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# Cooperative Stabilization of Microtubule Dynamics by EB1 and CLIP-170 Involves Displacement of Stably Bound $P_i$ at Microtubule Ends

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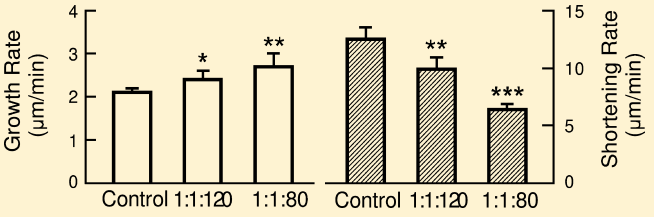
**ABSTRACT:** End binding protein 1 (EB1) and cytoplasmic linker protein of 170 kDa (CLIP-170) are two well-studied microtubule plus-end-tracking proteins (+TIPs) that target growing microtubule plus ends in the form of comet tails and regulate microtubule dynamics. However, the mechanism by which they regulate microtubule dynamics is not well understood. Using full-length EB1 and a minimal functional fragment of CLIP-170 (ClipCG12), we found that EB1 and CLIP-170 cooperatively regulate microtubule dynamic instability at concentrations below which neither protein is effective. By use of small-angle X-ray scattering and analytical ultracentrifugation, we found that ClipCG12 adopts a largely extended conformation with two noninteracting CAP-Gly domains and that it formed a complex in solution with EB1. Using a reconstituted steady-state mammalian microtubule system, we found that at a low concentration of 250 nM, neither EB1 nor ClipCG12 individually modulated plus-end dynamic instability. Higher concentrations (up to 2 $\mu$M) of the two proteins individually did modulate dynamic instability, perhaps by a combination of effects at the tips and along the microtubule lengths. However, when low concentrations (250 nM) of EB1 and ClipCG12 were present together, the mixture modulated dynamic instability considerably. Using a pulsing strategy with [$\gamma^{32}$P]GTP, we further found that unlike EB1 or ClipCG12 alone, the EB1−ClipCG12 mixture partially depleted the microtubule ends of stably bound $^{32}P_i$. Together, our results suggest that EB1 and ClipCG12 act cooperatively to regulate microtubule dynamics. They further indicate that stabilization of microtubule plus ends by the EB1−ClipCG12 mixture may involve modification of an aspect of the stabilizing cap.




Dynamic instability, a highly regulated functional property of microtubules, is characterized by switching between growth and shortening at the ends of the microtubules.[1] This behavior is thought to be associated with gain and loss of a still poorly understood stabilizing "cap", which is postulated to be composed of a short region of tubulin-GTP or tubulin-GDP-$P_i$ subunits at the microtubule ends (reviewed in refs 2 and 3). The ends grow when the cap is present and shorten when it is lost. An important group of proteins that regulates dynamic instability is the plus-end-tracking proteins (+TIPs), specialized microtubule-associated proteins (MAPs) that function as protein complexes.[4] In cells, the +TIPs localize to growing plus ends in the form of "comet tails" and "track" the plus ends as the microtubules grow.[4−8] They are lost when the ends shorten. Because of their ability to track growing microtubule plus ends, +TIPs are precisely positioned to regulate dynamic instability behavior. By regulating microtubule dynamics, they are thought to facilitate cytoskeletal remodeling, to modulate cell signaling, and to assist with the stabilization of microtubules at leading edges of cells.[7,8]

End binding protein 1 (EB1) and cytoplasmic linker protein of 170 kDa (CLIP-170) are two such +TIP proteins. EB1 and its family members localize to growing microtubule plus ends and track the ends in the shape of comet tails.[9,10] EB1 is indispensable for the recruitment of many other +TIP proteins to plus ends, including CLIP-170 and p150$^{glued}$ [8]. The N terminus of EB1 interacts with the microtubules and is sufficient to mediate plus-end-binding specificity of the molecule.[11] The C terminus of EB1 interacts with the other +TIP proteins to enable the formation of +TIP interaction networks at growing plus ends.[8] CLIP-170 is the first reported +TIP.[12] It possesses two N-terminal CAP-Gly domains that interact with microtubules.[13−19] CLIP-170 binds the C-terminal tails of $\alpha$-tubulin and EB1 through the EEY/F motifs present at the C termini of these proteins.[13,14] In addition, CLIP-170 interacts with the H12 helices of both $\alpha$-tubulin and $\beta$-tubulin.[17] It is established that CLIP-170 depends on EB1 to target growing microtubule ends.[10,18] Formation of a composite binding site involving the EEY/F motifs of both EB1 and $\alpha$-tubulin is reported to be essential for localization of CLIP-170 to microtubule plus ends.[17,18] Even though the interaction of CLIP-170 and EB1 with microtubules has been reported, the mechanisms by which EB1 and CLIP-170 regulate plus-end dynamics are not well understood.

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In the present study, we analyzed the interaction of EB1 and CLIP-170 in solution by small-angle X-ray scattering and analytical ultracentrifugation. We also analyzed the effects of the two proteins, when present individually or as a mixture, on dynamic instability with a purified reconstituted mammalian microtubule system. Because full-length CLIP-170 can be autoinhibited through an intramolecular interaction between its two N-terminal cytoskeleton-associated protein glycine-rich (CAP-Gly) domains and its C-terminal zinc-knuckle motifs,[13,16,18] we used an N-terminal fragment of CLIP-170 comprising the two CAP-Gly domains, here called ClipCG12 (CLIP CAP-Gly domains 1 and 2).[13] ClipCG12 or a closely similar CLIP-170 fragment contains all features necessary to mediate microtubule plus-end tracking and thus represents a minimal functional fragment to study CLIP-170 behavior.[13,19] Whether ClipCG12 has an effect on microtubule dynamics has been, however, unclear. Finally, we determined the effects of EB1, ClipCG12, and a mixture of the two proteins on the stoichiometry of nonexchangeably bound $^{32}P_i$ at microtubule ends, which may represent an aspect or remnant of the stabilizing cap at microtubule ends.[20] Our data support the emerging consensus that +TIP proteins act cooperatively at microtubule tips to regulate microtubule dynamics.

