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Probing interactions between CLIP-170, EB1, and microtubules

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Abstract

CLIP-170 is a microtubule (MT) plus-end tracking protein (+TIP) that dynamically localizes to the MT plus end and regulates MT dynamics. The mechanisms of these activities remain unclear because the CLIP-170-MT interaction is poorly understood, and even less is known about how CLIP-170 and other +TIPs act together as a network. CLIP-170 binds to the acidic C-terminal tail of β -tubulin. However, the observation that CLIP-170 has two-CAP-Gly motifs and multiple serine-rich regions suggests that a single CLIP-170 molecule has multiple tubulin binding sites, and that these sites might bind to multiple parts of the tubulin dimer. Using a combination of chemical cross-linking and mass spectrometry, we find that CLIP-170 binds to both α - and β -tubulin, and that binding is not limited to the acidic C-terminal tails. We provide evidence that these additional binding sites include the H12 helices of both α - and β -tubulin and are significant for CLIP-170 activity. Previous work has shown that CLIP-170 binds to EB1 via the EB1 C-terminus, which mimics the acidic C-terminal tail of tubulin. We find that CLIP-170 can utilize its multiple tubulin binding sites to bind to EB1 and the MT simultaneously. These observations help to explain how CLIP-170 can nucleate MTs and alter MT dynamics, and they contribute to understanding the significance and properties of the +TIP network.

Keywords

microtubule; CLIP-170; EB1; +TIP; tubulin

Introduction

Microtubules are essential components of the cytoskeleton and play vital roles in multiple cellular processes including cell division and intracellular transport.^{1–4} MTs are highly dynamic and alternate between periods of growing and shortening in a process termed “dynamic instability.”² The dynamic behavior of MTs is critical to their function and is carefully controlled by regulatory proteins.^{2,3} However, the molecular mechanisms of this

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regulation remain poorly understood. To elucidate this regulation it is necessary to characterize how other proteins interact with MTs and affect their dynamics.

The dynamic behavior of MTs is governed primarily by the fast growing MT plus end, and many proteins that control MT dynamics localize specifically to this end.^{5–7} The group of proteins that dynamically track growing MT plus ends are known as MT plus-end tracking proteins (+TIPs).^{6,7} A large number of +TIPs have now been identified, and they are involved in a variety of processes ranging from MT assembly to membrane transport.^{6–13}

Cytoplasmic linker protein 170 (CLIP-170) initially attracted attention as the first identified +TIP, and later as the first +TIP shown to be involved in regulating MT dynamics *in vivo* and *in vitro*.^{8,14–17} CLIP-170 participates in interactions between chromosomes and MTs,^{18,19} and it has been implicated in endosome-MT interactions.²⁰ Furthermore, CLIP-170 interacts with a series of other +TIPs, most significantly EB1, which is proposed to form the core of the MT plus end protein network.^{21–25} Native CLIP-170 consists of an N-terminal MT-binding domain, a central coiled-coil domain that drives homodimerization, and a C-terminal metal-binding domain.^{26–28} The N-terminus of each CLIP-170 monomer (H1^{1–350}) contains two conserved CAP-Gly (cytoskeleton-associated protein glycine-rich) domains surrounded by three basic serine-rich regions (Fig. 1a). The CLIP-170 CAP-Gly domains are well-conserved from yeast to humans, while the surrounding serine-rich regions are poorly conserved at the primary sequence level but are well-conserved in terms of amino acid composition.^{29–32} Although earlier reports indicated that the binding of CLIP-170 to MTs is mediated by the CAP-Gly domains, the serine-rich regions are also important in both +TIP behavior and tubulin polymerization activity.^{11,26,33} Indeed, analysis of the behavior of CLIP-170 fragments revealed that each CAP-Gly and serine-rich region is capable of binding MTs independently.²⁶

Previous studies have indicated that CLIP-170 binds to MTs through the acidic C-terminal tail of β -tubulin.^{21,34,35} The presence of the β -tubulin terminal tyrosine residue was found to be crucial for the +TIP behavior of CLIP-170 *in vivo*.³⁴ However, a recent report showed that binding of CLIP-170 to both the MT lattice and plus ends was reduced but not abolished when MTs were assembled from detyrosinated tubulin,²¹ suggesting that CLIP-170 might have additional binding sites on tubulin. In addition, CLIP-170 cross-links to both α - and β -tubulin, suggesting a binding site on β -tubulin.¹⁴ The sum of this evidence suggests that CLIP-170 binds at sites in addition to the β -tubulin tail, but the identity of these sites is not known.

Given the fact that the molecular details underlying the CLIP-170-MT interaction are poorly understood, characterizing the binding sites of CLIP-170 on MTs both alone and in combination with other +TIPs will contribute to understanding its role in regulating MT dynamics. In the present study, by using a combination of chemical cross-linking and mass spectrometry we have dissected interactions between the subdomains of CLIP-170 and tubulin. We have found that the individual CAP-Gly domains and serine-rich regions of the CLIP-170 microtubule binding region (H1^{1–350}) each bind to both α - and β -tubulin. Moreover, we observe that H1^{1–350} binds to subtilisin-treated MTs (which lack the acidic tail) with reduced but still moderate affinity, demonstrating that the CLIP-170-MT interaction occurs through sites besides the already established α -tubulin acidic tail. We provide evidence that these additional binding sites include the H12 helices of both α - and β -tubulin. The existence of multiple interaction interfaces between CLIP-170 and tubulin helps to explain how both full-length CLIP-170 and the monomeric CLIP-170 fragment (H1^{1–350}) are able to nucleate MTs and alter MT dynamics. Further, the presence of multiple tubulin binding sites in CLIP-170 suggests that a CLIP-170 molecule might be able to bind to a MT and to the +TIP EB1 simultaneously. EB1 forms the core of the +TIP network and binds to

CLIP-170 via its C-terminus, which mimics the acidic C-terminal tail of α -tubulin. Our microtubule cosedimentation experiments with fragments of EB1 and CLIP-170 confirm this prediction, explaining how EB1 and CLIP-170 can each recruit the other to MT tips and providing insight into the network of interactions between MTs and +TIPs.

