Microtubule plus-end tracking by CLIP-170 requires EB1

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Microtubules are polarized polymers that exhibit dynamic instability, with alternating phases of elongation and shortening, particularly at the more dynamic plus-end. Microtubule plus-end tracking proteins (+TIPs) localize to and track with growing microtubule plus-ends in the cell. +TIPs regulate microtubule dynamics and mediate interactions with other cellular components. The molecular mechanisms responsible for the +TIP tracking activity are not well understood, however. We reconstituted the +TIP tracking of mammalian proteins EB1 and CLIP-170 in vitro at single-molecule resolution using time-lapse total internal reflection fluorescence microscopy. We found that EB1 is capable of dynamically tracking growing microtubule plus-ends. Our single-molecule studies demonstrate that EB1 exchanges rapidly at microtubule plus-ends with a dwell time of <1 s, indicating that single EB1 molecules go through multiple rounds of binding and dissociation during microtubule polymerization. CLIP-170 exhibits lattice diffusion and fails to selectively track microtubule ends in the absence of EB1; the addition of EB1 is both necessary and sufficient to mediate plus-end tracking by CLIP-170. Single-molecule analysis of the CLIP-170–EB1 complex also indicates a short dwell time at growing plus-ends, an observation inconsistent with the copolymerization model of this complex with tubulin for plus-end-specific localization. GTP hydrolysis is required for +TIP tracking, because end-specificity is lost when tubulin is polymerized in the presence of guanosine 5’-[^α,β-methylene]triphosphate (GMPCPP). Together, our data provide insight into the mechanisms driving plus-end tracking by mammalian +TIPs and suggest that EB1 specifically recognizes the distinct lattice structure at the growing microtubule end.

+TIP | single molecule | total internal reflection fluorescence (TIRF) microscopy | dynamic instability

Microtubules are highly dynamic polymers of tubulin that are essential for various fundamental processes within eukaryotic cells, including vesicle transport, cell division, and cell motility. In most mammalian cells, microtubules are nucleated at the microtubule organizing center (MTOC), where their minus-ends may remain stably anchored (1). Microtubule plus-ends growing out from the MTOC are highly dynamic, undergoing stochastic switching between growing and shortening phases (2). These dynamics allow microtubule plus-ends to explore the cellular environment. A “search-and-capture” model has been proposed in which the randomly directed outgrowth of a microtubule from the MTOC may lead to a chance encounter with a binding target, such as an organelle or the cell cortex (3). Interaction of the microtubule plus-end with a target protein may lead to preferential stabilization of end dynamics; the transiently stabilized microtubule may then serve as a marker for the establishment of cell polarity or act to stabilize a cellular protrusion or extension (4). Stabilized microtubules also may serve as preferential tracks for intracellular transport, allowing localized delivery within the cell (5). Locally captured microtubules may play a more active role, transmitting a force between the cell cortex and the MTOC. This generation of force may further facilitate cell polarization (6). Thus, spatial and temporal regulation of microtubule plus-end dynamics is critical for controlling microtubule architecture and function in the cell.

Cellular studies have revealed a specialized class of microtubule-binding proteins, known as plus-end tracking proteins (+TIPs), that accumulate at and track with growing microtubule plus-ends (7). These proteins participate in various processes, including regulation of microtubule dynamics, interactions between microtubules and other cellular structures, and delivery of signaling molecules (8). The cytoplasmic linker protein-170 (CLIP-170) was the first +TIP identified (9, 10). Subsequently, numerous structurally unrelated +TIP proteins have been identified that are evolutionarily conserved in most eukaryotes. Genetic and biochemical studies have revealed that many of these proteins interact with one another and may form higher-order plus-end complexes (11). One member in particular, the end-binding 1 (EB1) protein, interacts directly with +TIPs and, consequently, has been proposed to form the core of the microtubule plus-end complex (8, 12).

Most +TIPs can directly bind to microtubules, but the molecular basis for their dynamic plus-end localization remains unresolved. The copolymerization model for +TIP localization suggests that +TIPs copolymerize with tubulin and thus incorporate preferentially at growing microtubule plus-ends (13–17). Alternatively, the end-recognition model posits that +TIPs have an increased affinity for the microtubule plus-end because of its distinct structural and/or biochemical state (18, 19).

