

A novel kinesin-like protein with a calmodulin-binding domain

030.368

W. Wang¹, D. Takezawa¹, S. B. Narasimhulu², A. S. N. Reddy² and B. W. Poovaiah^{1,*}

¹ Laboratory of Plant Molecular Biology and Physiology, Department of Horticulture, Washington State University, Pullman, WA 99164-6414, USA (*author for correspondence); ² Department of Biology and Program in Cell and Molecular Biology, Colorado State University, Fort Collins, CO 80523, USA

Received 27 November 1995; accepted in revised form 12 February 1996

Key words: calcium, calmodulin, calmodulin-binding protein, kinesin, motor protein, microtubule

Abstract

Calcium regulates diverse developmental processes in plants through the action of calmodulin. A cDNA expression library from developing anthers of tobacco was screened with ³⁵S-labeled calmodulin to isolate cDNAs encoding calmodulin-binding proteins. Among several clones isolated, a kinesin-like gene (*TCK1*) that encodes a calmodulin-binding kinesin-like protein was obtained. The *TCK1* cDNA encodes a protein with 1265 amino acid residues. Its structural features are very similar to those of known kinesin heavy chains and kinesin-like proteins from plants and animals, with one distinct exception. Unlike other known kinesin-like proteins, *TCK1* contains a calmodulin-binding domain which distinguishes it from all other known kinesin genes. *Escherichia coli*-expressed *TCK1* binds calmodulin in a Ca²⁺-dependent manner. In addition to the presence of a calmodulin-binding domain at the carboxyl terminal, it also has a leucine zipper motif in the stalk region. The amino acid sequence at the carboxyl terminal of *TCK1* has striking homology with the mechanochemical motor domain of kinesins. The motor domain has ATPase activity that is stimulated by microtubules. Southern blot analysis revealed that *TCK1* is coded by a single gene. Expression studies indicated that *TCK1* is expressed in all of the tissues tested. Its expression is highest in the stigma and anther, especially during the early stages of anther development. Our results suggest that Ca²⁺/calmodulin may play an important role in the function of this microtubule-associated motor protein and may be involved in the regulation of microtubule-based intracellular transport.

Introduction

Calmodulin (CaM), a Ca²⁺-binding multifunctional regulatory protein, is known to be a primary transducer of the intracellular Ca²⁺ signal [35, 39, 47]. Calmodulin regulates many cellular

or subcellular processes through CaM-binding proteins. A number of CaM-binding proteins have been identified and characterized in animals, including metabolic enzymes, protein kinases, membrane transporters, and structural proteins [11]. Although CaM-binding proteins are widely

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U52078.

distributed in plants [19, 30] and are believed to be involved in the diverse functions in plants [35, 39], little is known about the identities and functions of these CaM-binding proteins. In recent years, several genes encoding CaM-binding proteins have been cloned and identified, for example, glutamate decarboxylase [1, 20], Ca^{2+} /CaM-dependent protein kinase [33], and some proteins with unknown identities [21, 38]. Further identification and characterization of CaM-binding proteins should broaden our knowledge of Ca^{2+} -mediated signal transduction mechanisms in plants.

Using recombinant ^{35}S -labeled potato calmodulin as a ligand probe, a cDNA expression library from developing anthers of tobacco was screened to obtain CaM-binding clones. Several positive clones were isolated and characterized [54]. The sequence comparison revealed that one of the clones had homology with kinesin heavy chain and kinesin-like genes.

Kinesin, a large superfamily of the microtubule-based motor proteins, consists of kinesin heavy chains (i.e., conventional kinesins) and kinesin-like proteins (KLPs) [34]. Since kinesin was first identified from squid [48], a number of kinesin heavy chains and kinesin-like genes have been cloned and characterized from various eukaryotic organisms [10, 34]. The common feature of both conventional kinesin heavy chains and kinesin-like proteins is that they contain a motor domain, which contains a microtubule-activated ATPase activity that converts the chemical energy stored in ATP into mechanical forces to move them along the surface of microtubules [62]. In addition to the mechanochemical motor domain, most kinesins contain an α -helical coiled-coil stalk and a globular tail domain. However, except for the conventional kinesin heavy chain, the sequences of kinesin-like proteins in the stalk and tail domains do not share obvious homology. Kinesin appears to be a major intracellular motility molecule, which plays essential roles in membrane-bound organelle transport [29, 31, 48], mitotic and/or meiotic spindle organization [9, 50] and chromosome positioning [50]. In plants, an immunoreactive kinesin homolog was detected

in the pollen tubes of tobacco by using a monoclonal antibody to the heavy chain of the calf brain kinesin molecule [44]. A gene family encoding kinesin-like proteins has also been cloned from *Arabidopsis thaliana* [25, 26]. Matthies *et al.* [23] reported that kinesin light chains from bovine brain bind calmodulin in a calcium-dependent manner. However, all of the known kinesin heavy chains and kinesin-like proteins characterized from plants and animals do not bind calmodulin. We cloned a kinesin-like gene (*TCK1*) by screening a cDNA expression library using ^{35}S -labeled calmodulin. Sequence analysis and biochemical studies revealed that TCK1 has all the common characteristics of kinesin-like proteins. In addition, TCK1 binds to calmodulin in the presence of Ca^{2+} . The presence of a CaM-binding domain in TCK1 makes it a unique addition to the kinesin superfamily. Since calmodulin is a multifunctional regulatory protein, it is likely that it plays a unique regulatory role in controlling the function of TCK1.

Materials and methods

Plant materials

Nicotiana tabacum cv. Petit Havana SR-1 was grown under greenhouse conditions. Anthers of different stages and various organs were dissected from the tobacco plants, immediately frozen in liquid N_2 , and stored at -70°C until use. The stages of flowers were determined by measuring the distance from the base of the pedicel to the tip of the sepal or corolla depending on which was longer.

Screening of tobacco anther cDNA library with ^{35}S -calmodulin

Total RNA was extracted from developing anthers of tobacco flowers 0.5 to 1.0 cm in length [51], and poly(A)⁺ RNA was isolated using oligo(dT) cellulose column chromatography [40]. A tobacco anther cDNA library was con-

structed in the λ ZAPII vector (Stratagene) using a cDNA synthesis kit from Pharmacia. Potato calmodulin PCM6 cDNA [43] was cloned into *Nde*I/*Bam*HI sites of the pET3b expression vector (Novagen) and 35 S-labeled calmodulin was prepared according to Fromm and Chua [7]. About 6×10^5 plaques were screened essentially as described [38]. The positive plaques obtained in the first round of screening were purified by two additional rounds of screening.

DNA sequence analysis

The pBluescript SK(–) plasmid containing the cDNA clone was *in vivo* excised from the λ ZAPII vector according to the protocol described by Stratagene. The DNA sequences were determined by the dideoxynucleotide chain termination method using the Sequenase 2.0 kit (US Biochemical Corp.) and the PCR *fmol* sequencing kit (Promega). Sequence data analyses were performed using the Wisconsin Genetics Computer Group (GCG package, version 8.0) software.

Expression of fusion proteins of TCK1 in *E. coli*

The fragments of the *TCK1* cDNA encoding amino acids 824–1265 and 1214–1265 were fused in frame to glutathione *S*-transferase (GST) in a pGEX-3X vector. *E. coli* strain BL21 (DE3) containing the expression plasmids was grown at 30 °C in M9 minimal medium supplemented with 2 g/l NZ amine and 100 mg/l ampicillin. Proteins were induced by adding 0.1 mM IPTG when OD_{600} reached 0.6 unit. Three hours after induction, *E. coli* cells were collected by centrifugation at $3000 \times g$ and sonicated in PBS to prepare bacterial lysates. Insoluble materials were removed by centrifugation at $15000 \times g$ and the fusion proteins were purified using glutathione-Sepharose column (Pharmacia) according to the manufacturer's instructions. Alternatively, the fusion proteins were purified by calmodulin-Sepharose column chromatography. $CaCl_2$ (1 mM) was added to the soluble fractions of *E. coli* lysate before

loading onto the calmodulin-Sepharose column. The column was washed thoroughly with a buffer containing 50 mM Tris-Cl pH 7.5, 1 M NaCl, 0.1% (w/v) Triton X-100, 1 mM DTT and 1 mM $CaCl_2$, and fusion proteins were eluted with 50 mM Tris-Cl pH 7.5, 200 mM NaCl, 1 mM DTT, 0.1% (w/v) Triton X-100 and 2.5 mM EGTA. Fusion proteins containing amino acid residues 824–1265 and 1214–1265 of TCK1 were used to perform ATPase assays and calmodulin-binding assays, respectively.