Results

Binding of CLIP-170 to subtilisin-treated MTs

Recent reports suggest that the CLIP-170 binding region on MTs is located on the –EEY or –EExEEY motif of the α -tubulin acidic tail.^{21,34,35} However, the observations that CLIP-170 has multiple MT-binding domains²⁶ and cross-links to both α - and β -tubulin¹⁴ suggest that CLIP-170 binds to multiple parts of the tubulin dimer. β -tubulin has an acidic tail (also called an E-hook for its many E residues) similar to that of α -tubulin (Fig. 2a). Preceding the unordered E-hook of both α - and β -tubulin is a structure called the H12 helix (Fig. 2a). Both the H12 helices and C-terminal tails are involved in the binding of a variety of MAPs and motors and therefore are reasonable candidates for CLIP-170 binding.^{36–40}

To investigate the possibility that CLIP-170 binds to multiple regions of $\alpha\beta$ -tubulin, we compared the affinity of the CLIP-170 MT-binding domain fragment (H1^{1–350}) for normal untreated MTs and for MTs that have had their E-hooks removed by subtilisin treatment. To specifically address the question of whether CLIP-170 binds to the β -tubulin E-hooks, we took advantage of the differential susceptibility of α - and β -tubulin to subtilisin: treatment for a short time removes only the β -tubulin tail ($\alpha\beta_s$ -MTs), while longer treatment results in MTs lacking both C-terminal tails ($\alpha_s\beta_s$ -MTs) (Supplemental Fig. 1). Consistent with earlier reports,^{17,26} H1^{1–350} binds to undigested MTs ($\alpha\beta$ -MTs) with a K_d of $0.51 \pm 0.25 \mu\text{M}$, while the K_d values of H1^{1–350} for $\alpha\beta_s$ - and $\alpha_s\beta_s$ -MTs were 0.56 ± 0.07 and $3.25 \pm 1.19 \mu\text{M}$, respectively (Fig. 2b). The observation that removal of the β -tubulin C-terminal tail has little effect on the binding of H1^{1–350} suggests that the cleaved portion of the β -tubulin tail is not involved in CLIP-170-MT interactions, which is in agreement with previous work showing that a peptide derived from the β -tubulin tail does not bind to CLIP-170.³⁵ However, the observation that CLIP-170 retains significant affinity for MTs even after complete removal of both α - and β -tubulin C-terminal tails (see Supplemental Fig. 1 for demonstration that digestion was complete) indicates that CLIP-170 does have binding sites in addition to the α -tubulin C-terminal tail.

Cross-linking of CLIP-170 fragments to MTs

In order to clarify whether the additional binding regions for CLIP-170 are located on β -tubulin, β -tubulin, or both, chemical cross-linking reactions were performed on mixtures of CLIP-170 and MTs using the zero-length covalent cross-linker, EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide).^{14,41} To determine which regions of CLIP-170 are involved in these interactions, we used H1^{1–350} as well as five smaller constructs corresponding to individual subdomains (Fig. 1a). We also examined the effect of removing the tubulin acidic tails via subtilisin treatment (Fig. 3). In control samples lacking MTs, the CLIP-170 fragments did not cross-link to themselves, nor did they cross-link to the negative control carbonic anhydrase (Fig. 3; Supplemental Fig. 2). In contrast, EDC treatment of mixtures of CLIP-170 fragments and MTs (both untreated and subtilisin-treated) generated a series of cross-linked products as observed after separation in the SDS gels (Fig. 3a and b). Mass spectrometry of the bands excised from these SDS gels indicated that multiple subdomains of CLIP-170 can bind to both α - and β -tubulin, and that they can do so even when the E hooks have been removed by subtilisin (Supplemental Table 1).

The specific experiments that lead to these conclusions are as follows: First, when the full-length head domain (H1¹⁻³⁵⁰) is incubated with $\alpha\beta$ -MTs (i.e., MTs that have not been treated with subtilisin) and then subjected to EDC cross-linking, two major gel bands (~90 and ~140 kDa) are produced (Fig. 3a, lane 8). Mass-spectrometry analysis of the tryptic digests of the excised bands revealed that both bands contain peptide fragments indicating the presence of H1¹⁻³⁵⁰, α -, and β -tubulin (Supplemental Table 1). This observation indicates that the smaller band (~90 kDa) is actually a doublet corresponding to the cross-linked products of H1¹⁻³⁵⁰ and each of the tubulin monomers (i.e., H1¹⁻³⁵⁰- α and H1¹⁻³⁵⁰- β). The larger band (140 kDa) corresponds to a cross-linked trimer of ambiguous composition (it could contain H1¹⁻³⁵⁰- α - β -tubulin or a mixture of H1¹⁻³⁵⁰- α - β -tubulin and H1¹⁻³⁵⁰- β - β -tubulin) (supplemental Table 1).

Similarly, two major bands (~85 and ~135 kDa) were seen in the reaction with H1¹⁻³⁵⁰ and $\alpha_s\beta_s$ -MTs (i.e., MTs that have had both E-hooks removed by subtilisin) (Fig. 3b). Consistent with the observations above, the in-gel digests of both bands contain peptide fragments from H1¹⁻³⁵⁰, α -, and β -tubulin sequences (Supplemental Table 1). The faster migration of these bands is due to the loss of the C-terminal tail of each tubulin subunit which is ~2 kDa.

Taken together, these observations suggest that H1¹⁻³⁵⁰ can cross-link to both α - and β -tubulin and that the additional (non-C-terminal tail) binding sites for H1¹⁻³⁵⁰ are distributed on both α - and β -tubulin. It is interesting to note that the tubulin-tubulin dimer product formed in both the $\alpha\beta$ -MT (~100 kDa) and $\alpha_s\beta_s$ -MT (~95 kDa) samples completely disappeared in the presence of H1¹⁻³⁵⁰, suggesting that CLIP-170 binding alters or occurs near interdimer and/or intradimer tubulin-tubulin interactions.

