

The Microtubule Plus-End Proteins EB1 and Dynactin Have Differential Effects on Microtubule Polymerization

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Several microtubule-binding proteins including EB1, dynactin, APC, and CLIP-170 localize to the plus-ends of growing microtubules. Although these proteins can bind to microtubules independently, evidence for interactions among them has led to the hypothesis of a plus-end complex. Here we clarify the interaction between EB1 and dynactin and show that EB1 binds directly to the N-terminus of the p150^{Glued} subunit. One function of a plus-end complex may be to regulate microtubule dynamics. Overexpression of either EB1 or p150^{Glued} in cultured cells bundles microtubules, suggesting that each may enhance microtubule stability. The morphology of these bundles, however, differs dramatically, indicating that EB1 and dynactin may act in different ways. Disruption of the dynactin complex augments the bundling effect of EB1, suggesting that dynactin may regulate the effect of EB1 on microtubules. In vitro assays were performed to elucidate the effects of EB1 and p150^{Glued} on microtubule polymerization, and they show that p150^{Glued} has a potent microtubule nucleation effect, whereas EB1 has a potent elongation effect. Overall microtubule dynamics may result from a balance between the individual effects of plus-end proteins. Differences in the expression and regulation of plus-end proteins in different cell types may underlie previously noted differences in microtubule dynamics.

INTRODUCTION

The microtubule network in the cell is highly dynamic. Microtubules grow and retract, continually probing the cellular environment. Several proteins including CLIP-170, APC, EB1, and dynactin have been localized to the plus-ends of growing microtubules (reviewed in Tirnauer and Bierer, 2000). Evidence for interactions among these plus-end proteins (Su *et al.*, 1995; Berrueta *et al.*, 1999; Valetti *et al.*, 1999; Vaughan *et al.*, 1999) has led to the hypothesis of a microtubule plus-end complex (Schroer, 2001). It is not clear, however, if such a complex exists in vivo and if it does, whether all plus-end proteins are obligate members of the complex. All plus-end proteins thus far identified have microtubule-binding domains and may, therefore, be independently targeted to microtubules. But interactions among plus-end proteins may also allow one protein to recruit others to the complex. Specific binding interactions among plus-end proteins and between plus-end proteins and mi-

croto- tubules remain unclear, as does the temporal sequence with which plus-end proteins are recruited to microtubules.

The specific functions of individual plus-end proteins as well as that of a plus-end complex also remain unclear. Plus-end proteins are perfectly positioned to regulate microtubule growth and dynamics as well as to act as sensors to mediate interactions between probing microtubules and potential targets. EB1 has been suggested to play a role in microtubule dynamics (Tirnauer *et al.*, 1999; Rogers *et al.*, 2002), but the precise nature of that role remains unclear. In addition, it has been suggested that EB1 may only act in concert with APC (Nakamura *et al.*, 2001).

One potential target for probing microtubule plus-ends is the cell cortex, in particular specific cortical domains such as migrating edges or areas of contact with the other cells or the matrix. For example, interactions between microtubule plus-ends and the cell cortex are thought to be important for the proper orientation and function of the mitotic spindle (Schuyler and Pellman, 2001). During mitosis in budding yeast, Bim1, the yeast EB1 homologue is localized to the tips of astral microtubules. When growing microtubules reach the bud cortex, Bim1 interacts with the cortical protein Kar9. This interaction tethers the microtubules and is critical for correct spindle positioning and the movement of the nucleus to the bud neck (Adames and Cooper, 2000; Korinek *et al.*,

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2000; Lee *et al.*, 2000; Miller *et al.*, 2000). A second step involving the microtubule motor dynein and its accessory complex dynactin is thought to be involved in the subsequent movement of the nucleus into the bud neck (Carniati and Stearns, 1997; Adames and Cooper, 2000).

Although these two steps are genetically separable in yeast, there is evidence suggesting that EB1 may interact more directly with components of the dynein and/or dynactin complexes in higher eukaryotes (Berrueta *et al.*, 1999), but the precise nature and function of this interaction was unclear. Here we have investigated the interaction between EB1 and dynein and dynactin and shown that EB1 binds directly to the p150^{Glu} subunit of dynactin. EB1 and p150^{Glu} each seem to play a role in microtubule dynamics and/or stability *in vitro* and *in vivo*, but these roles are distinct. EB1 appears to have a potent microtubule elongation effect, whereas p150^{Glu} appears to have a potent microtubule nucleation effect. These data suggest that the balance between EB1 and p150^{Glu} may regulate the state of microtubule dynamics within the cell, and regulatory differences between cell types may account for differences seen in microtubule organization and dynamics.

MATERIALS AND METHODS

Generation of EB1 and Dynactin cDNA Constructs

The EST for human EB1 (AAI285786) was obtained from ATCC and subcloned into pGEX(6p-2) (Amersham Pharmacia Biotech, Piscataway, NJ), pEGFP-N1 (Clontech, Palo Alto, CA), pDsRed2-N1 (Clontech), and pET-15b (Novagen, Madison, WI). The p150^{Glu} and p135 human brain cDNA library clones were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA; Tokito *et al.*, 1996) and p150^{Glu} deletion constructs pMT7-1 (aa 237-1255) and pMT1-1 (aa 345-1255) were subcloned into pBluescript SK⁻ (Stratagene, La Jolla, CA). To make a GST-p150 fusion protein, p150 MTB-DICB (aa 8-566) from rat full-length cDNA clone (Holzbaur *et al.*, 1991) was subcloned into pGEX-6p-1 (Pharmacia). pET21a-p150[1-330]wt was a gift from Kevin Vaughan (Vaughan *et al.*, 2002). Other dynactin constructs were made as previously described: Arp1 (Holleran *et al.*, 1996), p50/dynamitin (LaMonte *et al.*, 2002), p62 (Karki *et al.*, 2000), and p22 (Karki *et al.*, 1998).

Antibodies

Affinity-purified rabbit polyclonal antibodies to dynactin subunits p150^{Glu} (Tokito *et al.*, 1996), Arp1 (Holleran *et al.*, 1996), p62 (Karki *et al.*, 2000), p50/dynamitin (Tokito *et al.*, 1996), and p22 (Karki *et al.*, 1998) were produced as previously described. An additional polyclonal antibody to p150^{Glu} was generously provided by Kevin Vaughan (University of Notre Dame). Monoclonal antibodies to EB1 (E4620 from Transduction Laboratories), tubulin (YL1/2 from Serotec and DM1A from Sigma), p150^{Glu} (P41920 from Transduction Laboratories, Lexington, KY), dynamitin (D74620 from Transduction Laboratories), and dynein IC (MAB1618 from Chemicon, Temecula, CA) were obtained commercially. Peroxidase-conjugated secondary antibodies were obtained from Jackson Immunochemicals (West Grove, PA) and Alexa 350-, 488-, and 594-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR).

Dynein Purification from Rat Brain

Frozen rat brains (Pel Freez, Rogers, AZ) were dounce homogenized at a 1:1 wt/vol ratio in ice-cold PHEM with 10 mM NaCl (50 mM Na-PIPES, 50 mM Na-HEPES, 1 mM EDTA, 2 mM MgCl₂, pH 6.9) containing leupeptin (10 μg/ml), pepstatin (1 μg/ml), *n*-tosyl-L-

arginine methylester (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, and the resulting homogenate was clarified by centrifugation at 39,000 × *g* for 20 min. The supernatant was further clarified by high-speed centrifugation (135,000 × *g*, 1 h) to obtain cytosol. Cytoplasmic dynein and dynactin complexes were obtained by microtubule affinity followed by ATP release essentially as described by (Paschal *et al.*, 1991). ATP extracts containing dynein and dynactin were then dialyzed at 4°C into the appropriate buffer for use in affinity experiments.

