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Function of the p86 subunit of eukaryotic initiation factor (iso)4F as a microtubule-associated protein in plant cells

CAROL L. BOKROS*, JEFFREY D. HUGDAHL*, HYONG-HA KIM*, VIRGINIA R. HANESWORTH*, ANN VAN HEERDEN†, KAREN S. BROWNING†, AND LOUIS C. MOREJOHN*‡

*The Department of Botany and †The Department of Chemistry and Biochemistry, University of Texas, Austin, TX 78713

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ABSTRACT **The isozyme form of eukaryotic initiation factor 4F [eIF-(iso)4F] from wheat germ is composed of a p28 subunit that binds the 7-methylguanine cap of mRNA and a p86 subunit having unknown function. The p86 subunit was found to have limited sequence similarity to a kinesin-like protein encoded by the *katA* gene of *Arabidopsis thaliana*. Native wheat germ eIF-(iso)4F and bacterially expressed p86 subunit and p86–p28 complex bound to taxol-stabilized maize microtubules (MTs) *in vitro*. Binding saturation occurred at 1 mol of p86 per 5–6 mol of polymerized tubulin dimer, demonstrating a substoichiometric interaction of p86 with MTs. No evidence was found for a direct interaction of the p28 subunit with MTs. Unlike kinesin, cosedimentation of eIF-(iso)4F with MTs was neither reduced by MgATP nor enhanced by adenosine 5′-[γ-imido]triphosphate. Both p86 subunit and p86–p28 complex induced the bundling of MTs *in vitro*. The p86 subunit was immunolocalized to the cytosol in root maize cells and existed in three forms: fine particles, coarse particles, and linear patches. Many coarse particles and linear patches were colocalized or closely associated with cortical MT bundles in interphase cells. The results indicate that the p86 subunit of eIF-(iso)4F is a MT-associated protein that may simultaneously link the translational machinery to the cytoskeleton and regulate MT disposition in plant cells.**

Numerous studies have provided evidence for interactions between the cytoskeleton and the eukaryotic protein synthesis machinery. Polysomal mRNAs, ribosomes, and certain translation factors are associated with the cytoskeleton from detergent-extracted animal cells (1–4). Treatment of HeLa cells with the microfilament-disrupting agent cytochalasin releases mRNA, ribosomes, and initiation factors from the cytoskeletal framework (1, 3, 4). Polyribosomes have been found associated with cytoskeleton preparations from plants as well (5–7). Certain translation factors have been immunolocalized to the cytoskeleton of animal (8–10) and plant (11) cells. The translational machinery appears to have a relatively stable association with the cytoskeleton in animal cells, because detergent-extracted cytoskeletons (12) and saponin-permeabilized cells (13) engage in efficient translation without the addition of macromolecular translation components. Thus, these observations indicate that the translational machinery may be bound to and regulated by the cytoskeleton.

A different perspective is provided, however, by mounting evidence that protein synthesis factors may influence cytoskeletal function. For example, the existence of homologs of elongation factor 1α (EF-1α) in putative centrosome precursor particles from CHO cells (14) and in microtubule (MT)-organizing centers of sea urchin mitotic spindles (15–17) suggests binding of EF-1α to the minus ends of MTs and a function in MT nucleation. A similar MT-organizing role of a putative EF-1α in tobacco cells was deduced from immunolocalization patterns produced by antibodies against a sea urchin centrosomal EF-1α homolog (11). However, animal EF-1α was recently reported to sever MTs deficient in MT-associated proteins (MAPs) (18), a result we have confirmed with plant EF-1α and MTs (L. E. Littlepage and L.C.M., unpublished data). Thus, these observations indicate that, under particular conditions, EF-1α may modulate the disposition of MTs. Other than EF-1α, however, no other translation factor has been demonstrated to affect MTs.