## EXPERIMENTAL PROCEDURES

**Protein Preparation.** The human CLIP-170 fragment ClipCG12 (residues 48−300) was cloned into pDEST17-O/I using the Gateway cloning system (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* strain C41 (DE3) (Lucigen, Middleton, WI, USA). Cells were grown at 37 °C in Luria−Bertani media to an $OD_{600}$ of 0.7. Expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and performed overnight at 20 °C. The 6×His-tagged fusion protein was affinity purified by immobilized metal-affinity chromatography on $Ni^{2+}$ Sepharose (GE Healthcare, Uppsala, Sweden) at 4 °C according to the manufacturer's instructions using reducing conditions (1 mM β-mercaptoethanol). The 6×His-tag was removed by proteolytic cleavage using human thrombin (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C in thrombin cleavage buffer (150 mM NaCl, 2.5 mM $CaCl_2$, 20 mM Tris/HCl, pH 8.4, 1 mM β-mercaptoethanol). Processed protein was subjected to a second $Ni^{2+}$ Sepharose column and further purified by size exclusion chromatography on a Superdex-200 column (GE Healthcare) equilibrated in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.3 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, pH 7.4) supplemented with 1 mM β-mercaptoethanol.

Untagged human EB1 was cloned into pET3d vector (EMD4Biosciences, Rockland, MA, USA) and overexpressed in the *E. coli* strain BL21(DE3) at 24 °C for 40 h in ZY autoinduction media.[21] The protein was bound by ion exchange chromatography on a Resource Q column (GE Healthcare) equilibrated in 20 mM BisTris, pH 6.0, and subsequently eluted by applying a linear concentration gradient of NaCl from 0 to 500 mM over 20 column volumes. Enriched EB1 fractions were pooled, concentrated, and applied onto a Superdex 200 size exclusion chromatography column (GE Healthcare) equilibrated in 20 mM Tris/HCl and 300 mM NaCl, pH 7.5. All EB1 concentrations used in this work are reported as EB1 dimers.[22]

The 10×His-tagged EB1 version for analytical ultracentrifugation experiments was cloned into a PositiveSelection vector as described in ref 23. The protein was overexpressed in Terrific broth medium for 24 h at 24 °C using the *E. coli* BL21(DE3) strain. Expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at $OD_{600}$ of 1.5. Cells were lysed in binding buffer (50 mM Tris, pH 8.0, 500 mM NaCl, and 4 mM imidazole) and supplied on a HisTrap column (GE Healthcare). Eluted protein fractions were pooled, concentrated, and applied onto a Superdex 200 size exclusion chromatography column (GE Healthcare) equilibrated in PBS. The homogeneity of the recombinant proteins (>95%) was assessed by SDS-PAGE, and their identities were confirmed by mass spectral analysis. Exact concentrations of protein solutions for biophysical experiments were determined by absorbance at 280 nm in 6 M guanidine−HCl.[24]

**Small-Angle X-ray Scattering (SAXS).** X-ray scattering data were collected at the cSAXS (×12sa) beamline at the Swiss Light Source (SLS, Paul Scherrer Institut, Villigen, Switzerland). The distance sample-to-detector (PILATUS 2M) was set at 2.15 m, and the beam energy was adjusted at 12.4 keV (1.0 Å wavelength). Different concentrations of the ClipCG12 protein sample (1.3, 5.2, 7.5, and 9.7 mg/mL) and PBS buffer supplemented with 5% glycerol and 2.5 mM dithiothreitol were measured in borosilicate glass capillaries of 1 mm diameter at 10 °C. To minimize radiation damage, scattering intensities were acquired at 10 different points (0.5 mm distance) in the capillary for 0.5 s each, and the scans were repeated 20 times. The collected frames were radially averaged using MATLAB scripts (J. Missimer, Paul Scherrer Institut, unpublished data). Data reduction was performed according to standard procedures using the program PRIMUS from the ATSAS software package.[25] The flexibility analysis was performed using the ensemble optimization method,[26] using default parameters. The crystallographic structures with PDB codes 2E3I and 2E3H, corresponding to the first and second CAP-Gly domains of CLIP-170, respectively, were used for this analysis. The theoretical PDFs of the isolated CAP-Gly domains were calculated using HYDROPRO.[27]

**Analytical Ultracentrifugation (AUC).** Sedimentation velocity AUC experiments were performed using a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA, USA) equipped with a four-hole rotor and standard velocity centerpiece. Proteins (EB1 and ClipCG12) in PBS buffer supplemented with 1 mM β-mercaptoethanol were analyzed at 45000 rpm and 10 °C. Sedimentation profiles were measured by absorption at 280 nm and analyzed using the SedFit software package[28] using a continuous $c(S)$ distribution model.

**Tubulin Purification.** Tubulin was purified as described by Miller and Wilson.[29] Briefly, bovine brain microtubule protein (tubulin plus microtubule-associated proteins) was isolated by two cycles of warm polymerization and cold depolymerization. Tubulin was purified from the microtubule protein by phosphocellulose chromatography, concentrated to 10 mg/mL in 100 mM 1,4-piperzinediethanesulfonic acid (Pipes), 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8 (PEM buffer) at 30 °C, drop-frozen in liquid nitrogen, and stored at −70 °C until used. The tubulin was >99% pure as evidenced by the absence of MAPs on heavily overloaded SDS−polyacrylamide gels stained with Coomassie Blue. Protein concentration was determined according to the method of Bradford[30] using bovine serum albumin as the standard.

**Video-Enhanced Differential Interference Contrast Microscopy.** Purified tubulin (20 μM) was assembled onto sheared sea urchin (*Strongylocentrotus purpuratus*) axonemes in PMEM buffer (87 mM Pipes, 36 mM MES, 1 mM EGTA, 2

