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A Calmodulin-Sensitive Interaction between Microtubules and a Higher Plant Homolog of Elongation Factor-1α

Neil A. Durso [1] and Richard J. Cyr

Department of Biology, 208 Mueller Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

The microtubules (MTs) of higher plant cells are organized into arrays with essential functions in plant cell growth and differentiation; however, molecular mechanisms underlying the organization and regulation of these arrays remain largely unknown. We have approached this problem using tubulin affinity chromatography to isolate carrot proteins that interact with MTs. From these proteins, a 50-kD polypeptide was selectively purified by exploiting its Ca^{2+}-dependent binding to calmodulin (CaM). This polypeptide was identified as a homolog of elongation factor-1α (EF-1α)—a highly conserved and ubiquitous protein translation factor. The carrot EF-1α homolog bundles MTs in vitro, and moreover, this bundling is modulated by the addition of Ca^{2+} and CaM together (Ca^{2+}/CaM). A direct binding between the EF-1α homolog and MTs was demonstrated, providing novel evidence for such an interaction. Based on these findings, and others discussed herein, we propose that an EF-1α homolog mediates the lateral association of MTs in plant cells by a Ca^{2+}/CaM-sensitive mechanism.

INTRODUCTION

Microtubules (MTs) participate in a number of essential processes in eukaryotes (MacRae, 1992b). In cells of higher plants, MTs are generally arranged into four structurally and functionally distinct, cell cycle–dependent MT arrays (Goddard et al., 1994; Lambert and Lloyd, 1994). During interphase, the MT array in a cell's cortex is involved in the extracellular deposition of cellulose microfibrils (Giddings and Staehelin, 1991). During G_2 of the cell cycle, this array is succeeded by a narrower preprophase band involved in establishing the site of the incipient division plane (Palevitz, 1991; Wick, 1991). The preprophase band resolves into the mitotic spindle apparatus as M phase progresses (Lambert et al., 1991; Palevitz, 1993). The fourth MT array, the phragmoplast, appears as cytokinesis begins and is involved in depositing the new cell plate at the position previously marked by the preprophase band (Lloyd, 1991).

The bases by which plant MTs are organized into higher order arrays and by which those arrays are regulated remain largely unknown (Lambert, 1993). MTs are hollow cylinders with 25-nm diameters, and tubulin is the principal protein subunit of MTs. MTs assembled from pure tubulin have a limited range of behavior—they assemble or disassemble. This limited range of behavior can only partly account for the diverse structures and functions of MT arrays.

Nearly all species have multiple tubulin genes encoding various tubulin isotypes (for plant genes, see Goddard et al., 1994), and it has been proposed that the isotypes may provide a means for serving different MT functions (Fulton and Simpson, 1976). However, nearly all existing evidence indicates that tubulin isotypes are functionally interchangeable (Ludueña, 1993). In general, tubulins are conserved even between kingdoms (Fosket and Morejohn, 1992; Burns and Surridge, 1994). In fact, animal brain tubulin that is microinjected into living plant cells incorporates into the plant MT arrays (Zhang et al., 1990, 1993; Cleary et al., 1992; Hepler et al., 1993). In addition to isotypic variation, many post-translationally modified isoforms of tubulin have been described (Greer and Rosenbaum, 1989; also see MacRae, 1992b; Ludueña, 1993). Although specific isotypes or isoforms may be required for particular MT functions, it remains unclear how this variation is causally sufficient to produce the diverse structures or functions of MT arrays.

A third means by which MT arrays may achieve their structural and functional diversity is by interactions with non-tubulin proteins—loosely termed microtubule-associated proteins (MAPs). MAPs are generally divided into two classes. Structural MAPs affect the assembly properties of MTs (Chapin and Bulinski, 1992) and the structural arrangements of MT arrays (Lee, 1993). "Motor" MAPs are mechanochemical enzymes that transduce the energy of nucleotide hydrolysis into MT-directed motility of subcellular structures (Vale, 1992; Skoufias and Scholey, 1993). Although MAPs from animal cells have been well characterized biochemically and genetically (Wiche et al., 1991; Lee, 1993), knowledge of MAPs from plants is limited (Cyr, 1991b; Schellenbaum et al., 1992; Goddard et al., 1994).

The handful of studies in which plant MAPs are reported have employed as an affinity substrate MTs obtained by two general methods: (1) stabilized MTs from tubulin-rich, animal

[1] To whom correspondence should be addressed.

sources and (2) MTs assembled from endogenous plant tubulin by extraordinary experimental conditions. The first method was used to isolate carrot MT binding proteins that bind MTs at periodic intervals along an MT, promote tubulin assembly, and bundle MTs (Cyr and Palevitz, 1989). Furthermore, a major polypeptide in these proteins immunocytochemically colocalizes with MTs of the cortical array. The latter method was utilized to isolate maize MAPs that bind and copurify with MTs, promote tubulin assembly, and bundle MTs (Vantard et al., 1991; Schellenbaum et al., 1993). In addition, a polypeptide in these proteins is antigenically related to a neuronal, structural MAP. The second method was also used to isolate tobacco MAPs that bind, copurify with, and bundle MTs (Chang-Jie and Sonobe, 1993). Antibodies raised against a polypeptide present in these proteins and implicated in MT bundling immunocytochemically colocalized with the four plant MT arrays.

We have employed an alternative method—tubulin affinity chromatography—for isolating putative MAPs from plant cells. During the course of this study, Jablonsky et al. (1993) reported the use of tubulin affinity chromatography to purify a 34-kD mung bean MAP that appears to bind a tubulin domain involved in MT assembly. Here, we report on the use of tubulin affinity chromatography to isolate from crude soluble extracts of carrot cells proteins that also bind MTs. From these, a 50-kD polypeptide (pp50) was highly purified and found to be similar to elongation factor-1α (EF-1α)—a protein essential for protein translation and for which homologs exist in all organisms. The homolog described here has a physiologically pertinent bundling activity. In addition, we found that this activity is modulated by Ca^{2+}/calmodulin (Ca^{2+}/CaM), which is a significant regulatory complex ubiquitous in living organisms.

RESULTS

Tubulin Binding Proteins Isolated from Carrot Soluble Proteins by Tubulin Affinity Chromatography

Classical methods for identifying MAPs exploit their copurification with MTs self-assembled from endogenous tubulin in protein extracts, and these methods have worked successfully using animal tissues rich in tubulin. The application of these procedures to plant material is problematic for reasons not entirely clear, although the lower concentration of tubulin in plant protein extracts is a plausible explanation. Although this limitation has been experimentally circumvented for maize (Vantard et al., 1991; Schellenbaum et al., 1993) and tobacco (Chang-Jie and Sonobe, 1993), we have encountered difficulty in consistency and yields with carrot proteins. Therefore, an alternative method was developed.