The isozyme form of eukaryotic initiation factor 4F [eIF-(iso)4F] from wheat germ is composed of equimolar amounts of a small subunit (p28) that binds the 7-methylguanine (m^7G) cap of mRNAs and a large subunit (p86) that has unknown function during translation and limited sequence similarity to sea urchin kinesin (19). The p86 subunit has been immunologically identified in wheat, cauliflower, and maize (20), but no structural homolog of p86 has been identified in animal or yeast cells.

Here, eIF-(iso)4F was investigated further for structural and functional similarities to MAPs. We show that p86 binds substoichiometrically to polymerized tubulin, induces MT bundling *in vitro*, and is colocalized or closely associated with MTs in plant cells.

MATERIALS AND METHODS

Protein Purification. Tubulin was isolated from cultured cells of maize (*Zea mays* cv. Black Mexican Sweet) as described (21, 22) by using an isolation buffer (IB = 50 mM Pipes·KOH, pH 6.9/1 mM EGTA/0.5 mM $MgSO_4$) supplemented with 1 mM dithiothreitol/0.1 mM GTP/protease inhibitor mixture [N^{α}-*p*-tosyl-L-arginine methyl ester (50 μg/ml)/pepstatin A (5 μg/ml)/leupeptin hemisulfate (5 μg/ml)/aprotinin (5 μg/ml)]. Taxol-stabilized MTs were purified as described (22). Native eIF-(iso)4F was purified from wheat germ as described (20). Individual subunits of wheat germ eIF-(iso)4F were expressed from cDNAs in *Escherichia coli*, and p28 and p86 were purified by m^7GTP-Sepharose chromatography and phosphocellulose chromatography, respectively (23). Equimolar amounts of expressed p86 and p28 were combined to form the p86–p28 complex. Each eIF-(iso)4F protein was equilibrated in IB/protease inhibitor mixture by centrifugation on a Sephadex G-50 spin column. MAP2 from bovine brain was prepared as described (24).

Electron Microscopy (EM), Polymer Sedimentation Analysis, Protein Determination, SDS/PAGE, and Quantitative Densitometry. Polymer sedimentation analysis and EM were as described (25). Protein determinations were done by using dye binding (26) as described (27). Masses of p28, p86, and tubulin dimer were assumed to be 24, 86, and 100 kDa,

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Abbreviations: eIF-(iso)4F, eukaryotic initiation factor (iso)4F; EF-1α, elongation factor 1α; MAP, microtubule-associated protein; pNppA, adenosine 5′-[γ-imido]triphosphate; EM, electron microscopy; MT, microtubule.

‡To whom reprint requests should be addressed.

respectively (19, 28). Proteins were analyzed by SDS/PAGE (8 or 10% polyacrylamide) (29). Gels were stained with Coomassie blue and analyzed by quantitative densitometry as described (25). In cases where bands were distorted, gel images were produced with a Color OneScanner (Apple, Cupertino, CA), saved as 8-bit TIFF files, and analyzed with NIH IMAGE program (version 1.52) (30). Gray scales were calibrated to optical density with a photographic no. 2 step tablet (Kodak), and peak areas of entire bands were integrated. Quantities of proteins cosedimenting with MTs were corrected for the amount of protein sedimenting in the absence of MTs.

Immunoblot Analysis. Polypeptides were prepared from the distal 1- to 2-cm root tips of 3-day-old seedlings of maize (*Zea mays* cv. Funk). Tips were excised, frozen in liquid N_2, ground into a fine powder, and suspended in IB/1 mM dithiothreitol/protease inhibitor mixture/5 mM *N*-ethylmaleimide/*N*-tosyl-L-phenylalanine chloromethyl ketone (25 μg/ml)/N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (25 μg/ml)/phenylmethylsulfonyl fluoride (25 μg/ml). The sample was mixed with an equal volume of hot SDS sample buffer (29), boiled, and clarified by centrifugation. Electrophoretically separated proteins were transferred to a nitrocellulose filter (0.45-μm pore size) (31), which was blocked overnight at 4°C in PBS/10% (wt/vol) nonfat milk/0.05% Tween 20/0.02% NaN_3. Filter strips were incubated overnight at 4°C with a 1:1000 dilution of affinity-purified rabbit IgG to p86 (anti-p86) (32) or a 1:750 dilution of mouse monoclonal IgG to β-tubulin (DM1B) (33) in PBS/1% milk/0.05% Tween 20/0.02% NaN_3. Blots were washed once in PBS/1% milk/0.05% Tween 20 for 10 min and twice in PBS. Horseradish peroxidase-conjugated secondary antibodies (Sigma) were diluted (1:8000 for goat anti-mouse IgG or 1:5000 for goat anti-rabbit IgG) in PBS/1% milk/0.05% Tween 20 and incubated with strips for 1 h at 25°C. Filters were washed in the same solution and bound antibodies were detected by using enhanced chemiluminescence (ECL; Amersham) and exposure to x-ray film.

