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Microtubule +TIP Protein EB1 Binds to GTP and Undergoes Dissociation from Dimer to Monomer on Binding GTP

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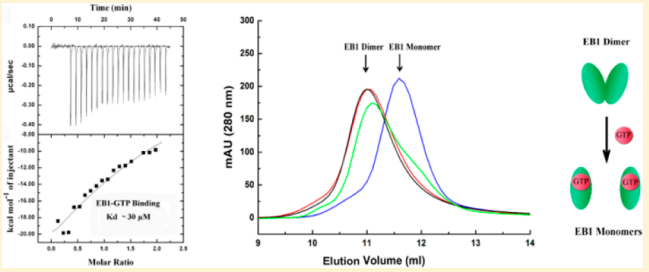
ABSTRACT: The +TIP protein EB1 autonomously tracks the growing plus end of microtubules and regulates plus-end dynamics. Previous studies have indicated that EB1 can recognize GTP-bound tubulin structures at the plus end, and it localizes on the microtubule surface at a site close to the exchangeable GTP-binding site of tubulin. Although the GTP-dependent structural change in tubulin has been demonstrated to be a critical determinant for recognition of plus ends by EB1, the effect of GTP on the structure of EB1 has remained unclear. Here, we have used spectroscopic, calorimetric, and biochemical methods to analyze the effect of GTP on EB1 *in vitro*. Isothermal titration calorimetry and tryptophan fluorescence quenching experiments demonstrated that EB1 binds to GTP with a dissociation constant ~30 μM. Circular dichroism measurements showed that EB1 undergoes changes in its secondary structure on binding GTP. Size-exclusion chromatography and urea-induced unfolding analyses revealed that GTP binding induces dissociation of the EB1 dimer to monomers. Size-exclusion chromatography followed by biochemical analysis further determined that EB1−GTP binding involves association of approximately one molecule of GTP per EB1 monomer. The results reveal a hitherto unknown GTP-dependent mechanism of dimer-to-monomer transition in EB1 and further implicate its possible role in regulating the stability of the EB1 dimer vs monomer as well as plus-end regulation in cells.



Microtubules are major cytoskeletal components that are essential for cell division, cell polarity, and cellular morphogenesis. They are intrinsically polar tubular structures consisting of two structurally and functionally distinct ends: a fast-growing plus end and a slow-growing minus end. In cells, the plus ends are highly dynamic and stochastically switch between phases of growth and shortening, which is a phenomenon known as dynamic instability, whereas the minus ends are often anchored to the microtubule organizing center and thus are less dynamic.[1−3] Microtubules polymerize through the addition of guanosine triphosphate (GTP)-bound tubulin subunits to their plus ends. GTP−tubulin subunits enriched at the plus end of the polymerized lattice protect the end from depolymerization by forming a stabilizing structure at the end.[4] The hydrolysis of GTP−tubulin to guanosine diphosphate (GDP)−tubulin leads to the loss of this stabilizing structure, which causes destabilization in the lattice and leads to depolymerization of the microtubule protofilaments. The stochastic addition or loss of GTP−tubulin subunits at the plus ends contributes to the dynamic instability of the microtubules.[1,3,5] Among the proteins known to regulate dynamic instability, tip tracking proteins (+TIPs) are special in that they selectively target the growing plus ends of microtubules, track the ends, and regulate numerous microtubule-mediated processes.[6]

Recent studies have emphasized the central role of EB1 among the +TIPs proteins.[7] It is evolutionarily conserved and is the core component of multiprotein complexes at the plus ends.[8] It can track the plus ends autonomously, independent of other factors. Additionally, it interacts with numerous other plus-end modulators and stabilizes their accumulation at the plus ends.[9−13] Several studies have demonstrated the critical role of EB1 in regulating microtubule plus-end dynamics.[14−17] However, the mechanism through which it targets the plus ends is yet to be fully understood. EB1 consists of a globular N-terminal microtubule-binding domain known as the calponin homology (CH) domain[13,18] and a C-terminal coiled-coil EB homology (EBH) domain that has been shown to be involved in dimerization of EB1 monomers[19] and also in binding with other +TIPs.[20,21]

Recent high-resolution microscopy and biochemical analyses with the fission yeast EB1 homologue, Mal3, showed that it preferentially recognized GTP-loaded tubulin structures at the plus ends.[22,23] Specifically, Mal3 binds to the outer microtubule surface in the region close to the exchangeable GTP-binding site of tubulin. In addition to binding to these sites, it has been shown to sense GTP-hydrolysis-mediated conformational

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changes in the microtubule lattice.[24] Subsequent studies have indicated that EB1 binding promotes conformational maturation of the polymerizing ends, presumably by accelerating the reactions of GTP hydrolysis cycle at the ends.[24] Although the GTP-dependent structural change in the tubulin dimer has been demonstrated to be a critical determinant for recognition of the plus ends by EB1, little is known about how GTP affects the structure of EB1. The close association of EB1 at the GTP-binding site of tubulin in the microtubule prompted us to investigate the effect of GTP on the structure of EB1.

In this study, we investigated the effect of GTP on the structural stability of EB1 *in vitro*. Isothermal titration calorimetry and tryptophan fluorescence quenching experiments demonstrated that EB1 binds to GTP with moderate affinity. Through biochemical analysis, we further determined the binding stoichiometry between EB1 and GTP. Measurements of circular dichroism (CD) spectra showed that EB1 undergoes changes in its secondary structure on binding GTP. Size-exclusion chromatography and urea-induced unfolding experiments revealed that EB1 undergoes dissociation from its dimer to the monomer form on binding GTP. The results revealed, for the first time, that GTP substantially modulates the structure of EB1 and plays a critical role in regulating its transition from the dimer to the monomer form. The results also suggest that the GTP-dependent structural transition in EB1 may regulate the levels of EB1 dimer vs monomer in cells.