Purification of Recombinant Proteins

GST, GST-EB1, GST-p150, and pET-EB1 His-tagged recombinant proteins were expressed in *Escherichia coli* strain BL21, induced with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG). Cells were harvested by centrifugation and pellets were resuspended in one tenth volume phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM KH₂PO₄, pH 7.3) with 1 mg/ml lysozyme, lysed by freeze/thaw at -80°C, incubated with 1 mg/ml DNase and RNase, and sonicated 3 × 15 s if necessary, and lysates were clarified by centrifugation at 39,000 × *g*. GST-tagged protein-soluble supernatants were filtered with a 25-mm Aerodisc syringe filter of 0.45 μm (Gelman Laboratories, East Hills, NY), loaded onto glutathione Sepharose 4B beads (Amersham Pharmacia Biotech), and washed extensively with phosphate-buffered saline. Proteins were eluted with modified glutathione elution buffer (20 mM reduced glutathione, 50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, pH 8.0) and dialyzed into the appropriate buffer. When necessary the GST group was removed from recombinant EB1 and p150^{Glu} proteins using on-column cleavage by PreScission Protease (Amersham Pharmacia Biotech) as described in the product literature. pET21a-p150[1-330]wt was expressed in Rosetta Blue (DE3) cells (Novagen) and induced with 1 mM IPTG. Cells were harvested by centrifugation and lysed using a French Pressure Cell Press. His-tagged recombinant proteins were purified on a Ni²⁺ affinity column. Purified proteins were eluted with imidazole and transferred to the appropriate buffer by dialysis or a PD-10 Desalting column (Amersham Pharmacia Biotech).

Affinity Chromatography

ATP extracts containing dynein and dynactin or purified recombinant protein, with 0–0.5% Triton X-100, were loaded onto the appropriate affinity matrix and incubated for 10–15 min at 25°C. The columns were washed extensively with phosphate-buffered saline, and specifically retained proteins were eluted with modified glutathione elution buffer. Fractions were collected and resolved by SDS-PAGE, transferred to Immobilon-P (Millipore, Bedford, MA), blocked using 5% nonfat milk in Tris-buffered saline, pH 8.0, 0.05% IGEPAL, and 0.05% sodium azide, and probed with the appropriate antibody detected by Renaissance Western blot chemiluminescence reagent (NEN, Boston, MA; Karki and Holzbaur, 1995).

In Vitro Transcription/Translation

The Promega TNT T7 Quick and T3 coupled transcription/translation systems (Promega, Madison, WI) were used to express dynactin constructs. TNT T7 Master Mix, [³⁵S]methionine, and 1 μg of cDNA were incubated at 30°C for 1–1.5 h. *In vitro* synthesized proteins were loaded onto GST or GST-EB1 column or batch-bound beads and incubated for 10–15 min at 25°C. Columns were washed extensively with phosphate-buffered saline containing 0–0.5% Triton X-100 and eluted with glutathione elution buffer or denaturing buffer (2% SDS and 5% β-mercaptoethanol). Fractions were analyzed by SDS-PAGE and autoradiography (Holleran *et al.*, 2001).

Cell Culture, Immunocytochemistry, and Transient Transfection

PtK2 epithelial cells or Rat2 fibroblasts were seeded onto 18 × 18-mm square or 40-mm round glass coverslips. Cells for immuno-

cytochemistry were grown to ~75% confluency, rapidly fixed in -20°C 100% methanol with 1 mM EGTA for 10 min, and then processed for immunocytochemistry as previously described (Karki *et al.*, 1998). Cells for transient transfection experiments were grown to ~35% confluency and transfected with the appropriate cDNA using the lipid-mediated transfection reagent Eugene (Roche, Indianapolis, IN). Forty-eight hours after transfection, cells were fixed and prepared for immunocytochemistry as described above. Fluorescent images were acquired with an Orca ER CCD camera (Hamamatsu, Bridgewater, NJ) controlled by OpenLab image acquisition software (Improvision, Lexington, MA).

To investigate the effects of nocodazole and cold on EB1-induced microtubule bundles, cells were either treated with nocodazole (5 μ g/ml) for 30–60 min at 37°C or placed at 4°C for 60 min. Cells were then immediately fixed in cold methanol and processed for immunocytochemistry as above. To assess the effect of disruption of the dynactin complex on EB1-induced bundles, cells were transfected with both EB1 and the p50 subunit of dynactin. Fifty cells from each of three different experiments (150 cells total) were scored for the expression level of EB1 (high or low), p50 (high or low) and presence or absence of microtubule bundles.

Tubulin Purification

Tubulin was isolated from porcine brain by three cycles of assembly/disassembly, phosphocellulose ion exchange purification, and glutamate cycling and then aliquoted and stored at -80°C (Vasquez *et al.*, 1997). No microtubule-associated proteins were detectable by Coomassie staining of heavily loaded SDS-PAGE gels. Additional bovine tubulin, porcine tubulin, rhodamine- and fluorescein-labeled tubulin were purchased from Cytoskeleton (Denver, CO).

MT Assembly Assay and MT Pelleting

Purified tubulin and recombinant proteins were thawed and clarified by centrifugation before use. Assembly assays were carried out in PHEM with 50 mM NaCl containing 1 mM MgGTP, 16 μ M tubulin (1:40–1:150 rhodamine-labeled to unlabeled tubulin), 2.4 μ M p150[1–330]wt and/or 2.4 μ M pET-EB1 in the presence or absence of 1–100 μ g of microtubule seeds in an 80- μ l reaction volume. After incubation, reactions were centrifuged at 39,000 \times g and gel samples were made of the supernatant and pellet fractions. Samples were analyzed by SDS-PAGE and either scanned with a Molecular Dynamics phosphorimager (Sunnyvale, CA) or analyzed with Coomassie blue staining. Alternately, after assembly microtubules were fixed in 1% glutaraldehyde, pelleted onto poly-L-lysine-coated coverslips (Evans *et al.*, 1985), and processed for immunocytochemistry as previously described (Karki *et al.*, 1998).

Microtubule seeds were prepared by polymerization of tubulin with 20 μ M paclitaxel (Cytoskeleton), 1 mM MgGTP, and incubated at 37°C for 10 min. Microtubules were pelleted at 39,000 \times g, resuspended to 10 μ g/ μ l in PHEM with 50 mM NaCl, and then sheared with a 26G needle. Microtubule assembly was performed in a 125- μ l reaction volume in a water-jacketed cuvette at 37°C measuring absorbance at 350 nm using a UV-160 spectrophotometer (Shimadzu, Columbia, MD).

RESULTS

EB1 Binds Directly to the p150^{Glued} Subunit of Dynactin

Several proteins, including EB1, p150^{Glued}, and APC have been localized to the plus-ends of growing microtubules, and evidence for interactions among these plus-end proteins has led to the hypothesis of a microtubule plus-end complex (Schroer, 2001). EB1 has been reported to interact with components of the dynein and/or dynactin complexes, but the

precise nature of this interaction was unclear (Berrueta *et al.*, 1999). To explore this interaction further, we used affinity chromatography. A GST-EB1 fusion protein was bound to glutathione Sepharose beads and an extract of ATP-releasable microtubule-binding proteins from rat brain cytosol was applied to the column. This ATP extract is highly enriched in both dynein and dynactin (Paschal *et al.*, 1991). Fractions were then analyzed by gel electrophoresis and Western blotting with antibodies to subunits of dynein and dynactin. Dynein was not retained on the GST-EB1 column, but subunits of dynactin were retained, suggesting that EB1 interacts specifically with dynactin, but not dynein (Figure 1A). An alternatively spliced isoform of the p150^{Glued} subunit of dynactin that does not contain the amino terminal microtubule-binding domain (p135) is also expressed in brain (Tokito *et al.*, 1996). Strikingly, p135 was not retained on the GST-EB1 column, suggesting that only a subset of dynactin may interact with EB1 (Figure 1A).