Immunofluorescence Microscopy. Root tips (1–2 cm) from 3-day-old maize seedlings were excised and fixed for 1 h (23°C) in 4% (wt/vol) paraformaldehyde in IB/0.4 M sorbitol/protease inhibitor mixture. Root tips were washed for three 5-min periods and digested overnight at 4°C with 2% (wt/vol) cellulysin/0.25% pectolyase in the same solution. Root tips were washed for three 5-min periods with 10 mM Tris·HCl, pH 7.4/5 mM EDTA/0.15 M NaCl (TEN) supplemented with protease inhibitor mixture. Cells were dissociated on slide wells coated with poly(L-lysine) (>300 kDa) (1 mg/ml). To adhere cells to the surface and permeabilize membranes without extraction, slides were dried for 1 h at 37°C. Cells were rinsed in distilled water, blocked 15 min in TEN/1% bovine serum albumin, and incubated 1 h at 23°C with a 1:50 dilution of anti-p86 (32) and a 1:750 dilution of DM1B (33). Cells were washed for three 5-min periods and incubated 1 h with 1:50 dilutions of affinity-purified dichlorotriazinylaminofluorescein-conjugated donkey anti-rabbit IgG and affinity-purified tetramethylrhodamine isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). Cells were washed 5 min in TEN and DNA was stained for 5 min with 4′,6′-diamidino-2-phenylindole (0.2 μg/ml). Cells were mounted with Mowiol 4-88 containing 2% (wt/vol) *N*-propyl gallate and photographed with T-Max 400 film (Kodak) under epifluorescence illumination on an Olympus BH-2 microscope.

RESULTS AND DISCUSSION

Structural Similarities of the p86 Subunit of eIF-(iso)4F and a Kinesin-Like Protein. When the sequence of the p86 subunit of wheat germ eIF-(iso)4F and its limited similarity to sea urchin kinesin were reported (19), no plant kinesin sequence was available for comparison. Subsequently, however, the primary structure of a kinesin-like protein from *Arabidopsis thaliana* was deduced from sequencing of the *katA* gene (34). The predicted 89-kDa protein belongs to the *KAR3* family of minus-end-directed kinesin-like proteins that may bundle and slide MTs (34–36). These proteins have a globular head domain with an ATP binding site and a MT binding site in the C terminus, an α-helical rod domain in the central region, and a globular tail domain in the N terminus that may also bind MTs (34–38). Sequences of p86 and the katA protein were aligned using P/C GENE PALIGN programmed with rules for grouping amino acids by their physical and chemical properties and frequency of exchange (39). The p86 and katA proteins have 787 and 793 aa, respectfully, and only eight gaps were used for an alignment. N-terminal positions 1–81 of both proteins have 15% identity and 43% similarity, and C-terminal positions 625–731 of p86 have 15% identity and 41% similarity to C-terminal positions 625–736 of the katA protein. Although p86 has no consensus ATP binding site similar to that of kinesin, the C-terminal region of similarity resides mostly within the MT binding site of the kinesin head domain (35, 37, 38), suggesting that p86 could bind to MTs (data not shown).