To dissect the mechanisms underlying plus-end tracking, we have developed a cell-free in vitro reconstitution system to observe mammalian EB1 and CLIP-170 proteins and their interactions with microtubules. Using time-lapse total internal reflection fluorescence (TIRF) microscopy, we found that the mammalian EB1 can dynamically track growing microtubule plus-ends. Localization of CLIP-170 specifically to the plus-end requires the presence of mammalian EB1; in the absence of EB1, CLIP-170 moves diffusively along the microtubule lattice. Single-molecule analysis indicates that the CLIP-170–EB1 complex undergoes multiple cycles of binding and dissociation from growing microtubule ends, an observation inconsistent with a copolymerization model. We also found that the plus-end specificity of the CLIP-170-EB1 complex requires GTP hydrolysis. Together, these observations provide new insight into the dynamic and specific localization of these proteins seen in vivo and indicate that EB1 binds preferentially to a distinct conformation at growing microtubule plus-ends.

Results

EB1 Targets Growing Microtubule Plus-Ends Independently of Other Proteins. To study the +TIP activity of human EB1 protein, we fluorescently tagged recombinant EB1 protein with Alexa


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488. In vitro binding experiments demonstrated that the Alexa 488-tagged EB1 protein bound to microtubules to a similar extent as untagged recombinant protein [supporting information (SI) Fig. S1A].

Reconstitution experiments with dynamic microtubules and EB1-Alexa 488 using TIRF microscopy demonstrated that EB1-Alexa 488 alone was capable of +TIP activity. In these experiments, EB1-Alexa 488 labeled all growing microtubule plus-ends; in contrast, we observed no EB1-Alexa 488 labeling at depolymerizing microtubule ends (Fig. 1A and Movie S1). We did not reproducibly detect EB1-Alexa 488 labeling of the more slowly growing microtubule minus-ends in our experiments. Increasing the EB1-Alexa 488 concentration in these experiments resulted in enhanced accumulation of EB1 at growing microtubule plus-ends (Fig. 1B). The robust and specific comet-like labeling of growing microtubule plus-ends indicates that +TIP activity is an intrinsic property of mammalian EB1, similar to previous observations on the Schizosaccharomyces pombe EB1 homolog Mal3 (18).

**CLIP-170 Requires EB1 for +TIP Activity.** We next examined the interaction of CLIP-170 with microtubules. In CLIP-170, the central coiled-coil domain allows the formation of intramolecular contacts between the N-terminal CAP-Gly microtubule-binding domain and the C-terminal zinc-finger motifs, resulting in a “closed” conformation of CLIP-170 that inhibits microtubule binding (20). Because the amino terminus alone is sufficient for +TIP activity in the cell, to avoid autoinhibition, we used a well-characterized carboxyl-terminal truncation construct of CLIP-170 designated as the H2 fragment (13). The H2 fragment is dimeric, like full-length CLIP-170 (21). We purified recombinant GFP-tagged CLIP-170(H2) and found that CLIP-170(H2)-GFP did not differ significantly from untagged CLIP-170(H2) in microtubule-binding affinity; we found a $K_d$ of 0.9 ± 0.5 µM for untagged H2 and 1.5 ± 0.6 µM for H2-GFP under our experimental conditions (Fig. S1A).

In contrast to EB1, CLIP-170(H2)-GFP alone exhibited no +TIP activity over a broad range of concentrations. Instead, CLIP-170(H2)-GFP bound nonspecifically along the length of the microtubule. At relatively high concentrations (150 nM), CLIP-170(H2) decorated dynamic microtubules uniformly during both the growth and shortening phases (Fig. S1B). Control experiments with GFP alone at similarly high concentrations demonstrated no detectable microtubule binding (Fig. S1C). At lower concentrations (25 nM), CLIP-170(H2) was bound nonspecifically along the microtubule length, and time-lapse (Movie S2) and kymograph analyses (Fig. 2A) indicated that CLIP-170(H2) moved bidirectionally along the microtubule lattice. Mean squared displacement analysis of single CLIP-170(H2)-GFP molecules moving over the microtubule surface indicated that this movement is consistent with a 1-dimensional random walk with an average diffusion coefficient, $D$, of 3.5 ± 0.1 × 10$^{-11}$ cm$^2$ s$^{-1}$ (Fig. 2B). This diffusive motion of CLIP-170 is similar to that of other microtubule end-binding proteins (22–24), but it did not result in plus-end accumulation of CLIP-170(H2)-GFP over time.

Because EB1 and CLIP-170 interact directly with each other (16, 25, 26), we explored whether EB1 could significantly alter the microtubule association of CLIP-170. In the presence of unlabeled EB1, CLIP-170(H2)-GFP dramatically changed localization, from nonspecific lattice labeling to selective targeting of growing microtubule plus-ends (Fig. 2C). Under these conditions, CLIP-170(H2)-GFP preferentially labeled all actively growing microtubule plus-ends (Fig. 2D).