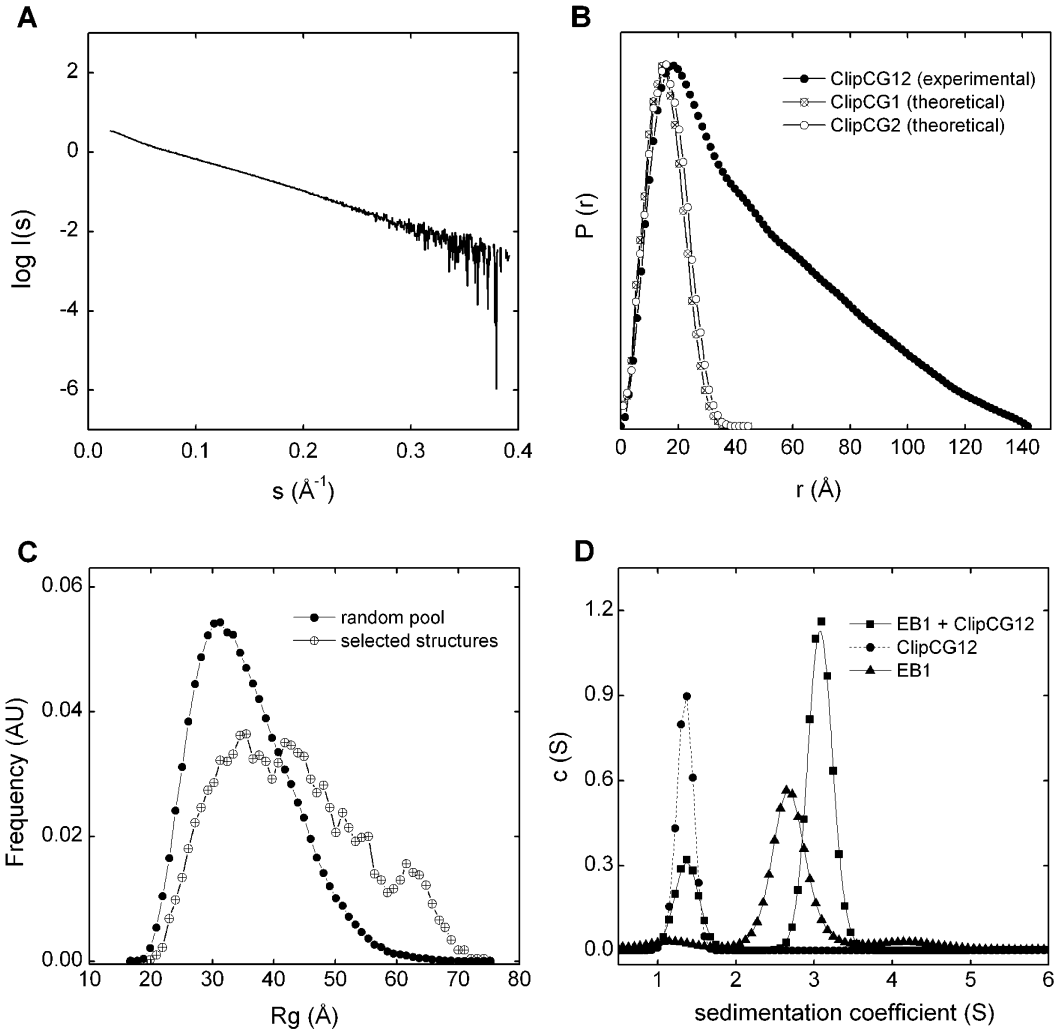


**Figure 1.** Biophysical characterization of the EB1−ClipCG12 complex. (A) SAXS profile of ClipCG12 extrapolated to zero protein concentration. s, scattering vector; log $I$(s), log of the intensity. (B) Experimental PDF of ClipCG12 (solid circles) and theoretical PDFs of the first (open crossed circles) and second (open circles) CAP-Gly domains of CLIP-170. Protein Databank (PDB) entries 2E3I (ClipCG1 structure) and 2E3H (ClipCG2 structure) were used for calculating the theoretical pair distribution function (PDFs). The PDF represents a histogram of interatomic distances in a molecule. $P(r)$, pair distribution function; $r$, distance. (C) Distribution of the radius of gyration ($R_g$) values of the 10000 structures in the randomly generated pool (solid circles) versus the ensemble of structures (open crossed circles) that optimally fits the experimental data according to the ensemble optimization method.[26] (D) Sedimentation profiles of EB1 (solid triangles), ClipCG12 (solid circles), and EB1−ClipCG12 (solid squares) recorded by AUC at 10 °C in PBS buffer. Protein concentrations were 7.5 $\mu$M for each sample. The data represent the distribution of sedimenting molecule species during the experiment. $c(S)$, protein concentration as a function of the sedimentation coefficient.

mM $MgCl_2$, pH 6.8) in the presence of 2 mM GTP as described.[31−33] To determine the effects of EB1 or ClipCG12 individually on dynamic instability, tubulin-containing reaction mixtures were incubated at 30 °C for 30 min in the presence or absence of 250 nM, 1 $\mu$M, or 2 $\mu$M EB1 to obtain 1:80, 1:20, or 1:10 molar ratios of EB1 to tubulin, respectively, or 250 nM, 1 $\mu$M, or 2 $\mu$M ClipCG12 to obtain molar ratios of 1:80, 1:20, or 1:10 of ClipCG12 to tubulin, respectively. To determine the effects on the dynamic instability of mixtures of EB1 and ClipCG12, either a mixture of 165 nM EB1 plus 165 nM ClipCG12 or a mixture of 250 nM EB1 plus 250 nM ClipCG12 was used with 20 $\mu$M tubulin. Thus, the molar ratios of EB1/ClipCG12/tubulin were 1:1:120 or 1:1:80, respectively. We confirmed by a light scattering assay that incubation for 30 min at 30 °C was sufficient for polymerization to reach steady state, and all dynamic instability assays were carried out at steady state, conditions at which the free tubulin concentration and polymer concentration remain constant. Plus-end dynamic instability parameters were determined as described previously.[33] Microtubules were considered to be growing if they increased in length >0.3 $\mu$m at a rate >0.3 $\mu$m/min. Shortening events were identified by a >1 $\mu$m length reduction at a rate of >2 $\mu$m/min. Changes in length of <0.3 $\mu$m were considered periods of attenuated dynamics or pause. The catastrophe frequency is the frequency of transition from the growth or attenuated state to shortening. The rescue frequency is the frequency of transition from shortening to the growing or attenuated state. Dynamicity is the sum of the total growth length and the total shortening length divided by the total time.[32] Between 25 and 30 microtubules were analyzed for each condition, a sufficient number of events to achieve good statistical significance.