MATERIALS AND METHODS

Materials. GTP, GDP, GTP-γS, PIPES, and EGTA were obtained from Sigma (St. Louis, MO, USA).

Plasmids and Proteins. Full-length human EB1 cDNA (generously provided by Stephen Doxsey, UMass Medical School, Worcester, MA) was used to clone and purify recombinant EB1. Briefly, the full-length EB1 insert obtained by PCR from its cDNA was ligated into the pET28a vector (Novagen) and transformed into BL21 (DE3) cells. Cells expressing 6-His-tagged EB1 were cultured under induction with IPTG (1 mM) for 6 h prior to cell lysis. EB1 was purified after passing the lysate through a Ni^{2+}-NTA column, followed by elution using 0.5 M imidazole.[15] Near 100% pure EB1 was obtained. Protein concentrations were estimated using the Bradford method with BSA as the standard.[25]

Isothermal Titration Calorimetry (ITC). Thermodynamic measurements were performed using ITC-200 microcalorimeter from Microcal (Northampton, MA, USA). EB1 (15 μM), after dialysis with 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 7.0 (PEM buffer), was titrated against GTP (150 μM), which was dissolved in the final dialysate. A typical titration involved injecting 20 injection volumes (2 μL) of GTP into the sample cell containing EB1 (201.6 μL) at 2.0 min intervals with continuous stirring.[26] The heat of dilution data corresponding to individual injections were analyzed using a binding model of one set of sites considering one GTP binding site per EB1 monomer with the system running Microcal Origin 7.0 software. The ΔH and ΔS values were obtained using a nonlinear least-squares fit of the data. Gibbs free energy (ΔG) was calculated by using Gibbs equation: $\Delta G = \Delta H - T\Delta S$.

Fluorescence Measurements and Determination of K_d. Fluorescence measurements were performed using a HORIBA Fluorolog-3 spectrofluorimeter. A cuvette with a 1 cm path length was used for all fluorescence measurements. EB1 (1.5 μM) was incubated with varying concentrations (5−40 μM) of GTP in PBS, 50 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, and 1 mM $MgCl_2$ at room temperature for 30 min. The tryptophan fluorescence measurements were performed using 290 nm as the excitation wavelength.[27] When excited at 290 nm, EB1 displayed its characteristic emission spectrum with a maximum at 340 nm. The intrinsic tryptophan fluorescence of EB1 in the presence and absence of gradients of GTP concentrations was measured. The inner filter effect was corrected for the observed fluorescence values using the equation $F_{corrected} = F_{observed}$ antilog $[(A_{ex} + A_{em})/2]$, where A_{ex} and A_{em} are the absorbance at the excitation and emission wavelengths, respectively.[28] The dissociation constant (K_d) was determined by the equation $1/X = 1 + K_d/[L_f]$, where X is the fractional occupancy and L_f is the free GTP concentration. The fraction of binding sites (X) of GTP on EB1 was determined by using an equation, $X = \Delta F/\Delta F_{max}$, where ΔF is the change in fluorescence intensities at different GTP concentrations and ΔF_{max} is the value of maximum fluorescence change when EB1−GTP binding is completely saturated. ΔF_{max} was obtained by extrapolating 1/[GTP] to zero in the $1/\Delta F$ vs 1/[GTP] plot.[29,30] The K_d is represented as mean ± SEM.

Size-Exclusion Chromatography. For analysis of dimer vs monomer form of EB1, EB1 (30 μM) control or the mixtures of EB1 (30 μM) and GTP (0.5 to 1.5 mM) or GTP-γS (0.5 to 1.5 mM) or GDP (1.5 mM) in PBS buffer, pH 7.4, containing 1 mM $MgCl_2$ were run through a Superose-12 size-exclusion column (Akta 10, GE Life Sciences, USA) at 4 °C. The column was pre-equilibrated with the same buffer at 4 °C prior to loading the protein samples. The elution profiles were obtained by measuring absorbance at 280 nm. Bovine serum albumin (BSA) (66 kDa) and carbonic anhydrase (29 kDa) were run as standards. The elution volumes of the EB1 dimer and monomer were 11.0 and 11.6 mL, respectively.

For determination of binding stoichiometry between the EB1 and GTP, a 1:50 molar mixture of EB1 (30 μM) and GTP (1.5 mM) in PBS buffer, pH 7.4, containing 1 mM $MgCl_2$ was loaded onto the Superose-12 size-exclusion column, and the eluted samples were collected in 0.5 mL fraction volumes. The column was pre-equilibrated with the same buffer at 4 °C prior to loading the protein samples. Each of the eluted protein fractions was equally divided into two fraction volumes: in one-half, the amount of GTP was estimated by measuring the absorbance of GTP at 252 nm after precipitating EB1 protein by ice-cold 4 M perchloric acid followed by centrifugation at 13 000 rpm at 4 °C; in the other half of the fraction, the amount of EB1 was estimated by Bradford assay. The estimated amounts of EB1 and GTP present in the eluted fractions were used to determine the molar stoichiometry between EB1 monomer and GTP. The data shown are based on the analysis of three independent experiments.

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectroscopy was used to discern the GTP- or GDP-induced conformational changes in EB1 using a CD spectrophotometer (JASCO 815). A 0.2 cm path length cuvette was used for the measurements. CD spectra of EB1 (3 μM) in the presence and absence of gradients of GTP concentrations in PBS buffer, pH 7.4, containing 1 mM $MgCl_2$ were measured in the 215−240 nm range. The control spectrum of GTP or GDP alone was subtracted from the sample data to obtain the final EB1 spectrum.

