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Conformational changes in CLIP-170 regulate its binding to microtubules and dynactin localization

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Cytoplasmic linker protein (CLIP)-170, CLIP-115, and the dynactin subunit p150^{Glued} are structurally related proteins, which associate specifically with the ends of growing microtubules (MTs). Here, we show that down-regulation of CLIP-170 by RNA interference results in a strongly reduced accumulation of dynactin at the MT tips. The NH_2 terminus of p150^{Glued} binds directly to the COOH terminus of CLIP-170 through its second metal-binding motif. p150^{Glued} and LIS1, a dynein-associating protein, compete for the interaction with the CLIP-170 COOH terminus, suggesting that LIS1 can act to release dynactin from the MT tips. We also show that the NH_2-terminal part of CLIP-170 itself associates with the CLIP-170 COOH terminus through its first metal-binding motif. By using scanning force microscopy and fluorescence resonance energy transfer-based experiments we provide evidence for an intramolecular interaction between the NH_2 and COOH termini of CLIP-170. This interaction interferes with the binding of the CLIP-170 to MTs. We propose that conformational changes in CLIP-170 are important for binding to dynactin, LIS1, and the MT tips.

Introduction

Microtubules (MTs) are highly dynamic cytoskeletal elements, which undergo alternating phases of growth and shrinkage (Desai and Mitchison, 1997). This dynamic behavior allows MTs to search the cellular space and to establish and remodel contacts with various cellular components. Therefore, interactions of MT tips with different structures play an important role in many cellular processes and are regulated by a large number of factors. Recently, a diverse group of proteins has attracted general interest by its ability to bind specifically to the plus ends of growing MTs. These proteins have been designated plus end–tracking proteins (+TIPs; for reviews see Schuyler and Pellman, 2001; Carvalho et al., 2003; Galjart and Perez, 2003; Howard and Hyman, 2003).

The first identified +TIP was the cytoplasmic linker protein (CLIP)-170 (Pierre et al., 1992). CLIP-170 contains two CAP-Gly motifs, which are surrounded by serine-rich regions at its NH_2 terminus, followed by a long coiled-coil structure and two putative metal binding domains ("CCHC zinc fingers" or "zinc knuckles") at the COOH terminus (Fig. 1 A). The closest homologue of CLIP-170 in vertebrates is CLIP-115, which is similar to CLIP-170 in its

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Abbreviations used in this paper: CLIP, cytoplasmic linker protein; FRET, fluorescence resonance energy transfer; HIS, 6X histidine; IP, immunoprecipitation; MT, microtubule; RNAi, RNA interference; siRNA, small interfering RNA; SFM, scanning force microscopy; +TIP, plus end–tracking protein.

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structure, but lacks the COOH-terminal metal binding motifs (De Zeeuw et al., 1997; Fig. 1 A).

Another, more distant CLIP-170 family member, is $p150^{Glued}$, which contains one CAP-Gly domain, followed by coiled-coil regions (Holzbaur et al., 1991; Fig. 1 A). $p150^{Glued}$ is the MT-binding subunit of dynactin, a large protein complex, which functions as an accessory factor for cytoplasmic dynein (Karki and Holzbaur, 1999; Allan, 2000).

In many cultured cells CLIPs and dynactin are observed to form cometlike accumulations at the MT tips (Perez et al., 1999; Vaughan et al., 1999). The motifs necessary and sufficient for the MT plus end localization include the CAP-Gly domains and the surrounding serine-rich regions of CLIPs and $p150^{Glued}$ (Diamantopoulos et al., 1999; Hoogenraad et al., 2000; Vaughan et al., 2002).

A large body of data links CLIP-170 to the function of the dynein–dynactin complexes. In budding yeast, genetic analysis of spindle positioning placed Bik1p, the CLIP-170 homologue, in the dynein pathway and has shown that Bik1p contributes to the targeting of dynein to the astral MTs (Sheeman et al., 2003). In mammalian cells, overexpression of CLIP-170 enhances the accumulation of dynactin at the MT ends (Valetti et al., 1999; Hoogenraad et al., 2002; Goodson et al., 2003). This effect depends on the second metal binding motif of CLIP-170 (Goodson et al., 2003). In a separate line of investigation, it was shown that CLIP-170 binds to kinetochores of unattached chromosomes in a dynein–dynactin-mediated manner (Dujardin et al., 1998).

The only direct binding partner of the CLIP-170 COOH terminus, identified so far, is LIS1 (Coquelle et al., 2002). LIS1 is a multifunctional protein, which plays an essential role in brain development and interacts with dynein–dynactin (for reviews see Vallee et al., 2001; Wynshaw-Boris and Gambello, 2001). The binding of LIS1 to CLIP-170 depends on the second zinc knuckle of CLIP-170, and therefore, it has been proposed that the interaction between dynein–dynactin and CLIP-170 is mediated by LIS1 (Coquelle et al., 2002). Although attractive, this proposal is contradicted by a number of observations. First, endogenous CLIP-170 and dynactin can be readily observed at the ends of MTs in interphase cells, whereas endogenous LIS1 is found in a dotlike pattern along the MTs (Smith et al., 2000). Second, overexpression of LIS1 displaces dynactin from the MT ends (Faulkner et al., 2000). Third, endogenous LIS1 can be displaced from kinetochores by overexpressing a LIS1 deletion mutant, without removing either dynactin or CLIP-170 (Tai et al., 2002).

A simple explanation for all these observations can be offered by proposing a direct link between CLIP-170 and dynactin. Here, we provide experimental support for this idea and show that normal dynactin accumulation at MT tips depends on CLIP-170. We also show that CLIP-170 can adopt a folded conformation through an association between its NH_2 and COOH termini. This conformation is likely to be inhibitory for the binding of CLIP-170 to its partners, including dynactin, LIS1, and MTs.

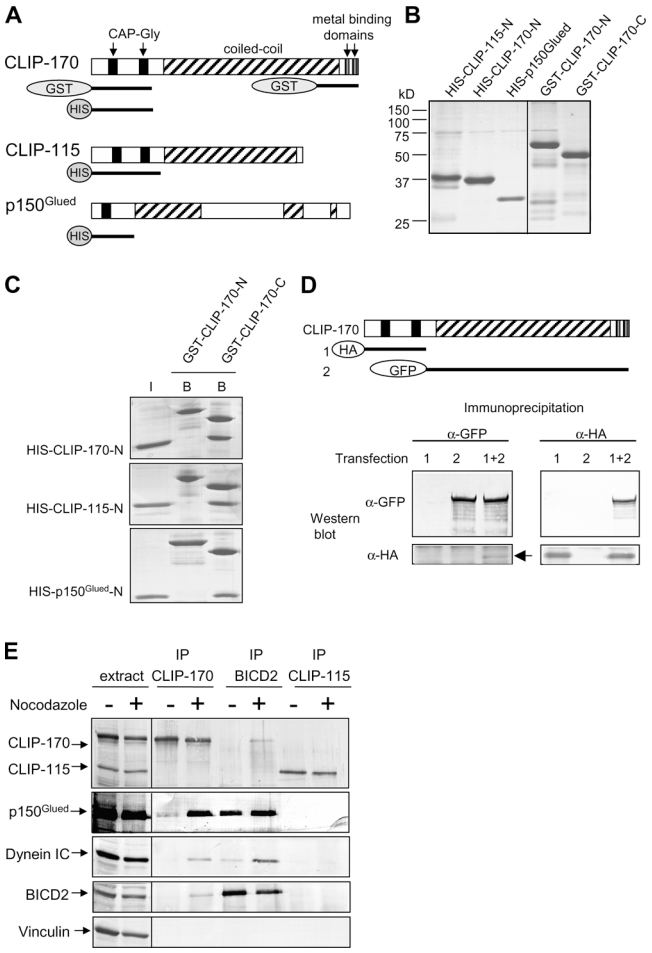

Figure 1. **The COOH terminus of CLIP-170 interacts with the NH_2 termini of both CLIPs and $p150^{Glued}$.** (A) Schematic representation of the protein fragments used in this work. (B) Purified fusion proteins shown on a Coomassie-stained gel. (C) GST pull-down assays in low salt conditions with the NH_2 termini of the two CLIPs and $p150^{Glued}$. Coomassie-stained gels are shown. I, 10% of the input; B, 25% of the protein retained on beads. (D) IPs from COS-1 cells transfected either singly or simultaneously with the two indicated constructs. Anti-GFP or anti-HA antibodies were used for IP in low salt conditions. (E) IPs performed in low salt conditions from untransfected CHO cells that were either untreated or incubated for 1 h with 10 μM nocodazole. Antibody against vinculin served as a negative control.