Binding of Native eIF-(iso)4F to MTs *in Vitro*. To determine whether native wheat germ eIF-(iso)4F binds to MTs *in vitro*, polymer sedimentation analysis was performed. eIF-(iso)4F was incubated with taxol-stabilized maize MTs and either MgATP or the nonhydrolyzable analogue adenosine 5′-[γ-imido]triphosphate (pNppA) (35, 38). Control samples of eIF-(iso)4F were incubated with MTs and $MgSO_4$ or with buffer alone. Samples were sedimented through a 20% sucrose cushion, and supernatant and pellet fractions were analyzed by SDS/PAGE. Fig. 1 shows that very little eIF-(iso)4F sedimented in the absence of MTs but that the bulk of eIF-(iso)4F sedimented in the presence of MTs, with excess unbound eIF-(iso)4F remaining in supernatants. Unlike kinesin, eIF-(iso)4F binding to MTs was neither reduced by MgATP nor enhanced by pNppA (Fig. 1). Thus, eIF-(iso)4F exhibits ATP-independent binding to MTs.

Quantitative densitometry showed that eIF-(iso)4F bound substoichiometrically to tubulin, with 1 mol of eIF-(iso)4F bound per 5–6 mol of tubulin. Because eIF-(iso)4F is eluted from a calibrated molecular sieving column at ≈375 kDa (40) and has a 1:1 stoichiometry of p86 and p28 subunits with a combined mass of 110 kDa (19), the holoenzyme is predicted

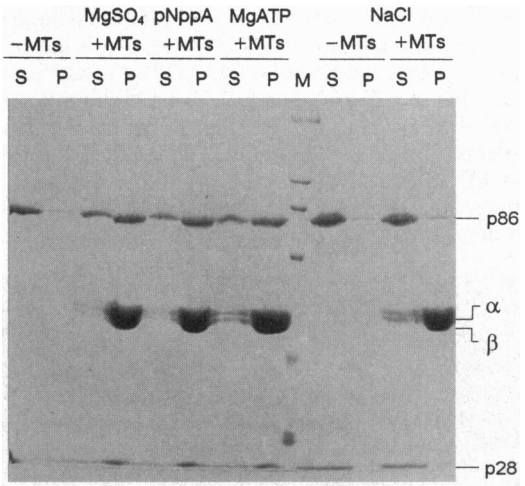

FIG. 1. ATP-independent binding of eIF-(iso)4F to maize MTs. Native eIF-(iso)4F (2.7 μM) was incubated with (+) or without (−) MTs (8 μM tubulin dimer) in the presence of 0.5 mM $MgSO_4$, 0.5 mM pNppA, 0.5 mM MgATP, or 75 mM NaCl, as indicated, and sedimented by centrifugation. Supernatant (S) and pellet (P) fractions were analyzed by SDS/PAGE (8% gels) and Coomassie blue staining. Lane M contains molecular mass marker proteins, and positions of p28, p86, and tubulin α and β subunits are indicated.