Biotinylated calmodulin overlay assay

E. coli-expressed recombinant fusion proteins were separated on the SDS-polyacrylamide gel [16] and electrophoretically transferred onto a nitrocellulose filter [46]. The filter was blocked in TBS containing 3% (w/v) non-fat dry milk at room temperature for 2 h. The filter was then incubated with 100 ng/ml biotinylated calmodulin (BRL) in binding buffer (TBS containing 1% w/v BSA, and 1 mM $CaCl_2$) at room temperature for 2 h. After washing in TBS containing 1 mM $CaCl_2$, the filter was incubated with avidin-conjugated alkaline phosphatase in the binding buffer for 2 h. The bound biotinylated-calmodulin was detected by NBT/BCIP reagent (Amresco).

Southern blot analysis

Tobacco genomic DNA (10 μ g) was digested with different restriction enzymes, run in 0.8% agarose gel, and transferred onto a nylon membrane. The membrane was incubated with random-primed 32 P-labeled probe using a *Pst*I/*Xba*I fragment (nt 2827–3946, Fig. 1) as a template [40]. The hybridization was performed at 42 °C in a solution containing 50% formamide, $6 \times$ SSC, $5 \times$ Denhardt's solution, 0.1% w/v SDS and 100 μ g/ml herring sperm DNA. The membrane was washed at 60 °C in $0.5 \times$ SSC and 0.1% w/v SDS, then exposed to Kodak XAR-5 film.

Northern blot analysis

Poly(A)⁺ RNA from various organs and developing anthers of different stages were isolated as described above. Five μg of poly(A)⁺ RNA was denatured by glyoxal and DMSO [40], electrophoresed in 1% agarose gel, and then transferred onto a nylon membrane. Hybridization was performed at 42 °C in a solution containing 50% formamide, 5 \times SSPE, 5 \times Denhardt's solution, 0.1% w/v SDS and 100 $\mu\text{g}/\text{ml}$ herring sperm DNA. Random-primed ³²P-labeled cDNA probes were applied using a *HindIII/PstI* fragment (nt 1098–1940, Fig. 1) as a template. After hybridization, the membrane was washed with 0.1 \times SSC and 0.1% w/v SDS at 65 °C and exposed to Kodak XAR-5 film. The same membrane was hybridized with a tobacco α -tubulin probe under similar conditions.

Isolation, purification, and polymerization of sheep brain tubulin

Tubulin was purified from the gray matter of fresh sheep brain according to the protocol of Williams and Lee [57]. The tissue was homogenized in a buffer containing 100 mM Pipes-NaOH pH 6.9, 2 mM EGTA, 1 mM MgSO₄, 4 M glycerol, and 2 mM DTT. The supernatant obtained after spinning at 96 000 $\times g$ at 4 °C for 75 min was used for the assembly of the tubulin polymer at 34 °C in the presence of 1 mM GTP and disassembly through cold treatment. Twice-cycled tubulin protein was further purified from microtubule-associated proteins by passing through a phosphocellulose column. The void containing the pure tubulin was adjusted to a concentration of 1.3 mg/ml, frozen in liquid N₂ and stored at –80 °C in small aliquots. For preparing microtubules, the frozen tubulin was thawed, equilibrated with buffer, supplemented with 1 mM GTP

and 20 μM taxol, and incubated at 34 °C. The process of polymerization was monitored continuously following the change in absorbance at 350 nm.

ATPase assay of kinesin protein

ATPase activity of the kinesin protein was measured according to the protocol of Chandra and Endow [3]. The reaction mixture contained 20 $\mu\text{g}/\text{ml}$ purified protein, 4 mM Mg ATP, 15 mM imidazole pH 6.9, 1 mM EGTA, 1 mM DTT, 1.5 μCi of ³²P-ATP and with or without 1 mg/ml taxol-stabilized microtubules of sheep brain in a reaction volume of 150 μl . The reaction was allowed to proceed for 15 min at 24 °C. Aliquots of 40 μl were withdrawn into a microcentrifuge tube containing 760 μl of 5% activated charcoal suspended in 50 mM NaH₂PO₄. After 15 min incubation on ice, tubes were centrifuged for 5 min at 14 000 rpm. The supernatants were collected and recentrifuged to remove charcoal particles. A 10 μl fraction of the supernatant was measured for inorganic ³²P in a scintillation counter. Control assays without kinesin and microtubules alone were performed to determine the background counts. The ATPase activity is expressed in nmol of inorganic phosphate released per min per mg protein.

Results

Isolation of a cDNA encoding a CaM-binding protein with homology to kinesin

A tobacco anther cDNA expression library was screened with ³⁵S-labeled calmodulin. After three rounds of screening, several positive clones were identified. Among these clones, one partial cDNA clone was sequenced (3510 bp, nt 692–4201,

Fig. 1. Nucleotide and deduced amino acid sequences of the *TCK1* gene. The nucleotides are numbered on the left, and the corresponding amino acids are numbered on the right. The predicted α -helical coiled-coil region are underlined with solid lines. Amino acids indicated by arrow heads show the leucine zipper motif. The ATP-binding consensus sequence is marked with dashed lines. The putative CaM-binding domain is boxed.