To determine the specific dynactin subunit that interacts with EB1, we generated [³⁵S]methionine-labeled p150^{Glued}, Arp1, p62, dynamitin (p50), and p22 subunits using a reticulocyte lysate *in vitro* transcription/translation system (Promega). Only p150^{Glued} bound to GST-EB1 (Figure 1B), suggesting that EB1 interacts with the dynactin complex through p150^{Glued}.

Because EB1 interacts with p150^{Glued}, but not p135, we hypothesized that the site of interaction may be at the N-terminus of p150^{Glued}. The microtubule-binding site of p150^{Glued} is also near the N-terminus and the proximity of these two sites of interaction raises the possibility of cooperative binding interactions between p150^{Glued}, EB1, and microtubules. To identify the EB1 binding domain, we generated a series of N-terminal deletion constructs of p150^{Glued}, synthesized [³⁵S]methionine-labeled peptides, and applied the peptides to the GST-EB1 column. Only the full-length p150^{Glued} construct showed appreciable binding to the column in comparison to the GST control column. Constructs missing the N-terminal domain showed little interaction with EB1, confirming that the site of interaction is near the N-terminus (Figure 2A).

The interaction between EB1 and p150^{Glued} observed in these assays may not, however, be direct, as other proteins in cytosol or the reticulocyte lysate could mediate the interaction. To determine if the two proteins can interact directly, we isolated and purified a recombinant fragment of p150^{Glued} consisting of the first 330 amino acids and applied it to a GST-EB1 column. Recombinant p150^{Glued}[1–330] was specifically retained on the GST-EB1 column, indicating that the two proteins can interact directly (Figure 2B). The converse experiment was also performed and showed that recombinant EB1 bound to a GST-p150^{Glued} column (unpublished data).

EB1 and p150^{Glued} Exhibit Different Patterns of Microtubule Localization

Immunocytochemistry shows that EB1 and p150^{Glued} exhibit different patterns of microtubule localization and, further, that these patterns may vary with cell type. In cultured PtK2 epithelial cells, EB1 is localized to a discrete point at the most distal tip of the plus-end of the microtubule (Figure 3A). In cultured Rat2 fibroblasts, however, EB1 exhibits a more comet-tail expression pattern with a bright spot at the tip

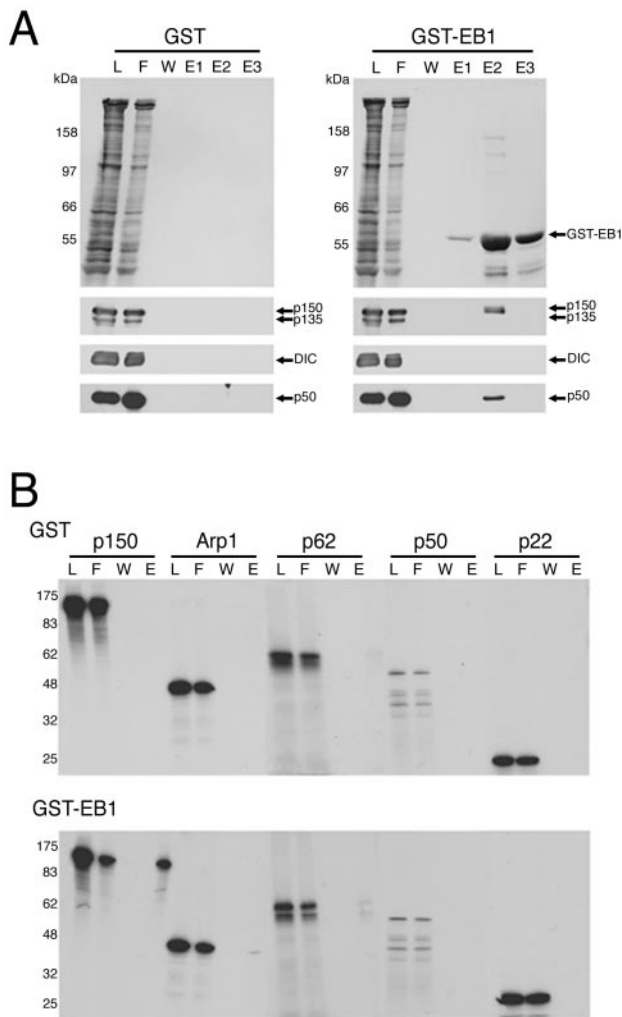


Figure 1. EB1 interacts with the p150^{Glued} subunit of dynactin. (A) Affinity chromatography of ATP extract from rat brain microtubules over GST-EB1 and GST control columns resulted in the binding of dynactin but not dynein to GST-EB1. The top panel was stained for total protein, and the bottom panels show Western blots. Dynactin subunits p150^{Glued} and p50 as well as Arp1 and p62 (unpublished data) were retained by GST-EB1 but not by GST alone. The p135 isoform was not retained by GST-EB1, suggesting p150^{Glued} binds EB1 through its amino-terminal domain. Dynein IC (DIC) was not retained on the GST-EB1 column. (B) Affinity chromatography of in vitro-translated dynactin subunits over GST-EB1 and GST control columns showed that the dynactin subunit p150^{Glued} bound to GST-EB1 but not to GST alone. Dynactin subunits Arp1, p62, p50, and p22 did not bind significantly to either GST or GST-EB1.

and tapering label that extends 1–2 μm toward the minus-end of the microtubule (Figure 3B). This comet-tail pattern of EB1 labeling has also been seen in another fibroblast-like cell line (COS-7; Morrison *et al.*, 1998) and in a fibrosarcoma cell line (HT 1080; Juwana *et al.*, 1999).

p150^{Glued} is more broadly distributed in both PtK2 and Rat2 cells (Figure 3, C and D). Consistent with the essential role of dynactin in dynein-mediated vesicular transport

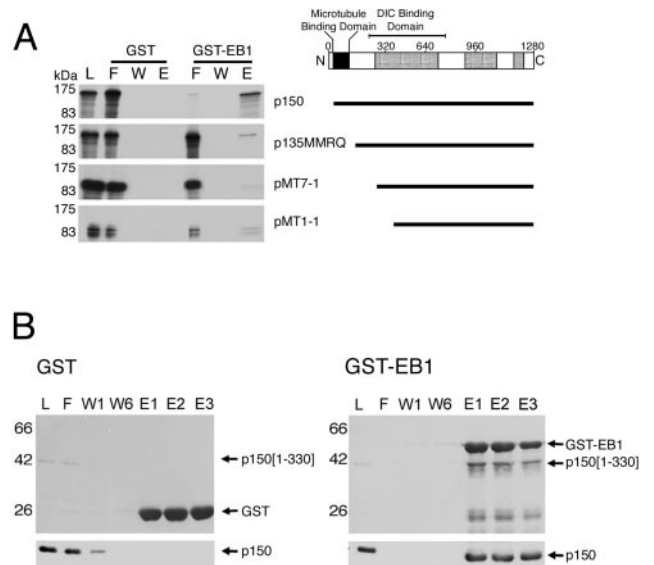


Figure 2. EB1 binds directly to the N-terminal domain of p150^{Glued}. (A) In vitro-translated full-length p150^{Glued} was retained on a GST-EB1 column, but amino-terminal p150^{Glued} deletion constructs p135MMRQ, pMT7-1, and pMT1-1 did not bind significantly to GST-EB1. (B) A recombinant amino-terminal fragment of p150^{Glued} (residues 1–330) bound to a GST-EB1 column, but not significantly to a GST control column. The top panel was stained for total protein, and the bottom panel was blotted with an anti-p150^{Glued} antibody. This result shows EB1 interacts directly with the amino-terminal domain of p150^{Glued}, which also includes the microtubule-binding motif.