In order to analyze which regions of H1¹⁻³⁵⁰ interact with the different tubulin monomers, we expressed and purified five different fragments corresponding to each of the individual subdomains of H1¹⁻³⁵⁰: the CAP-Gly containing fragments H1⁵⁸⁻¹⁴⁰ and H1²⁰⁶⁻²⁸⁸, and the serine-rich domain fragments H1¹⁻⁷⁰, H1¹²²⁻²¹⁹, and H1²⁷⁶⁻³⁵⁰ (Fig. 1a). These CLIP-170 fragments were incubated with MTs followed by EDC cross-linking. All fragments produced a single major band (~65 kDa) after cross-linking to $\alpha\beta$ -MTs (Fig. 3a), corresponding to the weight of a tubulin monomer cross-linked to a CLIP-170 fragment. Additional products (~115 kDa) were also seen in the case of H1⁵⁸⁻¹⁴⁰, H1²⁰⁶⁻²⁸⁸ and H1¹⁻⁷⁰ (Fig. 3a). As was seen with the cross-linking of H1¹⁻³⁵⁰, formation of cross-linked tubulin-tubulin dimers was suppressed by all of the CLIP-170 fragments.

To investigate the identity of the tubulin monomer(s) in the ~65 kDa cross-linked bands and also determine the relative ratio of α - and β -tubulin in this band, we utilized carboxymethylation reaction (Fig. 3a, lower panel). α - and β -tubulin are differentially susceptible to carboxymethylation, allowing for their electrophoretic separation.^{42,43} As shown in Fig. 3a lower panel, all of the bands at ~65kDa (corresponding to the cross-linked products of the various H1¹⁻³⁵⁰ subfragments and tubulin-monomer) were separated into two different bands corresponding to α and β -tubulin, indicating that each of the H1¹⁻³⁵⁰ subdomains is capable of cross-linking to both α - and β -tubulin monomers. (Note: because each sample is diluted 5-fold during the carboxymethylation process, the carboxymethylated products of the higher molecular weight bands are not visible). The identity of the upper and lower carboxymethylated bands as cross-linked products of CLIP-170 with alpha and beta tubulin respectively was further confirmed by mass spectrometry analysis of one of the CLIP-170 fragments (H1²⁰⁶⁻²⁸⁸) (Supplemental Table 1).

Similar cross-linking patterns were obtained with subtilisin-treated MTs ($\alpha_s\beta_s$ -MTs) (Fig. 3b). However, the intensity of the cross-linked bands at ~60 kDa was generally weaker than observed with normal MTs, and the ~100kDa band was often hard to discern; the band at

~100 kDa is likely cross-linked tubulin dimer based on the observation that it does not shift with different CLIP-170 fragments. These observations are consistent with the lower affinity of CLIP-170 for MTs lacking E-hooks (Fig. 2a), and with the fact that the glutamate-rich E-hooks are likely a “hotspots” for cross-linking. While we were unable to perform the carboxymethylation reaction on subtilisin-treated MTs due to the low intensity of the cross-linked products, the mass spectrometry analysis of the ~60kDa band from one fragment (H1^{206–288}) revealed the presence of the peptide fragments from the H1 fragment and both α - and β -tubulin, indicating that this CAP-Gly containing fragment can bind both α - and β -tubulin (Supplemental Table 1).

Taken together, these data indicate that a CLIP-170 molecule can use its multiple MT-binding domains to associate with multiple sites on both α - and β -tubulin. From our binding experiments with subtilisin-treated MTs, it is clear that one of the binding sites is located in the C-terminal tail of α -tubulin (Fig. 2b), while cross-linking experiments indicate that the additional sites are located in both the α - and β -tubulin subunits upstream of the subtilisin cleavage sites (Fig. 3b). Our next goal was to try to identify these additional binding sites on both α - and β -tubulin.

Effects of salt on the binding of CLIP-170 to subtilisin-treated MTs

The binding of H1^{1–350} to subtilisin-treated MTs indicates the involvement of additional binding sites upstream of (perhaps close to) the subtilisin cleavage sites (Fig. 2). To investigate the nature of these additional binding sites, we tested the effect of salt on H1^{1–350}- $\alpha_s\beta_s$ -MT binding. Normal undigested MTs ($\alpha\beta$ -MTs) were also used for comparison. H1^{1–350} began to dissociate from the $\alpha\beta$ -MTs at 100 mM NaCl (~30%) and was completely dissociated at 500 mM NaCl (Fig. 4a and b), consistent with previous observations.³⁴ However, the interaction of H1^{1–350} with $\alpha_s\beta_s$ -MTs was found to be more sensitive. For example, at 100 mM NaCl, ~42% H1^{1–350} dissociated from $\alpha_s\beta_s$ -MTs, while complete removal occurred at 500 mM NaCl (Fig. 4a and b). These observations suggest that the H1^{1–350} binding to $\alpha_s\beta_s$ -MTs, like $\alpha\beta$ -MTs, is electrostatic and might involve a charged (acidic) domain on the $\alpha_s\beta_s$ -MTs.

Interaction between CLIP-170 fragments and synthetic peptides derived from the H12 helices of α - and β -tubulin

Based on tubulin structure and the evidence that CLIP-170 binding to MTs is electrostatic in nature, one good candidate for binding to the CLIP-170 fragments is the H12 helix, which is an acidic surface helix found in both α - and β -tubulin (Fig. 2a). The H12 helix is also important for binding to motors.³⁹ To investigate whether the H12 helix might be involved in binding to CLIP-170, we used synthetic peptides corresponding to the H12 helices from porcine brain α - and β -tubulin monomers (Fig. 2a) and tested their ability to cross-link to CLIP-170. More specifically, the H12 peptides and various CLIP-170 fragments were cross-linked by EDC as described above, followed by the separation of the cross-linked products by SDS-PAGE. Since the molecular mass of each peptide is ~2 kDa, the cross-linked products formed between CLIP-170 fragments and peptide can be distinguished by the generation of ~2 kDa higher-molecular mass products. In addition to the constructs containing full-length H1^{1–350} or single domains of H1^{1–350} as described above, we also created and tested a group of constructs with two domains (a CAP-Gly and a serine-rich region) that are adjacent to each other (Fig. 1a).