1 TCCAAAGCTCTTTTTCATCA
 24 TTTTACTTTGGTAACTGAGGAAGCTATTGAAGATTAAAGGTCAGCAAAATATTCCTTA
 84 ATTGAGAAGTTGGATGGCTCTTTCTAATAGTTGTATAGAGTTGAGACTATAATTTTGAAC
 144 ATGACTTCTGATATGCCACCAAGTTAGCATGAGGTCAAGCAGGCTCTCTTTGGCTCAAGT
 M T S D M P F V S M R S S R S S Y G S S 20
 204 AACGGATATGAAGACCTTCACACTATTCTTTTGAACCTCAAAATGGGGATGATTATGAT
 N G Y E R P S H Y S F A T S N G D D Y D 40
 264 AGTGATGGTTCCAAATTTTGGTCCACCCACCCCACTACTCTCTCATCAGTTCTGCTCACCC
 S D G S N F A P P T T T T L S V L S P 60
 324 GAACCTGGTGGCGCATACCATATATTGACAGATTCCAGGTTGAGGGTTTTCGAAGGCT
 E L A G A I P Y I D R F Q V K G F L K A 80
 384 ATGCAAAAGCAGCTTCAATCTGCTGGAAACCTGGGTTCTTTTAAAAAATCCGTTGGG
 M Q K Q L Q S A G K R G P F L K K S V G 100
 444 CCACAAGTTCGGGAAAAGTTCCACATTTGAGGATATGTTGTTTCCAAAGGGAACCCATT
 P Q V R E K F T F E D M L C F Q R E F I 120
 504 CCAACATCAATTTGAAATTAATGGCGATCTCGTTGGCAGGACAGTTAAGTTATTTCAG
 P T S I L K I N G I T T V K L F 140
 564 TCCATTCTGAAGTATATGGGTATTGATTCCTATGATAGACAGCTCCAACTCAGCTGGAT
 S I L K Y M G I D S Y D R A A P I S L D 160
 624 GAGCAATCGAGCTTGTGGCAGCTATTTAAGCAGGCGTTGAAGCGGTCTGAGCTTCGT
 E R I E L V E A N N Q V L Q K I Q E E L R 180
 684 GATGAATGTTTCCCAAAATTTCAAGCAAAACAGGAATAATCCGAGAGGCATTCTTTG
 D E H F A Q I S K Q T X N N F E R H S L 200
 744 ATTAAGCATGGAGCTTAATGTACTTGTGTCATCTTCATGCGCTCCGAGCAAGAAAT
 I K A W E L M Y L C A S C H P F S K E I 220
 804 GGTGGATCTTGTGAGATATATTCTACTGTGTCACATGGAATTAATCTGATCTGTAG
 G G Y L S E Y I H T V A H G I N T D S E 240
 864 GTTCAAGTTTATGCAATTAATCTCTTAATGCGTTGAACGTTCTTATTAAGGCTGAGCT
 V Q V Y A I N T L N A L K R S I K A G P 260
 924 AGGCACACGATACCTGGTCTGAGGAGATTGAAGCTCTCTTAATCTGTTAAAGCTTACT
 R H T I P G R E E I E A L L T G K K L T 280
 984 ACAATAGTGTTTTCTGGATGAACCTGCGAAGAAATACATATGACATGGCCACACG
 T I V F F L D E T F E E I T Y D M A T T 300
 1044 GTAGCTGATGCTATTGAGGAGTTGCGAGGATAATCAAAATGTCTGCTCATGCAAGCTTC
 V A D A I E V A G I I K L S A H A S P 320
 1104 AGTCTGTTGCGAGTCCCTAAGGTTGTTTACTGGGTCTTCAATCTCCAGATCTCGGAATGAG
 S L F E C R K V V T G S K S P D P G N E 340
 1164 GAGTACATTTGTTGGATGAATAAGTATGAGAGATCTGTTGGAGGACTTTAAGGCA
 E Y I C L D L E N K Y I G D L L E D F K A 360
 1224 CTAAAGACCGAAGTAAGGAGGAGATTTTGCATGTAACCTTAAGTTCAAAAGAGTTG
 L K D R S K G E I L H C K L S F K K K L 380
 1284 TTTGGGAGTCAGATGAAGCTGTTCAGAACCAATGTTCTGTCATTTGTCATGTTTCAA
 F R E S D E A V T E P M F L S Y V Q 400
 1344 TTACAACATGATTACATAATGGGCAATATCTCTGTTGGCAAGGATGATGCAGCAGATG
 L Q H D Y I M G N Y P V G K D D A A Q M 420
 1404 TCTGCTCTTCAGTACTGTTGATGATGATGTTGATGAGGCTGATCTTCTGCTGAC
 S A L Q I L V D I G Y V D G P E S C T D 440
 1464 TGGACATCACTGCTGGAGCGTTTCTACCCAGCAAAATTCGAATGACACGGGCAAGAGG
 W T S L L E R F L P R Q I A M T R A K R 460
 1524 GAATGGGAATTTGGATATACTTTCTGCTTCAAAATGTTGGAATACTGACAAAGATGAT
 E W E L D I L S R Y K L M E N L T K D D 480
 1584 GCCAAACAACATTTTGGGATTTCTGAGGACACTTCTTATGGAATTCAGTTTCTTT
 A K Q Q F L R I L R T L P Y G N S V F F 500
 1644 GCTGTTGGAAGATGATGATCTTCTGAGCTTTTCTGCTGGGAAATCATATTGGGCATT
 A V R K I D L D P I G L L P G K I I L G I 520
 1704 AATAAAGTGGGTTTCAATTTTCCGTCAGTTCCAAAGAGGATTTTGCACCTCAGCTGAG
 N K R G V H F F R P V P K E Y L H S A E 540
 1764 TTGAGGACATAATGCAATTTGGTAGCAGCAACCTGCTGTTCTTTAAGATGAGAGTT
 L R D I M Q F G S S N T A V F F K M R V 560
 1824 GCTGCTGCTTGCATATCTTTCAAGTTCAGAACAAACAGGAGAGGAAATTTGTTGCT
 A G V L H I F Q F R T K Q G E E I C V A 580
 1884 CTACAGACATATTAATGATGATGATTACGCCCTACTCAAAAGCCGCTTCTGACGCT
 L Q T H I N D V R L S K A R I S C A 600
 1944 AATGGTTGCTTAATGAGATGTTTCCAAATTAATCTCAAACTGCAAACTGACATTAAAT
 N G C V N A D V P N N L K T A N T D I N 620
 2004 GAAGAGCAGCTTCAAGATTTGCTGCGGCTTGAAGAAATCTCAGAAGAAAGTCAATGAT
 E R I Q D L S R A L E E S Q K K V N D 640
 2064 TTACTGGAAGATTATCATGAAGCAAGAGAAATTCGAAGATGCAAGAAAGATTGGAT
 L L E D L H E R Q R E E S K M Q E E L D 660
 2124 AGCTTAAAGATAACTTGAGATCAGAGAAAGCAAAATTTAGCAGCTGCTGTTATTGATGT
 S L K D N L R S E K Q N L A A A Y D C 680
 2184 GAAAAATTTAGATCTCTATGCAATGAAAAAGATGAGAGCTTCAGGCTGCATCAACGGAG
 E K F R S L C N E K D A E L T E 700
 2244 AAGCAGAACTTGGAAATGCGACTTTCAAAATTAAGTTCTAAAGGTTTGGAGAAAAATATT
 K Q N L E M R L S K L S S K G L E K N I 720
 2304 AGAAAGGAGTTGGTTGAAGCAATAACCAAGGCTTACAGAGATCCAGGAAGAGTTGAGA
 R K E L V E A N N Q V L Q K I Q E E L R 740
 2364 GCTCGTACTATGATGCTGCGCGCTGCAGAGAAACAAAGAGGAAGCTTTTGAAGTAAAGA
 A R T M D V R A A E E T K R K L L S E R 760
 2424 ACATCACTTGGAGAAAAATCATAGGCTAGAAAAAGAAATCAAGTGAAGTGAAGAAC
 T S L E E K I I G L E K K K S E M E N 780
 2484 CTTCAAGAGATTGTAAGAAAGATGCAAGCGCTGAGGCTCCAAGTCTCTGAATCTCA
 L Q K D F E K E C K A L R L Q V S E L Q 800
 2544 AGGAAGGAGGAGGAGGCAACATGATTGTTGTCGACGCTGAGGCTGGAAGCTAAA
 R K L E E A K H D L V V A R S G L E A 820
 2604 GACAGGGAATGAAATGCTACAGAAATTAATTAAGGAGCTCGAGGCTTAAGAGAAATG
 D R E L E M L Q N N L K E L E E L R E M 840
 2664 AAAGAGGAGCTTGAATGAAAGAAATGAACAACTGCCCACTTTGAAATGCAAGGAGGCT
 K E D I D R K N E Q T A T I L K M Q A 860
 2724 CAATTAGCTGGAATGGAAGCGCTTTACCGAGAGGAAACAAAGTTCTAAGGAAAAAGTCTTC
 Q L A G H E A L Y R E E Q V L R K K Y F 880
 2784 AACCAATGAGATATGAAAGGCAAGATCAGAGCTGAGCTGAGATTAAGACCTCTTTGT
 H T I E D M K G K I R V Y C R L R P L C 900
 2844 GAAAGGAAATATAGCGAAGGAAAGAAATGTAATGAGAGTGTGTGATGATTTACTATT
 E K E I I A K E R N V N R S V D E F T I 920
 2904 GAACATATATGGAAGATGATAAGCAAAACACACATGATGATGATGCTTTGACGGA
 E H I W K D D K A K Q H M Y D R V F D G 940
 2964 AATCCACTCAAGATGATGTTGTTGAAGACACTAAGTATTGGTGCAGTCAAGCTGCTGAT
 N S T Q D D V F E D T K Y L V Q S A A D 960
 3024 GGATATAATGTTGATATTGTCATATGCAACAACTGGATCTGGCAAGACATTCAACATC
 G Y N V C I F A Y G Q T G S G K T F T I 980
 3084 TATGGAGCGGATGATTAATCAGGAGCTGACCAAGAGCTATATCTGAATCTTTAGATT
 Y G A D S N P G L T P R I S E L I 1000
 3144 ATGAAGCGAGATAGTAATTAAGTTTCTTTCTCTTTAAAGGCAATCATGTTGAGATTGAT
 M K R D S N K F S F S L K A Y M V E L Y 1020
 3204 CAGGATACATTTGGTGGACCTCTTATTGCCAAAGAAATGCAAGCGCTTGAAGTTGATTA
 Q D T L V D L L L P K N A K R L D I 1040
 3264 AAAAAGATTCAAGAGGCTGTTTCTGTTGAAAAATGTCAGTGGTGTCTATTTCACAG
 K K D S K G M V S V E N V T V V S I S T 1060
 3324 YATGAGGAATTAAGCAATTAATCCAAAGAGGATCTGAACAACTCATACGATGGAACC
 T E E L K T I I Q R G S E Q R H T T G 1080
 3384 TTGATGAATGAGCAGAGTTCAAGATCTCATCTTATAGTTTCAAGTTATTGAGAGTACC
 L M N E Q S S R S H L I V S V I I E S T 1100
 3444 AATCTTCAACCGAGGCAATTCAGAGGGAAGCTAAGTTTGTGGATTTGCTGGCTCA
 N L Q T Q A I A R G K L S F V S G N Q H I 1120
 3504 GAAAGAGTTAAGAAATCTGGCTCAGCTGGCAATCAATTAAGAAAGCTCAAGCATTAA
 E R V K K S G S A G N Q L K E A Q S I N 1140
 3564 AAGTCACTGTCAGCACTTGGTGTGATGATGATGATGATGATGATGATGATGATGATGAT
 K S L S A L G D V I S A L S S G N Q H I 1160
 3624 CCTTATCGGAATCAAGCTAACCATGTTGATGAGGAGCTGTTAGTGGAAATGCTAAA
 P Y R N H K K L T M L M S D S L G G N A K 1180
 3684 ACTCTTATGTTTGAATCACTCTCCAGCAGAACTCAAACTTGGATGAGACTCAACATCTC
 T L M F V N I S P A E S N L T H N S 1200
 3744 TTGACGATGATCAAGAGTCCGTTCCATGTAATGATCCAGCAAAATGTTTCTATCT
 L T Y A S R V R S I V N D P S K N V S S 1220
 3804 AAGGAAGTCCGCTGCTTAAAGAGCTAGTGGGATTTGGAAGGAACAAGCTGGTAGAAA
 K E V A R L K K L V G Y W K E Q A G R K 1240
 3864 GGGGATGATGAGGATTTAGAGGAAATCCAAAGATGAACGCCCACTAAGAGAGAGATGAT
 G D D E D L E E I Q D E R P T K E K T D 1260
 3924 GGTGCTCATTCGATGTAACATCTAGAATTAATGCGTAGAACGAGTGAATCAAGTTGA
 G R H S M 1285
 3984 GCATCTTGTGTTAGTTGCTCCAAATATGAGGAGGAATGGTGAACATTGTAAGCTGCTGT
 4044 TCAGTGGTTTGTACTGCAAGTAAATGTTGAGATTTTCTTCCAGAAATTTTGTGTC
 4104 ATATAGGAGGCTTAAAGTATTAGCTTAGGAGCTCTAATCTCTCATGTTGTATAGAGCT
 4164 ATATGTTATATTGTAATCAATCAATCAATGTTA