**Measurement of the Quantity of $^{32}P_i$ Stably Bound to Microtubules.** To measure the stoichiometry of $^{32}P_i$ per microtubule derived from [$\gamma^{32}$P]GTP that remained stably bound at the tips of the microtubules in the presence and


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absence of EB1, ClipCG12, or mixtures of the two proteins, MAP-rich microtubules were assembled to steady state with 1 $\mu$M EB1 (a 1:20 molar ratio of EB1 to tubulin), 1 $\mu$M ClipCG12 (a 1:20 molar ratio), or a mixture of 1 $\mu$M EB1 plus 1 $\mu$M ClipCG12 (a ratio of 1:1:20 of EB1/ClipCG12/tubulin). At steady state, samples were pulsed with [$\gamma^{32}$P]GTP for 25−30 min at 30 °C and sedimented at 200000*g* through 50% sucrose cushions for 2 h to remove all nonspecifically associated radiolabel as described previously.[20] At the end of the 2 h centrifugation, the microtubules were collected, and the amount $^{32}P_i$ remaining bound per microtubule was determined.[20] In this assay, the [$\gamma^{32}$P]GTP has completely hydrolyzed to GDP and $^{32}P_i$. The $^{32}P_i$ is stably bound after centrifugation through the sucrose cushions and does not exchange with exogenous $P_i$. Because as much as 25% of the [$\gamma^{32}$P]GTP added to the microtubule suspension was hydrolyzed during the pulse (data not shown), the specific activity of the [$\gamma^{32}$P]GTP and the bound $^{32}P_i$ was corrected to reflect this loss. Microtubule mean lengths were determined by electron microscopy to enable calculation of the number of stably bound molecules of $^{32}P_i$ per microtubule as described previously.[20]

## RESULTS

**Biophysical Characterization of EB1 and ClipCG12.** We sought to analyze the effects of EB1 and ClipCG12 individually and together on microtubule plus-end dynamic instability. First, we wanted to characterize the structures and activities of our recombinantly produced EB1 and ClipCG12 proteins by SAXS and AUC under conditions similar to the ones used to perform the microtubule dynamics studies. Figure 1A shows the SAXS profile extrapolated to zero protein concentration of ClipCG12. The PDF, which represents a histogram of interatomic distances of ClipCG12, is characterized by the presence of a main peak centered at ∼18 Å and wide tailing toward longer distances (Figure 1B). The distance values covering the main peak are in good agreement with the interatomic distances of a single CAP-Gly domain (Figure 1B). The tailing seen in the PDF, with a maximum particle distance, $D_{max}$, of 143 Å, is indicative of a wide range of interatomic distances between the two CAP-Gly domains and between them and the interdomain linker region. From the PDF, the radius of gyration, $R_g$, was determined to be 39.4 ± 3.0 Å. A flexibility analysis was further performed using the ensemble optimization method,[26] which is able to distinguish between rigid or flexible linkers interconnecting multidomain proteins. This analysis first generates a pool of 10000 conformers using high-resolution structures of CAP-Gly domains and randomly models the linker between them. A genetic algorithm then selects the set of conformers that fit best the experimental data. As shown in Figure 1C, the distribution of $R_g$ values of the selected structures is similar or even broader compared to the distribution of $R_g$ values of the randomly generated conformers, suggesting a flexible, nonstructured linker interconnecting the two CAP-Gly domains. These SAXS data indicate that ClipCG12 adopts a largely extended conformation with two noninteracting CAP-Gly domains. The conformation of EB1 in solution was previously assessed by analytical ultracentrifugation and SAXS; the data were consistent with EB1 adopting an elongated dimeric form.[22,34]

Next, we analyzed EB1, ClipCG12, and mixtures of the two proteins by sedimentation velocity AUC to determine whether these proteins form a complex in solution. The sedimentation profiles of EB1 (7.5 $\mu$M dimer equivalents) and ClipCG12 (7.5

**Table 1. Effects of EB1 Alone and ClipCG12 Alone on Dynamic Instability**[a]

| | | EB1/tubulin | | | ClipCG12/tubulin | | |
|---|---|---|---|---|---|---|---|
| dynamic instability parameter | control | 1:80 250 nM | 1:20 1 $\mu$M | 1:10 2 $\mu$M | 1:80 250 nM | 1:20 1 $\mu$M | 1:10 2 $\mu$M |
| growth rate ($\mu$m/min) | 2.1 ± 0.1 | 2.04 ± 0.1 (−3%) | 2.7 ± 0.2** (+29%) | 3.3 ± 0.3*** (+57%) | 2.07 ± 0.2 (−1%) | 2.5 ± 0.2* (+19%) | 3 ± 0.2** (+43%) |
| shortening rate ($\mu$m/min) | 12.5 ± 1 | 11.5 ± 0.7 (−8%) | 9.9 ± 0.7* (−21%) | 7.2 ± 1.6*** (−42%) | 12.5 ± 1 (0%) | 10.2 ± 0.8* (−18%) | 9.8 ± 0.6* (−22%) |
| percentage of time | | | | | | | |
| growing | 39 | 45 (+15%) | 36 (−8%) | 39 (0%) | 42 (+8%) | 40 (+3%) | 44(+13%) |
| shortening | 21 | 19 (−10%) | 13 (−38%) | 11 (−48%) | 18 (−14%) | 19 (−10%) | 16 (−24%) |
| attenuated | 40 | 36 (−10%) | 51 (+28%) | 50 (+25%) | 40 (0%) | 41 (+3%) | 40 (0%) |
| catastrophe frequency (per min) | 0.44 ± 0.02 | 0.4 ± 0.1 (−9%) | 0.29 ± 0.03** (−34%) | 0.27 ± 0.01** (−39%) | 0.39 ± 0.05 (−11%) | 0.41 ± 0.03 (−7%) | 0.38 ± 0.04 (−14%) |
| rescue frequency (per min) | 1.1 ± 0.03 | 1.19 ± 0.2 (+8%) | 1.77 ± 0.06*** (+61%) | 1.88 ± 0.05*** (+71%) | 1.05 ± 0.2 (−5%) | 1.24 ± 0.06 (+13%) | 1.43 ± 0.05** (+30%) |
| dynamicity | 2.3 | 2.52 (+10%) | 1.88 (−18%) | 1.89 (−18%) | 2.61 (+13%) | 2.32 (+1%) | 2.39 (+4%) |

[a]Phosphocellulose purified bovine brain microtubules were assembled to steady state in the presence of EB1 or ClipCG12, and the dynamic instability parameters were determined as described under Experimental Procedures. Twenty-five to 30 microtubules were measured for each condition. *, **, and *** represent significance of differences at >0.05, >0.01, and >0.001, respectively, as determined with a Students' *t* test. Data = mean ± SEM.