The covalent immobilization of tubulin (Mithieux and Rousset, 1989; Balaban and Goldman, 1992; Jablonsky et al., 1993) or MTs (Michalik et al., 1991, 1992; Miller et al., 1991) onto a chromatographic resin has been used previously to identify tubulin- or MT-interacting proteins, respectively. The method is particularly useful for identifying MAPs from sources where endogenous tubulin concentrations are low. Hence, we adapted one of these protocols for use in isolating proteins from carrot cells.

Figure 1 shows the results of tubulin affinity chromatography using soluble proteins obtained from suspension-cultured cells of carrot (lane a). "Tubulin binding proteins" adsorbed to the column matrix were eluted by a single 325-mM NaCl step (lane b).

Because we were interested in obtaining putative MAPs, we initially tried an established protocol for MT affinity chromatography (Miller et al., 1991). However, the polymer state of the tubulin in the matrix cannot be ascertained, and we found that

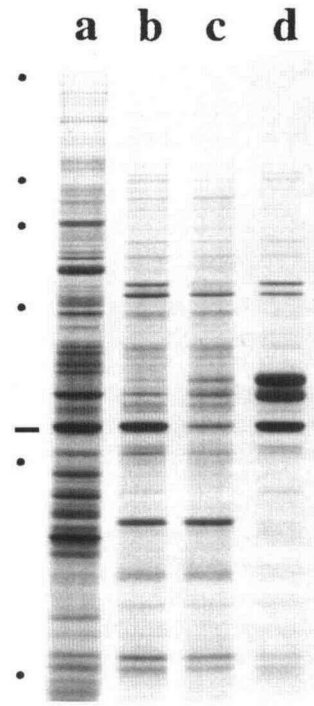

Figure 1. SDS-PAGE Analysis of Carrot Proteins Isolated by Tubulin Affinity Chromatography and of Cosedimentation Assays for MT Binding Proteins Present in the Affinity-Isolated Proteins.

Total soluble proteins from carrot cells (lane a) were chromatographed using a matrix of covalently immobilized tubulin. Proteins that required 0.3 M NaCl for elution from the matrix were designated "tubulin binding proteins" (lane b). These proteins were assayed by cosedimentation with taxol-stabilized MTs free in solution to determine which tubulin binding proteins are also MT binding proteins. Proteins that did not bind MTs tightly remained in the cosedimentation supernatant (lane c) when MTs were pelleted by centrifugation. Proteins found in the pellet (lane d) bound to MTs, provided that their sedimentation was dependent on the presence of MTs in the assay. Dots in the left margin indicate the positions of molecular mass standards (in kilodaltons from top: 205, 116, 97, 66, 45, and 29, respectively), and the dash indicates the position of pp50.

using an unassembled tubulin solution in place of taxol-stabilized MTs yielded nearly identical, polypeptide elution profiles (data not shown). In addition, increasing the tubulin quantity per volume of matrix also increased the yield of tubulin binding proteins (data not shown).

Control columns containing either a BSA or ligandless matrix were used to assess the specificity of tubulin affinity chromatography. From the BSA column, a single 40-kD polypeptide was eluted (data not shown); a 40-kD polypeptide present in tubulin binding proteins does not cosediment with MTs (see below). Using the ligandless column, negligible binding of soluble carrot proteins was observed (data not shown). The results of these two experiments indicated that tubulin affinity chromatography is specific and that proteins obtained thereby are not simply interacting with an acidic protein or the column resin.

Our isolation method permits the recovery of tubulin binding proteins in quantities amenable to biochemical analyses. Following dialysis against a buffer commonly used for tubulin biochemistry, these proteins were analyzed by functional assays for characteristics common to reported plant MAPs (reviewed in Vantard et al., 1993), including binding to MTs and induction of MT bundles.

Characterization of Tubulin Binding Proteins

To determine whether the tubulin binding proteins also bind to assembled MTs, the proteins were subjected to cosedimentation assays using taxol-stabilized MTs preassembled from pure brain tubulin. The enrichment of a polypeptide in the cosedimentation pellet (versus the corresponding supernatant) implies that the polypeptide has an affinity for MTs that are sedimented under the assay conditions. Some polypeptides present in the tubulin binding proteins (Figure 1, lane b) were depleted from the supernatant (lane c) and cosedimented with MTs (lane d). In controls, no polypeptides of the tubulin binding proteins sedimented in the absence of MTs under otherwise identical conditions (data not shown).

When tubulin binding proteins (Figure 1, lane b) are introduced to taxol-stabilized MTs preassembled from either brain or carrot tubulin, bundles similar to those shown in Figure 2A could be observed by dark-field microscopy. In fact, these bundles were observed prior to centrifugation in the cosedimentation assay; thus, it is likely that most tubulin that is sedimented into the pellet (Figure 1, lane d) is in the form of MT bundles because (1) virtually no single MTs are observed either before centrifugation or in the post-sedimentation supernatant and (2) in control experiments of MTs alone, single MTs are observed both before centrifugation and in the post-sedimentation supernatant (data not shown).

The most prominent polypeptide in the tubulin binding proteins that cosediments with MTs has an apparent molecular mass of 50 kD and is identified by the dash in Figure 1. This polypeptide was preliminarily designated pp50 (polypeptide, 50-kD). Because of pp50's abundance, we hypothesized that it was responsible for the MT bundling activity. Testing this hypothesis required separation of pp50 from other tubulin binding proteins.

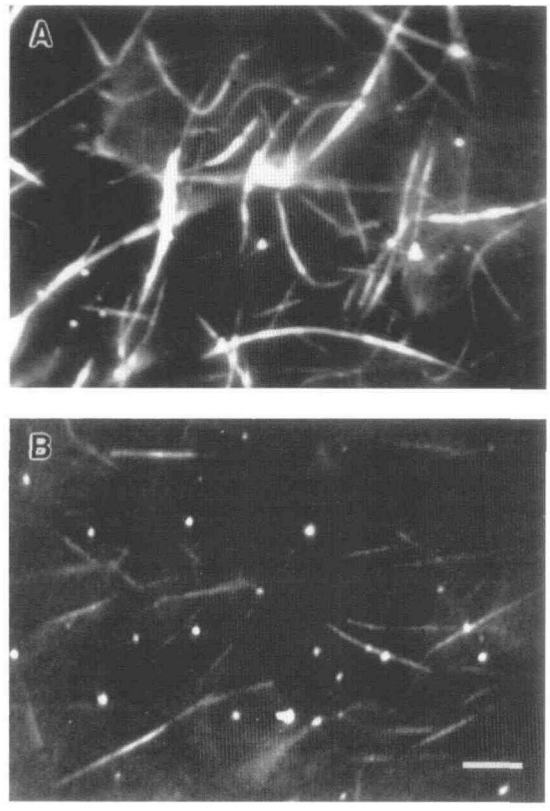

Figure 2. Dark-Field Microscopy of the MT Bundles Induced by the Presence of pp50-Containing Protein and the Ca^{2+}/CaM–Dependent Dissociation of These Bundles.