For CD analysis under urea-induced denaturation conditions, EB1 (3 μM) in the absence or presence of GTP (1 mM) in PBS buffer, pH 7.4, containing 1 mM $MgCl_2$ was denatured with varying concentrations (1−9 M) of urea, and the far-UV

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CD spectra (215−240 nm) of the denatured protein samples were measured. CD data at 222 nm were plotted as a function of urea concentration in order to obtain the stability curves. Transition midpoints of unfolding were determined by using the growth/sigmoidal function under nonlinear curve fitting in Origin 8.1 software.

Data Analysis and Fitting. The data were plotted and fitted using Origin 8.1 software. The figures were organized using Adobe Illustrator. SEM refers to standard error of the mean.

RESULTS

EB1 Binds to GTP *in Vitro*. We first investigated the binding of EB1 with GTP using isothermal titration calorimetry (ITC). EB1 was titrated with GTP through a series of mixing reactions until the heat changes reached close to saturation. The results showed that EB1 bound to GTP with moderate affinity. The association constant (K_a) of GTP−EB1 binding was 3.12×10^4 M^{-1}, which corresponded to a dissociation constant (K_d) of ~32 μM. The reaction involved an enthalpy change of approximately −40 ± 8.7 kcal/mol and an entropy change of −117 cal/mol (Figure 1A,B). The Gibbs free energy

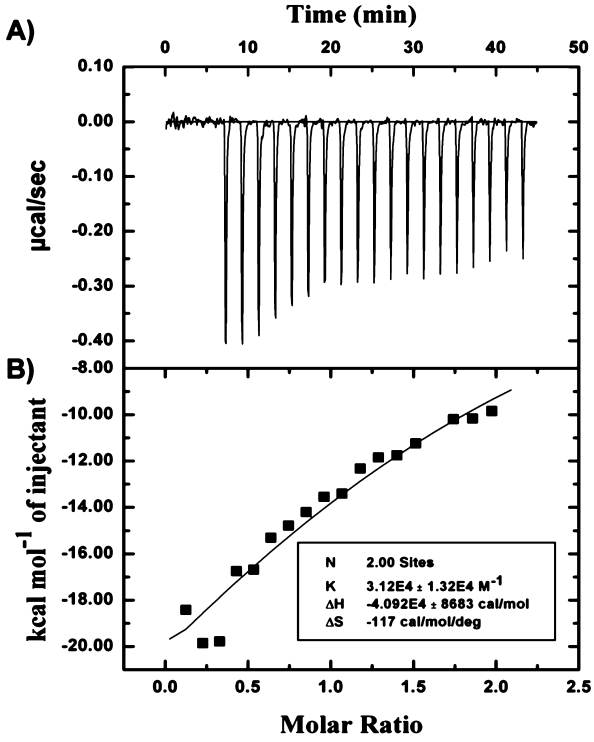

Figure 1. Isothermal calorimetric titration of EB1 by GTP. (A) Raw data obtained from 20 injections of 150 μM GTP to 15 μM EB1 in PEM buffer at 25 °C. (B) Nonlinear least-squares fit of the heat changes per mole of the added ligand in the titration shown in panel A as a function of the molar ratio of GTP and EB1 using Origin 7. Data are representative of three independent experiments.

change was −6 kcal/mol. Such a large negative enthalpy change in the absence of any positive entropy change indicates that the binding reaction was predominantly enthalpy-driven. We also performed titration of EB1 with GDP. No effective heat change characteristic of stable binding was detected with GDP (data not shown).

GTP binding was further determined by a fluorescence-based quenching experiment. EB1 contains two tryptophan residues, at amino acid positions 23 and 110, that exhibit intrinsic fluorescence upon excitation (Figure 2A). The intrinsic

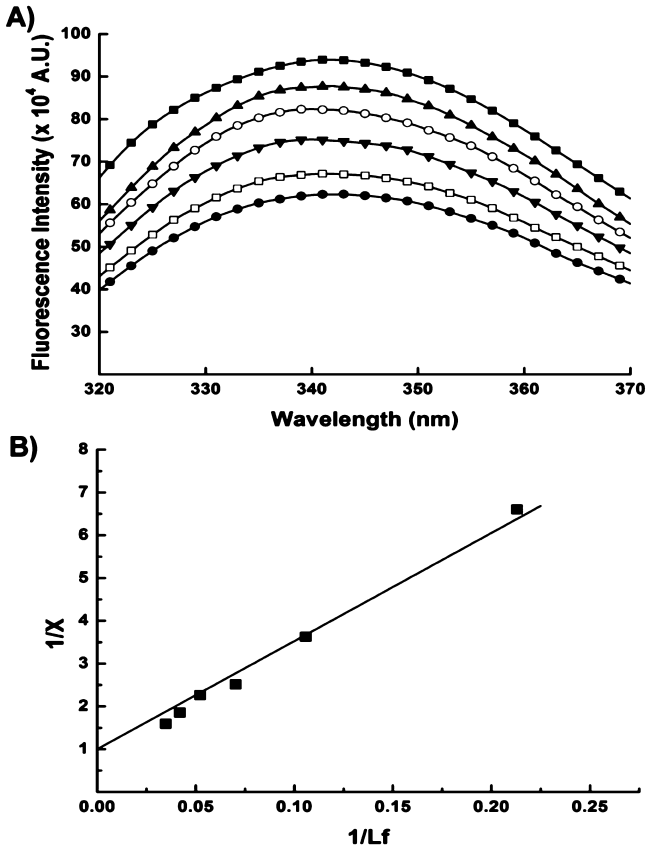

Figure 2. Effect of GTP on intrinsic tryptophan fluorescence of EB1. (A) EB1 (1.5 μM) was mixed with varying concentrations of GTP from 0 (■), 5 (▲), 10 (○), 20 (▼), 30 (□), and 40 μM (●) in PBS (pH 7.4) with 1 mM $MgCl_2$, and the emission spectra were recorded after 30 min of incubation at 25 °C. The emission spectra (320−370 nm) were measured by exciting the protein samples at 290 nm. (B) Double reciprocal plot of binding of GTP to EB1. L_f refers to the free GTP concentration (Materials and Methods). Data are representative of three independent experiments.