Fig. 1) and the sequence comparison using the GenBank database revealed that it encodes a protein containing a stretch of amino acids at the carboxyl terminal end (amino acids 888–1265, Fig. 1) with high homology to the motor domains of kinesin heavy chains and kinesin-like proteins.

In order to clone the 5' region, the cDNA library was rescreened with ^{32}P -labeled random-primed DNA probes using an 843 bp *Hind*III/*Pst*I fragment (nt 1098–1940, Fig. 1) of the original clone as a template. After three rounds of plaque purification, one positive clone was obtained which contained the complete 5' region. The complete nucleotide sequence and the deduced amino acid sequence of *TCK1* are shown in Fig. 1. The translation initiation codon ATG at position 144 is in the 3795 bp long open reading frame. In this reading frame, there are two translation stop codons at positions 60 and 132 in the 5'-untranslated region, indicating that *TCK1* cDNA contains the complete coding region. The full-length *TCK1* codes for a 1265 amino acid long protein with a predicted molecular mass of 144 kDa.

The deduced TCK1 protein contains a motor domain of kinesin

Members of the kinesin superfamily have a distinct motor domain, a sequence of conserved 340 amino acids, which specifies force generation, and motility [10]. This motor domain can be located in the N-terminal [28, 31, 61], the C-terminal [24, 50] or the central region [29] of the molecule. A search of the GenBank protein sequence database revealed that the C-terminal region (aa 888–1265) of *TCK1* has extensive sequence homology with motor domains of kinesin heavy chains and kinesin-like proteins. The secondary structure prediction is consistent with this result (see below). The amino acid sequence was aligned with presumptive motor domains of a kinesin heavy chain and several kinesin-like proteins as shown in Fig. 2.

The predicted motor domain of *TCK1* shares highest sequence homology (78.3% identity) with

the corresponding region of *AKCBP*, a CaM-binding kinesin-like gene that was recently isolated from *Arabidopsis* [36, 37]. It also has appreciable sequence similarity to the motor domain of the kinesin-like protein (40.5% identity) and kinesin heavy chain (36.5% identity) of *Drosophila melanogaster*. Although *KatA* is another kinesin-like gene isolated from *Arabidopsis thaliana* [25], the motor domain of *TCK1* shows much lower sequence homology (39.8% identity) with that of *KATA* than *AKCBP*. This result indicates that *TCK1* and *AKCBP* belong to a new subfamily of kinesin-like proteins in eukaryotes.

Like other kinesin-like proteins, *TCK1* protein also contains an ATP-binding consensus sequence. The amino acid residues 964–984 have high similarity to the presumptive ATP-binding consensus sequences of kinesin heavy chain and kinesin-like proteins (Fig. 2). This stretch of the amino acid sequence has similarity to the ATP-binding consensus sequence previously proposed [53]. There is another region (amino acids 188–320, see Fig. 2) in the motor domain of *TCK1* which shows significantly higher homology with the corresponding region of other motor domains. This region has been implicated in microtubule-binding activity [26, 61].

Secondary structure prediction of TCK1 protein

In addition to the motor domain, most members of the kinesin superfamily share two other conserved structural features: a tail which forms a globular domain and a stalk-like region consisting of a heptad repeat sequence of amino acids capable of forming α -helical coiled-coil structures [50, 61]. However, homology of the amino acid sequences is very poor outside the motor domain among different kinesin-like proteins [10]. When we first obtained the sequence of a segment (amino acids 751–833, Fig. 1) in the stalk region of *TCK1*, a sequence search for homologues in the GenBank database was performed by using TFASTA program in GCG. It was found to share homology (around 30% identity) with a number

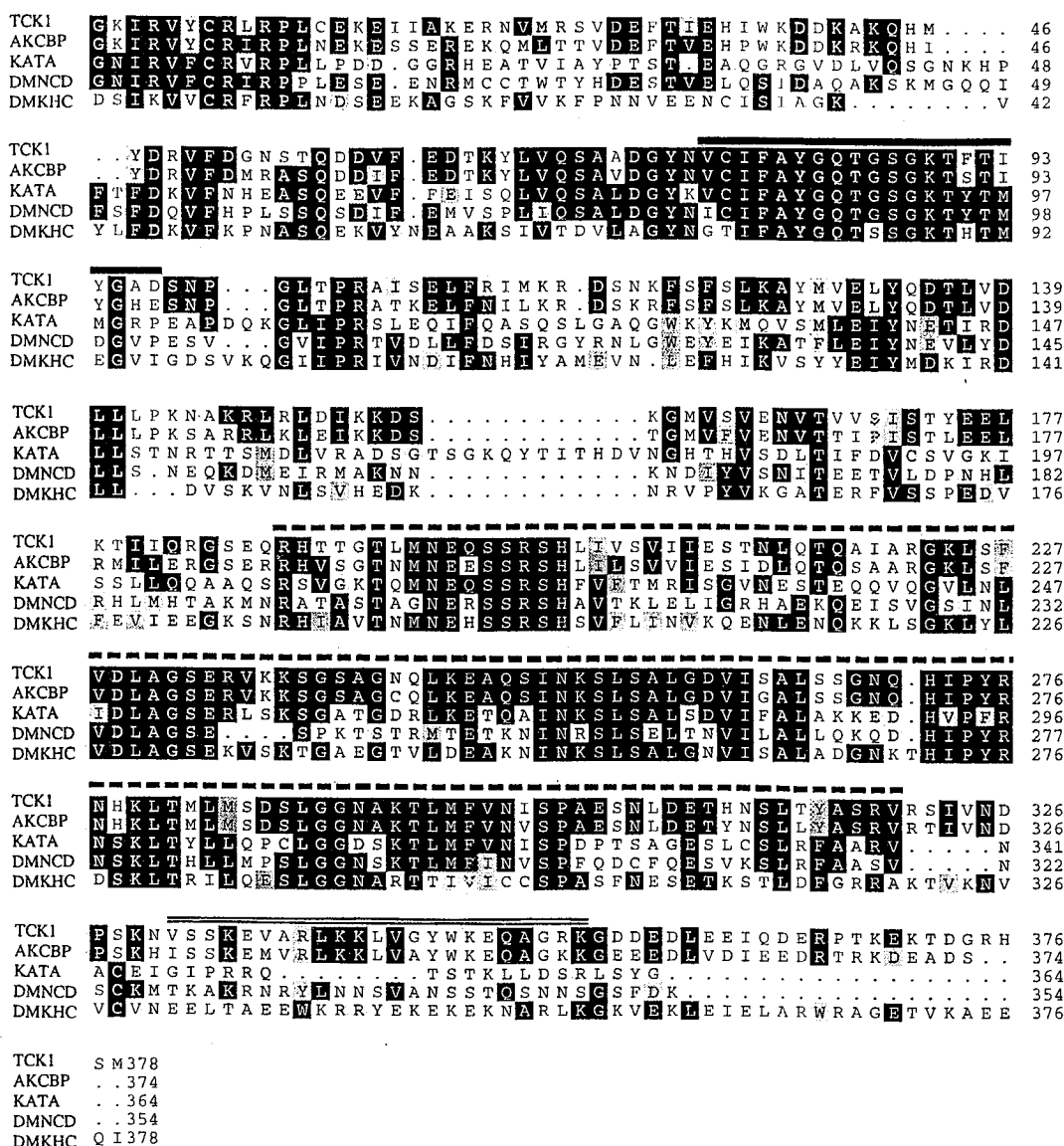


Fig. 2. Comparison of the predicted motor domain of *TCK1* to those of the kinesin heavy chain and kinesin-like proteins. The ATP-binding consensus sequences are indicated by a solid line. The putative tubulin-binding regions are marked by a dashed line. The predicted CaM-binding domain is indicated by a double line. AKCBP, an *Arabidopsis* kinesin-like protein [37]; KATA, an *Arabidopsis* kinesin-like protein [35]; DMNCD, a *Drosophila* kinesin-like protein [24]; DMKHC, a *Drosophila* kinesin heavy chain [61].