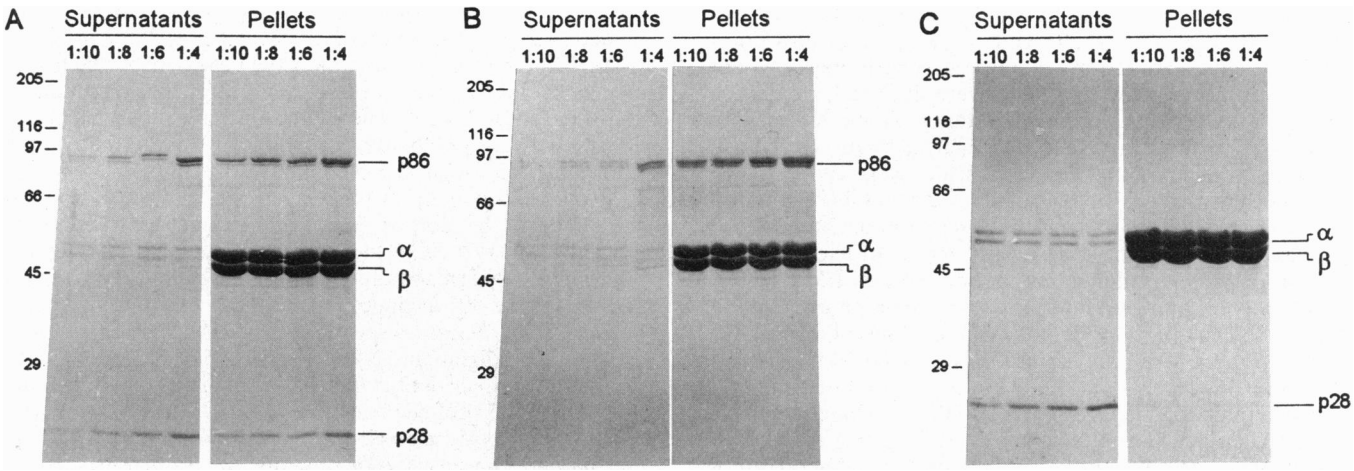

FIG. 2. Saturation binding to MTs of bacterially expressed p86 subunit and p86–p28 complex. Different concentrations (0.4–1.0 μM) of p86–p28 complex (*A*), p86 subunit (*B*), or p28 subunit (*C*) were incubated with MTs (4 μM tubulin dimer) to provide the indicated molar ratios of protein to tubulin dimer and were sedimented by centrifugation. Corresponding supernatant and pellet fractions were analyzed by SDS/PAGE (10% gels) and Coomassie blue staining. To enhance visualization of p28 in *C*, twice as much total protein was loaded in *C* as in *A* or *B*. Positions of molecular mass markers, p28, p86, and tubulin α and β subunits are indicated.

to be a heterohexamer of ≈330 kDa. Thus, a 1:5–6 molar binding stoichiometry of eIF-(iso)4F and polymerized maize tubulin results from the binding of one eIF-(iso)4F molecule per 15–18 dimers on the MT surface.

To determine whether eIF-(iso)4F binding to MTs was dependent upon electrostatic interactions (40), eIF-(iso)4F was incubated with 75 mM NaCl in the presence or absence of MTs and sedimented through a sucrose cushion. Fig. 1 shows that NaCl inhibited eIF-(iso)4F binding to MTs. Because MAP2 binding to plant and animal MTs is little affected by 75 mM KCl (24, 25), eIF-(iso)4F binding to MTs must be of relatively low affinity.

Binding of the p86 Subunit of eIF-(iso)4F to MTs *in Vitro*. To examine the specificity of eIF-(iso)4F subunit binding to MTs, individually purified p86 and p28 subunits expressed in bacteria were used (23). Mixing stoichiometric amounts of p86 and p28 provides a p86–p28 complex with translation initiation activity equal to that of native eIF-(iso)4F (23). Different concentrations of expressed p86, p28, or p86–p28 complex were incubated with taxol-stabilized maize MTs providing 1:10 to 1:4 molar ratios of each protein and tubulin. Samples were sedimented and analyzed by SDS/PAGE. Both the p86–p28 complex and p86 subunit bound to MTs in a concentration-dependent manner (Fig. 2*A* and *B*). The p28 subunit alone did not bind to MTs (Fig. 2*C*). Parallel polymer sedimentation experiments with p86–p28 complex, p86, and p28 showed no effect of MgATP or pNppA on MT binding (data not shown), confirming an absence of kinesin-like activity (35, 38). Quantitative densitometry of pellet lanes from saturated samples (1:4 molar ratios) afforded a binding stoichiometry of 1 mol of p86 per 5–6 mol of tubulin dimer, in the presence or absence of p28. The results indicated that eIF-(iso)4F binding to MTs is mediated by p86, with no detectable steric hindrance by p28.