tryptophan fluorescence of EB1 (1.5 μM) was measured after mixing it with increasing concentrations of GTP (5−40 μM). GTP quenched the intrinsic tryptophan fluorescence of EB1 in a concentration-dependent manner, indicating that GTP altered the moiety of tryptophan residues in EB1 through its binding (Figure 2A). A double reciprocal plot of the fluorescence data yielded a dissociation constant (K_d) of 30.8 ± 5.5 μM (Figure 2B) (Materials and Methods). The K_d determined by this method was in good agreement with the K_d obtained from ITC analysis (Figure 1). We also found that addition of GDP did not cause significant quenching of tryptophan fluorescence of EB1, indicating that the interaction of GDP is too weak to induce quenching.

Binding of GTP Induces Changes in the Secondary Structure of EB1. Because EB1 can bind to GTP, we then investigated whether GTP binding induces any conformational changes in EB1. We assessed conformational changes in EB1 (3 μM) by measuring the far-UV CD spectra of EB1 in the presence and absence of GTP concentration gradients (20 μM

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to 1.5 mM). The results showed a dose-dependent decrease in molar ellipticity in the 215−225 nm region, indicating that GTP induced a loss of secondary structure in EB1 (Figure 3A).

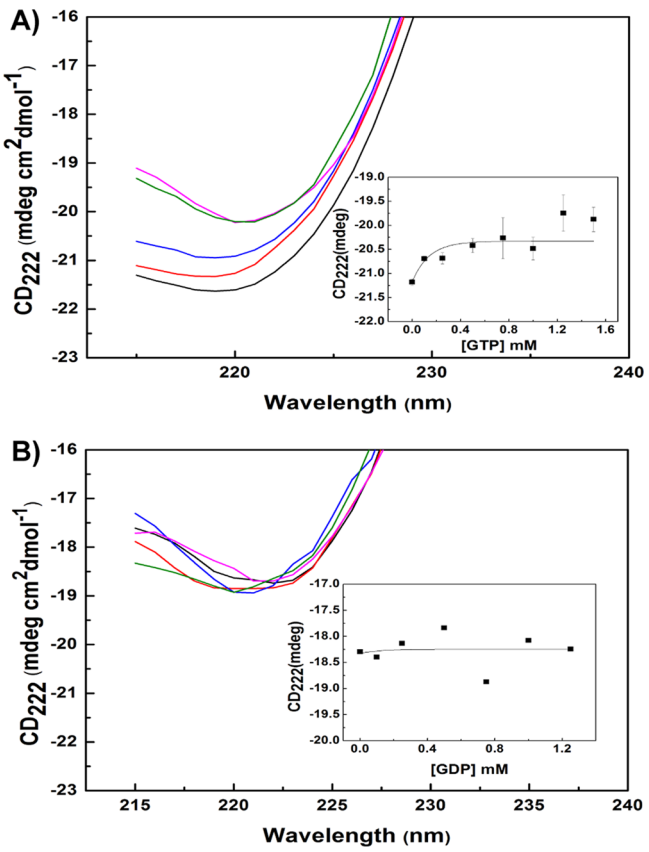

Figure 3. Effects of GTP and GDP on the secondary structure of EB1. (A) Far-UV CD spectra of EB1 (3 μM) in the absence (black) and presence of 0.1 (red), 0.25 (blue), 1 (pink), and 1.5 mM (green) GTP in PBS (pH 7.4) with 1 mM $MgCl_2$. (B) Far-UV CD spectra of EB1 (3 μM) in the absence (black) and presence of 0.1 (red), 0.25 (blue), 1 (pink), and 1.25 mM (green) GDP in PBS (pH 7.4) with 1 mM $MgCl_2$. Data are representative of three experiments. Insets in panels A and B show the plots of CD values at 222 nm vs GTP and GDP concentrations, respectively.

CD spectra at GTP concentrations below 0.1 mM are not shown, as they were close to the control spectrum in the absence of GTP. Plot of the molar ellipticity values at 222 nm (minima), which represents α-helix structure, as a function of GTP concentrations showed a concentration-dependent decrease of molar ellipticity as the GTP concentration increased (inset, Figure 3A). We also verified the effect of GDP on the conformation of EB1 by far-UV CD. GDP did not significantly alter the CD spectrum of EB1, indicating that it did not exert any effect on the secondary structure of EB1 (Figure 3B).

Binding of GTP Induces Dissociation of the EB1 Dimer to Monomers. We then questioned whether GTP binding and the GTP-induced conformational changes in EB1 had any effect on the stability of the EB1 dimer. We first examined this using size-exclusion chromatography. EB1 (30 μM) premixed with increasing concentrations of GTP was loaded onto a Superpose-12 size-exclusion column, and the relative abundance of EB1 dimer vs monomer was assessed by the absorbance of the eluted proteins. EB1 in the absence of GTP was eluted at a volume of 11.0 mL, corresponding to the size of the EB1 dimer (Figure 4A). EB1 (30 μM) mixed with GTP (0.5 mM) (corresponding to ~1:17 molar ratio of EB1 and GTP) was also eluted in the dimer form. Interestingly, when mixed with 1 mM GTP (corresponding to a 1:33 molar ratio of EB1 and