(Waterman-Storer *et al.*, 1997), p150^{Glued} shows a punctate “vesicular” distribution in the cytoplasm of both cell types. A population of the protein is also localized to microtubules in both cell types, but the pattern of this localization differs strikingly between the two. In Rat2 cells, p150^{Glued} is prominently localized to microtubule plus-ends with a comet-tail like distribution (Figure 3D), similar to that previously reported in COS-7 fibroblasts (Vaughan *et al.*, 1999). In PtK2 epithelial cells, however, p150^{Glued} decorates microtubules, but is not limited to the plus-ends. Rather, labeling is distributed along the shaft of the microtubule (Figure 3C).

Overexpression of both EB1 and p150^{Glued} Induces Microtubule Bundling

Although the localization of endogenous EB1 is restricted to the plus-ends of microtubules, overexpression of recombinant EB1 suggests that the distribution of the protein depends on the level of expression. We and others have observed that at low expression levels, exogenous EB1 mimics the distribution of the endogenous protein and is concentrated at microtubule tips, but at higher levels of expression, exogenous EB1 can decorate the entire microtubule array (unpublished data; Mimori-Kiyosue *et al.*, 2000; Bu and Su, 2001). At very high levels of EB1 expression, the microtubules become bundled and EB1 is intensely localized to the bundles (Figure 4A). Often these EB1-microtubule bundles

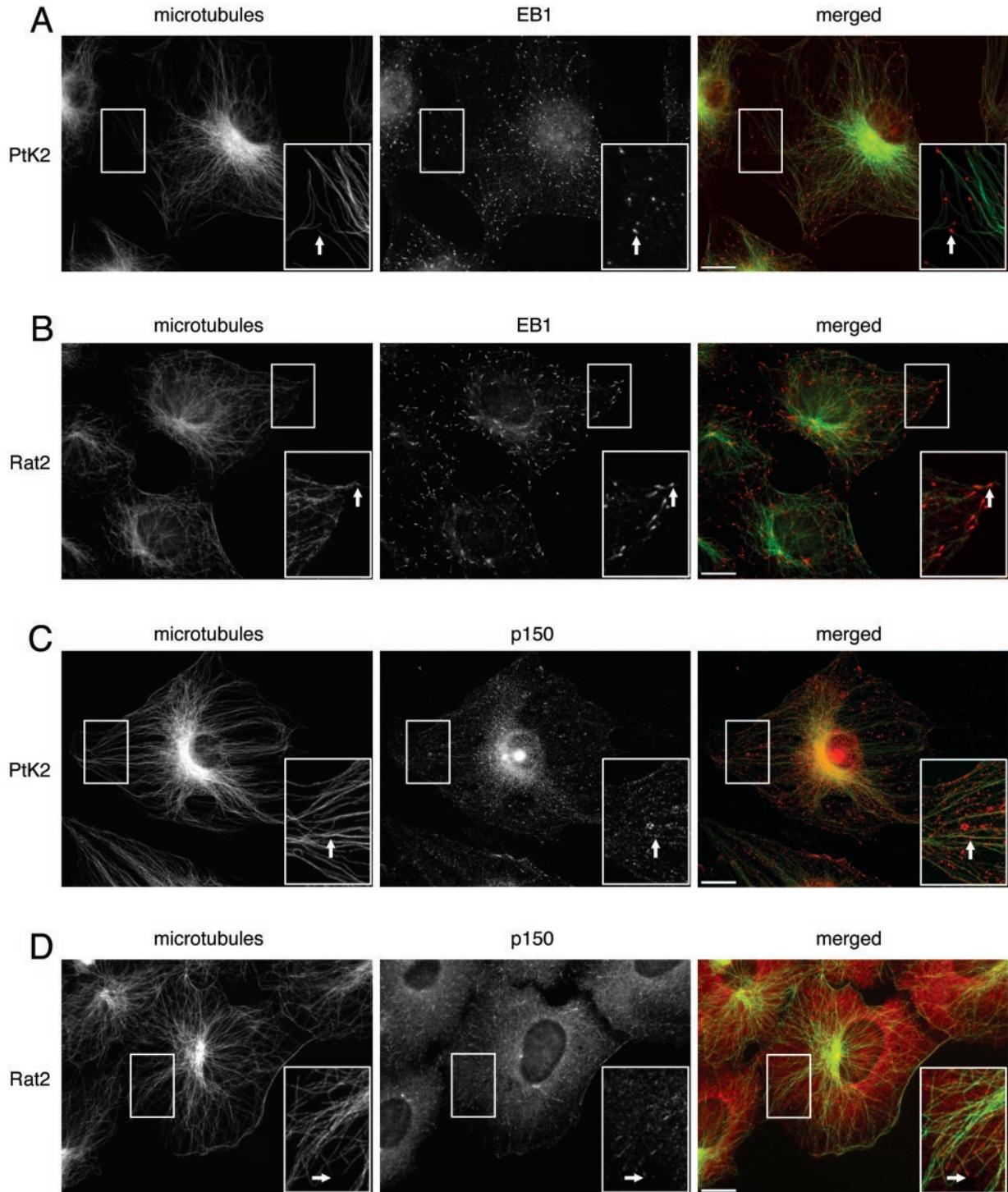


Figure 3. EB1 and p150^{Glued} both localize to microtubules in epithelial cells and fibroblasts, but the pattern of localization differs. For each panel, the individual labels are shown in black and white, whereas the overlays are shown in color. The area in the box is shown magnified in the inset. Scale, 10 μ m. (A) Cultured PtK2 epithelial cells labeled with antibodies to EB1 and tubulin. Arrows show EB1 (red) localized to discrete points at the plus ends of microtubules (green). (B) Cultured Rat2 fibroblasts labeled with antibodies to EB1 and tubulin. Arrows show EB1 (red) localized to comet-tails at the plus-ends of microtubules (green). (C) PtK2 cells labeled with antibodies to p150^{Glued} and tubulin. Arrows show microtubule (green) decoration by p150^{Glued} (red), but decoration was not limited to microtubule tips. (D) Rat2 cells labeled with antibodies to p150^{Glued} and tubulin. Arrows show p150^{Glued} labeling (red) was localized to comet tails at the plus-ends of microtubules (green).