of myosin heavy chains [54]. The secondary structure of TCK1 was predicted with the PEP-COIL program in GCG [22]. Two stretches of amino acid residues 614–715 and 729–887 have a high tendency to form α -helical coiled-coil structures (Fig. 3). Between these two regions,

there is a disruption of a short segment (amino acids 716–728) which is unable to adopt an α -helical coiled-coil conformation based on the prediction. The disruption (bend) also exists in the presumptive stalk regions of kinesin heavy chains and other kinesin-like proteins [24, 62]. A

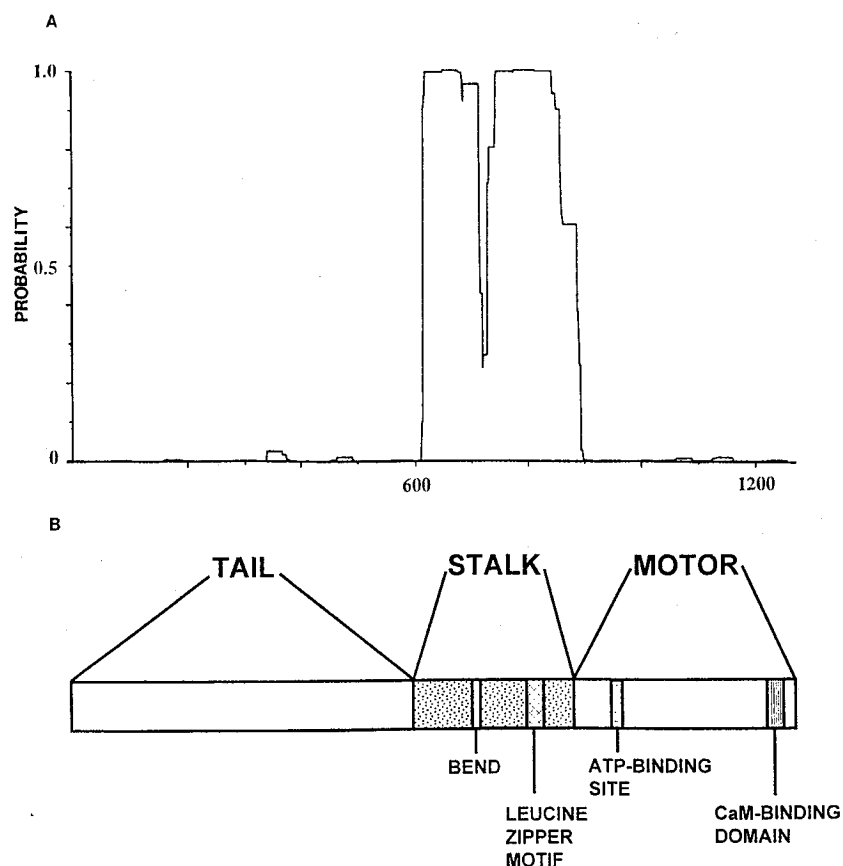


Fig. 3. Structural features of TCK1. A. α -helical coiled-coil structure was predicted using PEPKOIL in GCG for a window of 28 amino acid residues [22]. The numbers on the Y-axis indicate the probability of forming coiled-coil structure. B. A schematic representation of the structure of the *TCK1* protein.

schematic diagram illustrating the structural features of TCK1 is shown in Fig. 3B. As shown in the figure, TCK1 has three distinct domains: a tail, stalk, and a motor domain.

Examination of the TCK1 sequence revealed the presence of a leucine zipper motif in the stalk region (Fig. 1). Five leucine residues occur in the heptad repeat from the amino acid residue 803 to 837. One of the known functions of the leucine zipper motif is to participate in the formation of dimers in some proteins [52]. The presence of a leucine zipper motif strongly indicates that the stalk region may be involved in dimerization.

The deduced TCK1 protein contains a calmodulin-binding domain

The fact that *TCK1* gene was isolated by screening a cDNA expression library using ^{35}S -labeled calmodulin as a probe strongly suggests that *TCK1* encodes a calmodulin-binding protein. In a recently characterized kinesin-like protein, a calmodulin-binding domain was mapped to a stretch of 52 amino acids in the C-terminal region [36, 37]. To determine if the calmodulin-binding domain is located in the C-terminal domain of the *TCK1* gene product, a fusion protein of glutathione-S-transferase and amino acids 1214 to 1265 was prepared using a pGEX-3X fusion protein expression vector. The *E. coli* expressed fu-

sion protein was affinity-purified using a glutathione-Sepharose column. SDS-PAGE revealed that the molecular weight of purified protein GST(1214–1265) was 33 kDa (Fig. 4A). The GST alone was expressed, purified, and used as a negative control in our binding experiments. These proteins were electrophoretically transferred from the polyacrylamide gel onto a nitrocellulose filter, and used for a biotinylated calmodulin overlay assay. The purified GST(1214–1265) bound to Ca^{2+} /calmodulin while GST

alone did not show calmodulin binding (Fig. 4B). The GST(1214–1265) also bound to calmodulin-Sepharose column in the presence of Ca^{2+} . After washing the column with a buffer containing 0.5 M NaCl, the protein was still bound to the column. The fusion protein was eluted from the column by adding 2 mM EGTA, indicating that the fusion protein binds to calmodulin in a Ca^{2+} -dependent manner (Fig. 4C). These results further confirm that the C-terminal region of the *TCK1* gene product contains a calmodulin-