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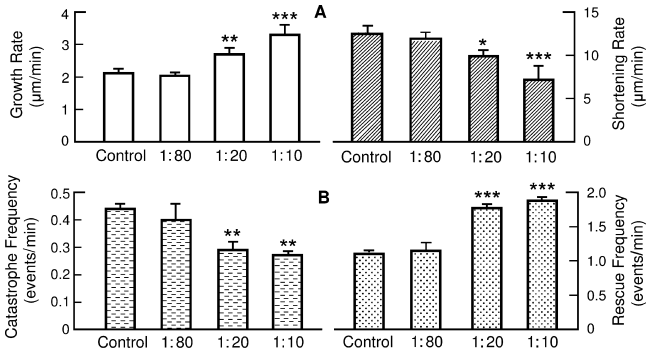


**Figure 2.** Effects of EB1 alone on dynamic instability: (A) effects on the growth and shortening rates; (B) effects on the catastrophe and rescue frequencies. Two hundred and fifty nanomolar EB1 (a 1:80 molar ratio to tubulin), 1 $\mu$M EB1 (a 1:20 molar ratio to tubulin), or 2 $\mu$M EB1 (a 1:10 molar ratio to tubulin) was mixed with 20 $\mu$M tubulin and 2 mM GTP in PMEM buffer, pH 6.8, added to sea urchin axoneme seeds, and incubated at 30 °C for 30 min to polymerize the microtubules to steady state. The dynamic instability parameters were analyzed as described under Experimental Procedures. Error bars = SEM.

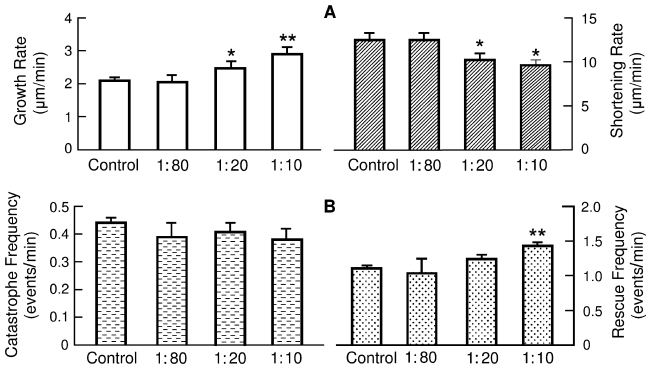


**Figure 3.** Effects of ClipCG12 alone on dynamic instability: (A) effects on the growth and shortening rates; (B) effects on the catastrophe and rescue frequencies. Two hundred and fifty nanomolar ClipCG12 (a 1:80 molar ratio to tubulin), 1 $\mu$M ClipCG12 (a 1:20 molar ratio to tubulin), or 2 $\mu$M ClipCG12 (a 1:10 molar ratio to tubulin) was mixed with 20 $\mu$M tubulin and 2 mM GTP in PMEM buffer, pH 6.8, added to sea urchin axoneme seeds, and incubated at 30 °C for 30 min to polymerize the microtubules to steady state. The dynamic instability parameters were analyzed as described under Experimental Procedures. Error bars = SEM.

$\mu$M monomer equivalents) revealed only single peaks for each protein centered at ~2.7 and ~1.4 S, respectively, which we assigned to an EB1 dimer (61 kDa) and a ClipCG12 monomer (27 kDa) (Figure 1D). A mixture containing 7.5 $\mu$M EB1 and 7.5 $\mu$M ClipCG12 yielded a sedimentation profile with two peaks. The first peak, centered at ~1.4 S, corresponded to ClipCG12 (Figure 1D). The second peak, centered at ~3.1 S, was significantly shifted to a higher sedimentation value compared to the peak obtained with EB1 alone, indicative of the formation of a complex between EB1 and ClipCG12 (Figure 1D). Collectively, these results provide molecular insights into the EB1−ClipCG12 interaction and document the quality and functional properties of the recombinant protein samples used to perform the following microtubule dynamic experiments.

**Effects of EB1 and ClipCG12 Individually on Plus-End Microtubule Dynamic Instability.** We first evaluated the effects of EB1 or ClipCG12 alone on steady-state dynamic instability. As described under Experimental Procedures, all EB1 concentrations were calculated as EB1 dimers. A concentration of 250 nM EB1 (a 1:80 molar ratio of EB1 to tubulin) did not significantly affect plus-end dynamic instability (Table 1). At higher concentrations as shown in Table 1 and Figure 2A, 1 $\mu$M EB1 (a molar ratio of 1:20 to tubulin) moderately increased the growth rate (29%) and decreased the shortening rate (21%). It also suppressed the catastrophe frequency (34%) and more strongly increased the rescue frequency (61%) (Figure 2B). Stronger stabilization occurred at 2 $\mu$M EB1 (a 1:10 molar ratio; Figure 2 and Table 1). These results, which are similar to the previously published results using this purified reconstituted mammalian system at steady state,[35] indicate that relatively high molar ratios of EB1 to tubulin (1:20 and 1:10) stabilize microtubules by promoting growth events and suppressing shortening events. Because EB1 binds along the length of the microtubule at higher protein concentrations, stabilization of dynamics at 1 and 2 $\mu$M EB1 is probably due to a combination of tip binding and binding along the length of the microtubule.

CLIP-170 does not autonomously track growing microtubule plus ends but binds directly to the microtubule walls through its CAP-Gly domains.[36] We analyzed the effects of the CAP-Gly domain containing the CLIP-170 fragment ClipCG12 on steady-state dynamic instability. As shown in Table 1 and Figure 3, 250 nM ClipCG12 also did not affect microtubule dynamic instability. Similar to EB1, ClipCG12 did modulate dynamics at higher concentrations. Specifically, 1 $\mu$M ClipCG12 (1:20 molar ratio of ClipCG12 to tubulin) moderately increased the growth rate (19%) and moderately decreased the shortening rate (18%). There was a slight increase in the rescue frequency (13%), but this increase was not statistically different from the control. The effects of 2 $\mu$M ClipCG12 (a molar ratio of 1:10 to tubulin) were considerably stronger (Table 1 and Figure 3). It is likely that the effects of high ClipCG12 concentrations on dynamic instability, like those of EB1, are also due to the binding of ClipCG12 along the microtubule lengths.[10]

**Cooperative Modulation of Dynamic Instability by Mixtures of EB1 and ClipCG12.** EB1 is known to synergize with other +TIPs in modulating microtubule dynamic instability,[35,37−39] and we wanted to analyze the effects of EB1 and ClipCG12 mixtures on dynamic instability with our reconstituted system as compared with the effects of each protein individually. We found that in the presence of ClipCG12, EB1 modulated dynamic instability at a concentration considerably lower than when it was reconstituted with microtubules alone. Specifically, a mixture of 165 nM EB1 and 165 nM ClipCG12 (1:120 molar ratio of each protein to tubulin) increased the growth rate 14% and decreased the shortening rate 21% (Table 2 and Figure 4A), statistically significant differences from control microtubules. The low concentrations of EB1 and ClipCG12 used in this mixture also decreased the catastrophe frequency (43%) and increased the rescue frequency (36%) (Table 2 and Figure 4B). The dynamicity was also reduced from 2.3 to 1.62 (a 30% reduction; Figure 4C and Table 2). At 250 nM EB1 and 250 nM ClipCG12, a molar ratio of each protein to tubulin of 1:80, the growth rate was increased 29% and the shortening rate was reduced 49% (Figure 4A). The microtubules also spent 52% less time shortening than control microtubules. The catastrophe frequency was reduced by approximately 60%