Full-length head domain (H1^{1–350}) efficiently cross-linked with both the α - and β -H12 peptides (Fig. 5a and b). However, weak or negligible cross-linking was observed when peptides were incubated with the CLIP-170 fragments containing either single CAP-Gly domains or serine-rich regions (Fig. 5a' and b'). The observation that these CLIP-170

fragments bind to MTs with moderate affinity²⁶ and cross-link to intact MTs (Fig. 3) suggests that the poor cross-linking of these domains to the peptides is due to lack of helix-binding activity instead of misfolding or lack of cross-linking activity.

To test the possibility that multiple domains of CLIP-170 are necessary to interact with the H12 helices, we cross-linked the H12 helices to CLIP-170 fragments containing two adjacent domains (Fig. 1a). As shown in Fig. 5a and b, the fragments derived from the proximal half of the H1¹⁻³⁵⁰ fragment (H1¹⁻¹⁴⁰ and H1⁵⁸⁻²¹⁹) cross-linked weakly with the tubulin peptides, consistent with our previous evidence that the first half of CLIP-170 binds weakly to MTs and has little effect on tubulin polymerization.²⁶ However, the fragments derived from the distal half of the head domain (H1¹²²⁻²⁸⁸ and H1²⁰³⁻³⁵⁰) cross-linked with the peptides efficiently, further supporting the idea that the second CAP-Gly and adjacent serine-rich regions play a dominant role in MT binding (Fig. 5a and b).²⁶ No cross-linking of the peptides is seen with the negative control carbonic anhydrase (Fig. 5, all panels), indicating that the binding between CLIP-170 and both peptides is specific.

Inhibition of CLIP-170-induced tubulin polymerization by tubulin peptides

To examine whether the interaction between the H12 helices and CLIP-170 is important for CLIP-170 function *in vitro*, we tested the effect of the H12 peptides on CLIP-170-induced tubulin polymerization using a light scattering assay. As expected, the addition of H1¹⁻³⁵⁰ to tubulin induced MT polymerization as indicated by a rapid increase in absorbance (Fig. 6). The addition of either peptide at lower concentration (100 μ M) inhibited tubulin polymerization weakly, while higher concentration (200 μ M) significantly inhibited assembly. The β -tubulin peptide was less effective in comparison to the α -tubulin peptide. However, a combination of both peptides at low concentrations (100 μ M each) was more inhibitory than either individual one alone at higher concentration (200 μ M). These data suggest that the combination of the peptides can produce synergistic effects and that the binding of H1¹⁻³⁵⁰ to both the α - and β -tubulin H12 helices is functionally significant.

Simultaneous binding of CLIP-170 to EB1 and MTs

As discussed above, the CAP-Gly domains of CLIP-170 are important for its interactions with MTs. However, these CAP-Gly domains are also responsible for interacting with EB1, and the concomitant binding of CLIP-170 to both MTs and EB1 is thought to be crucial for its +TIP behavior.^{21,23} How CLIP-170 could use its CAP-Gly domains to bind simultaneously to both EB1 and MTs is not yet established. There is evidence that the first half of the H1¹⁻³⁵⁰ domain is specialized for binding to EB1, and the second is specialized for binding to MTs,^{26,31,35} but as yet there is no biochemical evidence for the formation of a CLIP-170-EB1-MT ternary complex.

To investigate the formation of this predicted CLIP-170-EB1-MT ternary complex, cosedimentation assays were used. Since EB1 interacts with MTs via its N-terminal calponin homology (CH) domain and with CLIP-170 via its C-terminal tail,^{23,31} we utilized an EB1 construct lacking the MT binding domain and having only CLIP-170 binding sites (EB1¹⁹⁴⁻²⁶⁸) to avoid the complication introduced by direct binding of EB1 to MTs. We also used both monomeric (H1¹⁻³⁵⁰) and dimeric (H2¹⁻⁴⁸¹) CLIP-170 fragments to observe any changes in the binding behavior due to dimerization.¹⁷ We tested whether CLIP-170 can recruit EB1¹⁹⁴⁻²⁶⁸ onto MTs in a cosedimentation assay. Note that EB1¹⁹⁴⁻²⁶⁸ alone does not bind significantly to MTs (Fig. 7a and b; see also Supplemental Fig. 3. We do not know if the weak apparent binding seen at high concentrations of EB1¹⁹⁴⁻²⁶⁸ in Fig. 7 is real or due to artifacts such as sticking to the tube). Our data clearly indicate that addition of monomeric or dimeric CLIP-170 fragments shifts the EB1¹⁹⁴⁻²⁶⁸ protein from supernatant to pellet, indicating that CLIP-170 protein can bind to both EB1¹⁹⁴⁻²⁶⁸ and MTs (Fig. 7a

and b). However, addition of increasing amounts of EB1^{194–268} to the CLIP-170-MT mixture also leads to the partial removal of the CLIP-170 fragments from MTs, as would be expected if both CLIP-170 CAP-Gly domains become occupied by the EB1^{194–268} when concentrations of EB1^{194–268} are high relative to the concentration of MTs. Collectively, these data indicate that CLIP-170 and EB1 can form a tripartite complex with MTs.

Discussion

The focus of this work was to elucidate interactions between CLIP-170 and tubulin, with the purpose of beginning to establish how CLIP-170 might function as part of the +TIP network. The major goal was to test the hypothesis that CLIP-170 has multiple binding sites on the tubulin dimer, and if so, to define which regions of CLIP-170 and tubulin are involved in these interactions. Assuming that CLIP-170 has multiple MT binding sites, we were also interested in testing whether CLIP-170 can bind to EB1 and MTs simultaneously. Our main approach was to use a zero-length chemical cross-linker, EDC, to entrap various interacting domains in a given protein complex. EDC allows identification of regions that associate very closely with one another by specifically linking lysine residues with either aspartate or glutamate.⁴¹ The presence of these residues in interacting protein domains is crucial for EDC cross-linking, because lack of these amino acids in either participating protein domain could lead to the “false negative” result. Fortunately, both tubulin and H1^{1–350} contain large numbers of lysine and acidic amino acids, thus allowing the efficient cross-linking of various subdomains of H1^{1–350} with MTs by EDC.