Results

The COOH terminus of CLIP-170 interacts directly with the NH_2 termini of both CLIPs and $p150^{Glued}$

Our previous work showed that overexpression of the CLIP-170 COOH-terminal domain had a dominant negative effect: namely, it led to a highly decreased MT tip association of all three CAP-Gly–containing proteins (Komarova et al., 2002). We reasoned that the underlying mechanism might be the sequestration by the CLIP-170 COOH terminus of $p150^{Glued}$ and the CLIPs by binding to their NH_2-terminal domains, which are necessary for their interaction with MTs. To test this possibility, we purified GST- and 6X histidine (HIS)-tagged fusions of the NH_2-terminal domains of CLIP-115, CLIP-170, $p150^{Glued}$, and the COOH terminus of CLIP-170 (Fig. 1, A and B). We

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investigated by GST pull-down assays if these proteins could directly interact with each other and found that HIS-tagged NH_2 termini of both CLIPs and $p150^{Glued}$ bound to the GST-tagged COOH terminus, but not the NH_2 terminus of CLIP-170 (Fig. 1 C).

In apparent contradiction with the in vitro data, endogenous CLIP-170 was never coprecipitated with the CLIP-170 COOH terminus (Komarova et al., 2002). This could be explained if the NH_2 and COOH termini of CLIP-170 interacted with each other within the same molecule. In this way, endogenous CLIP-170 could "titrate itself" out of the complex with the overexpressed CLIP-170 COOH terminus. This explanation predicts that the NH_2- and COOH-terminal domains of CLIP-170, expressed as separate proteins, should form a complex. Indeed, GFP-CLIP-170-N and HA-CLIP-170-C coexpressed in COS-1 cells, coprecipitated with each other (Fig. 1 D), supporting the data from the in vitro binding assays.

Endogenous CLIP-170 and dynactin interact in vivo

To investigate if the interaction between the COOH terminus of CLIP-170 and endogenous CAP-Gly proteins occurs in vivo, we used CLIP-170 antibodies to perform immunoprecipitations (IPs) on extracts from untransfected CHO cells. As a control, we used antibodies against CLIP-115, which is not expected to interact with dynactin, and Bicaudal D2 (BICD2), a dynein–dynactin binding protein, which does not coprecipitate with CLIP-170 under normal conditions (Hoogenraad et al., 2001). $p150^{Glued}$, but not CLIP-115, coprecipitated with both CLIP-170 and BICD2 (Fig. 1 E). As expected, CLIP-115 and $p150^{Glued}$ did not coprecipitate with each other (Fig. 1 E). This experiment shows that CLIP-170 binds to dynactin under physiological conditions, whereas no association between CLIP-170 and CLIP-115 is detected. We cannot exclude, however, that CLIP-170 titrates itself out of the complex with CLIP-115, similar to what is likely to occur with the overexpressed CLIP-170 tail.

Interestingly, the coprecipitation between CLIP-170 and dynactin was enhanced by nocodazole treatment. After MT depolymerization, some coprecipitation was also observed between CLIP-170, cytoplasmic dynein, and BICD2, and the association of BICD2 with dynein was also increased (Fig. 1 E). This is in line with our previous observation that nocodazole treatment results in formation of dynein–dynactin–BICD2–containing aggregates (Hoogenraad et al., 2001). We observed that CLIP-170, but not CLIP-115, was also present in these structures, which apparently result from aggregation of dynein–dynactin with their binding partners after MT depolymerization (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200402082/DC1).

The CLIPs and $p150^{Glued}$ preferentially bind to distinct zinc knuckles of CLIP-170

Next, we sought to determine the sites in the COOH-terminal domain of CLIP-170, important for the identified interactions. The deletion of metal-binding motifs abolished the dominant-negative action of the CLIP-170 COOH terminus (unpublished data), indicating that these motifs play a significant role in the association with CAP-Gly proteins. Therefore, we used mutated versions of the

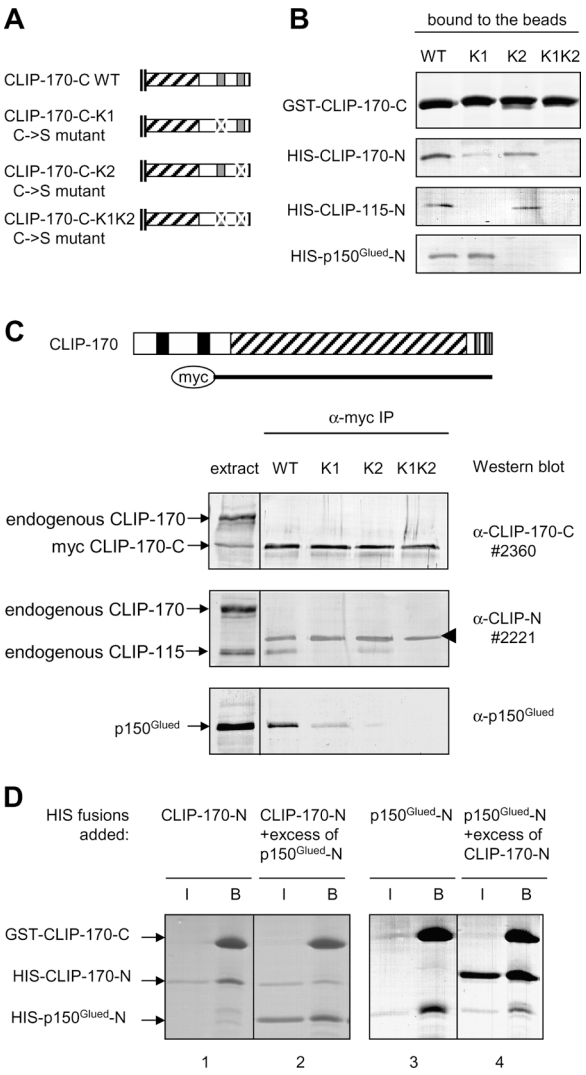

Figure 2. **Distinct zinc knuckles of the CLIP-170 COOH terminus are responsible for its binding to the NH_2 termini of the two CLIPs and $p150^{Glued}$.** (A) Schematic representation of the CLIP-170 zinc knuckle mutants. (B) Pull-down assays with the GST fusions of CLIP-170 zinc knuckle mutants and the NH_2 termini of the three CAP-Gly proteins, performed with purified proteins in high salt conditions. Coomassie staining is shown for the GST fusions, Western blots with anti-HIS antibodies for the HIS-tagged fusions. (C) IPs of CLIP-170 COOH-terminal constructs. Extracts were prepared from CHO cells transfected with the myc-tagged CLIP-170 COOH-terminal constructs, either wild type or with the zinc knuckle mutations, and IPs were performed with anti-myc antibodies in low salt conditions. Note that the "CLIP head" antibody 2221 cross-reacts with the CLIP-170-C proteins (arrowhead). (D) Competition assay between CLIP-170 and $p150^{Glued}$ NH_2 termini for the binding to the CLIP-170 COOH terminus. The assay was performed in low salt with either a low amount of HIS-CLIP-170-N (1 and 2) and an 8× M excess of HIS-$p150^{Glued}$-N (2) or with a low amount of HIS-$p150^{Glued}$-N (3 and 4) and an 8× M excess of HIS-CLIP-170-N (4). I, 10% of the input, B, 25% of the proteins retained on the beads.

CLIP-170 COOH terminus, in which two cysteines of either the first, the second or both metal binding domains were exchanged for serines (CCHC to SSHC mutations [Goodson et al., 2003], designated K1, K2, and K1K2 mutants; Fig. 2 A). Using GST pull-down assays, we found that in low salt conditions (100 mM NaCl) the wild type

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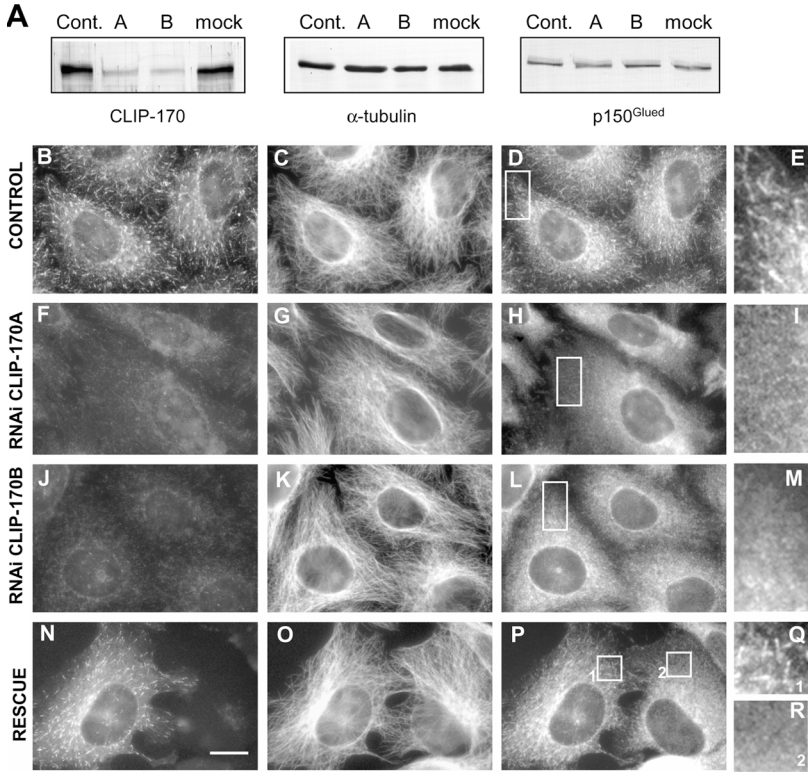