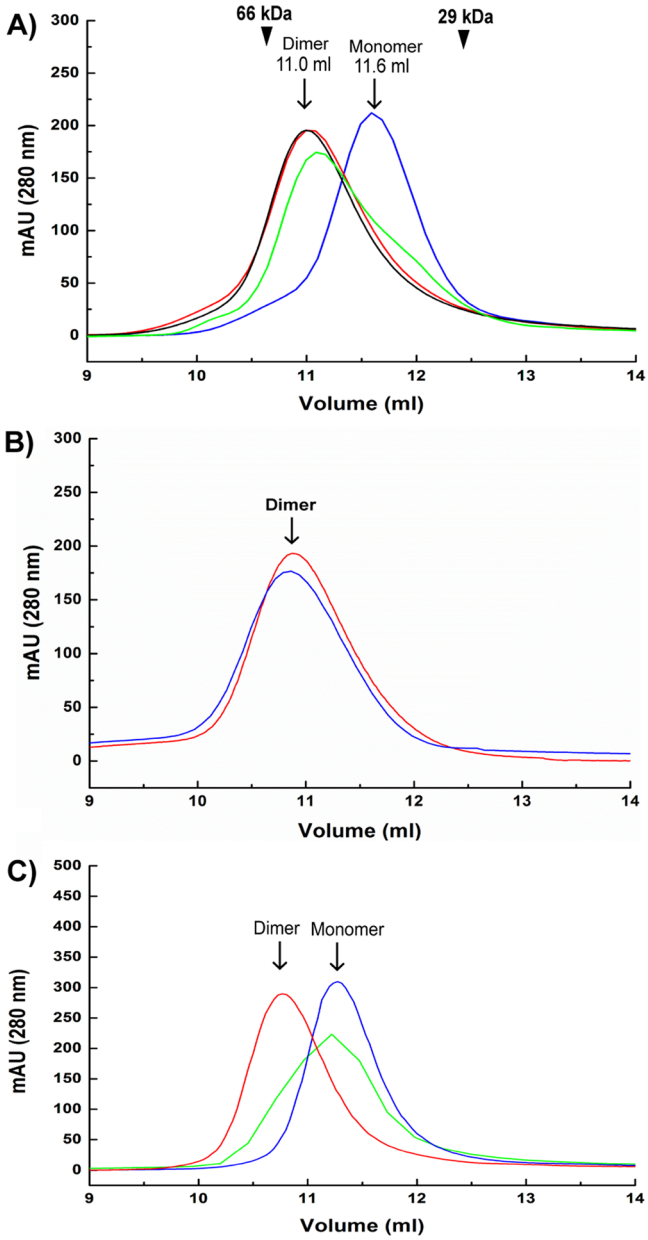

Figure 4. Addition of GTP induces dissociation of EB1 dimer to monomers. (A) Elution profiles of 30 μM EB1 in the absence (black) and presence of 0.5 mM (red), 1 mM (green), and 1.5 mM GTP (blue) in PBS buffer (pH 7.4) with 1 mM $MgCl_2$ after passing through Superose-12 size-exclusion column. Elution volumes 11.0 and 11.6 mL refer to the EB1 dimer and monomer, respectively. Data are representative of three experiments. Molecular weight standards, BSA (66 kDa) and carbonic anhydrase (29 kDa), are shown by arrows. (B) Elution profiles of 30 μM EB1 in the absence (red) and presence of 1.5 mM (blue) GDP in PBS buffer (pH 7.4) with 1 mM $MgCl_2$. (C) Addition of GTPγS induces dissociation of EB1 dimer to monomers. Elution profiles of 30 μM EB1 in the absence (red) and presence of 1 mM (green) and 1.5 mM (blue) GTPγS in PBS buffer (pH 7.4) with 1 mM $MgCl_2$.

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GTP), an additional peak corresponding to the EB1 monomer (11.6 mL) was observed with a concomitant decrease of the EB1 dimer peak (Figure 4A). The relative abundance of the EB1 monomer over the dimer was further increased with increasing GTP concentrations. At 1.5 mM GTP (1:50 molar ratio of EB1 and GTP), 100% of EB1 was eluted as the monomer form. These results indicate that GTP binding induces the dissociation of the EB1 dimer to monomer form. We also verified the effect of GDP on the stability of the EB1 dimer by size-exclusion chromatography. We found that GDP could not induce the dissociation of the EB1 dimer to monomers (Figure 4B).

To determine whether the hydrolysis of GTP is required for the dissociation of the EB1 dimer, size-exclusion chromatography was performed using a very slowly hydrolyzable analogue of GTP with a modification at the γ-phosphate, GTPγS. Similar to GTP, the addition of GTPγS induced the dissociation of the EB1 dimer to monomer form (Figure 4C), indicating that GTP binding only was sufficient to induce the dissociation of the EB1 dimer.

The GTP-induced dissociation of EB1 dimer to monomers was also confirmed by analyzing the structural stability of EB1 against urea-induced unfolding in the presence and absence of GTP. EB1 in the presence or absence of GTP was denatured with urea concentration gradients (1−9 M), and the structure of EB1 at different urea concentrations was assessed by recording the CD spectra. On the basis of the results of the size-exclusion chromatography analysis (Figure 4A), we used GTP (1 mM) well in excess over EB1 (3 μM) to ensure complete dissociation of the EB1 dimer to the monomer form. The molar ellipticity values plot at 222 nm of EB1 (− GTP) samples as a function of urea concentrations showed two structural transition midpoints (T_{m1} and T_{m2}) at 4.18 and 7.08 M, corresponding to the presence of the EB1 dimer and monomer, respectively. However, EB1 preincubated with GTP showed a single structural transition with a midpoint (T_m) at 5.3 M urea, indicating that it was present predominantly in the monomer form (Figure 5A,B). It should be noted that the structural unfolding of EB1 when preincubated with GTP occurred at a relatively lower urea (5.3 M) concentration than the urea concentration (7.08 M) required for unfolding of the EB1 monomer in the absence of GTP. This could be due to the difference in the conformational stability of the GTP-bound monomer form vs the monomer form in the absence of GTP.