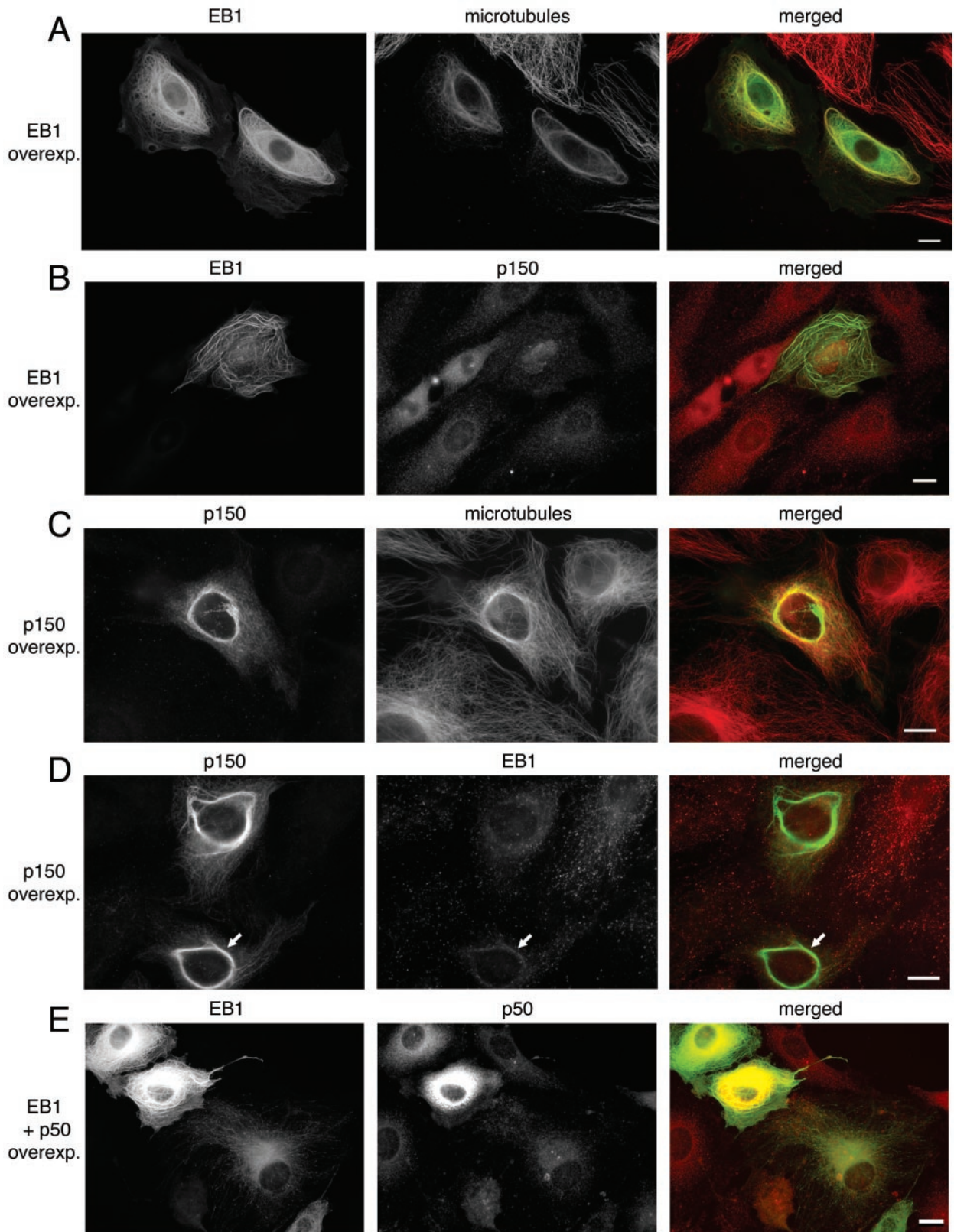


Figure 4.

are very long and loop throughout the cell. Some EB1-microtubule bundles grow very large and encircle the cell. Similar results were obtained with transient transfection of cDNAs for a GFP-EB1 fusion protein (Figure 4A), a DsRed2-EB1 fusion protein (unpublished data), and EB1 without a fluorescent tag (unpublished data).

It has previously been reported that microtubule bundles induced by the overexpression of EB1 are very stable (Bu and Su, 2001). In these experiments, we saw that EB1-microtubule bundles persisted after a 60-min incubation with the microtubule-depolymerizing drug nocodazole (Figure 5A) or a 60-min incubation at 4°C (unpublished data), suggesting that these bundles are both drug- and cold-stable.

Overexpression of p150^{Glued} has also been shown to bundle microtubules (Waterman-Storer *et al.*, 1995). However, the microtubules bundled by p150^{Glued} look very different from those bundled by the overexpression of EB1. p150^{Glued}-microtubule bundles are generally short extensions from the centrosome. In some cells, thick bundles encircle the nucleus, but rarely do these bundles extend toward the periphery of the cell (Figure 4C). p150^{Glued}-microtubule bundles are also very stable and resist nocodazole treatment (Figure 5C and see Waterman-Storer *et al.*, 1995).

A small population of highly stable microtubules in PtK2 cells resists both cold and drug treatment. In most cells, this population consists of a few microtubules emanating from the centrosome, but some cells have a population of stable microtubules that appears to be noncentrosomal. These noncentrosomal stable microtubules, shown here in nocodazole-treated cells, are highly decorated with EB1, whereas the centrosomal stable microtubules are not (Figure 5B). p150^{Glued} was not observed to be associated with either population of stable microtubules in this cell type (unpublished data).

EB1 Does Not Recruit Dynactin to Microtubules, But p150^{Glued} May Recruit EB1 to Microtubules

Because we had observed a direct interaction between EB1 and p150^{Glued} *in vitro*, we sought to determine if p150^{Glued} or other dynactin components were recruited to the microtubule bundles induced by EB1 overexpression and if EB1 was recruited to bundles induced by p150^{Glued} overexpression. Immunocytochemistry with several different antibodies to p150^{Glued} (monoclonal and polyclonals; one polyclonal is shown in Figure 4B) and antibodies to the p62, p22,

dynamitin (p50), and Arp1 (unpublished data) subunits of dynactin showed that little or no dynactin was present on EB1-microtubule bundles, suggesting that EB1 does not recruit dynactin to microtubules.

Immunocytochemistry with an antibody to EB1 shows some recruitment of EB1 to p150^{Glued}-microtubule bundles, but these results were variable. Some bundles showed prominent EB1 recruitment (unpublished data), but most showed a weak recruitment (Figure 4D). These data suggest that although p150^{Glued} may recruit EB1 to microtubules, this process is likely to be tightly regulated within the cellular environment.

Dynactin May Regulate the Function of EB1

Overexpression of the dynamitin subunit of dynactin has been shown to disrupt the dynactin complex and interfere with its functions (Echeverri *et al.*, 1996). To determine if the disruption of dynactin affects the function of EB1, we transiently transfected cells with cDNAs for both dynamitin and EB1. We qualitatively scored the level of expression (low or high) of each protein (EB1 and dynamitin) in 150 cells (50 cells from each of three separate experiments) and correlated these data with the presence or absence of microtubule bundles. Thirty-three percent of cells with high levels of exogenous EB1 but low levels of dynamitin had bundles (7/21), but 68% of cells with high levels of both EB1 and dynamitin had bundles (21/31; Figure 4E). Dynamitin alone does not induce microtubule bundling in these cells (unpublished data), suggesting that the disruption of the dynactin complex enhances the bundling effect of EB1.

p150^{Glued} and EB1 Have Different Effects on Microtubule Polymerization *In Vitro*

Because both EB1 and p150^{Glued} appear to affect microtubule polymerization and/or stability within the cell, we tested the effects of these two proteins in *in vitro* microtubule polymerization and sedimentation assays. First, we polymerized tubulin either alone, in the presence of recombinant EB1, in the presence of recombinant p150^{Glued} or in the presence of both EB1 and p150^{Glued}. Under these conditions (no seeds or other nucleation factors), tubulin alone polymerizes very inefficiently. A small amount of polymerized microtubules was seen in the pellet after centrifugation, but most of the tubulin remained in the supernatant (Figure 6). The addition of EB1 did not significantly increase the extent of microtubule polymerization, but the addition of p150^{Glued} dramatically enhanced polymerization. The addition of the two proteins in combination, however, had no effect beyond that of p150^{Glued} alone (Figure 6).

Tubulin polymerizes more efficiently in the presence of nucleating microtubule seeds. To examine the effect of EB1 and p150^{Glued} on microtubule polymerization in the absence and in the presence of seeds, we used a spectrophotometric light scattering assay. In the absence of microtubule seeds, the addition of either recombinant EB1 or p150^{Glued} alone was very similar to that seen with the microtubule pelleting assay. The addition of EB1 increased light scattering only slightly over that seen with tubulin alone, whereas the addition of p150^{Glued} caused a significant increase in light scattering (Figure 7A). In this assay, the addition of p150^{Glued} and EB1 in combination appeared to show an

Figure 4 (facing page). Overexpression of both EB1 and p150^{Glued} can bundle microtubules. (A) PtK2 cells transiently transfected with a cDNA for GFP-EB1 (green) and labeled with an antibody to tubulin (red) show extensive bundling of microtubules. (B) PtK2 cells expressing GFP-EB1 and labeled with an antibody to p150^{Glued} (red) show that p150^{Glued} is not recruited to EB1-microtubule bundles. (C) PtK2 cells transiently transfected with a cDNA for p150^{Glued} and labeled with antibodies to p150^{Glued} (green) and tubulin (red) show short circumnuclear microtubule bundles. (D) PtK2 cells expressing p150^{Glued} and labeled with antibodies to p150^{Glued} (green) and EB1 (red) show that EB1 is weakly recruited to p150^{Glued}-microtubule bundles (arrows). (E) Overexpression of p50 (dynamitin) disrupts the dynactin complex. In cells overexpressing high levels of both GFP-EB1 (green) and p50 (red), an increased bundling effect was evident when compared with cells overexpressing GFP-EB1 alone. Scale, 10 μ m.