Figure 3. **CLIP-170 recruits dynactin to the MT plus ends in HeLa cells.** (A) Depletion of CLIP-170 by RNAi. Western blot analysis of the extracts of cells, treated with the RNAi duplex against luciferase (Cont.), CLIP-170A (lane "A"), CLIP-170B (lane "B"), or mock transfected. (B–M) Immunofluorescent staining of siRNA-treated cells. Cells were stained for CLIP-170 (B, F, and J), α-tubulin (C, G, and K), and $p150^{Glued}$ (D, H, and L). Enlarged portions of the $p150^{Glued}$-stained cells, indicated by white rectangles, are shown in E, I and M. (N–R) Rescue of dynactin localization by CLIP-115(+tail) fusion. HeLa cells were transfected with the CLIP-170B duplex, and 60 h later were transfected with GFP-CLIP-115(+tail). (N) GFP signal. (O) Staining for α-tubulin. (P) Staining for $p150^{Glued}$. (Q and R) Enlarged portions of the two cells, either transfected or untransfected with the rescue construct, respectively. Bar, 10 μm.

and all three mutants were still able to bind to the NH_2 termini of all three CAP-Gly proteins (not depicted). However, at 400 mM NaCl, the $p150^{Glued}$ NH_2 terminus bound only to the CLIP-170 COOH-terminal fragments with an intact second metal binding domain (K1; Fig. 2 B). In contrast, an efficient interaction with the NH_2 termini of both CLIPs depended on the first zinc knuckle (Fig. 2 B). These data are consistent with the fact that the CLIP-170–mediated relocalization of dynactin depends on the second metal binding domain, whereas the self-association of CLIP-170, revealed as aggregate formation upon overexpression, relies on the first zinc knuckle (Coquelle et al., 2002; Goodson et al., 2003).

The selective binding of the CLIP NH_2 termini and dynactin to the two different zinc knuckles of CLIP-170 was further supported by IP of CLIP-170 COOH-terminal mutants from transfected CHO cells. Coprecipitation of CLIP-115 occurred only if the first zinc knuckle was intact (Fig. 2 C). $p150^{Glued}$ displayed very little coprecipitation with the K2 and K1K2 mutants, underscoring the importance of the second metal binding domain of CLIP-170 for the association with dynactin. The binding between $p150^{Glued}$ and the K1 mutant was reduced, compared with the nonmutated protein, suggesting that in cells the first zinc knuckle also contributes to the CLIP-170–dynactin interaction.

Although the NH_2 termini of CLIP-170 and $p150^{Glued}$ associate with different zinc knuckles of CLIP-170, their binding sites are likely to overlap. Indeed, the addition of an excess of $p150^{Glued}$ NH_2 terminus reduced the binding of CLIP-170 NH_2 terminus to CLIP-170 COOH terminus, and vice versa (Fig. 2 D). These data show that the two CAP-Gly proteins can interfere with each other's binding to the CLIP-170 COOH terminus.

CLIP-170 targets dynactin to the MT tips

We have demonstrated that CLIP-170 binds $p150^{Glued}$ in vivo and in vitro. To address the role of CLIP-170 in the cytoplasmic distribution of dynactin, we knocked down CLIP-170 by RNA interference (RNAi) in HeLa cells, which express CLIP-170, but not CLIP-115 (unpublished data). We used two small interfering RNA (siRNA) duplexes (CLIP-170A and CLIP-170B), directed against different regions of CLIP-170 mRNA. Significant down-regulation of CLIP-170 was detected 3 d after treatment of cells with either duplex as evaluated by Western blotting and immunostaining of transfected cells (Fig. 3, A, F, and J). Densitometry analysis of Western blots indicated that the reduction in CLIP-170 level was >80% for the A duplex and >90% for the B duplex. Control treatment with a duplex directed against luciferase did not result in a decrease of CLIP-170 amount compared with mock-transfected cells. Down-regulation of CLIP-170 did not cause any change in the expression level of $p150^{Glued}$ (Fig. 3 A).

Next, we examined the localization of $p150^{Glued}$ in siRNA-treated cells. We detected prominent MT tip labeling by $p150^{Glued}$ antibodies in cells treated with the control siRNA (Fig. 3, B–E). However, in cells treated with either CLIP-170A or CLIP-170B siRNA, a much more diffuse pattern of $p150^{Glued}$ was observed (Fig. 3, F–M). We performed a rescue experiment by expressing a fusion construct, in which the COOH-terminal metal binding domain of CLIP-170 was attached to the end of CLIP-115 (GFP-CLIP-115(+tail)). This construct is not sensitive to the treatment with the CLIP-170A or CLIP-170B siRNA duplexes. Previously, we have shown that overexpression of GFP-CLIP-115(+tail) enhanced dynactin accumulation at the growing plus ends (Hoogenraad et al., 2002). Here, we observed that the expression of this fusion protein restored the normal

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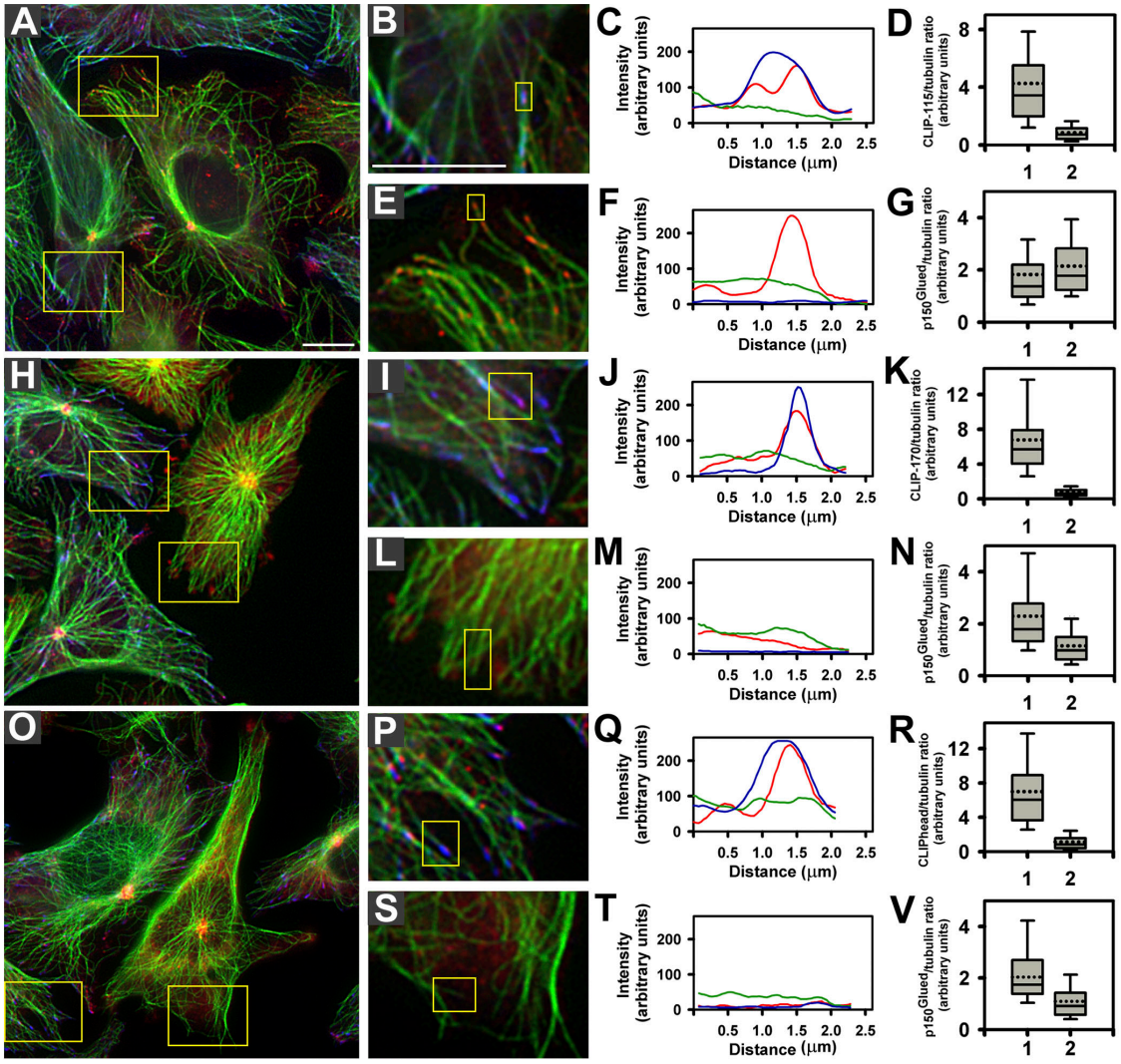

