., 1996). Immunolocalization results show that calcium and calmodulin are present in the cytoskeleton and mitotic apparatus of plant and animal cells (Brady et al., 1986; Cyr, 1991; Fisher et al., 1996; Hepler, 1992; Vantard et al., 1985; Wick et al., 1985). During mitosis of plant cells, calcium and calmodulin show identical distribution; both are localizing to the mitotic apparatus such as the asterlike structure (so called

the pericentriolar material), spindle microtubules, and phragmoplast, but not the preprophase band (Vantard et al., 1985; Wick et al., 1985). The calcium-dependent calmodulin is distributed along plant kinetochore fibers, suggesting that the kinetochore-microtubule organization might be related or controlled by calcium and calmodulin (Vantard et al., 1985). Calcium-independent calmodulin is characterized and localized on the yeast spindle-pole body and required for chromosome segregation (Geiser et al., 1993), implying that calmodulin might have a role in microtubule-related organization. Because calmodulin does not bind to tubulin or microtubules directly (Lee and Wolff, 1984; Reddy et al., 1996b), the binding between calmodulin and microtubules might be mediated by the microtubule associated proteins (MAPs). A number of MAPs including calmodulin-regulated MAPs have been characterized in animals (Wolenski, 1995). There are two classes of MAPs: structural MAPs that affect the assembly and arrangement of microtubules (Chapin and Bulinski, 1992; Lee, 1993) and motor MAPs that are involved in motility (Vale, 1992).

Calcium- or calmodulin-regulation of motor protein activity has been commonly accepted. The neck domain of myosin heavy chain contains one or more IQ motifs that can bind light chains with EF-hand motif, such as calmodulin (Asada and Collings, 1997; Wolenski, 1995). It provides a way of regulating the activity of these motors by calcium/calmodulin that the heavy-chains and light-chains of some actin-based myosin motors show calcium/calmodulin binding ability (Wolenski, 1995). With exceptions, some myosin IQ-motifs bind calmodulin in the absence of calcium (Bahler et al., 1994). The conventional two headed myosin II lacks calcium-binding sites but contains two light chains sharing sequence similarity to calmodulin-binding proteins with EF-hand motif, including calmodulin (Sellers et al., 1996; Wolenski, 1995). Unconventional myosins differ from conventional myosin II in containing variable numbers of light chains, which are calmodulin-light chains frequently. The

regulation can be either by dissociation of one or more calmodulins from the heavy chains or by calcium-induced effects on calmodulin light chains (Wolenski, 1995). The activity of light chains in microtubule-based kinesin motors is regulated by calcium/calmodulin (Matthies et al., 1993). Co-pelleting complex of bovine brain kinesin and microtubules bind calmodulin, but only the kinesin light chain (KLC), not heavy chain (KHC), can bind calcium-dependent calmodulin (Matthies et al., 1993). Calcium-dependent binding of calmodulin to KLP inhibits the ATPase activity of native kinesin suggesting that KLC possesses the regulatory functions in kinesin via a signal transduction pathway (Matthies et al., 1993). However, all the characterized KHCs of conventional kinesins or kinesin-like proteins from animals do not bind calmodulin.

Kinesin-like calmodulin-binding protein (KCBP) is a novel kinesin-like protein that appears to be unique to and ubiquitous in plants (Reddy et al., 1996b). KCBP is the only member of the kinesin superfamily (more than one hundred KLPs) (Moore and Endow, 1996) that has been shown to have a calmodulin-binding domain so far. Homologs of KCBP have also been isolated from potato and tobacco, suggesting that KCBP is ubiquitous in plants (Reddy et al., 1996a; Wang et al., 1996). The 23 amino acid calmodulin-binding domain (CBD) is located at the carboxy-terminus adjacent to the motor domain. The presence of a calmodulin-binding domain on KCBP permits regulation of KCBP by calcium, through calmodulin (Narasimhulu and Reddy, 1998; Narasimhulu et al., 1997; Reddy et al., 1996a; Wang et al., 1996). None of the other KHCs or KLPs binds calmodulin. The presence of a calmodulin-binding domain as a short stretch of amino acids next to the motor domain suggests that the calmodulin-binding domain might act as a modular domain and may confer calcium/calmodulin regulation to other KLPs if the calmodulin-binding domain is placed adjacent to the motor domain. To test this possibility, a C-terminal motor (NCD) and an N-terminal motor (DK, *Drosophila* kinesin) from *Drosophila* were constructed and used in

microtubule pelleting assay. In addition, the AKCBP motor domain (1.0 C) and calmodulin-binding domain (0.4 C) were expressed to determine if these protein domains work together in regulating KCBP.

Materials and methods

Construction of NCD-CBD and DK-CBD fusion proteins and truncated AKCBP

To perform these experiments, four plasmid constructs were prepared: i) NCD-CBD (motor domain of *Drosophila* C-terminal motor fused to calmodulin-binding domain), ii) DK-CBD (motor domain of *Drosophila* N-terminal motor fused to calmodulin-binding domain), iii) 1.0 C KCBP (motor domain of *Arabidopsis* KCBP), iv) 0.4 C KCBP (calmodulin-binding domain of *Arabidopsis* KCBP). The preparation of 1.0 C of AKCBP was described in materials and methods of chapter 2 "Interaction of *Arabidopsis* kinesin-like calmodulin-binding protein with tubulin subunits: modulation by Ca^{2+}-calmodulin". The 0.4-kb region of the cDNA was amplified with sense (GATCGTGAATGATCCCAGCAAAC) and antisense (GAGACATATAGGACTACTCTTCG) primers that contained restriction sites for *EcoR*I and *Hind*III, respectively, and cloned in-frame in a pET-28b vector (Reddy et al., 1996b).

NCD-CBD: The Ncd sequence was amplified from the pET28-MC1 clone (containing the motor domain) using 5' T7 and 3' Ncd reverse primers that contain *NdeI* and *EcoRI* sites, respectively. The PCR product of Ncd and pET28 containing the calmodulin-binding domain (CBD, 0.4 C construct) were digested with *Nde*I and *EcoR*I and ligated.

DK-CBD: The motor domain of *Drosophila* kinesin was amplified from pET21-DK375 plasmid using T7 and DK reverse primers containing *Nco*I and *EcoR*I sites, respectively. The amplified product of DK and pET28 containing the calmodulin-

binding domain (CBD, 0.4 C construct) were digested with *Nco*I and *EcoR*I and ligated.

These constructs were introduced into *E. coli* BL21 (DE3) strain by transformation, grown to OD_{600} of 0.6 in LB medium containing kanamycin (30 μg/mL). The fusion protein was induced by adding 0.2mM IPTG to the culture and growing the culture for overnight at room temperature. Soluble and insoluble protein fractions from uninduced and induced cultures were isolated as described below.

Protein Induction and purification

Bacteria cells were grown at 37°C to an OD_{600} of 0.6, then the fusion proteins were induced by 0.2 mM IPTG and growing the culture overnight at room temperature in the presence of IPTG. The bacterial cells containing the NCD-CBD, DK-CBD or 0.4 C were spun, washed and resuspended in buffer A (containing 50mM Tris, pH 7.5, and 150mM NaCl), and the "Complete" protease inhibitor cocktail (Boehringer-Manheim). The suspension was lysed by incubating on ice for a period of one hour in the presence of 200μg/mL lysozyme followed by 10 pulses of sonication on ice. The lysate was spun at 100,000g for 45 min and the supernatant was added with 2mM $CaCl_2$ and passed through the Calmodulin Sepharose-4B column. Calmodulin Sepharose column was equilibrium with buffer A (50mM Tris, pH 7.5, and 150mM NaCl). The unbound proteins were washed with several volumes of binding buffer (buffer A containing 2mM $CaCl_2$) and the bound-protein was eluted with binding buffer except that $CaCl_2$ was replaced with 2mM EGTA.

Protein expressed from the 1.0 C was extracted as above except that the buffer B (20mM Tris, pH7.9, 0.5M NaCl, and 5mM imidazole) supplemented with 200μg/ml lysozyme was used to lyse the cells. 1.0 C constructed protein contained a histidine-Tag sequence at its N-terminus, permitting purification on a His-Bind affinity column. The high-speed supernatant was allowed to bind to His-Bind resin in the

presence of buffer B and washed extensively with the same buffer containing an additional 15mm imidazole to remove unbound proteins. The bound proteins were eluted in buffer B supplemented with 60mM imidazole. All four eluted proteins were dialyzed in a buffer containing 50mM Tris, pH 7.5, and 250mM NaCl to remove EGTA and imidazole and prevent aggregation. Protein concentration was measured using Bio-Rad dye reagent using BSA as a standard and by running on SDS gel and staining with Coomassie Brilliant Blue R-250.

Calmodulin interaction with fusion proteins

NCD-CBD and DK-CBD were separated on SDS-PAGE (Laemmli, 1970), blotted onto a nitrocellulose membrane, tested for their ability to bind calmodulin according to the method modified from Fordham-Skelton et al. (1994). The nitrocellulose membrane was blocked for 1 hour in 3% gelatin in TBS/Ca/Mg buffer (50mM Tris, pH 7.5, 0.2M NaCl, 0.5mM $CaCl_2$, 50mM $MgCl_2$) at 30°C. Rinsed the membrane with TBS/Ca/Mg buffer and incubated in avidin solution (ten drops of avidin solution to 5mL of TBS/Ca/Mg/0.1% Tween-20 buffer) for 1 hour at 30°C. Rinsed the membrane with TBS/Ca/Mg buffer again and incubated in biotin solution (ten drops of biotin solution to 5mL of TBS/Ca/Mg/0.1% Tween-20 buffer) for 1 hour at 30°C. Then the membrane was washed with TBS/Ca/Mg buffer and incubated with 800ng/mL biotinylated calmodulin in TBS/Ca/Mg/0.05% Tween-20 buffer containing 1% gelatin for 2 hours at 30°C. The blot was then washed in TBS/Ca/Mg buffer and incubated with Vectastain ABC-HRP (2 drops of reagent A-Avidin-DH and two drops of reagent B-biotinylated horseradish peroxidase H in TBS/Ca/Mg/0.1% Tween-20 buffer) 1 hour at 30°C. The calmodulin-binding proteins on membrane were detected colorometrically on room temperature by immersing it in substrate solution (200 µL of 40mg/mL diaminobenzidine (DAB), 50µL of 80mg/mL $NiCl_2$, 1mL of 1M Tris, pH 7.5, 7.75 mL of water and 2µL of 30% H_2O_2).

Microtubule binding assay

To prepare polymerized microtubules, thawed frozen tubulin (Cytoskeleton Inc.) in 80mM Pipes, pH6.8, 1mM $MgCl_2$, 1mM EGTA, 1mM GTP and 3% glycerol was incubated at 35°C for 30min, then supplemented it with 20μM Taxol (Paclitaxel; Sigma) and incubated at 35°C for 2 hours or longer. The polymerized MTs were separated from unpolymerized tubulin by centrifuging at 35°C in TLA 100.3 rotor (Beckman Instruments Inc., Palo Alto, CA) for 1hr at 100,000 g. The binding reactions contained dialyzed NCD-CBD, DK-CBD, 1.0 C or 1.0 C+0.4 C proteins with or without polymerized microtubules. Equimolar concentration of bacterially expressed proteins and MTs were used in each assay. The reaction buffer contained 20mM Pipes, pH6.8, 2mM $MgCl_2$, 1mM DTT, 60μM Taxol, 5mM AMP-PNP, and 150mM NaCl. 5mM $CaCl_2$ and/or 10μM bovine-calmodulin were added to the appropriate reaction. After a 25-min incubation at 37°C, the samples were centrifuged in TLA 100.3 rotor at 100,000 g for 25 min at 35°C. The supernatant and pellet fractions were separated immediately after the centrifugation and mixed with SDS sample buffer and analyzed by SDS-PAGE. Microtubules and the fusion proteins were visualized by staining the gels with Coomassie Brilliant Blue R250. A duplicate gel was run, blotted, and probed with biotinylated calmodulin or T7-tag antibodies for detecting the proteins.

Results and discussion

DK-CBD and NCD-CBD chimeric fusion proteins bind calmodulin

Here the KCBP calmodulin-binding domain (CBD) was added to kinesin motors (N-terminal and C-terminal motors) that do not possess CBD or calmodulin-binding ability and asked i) if the addition of calmodulin-binding domain makes them

calmodulin-binding and ii) if the addition of calmodulin-binding domain to these motors confers calcium/calmodulin regulation as in the case of KCBP. Two non calmodulin-binding motor proteins (kinesin and Ncd) from Drosophila were fused to calmodulin-binding domain from KCBP. These proteins were expressed in bacteria and purified on bovine calmodulin-Sepharose column. As shown in Figures 4.1a and 4.2a, both proteins bound to calmodulin-Sepharose and eluted with EGTA, a calcium chelator, suggesting that DK-CBD and NCD-CBD bind to calmodulin in a calcium-dependent manner.

The proteins eluted from bovine calmodulin-Sepharose column were run on a SDS gel and transblotted to nitrocellulose membrane. The protein blots were probed with biotinylated calmodulin and detected DK-CBD and DK-CBD proteins (Figures 4.1b and 4.2b), indicating that these two fusion proteins contain a complete and functional calmodulin-binding domain.