The data presented here demonstrate that the binding of CLIP-170 to MTs is not only limited to the –EEY motif of α -tubulin, as previously reported, but involves multiple regions of tubulin (Fig. 2 and 3).^{21,34,35} Our results indicate that CLIP-170 can bind not only to the α -tubulin C-terminal tail but also to the H12 helices of both α - and β -tubulin (Fig. 3 and 5). These results are in agreement with previous studies showing that CLIP-170 can cross-link to both α - and β -tubulin subunits.¹⁴ Although β -tubulin has an acidic tail similar to that of α -tubulin, CLIP-170 does not appear to bind significantly to it. This conclusion is supported both by previous evidence that CLIP-170 binds with very weak affinity to a β -tubulin peptide,³⁵ and also by our evidence that subtilisin removal of the β -tubulin tail has little if any detectable effect on affinity of CLIP-170 for taxol-MTs. These experiments do not rule out the possibility that additional CLIP-170 binding sites on tubulin exist.

One limitation of our data is that the CLIP-170 proteins used in this work have His-tags. His-tags, which are expected to be partially positively charged under standard conditions for analysis of MT binding (pH 6.8), might increase binding to the negatively charged surface of MTs. We cannot straightforwardly exclude this possibility: while we have previously shown that cutting off the tag does not alter detectably H1^{1–350} behavior¹⁷, we cannot cut the tags off of the smaller pieces because the serine-rich fragments are too sensitive to thrombin cleavage (data not shown). However, while the His-tags may contribute to binding, we conclude that they are not responsible for it on the basis of two observations: a) different CLIP-170 fragments have different binding behavior, implying that binding is mediated by different structures (Fig. 3 and 5); b) control his-tagged proteins do not significantly bind to MTs, indicating that the presence of a his-tag alone is not sufficient for binding (see Supplemental Fig. 3 for an example). Similarly, one could ask about whether binding occurs at physiological salt concentration. The PEM buffer used in these experiments was titrated with 136 mM KOH, which is close to the physiological K⁺ concentration (~140 mM KCl), and also close to the overall physiological salt concentration (~150 mM total)⁵¹. Therefore, while it is possible that the His-tags and/or inexact salt concentrations alter the observed strength of the binding of the CLIP-170 fragments, we find it very unlikely that they create

MT binding where it would not otherwise exist. Even weak MT binding should be important in the context of a protein with multiple MT binding sites.

Our work has relevance for how CLIP-170 alters MT dynamics. First, the observation that each of the five different domains of the CLIP-170 MT binding region can independently bind to MTs means that a CLIP-170 dimer has as many as ten different MT binding sites and so would be expected to contact many different tubulin dimers. The ability to cross-link multiple dimers provides an obvious mechanism for suppressing catastrophe or enhancing rescue (Fig. 3).^{15,16} Interestingly, even a small fragment of CLIP-170 (H1^{203–350}) containing only a single CAP-Gly and serine-rich region was found to promote MT polymerization, indicating that presence of multiple domains together in a CLIP-170 molecule could potentially generate profound effects on MT dynamics.²⁶ Further, CLIP-170's ability to bind both α - and β -tubulin suggest that it might be able to alter the angle between monomers or dimers and/or suppress the tubulin GTPase. An effect of CLIP-170 on the GTPase is also predicted by the observation that CLIP-170 has higher affinity for GMPCPP MTs than for GTP MTs.¹⁷ However, we cannot yet predict how such effect might occur at the molecular level due to the lack of structural information on the CLIP-170-MT/tubulin complex.

These data help to explain some discrepancies in the CLIP-170 literature. For example, CLIP-170 was shown to exchange slowly *in vitro* on taxol-stabilized MTs,¹⁷ while rapid exchange kinetics were reported both *in vivo*^{8,23,44} and *in vitro*²¹ on dynamic MTs. The presence of multiple MT binding sites, each with significant affinity, would be expected to strongly suppress the dissociation of CLIP-170 from the MTs, leading to the slow exchange dynamics observed *in vitro* on taxol-stabilized MTs. To account for the rapid CLIP-170 dynamics observed *in vivo* and in dynamic systems *in vitro*, we suggest that some of the MT binding sites are turned off by regulation (*in vivo*) and/or are occupied by other proteins such as EB1 or unpolymerized tubulin dimers (*in vivo* or *in vitro*).

The present work also has relevance for how the network of proteins at the MT plus end is assembled. Our data show that CLIP-170 and tubulin each have multiple sites for binding the other. This observation suggests that CLIP-170 and MAPs could each affect the binding of the other in complex ways: these proteins could compete for some binding sites on tubulin but not all, leading to partial competition that might reduce affinity of individual sites but leave binding of the complex intact or even enhanced. The situation is even more complex for EB1, a MT-binding protein that directly binds to CLIP-170 by mimicking the β -tubulin tail. One might expect that EB1 would interfere with CLIP-170 binding to MTs, and indeed, at high concentrations it does (Fig. 7). However, at more moderate EB1 concentrations, we show that CLIP-170 is able to bind both EB1 and MTs simultaneously, presumably by utilizing its first half to bind to EB1, and its second half to bind to tubulin.²⁶ This observation suggests that these proteins could have synergistic effects on MT dynamics.

Materials and Methods

Materials

PIPES (1,4-piperazinediethanesulfonic acid), subtilisin, EDC and taxol were obtained from Sigma (MO, USA). NHS (N-Hydroxysulfosuccinimide) was purchased from Fluka (MO, USA). Anti- α -tubulin (TUB-1A2) antibody was from the Kreis lab.¹⁴ Both α - and β -tubulin peptides (>98% purity level) containing the sequences derived from the H12 helix (Fig. 2a) were synthesized by GenScript Corporation (Piscataway, NJ). All other chemicals were of analytical grade.