Figure 4. **CLIP-170 recruits dynactin to the MT plus ends in CHO cells.** RNAi experiments in CHO cells. In the fluorescent images (A, B, E, H, I, L, O, P, and S) and the line scans (C, F, J, M, Q, and T), the α-tubulin signals are shown in green, CLIP-specific signals in blue, and $p150^{Glued}$ in red. Line scans correspond to individual MT plus ends, starting at ~2 μm away from the MT end. The ratio of the signal of the plus end–tracking protein to tubulin in experimental cells was expressed as a percentage of the same ratio in control cells, which was taken for 100%. The box plot graphs (D, G, K, N, R, and V) are used to demonstrate statistical analysis of $p150^{Glued}$/tubulin or CLIP/tubulin ratio at the MT tip in control (1) and knockdown (2) cells. The boundaries of the box indicate the 25th and the 75th percentile, whiskers indicate the 90th and 10th percentiles. The median and mean are shown by a straight and a dotted line, respectively. The differences between the measured ratios are significant ($P < 0.0001$ for D, K, N, R, V; $P < 0.03$ for G, determined with the *t* test). In all cases 95% confidence intervals are nonoverlapping. (A–G) Knockdown of CLIP-115. (A, B, and E) Knockdown cells were identified by staining with CLIP-115–specific antibodies. (B and C) Control (untransfected) cells. (E and F) CLIP-115 knockdown cells. (H–N) Knockdown of CLIP-170. (H, I, and L) Knockdown cells were identified by staining with CLIP-170–specific antibodies. (I and J) Control (untransfected) cells. (L and M) CLIP-170 knockdown cells. (O–V) Knockdown of both CLIPs. (O, P, and S) Knockdown cells were identified by staining with the antibody against both CLIPs. (P and Q) Control (untransfected) cells. (S and T) CLIP-115 and CLIP-170 knockdown cells. Note that the centrosomal localization of dynactin is not affected by CLIP-170 knockdown. Bars, 10 μm.

dynactin pattern when endogenous CLIP-170 was knocked down (Fig. 3, N–R). This experiment shows that the metal binding domains of CLIP-170 are involved in targeting of $p150^{Glued}$ to the MT plus ends.

The large number of MT tips and other dynactin-positive granules made it difficult to quantify dynactin accumulation at MT tips in HeLa cells. Therefore, we switched to CHO cells, which have a sparser MT cytoskeleton. CHO cells co-express both CLIP-170 and CLIP-115; therefore, the effect of the knockdown of each single CLIP protein or both CLIPs simultaneously could be analyzed in this system. Transfection of RNA duplexes did not work efficiently in CHO cells in our hands. Consequently, we used plasmid-based RNAi instead (Brummelkamp et al., 2002). The target sequences for CLIP-170 and CLIP-115 RNAi were chosen based on human-mouse homology. To confirm their identity with the hamster sequences, the corresponding portions of CLIP-170 and CLIP-115 cDNAs were obtained by RT-PCR from CHO cells and sequenced. To knock down both CLIPs, we combined two RNAi cassettes in the same vector. As a control, we used a GFP-expressing RNAi vector directed against luciferase.

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CHO cells were transfected with different RNAi vectors and 4 d after transfection the cells were stained for CLIPs, p150^{Glued}, and tubulin (Fig. 4). The knockdown cells were identified by the strongly reduced CLIP staining, whereas the surrounding, nontransfected cells served as an internal control. CHO cells, transfected with the control vector, were detected by staining with anti-GFP antibody.

Line scan analysis (plots of intensity vs. distance), demonstrated that knockdown of CLIP-115 had no strong effect on the p150^{Glued} distribution (Fig. 4, A–G). RNAi-treated cells showed a reduction of CLIP-115–specific signal to 20 ± 5.8% (SEM) compared with control surrounding cells whereas the p150^{Glued} signal was slightly elevated to 117 ± 3% (n = 197 MT ends in 18 knockdown cells; 156 ends in 23 control cells). In contrast, knockdown of CLIP-170 (12 ± 5% remaining) resulted in a significant reduction (50 ± 3.5% remaining) of the p150^{Glued} signal at the MT tip (Fig. 4, H–N; n = 271 MT ends in 18 knockdown cells; 168 ends in 19 control cells). A similar result was obtained in cells where both CLIP species were knocked down simultaneously (Fig. 4, O–V). Quantification of the signals for the CLIPs (Fig. 4 R) and p150^{Glued} (Fig. 4 V) gave values of 16 ± 6% and 52 ± 5%, respectively, compared with control levels (n = 207 MT plus ends in 19 knockdown cells; 157 ends in 20 control cells). No change in the distribution of either CLIPs or p150^{Glued} was observed in GFP-positive cells transfected with the control RNAi construct (unpublished data). All these data were confirmed by using an antibody against another dynactin subunit, dynamitin (unpublished data).

Furthermore, we observed no differences in the localization of endosomes, the Golgi apparatus, and mitochondria after CLIP-170 knockdown (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200402082/DC1 and not depicted), indicating that a 50% reduction of dynactin association with MT tips had no significant consequences for the steady-state distribution of these organelles.

p150^{Glued} and LIS1 compete for the binding to the second metal binding domain of CLIP-170

Previous works have shown that LIS1 binds to the CLIP-170 COOH terminus through its second zinc knuckle (Coquelle et al., 2002). Using purified HIS-tagged mouse LIS1 protein (Fig. 5 A) and the GST fusions of CLIP-170 COOH terminus and its K1, K2, and K1K2 mutants, we confirmed this observation in an in vitro experiment with purified proteins (Fig. 5 B). Because both LIS1 and p150^{Glued} associate directly with the second metal binding motif of CLIP-170, we tested whether the addition of an excess of the NH_2-terminal fragments of CLIP-170 or p150^{Glued} could inhibit the interaction between CLIP-170 COOH terminus and LIS1. In the presence of the NH_2 terminus of CLIP-170, the association of LIS1 with CLIP-170 COOH terminus was somewhat reduced (Fig. 5 C). More strikingly, the addition of an excess of the p150^{Glued} NH_2 terminus completely abolished the interaction (Fig. 5 C). Because LIS1 and p150^{Glued} NH_2 terminus do not bind to each other (unpublished data), this result demonstrates that p150^{Glued} efficiently competes with LIS1 for binding to CLIP-170. We were not able to reduce the amount of p150^{Glued} bound to the COOH terminus of CLIP-170 by addition of an excess of LIS1 (unpublished data). This indicates that at least in vitro, the CLIP-170 COOH terminus has a higher affinity for p150^{Glued} than for LIS1.

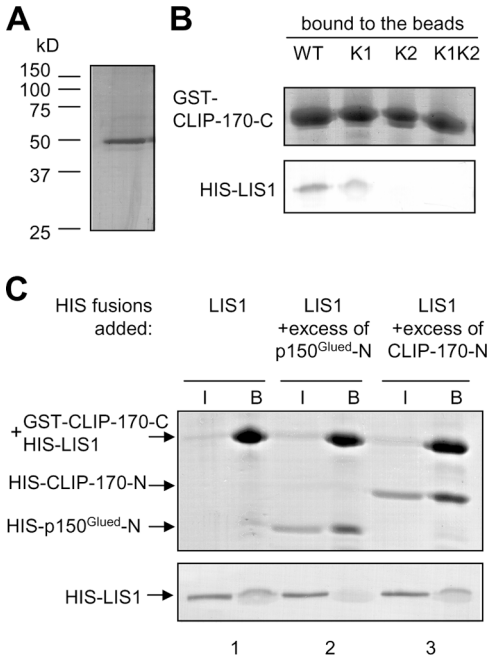

Figure 5. **LIS1 and p150^{Glued} compete for binding to the COOH terminus of CLIP-170.** (A) Coomassie-stained gel with purified HIS-LIS1 protein. (B) GST pull-down assay using purified HIS-LIS1 in low salt conditions with the wild type and the zinc knuckle mutants of the CLIP-170 COOH terminus. GST fusions are detected by Coomassie staining; HIS-tagged LIS1 detected by Western blotting with anti-HIS antibodies (note that HIS-LIS1 and GST-CLIP-170 have exactly the same size, 50 kD). (C) Competition between LIS1 and p150^{Glued} or CLIP-170 NH_2 termini for the binding to the CLIP-170 COOH terminus. GST pull-down assays were performed in low salt conditions with a low amount of HIS-LIS1 and an 8× M excess of either HIS-p150^{Glued}-N (2) or HIS-CLIP-170-N (3). The top panel shows a Coomassie-stained gel and the bottom panel shows a Western blot with anti-HIS antibodies. I, 10% of the input; B, 25% of the proteins bound to the beads.