We then determined the binding stoichiometry between the EB1 monomer and GTP. EB1 (30 μM) preincubated with GTP (1.5 mM) at a 1:50 molar ratio, at which EB1 predominantly exists in the monomer form (Figure 4A), was loaded onto the Superose-12 size-exclusion column, and the amounts of EB1 and GTP in the eluted protein fractions were estimated by biochemical measurements (Materials and Methods). GTP was present in all of the eluted EB1 fractions, indicating that EB1 was eluted in the GTP-bound form. We determined the binding stoichiometry from the estimated amounts of EB1 and GTP present in the eluted fractions. The binding stoichiometry (molar) between the EB1 monomer and GTP was 1:(1.2 ± 0.35) (SEM), which indicates that each EB1 monomer binds to approximately one molecule of GTP.

DISCUSSION

We have shown in this study that EB1 binds to GTP and undergoes conformational changes that induce dissociation of the EB1 dimer to monomer form. It has been demonstrated

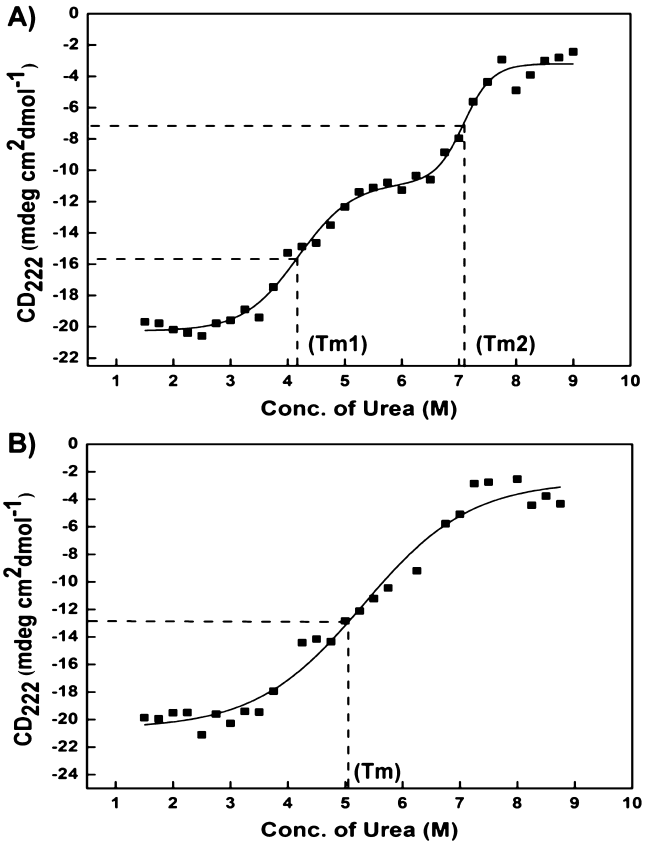

Figure 5. Urea-induced unfolding of EB1 in the absence (A) and presence (B) of GTP. EB1 (3 μM) in the absence and presence of GTP (1 mM) in PBS (pH 7.4) with 1 mM $MgCl_2$ was denatured with gradients of urea concentrations (1−9 M), and the far-UV CD spectra were measured. The molar ellipticity values at 222 nm (CD_{222}) were plotted against the urea concentration. Transition midpoints (T_m) in panel A are 4.18 and 7.08 M, respectively, and that in panel B is 5.3 M. Data are representative of three independent experiments.

previously that EB1 exists as an obligatory dimer under normal buffer conditions.[31] The results of the present study additionally revealed that the stability of the EB1 dimer changes drastically in the presence GTP. Far-UV CD data showed a consistent loss of secondary structure in EB1 as a result of GTP binding (Figure 3A). We found that the percent decrease of molar ellipticity (CD_{222} value) of EB1 at 0.1 mM GTP, at which the molar ratio of EB1 and GTP was ∼1:33, was about 25% compared to the total change of molar ellipticity resulted at 1.5 mM GTP (Figure 3A). This indicated that a significant conformational perturbation occurred at 0.1 mM GTP. We also observed that the EB1 dimer was dissociated to monomers to a detectable extent at the ∼1:33 molar ratio of EB1 and GTP (Figure 4A), suggesting that the structural perturbation occurring at this molar ratio was sufficient to induce the dissociation. It has been shown previously that GTP binding induces only local conformational changes in proteins.[32−35] Consistently, we found that the amount of secondary structural change induced in EB1 due to GTP binding was comparable to the extent of GTP-induced secondary structural changes reported in other GTP-binding proteins.[32,33]

The ITC and fluorescence quenching data indicated that EB1−GTP binding can occur at a relatively lower stoichiometry of EB1 and GTP than the that required to trigger the dissociation of the EB1 dimer to monomers (Figures 1, 2, and

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4). For example, in the ITC experiment, we detected the binding through titration of EB1 with only a 10-fold molar excess of GTP (Figure 1). In the fluorescence quenching experiment, the binding was detected by addition of about a 10-fold molar excess of GTP over EB1 (Figure 2). However, the dimer-to-monomer dissociation was detectable only at a molar ratio of EB1 and GTP of ∼1:30 or above (Figure 4A). It is possible that some additional structural perturbation is induced under the high GTP conditions that trigger the dissociation of the dimer. Consistent with this mechanism, we found that there was a significant loss of ellipticity (CD value) in EB1 when the GTP concentration was increased from 0.1 to 1 mM (Figure 3A).