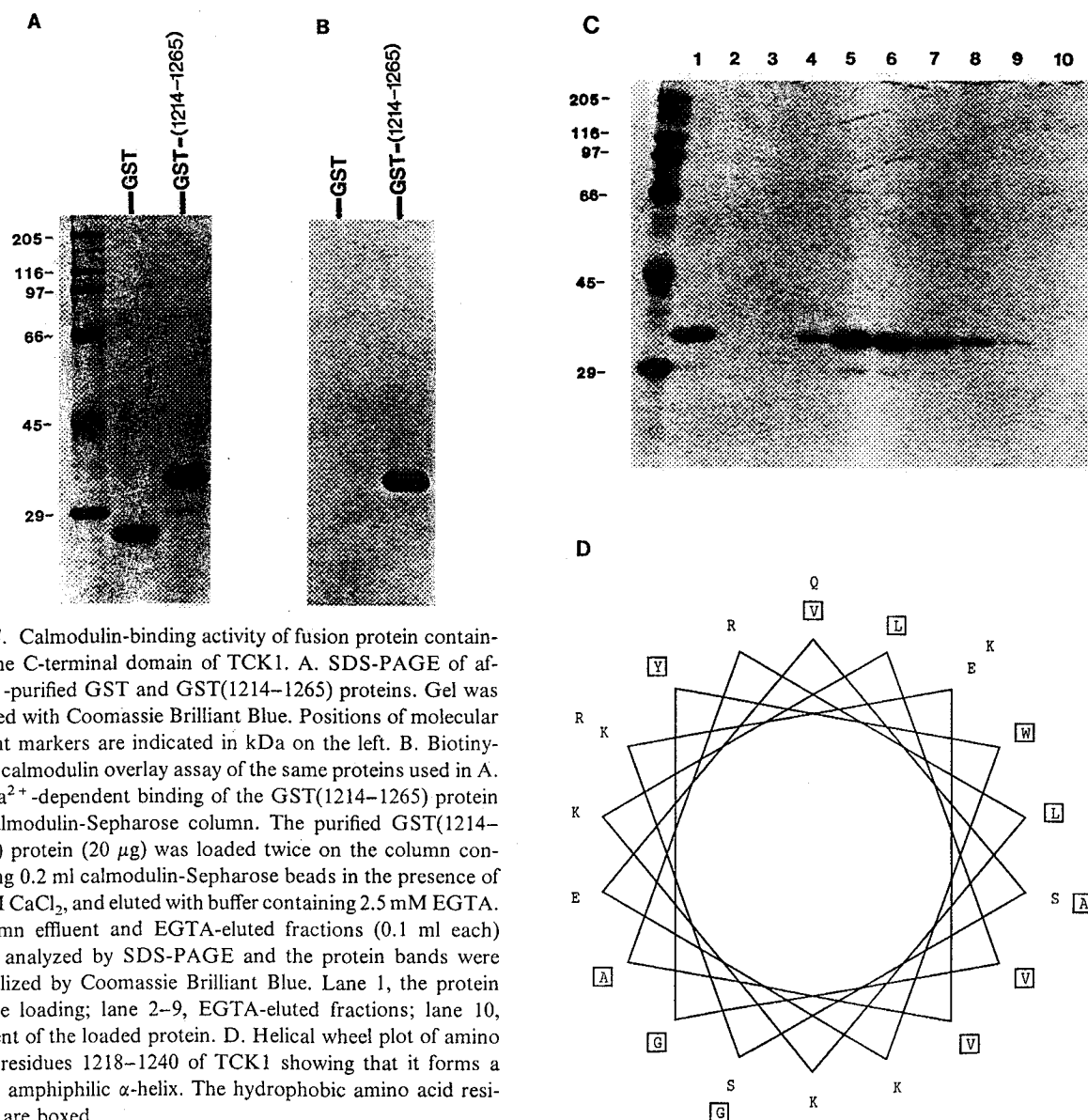


Fig. 4. Calmodulin-binding activity of fusion protein containing the C-terminal domain of TCK1. A. SDS-PAGE of affinity-purified GST and GST(1214–1265) proteins. Gel was stained with Coomassie Brilliant Blue. Positions of molecular weight markers are indicated in kDa on the left. B. Biotinylated calmodulin overlay assay of the same proteins used in A. C. Ca^{2+} -dependent binding of the GST(1214–1265) protein to calmodulin-Sepharose column. The purified GST(1214–1265) protein (20 μg) was loaded twice on the column containing 0.2 ml calmodulin-Sepharose beads in the presence of 1 mM CaCl_2 , and eluted with buffer containing 2.5 mM EGTA. Column effluent and EGTA-eluted fractions (0.1 ml each) were analyzed by SDS-PAGE and the protein bands were visualized by Coomassie Brilliant Blue. Lane 1, the protein before loading; lane 2–9, EGTA-eluted fractions; lane 10, effluent of the loaded protein. D. Helical wheel plot of amino acid residues 1218–1240 of TCK1 showing that it forms a basic amphiphilic α -helix. The hydrophobic amino acid residues are boxed.

binding domain. Furthermore, the helical-wheel plot of the amino acids 1218–1240 of TCK1 forms a basic amphiphilic α -helix (Fig. 4D), commonly found in the calmodulin-binding sites of many proteins [32].

ATPase activity is associated with the motor domain

It has been shown that the motor domain of kinesin heavy chain contains ATPase activity that is stimulated by microtubules [3]. To test if the motor domain of TCK1 has microtubule-stimulated ATPase activity, the *E. coli* expressed motor domain (824–1265) was purified (Fig. 5A) assayed for ATPase activity in the presence and absence of microtubules. As shown in Fig. 5B, the motor domain has basal ATPase activity which was stimulated by about three-fold in the

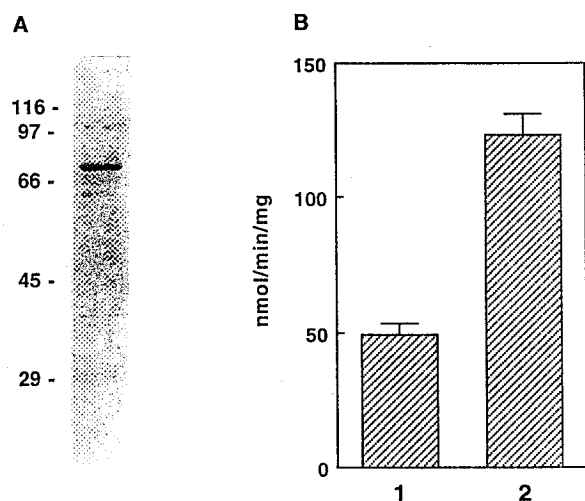


Fig. 5. ATPase activity of the motor domain of TCK1. A. SDS-PAGE of the affinity-purified GST(824–1265) protein which was used for ATPase assays. The gel was stained with Coomassie Brilliant Blue. B. ATPase activity of motor domain in the presence and absence of microtubules. Assays were performed in triplicate. Control assays without motor domain and microtubules alone were performed to determine background counts and were subtracted prior to calculating the ATPase activity of motor domain alone (1) and motor domain plus microtubules (2). The background counts for without motor domain and microtubules alone were 514 ± 10 and 918 ± 28 cpm, respectively. SD of triplicate assays are presented.

presence of microtubules, indicating that like other kinesin heavy chains, TCK1 has microtubule-stimulated ATPase activity.

TCK1 is a single-copy gene and expressed in all organs

To determine the approximate copy number of *TCK1*, Southern blot analysis of tobacco genomic DNA was carried out (Fig. 6). Both *EcoRI*- and *HindIII*-digested DNAs have one hybridizing band, indicating that *TCK1* was coded by a single-copy gene. However, *EcoRV*-digested DNA showed two hybridizing bands. This is most likely due to the presence of an intron containing *EcoRV* sites in the genomic DNA corresponding to the cDNA probe or a minor polymorphism in two sets of tobacco genomes.

To study the expression of *TCK1*, northern blot analysis was carried out using poly(A)⁺ RNA. The length of the *TCK1* transcript is ca. 4.5 kb and matches the length of the cDNA. The tran-

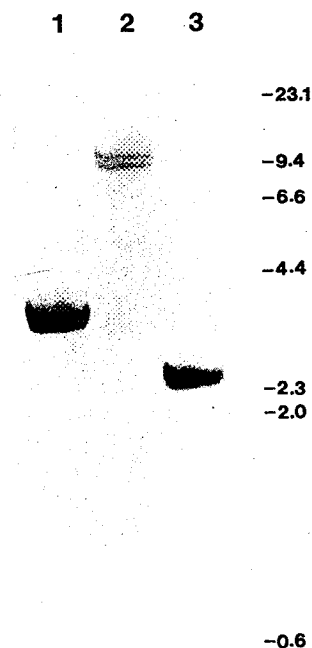


Fig. 6. Genomic Southern blot analysis of *TCK1*. The size of standard markers is shown in kb on the right. Lane 1, *EcoRI*; lane 2, *EcoRV*; lane 3, *HindIII*.

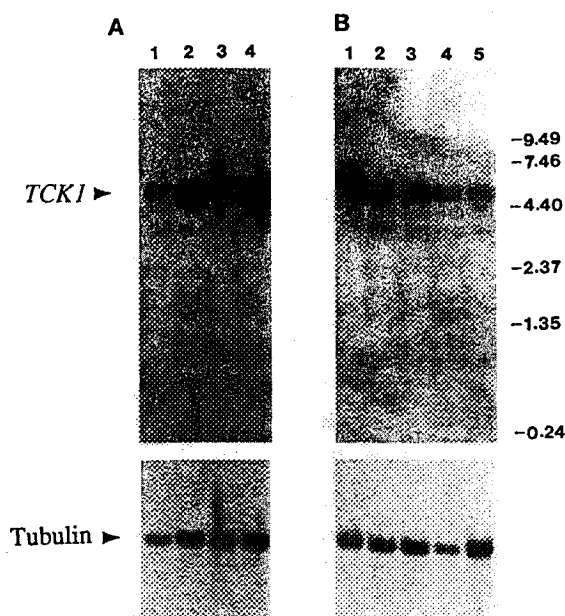


Fig. 7. Expression of *TCK1* mRNA. A. Expression of *TCK1* in different organs. Northern blot analysis of poly(A)⁺ RNA (5 μ g) from leaf (lane 1), stem (lane 2), stem apex (lane 3), and inflorescence (lane 4). B. Expression of *TCK1* in stigma and developing anthers. Northern blot analysis of poly(A)⁺ RNA (5 μ g) from stigma (lane 1), 0.5–1.0 cm anther (lane 2), 1.1–2.0 cm anther (lane 3), 2.1–3.0 cm anther (lane 4), and \geq 3.1 cm mature anther (lane 5). The size of standard markers is shown in kb on the right. The expression of tubulin was used for comparison.

script of *TCK1* is expressed in all tobacco organs, but the mRNA levels varied with the highest expression in the inflorescence and stem apex (Fig. 7A). *TCK1* is highly expressed in the stigma and during the early stages of anther development (Fig. 7B).