Evidence for intramolecular head-to-tail interaction in CLIP-170

The interaction between the NH_2 and COOH termini of CLIP-170 could be either intramolecular or intermolecular, leading to oligomerization of CLIP-170. To address these possibilities, we have analyzed the structure of the purified full-length CLIP-170 by scanning force microscopy (SFM; Fig. 6, A–F). The majority of the molecules (68.9% of the 536 identifiable molecules counted) resembled straight or bent rods (Fig. 6, B–D), supporting previous observations that CLIP-170 is a rod-shaped protein (Scheel et al., 1999). The length of CLIP-170 molecule, measured by us (95.3 ± 16.5 nm) was shorter, than the one previously observed by electron microscopy (~135 nm), because the rat brain isoform, used in our work, misses 115 aa of the central heptad repeat region, compared with the CLIP-170 from human placenta, analyzed by Scheel et al. (1999). In contrast to the latter work, we could frequently distinguish the two ends of the molecule. One of the ends of CLIP-170 often displayed two globular heads, which likely represent the NH_2-terminal

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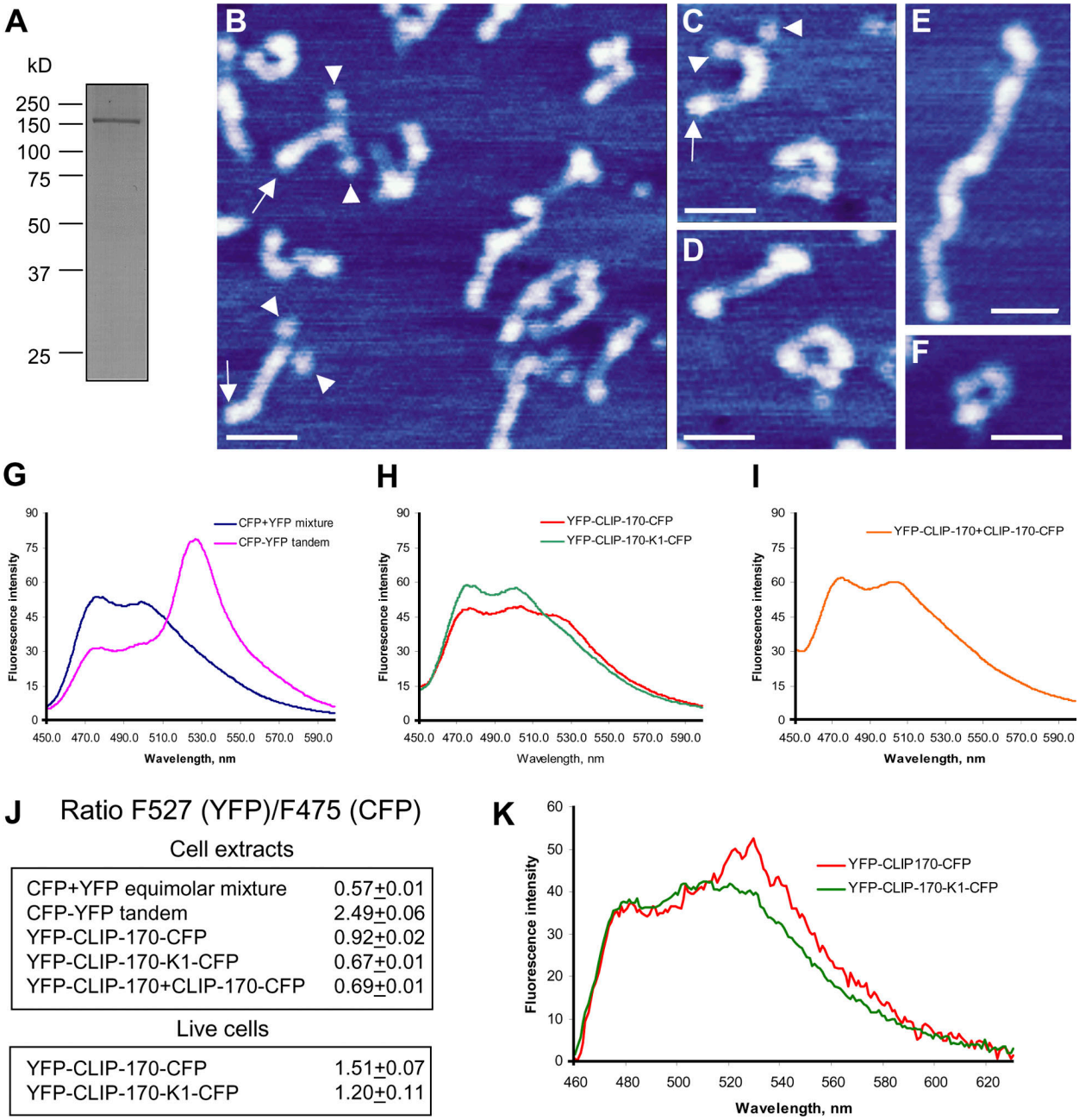

Figure 6. **Evidence for intramolecular interaction between the terminal domains of CLIP-170.** (A) Coomassie-stained gel with purified HIS-CLIP-170. (B–F) SFM images of the purified HIS-CLIP-170. Color represents height from 0 to 2 nm, blue to white. Bars, 50 nm. Globular head domains are indicated by arrowheads and the COOH-terminal domain is indicated by arrows. (G–I) Uncorrected emission spectra of the extracts of cells, transfected with the indicated constructs, measured with the excitation at 425 nm. Fluorescence intensity is shown in arbitrary units. (J) Ratios of emission at 527 nm (YFP acceptor) to 475 nm (CFP donor). Mean ± SD was determined from three independent measurements (extracts) or seven different cells (live cells). (K) Uncorrected emission spectra of single live cells, transfected with the indicated constructs after background subtraction. Fluorescence intensity is shown in arbitrary units.

MT-binding domains (~350 aa), because they are larger than the COOH-terminal domains (~90 aa). These data strongly support the view that CLIP-170 forms parallel dimers.

Furthermore, 10.6% of the molecules, classified as single dimers based on their length (average 100.2 ± 10.1 nm), displayed a donutlike shape (Fig. 6, B, C, D, and F). The presence of such molecules clearly demonstrates that the coiled-coiled tail of CLIP-170 is sufficiently flexible to allow the NH_2 and COOH termini to be brought into close proximity. In addition, we also observed CLIP-170 proteins forming oligomers due to intermolecular end-to-end association (20.5% of the molecules counted; Fig. 6 E). Observation of both inter- and intramolecular association of CLIP-170 NH_2 and COOH termini might suggest that these interactions are transient and dynamic. Alternatively, our preparation might contain a mixture of molecules with different posttranslational modifications, which preferentially stabilize different CLIP-170 conformations.

Next, we reasoned that attaching fluorophores to the NH_2 and COOH termini of CLIP-170 (CFP donor and YFP ac-