(A) MT bundles resulting from the addition of tubulin/CaM binding proteins (enriched in pp50) to taxol-stabilized MTs. Bundles were observed by dark-field microscopy without fixation.

(B) The dissociation of the bundles shown in **(A)** into single MTs with the addition of Ca^{2+} plus CaM.

In controls, no effect on the bundles shown in **(A)** was observed when the suspension was adjusted to the equivalent concentrations of $CaCl_2$ alone or CaM alone. In addition, none of these three treatments had an observable effect on taxol-stabilized brain MTs alone. To reduce light intensity to a level compatible with the video camera, the optical filtration required for obtaining the image shown in **(A)** was five times greater than that for **(B)**. Bar = 10 μm.

pp50 of the Tubulin Binding Proteins also Binds CaM in a Ca^{2+}-Dependent Manner

Two previous studies have reported major Ca^{2+}-dependent CaM binding polypeptides of 50 kD among carrot cell proteins (Cyr, 1991a; Oh et al., 1992); therefore, we hypothesized that pp50 in the tubulin binding proteins would also bind CaM in

the presence of Ca^{2+}. Accordingly, CaM was covalently immobilized onto an activated resin to construct a CaM affinity matrix similar to our tubulin affinity matrix. Figure 3 shows the results for CaM affinity chromatography of tubulin binding proteins (lane a) using a Ca^{2+}-dependent elution scheme. Most of the tubulin binding proteins did not bind CaM in the presence of Ca^{2+}, yet pp50 was depleted from these unbound proteins (lane b). That is, pp50 bound CaM in the presence of Ca^{2+}, and it was eluted from the matrix only when Ca^{2+} was removed from the immobilized CaM (lane c). Thus, pp50 is selectively purified from the tubulin binding proteins and can be classified as a Ca^{2+}-dependent CaM binding protein. These "tubulin/CaM binding proteins" provided a very highly purified (note that this is a silver-stained gel) preparation of pp50 for biochemical analyses.

We confirmed that the pp50 present in the tubulin/CaM binding proteins retained its MT binding and MT bundling properties. Figure 3 (lanes d and e) shows that pp50 cosediments with MTs, and Figure 2A is a dark-field photomicrograph of the MT bundles induced by the tubulin/CaM binding proteins. Because pp50 bound MTs as well as Ca^{2+}/CaM, the effect of Ca^{2+}/CaM on MT bundles induced in the presence of the tubulin/CaM binding proteins was investigated.

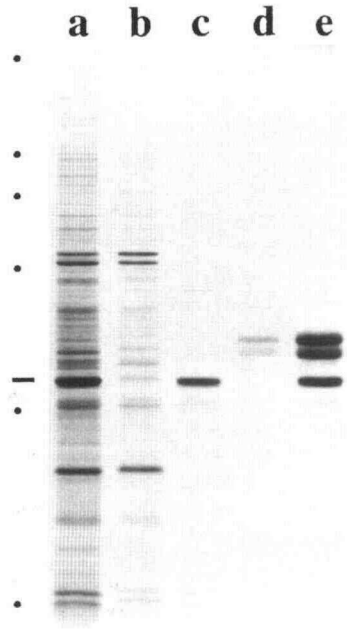

Figure 3. SDS-PAGE Analysis of the Selective Purification of pp50 Using CaM Affinity Chromatography and of the Retention of pp50's MT Binding Activity Assessed by Cosedimentation.

Tubulin binding proteins (lane a; as shown in Figure 1, lane b) were chromatographed using a matrix of covalently immobilized CaM. A fraction of the tubulin binding proteins did not bind to the CaM affinity matrix in the presence of Ca^{2+} (lane b). However, pp50 did bind CaM in the presence of Ca^{2+}, and it was among the proteins that eluted from the CaM affinity matrix when Ca^{2+} was removed from the matrix by EGTA. Such proteins are a Ca^{2+}-dependent CaM binding subfraction of tubulin binding proteins and, hence, are designated "tubulin/CaM binding proteins" (lane c). These tubulin/CaM binding proteins were assayed by cosedimentation to confirm that pp50 thus obtained retains its MT binding activity: lane d is the cosedimentation supernatant and lane e is the cosedimentation pellet. The symbols at left are as given in Figure 1.

MT Bundles Induced in the Presence of Purified pp50 Are Abolished by Ca^{2+}/CaM

We hypothesized that Ca^{2+}/CaM would affect the bundling function of the tubulin/CaM binding proteins. Figure 2A is a dark-field micrograph of the striking MT bundles induced in the presence of the tubulin/CaM binding proteins highly enriched in pp50. When this suspension of bundles was adjusted to 1 mM free Ca^{2+} and bovine brain CaM was added to 0.15 mg mL^{-1}, the MT bundles dissociated into predominantly single MTs, although a rather small proportion of finer bundles remained (Figure 2B). In control experiments, adjustment of the MT bundle solution to the equivalent concentration of Ca^{2+} alone or CaM alone had no observable effect, and none of these three treatments had an observable effect on taxol-stabilized brain MTs alone (data not shown). In addition, the use of taxol-stabilized MTs assembled from carrot tubulin produces similar results (Durso and Cyr, 1994).

Identification of pp50 as an EF-1α Homolog

To distinguish whether pp50 was related to other identified proteins, a sample of pp50 from the tubulin binding protein was proteolytically digested with trypsin (the N terminus was blocked), and peptide fragments were chromatographically separated. Amino acid microsequencing of five selected fragments revealed similarity to sequences of EF-1α from a number of species. EF-1α is a highly conserved protein translation factor that directs aminoacyl-tRNAs to their respective mRNA anticodons in the A site of ribosomes in a GTP-dependent reaction (Riis et al., 1990).