DK-CBD and NCD-CBD proteins have ability to bind microtubules

The purified DK-CBD or NCD-CBD protein was used in microtubule pelleting assays to test if the protein binds microtubules and if Ca^{2+}/calmodulin regulate its interaction with microtubules. With DK-CBD protein alone (Figure 4.3), a faint band showed in pellet part and suggesting that there is some amount of precipitation of this protein. In microtubule pelleting assay with DK-CBD, part of the protein bound to MTs and pellet with MTs (Figure 4.3, MT treatment), suggesting that the DK-CBD interacts with MTs. A part of the DK-CBD appeared in supernatant (unbound form) implying that part of it may be inactivate or lost its binding ability. In the absence of calcium/calmodulin or in the presence of calcium or calmodulin alone, the amount of DK-CBD is same in the pellet. However, most of the protein remained in the supernatant when both calcium and calmodulin were supplemented (Figure 4.3, MT

with Ca/CaM treatment) suggested that the protein is released from microtubule in the presence of calcium and calmodulin.

NCD-CBD was also used in microtubule binding assays to test if it still possesses the ability to bind microtubules and if its binding to microtubules is regulated by calcium and calmodulin. In the control (NCD-CBD protein alone), most protein was in the supernatant although there is also a degradation product. From the results of cosedimentation assay (Figure 4.4, MT treatment), it is clear that NCD-CBD binds to microtubules and sedimented in the pellet (bound product), suggesting that the fusion protein still contains its microtubule binding activity. A small fraction of NCD-CBD remains in the supernatant implying that part of NCD-CBD cannot bind on microtubules due to the inactivation. Calcium alone, calmodulin alone or calcium/calmodulin together were added to mixture of polymerized microtubule and NCD-CBD to see how calcium and calmodulin affect on the interaction of NCD-CBD with MTs. Calcium alone or calmodulin alone did not affect its binding ability because most of protein was in the pellet form. Both calcium and calmodulin together inhibited the binding of NCD-CBD to microtubules as much of the NCD-CBD was released from pellet form and stayed in the supernatant (Figure 4.4).

Microtubule pelleting assays in the presence of 1.0 C and 0.4 C of KCBP

It was previously shown that Ca^{2+} /calmodulin inhibit the interaction of KCBP with MTs through calmodulin-binding domain (CBD). Here I had tested if a similar regulation takes place if the motor domain and calmodulin-binding domain are on two separate peptides. 1.0 C (motor domain) and 0.4 C (calmodulin-binding domain) proteins of AKCBP were used in the microtubule pelleting assays to test if these two domains function when there are on two separated peptides. In the absence of MTs, 1.0 C and 0.4 C proteins remained in the supernatant but a fraction of 1.0 C was in the pellet (Figure 4.5). Incubating 1.0 C of AKCBP alone with microtubules, a major

protein of 1.0 C showed in the pellet although some of 1.0 C remained in the supernatant implying that 1.0 C of KCBP lost some of its binding activity (Figure 4.5, 1.0 C and MT). In the presence of calcium/calmodulin, the 1.0 C remained bound to microtubules as in the absence of calcium/calmodulin because there is no calmodulin-binding domain to respond to calcium and calmodulin (Figure 4.5, 1.0 C, MT and Ca/CaM). Addition of calmodulin-binding domain (0.4 C protein) together with Ca^{2+} /calmodulin did not affect the binding of 1.0 C to MTs where much of it was still present in the pellet, suggesting that the motor domain and calmodulin-binding domain have to be on the same protein in order to show Ca^{2+} /calmodulin modulation.

From the results of cosedimentation assays, DK-CBD and NCD-CBD show similar pattern of microtubule binding and calcium/calmodulin regulation (Figures 4.3 and 4.4). Both DK-CBD and NCD-CBD showed interaction with microtubules indicating that the addition of calmodulin-binding domain (CBD) did not affect their MT-binding activity. According to the biotinylated-calmodulin detection and microtubule-binding assays, the calmodulin-binding domain in DK-CBD or NCD-CBD can bind calmodulin and the calmodulin-binding domain confers Ca^{2+}/calmodulin regulation via calmodulin-binding domain. The binding response of the DK-CBD and Ncd-CBD fused proteins in the presence of calcium alone or calmodulin alone is similar to the results obtained with the AKCBP (Narasimhulu and Reddy, 1998).

The KCBP is the only known KLP with a calmodulin-binding domain. The presence of a calmodulin-binding domain in the KCBP allows the binding of calmodulin and regulates motor function by calcium, a well-recognized messenger molecule in plants (Poovaiah and Reddy, 1993). From these experiments, the calmodulin-binding domain from AKCBP can confer Ca^{2+} /calmodulin regulation to other motor proteins that are not normally CaM binding. The results that the calmodulin-binding domain from a minus-end motor protein (KCBP) can regulate the

other plus- and minus-end motor's function suggesting that the binding of CaM to calmodulin-binding domain is likely to influence MT-binding sites in the same manner in these different motors. Other effects of calmodulin-binding domain on motility and properties of these chimeras remain to be investigated.

The observation that the addition of 0.4 C to pelleting assays with 1.0 C protein did not confer Ca^{2+} /calmodulin regulation suggests that both motor and calmodulin-binding domain have to be on the same protein.

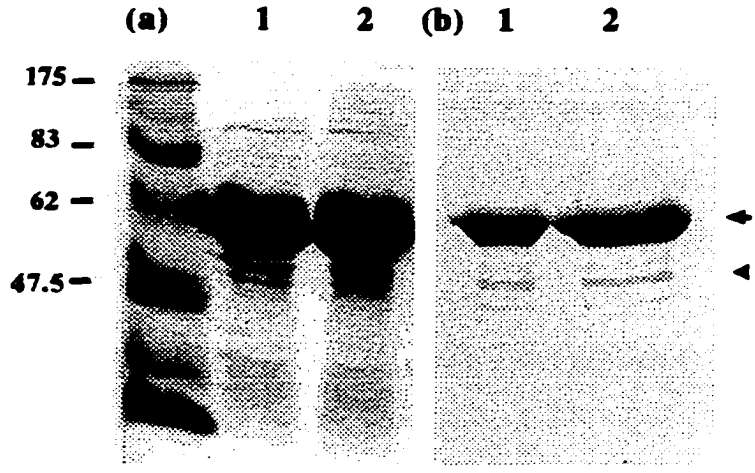


Figure 4.1: Purification and detection of DK-CBD fusion proteins.
Purified fusion proteins were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (a) or blotted and probed with biotinylated calmodulin (b). Lane 1 and 2 are two different fractions eluted from CaM-Sepharose column. Molecular weight markers are indicated (in kD) on the left. Location of DK-CBD fusion protein is indicated by an arrow and DK-CBD degraded product is marked as arrowhead.

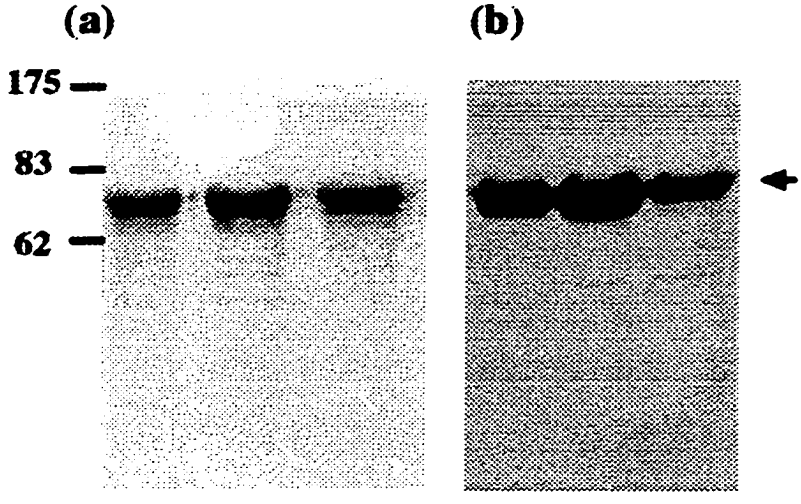


Figure 4.2: Purification and detection of NCD-CBD fusion proteins.
Purified fusion protein was separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (a) or blotted and probed with biotinylated calmodulin (b). Lane 1, 2 and 3 are different eluted fractions from CaM-Sepharose column. Molecular weight markers are indicated (in kD) on the left. Location of NCD-CBD fusion protein is indicated by an arrow.

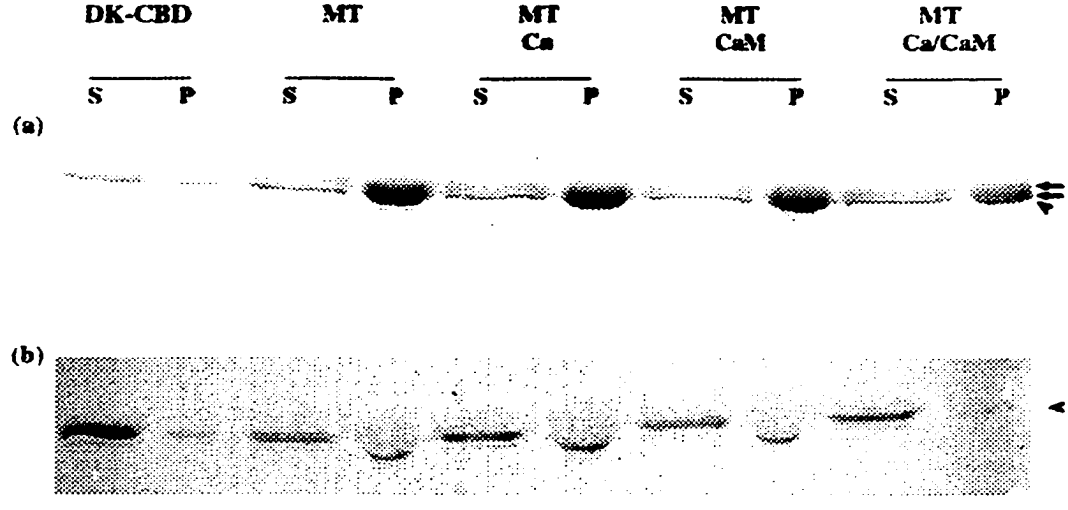


Figure 4.3: Ca^{2+}-calmodulin regulates the interaction of the DK-CBD fusion protein with microtubules.
DK fusion protein and taxol-stabilized MTs were incubated in the absence (MT) or presence of calcium (MT Ca), or calmodulin (MT CaM) or Ca^{2+}-calmodulin (MT Ca/CaM) for 30 min and centrifuged. As a control, the protein alone (DK-CBD) was also centrifuged. The resulting supernatant (s) and pellet (p) fractions from these microtubule pelleting assays were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (a) or blotted and probed with biotinylated calmodulin (b). Location of DK-CBD fusion protein is indicated by an arrowhead and tubulin subunits (α and β) are denoted by arrows.

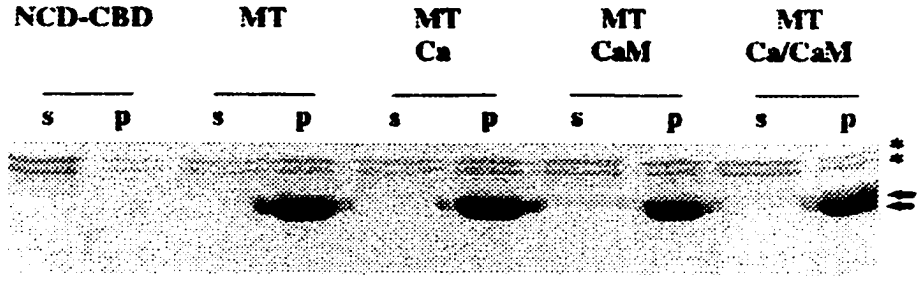


Figure 4.4: Ca^{2+}-calmodulin regulates the interaction of the NCD-CBD fusion protein with microtubules.
NCD-CBD fusion protein and taxol-stabilized MTs were incubated in the absence (MT) or presence of calcium (MT Ca), or calmodulin (MT CaM) or Ca^{2+}-calmodulin (MT Ca/CaM) for 30 min and centrifuged. As a control, the protein alone (NCD-CBD) was also centrifuged. The resulting supernatant (s) and pellet (p) fractions from these microtubule pelleting assays were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Location of NCD-CBD fusion protein and a degraded product are indicated by stars and the arrows denote tubulin subunits (α and β).