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**Table 2. Effects of EB1 and ClipCG12 Mixtures on Dynamic Instability[a]**

| | EB1/ClipCG12/tubulin | | |
|---|---|---|---|
| dynamic instability parameter | control | 1:1:120<br>165 nM EB1 + 165 nM ClipCG12 | 1:1:80<br>250 nM EB1 + 250 nM ClipCG12 |
| growth rate ($\mu$m/min) | 2.1 ± 0.1 | 2.4 ± 0.2* (+14%) | 2.7 ± 0.3** (+29%) |
| shortening rate ($\mu$m/min) | 12.5 ± 1 | 9.9 ± 1** (−21%) | 6.4 ± 0.5*** (−49%) |
| percentage of time | | | |
| growing | 39 | 44 (+13%) | 43 (+10%) |
| shortening | 21 | 11 (−48%) | 10 (−52%) |
| attenuated | 40 | 45 (+13%) | 47 (+18%) |
| catastrophe frequency (per min) | 0.44 ± 0.02 | 0.25 ± 0.04*** (−43%) | 0.18 ± 0.03*** (−59%) |
| rescue frequency (per min) | 1.1 ± 0.03 | 1.49 ± 0.04** (+36%) | 1.6 ± 0.05*** (+46%) |
| dynamicity | 2.3 | 1.62 (−30%) | 1.26 (−45%) |

[a]Phosphocellulose purified bovine brain tubulin assembled to steady state in the presence of equimolar concentrations of EB1 and ClipCG12, and the dynamic instability parameters were determined as described under Experimental Procedures. Twenty-five to 30 microtubules were measured for each condition. *, **, and *** represent significance at >0.05, >0.01, and >0.001, respectively, as determined with a Students' *t* test. Control data are the same as those shown in Table 1, included here for the convenience of the reader. Data = mean ± SEM.

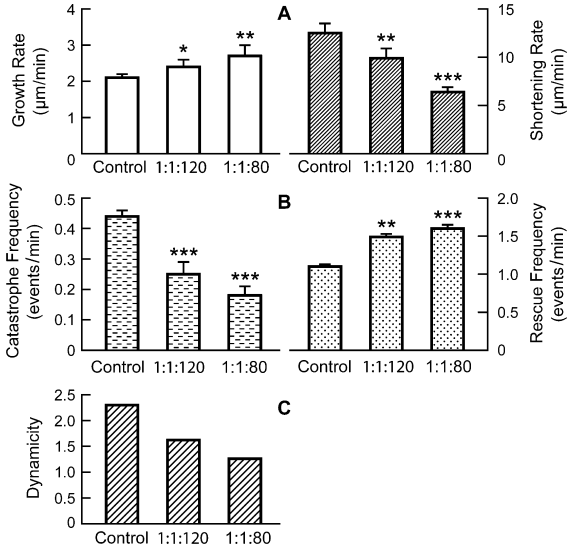


**Figure 4.** Effects of EB1 and ClipCG12 together on dynamic instability: (A) effects of the EB1−ClipCG12 mixture on the growth and shortening rates; (B) effects of the EB1−ClipCG12 mixture on the catastrophe and rescue frequencies; (C) effects of the EB1−ClipCG12 mixture on dynamicity. Either 165 nM EB1 and 165 nM ClipCG12 (1:120 molar ratio of each to tubulin) or 250 nM EB1 plus 250 nM ClipCG12 (a 1:80 molar ratio of each to tubulin) was mixed with 20 $\mu$M tubulin and 2 mM GTP in PMEM buffer, pH 6.8, added to sea urchin axoneme seeds, and incubated at 30 °C for 30 min to polymerize the microtubules to steady state. The dynamic instability parameters were analyzed as described under Experimental Procedures. Error bars = SEM.

(0.44 in control to 0.18 in ClipCG12−EB1-treated microtubules), and there was a 46% increase in the rescue frequency (Figure 4B). In addition, the EB1−ClipCG12 combination suppressed the dynamicity by 45% (Table 2 and Figure 4C), indicating that EB1 and ClipCG12 cooperatively modulate dynamic instability.

**Depletion of Stably Bound GDP-$P_i$ by EB1 and ClipCG12.** We previously found that when pulsed with [$\gamma^{32}$P] GTP, a small number of molecules of $^{32}P_i$ (~25), derived from the [$\gamma^{32}$P]GTP, remained stably bound to the microtubules after centrifugation through 50% sucrose cushions.[20] The stably bound $^{32}P_i$, which we determined was tubulin−GDP−$^{32}P_i$, that is, hydrolyzed GTP with the $^{32}P_i$ not

**Table 3. Effects of EB1, CLIPCG12, and an Equimolar Mixture of EB1 and ClipCG12 on the Number of Stably Bound Molecules of $^{32}P_i$ per Microtubule[a]**

| condition | no. of stably bound molecules of $^{32}P_i$ at microtubule ends |
|---|---|
| control (20 $\mu$M tubulin) | 23 ± 2 ($n$ = 18) |
| 1 $\mu$M EB1 + 20 $\mu$M tubulin | 24 ± 1 ($n$ = 9) |
| 1 $\mu$M ClipCG12 + 20 $\mu$M tubulin | 18 ± 1 ($n$ = 9) |
| 1 $\mu$M EB1 + 1 $\mu$M ClipCG12 + 20 $\mu$M tubulin | 11 ± 4* ($n$ = 12) |

[a]Microtubules (20 $\mu$M tubulin) were assembled to steady state for 30 min at 30 °C with 1 $\mu$M EB1 (a 1:20 molar ratio to tubulin), 1 $\mu$M ClipCG12 (a 1:20 molar ratio to tubulin), or a mixture of 1 $\mu$M EB1 + 1 $\mu$M ClipCG12 (a 1:1:20 molar ratio of EB1/ClipCG12/tubulin). The microtubule suspensions were then pulsed with [$\gamma^{32}$P]GTP for 25−30 min at 30 °C, and the stably bound GDP-$P_i$ was determined after sedimentation of the microtubules through 50% sucrose cushions for 2 h (Experimental Procedures). *, $p < 0.05$, versus control (Students' *t* test); *n*, number of samples per condition.