Bundling of MTs *in Vitro* by the p86–p28 Complex and p86 Subunit. Our preliminary negative-stain EM experiments with native eIF-(iso)4F and taxol-stabilized maize MTs showed the formation of bundles. To examine the role of eIF-(iso)4F subunits on MT bundle formation (38), taxol-stabilized MTs were incubated with concentrations of expressed p86–p28 complex, p86, or p28 subunits that were subsaturating (0.4 μM) or saturating (1 μM) for binding, and samples were negatively stained and examined by EM. Control MT samples were incubated alone or with subsaturating and saturating concentrations of bovine brain MAP2, which binds to plant MTs (24, 25) but does not spontaneously form bundles in solution, prior to centrifugation (24, 41). MTs were haphazardly arranged in the control sample incubated in buffer alone (Fig. 3*A*). The arrangement of MTs with subsaturating p86–p28 complex appeared similar to those in the control sample (data not shown). However, saturating p86–p28 complex induced a moderate amount of MT bundling (Fig. 3*B*). Subsaturating p86 induced some MT bundling but less than that produced by saturating p86–p28 complex (data not shown). Fig. 3*C* shows that saturating p86 caused extensive bundling of MTs. No bundling was observed by either p28 subunit or MAP2 (data not shown). The results summarized in Table 1 showed that p28 reduces the extent of MT bundling *in vitro* by p86.

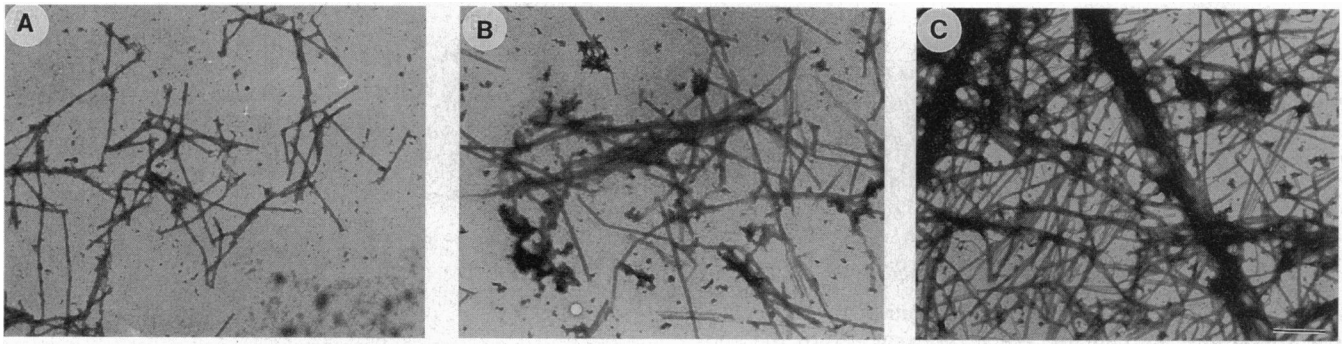

FIG. 3. Bundling of MTs *in vitro* by p86–p28 complex and p86 subunit. Negatively stained samples were from MT (4 μM tubulin dimer) samples containing no added protein (control) (*A*), saturating reconstituted complex (1 μM) (*B*), and saturating p86 subunit (1 μM) (*C*). (Bar = 0.5 μm for *A* to *C*.)

Table 1. Expressed p86–p28 complex and p86 subunit concentration-dependent bundling of microtubules *in vitro*

Protein added to microtubules	Molar ratio of added protein to polymerized tubulin	Relative amount of microtubule bundling*
None	0:1	−
p86–p28	1:10	−
	1:4	++
p86	1:10	+
	1:4	+++
p28	1:10	−
	1:4	−
MAP2	1:10	−
	1:2	−

*Scored qualitatively as none (−), some (+), moderate (++), or extensive (+++).

Association of p86 with Cortical MTs in Root Cells. The p86 subunit of eIF-(iso)4F was localized in maize seedling root tip cells by using double-stain indirect immunofluorescence microscopy. Antibody binding specificity was examined by immunoblot analysis of root tip polypeptides. Rabbit anti-p86 antibody bound to the 86-kDa polypeptide of eIF-(iso)4F (Fig. 4, lane A), and mouse DM1B antibody against β-tubulin bound to the ≈50-kDa β subunit of tubulin (Fig. 4, lane B). Neither antibody crossreacted with the other polypeptide.