**CLIP-170 Requires EB1 for +TIP Activity.** (A) Kymograph showing the microtubule binding and diffusive movement of CLIP-170(H2)-GFP at 25 nM. Note the transient binding of CLIP-170(H2)-GFP along the length of the microtubule lattice. (B) Plot of the mean squared displacement (MSD) of CLIP-170(H2)-GFP against time. A linear fit to the data yields a diffusion coefficient, $D$, of 3.5 ± 0.1 × 10$^{-11}$ cm$^2$ s$^{-1}$ (MSD = 2Dt). Error bars represent the SEMs of the squared displacement values ($n = 38$). (C) The montages at the top depict the specific decoration of growing microtubule plus-ends by 25 nM CLIP-170(H2)-GFP in the presence of 250 nM of unlabelled EB1 protein. The kymograph at the bottom shows the same microtubule over the duration of the observation period. Microtubule depolymerization is accompanied by loss of CLIP-170(H2)-GFP at the plus-ends and subsequent rescue of microtubule growth restores plus-end tracking of CLIP-170(H2)-GFP. The numbers on each frame represent time (in seconds). (Scale bar, 2 µm.)
Ends.

Both EB1 and CLIP-170 Exhibit Rapid Turnover at Microtubule Plus-Ends. Fluorescence intensity profiles along growing microtubule plus-end structures show that the spatial distribution of labeling are similar for both EB1–Alexa 488 and CLIP-170(H2)–GFP (Fig. 3A). These data are consistent with a model in which EB1 regulates CLIP-170 recruitment and dissociation at microtubule ends (16, 25). Based on the integrated fluorescence intensity in a 1 μm x 1 μm region at growing microtubule plus-ends, we estimate that an average of 10 EB1 molecules and 5 CLIP-170(H2) molecules are present in the comet-like structure at 250 nM of EB1 and 25 nM of CLIP-170(H2). To determine the turnover of individual EB1 and CLIP-170 proteins at microtubule plus-ends, we used single-molecule imaging conditions and demonstrated an intrinsic ability of mammalian EB1 to bind to the GTP-bound microtubule cap structure at growing plus-ends (27).

Microtubule polymerization with GMPCPP abolished the +TIP activity of CLIP-170(H2)–GFP in the presence of EB1 and led to nonspecific binding of CLIP-170(H2)–GFP along the entire microtubule lattice (Fig. 4 and Movie S4). Under these conditions, the CLIP-170(H2)–EB1 complex exhibited 1-dimensional diffusion along the microtubule surface with an average dwell time of 0.79 ± 0.02 s (n = 440). Thus, the dwell time measured along the sidewall of the GMPCPP lattice is similar to that observed at microtubule plus-ends formed in the presence of GTP. This similarity indicates that the dynamics of the plus-end complex depend on the nucleotide state of the lattice and do not require copolymerization with tubulin. Thus, we conclude that EB1 and CLIP-170 selectively accumulate at growing microtubule plus-ends because of their ability to recognize a structural or chemical discontinuity along the polymer generated by nucleotide hydrolysis.

Discussion

In this work, we reconstituted the microtubule plus-end tracking of the +TIPs EB1 and CLIP-170 in vitro in a minimal system and demonstrated an intrinsic ability of mammalian EB1 to bind specifically to the growing plus-end of a microtubule. A similar result was found previously with the S. pombe EB1 homolog Mal3 (18), highlighting the functional conservation of EB1 as a master regulator of +TIP function (7). We also found that an interaction between EB1 and CLIP-170 (16, 25, 26) is sufficient to recruit CLIP-170 to dynamic microtubule plus-ends in vitro, an observation consistent with the findings of RNAi studies in

and unbinding over the lifetime of the microtubule plus-end structure.

In the absence of EB1, single CLIP-170(H2)–GFP molecules had an average dwell time on the microtubule of 2.69 ± 0.16 s (n = 800) as they diffused along its surface. But when these molecules were imaged together with unlabeled EB1, their average dwell time dropped to 1.03 ± 0.03 s (n = 753), similar to that of single EB1–Alexa 488 molecules. The dwell time of the CLIP-170–EB1 complex was not affected by the addition of unlabeled CLIP-170(H2) to a concentration of 25 nM (see Materials and Methods). Thus, EB1 appears to directly influence the turnover of CLIP-170 at microtubule plus-ends. In contrast to the effects of EB1 on CLIP-170 dynamics, the presence of unlabeled CLIP-170(H2) did not significantly affect either the comet tail length or average dwell time of EB1–Alexa 488 at microtubule plus-ends (data not shown). This indicates that the inherent +TIP dynamics of EB1 were not altered by an association with CLIP-170.