Although the dissociation constant of the EB1 dimer-to-monomer dissociation reaction is unknown, competitive binding studies between the EB1 C-terminus (EB1-C) dimer and the binding region of EB1-C binding protein, adenomatous polyposis coli (APC),[20] suggested that the dissociation constant of EB1 monomer−monomer interaction could be in the micromolar range. Our results showed that EB1 bound to GTP with a relatively high dissociation constant ∼30 μM (Figures 1 and 2). Thus, it is reasonable to think that a significantly higher amount of GTP over that of EB1 would be required to destabilize the monomer−monomer interaction in the EB1 dimer. Consistent with this idea, we found that a minimum of a ∼25−30-fold molar excess of GTP over that of the EB1 dimer was required to induce the dissociation of the EB1 dimer to monomer form (Figure 4).

Previous cellular studies on EB family proteins have identified the formation of heterodimers of EB proteins in cells under specific conditions, although the individual EB proteins are homodimers.[17] This suggested that the dimers of EB proteins can dissociate to monomers and then the monomer of one EB can reassociate with the monomer of another EB. Reassociation of the monomer of one EB with the monomer of the same EB, i.e., formation of the homodimer again, could also be possible. This also implicated that the monomer form can exist in cells and that the transition between the EB dimer and monomer is tightly regulated in cells. Our results provide an additional implication, namely, that GTP could be an important factor involved in regulating the dimer−monomer transition of EB1 in cells. GTP concentration at or near the growing ends of microtubules can regulate the relative levels of the EB1 dimer vs monomer at the ends and modulate the function of EB1 at the ends.

The form in which EB1 is recruited onto the microtubules, dimer or monomer, has not been determined. Recent cryo-electron microscopy studies on the interaction of the fission yeast homologue of EB1, Mal3, with purified microtubules have shown that the N-terminal calponin homology (CH) domain of EB1, which exists in the monomer form,[17,18] can bind to microtubules independently. Specifically, it binds to microtubules in the interprotofilament regions near their plus ends.[23] This suggested that the dimerization in EB1 is not an absolute requirement for its interaction with microtubules to occur and that the monomer itself has the ability to bind to microtubules. It could be possible that both the dimer and monomer interact with microtubules, but with variable affinity, and their mechanisms of action at the plus ends are distinct. The GTP-dependent dimer-to-monomer transition of EB1 shown in this study could be a mechanism through which the relative amount of EB1 dimer vs monomer recruited onto the plus ends is restrained. However, the relative ability of the EB1 dimer vs the GTP-induced monomer form to bind the tubulin dimer and microtubules awaits further study.

In conclusion, our findings revealed that GTP plays a critical role in the regulation of the dimer-to-monomer transition in EB1. However, the binding site of GTP in EB1 and the details of the molecular and structural determinants responsible for EB1−GTP binding need to be further probed. It will also be of interest to understand how the GTP-induced dimer-to-monomer transition in EB1 influences its ability to regulate the plus ends and other +TIPs proteins.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

GTP, guanosine triphosphate; GTP-γS, guanosine 5′-*O*-[gamma-thio]triphosphate; GDP, guanosine diphosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′ tetraacetic acid; ITC, isothermal titration calorimetry

REFERENCES

(1) Desai, A., and Mitchison, T. J. (1997) Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol. 13*, 83−117.

(2) Rodionov, V. I., Nadezhdina, E. S., and Borisy, G. G. (1999) Centrosomal control of microtubule dynamics. *Proc. Natl. Acad. Sci. U.S.A. 96*, 115−120.

(3) Mitchison, T. J., and Kirschner, M. W. (1984) Dymanic instability of microtubule growth. *Nature 312*, 237−242.

(4) Panda, D., Miller, H. P., and Wilson, L. (2002) Determination of the size and chemical nature of the stabilizing cap at microtubule ends using modulators of microtubule dynamics. *Biochemistry 41*, 1609−1617.

(5) Luduena, R. F., Banerjee, A., and Khan, I. A. (1992) Tubulin structure and biochemistry. *Curr. Opin. Cell Biol. 4*, 53−57.

(6) Akhmanova, A., and Steinmetz, M. O. (2010) Microtubule +TIPs at a glance. *J. Cell Sci. 123*, 3415−3419.

(7) Akhmanova, A., and Steinmetz, M. O. (2008) Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat. Rev. Mol. Cell Biol. 9*, 309−322.

(8) Honnappa, S., Okhrimenko, O., Jaussi, R., Jawhari, H., Jelesarov, I., Winkler, F. K., and Steinmetz, M. O. (2006) Key interaction modes of dynamic +TIP networks. *Mol. Cell 23*, 663−671.

(9) Komarova, Y., Lansbergen, G., Galjart, N., Grosveld, F., Borisy, G. G., and Akhmanova, A. (2005) EB1 and EB3 control CLIP dissociation from the ends of growing microtubules. *Mol. Biol. Cell 16*, 5334−5345.

(10) Askham, J. M., Vaughan, K. T., Goodson, H. V., and Morrison, E. E. (2002) Evidence that an interaction between EB1 and p150 (Glued) is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. *Mol. Biol. Cell 13*, 3627−3645.

(11) Slep, K. C., Rogers, S. L., Elliott, S. L., Ohkura, H., Kolodziej, P. A., and Vale, R. D. (2005) Structural determinants for EB1-mediated recruitment of APC and spectraplakins to the microtubule plus end. *J. Cell Biol. 168*, 587−598.