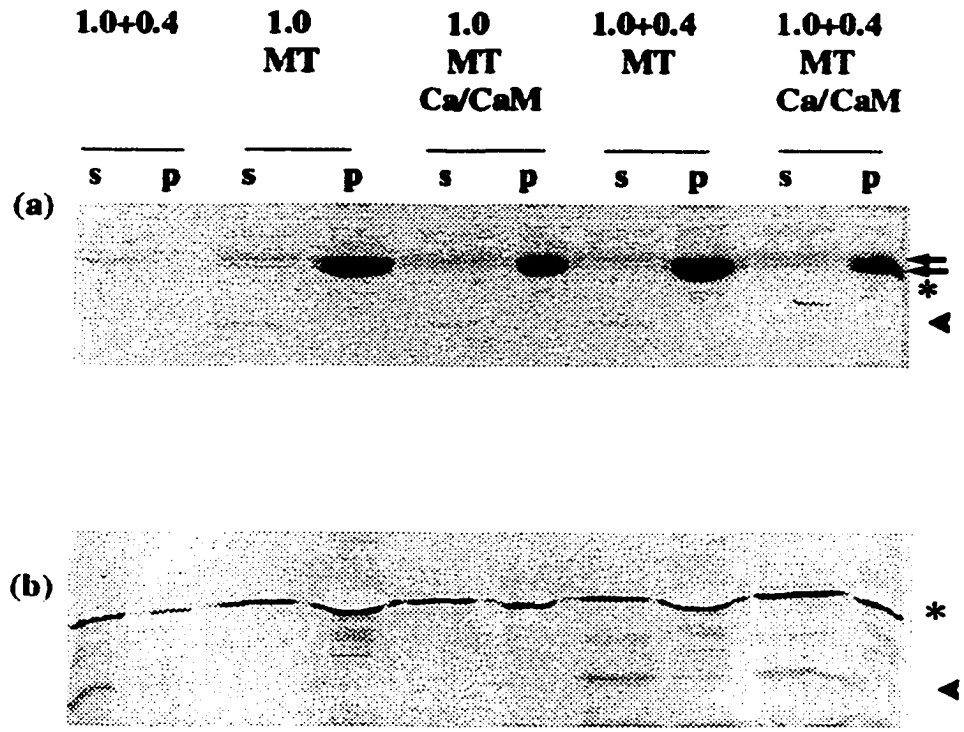


Figure 4.5: Effect of Ca^{2+}-calmodulin on the binding of the 1.0 C alone or together with 0.4 C KCBP to microtubules.
1.0 C KCBP alone (1.0) or both of 1.0 C and 0.4 C (1.0+0.4) were incubated with taxol-stabilized MTs in the absence (MT) or presence of calcium (MT Ca) or Ca^{2+}-calmodulin (MT Ca/CaM) for 30 min and centrifuged. The resulting supernatant (s) and pellet (p) fractions from these microtubule-pelleting assays were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (a) or blotted and probed with biotinylated calmodulin (b). Location of 1.0 C KCBP is indicated by star, 0.4 C KCBP by an arrowhead, and tubulin subunits by arrows.

CHAPTER 5

Regulation of KCBP interaction with CaM Isoforms

Abstract

In *Arabidopsis*, there are six calmodulin genes encoding six CaM-isoforms. The role of these proteins in regulating the activity of target proteins is poorly understood. It was shown previously that bovine CaM modulates the functions of *Arabidopsis* KCBP through calmodulin-binding domain (CBD) present in the KCBP. However, the interaction of *Arabidopsis* KCBP with plant calmodulins, especially CaM isoforms from *Arabidopsis*, remains unclear. Here I have used three isoforms of *Arabidopsis* CaM to study Ca^{2+} /calmodulin modulation of KCBP interaction with microtubules (MTs). Three different *Arabidopsis* calmodulin isoforms (AtCaM-2, 4, and 6) and bovine-CaM were used in MT pelleting assays. All three isoforms inhibit the binding of KCBP to MTs in a calcium-dependent manner. Comparison of bovine CaM with *Arabidopsis* CaMs, there are similar results in inhibiting KCBP binding microtubules. However, AtCaM-2 appears to be more effectively in inhibiting KCBP binding to microtubules as shown in the pelleting assays.

Introduction

Calmodulin (CaM), a well-characterized calcium-binding protein, is present and highly conserved in all eukaryotes. Biochemical and physiological properties of calmodulins are similar between plants and animals. There are four calcium-binding motifs in plant calmodulin numbered from I to IV from the amino-terminal side of amino acid sequence (Zielinski, 1998). In animals, there are a number of distinct calmodulin genes that encode one identical protein. For example, there are three CaMs in human, mouse and rat (Fischer et al., 1988). In plants also, there are multiple genes encoding calmodulin (Perera and Zielinski, 1992; Zielinski, 1998). However, in plants different calmodulin genes do not encode identical calmodulins. The calmodulins encoded by different calmodulin genes differ in their amino acid sequences. The presence of distinct forms of calmodulins called calmodulin isoforms is a distinct feature in higher plants (Zielinski, 1998). In addition to CaM isoforms, calmodulin-like proteins with calcium-binding domains have been found in plants (Zielinski, 1998). Calmodulin-like proteins and calmodulin isoforms are not clearly defined. Zielinski suggests that proteins with more than 20% divergence from the nonvariant conserved amino acid residues of calmodulin are referred to as calmodulin-like proteins (Zielinski, 1998). CaM-like proteins contain more than 148 amino acids and contain variable numbers (3-6) of calcium binding EF-hand motifs (Zielinski, 1998).

In *Arabidopsis*, the expression of multiple calmodulin genes is regulated in different ways (Ling et al., 1991; Perera and Zielinski, 1992), suggesting that they contain different regulatory elements. There are six CaM genes (*AtCaM1 - 6*)

encoding at least four CaM isoforms in *Arabidopsis* (Gawienowski et al., 1993). The nucleotide sequences of these calmodulins are divergent; *AtCaM-2, 3* and *5* genes encode one protein that is identical to touch-stimulated TCH-1 gene (Braam et al., 1990; Gawienowski et al., 1993). Calmodulin isoforms of AtCaM-1, AtCaM-4, and AtCaM-6 differ from the proteins encoded by AtCaM-2, 3 and 5 by four, six, and two amino acid residue substitutions, respectively (Gawienowski et al., 1993; Ling et al., 1991). Most of the amino acid substitutions in *Arabidopsis* CaM isoforms are in the calcium-binding motif IV (Gawienowski et al., 1993). The expression of all *AtCaM* isoforms can be induced by touch signals, but each showed different expression level and pattern during developmental processes (Gawienowski et al., 1993; Ling et al., 1991; Perera and Zielinski, 1992). AtCaM-3 has been detected in leaves, flowers and developing siliques where the expression of AtCaM-3 is higher than AtCaM-1 and 2. AtCaM-1 mRNA is the only calmodulin isoform detected in roots (Perera and Zielinski, 1992). The mRNA of AtCaM-4, 5, and 6 is present in leaves, but only AtCaM-4 and 5 accumulate in siliques (Gawienowski et al., 1993). The results of expression on different organs suggest these calmodulin isoforms may have different physiological functions in plant cells (Gawienowski et al., 1993; Perera and Zielinski, 1992).

In potato, eight different cDNAs of calmodulin (*PCM1 to 8*) have been isolated and characterized (Takezawa et al., 1995). They encode one conserved calmodulin and another divergent calmodulin isoform group. The amino acid sequences of PCM5, 6, 7, and 8 are identical to each other and similar to AtCaM-2, and they belong to conserved calmodulin. The PCM1 differs from the conserved calmodulin

with unique amino acid substitutions, especially in the calcium-binding motif IV (Takezawa et al., 1995). The PCM1 mRNA is highest in the stolon tip and very low in leaves and decreases during tuber development indicating these genes might be involved in this process. The mRNAs of *PCM5* and 8 show similar expression pattern with *PCM1*. The expression of *PCM2* and 3 is higher in germinating pollen and flowers suggesting they might be responding to other signals and involved in other developmental processes (Takezawa et al., 1995). Of these calmodulin isoforms, only *PCM1* showed touch-induced gene expression in potato. These results suggest that different calmodulin isoforms can control and/or respond to different signals or developmental stages (Takezawa et al., 1995).

In soybean, there are five cDNAs encoding CaM (named *SCaM*-1 to 5) and belong to one conserved and one divergent calmodulin isoform group (Lee et al., 1995). The conserved group (SCaM-1, 2, and 3) shows high similarity to AtCaM-2. *SCaM-1* and *SCaM-3* encode the same calmodulin. SCaM-2 differs from SCaM-1 in only two amino acid substitutions. The divergent group contains two isoforms (SCaM-4 and 5) which differ from the conserved group at 32 amino acid residues, including at least 10 totally novel substitutions of non-conserved exchanges that are not seen in any animal or plant calmodulin. This divergent group cannot activate NAD kinase, a calcium/calmodulin target protein activated by conserved CaM (Lee et al., 1995). The domain I of calmodulin in SCaM-1 and SCaM-2 is critical in activating NAD kinase (Lee et al., 1997). SCaM-1 shows abundant expression in apical and elongation regions of hypocotyls and roots, whereas SCaM-4 appears about five times less in apical and elongation regions of hypocotyls and is rarely

detectable in roots (Lee et al., 1995), implying that calmodulin isoforms might be involved in different developmental stages. The SCaM-4 and 5 are the most divergent calmodulin isoforms found in plants or animals so far (Lee et al., 1995).

Calmodulin isoforms also have been found in wheat. There are ten CaM encoding genes classified into three distinct calmodulin isoforms. Seven of these genes encode the first group (TaCaMI) and are identical to wheat germ calmodulin and a barley calmodulin (Ling and Zielinski, 1989; Yang et al., 1996). Two genes encode the second group (TaCaMII) which differs from TaCaMI by two amino acid changes, and one gene for the third group (TaCaMIII) which differs in eighteen amino acid residues in the N-terminus from all other reported calmodulins (Yang et al., 1996).

In contrast to animals that produce an identical calmodulin protein, plants produce multiple calmodulin isoforms (Zielinski et al., 1998) and these isoforms might be involved in different cellular processes. It has been recently shown that calmodulin-binding domain in KCBP confers calcium/calmodulin modulation of KCBP interaction with microtubules by the effect of calcium/calmodulin on microtubule gliding assay and tubulin subunits (Deavours et al., 1998; Narasimhulu et al., 1997; Narasimhulu and Reddy, 1998; Song et al., 1997). In those experiments, bovine-CaM was used as calmodulin source because of high conservation of plant and animal calmodulins (Fosket and Morejohn, 1992). Furthermore, previous experiments of microinjection and biochemical studies with bovine CaM have shown that animal calmodulin can substitute plant calmodulin (Hepler and Hush, 1996; Zhang et al., 1990a; Zielinski, 1998). However, the

interaction of *Arabidopsis* KCBP with plant calmodulins, especially the CaM isoforms from *Arabidopsis*, has not been tested. In this study, I have used different *Arabidopsis* calmodulin isoforms (AtCaM-2, 4, and 6) in the MT pelleting assays to determine the effect of *Arabidopsis* CaMs on KCBP interaction with MTs.

Materials and methods

Arabidopsis isoforms preparation

AtCaM isoform (AtCaM-2, 4, and 6) expression vectors were provided by Dr. Zielinski, University of Illinois. The AtCaM isoforms were prepared as described earlier (Liao et al., 1996) with some modifications. The *E. coli* BL21 (DE3) cells containing recombinant pET CaM isoform construct were grown in NZY medium with 50μg/mL ampicillin and then induced by 1mM IPTG for 3 hours at 37°C. The cells were harvested, washed in buffer A (50mM Tris, pH 7.5), and resuspended in the extraction buffer (buffer A, 2mM EDTA, 1mM DTT, and 200 μg/mL lysozyme) on ice. DNA was removed by treating with DNase, then the cell extract was centrifuged and the supernatant was collected at 4°C. The supernatant was precipitated by adding 55% ammonium sulfate on ice. The proteins in the supernatant were reprecipitated with 50% H_2SO_4 for 30 minutes with constant stirring. The pellet was collected after centrifugation and resuspended in buffer A with 1mM DTT. The resuspension sample was dialyzed against distilled water first then in buffer A containing 100mM NaCl, 0.5mM EGTA and 1mM DTT. $CaCl_2$ was added to the sample to obtain a final concentration of 5mM and loaded onto a phenyl Sepharose column CL-4B (Pharmacia) that was equilibrated with buffer B

(buffer A containing 0.1mM $CaCl_2$ and 0.5mM DTT) previously. The column was washed with buffer B containing 5mM NaCl to remove the nonspecifically bound proteins. The AtCaM protein was eluted with an elution buffer (50mM Tris, pH 7.5, 1mM EGTA, 0.5mM DTT) and dialyzed against water.

Expression and Purification of 1.5C KCBP

For preparing the 1.5 C construct, a 1.5 kb *EcoRI* fragment was excised from a previously constructed pGEX/AKCBP plasmid (Song et al., 1997) and cloned in-frame into the *EcoRI* site in pET32b expression vector. The plasmid construct was transformed into BL21(DE3)pLysS host cells for expression of fusion protein. Bacterial cultures were allowed to grow to an OD_{600} of 0.6 at 37°C. The fusion protein was induced by adding IPTG to a final concentration of 0.2mM and the culture was allowed to grow for an additional 8 hours at 20°C. The induced bacterial cell suspension was spun, washed and resuspended in one-tenth volume of a resuspension buffer (50mM Tris, pH7.5, and 150mM NaCl). The resuspension buffer contained 200µg/mL lysozyme and "Complete" proteinase inhibitor (a commercial mixture of several proteinase inhibitors from Boehringer-Mannheim). Cells in the resuspension buffer were incubated on ice for an hour followed by 10 pulses of sonication, each of 12sec duration, in an iced water-bath, with 2 minutes intervals between each pulse. The sonicated extracts were centrifuged at 100,000g to separate the soluble and insoluble protein fractions. The 1.5 C fusion protein in soluble protein fraction was adjusted to 2mM $CaCl_2$ and purified by using a Calmodulin-Sepharose affinity column. To confirm the induction of fusion protein,

soluble and insoluble protein fractions of both uninduced and induced cultures were separated on SDS-containing gel. One gel was stained with Coomassie Blue and the other gel was blotted onto a nitrocellulose membrane using a Bio-Rad mini transfer cell for detection with an appropriate probe.