Figure 4A demonstrates the similarity of pp50 with EF-1α sequences from a wide variety of species. The five pp50 fragments correspond to EF-1α sequences scattered over greater than half the protein (typically ~455 amino acids in length). There are some clustered regions of dissimilarity between pp50 and the other EF-1α sequences; however, these regions are also variable among all EF-1α sequences. In addition to the sequence comparisons in Figure 4, two other points demonstrate the similarity of pp50 and EF-1α. First, the apparent molecular masses for EF-1αs are typically reported to be ~50 kD. Second, we examined the identity of pp50 using antiserum raised against ABP-50, which is an EF-1α homolog from *Dictyostelium discoideum*, that interacts with the actin cytoskeleton of this slime mold (Demma et al., 1990; Yang et al.,

A

pp50 fragment		VHINIVVIGHVDSGK		STTTGHLIYK		EVSSYLK		SVEMHHESLQ?ALPGDNVGF		NVSV?DLK
tomato	006	I..S.....	021	.....	173	.....	279	.....A..E.....	302	..A.K...
Arabidopsis	006	F.....	021	.....	173	.....	279	.....LE.....	302	..A.K...
mung bean					000	.....	000	.....A.IE.....	000	..A.K...
wheat	006	T.....	021	.....	173	.....	279	.....LE.....	302	..A.K...
barley	006	I.....	021	.....	173	.....	279	.....LE.....	302	..A.K...
Dictyo (ABP-50)	009	T.....A..	024	.....	176	....F..	282	.....Q.PE.R.....	305	....KEI.
Mucor racemosus	006	T.V.V.....	021	.....	173	...GFI.	291	.....T.TEG.....	314	....K.IR
S cerevisiae	006	S...V.....	021	.....	173	.T.NFI.	291	.....Q.EQGV.....	314	....KEIR
Euglena	006	...SL.....	021	.....	173	...G...	279	.....A.TE.V.....	302	....K.IR
Tetrahymena	007	....L.....	022	.....	174	.L.D...	280	.....Q.PE.V.....	303	G...K.IR
Artemia salina	006	I.....	021	.....	173	...A.I.	291	.....EQ.S.....	314	....KE.R
sea urchin					000	...G.I.	000	.....T.EK.....	000	...IK.IR
Drosophila F1	006	I.....	021	.....	173	.....I.	291	.....A..E.V.....	314	....KE.R
Xenopus EF-1αS	006	T.....	021	.....	173	...T.I.	291	.....A.TE.V.....	314	....K.VR
Xenopus EF-1αO	006	I.....	021	.....	173	...A.I.	291	.....A..E.....	314	.I..K.IR
Xenopus 42Sp50	009	T.L...I.....	024	.....	176	N.MV...	294	.I.....P..M.F..F.I..	317	.IAAKS..
human EF-1α	006	T.....	021	.....	173	...T.I.	291	.....A.SE.....	314	....K.VR
human EF-1α2	006	T.....	021	.....	173	...A.I.	291	.....A.SE.....	314	....K.IR

B

pp50 fragment		VHINIVVIGHVDSGK		STTTGHLIYK		EVSSYLK		SVEMHHESLQ?ALPGDNVGF		NVSV?DLK
carrot A/Z	006	.....	021	.....	173	.....	279	.....LE.....	302	....K...
carrot CEM1	006	I..S.....	021	.....	173	.....	279	.....A..E.....	302	..A.K...
carrot PIK-A49							000	.....A..R.....		

Figure 4. Alignment of Amino Acid Sequences of Tryptic Peptide Fragments of pp50 with Corresponding Sequences of EF-1α Homologs.

pp50 was proteolytically digested and fragments were separated by HPLC. The amino acid sequences for five selected fragments are shown (top lines).
(A) Interspecies comparison of corresponding sequences from tomato (LeEF-1; Pokalsky et al., 1989); Arabidopsis and mung bean (Axelos et al., 1989); wheat (Metz et al., 1992); barley (Dunn et al., 1993); *Dictyostelium discoideum* ABP-50 (Dictyo ABP-50; Yang et al., 1990); *Mucor racemosus* (Linz et al., 1986); *Saccharomyces cerevisiae* (Nagata et al., 1984); *Euglena* (Montandon and Stutz, 1990); *Tetrahymena* (Kurasawa et al., 1992); *Artemia salina* (van Hemert et al., 1984); sea urchin (Kuriyama et al., 1990); Drosophila (Hovemann et al., 1988); Xenopus (EF-1αS, Krieg et al., 1989; EF-1αO and 42Sp50, Djé et al., 1990); and human (EF-1α, Brands et al., 1986; EF-1α2, Knudsen et al., 1993).
(B) Intraspecies comparison of other carrot EF-1α sequences: A/Z from unpublished sequence of N. R. Apuya and J. Zimmerman (University of Maryland, Baltimore County, Catonsville, MD) entered in the EMBL data bank as accession number X60302 (locus DCEF1A); CEM1 (Kawahara et al., 1992); and PIK-A49 (Yang et al., 1993).
Numbers correspond to the first amino acid residue in each fragment shown; 000 denotes data derived from incomplete sequences. A dot indicates identity with the top line (pp50 fragment sequence). A question mark in pp50 sequences indicates that amino acid sequencing did not permit unambiguous identification of the residue. The two residues indicated by question marks are known to be post-translationally modified in some organisms (Merrick et al., 1990). The underlined residues are discussed in the text.

1990; Dharmawardhane et al., 1991). The use of this antiserum for the immunoblot shown in Figure 5A indicates the identity of pp50, which eluted from both the tubulin (lane 1) and CaM (lane 2) affinity matrices. We concluded that pp50 is an EF-1α homolog.

Figure 4B is a more specific comparison of the pp50 fragments with corresponding sequences of described EF-1α homologs also from cultured carrot cells. Here, the sequence identities provide even more striking evidence that pp50 is an EF-1α homolog. The A/Z and CEM1 sequences resulted from screens for genes preferentially expressed during carrot somatic embryogenesis (Kawahara et al., 1992; Zimmerman, 1993). The PIK-A49 sequence was obtained for a carrot protein that activates phosphatidylinositol 4-kinase and that has conventional EF-1α activity (Yang et al., 1993). There is evidence for an EF-1α gene family in carrot (Kawahara et al., 1992), and the data in Figure 4B are consistent with this proposal. In particular, the residues underscored in Figure 4B indicate that at least four genes are present in carrot.