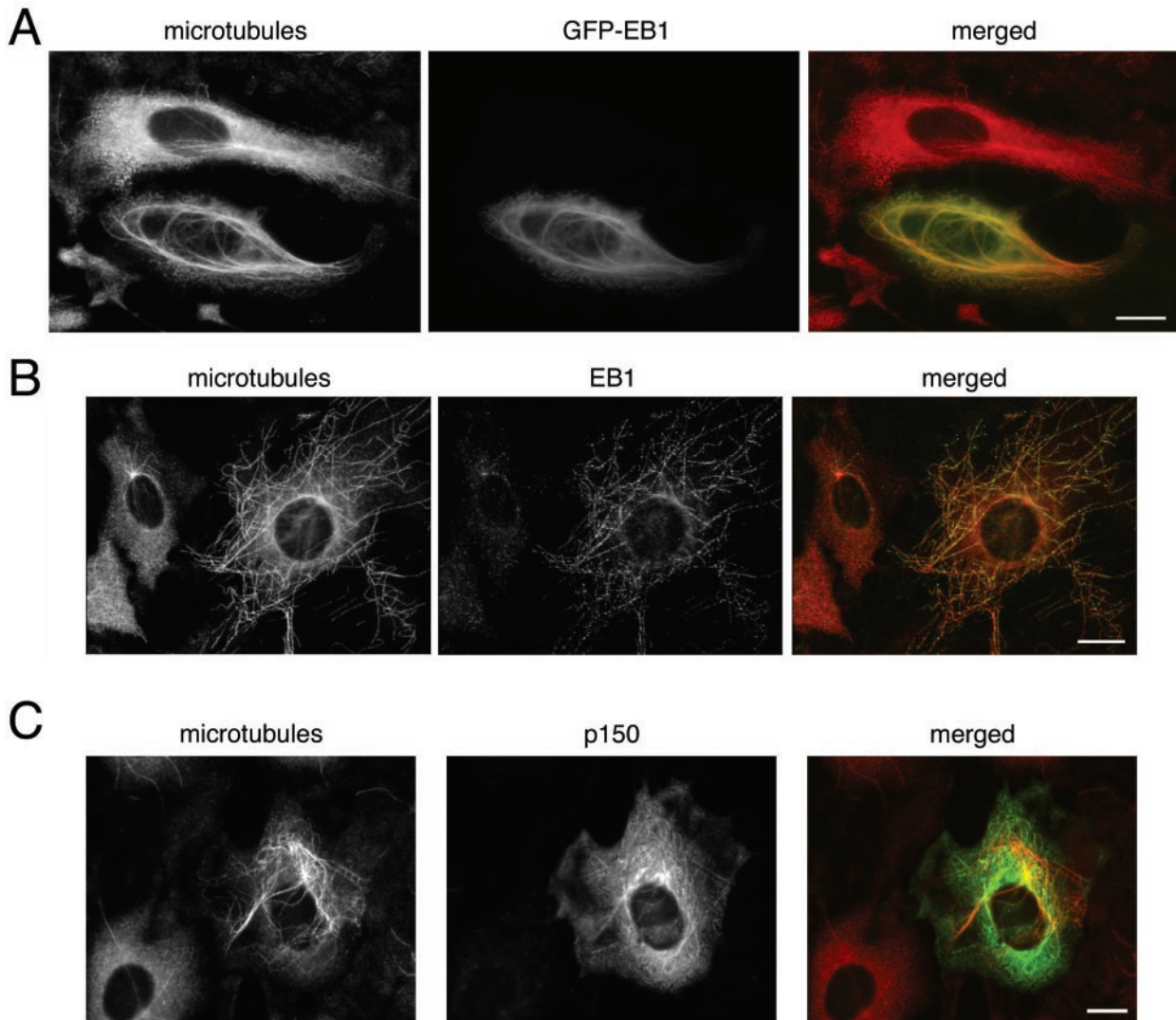


Figure 5. EB1 and p150^{Glued} are associated with stable microtubules. (A) PtK2 cells expressing GFP-EB1 were treated with the microtubule-depolymerizing drug nocodazole (5 $\mu\text{g}/\text{ml}$) for 30 min and then fixed and labeled with an antibody to tubulin (red). The EB1-microtubule bundles resist depolymerization. (B) A small population of stable microtubules persisted after nocodazole treatment in (untransfected) PtK2 cells. These stable microtubules were highly decorated with endogenous EB1. Cells were treated with nocodazole (5 $\mu\text{g}/\text{ml}$) for 30 min and then fixed and labeled with antibodies to tubulin (red) and EB1 (green). (C) PtK2 cells expressing p150^{Glued} were treated with the microtubule depolymerizing drug nocodazole as above, fixed, and labeled with antibodies to p150^{Glued} (green) and tubulin (red). p150^{Glued}-microtubules resisted depolymerization. Scale, 10 μm .

increased effect beyond that of p150^{Glued} alone. It is possible that this assay is more sensitive than the pelleting assay and can therefore identify smaller differences in the extent of polymerization. However, light scattering may also be enhanced by microtubule bundling. Because both EB1 and p150^{Glued} appear to bundle microtubules *in vivo*, we pelleted the microtubules onto coverslips after polymerization to examine their morphology (Figure 7C). Relatively short microtubules were pelleted after the polymerization of tubulin alone. Some bundling was seen, but it was not extensive. Microtubules pelleted after the addition of EB1 were slightly longer than those seen with tubulin alone, but the

difference was not large. The microtubules pelleted after the addition of p150^{Glued}, however, were dense tangles that appeared to consist of many short, splayed bundles of microtubules. The microtubules pelleted after the addition of EB1 and p150^{Glued} in combination were also densely tangled, but the tangles often contained long microtubule bundles, which could account for the increase seen in light scattering.

In the presence of microtubule seeds, the results were strikingly different (Figure 7B). The addition of p150^{Glued} slightly enhanced light scattering above that of tubulin alone, but the addition of EB1 caused a dramatic increase in

light scattering. Interestingly, the effect of the two proteins in combination was less than that of EB1 alone and was approximately the same as that seen without seeds. Microtubules pelleted after the polymerization of tubulin with seeds were longer than those polymerized without seeds, but did not appear to be more bundled (Figure 7D). The microtubules polymerized with EB1, on the other hand, were extremely long and thickly bundled, but the bundles did not show any of the splaying or tangling seen with p150^{Glued}. Microtubules polymerized with seeds in the presence of p150^{Glued} were similar to those polymerized without seeds, but the tangles were smaller and less complex. In addition, there appeared to be more bundles of very short microtubules. Microtubules polymerized with seeds in the presence of both EB1 and p150^{Glued} had characteristics of both. Many of the microtubule bundles were extremely long, but they often contained tangles of shorter microtubules as well (Figure 7D). Although the light scattering induced by the addition of EB1 to seeded assembly reactions increased by ~400% over that seen with tubulin alone, sedimentation assays indicate that the actual increase in polymer is only ~50% (unpublished data). The high degree of microtubule bundling observed by microscopy could account for the larger increase seen with light scattering.