Microtubule Cosedimentation Assay

To prepare microtubules, frozen tubulin (Cytoskeleton Inc.) in 80mM Pipes, pH6.8, 1mM $MgCl_2$, 1mM EGTA, 1mM GTP and 3% glycerol was thawed and incubated at 35°C for 30min, then supplemented with 1μM Taxol (Paclitaxel; Sigma). After 5 minutes, the concentration of Taxol was adjusted to 10μM and incubated at 35°C for 5 min more. Then the concentration of taxol was adjusted to 20μM and incubated for 5min at 35°C. The unpolymerized tubulin was separated by sedementing the microtubules in an ultra-centrifuge (TLA 100.3 rotor; Beckman Instruments Inc., Palo Alto, CA) for 1hr at 25000rpm at 35°C. The binding reactions were performed in a 60-μL reaction volume containing motor protein (1.5 C KCBP) with or without polymerized microtubules (1:1 molar ratio). The reaction buffer contained 20mM Pipes, pH6.8, 2mM $MgCl_2$, 1mM DTT, 60μM Taxol, 5mM AMP-PNP, and 150mM NaCl. CaCl2 (3mM), bovine-calmodulin (15μM), AtCaM-2 (15μM), AtCaM-4 (15μM), or AtCaM-6 (15μM) was added to the appropriate reactions. After a 25-min incubation at 37°C, the samples were centrifuged in TLA 100.3 rotor for 25min at 35°C. The supernatant and pellet fractions were separated immediately after the centrifugation and 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 R250.

Results and discussion

It was previously demonstrated that bovine calmodulin inhibits the interaction of KCBP with MTs. The interaction of plant CaMs including different isoforms of *Arabidopsis* with KCBP was shown recently (Reddy et al., 1999b). However, the effect of plant CaM isoforms on the interaction of KCBP with MTs was not tested. To address this question, I have used three purified isoforms of *Arabidopsis* CaMs in MT pelleting assays. The purity of the different CaM isoforms is shown in Figure 5.1. In the MT pelleting assays, I used 1.5 C KCBP that contains the motor domain and the calmodulin-binding domain and which was previously used in MT-pelleting assays. 1.5 C KCBP alone remains primary in the supernatant (Figure 5.2A). The presence of calcium or bovine-calmodulin alone or absence of calcium/calmodulin does not affect the binding between KCBP and microtubules, most of KCBP appear in the pellet together with microtubules (Figure 5.2B). In the presence of calcium/bovine-calmodulin, more KCBP is found in the supernatant as compared to the assay in which protein was incubated with MTs in the presence of calcium alone or CaM alone (Figure 5.2B). In the presence of calmodulin isoforms from *Arabidopsis* similar results were obtained. Calmodulin isoform alone (AtCaM-2, 4 or 6) does not affect KCBP binding ability, where most of the 1.5 C KCBP co-sedimented with the polymerized microtubules (Figures 5.2C, D and E). The presence of calcium together with calmodulin isoforms released the 1.5 C KCBP

which appeared in the supernatant fractions. Of the three isoforms, AtCaM-2 appears to be stronger in its inhibition of KCBP interaction with MTs (Figure 5.2C).

The conclusion that calmodulin-binding domain in KCBP confers calcium/calmodulin modulation of KCBP interaction with microtubules is supported by MT-gliding assays with KCBP in the presence of KCBP (Song et al., 1997), protein overlay assays with tubulin subunits (Narasimhulu et al., 1997) and MT pelleting assay (Narasimhulu and Reddy, 1998). In those experiments, bovine-CaM was used as the calmodulin source. However, the interaction of *Arabidopsis* KCBP with plant calmodulin especially those CaM isoforms from *Arabidopsis* has not been tested. For this purpose, *Arabidopsis* CaM isoforms (AtCaM-2, 4, and 6) were used to analyze the interaction of KCBP with microtubules. Bovine-calmodulin and *Arabidopsis* calmodulin isoforms (AtCaM-2, 4, and 6) showed similar modulation of KCBP interaction with microtubules binding.

There are at least four CaM isoforms in *Arabidopsis* (Gawienowski et al., 1993). The *AtCaM-2, 3* and *5* genes encoded an identical CaM (Gawienowski et al., 1993). Calmodulin isoforms of AtCaM-1, AtCaM-4, and AtCaM-6 differed from AtCaM-2 by four, six, and two amino acid substitutions, respectively (Gawienowski et al., 1993; Ling et al., 1991). AtCaM-1 was an incomplete calmodulin originally (Ling et al., 1991) and recovered by PCR amplification (Gawienowski et al., 1993). AtCaM-2, 4, and 6 were used in my study.

Interaction of AtCaM-2 with KCBP appears to be stronger than other *Arabidopsis* CaM isoforms (Figures 5.2, Reddy et al., 1999b). AtCaM-2 inhibition of KCBP binding to MTs is stronger than other two isoforms. A recent report on the

activation of pea NAD kinase with AtCaM isoforms showed similar results (Liao et al., 1996). AtCaM-2 differs from AtCaM-4 and AtCaM-6 only in a few amino acid substitutions. These substitutions are likely to contribute to these differences. Previous studies with CaM isoforms and mutants have shown that minor change in amino acids, including conservative amino acid substitution, influence the CaM target specificity or affinity for target proteins (Reddy et al., 1999b). The changes on single or two amino acids in calmodulin sequence can affect the interaction of calmodulin with target enzymes *in vivo* (Ling et al., 1991) or interaction with myosin light chain kinase and calmodulin-dependent protein kinase II (Weber et al., 1989). Amino acid differences within or adjacent to the calcium-binding sites might result in different affinities for calcium (Snedden and Fromm, 1998). AtCaM-4 and 6 containing six and two amino acid substitutions make the difference of affinity to target proteins (Reddy et al., 1999b) and might explain the modulation difference among these three calmodulin isoforms.

Based on the data presented here and the published reports, I conclude that different isoforms bind KCBP with different affinity. It would be interesting to test KCBP interaction with calmodulin-like proteins that exist in *Arabidopsis*.

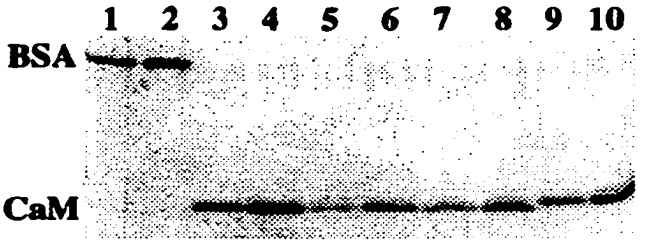
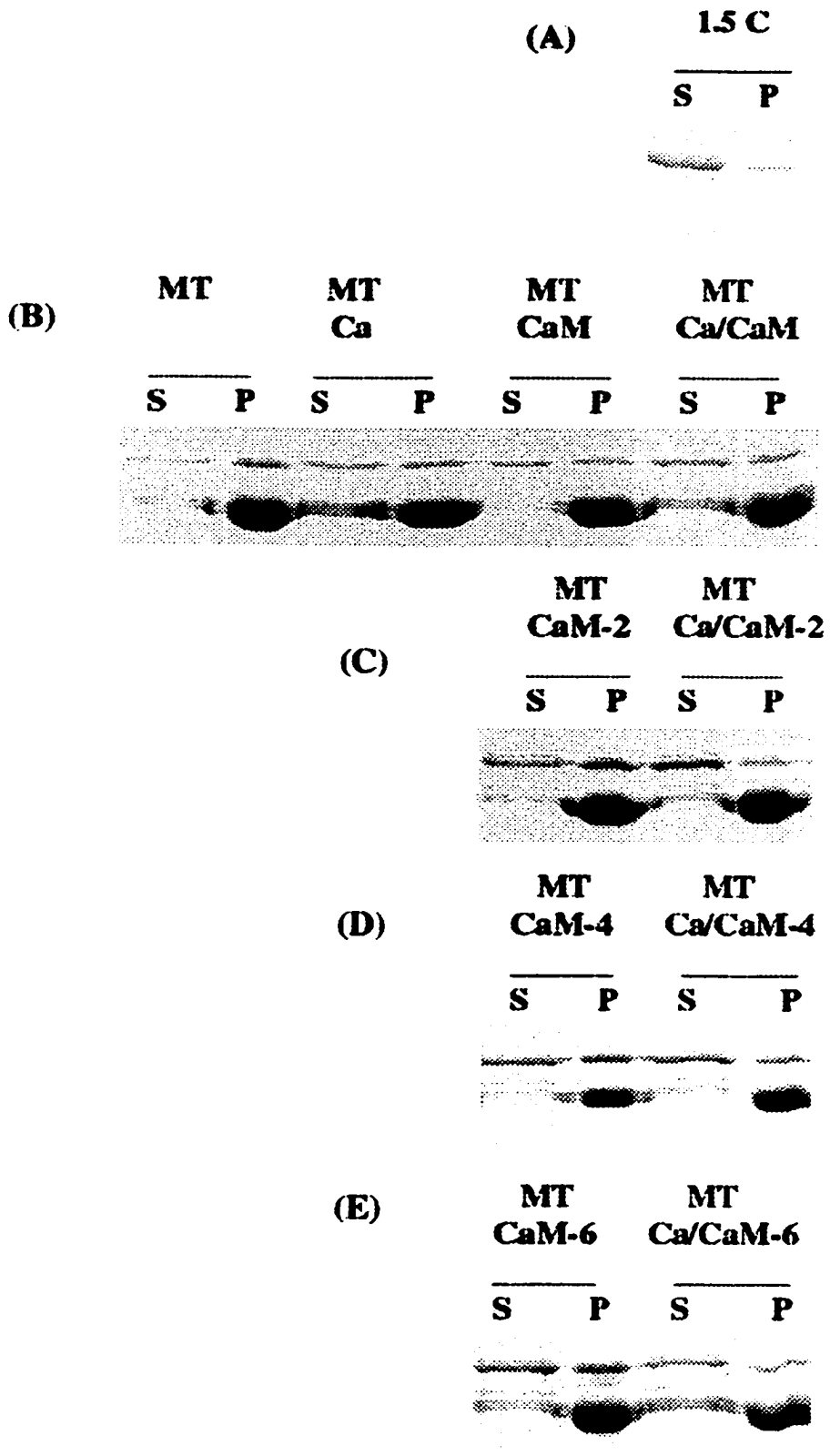


Figure 5.1: Quantification of AtCaMs and bovine CaM proteins by SDS-gel. Initially the concentration of *E. coli* expressed AtCaM isoforms were estimated by the Bradford's method (1976). The BSA was used as a standard in lane 1 (1.38μg) and 2 (2.76μg). AtCaM2 (lane 3, 1.2μg; lane 4, 2.4μg), AtCaM4 (lane 5, 0.36μg; lane 6, 0.84μg), AtCaM6 (lane 7, 0.7μg; lane 8, 1.4μg), bovine CaM (lane 9, 0.9μg; lane 10, 1.8μg). The concentration of each CaM was verified on 12% SDS-PAGE stained with Coomassie blue.

Figure 5.2: A SDS gel showing the regulation of the interaction of 1.5 C KCBP with microtubules by CaM isoforms.

- 1.5 C KCBP and taxol-stabilized MTs were incubated in the absence or presence of calcium alone, calmodulin (CaM) from bovine or *Arabidopsis* or Ca^{2+}/CaM for 30 min and centrifuged.

1.5 C: 1.5 C KCBP alone.
MT: KCBP plus MTs.
MT Ca: KCBP plus MTs in 3mM $CaCl_2$.
MT CaM: KCBP plus MTs in 15μM bovine-CaM.
MT Ca/CaM: KCBP plus MTs in 3mM $CaCl_2$ and 15μM bovine-CaM.
MT CaM-2: KCBP plus MTs in 15μM *Arabidopsis* CaM-2.
MT Ca/CaM-2: KCBP plus MTs in 3mM $CaCl_2$ and 15μM *Arabidopsis* CaM-2.
MT CaM-4: KCBP plus MTs in 15μM *Arabidopsis* CaM-4.
MT Ca/CaM-4: KCBP plus MTs in 3mM $CaCl_2$ and 15μM *Arabidopsis* CaM-4.
MT CaM-6: KCBP plus MTs in 15μM *Arabidopsis* CaM-6.
MT Ca/CaM-6: KCBP plus MTs in 3mM $CaCl_2$ and 15μM *Arabidopsis* CaM-6.
A) pelleting assay with 1.5 C KCBP protein alone.
B) pelleting assay of 1.5 C KCBP and MTs with bovine calmodulin.
C) pelleting assay of 1.5 C KCBP and MTs with *Arabidopsis* CaM-2.
D) pelleting assay of 1.5 C KCBP and MTs with *Arabidopsis* CaM-4.
E) pelleting assay of 1.5 C KCBP and MTs with *Arabidopsis* CaM-6.
1.5 C KCBP alone (1.5C) was also centrifuged as a control. The resulting supernatant (s) and pellet (p) fractions from these microtubule-pelleting assays were separated by SDS-PAGE and stained with Coomassie Blue.