released after hydrolysis, is located specifically at the microtubule plus ends. In other words, the GTP was hydrolyzed and the $^{32}P_i$ remained locked into its site in an unexchangeable form. To probe the mechanism by which EB1 and CLIP-170 might modulate dynamics at microtubule ends, we examined the effect of 1 $\mu$M EB1 and 1 $\mu$M ClipCGI2, individually and together, on the stably bound $^{32}P_i$ (molar ratio of the +TIPs to tubulin of 1:20 (1 $\mu$M EB1 or 1 $\mu$M ClipCG12 to 20 $\mu$M tubulin). Because the quantity of stably bound $^{32}P_i$ varies appreciably from experiment to experiment, we needed to use 1 $\mu$M concentrations of each protein to obtain statistically significant results. One micromolar EB1 alone or 1 $\mu$M ClipCG12 alone had no significant effect on the quantity of stably bound $^{32}P_i$ (Table 3). However, the EB1−ClipCG12 combination reduced the amount of stably bound $^{32}P_i$ by 52%, from 23 molecules per microtubule to 11 molecules per microtubule, indicating that the EB1−ClipCG12 combination, but not each protein individually, is acting at the microtubule ends through a mechanism distinct from that of the individual +TIP components (Table 3).

## DISCUSSION

In this work we analyzed the state in solution of two +TIP proteins, the major mammalian +TIP protein EB1, and

ClipCG12, the functional head domain of CLIP-170. We then analyzed the effects of the two proteins, both individually and together, on dynamic instability at plus ends of reconstituted mammalian steady-state microtubules in vitro. We also determined the effects of EB1 and ClipCG12, both individually and together, on the stoichiometry of stably bound $^{32}P_i$ at microtubule ends, which may be a remnant or component of the stabilizing cap.[20] We found that EB1 and ClipCG12, when together at low concentrations (250 nM), strongly modulated microtubule dynamic instability but had no effect on dynamic instability when they were present separately. We also found that 1 $\mu$M mixtures of the two proteins, but neither protein individually, can reduce the amount of stably bound $^{32}P_i$ at the microtubule ends, indicating that the two proteins cooperate to bind simultaneously to the ends and suppress dynamics by modulating a component or a remnant of the stabilizing cap.

We previously reported that EB1 exists as an extended dimer in solution.[22,34] Here, by using SAXS, we found that ClipCG12 also adopts an extended conformation with its CAP-Gly domains separated. Analytical ultracentrifugation experiments with equimolar mixtures of EB1 and ClipCG12 showed that these two proteins interact in solution and, together, the results indicate that EB1 and ClipCG12 form a fully functional and elongated complex.

**Effects of EB1 and ClipCG12 Individually on Steady-State Microtubule Dynamic Instability.** The effects of mammalian EB1 and its family members EB3 and the fission yeast *Schizosaccharomyces pombe* EB1 orthologue Mal3 on microtubule dynamic instability in cells and cell extracts and with purified microtubule systems have been described by many investigators.[10,35,37,38,40−42] The specific effects of EB1 family members on the various dynamic instability parameters have varied in different systems, most likely due to the influence of other regulatory factors present in cells and cell extracts and the use of different proteins and conditions in vitro. For example, in *Xenopus* extracts, which contain a mixture of +TIP proteins, EB1 stimulates microtubule polymerization and stabilizes microtubules by increasing the rescue frequency and decreasing the catastrophe frequency.[40] Suppression of the catastrophe frequency by the EB1 orthologue Mal3p, which is 28.9% identical to EB1,[43] also occurred in extracts of *S. pombe*.[41]

The effects of EB1 family members on dynamic instability of reconstituted microtubule systems have also been quite variable.[10,35,37,38,40,42] For example, Manna et al.[35] found that 2 $\mu$M EB1 reduced the catastrophe frequency, whereas Vitre et al.[42] found that it increased the catastrophe frequency at the same concentration. Similarly, Montenegro Gouveia et al.[37] and Komarova et al.[11] reported that the EB1 family member EB3 enhanced the catastrophe frequency. Specifically, using 75 nM mCherry-EB3, Gouveia et al.[37] found that the catastrophe frequency increased from 0.13 in control microtubules to 0.75 in EB3-treated microtubules in vitro.[37] Similarly, Komarova et al. found that the catastrophe frequency increased from 0.13 in control microtubules to 1.17 and 1.00 at 200 nM and 1 $\mu$M EB1-treated microtubules, respectively.[11] Bieling et al.[38] found that the EB1 orthologue Mal3 (200 nM) increases the catastrophe frequency. These results with very low EB1 family member concentrations are noteworthy because they differ from those of Dixit et al.[10] using a reconstituted mammalian system, who found no effects of 250 nM EB1 on microtubule dynamic instability. As also discussed by Komarova et al.,[11] these differences could be due to the use of different experimental conditions such as different temperatures or whether analysis is carried out at steady state or under non-steady state conditions or, possibly, use of different EB1 concentrations (see below). In addition, the use of His-tagged EB1, which clearly affects microtubule−EB1 binding and polymerization induction, might have accounted for some of the observed differences.[44]

Here we reconstituted microtubule +TIP complexes with bovine brain tubulin and analyzed their effects separately and together on dynamic instability at polymer mass steady state, conditions at which the soluble tubulin concentration and total polymer mass remain constant. Like Dixit et al.[10] with a reconstituted mammalian microtubule system, we found that low concentrations of EB1 did not significantly affect any dynamic instability parameters. Higher EB1 concentrations did modulate dynamics. For example, 2 $\mu$M EB1 (a 1:10 ratio to tubulin) stabilized plus ends by suppressing the shortening rate (42%) and the catastrophe frequency (39%) and increasing the growth rate (57%) and rescue frequency (71%). Because EB1 is known to bind along the microtubule surface at these relatively high concentrations, the effects may be due to a combination of plus tip binding and lattice binding.