Novel Evidence for the Direct Binding between an EF-1α Homolog and Tubulin or MTs

Indirect evidence indicates that EF-1α associates with MTs of the mitotic apparatus and that it may play a role in nucleating MTs (Ohta et al., 1988a, 1990; Toriyama et al., 1988; Kuriyama et al., 1990; Marchesi and Ngo, 1993). However, a direct interaction of EF-1α with MTs has not been demonstrated; it is unclear whether previous investigators have attained the high level of purity for EF-1α preparations that we show here (Figure 3, lane c). In conjunction with the anti–ABP-50 serum, our purified preparation allowed us to examine directly the binding of an EF-1α homolog to MTs.

Figure 5A demonstrates that the antiserum recognizes only the EF-1α homolog (pp50) in the tubulin/CaM binding proteins (lane 2). These proteins were introduced to MTs to induce bundles (as shown in Figure 2A), and the bundles were exposed to anti–ABP-50 serum in processing for indirect immunofluorescence microscopy. The fluorescence observed in Figure

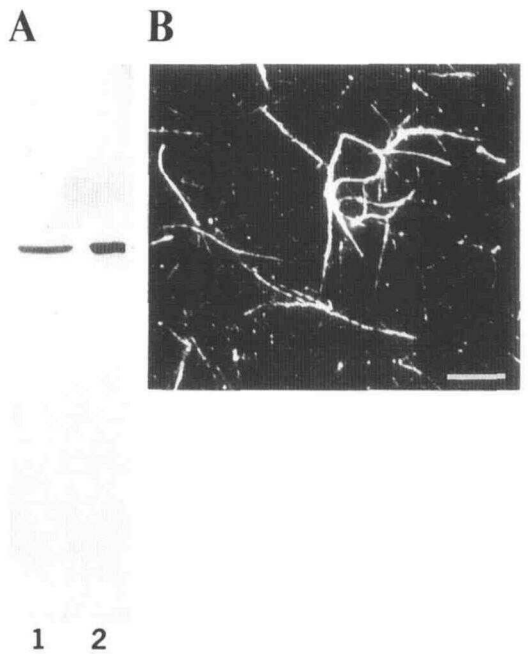

Figure 5. Evidence for Direct Binding between pp50 and MTs.

(A) Immunoblot analysis using antiserum raised against an EF-1α homolog (ABP-50) from *Dictyostelium*. The antiserum reacts monospecifically with pp50 in both the tubulin binding proteins (lane 1) and the tubulin/CaM binding proteins (lane 2).

(B) Indirect immunofluorescence microscopy using the anti–ABP-50 serum characterized in **(A)**. MT bundles were formed by mixing tubulin/CaM binding protein with taxol-stabilized MTs (as described in Figure 2A). These bundles were fixed, settled onto coated slides, and reacted with the anti–ABP-50 serum during processing for indirect immunofluorescence microscopy. The fluorescence observed in **(B)** indicates that pp50 is localized to the MT bundles. Bar = 10 μm.

5B clearly demonstrates that the EF-1α homolog is associated with the MT bundles. In control immunoblots, the antiserum shows absolutely no reactivity with tubulin (data not shown). This new evidence for a direct binding between an EF-1α homolog and tubulin or MTs extends and augments the indirect data for such an interaction.

The Carrot EF-1α Homolog also Interacts with Carrot MTs

Tubulin prepared from brain has been used because it can be more highly purified in greater yields, and studies on heterologous versus homologous MT–MAP interactions using brain or plant proteins show that either type of interaction produces similar results except for some quantitative variation (Hugdahl et al., 1993; Schellenbaum et al., 1993). Nevertheless, we investigated the interaction of pp50 with taxol-stabilized MTs of pure carrot tubulin.

As discussed above, the introduction of tubulin/CaM binding proteins to MTs assembled from carrot tubulin also induces bundling indistinguishable from bundling of brain MTs, and the modulation of these carrot MT bundles by Ca^{2+}/CaM was similar (Durso and Cyr, 1994). However, we encountered difficulty in analyzing cosedimentation of pp50 with carrot MTs by SDS-PAGE because a carrot tubulin polypeptide comigrates with pp50. This difficulty has been circumvented by use of the anti–ABP-50 serum in immunoblotting analyses.

Figure 6 shows the results of cosedimentation assays using carrot MTs and tubulin/CaM binding proteins. All pp50 is depleted from the supernatant (second lane) and cosediments with carrot MTs into the pellet (first lane). These results are similar to the cosedimentation analysis shown in Figure 3 (lanes d and e) in which MTs assembled from brain tubulin were used. Control immunoblots showed absolutely no reaction of anti–ABP-50 serum with carrot tubulin; neither was any reaction observed between pp50 and anti-tubulin antibodies (data not shown). The first and third lanes of Figure 6 also illustrate that the carrot tubulin polypeptide that comigrates with pp50 is an α-tubulin. These results suggest that EF-1α homologs may be present in many MT/MAP preparations but are masked by their comigration with tubulin polypeptides in SDS-PAGE.

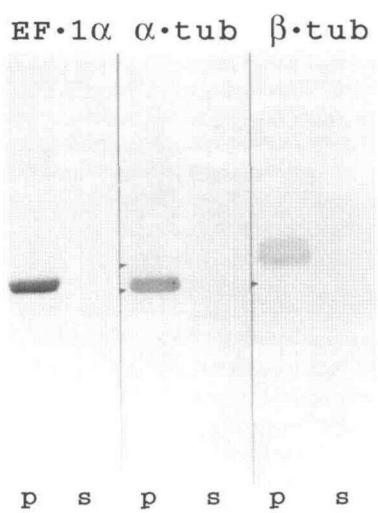

Figure 6. Analysis by SDS-PAGE Followed by Immunoblotting of pp50's Cosedimentation with Taxol-Stabilized MTs of Pure Carrot Tubulin and of pp50's Electrophoretic Comigration with Carrot α-Tubulin.

Tubulin/CaM binding proteins were assayed for binding to carrot MTs using cosedimentation. Replicates of the cosedimentation pellet (lanes marked p) and supernatant (lanes marked s) were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was temporarily stained for protein with Ponceau red and cut into three identical strips. Each of the three strips was independently immunoblotted using anti–ABP-50 serum (EF·1α; first and second lanes), anti–α-tubulin (α·tub; third and fourth lanes), or anti–β-tubulin (β·tub; fifth and sixth lanes).

DISCUSSION

We have adapted a method of tubulin affinity chromatography to circumvent difficulties typically associated with isolating putative MAPs from sources low in endogenous tubulin. By this procedure we isolated soluble carrot protein containing three major polypeptides. Importantly, tubulin binding proteins also bound and bundled MTs, two properties characteristic of identified plant MAPs (Schellenbaum et al., 1992; Chang-Jie and Sonobe, 1993), as well as structural MAPs of any origins (MacRae, 1992a).