Discussion

TCK1 belongs to a new subfamily of kinesin-like genes

More than 30 kinesin-like genes have been isolated and characterized from various eukaryotic organisms [10]. Multiple members of kinesin-like and conventional kinesin genes have also been found in one organism [28, 49]. Most kinesin-like

proteins have three structurally distinct domains [24]. However, the sequences within non-motor regions of kinesin-like proteins show very poor homology. A variety of tail domains may impart different functional specificities to the different motor proteins. The extreme diversity among the kinesin-like proteins suggests that a large number of superfamily members remain to be discovered [10].

We isolated a kinesin-like gene, *TCK1*, by screening a tobacco anther cDNA expression library using ³⁵S-labeled calmodulin [54]. Reddy *et al.* [36, 37] have also isolated a kinesin-like gene (*AKCBP*) from *Arabidopsis* by screening a cDNA expression library using biotinylated calmodulin. Sequence comparisons revealed that *TCK1* and *AKCBP* share extensive homology (71.7% identity) throughout the sequence and they both contain a CaM-binding domain near the carboxyl terminus (Figs. 1 and 2) which binds calmodulin in the presence of Ca²⁺. None of the other kinesin heavy chains or kinesin-like proteins characterized so far are known to bind calmodulin. The sequence comparison with the motor domains of a kinesin heavy chain and other kinesin-like proteins revealed that the calmodulin-binding region found in *TCK1* and *AKCBP* is not found in other kinesins (Fig. 2). This indicated that *TCK1* and *AKCBP* belong to a new subfamily of kinesin-like proteins that interact with calmodulin. The presence of a CaM-binding domain in the *TCK1* suggests that the mechanism of its motility regulation may differ from those of other kinesin-like proteins.

Ca²⁺/calmodulin, microtubule, and kinesin

Calmodulin is a ubiquitous protein in the eukaryotic cells known to have broad interactions with cytoskeleton [8, 13]. A number of cytoskeleton-associated proteins have been found to be calmodulin-binding proteins and modulated by Ca²⁺/calmodulin. Some microfilament-associated proteins were identified as calmodulin-binding, including caldesmon [60], spectrin and spectrin-like proteins [56], and the microfilament-

based motor protein, myosin [45]. Several microtubule-associated proteins also have been found to be calmodulin-binding, including MAP2, tau [18], STOP [12] and other proteins [2, 5, 42]. In addition to direct interactions with these cytoskeleton-associated proteins, calmodulin can modulate the activities of the cytoskeleton through other regulatory proteins such as myosin light-chain kinase [27]. The calcium- and calmodulin-dependent phosphorylation of some microtubule-associated proteins influences microtubule assembly and disassembly [5, 59]. These investigations suggested that calcium and calmodulin are involved in the regulation of cytoskeleton dynamics.

In plants, knowledge about the interaction between calmodulin and the cytoskeleton is very limited. Several myosin genes have been isolated from *Arabidopsis* and their deduced amino acid sequences contain CaM-binding IQ motifs, indicating that they may bind calmodulin [14, 15]. Calmodulin has been localized on the mitotic apparatus during plant cell division [55]. Ca^{2+} and calmodulin have also been found to affect microtubule stability in lysed plant protoplasts. It is proposed that calmodulin influences microtubule assembly through microtubule-associated and CaM-binding proteins [4]. However, the nature of the interaction of calmodulin with microtubules is largely unknown. Few microtubule-associated proteins are identified to be CaM-binding proteins [6].

Kinesin is believed to be involved in the organelle motility and microtubule organization in mammalian cells [10]. Evidence has also been accumulated to show Ca^{2+} and calmodulin involvement in these cellular processes in plants [4, 17, 41, 58, 63]. Isolation of the kinesin-like genes encoding Ca^{2+} /calmodulin-binding proteins implies that Ca^{2+} /calmodulin may be involved in the regulation of microtubule-based movement. Further characterization of this new member of kinesin-like proteins should reveal the mechanism of Ca^{2+} /calmodulin influencing subcellular movement, especially organelle transport or microtubule function.

Acknowledgement

The support of the National Science Foundation (Grant DCB 91-4586) and National Aeronautics and Space Administration (Grant NAG-10-0061) to B.W.P. is gratefully acknowledged.

References

1. Baum G, Chen Y, Arazi T, Takatsuji H, Fromm H: A plant glutamate decarboxylase containing a calmodulin binding domain, cloning, sequence and functional analysis. *J Biol Chem* 268: 19610–19617 (1993).
2. Brady RC, Cabral F, Dedman JR: Identification of a 52-kD calmodulin-binding protein associated with the mitotic spindle apparatus in mammalian cells. *J Cell Biol* 103: 1855–1861 (1986).
3. Chandra R, Endow SA: Expression of microtubule proteins in bacteria for characterization in *in vitro* motility assays. In: Wilson L, Matsudaira P (eds) *Methods in Cell Biology*, pp. 115–128. Academic Press, New York (1993).
4. Cyr RJ: Ca^{2+} /calmodulin affects microtubule stability in lysed protoplasts. *J Cell Sci* 100: 311–317 (1991).
5. Dinsmore JH, Sloboda RD: Calcium and calmodulin-dependent phosphorylation of a 62 kD protein induces microtubule depolymerization in sea urchin mitotic apparatuses. *Cell* 53: 769–780 (1988).
6. Durso NA, Cyr RJ: A calmodulin-sensitive interaction between microtubules and a higher plant homolog of elongation factor-1 α . *Plant Cell* 6: 893–905 (1994).
7. Fromm H, Chua NH: Cloning of plant cDNAs encoding calmodulin-binding proteins using ^{35}S -labeled recombinant as a probe. *Plant Mol Biol Rep* 10: 199–206 (1992).
8. Gratzer WB, Baines AJ: Calmodulin and the cytoskeleton. In: Cohen P, Klee CB (eds) *Molecular Aspects of Cellular Regulation*, vol 5, Calmodulin, pp. 329–340. Elsevier Biochemical Press, Amsterdam (1988).
9. Hagan Z, Yanagida M: Kinesin-like *cut 7* protein associates with mitotic and meiotic spindles in fission yeast. *Nature* 356: 74–76 (1992).
10. Hoyt MA: Cellular roles of kinesin and related proteins. *Curr Opin Cell Biol* 6: 63–68 (1994).
11. James P, Vorherr T, Carafoli E: Calmodulin-binding domains: just two-faced or multifaceted? *Trends Biochem Sci* 20: 38–42 (1995).
12. Job D, Rausch CT, Fisher EH, Margolis RL: Recycling of cold-stable microtubules: Evidence that cold-stability is due to substoichiometric polymer blocks. *Biochemistry* 21: 509–515 (1982).
13. Kakiuchi S, Sobue K: Control of the cytoskeleton by calmodulin and calmodulin-binding proteins. *Trends Biochem Sci* 8: 59–62 (1983).
14. Kinkema M, Schiefelbein J: A myosin for a higher plant