CHAPTER 6

Interaction of N-terminal tail-domain of KCBP with actin

Abstract

The KCBP contains three distinct domains: a highly conserved C-terminal motor domain which interacts with microtubules, a centrally coiled-coil region involved in dimerization and a tail domain for cargo binding at the N-terminus. A part of the tail domain in KCBP shows sequence similarity to tail domain of some conventional myosins, called MyTH4 domain. A part of the tail also shows similarity with talin, called talin-like domain. The talin-like domain in talin is involved in the interaction with membranes and actin. These two regions at the N-terminal tail domain make the KCBP unique in having features of actin-based motors (myosins) and microtubule-based motors (kinesins). For this reason, the 1.5 N KCBP containing tail domain with myosin features was tested for its interaction with G-actin and F-actin. 1.5 N can bind the unpolymerized actin (G-actin) and polymerized actin (F-actin), suggesting that the tail domain of KCBP can bind actin and connect microtubule and actin cytoskeletal systems. These results indicate that KCBP is constructed of part kinesin and part myosin.

Introduction

The KCBP contains three distinct domains: a highly conserved motor domain with ATP- and microtubule-binding sites at the C-terminus, a coiled-coil region involved in forming dimer and a globular N-terminal tail domain which is likely to interact with cargo. Narasimhulu and Reddy (1998) has been demonstrated that a second microtubule-binding site in the tail region of KCBP which interacts with microtubules in ATP-independent manner. There is no similarity between N-terminal tail domain of KCBP and known kinesins or other KLPs. The difference between N-terminal and C-terminal microtubule binding sites is their sensitivity to ATP and Ca^{2+}/CaM. The N-terminal tail domain is ATP- and Ca^{2+}/CaM-insensitive and it can bind to microtubules in the absence or presence of ATP and Ca^{2+}/CaM and C-terminal motor domain is not (Narasimhulu and Reddy, 1998). The presence of second microtubule binding region in the non-motor domain have been reported in other C-terminal and N-terminal motors such as Ncd, Kar3, CENP-E, and human "conventional" kinesin heavy chain (Chandra et al., 1993; Liao et al., 1994; Muleh and Rose, 1990; Navone et al., 1992).

In addition to the motor domain of Ncd, the N-terminal tail domain which was not conserved among the kinesin-like proteins binds microtubules and causes bundling. The binding of N-terminal domain to microtubules is ATP-insensitive (Chandra et al., 1993). The results of the Ncd and microtubule binding/pelleting assays suggest two or even three microtubule binding sites existed in Ncd (Karabay and Walker, 1999). The interaction of the N-terminal tail with microtubules maybe regulated *in vivo* by

association with light chains or other proteins. The C-terminal domain contains an ATP-sensitive microtubule-binding site (Chandra et al., 1993).

Kar3, a C-terminal KLP from *Saccharomyces cerevisiae*, functions in karyogamy. Localization of full-length, motor or tail domain of Kar3, to cytoplasmic microtubules in yeast indicates that both the motor domain and amino-terminal tail domain contain determinants for microtubule association (Muleh and Rose, 1990). In the absence of intact Kar3 protein, the amino-terminal tail domain of Kar3 is sufficient to stabilize and co-localize with cytoplasmic microtubules. A second microtubule-binding site in the tail domain of Kar3 is distinct from the MT-binding site in the motor domain. The Kar3 tail-domain is localized at the distal tips of MTs and the Kar3 proteins are evenly distributed along the length of the microtubules. The tip-specific association of the tail domain implies that Kar3 binds preferentially to GTP-tubulin at the ends of the microtubules or interacts with some other proteins showing uneven distribution along the microtubules. On the other hand, the kinesin-like motor domain may bind equally to either GTP-tubulin or GDP-tubulin (Muleh and Rose, 1990).

CENP-E (centromeric protein) is an essential kinetochore motor protein and has plus-end directed movement (Duesbery et al., 1997). CENP-E remains kinetochore-associated with chromosomes movement during cell division and dissociated when the chromosomes segregate in anaphase (Brown et al., 1996; Duesbery et al., 1997). CENP-E may cross-link the microtubules through binding sites at the N-terminus and the C-terminus (Liao et al., 1994). The identification of two distinct microtubule-binding sites in motor and non-motor domains of CENP-E supports its role as a microtubule cross-linker. Because CENP-E appears to have at least two distinct

microtubule-binding sites, the localization of CENP-E to the spindle mid-zone during anaphase may reflect cross-linking of the overlapping microtubules within this region. Microtubule cross-linking by CENP-E may help stabilize the overall structure of the anaphase spindle or serve to push the overlapping microtubules pass each other and elongate the spindle poles (Liao et al., 1994).

The N-terminal motor and C-terminal tail domain of human kinesin heavy chain (hKHC) also colocalized with microtubules (Navone et al., 1992). The finding that both the kinesin motor domain and the tail domain can interact with cytoplasmic microtubules raises the possibility that kinesin could crossbridge and induce sliding between microtubules. The whole KHC, KHC motor domain and KHC tail domain aligned with the taxol-induced microtubule bundles identically, causing polymerization of non-centrosomal microtubule networks, but not other cytoplasmic microtubules. The motor domain exhibits normal ATP-sensitive microtubule binding, but the tail domain is nucleotide insensitive. The hKHC tail domain binding to microtubules is direct and not through microtubule-associated proteins (Navone et al., 1992). Although the tail domain of KAR3, Ncd, and hKHC share no sequence similarity, they contain a large number of basic and proline-rich regions and relatively few acidic residues. These features are common in other microtubule-binding proteins (Chandra et al., 1993; Muleh and Rose, 1990; Navone et al., 1992). The localization and binding capability of motor and tail domains to microtubules raises the possibility that kinesin and KLPs may participate in movements of the microtubule cytoskeleton by forming crossbridges and by inducing sliding between microtubules.

The N-terminal tail domains of KCBP in tobacco, potato and *Arabidopsis* show significant sequence similarity to a region, referred as MyTH4 domain (myosin tail homology 4 domain), which was found in the tail of some myosins (Chen et al., 1996; Reddy et al., 1996b; Reddy and Reddy, 1999). The MyTH4 domain is a typical feature in myosin subfamilies of IV, VIIa, X, and XII (Chen et al., 1996; Horowitz and Hammer, 1990). There is a single MyTH4 domain in myosin IV and X, and two duplicate domains in VIIa and XII. MyTH4 domain does not function as a dimerization domain since the myosin IV is a monomer (Repezza et al., 1994). Even the genomic structure of MyTH4 in myosin VIIa is known, the functions of MyTH4 domain is unclear (Kelley et al., 1997).

In KCBP, there is also a region in the tail domain that is homologous to talin (Chen et al., 1996; Narasimhulu et al., 1997; Reddy and Reddy, 1999). Talin is known to be capable of interacting with membrane and actin (Niggli et al., 1994; Nuckolls et al., 1990), nucleating actin polymerization (Kaufmann et al., 1991; Kaufmann et al., 1992; Niggli et al., 1994), and increasing F-actin chain stiffness (Ruddies et al., 1993). Talin functions in actin-membrane-linkage because there are two identified domains involved in binding lipid and actin individually (Niggli et al., 1994). Mutation in the talin domain of Myosin VIIa might alter the membrane or cytoskeletal binding properties of this myosin and thereby interfere with its function in moving substrates within the cell (Kelley et al., 1997). These two regions of MyTH4 and talin-like sequence in the N-terminal tail domain have been found only in KCBP and not in any other known kinesins. In the tail domain of human myosin VIIa and bovine myosin X, there are regions of MyTH4 and talin homology (Chen et al., 1996; Solc et al., 1994).

Although myosin VIIa and X contain both of MyTH4 and talin-like domains, myosin IV and XII contain MyTH4 only, suggesting that the MyTH4 and talin may serve distinct functions.

There are two microtubule-binding sites at opposite ends of KCBP (Narasimhulu and Reddy, 1998) which are likely to bind and cross-link microtubules, thereby facilitating bundling of microtubules and connecting adjacent microtubules. The N-terminal tail region of KCBP with sequence similarity to a region in the tail of several myosins makes the KCBP the first known kinesin with myosin features (Chen et al., 1996; Reddy et al., 1996b; Reddy and Reddy, 1999). However, the significance of the conserved region and its function in the tail domain in the KCBP is still unknown. In this context, I have investigated the interaction of the KCBP tail domain with microtubules and actin. Whether the N-terminal tail domain of KCBP binds actin and connects the microtubule and actin cytoskeleton systems is addressed.

Materials and methods

Actin preparation

G-actin was extracted from chick muscle acetone powder and purified according to the protocol of Pardee and Spudich (1982) by Hui Chen and kindly provided by Dr. J. R. Bamburg, Department of Biochemistry, Colorado State University. G-actin was chromatographed over Sephadex G-150 gel filtration column in G-buffer containing 2mM Tris, pH8.0, 0.5mM DTT, 0.2mM ATP, 1mM NaN_3 and 0.2mM $CaCl_2$. F-actin was prepared in F-buffer including 200mM NaCl, 20mM Tris, pH7.5, 0.5mM DTT,

0.2mM ATP, and 2mM $MgCl_2$ and incubated at room temperature for 1 hour for polymerization.

Construction of 1.5 N

For preparing the 1.5 N KCBP construct, a 4.0-kb *SacI-NotI* fragment of the AKCBP cDNA, which contains the coding region of the *Arabidopsis thaliana* KCBP from amino acids 12 to 1261, was cloned into a pET32b vector. A 1.0-kb *XhoI* fragment from the 3′ end of this clone was deleted and religated to create a 3.0-kb *SacI-XhoI* clone. This new *SacI-XhoI* clone was digested with *BsiWI-XhoI* to remove 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-kb long clone for the N-terminal part of KCBP called 1.5 N. This 1.5 N clone contains amino acids from 12 to 503.

1.5 N protein preparation

Bacterial cultures in LB medium containing 30μg/mL kanamycin were allowed to grow at 37°C to an OD_{600} of 0.6. The fusion protein was induced by adding 0.2mM isopropyl β-D-thiogalactopyranoside (IPTG) to the culture and then growing the culture for at least 4 hrs at room temperature. Soluble and insoluble protein fractions from uninduced and induced cultures were prepared as described below. The bacterial cell pellet was resuspended in one-tenth volume of lysis buffer containing 20mM Tris, pH 7.9, 0.5M NaCl, 5mM imidazole, 200 μg/mL lysozyme and a protease inhibitor cocktail "Complete" (Boehringer-Manheim) to prevent the degradation of 1.5 N

KCBP protein. The suspension was lysed by incubating on ice for a period of one hour and followed by 10 pulses, 12 sec each, of sonication on ice. The supernatant obtained after high-speed centrifugation was passed through the His-Bind column. 1.5 N fusion protein contained a histidine-tag sequence at its N-terminus, permitting purification of the proteins on a His-Bind affinity column. The supernatant was allowed to bind to His-Bind resin in the presence of buffer. The column was washed with the same buffer containing 20mM imidazole. The bound proteins were eluted in the same buffer supplemented with 60mM imidazole. Protein concentrations were measured using Bio-Rad protein assay dye reagent using BSA as a standard and stained SDS-gels. The protein was stained with Coomassie Brilliant Blue R-250 to check the protein concentration and quality. Protein was dialyzed in a buffer containing 50mM Tris/Pipes, 500mM NaCl and 0.5mM DTT at pH 6.8, 7.5 and 8.0 to remove imidazole.

Sedimentation assay

F-actin was assembled at room temperature for 30 min in assembling buffer including 0.2M NaCl, 2mM $MgCl_2$, 0.5mM DTT, 0.2mM ATP and 3 different pH buffers (20mM Tris or Pipes, pH of 6.5, 7.5, or 8.0). Then 2.5μM of 1.5 N KCBP protein was mixed with polymerized 2.5μM F-actin and incubated for 60 min at room temperature. The samples were centrifuged for 10 min at 400,000xg (100,000rpm) in a Beckman TLA 100 rotor at 4°C. Sample buffer was added to both supernatants and pellets and analyzed on SDS-gel.

Native Assay

1.5 N KCBP protein and G-actin (2.5 μM each) were mixed and incubated for 30 min at room temperature and analyzed on native gels. Following the electrophoresis, the gel was rinsed in 0.5% SDS, transblotted onto nitrocellulose membrane and detect with S protein.