By the use of CaM affinity chromatography, we selectively purified from the tubulin/MT binding proteins an abundant 50-kD polypeptide that bundles MTs. The functional significance of pp50's binding to tubulin, MTs, and Ca^{2+}/CaM was examined in relation to pp50's MT bundling function. MT bundles induced by the presence of pp50 were abolished by Ca^{2+}/CaM.

Other MT/CaM–interacting proteins have been described. Besides the more abundant neuronal MAPs (Gratzer and Baines, 1988; Vera et al., 1988), brain spectrin binds both MTs and Ca^{2+}/CaM (Riederer and Goodman, 1990). The effects of Ca^{2+}/CaM on brain spectrin's MT bundling activity, however, are not known. Caldesmon exhibits Ca^{2+}/CaM–sensitive binding to MTs; in fact, it bundles MTs under sulfhydryl-reducing conditions (Ishikawa et al., 1992). However, unlike the activity we ascribe to pp50, a Ca^{2+}/CaM–sensitive bundling activity has not been reported for caldesmon nor is pp50's bundling activity dependent on reducing agents (data not shown). Stable-tubule-only-polypeptides (STOPs) are MT/CaM–interacting proteins, and they render MTs cold stable (Margolis and Job, 1994), whereas pp50 does not (data not shown). Hence, pp50's attributes appear unique.

pp50 was identified as a homolog of EF-1α, a ubiquitous protein with an essential role in protein translation. Binding between EF-1αs and Ca^{2+}/CaM has not been described previously, and evidence for interactions between EF-1αs and tubulin or MTs has been indirect (Ohta et al., 1988a, 1990; Toriyama et al., 1988; Kuriyama et al., 1990; Hasezawa and Nagata, 1993; Marchesi and Ngo, 1993). A direct binding between EF-1α and MTs is demonstrated in this report.

Physiological Significance of pp50's Properties

Although the molecular mechanisms by which MT arrays are established and regulated by higher plant cells are poorly understood, it appears likely that MAPs play an essential role (see Introduction). The significance of MT bundling MAPs lies in numerous observations that the MTs of plant arrays are coaligned and closely spaced to variable degrees (Ledbetter and Porter, 1963; Newcomb, 1969; Pickett-Heaps, 1974; Hardham and Gunning, 1977; Doohan and Palevitz, 1980; Gunning and Hardham, 1982; Bajer and Molè-Bajer, 1986; Lancelle et al., 1987; Baluška et al., 1992, 1993). Three of four studies on plant MAPs report MT bundling activity in the respective protein fractions (see Introduction). Also, an MT bundling MAP from a green alga in which photoresponsive MT bundles are present in vivo has been reported (Maekawa et al., 1990). MAPs from carrot (Cyr and Palevitz, 1989), an alga (Maekawa et al., 1990), and tobacco (Chang-Jie and Sonobe, 1993) have been immunocytochemically localized to MT structures in situ, thereby extending the in vitro MT association. For these MAPs, however, interactions of potential regulatory significance, such as that of Ca^{2+}/CaM with pp50, have not been reported.

We propose that an EF-1α homolog mediates lateral associations of MTs within the cells of higher plants by a Ca^{2+}/CaM–modulated mechanism. This proposal is supported by the direct, experimental evidence reported here and consistent with additional information reviewed below (see also Durso and Cyr, 1994).

Localizations of Tubulin, CaM, and EF-1α in Plants

Indirect evidence for the association of EF-1α with plant MT arrays has recently been reported. An antibody raised against a mitotic apparatus–associated EF-1α homolog from sea urchins (Ohta et al., 1988a, 1988b, 1988c, 1990; Toriyama et al., 1988) reacts with a 49-kD tobacco homolog that appears to be localized to MT structures of cultured tobacco cells (Hasezawa and Nagata, 1993). Significantly, CaM has been immunocytochemically localized to bundled MTs in the cortical array (Fisher and Cyr, 1993) and mitotic array (Vantard et al., 1985; Wick, 1985; Wick et al., 1985; Fisher and Cyr, 1993) in plant cells. MTs of the cortical array in carrot appear to be Ca^{2+}/CaM–sensitive, and MAPs have been implicated in this sensitivity (Cyr, 1991a). That pp50 is a Ca^{2+}/CaM binding MAP therefore suggests its involvement. It is noteworthy that a Ca^{2+}/CaM binding protein with an apparent molecular mass similar to pp50's may also be an animal MAP that mediates CaM's association with mitotic spindle MTs (Brady et al., 1986).

Gene Expression during Development

The developmentally modulated expression of EF-1α genes during carrot somatic embryogenesis has recently been covered in a review of that system as a model for studying plant development (Zimmerman, 1993). The mRNA of an EF-1α gene preferentially expressed in such embryos rose to a transient peak at earlier stages and then declined gradually (Kawahara et al., 1992), and post-transcriptional regulation of such EF-1α expression has been indicated (Apuya and Zimmerman, 1992). It is relevant that a carrot Ca^{2+}/CaM binding protein with an apparent molecular mass similar to EF-1α's increases in the later embryogenesis stages (Oh et al., 1992). In addition, increases in CaM (Oh et al., 1992) and tubulin (Cyr et al., 1987) levels occur during this period. Thus, it appears that the expression of genes for EF-1α, tubulin, CaM, and CaM binding

proteins (perhaps including an EF-1α homolog) is modulated during carrot development.

Additional studies in whole plants report developmental modulations of EF-1α gene expression. In tomato (Pokalsky et al., 1989), tobacco (Ursin et al., 1991; Marty et al., 1993), and possibly Arabidopsis (Curie et al., 1993), elevated levels of EF-1α gene expression were indicated in developing tissues. Auxin (Ursin et al., 1991), low temperature (Dunn et al., 1993), and light (Aguilar et al., 1991) appear to modulate expression of some EF-1α genes, and multigene families are present in several plants (Axelos et al., 1989; Pokalsky et al., 1989; Aguilar et al., 1991; Dunn et al., 1993). A gene family has been found in carrot cells as well (Kawahara et al., 1992), and Figure 4B presents data consistent with this finding.