Guanosine 5’-\([\alpha,\beta\text{-methylene}]\)triphosphate (GMPCPP) Abolishes +TIP Activity of EB1 and CLIP-170. During the reconstitution experiments, we observed that the +TIP activity of both EB1–Alexa 488 and CLIP-170(H2)–GFP did not require preincubation with tubulin dimers prior to microtubule polymerization. This observation and the dwell time dynamics discussed above suggest that although EB1 and CLIP-170 can form a complex with free tubulin dimers (16), the formation of such a complex is not required, and such formation does not enhance the targeted concentration of these proteins at plus-ends. Thus, our data support an end-recognition mechanism for +TIP activity, in which these proteins bind with higher affinity to the newly polymerized end of the microtubule because of biochemical and/or structural differences between the new end and the older lattice structure.

To test this mechanism directly, we conducted reconstitution experiments using GMPCPP instead of GTP. GMPCPP is a slowly hydrolyzable analog of GTP that promotes microtubule assembly and results in an overall microtubule lattice structure that mimics the GTP-bound microtubule cap structure at growing plus-ends (27).

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mammalian cells, including yeast and mammalian cells, have been shown to rely on plus-end-directed motors such as EB1 and CLIP-170 to dynamically track and regulate microtubule dynamics. EB1 alone, or the CLIP-170–EB1 complex, has a higher binding affinity for the tubulin structure found only at the growing plus-end. Interestingly, Biening et al. (18) reported a pronounced dependence of Mal3 comet tail length on the rate of microtubule polymerization, consistent with our observations on the comet tail lengths of EB1 and the CLIP-170–EB1 complex. Thus, the structural transition occurring after incorporation of newly polymerized subunits into the microtubule, resulting in significantly decreased affinity of EB1 for the lattice structure, is not tightly linked to subunit incorporation into the polymer, but instead occurs as a subsequent event. In this way, it is similar to the hydrolysis of bound GTP occurring with first-order kinetics on newly incorporated subunits, although whether these events occur at similar rates remains to be determined. The loss of plus-end specificity of binding observed when microtubules were polymerized in the presence of GMPCPP rather than GTP further supports the hypothesis that a conformational change in the microtubule lattice that occurs with, or subsequent to, GTP hydrolysis on incorporated tubulin subunits induces a significant change in the binding affinity of EB1 for the microtubule. These findings indicate that GTP hydrolysis serves as a critical regulatory switch for plus-end tracking.

EB1 enhances microtubule elongation (34), but the mechanisms involved remain unclear. EB1 has been proposed to promote microtubule sheet closure during microtubule polymerization (35), potentially through preferential binding along the seam (36). In vitro, EB1 has been reported to suppress the shortening rate and the frequency of catastrophe (37) or, alternatively, to promote both catastrophes and rescues (35). Much of this experimental variation is likely due to differences in EB1 concentrations used in the assays, because at higher concentrations, EB1 does not localize in a tip-specific manner but instead binds along the microtubule lattice both in vitro (16, 35) and in mammalian cells (34). In vivo, deletion analysis of Mal3 or Tip1 in S. pombe suggests that these proteins act to suppress the frequency of microtubule catastrophes (38–40). In Xenopus extracts, EB1 was shown to decrease the frequency of catastrophes and increase the frequency of rescues (30); in mammalian cells, CLIP-170 functions to promote rescue (41).

In our reconstitution experiments, however, neither EB1 nor CLIP-170, acting alone or in a complex, significantly altered the microtubule dynamic instability parameters that we observed in the presence of tubulin alone. These results suggest that in this minimal system, neither EB1 nor CLIP-170, nor their complex, is sufficient to modulate microtubule dynamics when localized to growing microtubule plus-ends, and that other cellular factors may be needed to drive this activity in the cell.

We propose a model in which EB1 dynamically recruits CLIP-170 to growing microtubule plus-ends by recognizing the distinct conformation of the plus-end (Fig. 5). This model is consistent with the increasing perception of EB1 as the master plus-end tracking protein, capable of recruiting multiple distinct +TIPs to the dynamic plus-end (8). The relatively weak binding affinities measured for direct associations among the +TIPs suggest that these proteins function in rapidly changing networks of interactions (7, 8), consistent with observations that +TIPs...
function in a wide array of cellular processes, including cell polarization, cell migration, and cell division. The enrichment of these +TIPs at the microtubule plus-end likely will facilitate microtubule search and capture, in which dynamic microtubules search the cellular space and become captured by specific +TIP protein that specifically decorates growing TIPs at the microtubule plus-end likely will facilitate microtubule search and capture, in which dynamic microtubules search the cellular space and become captured by specific +TIPs are likely to provide further insight into the cellular mechanisms of the plus-end complex.