Results

Expression and purification of 1.5N KCBP

1.5 N KCBP fusion protein contained a histidine-tag sequence at its N-terminus from the vector, hence a His-Bind affinity column was used to purify this protein. After passing the supernatant of 1.5 N protein crude extract through the column, unbound proteins were eluted and then bound 1.5 N KCBP was eluted sequentially with 60mM, 100mM, 200mM and 300mM imidazole. The concentration of 1.5 N protein in eluted fractions was measured using Bio-Rad protein assay using BSA as a standard and the purity was verified on a SDS-gel and western blot (Figures 6.1a and b). The eluted fractions contained a protein that could be detected by S protein (Figure 6.1b). The 1.5 N KCBP is expressed in pET32 vector as thioredoxin fusion protein and contains an additional S tag which is a 15-amino acid peptide which can bind to S protein from pancreatic ribonuclease A (McCormick and Mierendorf, 1994). From the results showed in Figure 6.1, the 1.5 N KCBP protein was eluted mostly from 60mM imidazole (Figures 6.1a and b, lane 3). Higher concentrations of imidazole (100mM) eluted more 1.5 N proteins and more contaminated proteins (Figure 6.1a and b, lane 4). Therefore I used 60mM imidazole to elute 1.5 N KCBP although a degraded band

of 1.5 N KCBP was also detected in the eluted fraction. The 1.5 N KCBP protein does not contain the calmodulin-binding domain, hence it is not detected by biotinylated calmodulin (Figure 6.1c) whereas the 1.4 C KCBP which contains the calmodulin-binding domain (CBD) is detected by biotinylated CaM. The 1.5N KCBP protein was eluted in several fractions and dialyzed in a buffer containing 50mM Tris/Pipes and 200mM NaCl to remove imidazole. The pH of the dialyzed protein was adjusted to 6.8, 7.5, 8.0, or 8.4 to meet the requirements of the following experiments. The concentration of protein in each fraction eluted from the His-Bind column was measured and a sample run on an SDS gel and western blotted (Figure 6.2). The fraction with the highest concentration of protein was used for the pelleting assay.

Actin-binding ability of 1.5 N KCBP

1.5N KCBP dialyzed in 50mM Tris, pH 8.4, and 500mM NaCl was mixed with G-actin and run on a native gel to check its binding to G-actin at pH 8.4. A faint band close to G-actin was detected by S protein in the mixture of 1.5 N KCBP and actin but not in 1.5 N KCBP or actin alone (Figure 6.3). The amount of 1.5 N KCBP and G-actin complex did not increase even in the presence of higher amounts of 1.5N KCBP and G-actin.

To test if the different pH conditions in the assay buffer affect the binding between actin and 1.5 N KCBP, the binding assays were performed at pH 6.8, 7.5 and 8.0. Unpolymerized G-actin, polymerized F-actin, 1.5 N KCBP and a mixture of 1.5 N and F-actin were incubated and centrifuged. The supernatants and pellets were separated immediately after the spin and both ran on the SDS gels and transblotted onto

nitrocellulose membranes. At pH 6.8, more than 80% of G-actin was formed in the supernatant. About 60% of F-actin was in the pellet. The control of 1.5 N KCBP alone appeared mostly in the supernatant. In the mixture of 1.5 N KCBP and F-actin, 1.5 N KCBP can be cosedimented together with F-actin and appeared in the pellet although there was still part of 1.5 N (about 50%) remained in the supernatant (Figure 6.4). At pH 7.5, all G-actin and 1.5 N KCBP stayed in the supernatant, and all F-actin appeared in the pellet. In the mixture of 1.5 N KCBP and F-actin, 1.5 N KCBP cosedimented together with F-actin and was found in the pellet. However, part of 1.5 N KCBP showed in the supernatant (Figure 6.5). At pH 8.0, most G-actin appeared in the supernatant, 70% of polymerized F-actin was in the pellet, and 1.5 N protein alone was in the supernatant only. In the mixture of 1.5 N KCBP and F-actin, some of the 1.5 N KCBP could be cosedimented together with F-actin and showed in the pellet (Figure 6.6).

Discussion

After His-Bind affinity column purification, 1.5 N KCBP and a degraded protein were eluted simultaneously with 60mM imidazole buffer. Both 1.5 N KCBP and the degraded protein contain the S-tag portion because both of these proteins are detected by S protein (Figures 6.1 and 6.2). Biotinylated calmodulin did not detect these proteins as they lack calmodulin-binding domain (Figure 6.1). A PEST motif, rich in proline, glutamate, serine and theronine that is present in proteins with a high turn-over rate, is found in the N-terminal tail domain of KCBP (Reddy et al., 1996b) and may contribute to the rapid turnover of this protein.

To analyze the effect of different pH's on KCBP binding to actin, the binding assays were performed at the different pH conditions, pH 6.8, 7.5 and 8.0. 1.5 N KCBP can bind polymerized F-actin at three pH conditions although there is difference of binding ability at different pH's (Figures 6.4, 6.5, and 6.6). At pH 7.5, G-actin and 1.5 N KCBP were found in the supernatant, and all F-actin was found in the pellet (Figure 6.5). In the mixture of 1.5 N KCBP and F-actin at pH 7.5, the 1.5 N KCBP bound F-actin, cosedimented with actin and appeared in pellet, but still part of 1.5 N KCBP remained in the supernatant (Figure 6.5) suggesting the affinity between two proteins may be weak or 1.5 N KCBP lost part of its binding ability in the process of purification or dialysis. At pH 6.8 or 8.0 condition (Figure 6.4 and 6.6), most of G-actin was found in the supernatant and part of F-actin in the pellet suggesting that these conditions may not be the best suitable for actin polymerization. The 1.5 N KCBP still can be cosedimented together with F-actin and was found in the pellet (Figure 6.4 and 6.6), suggesting that 1.5 N KCBP still binds to actin at these pH's.

1.5 N KCBP binds polymerized F-actin at different pH conditions (Figures 6.4, 6.5, and 6.6) and unpolymerized G-actin weakly at pH 8.4 (Figure 6.3) suggesting that there is an actin-binding site in the tail domain of KCBP. Myosin is an actin-based motor that binds actin and has a calmodulin-binding domain (IQ motifs) in its heavy chain (Coluccio and Bretscher, 1988; Wolenski, 1995). The 1.5 N KCBP protein does not contain any calmodulin-binding domain and cannot be detected by biotinylated calmodulin (Figure 6.1c).

A region in the tail domain of KCBP (in the sequence region of 1.5N) is similar to talin (Chen et al., 1996; Reddy and Reddy, 1999) which is capable of binding actin

and promoting its polymerization (Kaufmann et al., 1991; Kaufmann et al., 1992; Niggli et al., 1994; Nuckolls et al., 1990; Ruddies et al., 1993). The N-terminal tail domain of KCBP (1.5 N) shows significant sequence similarity to the MyTH4 domain in the tail domain of some myosins (Chen et al., 1996; Reddy et al., 1996b; Reddy and Reddy, 1999). The significance of MyTH4 in myosins needs to be identified but may relate to general functions of myosin because it appears in several myosin subfamilies IV, VIIa, X, and XII (Chen et al., 1996; Horowitz and Hammer, 1990). In human and bovine myosins, some myosin subfamilies possess both or either MyTH4 domain or talin domain, suggesting that the MyTH4 and talin-like domains may have different functions (Chen et al., 1996; Solc et al., 1994). These two regions of MyTH4 and talin are unique to the N-terminal tail domain of KCBP and have not found in any other kinesins or KLPs. Therefore, KCBP is the only molecular motor with a kinesin head and myosin tail (Reddy and Reddy, 1999). This myosin tail on the N-terminal part of KCBP possessing the actin-binding ability may crosslink the actin and microtubule cytoskeletons.

KCBP contains one microtubule-binding site in the C-terminal motor domain which is ATP-dependent and a second microtubule-binding site in the N-terminal tail domain which is ATP-independent (Narasimhulu and Reddy, 1998). The presence of two or more microtubule binding sites in the kinesin subfamilies have been found in Kar3, NCD, CENP-E, and human kinesin heavy chain (Chandra et al., 1993; Liao et al., 1994; Muleh and Rose, 1990; Navone et al., 1992). Two microtubule-binding sites in kinesins or KLPs might have a role in cross-linking, bundling, and stabilizing MTs, organizing the microtubule spindles, and inducing microtubule sliding. KCBP may be

involved in the formation of mitotic microtubule arrays such as preprophase bands, phragmoplasts and bipolar spindles (Baskin and Cande, 1990; Bowser and Reddy, 1997; Gunning, 1982). The presence of two microtubule-binding sites in the KCBP has important implications in the function of this protein in cross-linking mitotic microtubules.

The mutation in myosin can be complemented by a kinesin-like protein (Lillie and Brown, 1992) suggesting an interaction between actin- and microtubule-based motors. A direct interaction of actin-based myosin with microtubule-based kinesin has been found and suggests cooperation between actin and microtubule in the cytoskeleton (Huang et al., 1999). The KCBP with its microtubule- and actin-binding sites may connect two cytoskeleton systems, F-actin and microtubules.

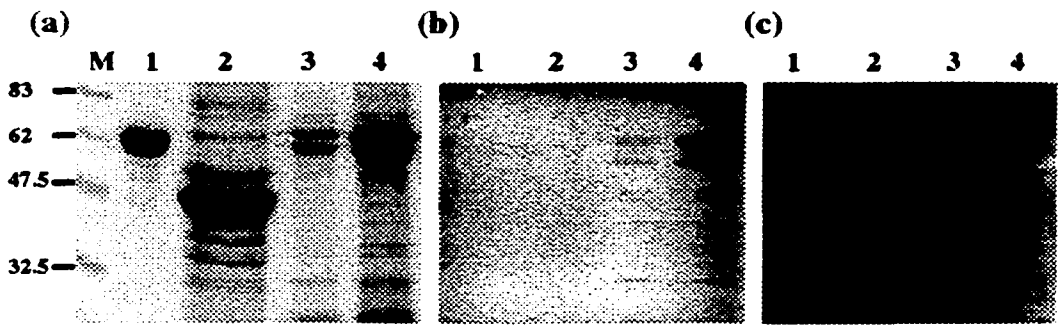


Figure 6.1: Purification and detection of 1.5 N KCBP.
1.5 N KCBP proteins purified from His-Bind column were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (a), blotted and probed with S protein (b) or biotinylated calmodulin (c). Molecular mass markers are indicated (in kD) at the left. Lane 1, BSA; lane 2, 1.4 C KCBP; lane 3 and 4 are different elution fractions of 1.5 N KCBP from His-Bind column.

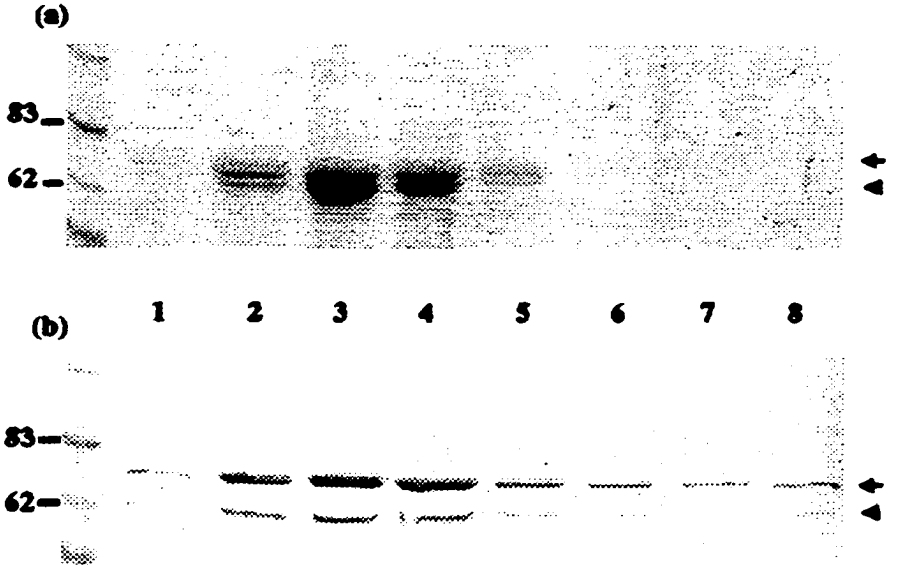


Figure 6.2: Purification and detection of 1.5 N KCBP in different elution fractions of His-Bind column.
1.5 N KCBP fractions purified with His-Bind column were separated by SDS-PAGE and stained with either Coomassie Brilliant Blue (a), or blotted and probed with S protein (b). Molecular mass markers are indicated (in kD) at the left. Lanes 1-8 are different fractions of purified 1.5 N KCBP. Location of 1.5 N KCBP is indicated by arrows and degraded 1.5 N KCBP product is indicated with an arrowhead.