Cloning and protein purification

The plasmids for bacterial expression of His-tagged CLIP-170 fragments and the EB1 tail (EB1^{194–268}) construct were generated in a manner similar to that used in our previous work.²⁶ Briefly, the coding sequences were PCR amplified from a pET15b vector (Novagen) already containing the CLIP-170 H1^{1–350} or full-length EB1 (EB1^{1–268}) sequence using the unique NdeI and BamHI restriction sites. The CLIP-170 H1^{1–350} and H2^{1–481} constructs were described previously.¹⁷ All constructs were verified by restriction enzyme digestion and DNA sequencing analysis.

The recombinant His-tagged CLIP-170 constructs and EB1 tail were expressed in *Escherichia coli* BL21 (DE3) and purified as described previously,²⁶ with the following modifications. Proteins were eluted from the nickel column in a buffer consisting of 20mM Tris, pH 7.9, 300mM NaCl, and 200mM imidazole. The pooled fractions containing protein were desalted and exchanged into PIPES buffer (pH 6.8) by a pair of consecutive columns pre-packed with Bio-Gel P-6DG resin (Bio-Rad), then flash-frozen and stored at -80°C . The purity of the purified proteins was assessed by SDS-PAGE (Fig. 1b). All expressed proteins were found in the soluble fraction and migrated on SDS-PAGE gels according to their expected molecular weights with the following exception: the constructs containing the first serine-rich region (H1^{1–140} and H1^{1–70}) lag during SDS-PAGE and appear larger than their expected molecular weights due to a higher proline content in the first serine-rich region compared to the other regions of the protein.⁴⁵ All proteins were quantified by comparison with standard amounts of bovine serum albumin on Coomassie blue stained SDS-PAGE gels.

Preparation of tubulin and subtilisin digested MTs

Porcine brain tubulin was prepared by two cycles of polymerization and depolymerization as described previously.¹⁷ Taxol (paclitaxel, Sigma) MTs were prepared from purified tubulin by the stepwise addition of taxol as described previously.¹⁷ As is common practice, we use the phrase “concentration of MTs” to mean the concentration of polymerized tubulin.

To remove the C-terminal tails of α - and β -tubulin, limited proteolysis by subtilisin was performed. Because β -tubulin is more sensitive to digestion than α -tubulin, different digestion times allow production of MTs that are either intact ($\alpha\beta$ -MT), missing the C-terminal tail of β -tubulin ($\alpha\beta_s$ -MT), or missing the C-terminal tail of both α - and β -tubulin ($\alpha_s\beta_s$ -MT).⁴⁶ To produce $\alpha\beta_s$ -MTs, taxol-MTs (2 mg/mL) were treated with subtilisin (80 $\mu\text{g/mL}$) at 37°C for 10 minutes. MTs missing both tails ($\alpha_s\beta_s$ -MTs) were produced by 120 min of digestion under similar conditions. In both cases, subtilisin cleavage was halted by the addition of 4 mM PMSF (in DMSO) for 20 min at 25°C , and the MTs were then pelleted at $100,000 \times g$ for 15 min. The pellet was washed once and resuspended to ~ 2.0 mg/mL tubulin concentration in PEM buffer (80 mM PIPES, pH 6.8, 2 mM MgCl_2 , and 1 mM EGTA) containing 1 mM GTP plus 20 μM taxol. To confirm that the C-terminal tails were either removed or left intact as expected, samples of each digest were carboxymethylated to allow separation of α - and β -tubulin (described below, see also Supplemental Fig. 1b). The status of the α -tubulin C-terminal tail was confirmed by western blotting using the monoclonal anti- α -tubulin antibody, 1A2, which reacts with the –EEEGEEY motif of α -tubulin (Fig. 2a; Supplemental Fig. 1a). This additional test was important because in early experiments, over-digestion of $\alpha\beta_s$ -MTs caused an artifactual reduction in CLIP-170 affinity due to partial loss of the α -tubulin tail.

Cosedimentation assays

The binding affinity of CLIP-170 for the different MT forms was measured by cosedimentation assays.¹⁷ Briefly, H1^{1–350} (2.0 μM) was incubated with varying

concentrations of MTs (0–10 μM polymerized tubulin) in PEM buffer. The samples were incubated for 30 min at 37 °C and then centrifuged at $165,000 \times g$ for 20 min. Equal fractions of both supernatants and pellets were separated on 10% SDS-PAGE gels. Proteins were stained with Coomassie blue, digitally scanned, and the band intensity of CLIP-170 was quantified with Image J (NIH, USA). The fraction of CLIP-170 in the pellet was assumed to be the fraction bound because each experiment included a control without MTs in which the CLIP-170 fragment remained entirely in the supernatant. The binding affinity was calculated as described previously.¹⁷ Data were fit to a bimolecular binding equation $Y = B_{\text{max}}X/(K_d + X)$, where Y is the fraction of CLIP-170 fragment in the pellet; X is the concentration of MTs, and B_{max} is the maximal achievable binding. All analyses were performed under the assumption of 1:1 stoichiometry (CLIP-170 fragment: tubulin dimer), and B_{max} values were set to 1.0. The poor fit of the high affinity curves in Fig. 2b is a common observation when a 1:1 binding ratio (H1 fragment: tubulin dimer) is used.¹⁷ The fit was improved when a 1:1.5 ratio was used (data not shown). However, we prefer to continue to assume a 1:1 binding ratio because we are suspicious that the deviation from 1:1 is an artifact due to MT bundling (bundling reduces the effective concentration of MTs).¹⁷ If this assumption is inaccurate, then the K_d values are even expected to be even lower (stronger).

To see the concurrent binding of both CLIP-170 fragments and EB1 to MTs, EB1^{194–268} (0–40 μM) was incubated in the presence of both MTs (4.0 μM) and CLIP-170 fragments (2.0 μM) for 30 min at 37 °C. Samples were sedimented as described above and further separated on a 10–14% SDS-PAGE gels followed by staining with Coomassie blue. Bands intensities and data calculation were performed as described above.