EF-1α Gene Families

Functions for EF-1α beyond its well-documented role in protein synthesis have been reported (e.g., Yang et al., 1993; Zhu et al., 1994; reviewed in Durso and Cyr, 1994). Evidence from other studies suggests that particular members of EF-1α families are responsible for additional EF-1α functions. For example, one Xenopus EF-1α homolog forms a phylogenetic outgroup to all eukaryotic EF-1αs (including three others also found in Xenopus), and this homolog's biochemical properties are distinct from those of EF-1α (Viel et al., 1991). In this same analysis, sea urchin EF-1α homologs implicated in nucleating mitotic apparatus MTs (see above) also appear divergent from other eukaryotic EF-1αs; the amino acid composition (Ohta et al., 1990) and sequence comparisons (Kuriyama et al., 1990) are consistent with this finding. In fact, Kuriyama et al. (1990) discuss that their EF-1α homolog may not be a typical EF-1α in sea urchin, but rather a gene family member serving the additional function in the mitotic apparatus. It was an antibody raised against one of these divergent sea urchin homologs that colocalized with MT structures in tobacco (see above). Finally, pp50 is also a member of a carrot EF-1α family (Figure 4B) and may play a role in the organization and regulation of the MT cytoskeleton.

These examples illustrate that EF-1α homologs with divergent sequences may not have the typical biochemical characteristics of EF-1αs (see Merrick, 1979) and that such distinction may indicate that a homolog has additional functions beyond translation. Experiments to characterize the activity of pp50 in protein translation are in progress.

Conclusion

Reports of EF-1α/cytoskeleton interactions often discuss possible cellular communication systems between the protein synthetic machinery and the cytoskeleton. We have hypothesized that EF-1α is involved in feedback regulation of cytoskeletal protein synthesis (Durso and Cyr, 1994). We are currently designing experiments to elucidate the relationships between MTs, tubulin, tubulin mRNA, and EF-1α in an in vitro translational system, and the effects of Ca^{2+}/CaM on this system will be studied.

In summary, we have isolated an EF-1α homolog from carrot cells by its affinity for both tubulin and Ca^{2+}/CaM. We propose that an EF-1α homolog mediates the lateral association of MTs within the cells of higher plants by a Ca^{2+}/CaM–sensitive mechanism. The abundance and versatility of EF-1α (reviewed in Durso and Cyr, 1994) present an opportunity to learn more about the integration of cellular processes such as Ca^{2+} signaling, cytoskeletal dynamics, and the cotranslational regulation of protein synthesis.

METHODS

Solutions

The solutions used in this study were as follows: buffer C (as given in Miller et al., 1991), 50 mM Hepes, pH 7.6, 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM EGTA; lysis buffer, 100 mM Hepes, pH 7.6, 5 mM $MgSO_4 \cdot 7H_2O$, 5 mM EGTA, 10 mM DTT, and protease inhibitors at 10 μg mL^{-1} each of leupeptin, chymostatin, pepstatin, antipain, and aprotinin, and 50 μg mL^{-1} each of Nα-benzoyl-L-arginine methyl ester (BAME) and Nα-*p*-tosyl-L-arginine methyl ester (TAME); buffer C.0, buffer C plus 25 mM NaCl, 0.5 mM DTT, and one-tenth the concentrations of protease inhibitors in lysis buffer; buffer C.3, buffer C.0 plus 0.3 M NaCl; PM, 50 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM $MgSO_4 \cdot 7H_2O$; PMCa, PM plus 3 mM $CaCl_2$; PMGTP, PM plus 0.5 mM GTP plus 0.5 mM $MgSO_4 \cdot 7H_2O$.

Preparation of Carrot Soluble Proteins

Suspension-cultured carrot cells were grown as described by Cyr and Palevitz (1989). Seven days after subculturing, the cells were collected using Miracloth (Calbiochem, La Jolla, CA), washed three times in ice-cold buffer C, and resuspended in ice-cold lysis buffer (1:2 [v/w]). Cells were lysed by explosive decompression from 3.4×10^7 Pa (5000 psi) using a French power laboratory press (American Instruments Co., Inc., Silver Spring, MD) with a prechilled cylinder (4°C). The lysate was centrifuged at 7700*g* at 4°C for 20 min, and lipids were skimmed from the supernatant surfaces using a Pasteur pipette. The supernatants were collected and recentrifuged at 48,000*g* at 4°C for 30 min, lipids were skimmed again, and supernatants were collected. Finally, the supernatant that was centrifuged at 48,000g was again centrifuged 3 to 4 hr at 100,000*g* at 4°C, the lipids were skimmed, and supernatants were collected as total soluble carrot proteins. The skimming at each step minimizes the buildup of lipids in the affinity column, which causes problematic back pressure during chromatography.

Preparation of Bovine Tubulin

Bovine brain tubulin was prepared as described by Cyr and Palevitz (1989). Briefly, neuronal microtubule (MT) protein (Shelanski et al., 1973) was cycled three times in 1 M glutamic acid (Hamel and Lin, 1981), desalted using a Biogel P-6DG (Bio-Rad) column, and chromatographed by phosphocellulose (Weingarten et al., 1975). No non-tubulin polypeptides were detectable in the resulting tubulin (in PMGTP) by

heavily loaded and silver-stained, SDS–polyacrylamide gels; therefore, this protein is termed "pure tubulin."

Tubulin Affinity Chromatography

Tubulin affinity columns were prepared by modifications of the protocol for constructing MT affinity columns (Miller et al., 1991). Equal-packed volumes of Affigel-10 (Bio-Rad) and Sepharose CL-6B (Sigma) were quickly stirred together in a column, rinsed with ≥ 4 volumes of ice-cold water, equilibrated with cold buffer C, and then resuspended in an equal volume of a highly purified solution of bovine brain tubulin adjusted to pH 7.6. The column was set at 4°C overnight to allow the covalent immobilization of tubulin onto Affigel's activated succinimidyl moiety. The column was washed under the most stringent conditions to which it was subsequently to be exposed, and the washes were assayed for unbound tubulin protein. Between uses, the column was stored in 10% glycerol in PM buffer plus 0.02% NaN_3, 1 mM DTT.

Our columns have ranged from 5 to 30 mL, with input tubulin at 2.5 to 20.0 mg mL^{-1}, resulting in 85 to 95% bound. We have used some columns for > 3 months, and the only noticeable change is some back pressure buildup after 5 to 7 uses, in which case the matrix is transferred to a column with a new bed support and successfully reused. The volume of soluble carrot proteins loaded into the column is typically four to five times the column volume, and it is loaded overnight at 0.25 to 0.35 column volume hr^{-1}. Unbound (or loosely bound) carrot protein is eluted from the column with 4 to 5 column volumes of buffer C.0 at 2 column volumes hr^{-1} until the absorbance of the eluate at 280 nm is stabilized. Then the column is eluted with buffer C.3 at the same flow rate to obtain "tubulin binding proteins."