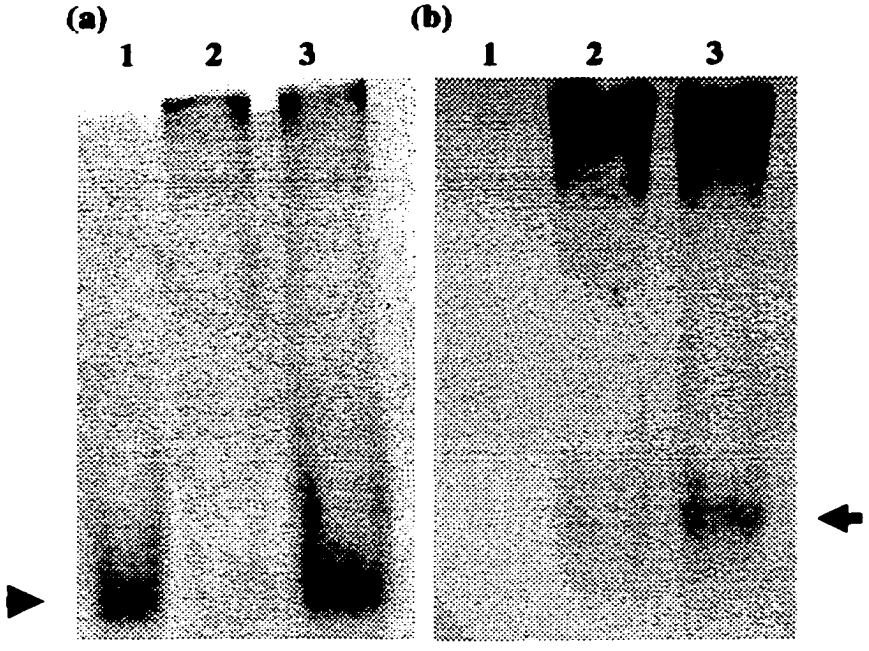


Figure 6.3: Actin-binding ability of 1.5 N KCBP on native gel at pH 8.4.
1.5 N KCBP was incubated with G-actin and run on native gel (pH 8.4). G-actin alone (lane 1), 1.5 N KCBP alone (lane 2), and mixture of G-actin and 1.5 N KCBP (lane 3) were separated on gel and stained with Coomassie Brilliant Blue (a) or blotted and probed with S protein to detect KCBP (b). Location of band 1.5 N KCBP is indicated by an arrow and G-actin with an arrowhead.

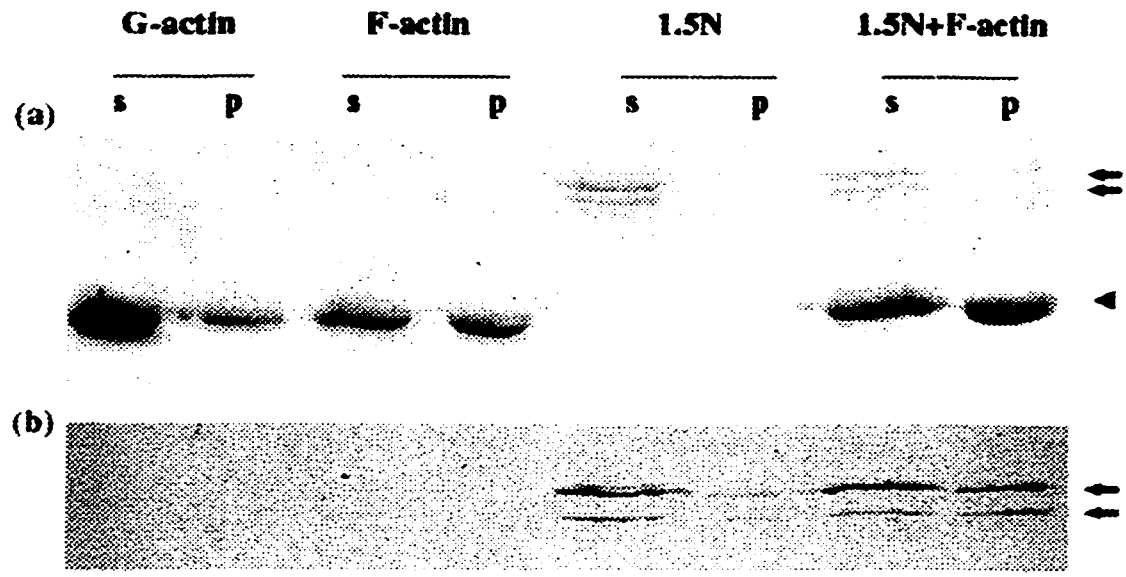


Figure 6.4: Actin-binding ability of 1.5 N KCBP at pH 6.8.
1.5 N KCBP was incubated with polymerized actin (F-actin) at pH 6.8 and centrifuged. The supernatant (s) and pellet (p) fractions from these binding assays were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (a) or blotted and probed to S protein (b). G-actin alone (G-actin), F-actin alone (F-actin), 1.5 N KCBP alone (1.5 N) at pH 6.8 were used as controls and compared with a mixture of 1.5 N KCBP and F-actin (1.5 N+ F-actin). Location of 1.5 N KCBP and actin are indicated by an arrow and an arrowhead, respectively.

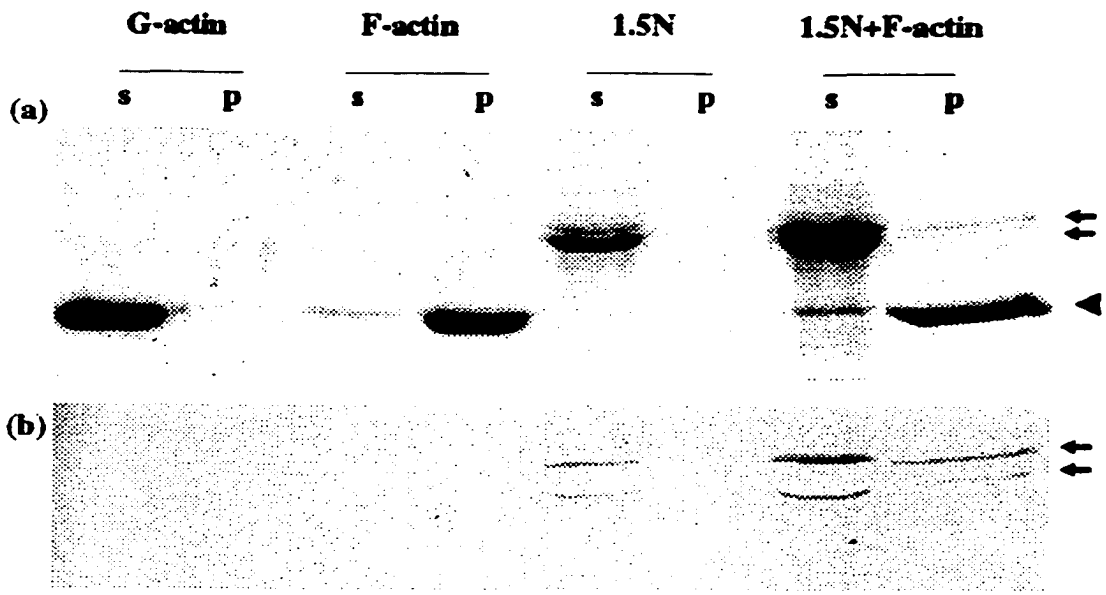


Figure 6.5: Actin-binding ability of 1.5 N KCBP at pH 7.5.
1.5 N KCBP was incubated with polymerized actin (F-actin) at pH 7.5 and centrifuged. The supernatant (s) and pellet (p) fractions from these binding assays were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (a) or blotted and probed with S protein (b). G-actin alone (G-actin), F-actin alone (F-actin), and 1.5 N KCBP alone (1.5 N) at pH 7.5 were used as controls and compared with a mixture of 1.5 N KCBP and F-actin (1.5 N+ F-actin). Location of 1.5 N KCBP is indicated by an arrowhead and actin with an arrow.

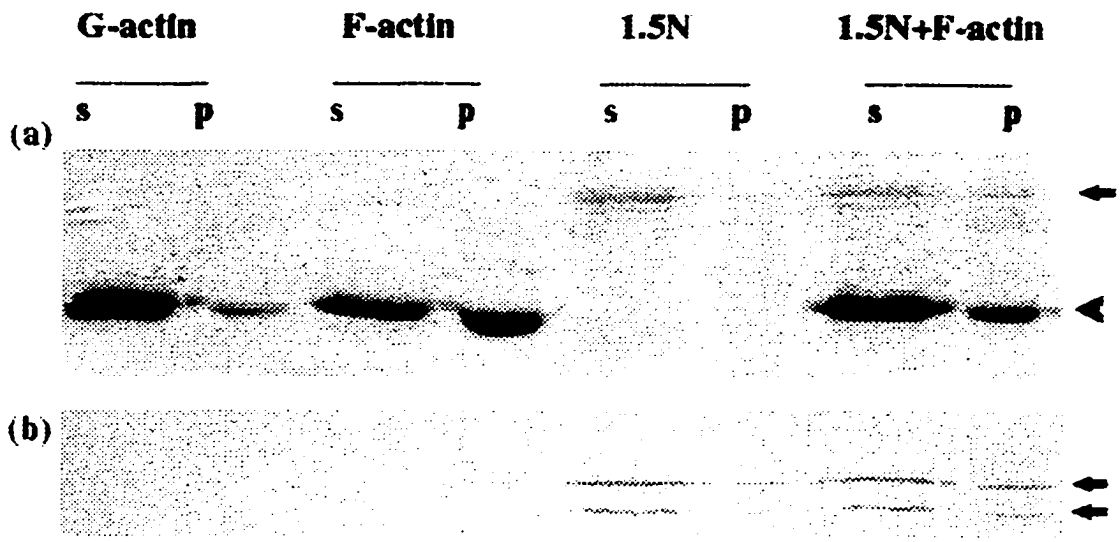


Figure 6.6: Actin-binding ability of 1.5 N KCBP at pH 8.0.
1.5 N KCBP was incubated with polymerized actin (F-actin) at pH 8.0 and centrifuged. The supernatant (s) and pellet (p) fractions from these binding assays were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (a) or blotted and probed to S protein (b). G-actin alone (G-actin), F-actin alone (F-actin), and 1.5 N KCBP alone (1.5 N) at pH 8.0 were used as controls and compared with a mixture of 1.5 N KCBP and F-actin (1.5 N+ F-actin). Location of 1.5 N KCBP is indicated by an arrowhead and an arrow denotes the position of actin.

CHAPTER 7

Discussion

Cell division and many cellular processes rely on molecular motors (kinesin, dynein and myosin) that move along cytoskeletal polymers, microtubule (MT) and actin (Goldstein, 1993; Moore and Endow, 1996; Mooseker and Cheney, 1995; Walker and Sheetz, 1993). Kinesin is a MT-based motor, and its superfamily consists of "conventional" kinesins and more than one hundred of kinesin-like proteins (KLPs) found so far that are involved in intracellular transport, cytoplasmic streaming, and cell division (Barton and Goldstein, 1996; Endow, 1999; Lloyd, 1991; Moore and Endow, 1996; Williamson, 1993; http://www.blocks.fhcrc.org/~kinesin/). Although "conventional" kinesins and KLPs share a highly conserved motor domain, KLPs vary in the location of the motor domain, motility properties and the presence of additional domains (Barton and Goldstein, 1996; Endow, 1999; Moore and Endow, 1996). Plant cells differ from animal cells by lacking defined centrosomes and by forming the preprophase band (PPB) and the phragmoplast during mitosis and cytokinesis (Baskin and Cande, 1990; Fosket and Morejohn, 1992). These plant-specific microtubule-organizations and differences in cell division between plants and animals imply that some plant-specific motors may be present and involve in cell division of plant cells.

A kinesin-like calmodulin-binding protein (KCBP) which possesses a C-terminal motor domain with minus-end motility (Song et al., 1997) has been found in *Arabidopsis* (AKCBP) (Reddy et al., 1996b), potato (PKCBP) (Reddy et al., 1996a),

tobacco (TCK1) (Wang et al., 1996), and maize (Abdel-Ghany and Reddy, unpublished data). Furthermore, an expected size protein detected in *Haemanthus* and *Lilium* using the antibody specific to KCBP (Bowser, 1998; Smirnova et al., 1998), suggest that KCBP is ubiquitous in both dicots and monocots. There is high homology among amino acid sequences and nucleotide sequences of these KCBPs (Abdel-Ghany and Reddy, unpublished data; Reddy et al., 1996a; b; Song et al., 1997; Wang et al., 1996). However, the homologue of KCBP has not been found in non-plant systems including *S. cerevisiae* and *C. elegans* whose genomes have been completely sequenced. Even though a new C-terminal motor protein, kinesin-C, found in embryos of a sea urchin (*Strongylocentrotus purpuratus*) shares 54% sequence identity with the motor domain and 35% identity with the calmodulin-binding domain of AKCBP, it contains a totally different tail domain (Rogers et al., 1999). These results suggest that KCBP is unique and ubiquitous in plants.

This unique *Arabidopsis* kinesin-like calmodulin-binding protein (AKCBP) was isolated during screening of an expression library for calmodulin-binding protein (Reddy et al., 1996b). KCBP is 1261 amino acids long with an estimated molecular weight of 143 KDa. There are three major domains in KCBP, a motor domain in the C-terminal end, a putative coiled-coil domain in the middle and a globular tail domain in the N-terminus (Figure 1.2). The motor domain on the C-terminus contains a stretch of 340 amino acids that show significant similarity with the kinesin motor domain containing ATP-binding sites and microtubule-binding sites. Outside the motor domain, the coiled-coil region and tail domain of KCBPs have no sequence similarity with kinesin or any other known KLPs. This coiled-coil domain has been

reported to help in dimerization (Reddy et al., 1996b). A complete coiled-coil domain makes kinesin (including KCBP) a dimer, shorter or absence of the coiled-coil domain makes it a monomer (Figure 3.4). In addition the coiled-coil region may also provide rotational freedom for the kinesin molecule (Bloom and Endow, 1994; Chandra et al., 1993). The tail domain of KCBPs has been predicted to link to cargoes. There is a PEST motif rich in proline, glutamate, serine and theronine located in the N-terminal tail domain (Reddy et al., 1996b). This PEST motif is a characteristic of proteins with a short half-life and it suggests that the KCBP is a rapidly turned over protein (Reddy et al., 1996b).