DISCUSSION

A class of microtubule-binding proteins has been identified that preferentially localize to the plus-ends of growing microtubules. Included among this class are CLIP-170, APC, EB1, and dynactin. Each of these proteins has a microtubule-binding domain and may be independently targeted to microtubules. However, interactions between individual plus-end proteins have also been identified: APC interacts with EB1 (Su *et al.*, 1995), EB1 interacts with dynein and/or dynactin (Berrueta *et al.*, 1999), and CLIP-170 may interact with dynactin (Valetti *et al.*, 1999; Vaughan *et al.*, 1999). These data have led to the hypothesis of a microtubule plus-end complex (Schroer, 2001), but the details of interactions among plus-end proteins and between plus-end proteins and microtubules have remained unclear. Here we have clarified the interaction between EB1 and the dynein and dynactin complexes. We have shown that EB1 interacts with the dynactin complex by binding directly to the p150^{Glued} subunit. EB1 binds to p150^{Glued} near the N-terminus of the protein, in close proximity to the CAP-Gly microtubule-binding site of p150^{Glued}, suggesting that EB1 and p150^{Glued} may form a ternary complex with microtubules. A recent crystal structure of the CAP-Gly motif has identified a groove in the protein structure that may mediate microtubule binding (Li *et al.*, 2002). It will be interesting to determine the structural relationship of the EB1 binding domain to this motif. While the present article was in review, another study was published that also noted a direct interaction between EB1 and p150^{Glued} (Askham *et al.*, 2002). Askham *et al.* demonstrated that the microtubule binding domain on EB1 is at the N terminus of the protein, whereas the dynactin binding domain is at the C terminus. However, both domains are necessary to increase microtubule stability and bundling. Together these data suggest a multipartite complex that might allow for tight regulation within the cellular environment.

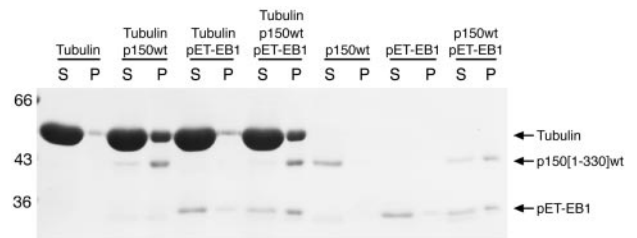


Figure 6. p150^{Glued} enhances microtubule polymerization, but EB1 appears to have little effect. Microtubules were polymerized in the presence of tubulin alone, tubulin with recombinant p150^{Glued}, tubulin with recombinant EB1, or tubulin with both EB1 and p150^{Glued} and then centrifuged at 39,000 × g. Supernatant and pellet fractions were analyzed by SDS-PAGE. The addition of p150^{Glued} increased the amount of tubulin in the pellet, but EB1 had little effect. The addition of EB1 and p150^{Glued} together had no apparent effect beyond that of p150^{Glued} alone. We note that the combination of EB1 and p150^{Glued} in the absence of tubulin results in the appearance of both proteins in the pellet. This could result from the formation of aggregates of EB1 and p150^{Glued} or a change in the solubility of the proteins in solution.

If a plus-end complex does exist, it may assemble in the cytoplasm and copolymerize with microtubules or it could assemble in situ on the microtubule. Here we have shown that purified recombinant EB1 and p150^{Glued} interact, suggesting that the proteins can associate independently of microtubules. It has also previously been shown that EB1 coimmunoprecipitates with dynein and dynactin from nocodazole-treated cells (Berrueta *et al.*, 1999). This is not a universal feature of interactions among plus-end proteins, however, because it has been shown that EB1 does not interact with APC in the absence of intact microtubules (Mimori-Kiyosue *et al.*, 2000). These data suggest that the formation of a microtubule plus-end complex may involve several distinct steps, some that occur in the cytoplasm and some that occur on the microtubule.

Interactions between plus-end proteins may allow one protein to recruit others to microtubules or a plus-end complex. For example, overexpression of CLIP-170 has been shown to recruit p150^{Glued} to microtubules, but the overexpression of p150^{Glued} does not recruit CLIP-170 to microtubules (Valetti *et al.*, 1999). It has also been shown that the overexpression of APC recruits EB1 to microtubules (Mimori-Kiyosue *et al.*, 2000). This finding is complicated, however, by the observation that EB1 is localized to microtubules in cells lacking functional APC (Berrueta *et al.*, 1998). The temporal sequence with which proteins are recruited and the hierarchies of binding are largely unknown. Here we have shown that the overexpression of p150^{Glued} may recruit EB1 to microtubules, but the overexpression of EB1 cannot recruit p150^{Glued} to microtubules. Interactions between plus-end proteins, however, are likely to be highly regulated, and overexpression of a protein may circumvent this regulation. Potential regulatory factors include the state of the microtubule (GTP cap, posttranslational modifications) and posttranslational modifications of the plus-end proteins. EB1 has few potential phosphorylation sites, but APC and p150^{Glued} are likely to be regulated by phosphorylation (Askham *et al.*,