BSA and ligandless control columns were prepared essentially as the tubulin columns. Because the pI of tubulin (e.g., Kenney et al., 1988) as well as BSA (e.g., Ghitescu et al., 1992) is acidic, a BSA matrix served as a control to assess whether tubulin columns functioned simply as cation exchange matrices. BSA columns are also useful controls for nonspecific protein interactions (Miller et al., 1991). The ligandless control column was prepared by permitting hydrolysis of the resin's activated group under the same conditions as for the tubulin and BSA matrices.

Assembly of Taxol-Stabilized MTs from Brain or Carrot Tubulin

Pure brain tubulin was diluted to ≤ 5 mg mL^{-1} in PMGTP. The assembly temperature and taxol (Sigma) concentration were step increased approximately as described by Kellogg et al. (1989) to 37°C and to a concentration equimolar to tubulin, respectively. The final suspension of assembled MTs are termed "taxol-stabilized MTs." When appropriately diluted, individual MTs are readily visible by dark-field microscopy.

Carrot tubulin fractions were prepared from soluble carrot protein as described by Cyr and Palevitz (1989). This preparation contains numerous proteins, but taxol-stabilized MTs of pure tubulin can be obtained as follows. Tubulin was assembled by the addition of taxol to an approximately equimolar concentration and incubated at room temperature. The assembled tubulin was centrifuged 50,000*g* for 10 min, the supernatant was removed, and the pellet was resuspended in PM plus 10 μM taxol. By dark-field microscopy, MTs indistinguishable from brain MTs were observed in such suspended pellets, and silver-stained, SDS–polyacrylamide gels and immunoblots indicated that tubulin is the only protein in such suspensions.

MT Bundling and Cosedimentation Assays

Tubulin binding proteins were dialyzed against a large volume of PM at 4°C overnight, or tubulin/calmodulin (CaM) binding proteins were used directly as eluted (see below). The following steps were performed at room temperature. Precipitates were removed by centrifugation at 100,000*g* for 15 min. The final assay solution was prepared to contain 0.12 mg mL^{-1} taxol-stabilized MTs plus 0.17 mg mL^{-1} non-tubulin protein plus 5 μM taxol, with PM as dilutant. The presence of MTs and the formation of MT bundles were verified by dark-field microscopy of aliquots. Mixtures were incubated 20 min and centrifuged 15 min at 100,000*g*; the cosedimentation supernatants were collected and adjusted to 4.5 M urea by adding a volume of 9 M urea (in PM) equal to the supernatant volumes. The cosedimentation pellets were solubilized in volumes of 9 M urea equal to respective supernatants and then subsequently adjusted to 4.5 M urea by the addition of PM; thus, equal volumes of supernatants and pellets could be directly compared by SDS-PAGE or immunoblotting.

Dark-Field Microscopy

Dark-field images of MTs were obtained using a Leitz Ortholux II microscope (Leica Inc, Malvern, PA) equipped with a PlanApo 40× objective fitted with an iris adjustable to a maximum numerical aperture of 1.0 (Olympus Corp., Lake Success, NY) and a Leitz D120A dark-field condenser. MTs were imaged using a silicon-intensified tube camera (Hamamatsu Photonic Systems, Bridgewater, NJ) and projected onto a video monitor, and the screen was photographed using Kodak Tri-X film. The MT bundles in Figure 2A were too bright to be directly imaged by the silicon-intensified tube camera; therefore, filters were inserted between the objective and the camera.

CaM Affinity Chromatography

Brain CaM columns were prepared, washed, and preequilibrated as described by Cyr (1991a). The tubulin binding protein (containing 325 mM NaCl) was supplemented to 3 mM $CaCl_2$ and applied to the column. Unadsorbed proteins were eluted by PMCa to obtain "flow-through," and this protein was saved. Loosely bound proteins were eluted with PMCa plus 1 mM 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) and discarded. Finally, Ca^{2+}/CaM binding proteins were eluted by PM to obtain tubulin/CaM binding protein. The flow-through fraction was repetitively chromatographed until SDS-PAGE indicated that no more polypeptides were being depleted from the flow-through and no additional proteins were being obtained by PM elution.

Protein Gels

SDS-PAGE was performed essentially as described by Laemmli (1970) using 9% resolving gels. For gels to be stained for protein (by silver nitrate; Morrissey, 1981), samples in sample buffer containing 5 mM DTT were boiled, and reduced sulfhydryls were alkylated by adjusting samples to 12.5 mM iodoacetamide and incubating 10 min at 50°C (a modification of Lane, 1978). However, samples used for protein gel blot transfers and immunoblotting (below) were not subjected to the alkylating reaction.

Immunoblotting and Immunofluorescence

The method of Towbin et al. (1979) was used for protein gel blot transfers and immunoblotting analysis. The serum used in Figure 5A and Figure 6 (first and second lanes) was raised against an elongation factor-1α (EF-1α) homolog, ABP-50, from *Dictyostelium discoideum* (Demma et al., 1990; Yang et al., 1990) and was generously provided by J. Condeelis (Albert Einstein School of Medicine, Bronx, NY). The primary antibody was anti-ABP-50, and secondary antibody was an anti-rabbit alkaline phosphatase conjugate (Sigma). Both were used at a 1:1000 dilution. The primary anti-α-tubulin and anti-β-tubulin antibodies (Figure 6, third through sixth lanes) are commercial monoclonal antibodies (Amersham Corporation) used at a 1:1500 dilution; the secondary antibody was an anti-mouse alkaline phosphatase conjugate (Sigma).

For indirect immunofluorescence, a suspension of MT bundles induced in the presence of the tubulin/CaM binding proteins was settled onto poly-L-lysine-coated slides and fixed with 3.7% formaldehyde plus 0.1% glutaraldehyde in PM. These slides were processed as described by Cyr (1991a). The primary antibody was anti-ABP-50, and the secondary antibody was anti-rabbit fluorescein conjugate (Sigma). Both were used at a 1:100 dilution. The slides were observed with an Axioskop (Zeiss, Oberkochen, Germany) using blue excitation and photographed using Tri-X film (Kodak).

Amino Acid Sequencing

A 50-kD polypeptide (pp50) of the tubulin binding protein was immobilized on a polyvinylidene fluoride membrane (Bio-Rad) and submitted to J. Leszyk of the Worcester Foundation for Experimental Biology's Protein Chemistry Facility (Shrewsbury, MA) for in situ tryptic digestion (the N terminus was blocked), HPLC separation of proteolytic fragments, and amino acid sequencing of selected, separated fragments.

Protein Concentration Assays

Protein concentrations were determined by Bio-Rad's protein assay kit using BSA as a standard.

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