Immunofluorescence microscopy showed three forms of p86 in the cytosolic compartment of all dividing and differentiating root tip cells: fine particles, coarse particles, and linear patches that were 1.4 ± 0.5 μm long (n = 38). Fine and coarse particles of p86 were preserved when fixed cells were extracted with methanol or Nonidet P-40, but linear patches were destroyed by these treatments, indicating that they are membrane-associated. Fig. 5 shows a focal plane through the upper cytosolic cortex of a portion of a permeabilized unextracted interphase cell. MTs subtending the plasma membrane were arranged in parallel bundles (Fig. 5*A*), and coarse particles and linear patches of p86 were colocalized or closely associated with MT bundles or lodged between MTs of a given bundle (Fig. 5*B*). All root cells had both fine and coarse particles of p86, whereas linear patches were found in the cortex of ≈5% of cells, most of which were in interphase and had transverse MT bundles similar to those described in cortex cells (42). No particles or linear patches were seen in control samples that omitted anti-p86 antibody (data not shown).

Unextracted cells often had low fluorescence signal-to-noise ratios, which interfered with our attempts to superimpose images of MTs and p86. However, computer-based processing of digital images of negatives gave much better resolution. The example shows a given focal plane through the upper cortex of an interphase cell containing MT bundles (Fig. 6*A*) and numerous large particles and linear patches of p86 (Fig. 6*B*) coaligned with cortical MTs. Image superimposition showed that among 49 resolved p86 structures, 10% were not associated with MTs and 90% were precisely colocalized or closely associated with MTs (Fig. 6*C*).

Our results indicate that the p86 subunit of eIF-(iso)4F is a MAP that shares certain structural and functional character-

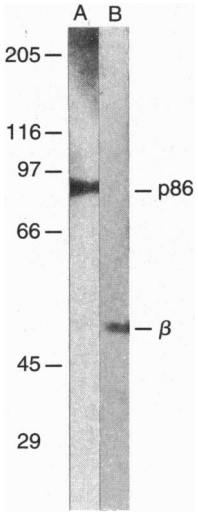

FIG. 4. Immunoblot analysis of the p86 subunit of eIF-(iso)4F in maize root polypeptides. Nitrocellulose filters blotted with electrophoretically separated whole cell polypeptides from maize seedling root were probed with anti-p86 antibody (lane A) and DM1B antibody (lane B). The signal at the top of lane A was an artifactual smear of materials extending from the well of the stacked gel and was not a polypeptide band. Positions of molecular mass marker proteins, p86, and β subunits of tubulin are indicated.