Tubulin polymerization assay

The effects of α - and β -tubulin peptides on CLIP-170-induced tubulin polymerization were monitored at 350 nm using a light scattering assay as previously described.^{26,47} Briefly, H1^{1–350} (1.0 μM) was preincubated with peptide in PEM buffer for 10 min at 0 °C. Tubulin (12 μM) and GTP (1 mM) were then added and the polymerization reaction was initiated by transferring the samples to a Perkin Elmer Lambda 2 spectrophotometer connected to a 37 °C water bath.

Cross-linking of MTs and CLIP-170 fragments by EDC

Cross-linking of the complex between the CLIP-170 fragments and MTs was performed using the zero-length covalent cross-linker, EDC.^{14,48} To generate the cross-linked products, CLIP-170 fragments (2.5–8.0 μM) were incubated with MTs (5.0 μM) in PEM buffer for 30 min at 37 °C. EDC (2 mM) and NHS (5 mM) were added and the mixture was incubated for another 30 min at 25 °C. The reaction was terminated by the addition of 10 mM hydroxylamine. Mixtures were diluted in 2X-SDS sample buffer and then separated on 10–14% SDS-PAGE gels as indicated.

Carboxymethylation of tubulin

Carboxymethylation was performed according to the method of Crestfield *et al.*, with minor modifications.^{42,43} Briefly, the cross-linked products of CLIP-170 fragments (2.5–8.0 μM) and MTs (5.0 μM) were mixed with a solution containing 0.6 M Tris-HCl, pH 8.5, 8 M urea, 6 mM EDTA, and 150 mM β -mercaptoethanol in a 1:4 ratio. The reaction was incubated at 25 °C for 2 h. One tenth volume of iodoacetic acid (250 mM in 1 N NaOH) was then added to each reaction mixture and samples were further incubated at 25 °C for 30 min in the dark. Reactions were stopped with 6X-SDS sample buffer and subjected to 10% SDS-PAGE.

In-gel digestion and mass spectrometry of tubulin-CLIP-170 complexes

Various MT-CLIP-170 fragment complexes were prepared by using EDC cross-linking reactions as described above. Cross-linked samples were separated on 10% SDS-PAGE gel, and proteins were negatively stained using a copper stain kit in accordance with the manufacturer's protocol (Bio-Rad).⁴⁹ The corresponding protein bands were excised from the copper stained gel and subsequently destained using the manufacturer's protocol (Bio-Rad). An in-gel digestion method slightly modified from a preparation described earlier was performed on the excised copper-stained gel bands.⁵⁰ Briefly, gel bands were rinsed with HPLC-grade water and cut into small pieces. The gel pieces were dried to completion in a speedvac. A 30 μ L aliquot of a mass spectrometry-grade trypsin gold (Promega, Madison, WI) at a concentration of 12.5 ng/ μ L in 25 mM ammonium bicarbonate was added to the gel pieces, which were then placed on ice for 10 min. Then, 25 mM ammonium bicarbonate was added to cover the gel pieces before placing them in a 37 C heating block overnight. After the overnight digestion, the aqueous supernatant was collected. The peptides were further extracted by adding 30 μ L of 50% acetonitrile/45% water/5% formic acid to the gel pieces, vortexing for 30 min, and collecting the organic supernatant. This step was repeated and the extracts were added to the aqueous fraction. The combined extracts were reduced to 10 μ L by speedvac and desalted and further concentrated using C18 Ziptips (Millipore, Billerica, MA).

A 0.5 μ L aliquot of the extract was spotted on a MALDI target with 0.5 μ L saturated 2,5-dihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO) in 50% acetonitrile/50% water. MALDI-TOF experiments of the digest extracts were performed on a Bruker Autoflex III mass spectrometer (Bruker Daltonics, Billerica, MA). Spectra were generated in the positive ion reflectron mode with a laser beam attenuation set between 53 and 67% (optimized for each extract) and a 100 Hz laser repetition rate. The ion source voltage 1 was 19 kV and voltage 2 was 16.49 kV, the lens voltage was 8.65 kV, the reflector voltage 1 was 21 kV and voltage 2 was 9.7 kV. The acquired mass range was m/z 0–5000 with masses $\leq$ 500 Da deflected. All mass spectra were analyzed using the BiotoolsTM and Sequence Editor software packages (Bruker).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

GTP	guanosine 5'-triphosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EB1	end binding protein-1
MAPs	microtubule associated proteins

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Fig. 1. Purification of recombinant CLIP-170 fragments

(a) Schematic representation of the CLIP-170 constructs. Each construct is named by numbers corresponding to the amino acids of CLIP-170 that are in the construct. The total molecular weight of each construct including the His-tag is shown in kDa. (b) SDS-PAGE of purified His-tagged proteins (~ 5.0 µg each). The additional lower bands in some samples are degradation products commonly observed in bacterially expressed CLIP-170 preparations.^{17,26,28}

Fig. 2. Removal of C-termini from MTs by subtilisin digestion reduces the MT-binding affinity of CLIP-170

(a) Schematic representation of the C-termini of porcine α - and β -tubulin, with the amino acid sequences, structural domains (above) and subtilisin cleavage sites (arrows) as indicated. The sequences of the synthetic peptides derived from the H12 helix of α - and β -tubulins are shown below. (b) Binding of H1¹⁻³⁵⁰ binding to normal and subtilisin-treated MTs. The binding of H1¹⁻³⁵⁰ (2.0 μ M) was measured as a function of MT concentration (0–10 μ M polymerized tubulin) by cosedimentation assay. The fraction of H1¹⁻³⁵⁰ bound (in the pellet) was plotted against the concentration of unbound polymerized tubulin (calculated from the total polymerized tubulin assuming a 1:1 ratio, see *Materials and Methods*), and the data were fit to the bimolecular binding curve to obtain the apparent K_d . Data are an average of three independent experiments and error bars are $\pm$ SD.

Fig. 3. Cross-linking of CLIP-170 fragments with MTs

Experiments were performed using the zero-length cross-linker, EDC, as described in the *Materials and Methods*. Briefly, MTs (5.0 μ M) were incubated with either H1¹⁻³⁵⁰ (2.5 μ M) or other CLIP-170 fragments (8.0 μ M) in PEM buffer for 30 min at 37 °C. The complexes were then cross-linked and resolved by 10% SDS-PAGE. To visualize the low molecular weight proteins, samples were also separated by 14% SDS-PAGE under similar conditions.