KCBP is different from all known KLPs because there are some unique domains that are not present in other kinesins or KLPs. At the end of the C-terminus, a unique calmodulin-binding domain (CBD) is mapped to 23 amino acids adjacent to the motor domain. In the amino-terminal part of KCBP, a region shows homology to a myosin, referred to as myosin tail homology domain (MyTH4) (see Figure 1.2) (Chen et al., 1996; Reddy et al., 1996b; Reddy and Reddy, 1999). The MyTH4 domain is typical in some myosin subfamilies such as myosins IV, VIIa, X and XII. Next to the MyTH4, a portion of 300 amino acids in the tail domain of KCBP shows significant homology to talin, which can interact with membrane and actin in cytoskeleton system (Niggli et al., 1994; Nuckolls et al., 1990). The functions of the MyTH4 and talin homology domains in the KCBP tail domain and myosins are poorly understood (Reddy and Reddy, 1999). The KCBP tail domain can bind to unpolymerized G-actin (Figure 6.3) and polymerized F-actin (Figures 6.4, 6.5 and 6.6) suggesting that KCBP can bind actin and connect microtubules and the actin cytoskeletal system. These

three specific regions (calmodulin-binding domain, MyTH4 and talin homology domain) have not been found in any other known members of the kinesin and KLP superfamily.

The C-terminal motor domain of KCBP contains an ATP-dependent microtubule-binding site and microtubule-stimulated ATPase activity (Reddy et al., 1996a) and the second ATP-independent microtubule-binding site is located in the N-terminal tail domain (Narasimhulu and Reddy, 1998). KCBP is a molecular motor, which translocates along the minus-end of microtubules with a velocity of 8-10 μm/min (Song et al., 1997). KCBP has the ability to induce microtubule bundling through the C-terminal motor domain (Figures 3.2, 3.3, 3.5, and 3.6) or the ATP-independent MT binding site in the N-terminal tail (Figure 3.8). KCBP homologs from different plants are grouped in the C-terminal motors subfamily based on sequence similarity in the motor domain with other C-terminal motors (Endow, 1999; Reddy et al., 1997; http://www.blocks.fhcrc.org/~kinesin/). In phylogenetic trees, the four reported KCBPs constitute a distinct group under the C-terminal motor family of kinesin subfamilies (Hirokawa, 1998; Reddy et al., 1998).

The complete motor domain of KCBPs with the calmodulin-binding domain can bind tubulin α and β subunits (Figure 2.3) except in the presence of both calcium and calmodulin (Figure 2.7). Similar modulation by calcium/calmodulin exists between KCBP and microtubules, the motor domain of KCBPs cannot bind to microtubules in the presence of calcium/calmodulin, but calcium alone or calmodulin alone has no effect (Deavours et al., 1998; Narasimhulu and Reddy, 1998). This inhibition of binding between the motor domain and microtubules in the presence of both calcium

and calmodulin is affected also by the concentration of calcium/calmodulin (Deavours et al., 1998). Calcium/ calmodulin modulation does not affect the basal ATPase activity of KCBP but reduces the microtubule-stimulated ATPase activity (Deavours et al., 1998). All these results indicate that the calcium/calmodulin might regulate the binding activity of KCBP by inhibiting its association with microtubules. In the N-terminal tail domain of KCBP there is a second microtubule-binding site which is ATP-independent and also calcium/calmodulin-independent (Narasimhulu and Reddy, 1998).

Through the calmodulin-binding domain, calcium/calmodulin not only inhibits the binding activity between KCBP and microtubules but also regulates other functions related to KCBP binding activity. The *in vitro* motility of AKCBP could be abolished by calcium/calmodulin by inhibiting the binding of the motor to microtubules (Song et al., 1997). *In vivo*, calcium/calmodulin may regulate the association between KCBP motor and microtubules by reducing the affinity of KCBP for microtubules (Deavours et al., 1998). The KCBP motor domain with (Figures 3.2, 3.3, and 3.5) or without the calmodulin-binding domain (Figure 3.6) or the tail domain alone (Figure 3.8) can sufficiently induce microtubule bundling. In the presence of the calmodulin-binding domain, calcium/ calmodulin can cause dissociation of the motor-domain-induced microtubule bundling (Figures 3.2, 3.3, and 3.5). However, calcium/ calmodulin could not dissociate those bundles caused by tail domain (Figure 3.8).

In *Arabidopsis*, there are six calmodulin isoforms (Gawienowski et al., 1993). *Arabidopsis* calmodulin isoforms (AtCaMs) bind to AKCBP through the calmodulin-

binding domain in a calcium-dependent manner, but with different affinities (Figure 5.2; Reddy et al., 1999b). Among these calmodulin isoforms (AtCaM2, 4 and 6), AtCaM2 has the highest affinity (Figure 5.2, Reddy et al., 1999b). The precise role of each *Arabidopsis* calmodulin isoform in regulating KCBP *in vivo* needs further investigation. Other plant systems such as potato, tobacco and maize, require further research on the interaction between their KCBP homologs and calmodulin isoforms.

The tail domain of kinesins has been predicted to bind specific cargo for transportation (Bloom and Endow, 1994). A kinesin-interaction-protein kinase (KIPK) has been found to interact with KCBP by using the tail and coiled-coil domains of KCBP as bait in a two-hybrid screen (Day et al., 1999; Reddy et al., 1999a). This has been confirmed by co-precipitation *in vitro* (Day et al., 1999; Reddy et al., 1999a). KIPK carries auto-phosphorylation activity. KCBP contains possible phosphorylation sites, however, which are not phosphorylated by KIPK (Day et al., 1999; Reddy et al., 1999a). There is accumulating evidence indicating that phosphorylation regulates kinesins and KLPs or modulates the interaction of kinesin motor and microtubules (Blangy et al., 1995; Liao et al., 1994; Nigg et al., 1996; Robertson and Allan, 1997; Sawin and Mitchison, 1995). The phosphorylation pathway between KIPK and KCBP is unclear. There are possible mechanisms that KIPK may need some modification steps in order to phosphorylate KCBP or KCBP-associated proteins (Day et al., 1999; Reddy et al., 1999a). To determine whether the native KCBP itself or KCBP-associated proteins are the targets for KIPK requires further research. The non-conserved tail domain in KCBP confers diversity to interact with specific cargoes and function in different cellular processes. Whether

KIPK is the only candidate of cargo or still other unknown cargoes interact with KCBP remains to be seen.

The highly dynamic-changes of microtubules suggests that the transition in microtubule arrays and changes in orientations may depend on polymerization/ depolymerization of microtubules rather than movement (Hepler and Hush, 1996; Mandelkow and Mandelkow, 1995). The mechanisms that regulate dynamic instability of depolymerization/repolymerization of microtubules remain unclear, but several microtubule-based motors are involved in these processes such as the Kar3 which can induce depolymerization of microtubules (Endow et al., 1994) and XKCM1 and XKIF2 which regulate microtubules (Desai et al., 1999; Walczak et al., 1996). Calcium and calmodulin are involved in stabilizing and destabilizing cortical microtubules in plants (Cyr, 1991; Fisher et al., 1996). Calmodulin regulates microtubules via calmodulin-binding microtubule-associated proteins rather than microtubules because calmodulin does not bind to tubulin (Durso and Cyr, 1994; Kotani et al., 1985; Lee and Wolff, 1984; Margolis et al., 1986; Pirollet et al., 1992; Reddy et al., 1996a).

Trichomes are specialized branched epidermal hair cells on the leaves of *Arabidopsis* (Hulskamp et al., 1994). The ZWICHEL (ZWI) gene is involved in controlling the final shape and branch number in trichome development and the gene product of ZWI has been identified as KCBP, suggesting that KCBP has a role in trichome morphogenesis (Oppenheimer et al., 1997). The *zwi*-3 mutant expresses a protein containing just the tail domain but lacking the coiled-coil region and motor domain of AKCBP and its phenotype has been rescued by *suppressor of zwichel*-3,

suz-3 (Krishnakumar and Oppenheimer, 1999). The *suppressors of zwichel* gene products are likely to interact with the tail domain of KCBP. KCBP may be involved in microtubule bundling and motility during trichome branch initiation. Trichome branch initiation depends on KCBP tail domain, but maybe the motor and coiled-coil domains are not necessary. How these genes, suz and zwi, cooperate and function in trichome morphology development remains to be seen.

KCBP is accumulated highly in flower tissue (Reddy et al., 1996a). Dynamic instability of microtubules are implicated in the pollen tube polarity and several MT-based motors are present in pollen tubes with unknown functions (Cai et al., 1993; Tiezzi et al., 1992). The double mutants of *suz* and *zwi* show male-sterility phenotype (Krishnakumar and Oppenheimer, 1999), suggesting that SUZ and KCBP may contribute to pollen germination and pollen tube growth. There is some vesicle accumulated in the pollen tube of these double mutants implying the KCBP may be involved in the vesicle movement. KLPs are involved in organelle/vesicle transport (KHC, KRP85/98, MCAK/KIF2 and Unc104 subfamilies) and mitotic and meiotic spindle movement and stabilization (C-terminal, BimC, Chromokinesin/KIF4, MCAK/KIF2 and MKLP1 subfamilies) (Moore and Endow, 1996). These results suggest that the functions of C-terminal KLPs may not only be restricted to aspects related to cell division and trichome morphogenesis.

From the research on the tobacco BY2 suspension system, KCBP is cell cycle regulated, which has been found to reach a high level in M-phase and a low level of KCBP in interphase (Bowser, 1998; Bowser and Reddy, 1997). Using the specific anti-KCBP antibody, KCBP is located on the pre-prophase band, mitotic spindle,

phragmoplast and cell plate in tobacco suspension cells, *Arabidopsis* culture cells and *Haemanthus* endosperm (Bowser and Reddy, 1997; Smirnova et al., 1998). Using *Haemanthus* endosperm as model, KCBP has been detected in the prophase spindle, on kinetochore microtubules in metaphase, at spindle polar regions in anaphase, and in the phragmoplast and cell plate in telophase (Smirnova et al., 1998). From the immunolocalization results, KCBP is likely to be involved in the formation and function of microtubule arrays and bipolar spindles (Bowser and Reddy, 1997; Smirnova et al., 1998).

The antibodies against the calmodulin-binding site of KCBP have been injected into *Tradescantia virginiana* stamen hair cells to inhibit calmodulin binding and to constitutively activate KCBP. The results reveal that activation of KCBP in late prophase induces nuclear envelope breakdown and progression into pro-metaphase before the cell cycle halts (Vos et al., 1999). Activation of KCBP results in abnormal phragmoplasts and delays the cytokinesis (Vos et al., 1999). The roles of KCBP in cell division change during progression of the cell cycle. The level of KCBP rises to induce nuclear envelope breakdown in late prophase, falls to allow the microtubule dynamics involved in chromosome alignment in metaphase, rises to maintain bipolar spindle formation by microtubule sliding and bundling during anaphase, and falls to permit the dynamic changes of microtubules in the phragmoplast during telophase (Vos et al., 1999). From the microtubule bundling assay, both the tail domain (Figure 3.8) and motor domain of KCBP (Figure 3.2, 3.3, 3.5, and 3.6) can bundle microtubules separately. This supports the hypothesis based on immunolocalization and antibody-injection results that KCBP bundles spindle microtubules to form the

preprophase band (PPB), spindle and phragmoplast. The dynamic spindle changes caused by microtubule instability may also be controlled by KCBP via calcium/calmodulin regulation, or it may cooperate with other motor proteins to cause polymerization/depolymerization on microtubules.

KCBP is unique to plants, and its absence in animal system strongly implies the possibility that it may be involved in aspects of cellular development that are specific to plant cells. KCBP which is the only member in the kinesin superfamily with a calmodulin-binding domain is a prototype calcium/calmodulin regulated KLP.

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Abbreviation list

ADP	adenosine 5'-diphosphate
AKCBP	*Arabidopsis* kinesin-like calmodulin-binding protein
AMP-PNP	Adenylylimidodiphosphate
AtCaM	Arabidopsis calmodulin
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BY2	Bright yellow tobacco cell culture
CaM	calmodulin
CBD	calmodulin-binding domain
CENEP	centromeric protein
CH	calponin homology domain
DAB	diaminobenzedine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
KAR	karyogamy related KLP
KCBP	kinesin-like calmodulin-binding protein
KHC	kinesin heavy chain
KIP	kinesin interacting protein
KLC	kinesin light chain
KLH	Keyhole Limpet Hemocyanin
KLP	kinesin-like protein
KRP	kinesin related protein
M phase	mitosis phase
MTs	microtubules
MyTH4	myosin tail homology domain

NBT	nitroblue tetrazolium
Ncd	non-claret disjunctional
NEM	N-ethylmaleimide
PCR	polymerase chain reaction
PKH	pollen kinesin homologue
PPB	preprophase band
RNA	ribonucleic acid
SPB	spindle pole body
S phase	synthesis phase
TCK1	tobacco KCBP
TKRP125	tobacco kinesin-related polypeptide of 125kDa
ZWI	ZWICHEL gene