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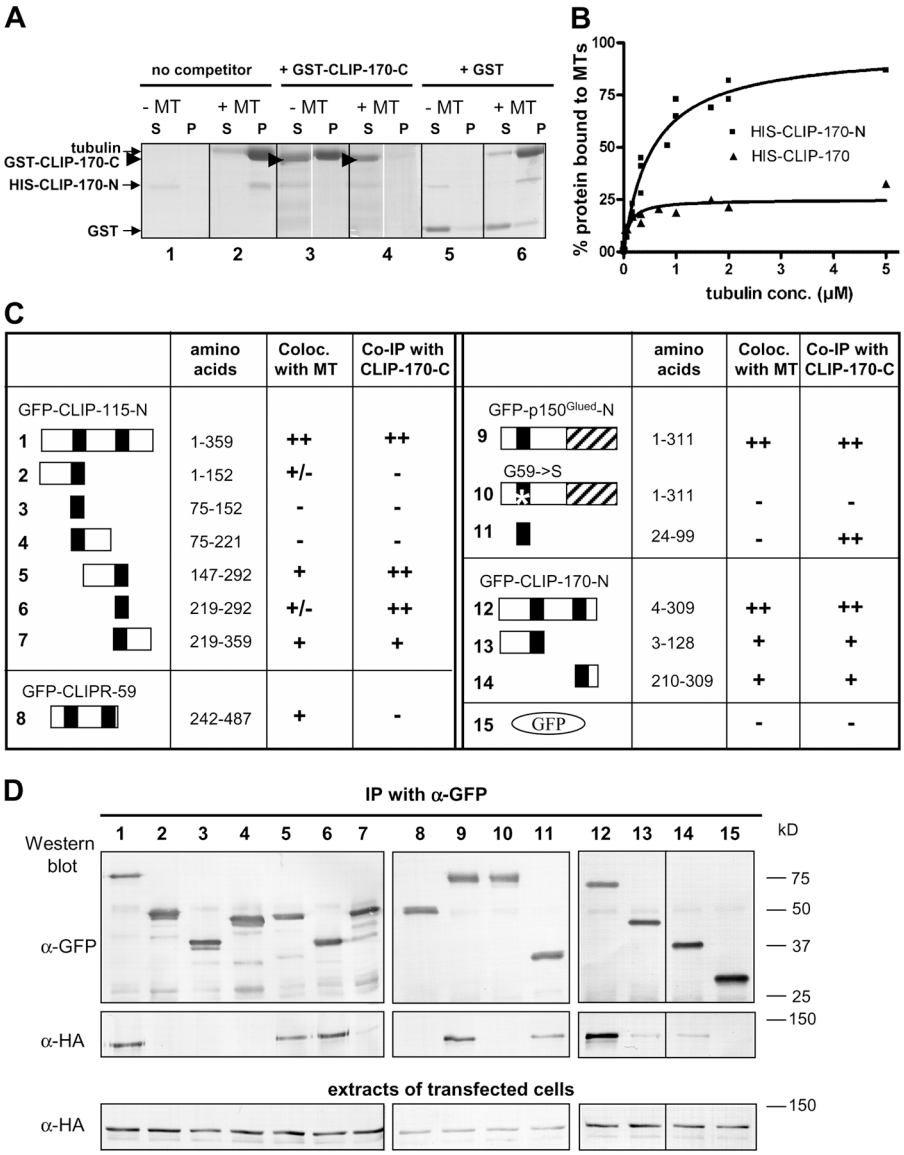

Figure 7. **The COOH terminus of CLIP-170 interferes with binding of the NH_2 terminus of CLIP-170 to MTs.** (A) MT pelleting assays. Coomassie-stained gels showing supernatants and pellets after incubation of MTs with the HIS-CLIP-170-N, either alone (1 and 2), preincubated with an 8× M excess of GST-CLIP-170-C (3 and 4) or with 15× M excess of GST (5 and 6). Note that GST-CLIP-170-C protein runs slightly below tubulin (arrowheads). S, supernatant; P, pellet. (B) MT pelleting assays with HIS-CLIP-170-N (~50 nM) or full-length CLIP-170 (~10 nM). The dissociation constant for HIS-CLIP-170-N binding to taxol-stabilized MTs, determined by best fit to the data, is 0.53 ± 0.09 μM (± SEM). Not more than 30% of the full-length CLIP-170 could be pelleted with MTs. (C) Schematic representation of different fragments of CLIP-115, CLIPR-59, $p150^{Glued}$, and CLIP-170 fused to GFP. Fusions were tested for colocalization with MTs in transfected COS-7 cells and their capacity to coprecipitate with the HA-CLIP-170 COOH terminus. (D) IPs with CLIP-170 COOH terminus. COS-1 cells were cotransfected with the HA-CLIP-170 COOH terminus together with the indicated GFP fusion proteins, and IP was performed with anti-GFP antibodies in a buffer with 150 mM NaCl. The expression of the HA-CLIP-170-C fusion was controlled by the Western blotting of the cell extracts with anti-HA antibodies (bottom).

ceptor) would allow us to detect fluorescence resonance energy transfer (FRET) if CLIP-170 indeed folds back and its NH_2 and COOH termini are brought within a 10-nm distance (Pollok and Heim, 1999). We generated a CLIP-170 fusion with YFP at the NH_2 terminus and a glycine linker, followed by CFP at the COOH terminus (YFP-CLIP-170-CFP). As a control, we used a mutated version of this CLIP-170 fusion protein, in which the first zinc knuckle, necessary for the interaction between the CLIP NH_2 and COOH termini, was disrupted (YFP-CLIP-170-K1-CFP).

COS-1 cells were transfected with plasmids, expressing either CFP or YFP, a CFP-YFP tandem fusion, YFP-CLIP-170-CFP or YFP-CLIP-170-K1-CFP and the fluorescence spectra of the resulting cell extracts were measured. A mixture of cell extracts, containing an equimolar amount of CFP and YFP, served as a negative FRET control, which displayed no significant emission of the YFP acceptor after the excitation of the CFP donor (Fig. 6 G). The CFP-YFP tandem, which served as a positive control, displayed marked sensitized YFP fluorescence after CFP excitation due to FRET (Fig. 6 G). A smaller, but significant YFP-sensitized emission was displayed by the YFP-CLIP-170-CFP but not by the YFP-CLIP-170-K1-CFP–containing cell extract (Fig. 6 H). The occurrence of FRET in the extract, containing the YFP-CLIP170-CFP fusion, is indicated by the higher ratio of fluorescence at 527 nm (YFP emission) to fluorescence at 475 nm (CFP emission) upon excitation at 425 nm, as compared with CFP+YFP mixture or the YFP-CLIP-170-K1-CFP control (Fig. 6 J). The ratio of YFP to CFP fluorescence in the extract, containing YFP-CLIP-170-CFP protein, did not change after it was diluted, suggesting that the binding between the CLIP-170 head and tail was intra- and not intermolecular (unpublished data). This conclusion is further supported by the fact that the extracts prepared from cells, cotransfected with YFP-CLIP-170 and CLIP-170-CFP, display no significant FRET signal (Fig. 6 I).

We have also measured the emission spectra of live COS-7 cells, expressing the same constructs. We selected cells with low expression levels, in which fluorescent CLIP proteins localized to the plus ends, but formed no patches or MT bundles. Again, after CFP excitation, YFP-CLIP-170-CFP displayed higher YFP/CFP fluorescence ratios, than

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the K1 mutant (Fig. 6, J and K), indicating that the interaction between NH_2 and COOH terminus of CLIP-170 occurs in vivo.

Inhibition of MT binding by head-to-tail interaction in CLIP-170

The proposed conformational change in CLIP-170 could regulate its association with MTs. We tested this idea by MT pelleting assay and found that the addition of an excess of the CLIP-170 COOH terminus abolished the pelleting of the CLIP-170 NH_2 terminus with MTs, whereas the addition of a control protein (GST) had no effect (Fig. 7 A). Similar results were obtained for MT pelleting of $p150^{Glued}$ NH_2 terminus (not depicted). We also observed that the full-length CLIP-170 pelleted with MTs much less efficiently than CLIP-170 NH_2 terminus (Fig. 7 B). This suggests that interaction of the COOH terminus of CLIP-170 either with its own NH_2 terminus or with $p150^{Glued}$ interferes with their binding to MTs, explaining the dominant negative effect of the CLIP-170 tail.

Next, we mapped the binding sites of the CAP-Gly proteins for the CLIP-170 COOH terminus. We initially used a collection of the CLIP-115 NH_2-terminal deletion mutants (Hoogenraad et al., 2000; Fig. 7 C) and found that only the fusions containing the second, but not the first CAP-Gly domain could coprecipitate CLIP-170 COOH terminus and associate with MTs in transfected cells (Hoogenraad et al., 2000; Fig. 7, C and D). Therefore, it appears that sites of interaction with MTs and with the COOH terminus of CLIP-170 are located in the second CAP-Gly domain of CLIP-115 and are in close proximity. Also in the case of $p150^{Glued}$, the CAP-Gly domain is the portion of the protein interacting with CLIP-170 COOH terminus (Fig. 7, C and D). Interestingly, the G59S mutation of $p150^{Glued}$, discovered in patients with neurodegenerative disease (Puls et al., 2003), abolishes the interaction of $p150^{Glued}$ with the CLIP-170 COOH terminus (Fig. 7, C and D), probably because it disrupts the folding of the CAP-Gly domain.

Mapping of the NH_2-terminal part of CLIP-170 uncovered differences between the two CLIPs: both CAP-Gly domains of CLIP-170 localized to MTs in transfected cells and coprecipitated, albeit weakly, with the CLIP-170 COOH terminus (Fig. 7, C and D). Interestingly, the YFP-CLIP-170-CFP molecule, containing only one (the second) CAP-Gly domain, could still produce a FRET signal (unpublished data). This indicates that the second CAP-Gly domain of CLIP-170 alone is sufficient for interaction with the COOH terminus.