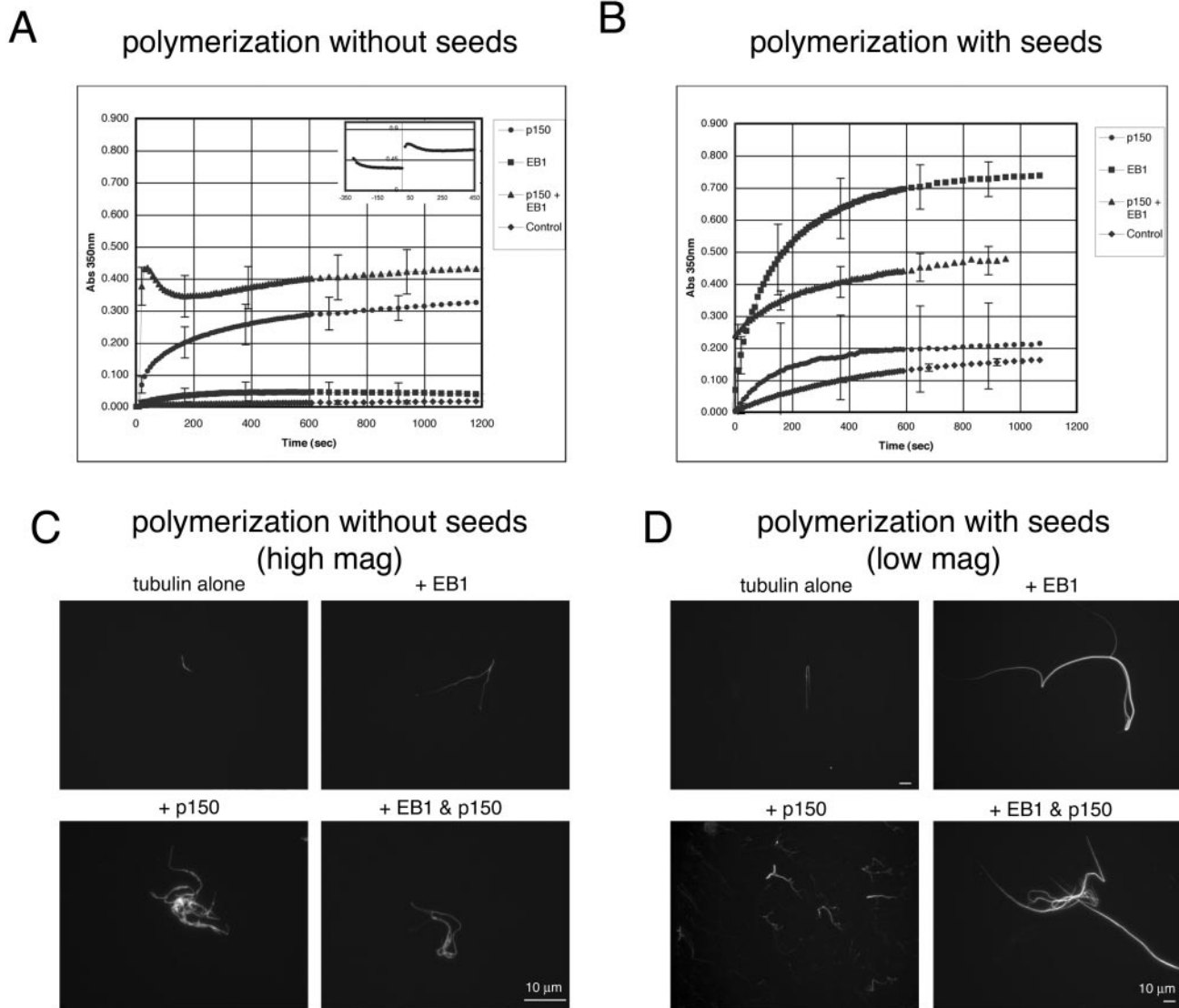


Figure 7. p150^{Glued} has a potent microtubule nucleation effect and EB1 has a potent microtubule elongation effect. (A) The effect of p150^{Glued}, EB1, both proteins, and tubulin only (control) on microtubule polymerization over time was examined using a light-scattering assay. When no microtubule seeds are present, EB1 has little effect over that of tubulin alone, but p150^{Glued} has a potent effect on the extent of polymerization. The combination of EB1 and p150^{Glued} had an even greater effect. However, the combination of EB1 and p150^{Glued} appeared to cause a confounding light-scattering aggregation that settled out of solution over time, similar to that seen in the pelleting experiment. The inset graph shows this effect before and after the addition of tubulin. (B) In the presence of microtubule seeds, p150^{Glued} had little effect on microtubule polymerization over that of the addition of tubulin alone, but the addition of EB1 had a potent effect. (C) Fluorescently labeled microtubules were pelleted onto coverslips after polymerization in the above conditions. Without seeds, very few microtubules were polymerized with tubulin alone. Long slender bundles were polymerized in the presence of EB1, and dense tangled bundles were polymerized in the presence of p150^{Glued}. Microtubules polymerized in the presence of both EB1 and p150^{Glued} had characteristics of both. (D) Microtubules polymerized much more efficiently in the presence of seeds, necessitating a larger microscopic field of view. Relatively short, simple microtubules were polymerized in the presence of tubulin alone, short, highly branched microtubule bundles were polymerized in the presence of p150^{Glued}, and extremely long microtubule bundles were polymerized in the presence of EB1. Again, microtubules polymerized in the presence of both EB1 and p150^{Glued} had characteristics of both. Scale, 10 μm .

2000; Vaughan *et al.*, 2000). The details of this regulation, however, remain an open question.

The function of a plus-end complex or plus-end proteins may be twofold: they may play a role in regulating micro-

tubule stability and/or dynamics and they mediate interactions between microtubules and cellular targets (for example, see Kaverina *et al.*, 1998; Ligon *et al.*, 2001). Here we have shown that both EB1 and p150^{Glued} appear to affect micro-

tubules growth and/or stability, but in very different ways. Increased levels of EB1 lead to the formation of cold- and drug-stable microtubule bundles. (This was also shown by Bu and Su [2001].) Likewise, increased levels of p150^{Glued} lead to the formation of stable microtubule bundles (shown here and in Waterman-Storer *et al.*, 1995). However, the microtubule bundles formed by the overexpression of EB1 and p150^{Glued} appear very different. Those induced by EB1 overexpression are very long and wrap around the cell, whereas those induced by p150^{Glued} overexpression are short and more circumnuclear. The overexpression of another plus-end protein, CLIP-170, also causes the formation of microtubule bundles (Pierre *et al.*, 1994). It is interesting to note that while CLIP-170 has a microtubule binding domain similar to that of p150^{Glued}, the bundles induced by its overexpression appear much more like those induced by EB1.

In addition, we have shown that high levels of endogenous EB1 are associated with a population of stable microtubules in epithelial cells, but we did not see p150^{Glued} associated with stable microtubules in this cell type. It has previously been reported that high levels of endogenous p150^{Glued} are associated with stable microtubules in fibroblasts (Vaughan *et al.*, 1999). In both of these experiments, however, it is not clear if increased levels of EB1 or p150^{Glued} caused the microtubules to be stable or if the proteins merely opportunistically bound to the microtubules remaining after drug treatment.

Both EB1 and p150^{Glued} have an effect on microtubule polymerization *in vitro*, but their effects are distinct. In the absence of microtubule seeds, the addition of EB1 does not increase microtubule polymerization much beyond that of tubulin alone, consistent with previous data (Nakamura *et al.*, 2001). p150^{Glued}, on the other hand, decreases the critical concentration for microtubule polymerization, thus increasing the extent of polymerization. In the presence of seeds, however, the results were very different. p150^{Glued} has little effect beyond that of tubulin alone, whereas the addition of EB1 results in an increase in light scattering in a spectrophotometric assay. Examination of the polymerized microtubules by microscopy suggests that p150^{Glued} may play a role in nucleating new microtubules. In the absence of seeds, this role is critical. But when seeds are present, this nucleating effect is not necessary. It is not clear if p150^{Glued} can bind to tubulin subunits and nucleate microtubules *de novo* or if it binds to and potentially stabilizes very short microtubules, thus enhancing their nucleating effect. Examination of microtubules polymerized in the presence of EB1, on the other hand, suggests that EB1 appears to have more potent elongation and bundling effects. In the absence of seeds, EB1 is ineffectual, but when seeds are present, the addition of EB1 results in extremely long microtubule bundles. It has been shown that EB1 can bind to tubulin in the absence of microtubules (Juwana *et al.*, 1999). Perhaps EB1 binds to free tubulin and this complex is more likely to add to polymer than tubulin alone.

These *in vitro* results also clarify the results observed in cells overexpressing EB1 and p150^{Glued}. The overexpression of EB1 causes very long microtubule bundles due to its elongation and bundling effects and the overexpression of p150^{Glued} causes many short microtubules due to its

nucleation effect. Together, these data suggests that the overall dynamics of the microtubule array in the cell may depend upon the balance of activities of various plus-end proteins, including EB1 and p150^{Glued}. We have shown that disruption of the dynactin complex enhances the effect of EB1 on microtubule bundling and stability, suggesting that the activities of these two plus-end proteins are linked, but other plus-end proteins may be involved as well.

Finally, differences in the expression and regulation of plus-end proteins may result in cell type differences in microtubule dynamics. Although EB1 and p150^{Glued} are both expressed in fibroblasts and epithelial cells and localize to microtubules in each cell type, the patterns of their localizations differ in the two cell types. It has long been known that the microtubule arrays of epithelial cells and fibroblasts differ in their organization and dynamics (Bershadsky *et al.*, 1979; Spiegelman *et al.*, 1979; Shelden and Wadsworth, 1993). In several cultured epithelial cell lines, microtubules have been shown to array from a single centrosome, whereas in cultured fibroblasts, multiple microtubule-nucleating sites are often seen in a cell, resulting in a large population of noncentrosomal microtubules (Bershadsky *et al.*, 1979; Spiegelman *et al.*, 1979). Differences in microtubule dynamics are more complicated. Microtubules in some epithelial cell types have been shown to be very dynamic on a short time scale, but stable on a longer time scale, *i.e.*, undergoing brief periods of growth and shortening, with little overall change. In contrast, microtubules in fibroblasts are more stable on a short time scale and more dynamic on a long time scale, *i.e.*, they undergo longer periods of growth and shortening, resulting in large net changes (Shelden and Wadsworth, 1993). EB1 and p150^{Glued} as well as other plus-end proteins are perfectly positioned to regulate these differences. The localization of the two proteins differs between the two cell types, they have differential effects on microtubule dynamics, and these proteins interact with one another. The function of differential microtubule dynamics in different cell types, however, remains an unanswered question.

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