A low concentration of ClipCG12 by itself, 250 nM, also did not affect any dynamic instability parameters. However, higher concentrations did. Specifically, 2 $\mu$M ClipCG12 (a 1:10 ratio to tubulin) was quite active, increasing the growth rate 43% (Table 1 and Figure 3). This is consistent with previous reports that CLIP-170 stimulates tubulin polymerization by binding to microtubule surfaces.[45,46] In addition, 2 $\mu$M ClipCG12 suppressed the shortening rate (22%), and consistent with previous results,[45,46] it increased the rescue frequency ~30% (Table 1). The slight suppression of the catastrophe frequency we observed (14%) may not be real because it was not statistically significant (Table 1 and Figure 3). The effects of ClipCG12 alone on dynamic instability are probably due to its binding along the microtubule lattice as this fragment is not able to autonomously localize to growing microtubule plus ends.[10,18,19]

**Cooperative Modulation of Dynamic Instability by EB1 and ClipCG12.** We previously found that a mixture of EB1 and the CAP-Gly domain of p150$^{glued}$ at plus ends appeared to be cooperative, increasing the growth rate and decreasing the shortening rate more strongly than either +TIP protein itself.[35] Thus, we wanted to investigate the generality of such cooperative activity between +TIP proteins and chose to analyze the effects of EB1 in the absence and presence of a different +TIP protein, ClipCG12, the minimal functional fragment of CLIP-170.

We found that a mixture of 250 nM EB1, 250 nM ClipCG12, and 20 $\mu$M tubulin (a 1:1:80 molar mixture of EB1/ClipCG12/tubulin) stabilized microtubule dynamics strongly, whereas neither +TIP protein significantly affected any dynamic instability parameter when present individually. For example, the mixture of EB1 and ClipCG12 increased the growth rate 29% (Table 2), which is comparable to the effects of EB1 and ClipCG12 individually on this parameter at 8-fold higher concentrations (see Table 1). The combination also strongly decreased the catastrophe frequency (59%) (Table 2). The 1:1:80 ratio of EB1/ClipCG12/tubulin also strongly suppressed the dynamicity, a parameter that describes the overall effects of the mixture on microtubule dynamics (Table 2 and Figure 4). Therefore, like mixtures of EB1 and the CAP-Gly domain of p150$^{glued}$,[35] mixtures of EB1 and ClipCG12 act

cooperatively and apparently synergistically to stabilize plus ends.

**Effects of ClipCG12 and EB1, Individually and Together, on the Stably Bound $^{32}P_i$ at Microtubule Ends.** We previously found that when microtubules are pulsed with [$\gamma^{32}$P]GTP, a small amount of $^{32}P_i$ remains bound at the ends of the microtubules (~25 molecules per microtubule[20]) after sedimentation of the microtubules for 2 h through warm 50% sucrose cushions. The $^{32}P_i$ is nonexchangeably incorporated into the microtubules at their ends (it does not exchange with added orthophosphate) and is derived from the E-site bound [$\gamma^{32}$P]GTP. The stably bound $^{32}P_i$ could have been hydrolyzed either as or after the tubulin-[$\gamma^{32}$P]GTP was added to the microtubule ends, or at any time during the 2 h sedimentation through the 50% sucrose cushion. Whereas 50% sucrose is well-known to strongly stabilize microtubules, the possibility cannot be excluded that some hydrolysis or phosphate loss occurred during sedimentation. The 2 h sedimentation through 50% sucrose cushions is essential to enable detection of such a small amount of microtubule-bound $^{32}P_i$. The stably bound $^{32}P_i$ (GDP-$P_i$) may represent a component or a remnant of the stabilizing cap. Whereas Caplow and Fee[47] have argued that the stably bound $P_i$ we measured was derived from the binding of $P_i$ itself to the microtubule lattice,[47] this is not the case, because the data in the original paper[20] and our recent data clearly demonstrate that the stably bound $P_i$ is indeed derived from [$\gamma^{32}$P]GTP and is located only at the microtubule ends. Specifically, we have shown that the number of molecules of stably bound $^{32}P_i$ per microtubule remains the same when microtubule lengths are varied between ~2.5 and 11.5 $\mu$m, and no $^{32}P_i$ binds to the microtubules when they are pulsed with $^{32}P_i$ (L. Wilson, H. P. Miller, and D. Panda, unpublished results). Therefore, to ask whether the mixture of EB1 and ClipCG12 might be affecting an aspect of the stabilizing cap, we determined if EB1 and ClipCG12, either individually or together, could modulate the quantity of stably bound $^{32}P_i$ after a [$\gamma^{32}$P]GTP pulse.

As shown in Table 3, 1 $\mu$M EB1 alone or 1 $\mu$M ClipCG12 alone (each at a 1:20 molar ratio to tubulin) did not influence the quantity of stably bound $^{32}P_i$ in a statistically significant fashion. However, a mixture of 1 $\mu$M EB1 and 1 $\mu$M ClipCG12 (each at a 1:20 molar ratio of EB1−ClipCG12 to tubulin) substantially reduced the amount of stably bound $^{32}P_i$ (Table 3). These data indicate that the mixture of EB1 and ClipCG12 exerts a unique effect at the tips of steady-state microtubules that is distinct from that of EB1 or ClipCG12 individually. Whereas EB1 is known to recognize a GTP-like conformation at microtubule tips,[48] we did not see any effect on the stably bound $^{32}P_i$ with EB1 alone. Perhaps when EB1 and ClipCG12 bind together to tips, they induce a conformational state in the tubulin that enhances the stabilizing GTP-like conformation of tubulin while permitting release of the $P_i$. Perhaps as well, specific recruitment of ClipCG12 through EB1 to the growing microtubule tip permits depletion of the stably bound GDP-$P_i$.

Interestingly, the reduction of stably bound $^{32}P_i$ by the mixture of EB1 and ClipCG12 is similar to the actions of low concentrations of colchicine and vinblastine.[20] Both drugs stabilize microtubule plus ends while reducing the amount of stably bound $^{32}P_i$. Although the end-stabilizing/acting microtubule targeted drugs and the +TIPs modulate microtubule dynamics in somewhat different ways, it seems likely that microtubule-end binding natural product drugs such as vinblastine[20] and maytansine[49] evolved to mimic the actions of natural regulatory proteins[50] including +TIPs. Moreover, a recent paper suggests that CLIP-170 promotes the binding of the microtubule-stabilizing anticancer drug paclitaxel to microtubules.[51] Such data indicate the possibility that +TIP proteins might act together with microtubule targeted anticancer drugs to regulate microtubule dynamics.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

PMEM buffer, 87 mM Pipes, 36 mM MES, 1 mM EGTA, and 2 mM $MgCl_2$, pH 6.8; EB1, end binding protein 1; CLIP-170, cytoplasmic linker protein-170; ClipCG12, CLIP CAP-Gly domains 1 and 2

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