Materials and Methods

Protein Purification and Labeling. His-tagged recombinant GFP-labeled CLIP-170(H2) was affinity-purified using a nickel column and then desalted using a PD-10 column (Amersham Biosciences) and exchanged into BRB80 buffer supplemented with 50 mM NaCl. Recombinant GST-tagged full-length EB1 protein was purified using the PreScission expression system (GE Healthcare). In brief, the fusion protein was purified using glutathione Sepharose beads, and the GST tag subsequently cleaved through site-specific cleavage with the PreScission protease. The released EB1 protein was further purified using a NAP-10 gel filtration column and exchanged into BRB80 buffer supplemented with 50 mM NaCl. Purified proteins were flash frozen and stored at -80 °C until use. Purified EB1 protein was fluorescently labeled at cysteine residues using Alexa–Fluor 488 maleimide (Invitrogen) in BRB80 buffer. The degree of labeling was estimated as ~70% by spectroscopic analysis.

Microtubule-Binding Assays. Microtubule-binding assays were conducted by coinoculating increasing concentrations of rhodamine-labeled microtubules with purified recombinant CLIP-170(H2), CLIP-170(H2)–GFP, EB1, or EB1–Alexa 488 proteins for 30 min at 37 °C. The samples were then centrifuged at 35,000 × g for 20 min, after which the resultant supernatant and pellet fractions were analyzed by SDS/PAGE and densitometry. Under our experimental conditions, both tagged and untagged EB1 were bound relatively weakly to microtubules and did not reach saturation.

Microtubule Plus-End Tracking Assays. The in vitro reconstitution experiments were conducted in flow cells (15 μl volume) constructed using slides and silanized coverslips (Repel silane, Amersham Biosciences) attached with double-sided adhesive tape. The flow cell was coated with 20% monoclonal anti-biotin antibody (clone BN-34, Sigma) and then blocked with 5% Pluronic F-127 (Sigma). Then 125 nM sheared GMPCPP microtubule seeds were introduced into the flow cell. Tubulin polymerization was initiated by introducing 22 μM 1:3:10 rhodamine-labeled bovine tubulin in BRB80 buffer supplemented with 0.1% methyl cellulose (4000 cp, Sigma), an oxygen-scavenging system containing glucose oxidase, catalase, glucose, 100 mM DTT, 1 mM Mg-GTP, and +TIP proteins. Unless stated otherwise, 250 nM EB1–Alexa 488 and 25 nM CLIP-170(H2)–GFP were used. Microtubule dynamics were visualized at 22 °C using a TIRF microscope fitted on an inverted microscope (Nikon TE-2000U). TIRF excitation was achieved using a 488-nm optically pumped semiconductor laser (Sapphire, Coherent) to visualize Alexa 488 and a 532-nm diode-pumped solid-state laser (CrystaLaser) to visualize rhodamine. The laser light intensities used for imaging were kept low, so that the photobleaching rates were slow compared with the dwell times. Images were captured with a back-illuminated electron-multiplying CCD camera (Cascade-512B, Photometrics) using either time-lapse capture at 2-sec intervals for 2-color microscopy or burst mode at 10 frames per second for the analysis of +TIP dynamics at the single-molecule resolution. Control experiments showed that the dwell times of single binding events of the CLIP-170–EB1 complex at 5 nM CLIP-170(H2)–GFP in the presence of 250 nM unlabeled EB1 or at 5 nM CLIP-170(H2)–GFP in the presence of both 25 nM unlabeled CLIP-170(H2) and 250 nM unlabeled EB1 were statistically indistinguishable.

Image Analysis and Statistics. Plus-end tracking of EB1–Alexa 488 and CLIP-170(H2)–GFP was analyzed using kymographs generated with the “Multiple Kymograph” plug-in for ImageJ submitted by J. Rietdorf and A. Seitz (European Molecular Biology Laboratory, Heidelberg, Germany). Microtubule growth rates and +TIP dwell times were extracted from these kymographs. The lengths of the +TIP comet tails, defined as the distances from the peak fluorescence intensity at the microtubule tips to the baseline lattice intensity, were measured from line profiles of fluorescence intensities of EB1–Alexa 488 and CLIP-170(H2)–GFP. To analyze the diffusional movement of CLIP-170(H2)–GFP, manual tracking was used to trace the displacement of single molecules on the microtubule lattice. Dwell time and mean squared displacement data were fitted using KaleidaGraph (Synergy Software), and statistical significance was determined using the Student’s t test.

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