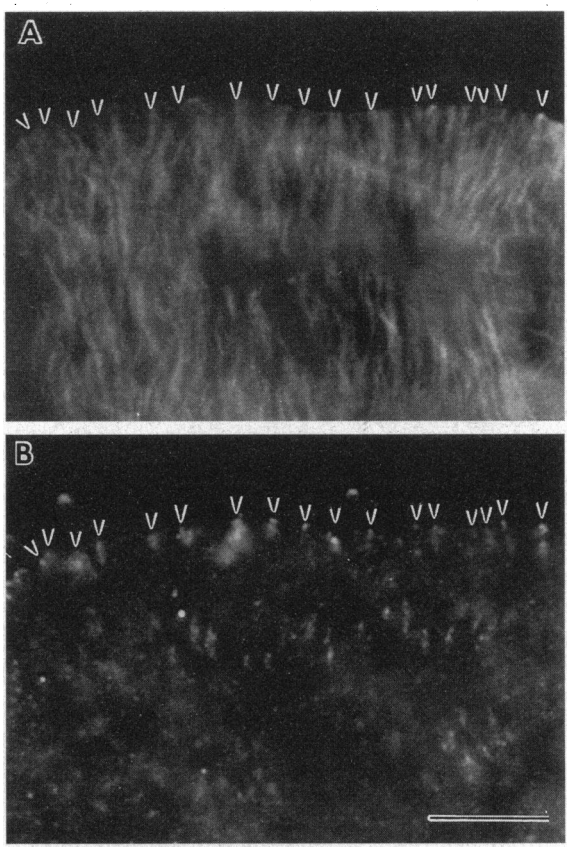

FIG. 5. Immunofluorescence localization of cortical MTs and p86 coarse particles and linear patches. Micrographs were taken at the same focal plane through the upper surface of a maize root interphase cell and reveal MTs stained with DM1B antibody (*A*) and p86 particles and linear patches stained with anti-p86 antibody (*B*). The positions of p86 staining near the cell edge are indicated in *B* and their positions relative to MTs are also indicated in *A*. (Bar = 10 μm for *A* and *B*.)

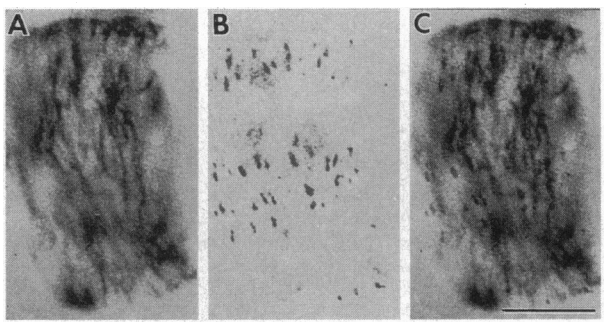

FIG. 6. Immunofluorescence localization of cortical MTs and p86 linear patches. Computer-enhanced digital images of negatives of a maize root cell show MT bundles (*A*) and p86 linear patches (*B*) in the same focal plane. Images are superimposed in *C*. Image contrast was enhanced by subtraction of signals from p86 fine particles, and doubly fluorescent markers were used to precisely superimpose the images for on-screen viewing. (Bar = 10 μm for *A–C*.)

istics of kinesin and fibrous MAPs. eIF-(iso)4F has the following similarities to kinesin. (*i*) It is composed of large and small subunits (35, 38). (*ii*) The large subunit, but not the small subunit, binds to MTs (35, 38). (*iii*) The large subunit has a molecular mass (86 kDa) similar to that of an *Arabidopsis* kinesin-like protein (89 kDa) (19, 34). (*iv*) The large subunit has limited primary sequence similarities to an *Arabidopsis* kinesin-like protein, including in the putative N- and C-terminal MT binding sites (19, 34, 35–38). (*v*) The large subunit is immunolocalized in plant cells to punctate structures coaligned, colocalized, and closely associated with MTs (43). The large subunit of eIF-(iso)4F has the following similarities to fibrous MAPs: (*i*) It binds substoichiometrically to tubulin in MTs (24, 25, 38, 41). (*ii*) It binds to MTs in an ATP-independent manner. (*iii*) It has a relatively low affinity for MTs, like MAP1B (44).

MAPs or MAP-like structures have been implicated in ribosome binding to MTs in rat neurons (45) and from sea urchin eggs (46), and a subset of mRNAs, polyribosomes, and poly(A)-binding proteins copurify with MTs from sea urchin embryos (47). Because p86 presumably binds to the 40S ribosomal subunit during translation initiation, fine particles and coarse particles of p86 in maize cells may represent p86 bound to 40S subunits and 80S ribosomes, respectively. However, because eIF-(iso)4F binding to MTs *in vitro* is mediated by the p86 subunit, with little or no steric hindrance by the p28 subunit that binds the m^7G cap of mRNAs, eIF-(iso)4F may anchor certain mRNAs to cortical MTs. The destruction of linear patches by membrane-disrupting agents suggests that this form of p86 may attach cortical tubular elements of rough endoplasmic reticulum to MTs (48). Thus, the p86 subunit of eIF-(iso)4F may simultaneously link components of the translational machinery to MTs and regulate the disposition of MTs.

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