- has structural similarities to class V myosins. *J Mol Biol* 239: 591–597 (1994).
15. Knight AE, Kendrick-Jones J: A myosin-like protein from a higher plant. *J Mol Biol* 231: 148–154 (1993).
 16. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685 (1970).
 17. Lambert AM, Vantard M: Calcium and calmodulin as regulators of chromosome movement during mitosis in higher plants. In: Trewavas AJ (ed) *Molecular and Cellular Aspects of Calcium in Plant Development*, pp. 175–183. Plenum, New York (1986).
 18. Lee YC, Wolff J: Calmodulin binds to both microtubule-associated protein 2 and tau proteins. *J Biol Chem* 259: 1226–1230 (1984).
 19. Ling V, Assman SM: Cellular distribution of calmodulin and calmodulin-binding proteins in *Vicia faba* L. *Plant Physiol* 100: 970–978 (1992).
 20. Ling V, Snedden WA, Shelp BJ, Assman SM: Analysis of a soluble calmodulin binding protein from fava bean roots: identification of glutamate decarboxylase as a calmodulin-activated enzyme. *Plant Cell* 6: 1135–1142 (1994).
 21. Lu Y, Harrington HM: Isolation of tobacco cDNA clones encoding calmodulin-binding proteins and characterization of a known calmodulin-binding domain. *Plant Physiol Biochem* 32: 413–422 (1994).
 22. Lupas A, Dyke MV, Stock J: Predicting coiled from protein sequences. *Science* 252: 1162–1164 (1991).
 23. Matthies HJG, Miller RJ, Palfrey HC: Calmodulin binding to and cAMP-dependent phosphorylation of kinesin light chains modulate kinesin ATPase activity. *J Biol Chem* 268: 11176–11187 (1993).
 24. McDonald HB, Goldstein LSB: Identification and characterization of a gene encoding a kinesin-like protein in *Drosophila*. *Cell* 61: 991–1000 (1990).
 25. Mitsui H, Yamaguchi-Shinozaki K, Shinozaki K, Takahashi H: Identification of a gene family (*kat*) encoding kinesin-like proteins in *Arabidopsis thaliana* and the characterization of secondary structure of KatA. *Mol Genet* 238: 326–368 (1993).
 26. Mitsui H, Nakatani K, Yamaguchi-Shinozaki K, Nishikawa K, Takahashi H: Sequencing and characterization of the kinesin-like gene *KatB* and *KatC* of *Arabidopsis thaliana*. *Plant Mol Biol* 25: 865–876 (1994).
 27. Nairn AC, Picciotto MR: Calcium/calmodulin-dependent protein kinases. *Sem Cancer Biol* 5: 295–303 (1994).
 28. Niclas J, Navone F, Hom-Booher N, Vale RD: Cloning and localization of a conventional kinesin motor expressed exclusively in neurons. *Neuron* 12: 1059–1072 (1994).
 29. Noda Y, Sato-Yoshitake R, Kondo S, Nangaku M, Hirokawa N: KIF2 is a new microtubule-based anterograde motor that transports membranous organelles distinct from those carried by kinesin heavy chain or KIF 3A/B. *J Cell Biol* 129: 157–167 (1995).
 30. Oh SH, Steiner HY, Dougall DK, Roberts DM: Modulation of calmodulin levels, calmodulin methylation, and calmodulin binding proteins during carrot cell growth and embryogenesis. *Arch Biochem Biophys* 297: 28–34 (1992).
 31. Okada Y, Yamazaki H, Sekine-Aizawa Y, Hirokawa N: The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell* 81: 769–780 (1995).
 32. O'Neil KT, DeGrado WF: How calmodulin binds its targets: sequence independent recognition of amphiphilic α -helicals. *Trends Biochem Sci* 15: 59–64 (1990).
 33. Patil S, Takezawa D, Poovaiah BW: Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visinin-like calcium-binding domain. *Proc Natl Acad Sci USA* 92: 4897–4901 (1995).
 34. Pereira A, Goldstein LSB: The kinesin superfamily in microtubules. In: Hyams JS, Lloyd CW (eds) *Microtubules*, pp. 269–284. Wiley-Liss, New York (1994).
 35. Poovaiah BW, Reddy ASN: Calcium and signal transduction in plants. *CRC Crit Rev Plant Sci* 12: 185–211 (1993).
 36. Reddy ASN, Safadi F, Narasimhulu SB, Golovkin M, Hu X: Isolation of a novel calmodulin-binding protein from *Arabidopsis thaliana*. *Plant Physiol* 108 (Suppl): 41 (1995).
 37. Reddy ASN, Safadi F, Narasimhulu SB, Golovkin M, Hu X: A novel calmodulin-binding protein with kinesin heavy-chain motor domain. *J Biol Chem* 271: 7052–7060 (1996).
 38. Reddy ASN, Takezawa D, Fromm H, Poovaiah BW: Isolation and characterization of two cDNAs that encode for calmodulin-binding proteins from corn root tips. *Plant Sci* 94: 107–117 (1993).
 39. Roberts DM, Harmon AC: Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. *Annu Rev Plant Mol Biol* 43: 375–414 (1992).
 40. Sambrook J, Fritsch EF and Maniatis T: *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
 41. Schleicher M, Iverson DB, Van Eldik LJ, Watterson DM: Calmodulin. In: Lloyd CW (ed) *The Cytoskeleton in Plant Growth and Development*, pp. 85–106. Academic Press, London (1982).
 42. Stirling DA, Welsh KA, Stark MJR: Interaction with calmodulin is required for the function of spc 110p, an essential component of the yeast spindle pole body. *EMBO J* 13: 4329–4342 (1994).
 43. Takezawa D, Liu ZH, An G, Poovaiah BW: Calmodulin gene family in potato: developmental and touch-induced expression of the mRNA encoding a novel isoform. *Plant Mol Biol* 27: 693–703 (1995).
 44. Tiezzi A, Moscatelli A, Cai G, Bartalesi A, Cresti M: An immunoreactive homolog of mammalian kinesin in *Nic-*

- otiana tabacum* pollen tubes. *Cell Motil Cytoskel* 21: 132–137 (1992).
45. Titus MA: Myosin. *Curr Opin Biol* 5: 77–81 (1993).
 46. Towbin H, Staehelin T, Gordon T: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 79: 4350–4354 (1979).
 47. Trewavas AJ, Gilroy S: Signal transduction in plant cells. *Trends Genet* 7: 356–361 (1991).
 48. Vale RD, Reese TS, Sheetz MP: Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* 42: 39–50 (1985).
 49. Vernos I, Heasman J, Wylie C: Multiple kinesin-like transcripts in *Xenopus* oocytes. *Devel Biol* 157: 232–239 (1993).
 50. Vernos I, Raats J, Hirano T, Heasman J, Karsenti E, Wylie C: Xklp1, a chromosomal *xenopus* kinesin-like protein essential for spindle organization and chromosome positioning. *Cell* 81: 117–127 (1995).
 51. Verwoerd TC, Dekker BMM, Hoekema A: A small-scale procedure for the rapid isolation of plant RNAs. *Nucl Acids Res* 17: 2362 (1989).
 52. Vinson CR, Hai TW, Boyd S: Dimerization specificity of the leucine zipper containing bZIP motif on DNA binding: prediction and rational design. *Genes Devel* 7: 1047–1058 (1993).
 53. Walker JE, Saraste M, Runswick MJ, Gay NJ: Distantly related sequences in the α - and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1: 945–951 (1982).
 54. Wang W, Takezawa D, Poovaiah BW: Isolation and characterization of cDNAs that encode calmodulin-binding proteins. *Plant Physiol* 108 (Suppl): 103 (1995).
 55. Wick SM: Immunolocalization of tubulin and calmodulin in meristematic plant cells. In: Thompson MP (ed) *Calcium Binding Proteins, Biological Functions*, vol 2, pp. 21–45. CRC Press, Boca Raton (1988).
 56. Widada JS, Asselin J, Colote S, Marti J, Ferraz C, Trave G, Haiech J, Liautard JP: Cloning and deletion mutagenesis using direct protein-protein interaction on an expression vector, identification of the calmodulin binding domain of α -fodrin. *J Mol Biol* 205: 455–458 (1989).
 57. Williams R, Lee JC: Preparation of tubulin from brain. *Meth Enzymol* 85: 376–385 (1982).
 58. Williamson RE: Calcium and the plant cytoskeleton. *Plant Cell Environ* 7: 431–440 (1984).
 59. Yamamoto H, Fukunaga K, Tanaka E, Miyamoto E: Ca^{2+} - and calmodulin-dependent phosphorylation of microtubule-associated protein 2 and tau factor, and inhibition of microtubule assembly. *J Neurochem* 41: 1119–1125 (1983).
 60. Yamashiro S, Matsumura F: Caldesmon. *Curr Opin Cell Biol* 5: 70–76 (1993).
 61. Yang JT, Laymon RA, Goldstein LSB: A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analysis. *Cell* 56: 879–889 (1989).
 62. Yang JT, Saxton WM, Stewart RJ, Raff EC, Goldstein LSB: Evidence that the head of kinesin is sufficient for force generation and motility *in vitro*. *Science* 249: 42–47 (1990).
 63. Zhang DH, Callaham DA, Hepler PK: Regulation of anaphase chromosome motion in *Tradescantia* stamen hair cells by calcium and related signaling agents. *J Cell Biol* 111: 171–182 (1990).