(12) Dixit, R., Barnett, B., Lazarus, J. E., Tokito, M., Goldman, Y. E., and Holzbaur, E. L. (2009) Microtubule plus-end tracking by CLIP-170 requires EB1. *Proc. Natl. Acad. Sci. U.S.A. 106*, 492−497.
(13) Bieling, P., Kandels-Lewis, S., Telley, I. A., van Dijk, J., Janke, C., and Surrey, T. (2008) CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. *J. Cell Biol. 183*, 1223−1233.
(14) Tirnauer, J. S., Grego, S., Salmon, E. D., and Mitchison, T. J. (2002) EB1-microtubule interactions in *Xenopus* egg extracts: role of EB1 in microtubule stabilization and mechanisms of targeting to microtubules. *Mol. Biol. Cell 13*, 3614−3626.
(15) Manna, T., Honnappa, S., Steinmetz, M. O., and Wilson, L. (2008) Suppression of microtubule dynamic instability by the +TIP protein EB1 and its modulation by the CAP-Gly domain of p150glued. *Biochemistry 47*, 779−786.
(16) Vitre, B., Coquelle, F. M., Heichette, C., Garnier, C., Chrétien, D., and Arnal, I. (2008) EB1 regulates microtubule dynamics and tubulin sheet closure *in vitro*. *Nat. Cell Biol. 10*, 415−421.
(17) Komarova, Y., De Groot, C. O., Grigoriev, I., Gouveia, S. M., Munteanu, E. L., Schober, J. M., Honnappa, S., Buey, R. M., Hoogenraad, C. C., Dogterom, M., Borisy, G. G., Steinmetz, M. O., and Akhmanova, A. (2009) Mammalian end binding proteins control persistent microtubule growth. *J. Cell Biol. 184*, 691−706.
(18) Hayashi, I., and Ikura, M. (2003) Crystal structure of the amino terminal microtubule-binding domain of end-binding protein1 (EB1). *J. Biol. Chem. 278*, 36430−36434.
(19) De Groot, C. O., Jelesarov, I., Damberger, F. F., Bjelić, S., Schärer, M. A., Bhavesh, N. S., Grigoriev, I., Buey, R. M., Wüthrich, K., Capitani, G., Akhmanova, A., and Steinmetz, M. O. (2010) Molecular insights into mammalian end-binding protein heterodimerization. *J. Biol. Chem. 285*, 5802−5814.
(20) Honnappa, S., John, C. M., Kostrewa, D., Winkler, F. K., and Steinmetz, M. O. (2005) Structural insights in to the EB1−APC interaction. *EMBO J. 24*, 261−269.
(21) Honnappa, S., Gouveia, S. M., Weisbrich, A., Damberger, F. F., Bhavesh, N. S., Jawhari, H., Grigoriev, I., van Rijssel, F. J., Buey, R. M., Lawera, A., Jelesarov, I., Winkler, F. K., Wüthrich, K., Akhmanova, A., and Steinmetz, M. O. (2009) An EB1-binding motif acts as a microtubule tip localization signal. *Cell 138*, 366−376.
(22) Maurer, S. P., Bieling, P., Cope, J., Hoenger, A., and Surrey, T. (2011) GTP-gammaS microtubules mimic the growing microtubule end structure recognized by end-binding proteins (EBs). *Proc. Natl. Acad. Sci. U.S.A. 108*, 3988−3993.
(23) Maurer, S. P., Fourniol, F. J., Bohner, G., Moores, C. A., and Surrey, T. (2012) EBs recognize a nucleotide-dependent structural cap at growing microtubule ends. *Cell 149*, 371−382.
(24) Maurer, S. P., Cade, N. I., Bohner, G., Gustafsson, N., Boutant, E., and Surrey, T. (2014) EB1 accelerates two conformational transitions important for microtubule maturation and dynamics. *Curr. Biol. 24*, 372−384.
(25) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem. 72*, 248−254.
(26) Gireesh, K. K., Rashid, A., Chakraborti, S., Panda, D., and Manna, T. (2012) CIL-102 binds to tubulin at colchicine binding site and triggers apoptosis in MCF-7 cells by inducing monopolar and multinucleated cells. *Biochem. Pharmacol. 84*, 633−645.
(27) Sharma, S., Poliks, B., Chiauzzi, C., Ravindra, R., Blanden, A. R., and Bane, S. (2010) Characterization of the colchicine binding site on avian tubulin isotype beta VI. *Biochemistry 49*, 2932−2942.
(28) Gupta, K., and Panda, D. (2002) Perturbation of microtubule polymerization by quercetin through tubulin binding: a novel mechanism of its anti-proliferative activity. *Biochemistry 41*, 13029−13038.
(29) Ward, L. D. (1985) Measurement of ligand binding to proteins by fluorescence spectroscopy. *Methods Enzymol. 117*, 509−525.
(30) Bhattacharyya, B., Kapoor, S., and Panda, D. (2010) Fluorescence spectroscopic methods to analyse drug−tubulin interactions. *Methods Cell Biol. 95*, 303−329.
(31) Sen, I., Veprintsev, D., Akhmanova, A., and Steinmetz, M. O. (2014) End binding proteins are obligatory dimers. *PLoS One 8*, e74448.
(32) Di Venere, A., Rossi, A., De Matteis, F., Rosato, N., Agro, A. F., and Mei, G. (2000) Opposite effects of Ca^{2+} and GTP binding on tissue transglutaminase tertiary structure. *J. Biol. Chem. 275*, 3915−3921.
(33) Lundin, B., Thuswaldner, S., Shutova, T., Eshaghi, S., Samuelsson, G., Barber, J., Andersson, B., and Spetea, C. (2007) Subsequent events of GTP binding by the plant PsbO protein: structural changes, GTP hydrolysis and dissociation from the photosystem II complex. *Biochem. Biophys. Acta 1667*, 500−508.
(34) Gebremichael, Y., Chu, J., and Voth, G. A. (2008) Intrinsic bending and structural rearrangement of tubulin dimer: molecular dynamics simulations and coarse-grained analysis. *Biophys. J. 95*, 2487−2499.
(35) Hung, K., Chang, Y., Eng, E. T., Chen, J., Chen, Y., Sun, Y., Hsiao, C., Dong, G., Spasov, K. A., Unger, V. M., and Huang, T. (2010) Structural fold, conservation and Fe(II) binding of the intracellular domain of prokaryote FEOB. *J. Struct. Biol. 170*, 501−512.