We also tested if the MT-binding CAP-Gly domains of a more distant CLIP relative, CLIPR-59 (Perez et al., 2002), could bind to the CLIP-170 COOH terminus and found this not to be the case (Fig. 7, C and D). This result illustrates that the COOH terminus of CLIP-170 interacts only with a subset of the existing CAP-Gly proteins.

Discussion

Association of CLIP-170, dynactin, and LIS1 with MT tips

Here, we have shown that the COOH terminus of CLIP-170 interacts directly with the NH_2 terminus of $p150^{Glued}$ and is required for efficient recruitment of dynactin to the MT tips. Previously it has been observed that expression of CLIP-170 lacking a functional tail interferes with dynactin localization to MT tips (Goodson et al., 2003). CLIP-170 binds to the MT tips in a dynactin-independent manner, because dynactin, but not CLIP-170, is displaced from the tips by overexpression of LIS1 (Faulkner et al., 2000). Moreover, down-regulation of $p150^{Glued}$ does not change the distribution of CLIPs (unpublished data). Therefore, we propose that a large portion of dynactin accumulated at the MTs tips is "hitchhiking" on CLIP-170, using its capacity to recognize the growing MT plus ends (Fig. 8; the term hitchhiking was introduced by Carvalho et al., 2003).

This conclusion appears to contradict the observations that the NH_2 terminus of $p150^{Glued}$ binds directly to MTs and to EB1, another +TIP, which is not dependent on CLIP-170 for its MT plus end accumulation (Askham et al., 2002; Komarova et al., 2002; Vaughan et al., 2002; Ligon et al., 2003). We believe that $p150^{Glued}$ can indeed recognize the growing MT tips independently of CLIP-170, because the knockdown of CLIP-170 reduced, but did not abolish

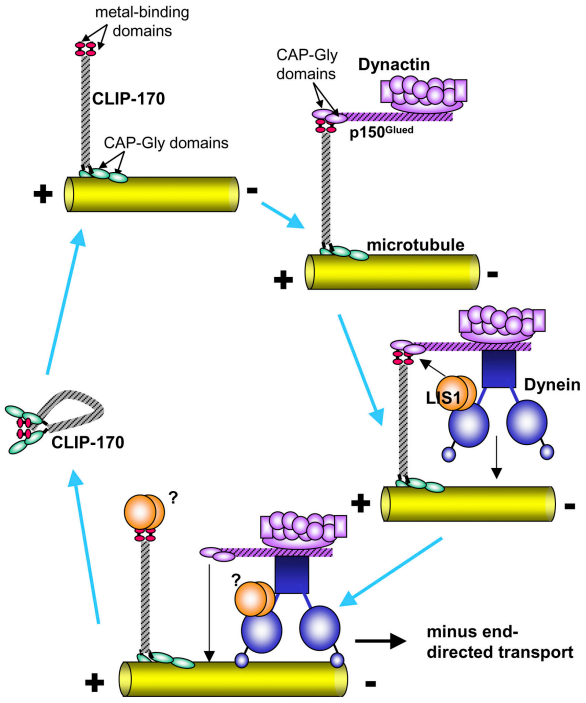

Figure 8. **A model of the possible interactions between CLIP-170, dynactin, dynein, and LIS1 at the plus end of a growing MT.** We propose that free, MT-unbound CLIP-170 can adopt a folded conformation through an intramolecular interaction of its terminal domains. Binding to MTs correlates with the unfolding of CLIP-170, which allows the interaction of the COOH-terminal domain with its binding partners, such as dynactin, resulting in their recruitment to the MT tip. Dynactin, in its turn, can subsequently recruit cytoplasmic dynein, together with LIS1. LIS1 might act to release dynactin from the complex with CLIP-170, facilitating MT minus end-directed transport. These interactions are probably coordinated with the association of dynein–dynactin complex with the cargo (omitted from the scheme). LIS1 may remain associated with dynein, or, alternatively, it may form a transient complex with CLIP-170. Other possible interactions not involving CLIP-170, such as direct binding of dynactin to the MT tips, are not depicted.

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dynactin accumulation at the MT tips. We propose that dynactin can interact with MT ends via three independent pathways: directly, via EB1 and through CLIP-170. In HeLa and CHO cells the third pathway contributes significantly to dynactin localization. It is possible that CLIP-170 serves as a transient facilitator of dynactin binding to MT ends.

We have found that LIS1 and $p150^{Glued}$, which show affinity for the same zinc knuckle of CLIP-170, cannot bind to this protein simultaneously. Although we were not able to compete $p150^{Glued}$ binding to CLIP-170 with an excess of LIS1 in vitro, it is likely that in cells such competition can occur, because overexpression of LIS1 displaces dynactin from the MT tips (Faulkner et al., 2000). It was shown that a phosphorylated form of LIS1 colocalizes with CLIP-170 very efficiently (Coquelle et al., 2002); it is possible, therefore, that in cells phosphorylated LIS1 can displace dynactin from MT plus ends.

The function of the competitive relationship between $p150^{Glued}$ and LIS1 is not yet clear. Although LIS1 was shown to interact with both dynein (through its heavy and intermediate chains) and dynactin (through dynamitin), most of the evidence suggests that LIS1 is primarily a partner of cytoplasmic dynein (Tai et al., 2002). We propose that a complex of dynein with LIS1 could bind to dynactin, which is targeted to the MT tip by CLIP-170. The dynein-associated LIS1 could then disrupt dynactin–CLIP-170 interaction, facilitating retrograde dynein transport (Fig. 8).

Alternatively, LIS1 could target dynein to MT tips through CLIP-170, but independently of dynactin. This sequence of interactions occurs in budding yeast, where Bik1p (CLIP-170 homologue) and Pac1p (LIS1 homologue) interact with each other and are both needed for efficient localization of dynein to the astral MTs (Lee et al., 2003; Sheeman et al., 2003). Removal of dynactin in this system enhances dynein localization to the astral MTs, because of a presumed decrease in dynein motor activity. However, it is possible that also in yeast, dynactin, and Pac1p compete with each other for binding to Bik1p, and in the absence of dynactin, the plus end localization of Pac1p and dynein is increased. It is likely that dynein interaction with the MT tips has essential functions, and it is not surprising that multiple, possibly redundant molecular links have evolved to ensure this interaction.

Function of dynactin accumulation at the MT tips

Our work has demonstrated that one of the functions of CLIP-170 is to concentrate dynactin at the tips of growing MTs. Several roles can be envisaged for this localization of dynactin. First, dynactin-bound MT ends can serve as sites for loading of cargos, such as cytoplasmic vesicles or endosomes (Vaughan et al., 1999). If this function were essential, knockdown of CLIP-170 would lead to mislocalization of membrane compartments. However, the steady-state organelle distribution appeared to be normal after CLIP-170 knockdown, as previously reported for other situations when $p150^{Glued}$ localization to plus ends was abolished (Goodson et al., 2003). It is still possible, however, that the kinetics of some types of dynein-mediated transport is affected in the absence of CLIP-170.

Second, dynein–dynactin complexes interact with the cell cortex (Dujardin and Vallee, 2002, Dujardin et al., 2003). By associating with cortical sites, dynein motors are believed to pull at the MTs to reorient the MT network (Burakov et al., 2003). This process is important for the positioning of the mitotic spindle and for directional cell migration. Therefore, it is possible that CLIP-170 and dynactin, targeted to the MT plus ends, contribute to cell motility or spindle positioning.

Finally, CLIP-170, LIS1 and dynein–dynactin localize to kinetochores in prometaphase. A possible role for CLIP-170 in mitotic progression is suggested by overexpression of the CLIP-170 COOH terminus, which displaces endogenous CLIP-170 from the kinetochore and causes a prometaphase delay (Dujardin et al., 1998).

Regulatory role of the CLIP-170 head-to-tail conformation

We have found that CLIP-170 can fold back upon itself through an interaction between its NH_2 and COOH termini. We propose that CLIP-170 alternates between an active, extended conformation, in which it can interact with MTs and its COOH-terminal partners, $p150^{Glued}$ and LIS1; and an inactive, folded conformation, in which it is not bound to other proteins (Fig. 8). The switching between the two conformations could be controlled by phosphorylation. This would explain the behavior of CLIP-170 in nocodazole-treated cells. MT depolymerization by nocodazole increases the rate of CLIP-170 dephosphorylation (Rickard and Kreis, 1991). Under these conditions CLIP-170 is probably in an "open" state, because it binds strongly to the few remaining nocodazole-resistant MTs and also to dynactin, accumulating in dynactin-positive aggregates.

The capacity of CLIP-170 to fold back is very reminiscent of conventional kinesin, the cargo-binding tail of which can associate with its MT-binding motor domain. This interaction prevents wasteful movement of motors not bound to cargo (for review see Verhey and Rapoport, 2001). Such autoinhibition appears to be a common theme in protein evolution with CLIP-170 providing an interesting example.