(a) Cross-linking of CLIP-170 fragments with normal MTs ($\alpha\beta$ -MTs). *Upper panel:* Coomassie blue stained gel (10%) showing cross-linked products of CLIP-170 fragments alone (lanes 1–6), MTs alone (lane 7), and CLIP-170 fragments in the presence of MTs (lanes 8–13). Bands at molecular weights expected from the cross-linked products of

CLIP-170 fragments and tubulin monomers are indicated by stars (*), while those corresponding to cross-linked products of CLIP-170 fragments and tubulin dimers (and/or cross-linked tubulin dimers alone) are indicated by asterisks (*). The numbers 1–5 refer to the names given to these samples in the MALDI-TOF analysis (Supplemental Table 1). The boxed region corresponds to the portion of the gel in the lower panel on which the carboxymethylated samples were run (discussed below). *Middle panel*: the same samples as in the upper panel run on a 14% gel to allow visualization of small proteins (only the region around 15kDa is shown). *Lower panel*: SDS PAGE gel (10%) of the cross-linked products after carboxymethylation, a chemical treatment that allows separation of α - and β -tubulin on SDS gels^{42,43} (only the part of the gel corresponding to the boxed region in the upper panel is shown, lanes are numbered according to the upper panel). Each lane shows the distinct migration of α - and β -tubulin as indicated by arrows. The identity of the bands marked “6” and “7” was confirmed by MALDI-TOF as described in *Materials and Methods*. (b) Cross-linking of CLIP-170 fragments with subtilisin- treated MTs ($\alpha_s\beta_s$ -MTs). Lanes, symbols and conditions are as in (a). The numbers 8–12 refer to the names given to these samples in the MALDI-TOF analysis (Supplemental Table 1).

Fig. 4.

Effects of salt (NaCl) on the CLIP-170-MT interactions. MT cosedimentation assay was performed as described above. Briefly, H1¹⁻³⁵⁰ (2.0 μ M) was incubated with $\alpha\beta$ -MTs (4.0 μ M) or $\alpha_s\beta_s$ -MTs (8.0 μ M; higher concentration chosen because of weaker affinity) in the presence of increasing concentrations of NaCl as indicated for 30 min at 37°C. After incubation, cosedimentation was performed and equal amounts of supernatant and pellet were resolved by 10% SDS-PAGE and stained with Coomassie blue. Intensities of H1¹⁻³⁵⁰ bands were quantified as described in the *Materials and Methods*. (a) Effects of NaCl (0–500 mM) on the binding of H1¹⁻³⁵⁰ to normal undigested $\alpha\beta$ -MTs and fully digested $\alpha_s\beta_s$ -MTs as indicated. (b) Quantitative analysis of H1¹⁻³⁵⁰ binding to both $\alpha\beta$ -MTs and $\alpha_s\beta_s$ -MTs at various NaCl concentrations. Data are the average of four independent experiments. Values are $\pm$ SD.

Fig. 5.

Cross-linking of CLIP-170 fragments with the tubulin H12 helix peptides. Cross-linking reactions were performed as described in Fig. 3. Briefly, the indicated peptide (100 μ M) was incubated with CLIP-170 fragments for 30 min at 25°C before the addition of EDC. All CLIP-170 fragments were used at 8.0 μ M, except full-length H1¹⁻³⁵⁰ which was used at 2.5 μ M in order to keep the band intensities balanced. Carbonic anhydrase (CA) (2.5 μ M) was used as a negative control. The stars (☆) indicate particular prominent cross-linked bands which are at the molecular weights expected from products of CLIP-170 fragments and tubulin H12 helix peptide. (a and a') Cross-linking of CLIP-170 fragments to the β -tubulin peptide (1.81 kDa). The -/+ represents the samples without/with the indicated H12 tubulin peptides; carbonic anhydrase (CA) is shown as a negative control. Larger fragments (those containing at least one GAP-Gly motif plus one serine rich region) are shown on the top (a), while smaller fragments (those containing only one motif) are shown on the bottom (a'). (b and b') Similar experiments conducted with the β -tubulin peptide (1.88 kDa).

Fig. 6.

Effects of the α - (α p) and β -H12 (β p) peptides on the H1¹⁻³⁵⁰ induced tubulin polymerization. Polymerization of tubulin (12 μ M) in the assembly buffer was monitored by change in the absorbance at 350 nm. H1¹⁻³⁵⁰ (1.0 μ M) was preincubated with the peptide at the indicated concentrations at 4°C for 10 min before mixing with the tubulin and GTP. Data were extracted at indicated time points and plotted using the GraphPad Prism 5 program. Each data point is the average from three independent experiments and the lines are provided as a guide.

Fig. 7. Detection of ternary complex between MT-CLIP-170-EB1

(a) Binding of EB1¹⁹⁴⁻²⁶⁸ to αβ-MTs in the absence (○) or presence (●) of constant H1¹⁻³⁵⁰ as measured by cosedimentation assays. The amount of H1¹⁻³⁵⁰ (▲) bound to MTs in these experiments is also shown. Briefly, EB1¹⁹⁴⁻²⁶⁸ (0–40 μM) was incubated with αβ-MTs (4.0 μM) in the absence or presence of H1¹⁻³⁵⁰ (2.0 μM) for 30 min at 37 °C. Samples were sedimented and equal fractions of supernatant and pellet were separated by SDS-PAGE followed by the staining with Coomassie blue. Bands intensities and data calculation were performed as described in Fig. 2b. Data are an average of three independent experiments. Values are ± SD. (b) Binding of EB1¹⁹⁴⁻²⁶⁸ to αβ-MTs in the absence (○) or presence (●) of a dimeric CLIP-170 fragment H2^{1-481.17}. Reactions were carried out as described above. The amount of H1¹⁻⁴⁸¹ (▲) bound to MTs in these experiments is also shown. The H2¹⁻⁴⁸¹ concentration is calculated as monomer protein concentration to allow comparison to (a). Data are an average of three independent experiments and error bars are ± SD.