Materials and methods

Protein purification and in vitro binding assays

Protein fragments of CLIP-170, CLIP-115, and $p150^{Glued}$ were produced in *E. coli*. To generate HIS-tagged fusions, the NH_2-terminal fragments of CLIP-170 (nt 183–1113 of the rat brain cDNA available from GenBank/EMBL/DDBJ accession no. AJ237670) and $p150^{Glued}$ (nt 270–890 of the rat cDNA available from GenBank/EMBL/DDBJ accession no. X62160) were subcloned into pET-28a (Novagen), the NH_2-terminal fragment of CLIP-115 (nt 290–1336 of the rat cDNA available from GenBank/EMBL/DDBJ accession no. AJ000485) was subcloned into pQE-9 (QIAGEN). HIS-tagged proteins were produced in Rosetta (DE3) pLysS *E. coli* (pET-28a) or BL21 *E. coli* (pQE-9) and purified using Ni-NTA agarose (QIAGEN) in nondenaturing conditions.

GST fusions of the CLIP-170 NH_2 terminus (nt 183–1046 of the rat cDNA available from GenBank/EMBL/DDBJ accession no. AJ237670) and COOH terminus (nt 3555–4142 of the rat cDNA available from GenBank/EMBL/DDBJ accession no. AJ237670) were generated in pGEX-2T and pGEX-3X, respectively. K1, K2, and K1K2 mutants of CLIP-170 COOH terminus (Goodson et al., 2003) were used to generate fusion proteins in pGEX-3X (the cDNA fragments corresponded to the nt 3832–4413 of the cDNA available from GenBank/EMBL/DDBJ accession no. NM_002956). The GST fusion proteins were produced in BL21 *E. coli* and purified using glutathione-Sepharose 4B (Amersham Biosciences).

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HIS-tagged LIS1 and CLIP-170 were purified using the Bac-to-Bac HT Baculovirus Expression System (Invitrogen) as described in the Online supplemental material.

All in vitro binding assays were performed in 20 mM Tris-HCl, pH 7.5, 100 mM (low salt) or 400 mM (high salt) NaCl, 1 mM β-mercaptoethanol and 1% Triton X-100 as described by Hoogenraad et al. (2000), using ~10 μg of a GST fusion protein and 0.5–10 μg of the HIS-tagged proteins. MT pelleting assays were performed using the MT-associated protein spin-down assay kit (Cytoskeleton, Inc.).

Antibodies

We used mouse mAbs against c-myc and vinculin (Sigma-Aldrich), penta-histidine tag (QIAGEN), p150^{Glued}, dynamitin, and GM130 (BD Biosciences), dynein intermediate chain (CHEMICON International, Inc.), GFP (Roche), HA tag (Babco), transferrin receptor (Boehringer), a rat mAb against α-tubulin (Abcam), rabbit antibody 2360 against CLIP-170 (Coquelle et al., 2002), antibody 2221, which recognizes both CLIPs, antibody 2238 against CLIP-115 (Hoogenraad et al., 2000) and antibody 2293 against BICD2 (Hoogenraad et al., 2001). The following secondary antibodies were used: alkaline phosphatase-labeled anti–rabbit and anti–mouse antibodies (Sigma-Aldrich); FITC-labeled goat anti–rabbit antibody (Nordic Laboratories); Alexa 594– and Alexa 350–labeled anti–rat and anti–mouse antibodies (Molecular Probes); TRITC- and FITC-conjugated donkey anti–mouse, anti–rabbit, and Cy5-coupled anti–rat antibodies (Jackson ImmunoResearch Laboratories).

Expression constructs, transfection, IP, and Western blotting

Human myc-tagged CLIP-170 COOH-terminal constructs were described by Goodson et al. (2003); HA-CLIP-170 NH_2 terminus, GFP-CLIP-170 COOH terminus and GFP-p150^{Glued}-N (Komarova et al., 2002); GFP-CLIP-115(+tail) (Hoogenraad et al., 2002); and the GFP-CLIP-115 NH_2-terminal deletion mutants (Hoogenraad et al., 2000). CFP-YFP tandem contained YFP, fused in frame downstream of CFP with a 20–amino acid linker. YFP-CLIP-170-CFP was derived from the GFP-CLIP-170 fusion, based on the rat brain CLIP-170 cDNA. To make this construct, the GFP was substituted for YFP, and the monomeric (A206K) CFP, preceded by a 7–amino acid linker, was fused to the end of the CLIP-170 ORF using a PCR-based strategy. PCR was also used to generate the YFP-CLIP-170-CFP mutants and G59S mutant of p150^{Glued}. GFP-CLIPR-59-MTB construct was provided by F. Perez (Institut Curie, Paris, France).

For IP, COS-1 cells were transfected by the DEAE-dextran method, as described by Hoogenraad et al. (2000). CHO cells were transfected with Lipofectamine 2000 (Invitrogen). Cells were lysed 24 h after transfection, and IPs and Western blotting were performed as described by Komarova et al. (2002).

Immunostaining, image acquisition, and analysis

Cell fixation and staining were performed as described by Komarova et al. (2002). Samples were analyzed either with a microscope (model DMRBE; Leica) with a PL Fluotar 100×, 1.3 NA objective, equipped with a CCD camera (C4880; Hamamatsu); or with a inverted microscope (model 200 Eclipse; Nikon) equipped with a Plan Fluor 100×, 1.3 NA objective, and a CH250 cooled CCD camera (Photometrics Ltd.). Images were prepared for presentation using Adobe Photoshop. Line scan analysis, measurements of fluorescence intensity, and densitometry analysis of Western blots were performed using MetaMorph. To estimate the accumulation of proteins at the MT end, the tip of MT was defined as a square box, four pixels on a side (0.36 μm). Integrated fluorescence intensities within the box in each channel were measured after subtracting background. The ratio of the signals was computed using SigmaPlot.

RNAi

The following target sequences were used to design siRNAs: CLIP-170A, GGAGAAGCAGCAGCACATT; CLIP-170B, TGAAGATGTCAGGAGATAA; CLIP-115A, GGCACAGCATGAGCAGTAT; CLIP-115B, CTGGAAATCCAAGCTGGAC; and luciferase, CGTACGCGGAATACTTCGA. siRNA duplexes were synthesized by QIAGEN and siRNA transfection was performed using Oligofectamine (Invitrogen).

RNAi vectors for CHO cells were designed based on the same target sequences, using the pSuper vector (Brummelkamp et al., 2002). The control vector was generated by inserting the luciferase RNAi cassette into the AseI site of pEGFP-C1. Cells were transfected using FuGene 6 (Roche).

SFM imaging of the CLIP-170 protein

The topographic images of purified CLIP-170, deposited on freshly cleaved mica, were made with a Digital Instruments NanoScope IV operating in tapping mode in air using NanoProbe silicone tips (Veeco/Digital Instruments). Images were collected as 1-μm^2 fields (512 × 512 pixels) and processed only by flattening to remove background slope using NanoScope software. The length of individual CLIP-170 proteins was determined by manually tracing the longest path from end to end.

FRET measurements in cell extracts and in cells

COS-1 cells were lysed 48 h after transfection in a buffer, containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, protease inhibitors (Complete; Roche), 1% Triton X-100, and 10% glycerol; the lysates were precleared by centrifugation at 13,000 rpm for 20 min at 4°C. Emission spectra were measured using a fluorescence spectrophotometer (model F-4500; Hitachi) with the excitation at 425 nm (CFP) and 485 nm (YFP). Spectra were not corrected for PMT sensitivity. Background fluorescence of a crude extract prepared in the same way was negligible. The concentration of the CFP, YFP, or CFP-YFP fusion proteins in cell lysates was adjusted by Western blotting and by measuring the YFP fluorescence.

Emission spectra of live cells were recorded on a multimodal microscopy platform described elsewhere (Vermeer et al., 2004). Basically, a slit-spectrograph with a CCD camera was coupled to the exit port of an inverted wide-field fluorescence microscope. Resulting images yielded spectral information in the x axis and spatial information in the y axis, enabling the recording of spectra multiple objects within the field of view. Observations were done using 436 nm excitation, and a 460-nm long-pass emission filter. Due to the long-pass filtering and wavelength dependency of the detector sensitivity, the 460–500-nm spectral region is underestimated resulting in higher YFP/CFP detected ratios. During measurements, cells were kept at 37°C using a heated culture chamber and an objective heater.

Online supplemental material

Fig. S1 shows the colocalization of CLIP-170, but not CLIP-115 with dynactin in nocodazole-treated cells. Fig. S2 shows the distribution of endosomes and the Golgi after CLIP-170 knockdown. Online supplemental materials is available at http://www.jcb.org/cgi/content/full/jcb.200402082/DC1.

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