Molecular characterization of two functional domains of CLIP-170 in vivo

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SUMMARY

CLIP-170 is a microtubule-binding protein isolated from HeLa cells that is involved in the interaction of endosomes with microtubules. The basic N-terminal domain of CLIP-170 binds to microtubules in vitro. To characterize further the functional domains of this cytoplasmic linker protein, we have transiently expressed intact and mutant forms of CLIP-170 in mammalian cells (HeLa and Vero cells) and show that the tandem repeat present in the N-terminal domain is essential for its binding to microtubules in vivo as previously found in vitro. With increasing levels of expression of CLIP-170, the sites with which the peripheral ends of microtubules interact enlarge, eventually forming large patches, which finally lead to the apparent bundling of microtubules. These patches do not form when the C-terminal domain is absent from the transfected protein.

INTRODUCTION

The interphase microtubules play a major role in intracellular membrane traffic and the spatial arrangement of cytoplasmic organelles. These functions are mediated by different groups of microtubule-binding proteins. The members of the growing families of the microtubule-based molecular motors, cytoplasmic dynein and kinesin, are the engines that can move components along microtubules (Vallee, 1993; Vale, 1993). Additional proteins are required, however, to regulate the precise spatial and temporal interaction of the various cytoplasmic organelles with microtubules. The dynactin complex, for example, is essential for the activity of cytoplasmic dynein (Schroer and Sheetz, 1991; Gill et al., 1991). Major components of this complex have been identified, including p150Glued (Holzbaur et al., 1991), dynactin (Gill et al., 1991) and proteins related to actin (Lees-Miller et al., 1992, Clark and Meyer, 1992). Furthermore, using in vitro organelle/microtubule-binding assays, a novel class of cytoplasmic linker proteins (CLIPs) has been identified, that mediates the interaction of cytoplasmic organelles with microtubules (van der Sluijs et al., 1990; Scheel and Kreis, 1991; Karecla and Kreis, 1992; Pierre et al., 1992). Different CLIPs may mediate an initial interaction of specific organelles with microtubules, establishing a linkage prior to their physical translocation along the microtubule track mediated by the motors (Scheel et al., 1993).

CLIP-170 has been shown to mediate the interaction of endocytic carrier vesicles with microtubules (Pierre et al., 1992). It was originally identified as a microtubule-binding protein in HeLa cells (Rickard and Kreis, 1990), and biochemical analysis has shown that microtubule binding is regulated in vitro by phosphorylation (Rickard and Kreis, 1991). The molecular characterization of CLIP-170 revealed a tripartite structure of the elongated homodimer, consisting of a basic N-terminal domain (350 amino acids), separated by a long coiled-coil rod (900 amino acids) with numerous heptad repeats from its rather acidic C terminal (100 amino acids), which possesses a potential metal-binding motif (Pierre et al., 1992). The N-terminal region of CLIP-170 contains two homologous motifs of 57 amino acids, one of which is also found with ~60% homology in other proteins (Pierre et al., 1992) that are implicated in microtubule function; in yeast, BIK1p (Berlin et al., 1990); in Drosophila, Glued protein (Swaroop et al., 1987); and in rat, p150 (Holzbaur et al., 1991). Analysis of co-sedimentation of in vitro translated intact and mutant CLIP-170 with microtubules indicated that this tandem repeat is essential for microtubule binding of the protein. Interestingly, all four proteins share similar overall structure (Pierre et al., 1992) and the putative metal-binding motif of CLIP-170 is also present in the BIK1 protein (Berlin et al., 1990).

To characterize the role of the different domains of CLIP-170 in vivo, we have transiently (over-)expressed intact or mutant forms of epitope-tagged CLIP-170 in mammalian cells (HeLa and Vero cells) and analysed their subcellular distribution and effects on microtubules and intermediate filaments. Here we show that the conserved repeated motif present in the N-terminal domain of CLIP-170 regulates its binding to micro-
tubules in vivo. Furthermore, analysis of the C-terminal domain of CLIP-170 revealed that it may be involved in anchoring of the protein to patches at the plasma membrane.

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies against CLIP-170 were obtained by immunization of rabbits with the bacterially expressed fusion protein of clone 55 (Pierre et al., 1992) and subsequent affinity purification with the recombinant protein transferred to nitrocellulose filters (Rickard and Kreis, 1990). The murine monoclonal antibody against the ‘Myc epitope’, 9E10 (Evan et al., 1985), was purified from ascites using a peptide containing the epitope coupled to CNBr-activated Sepharose (Pharmacia-LKB). The preparation and purification of the rabbit polyclonal antibodies against the cytoplasmic domain of vesicular stomatitis viral glycoprotein (VSV-G, anti-P4) and tyrosinated tubulin (anti-T13), and the murine monoclonal antibodies against tyrosinated tubulin (1A2) and vimentin (7A3) have been described elsewhere (Kreis, 1986, 1987; Matteoni and Kreis, 1987). The V9 monoclonal antibody against vimentin was a gift from Dr Mary Osborn (Osborn et al., 1984).

Epitope tagging of cDNA clones

Isolation of the CLIP-170 cDNA and associated cloning was done as described (Pierre et al., 1992). The plasmid pGEM-Myc (gift from Drs Vesa Olkkonen and Kai Simons, EMBL, Heidelberg, Germany) containing a sequence encoding the peptide MEQKLISEEDLGG (anti-T13), and the murine monoclonal antibodies against the cytoplasmic domain of vesicular stomatitis viral glycoprotein (VSV-G, anti-P4) and tyrosinated tubulin (1A2) and vimentin (7A3) have been described elsewhere (Kreis, 1986, 1987; Matteoni and Kreis, 1987). The V9 monoclonal antibody against vimentin was a gift from Dr Mary Osborn (Osborn et al., 1984).

Generation of mutant cDNA clones and eukaryotic expression

Construction of the mutants R1*, R2*, R1*/R2*, CΔH, H has been described elsewhere (Pierre et al., 1992). Briefly, the conserved sequence PXGKNDG of CLIP-170 was mutated to AXAENDA in each of the repeated motifs (R1*, R2* or R1*/R2*) by a PCR-based approach (Horton et al., 1989). Deletion of the C-terminal 30 kDa of CLIP-170 (CA1240) was performed by simple restriction digest and re-ligation at the XhoI site of the cDNA. All the constructions were sequenced and then tested using in vitro transcription and translation followed by immunoprecipitation.

RESULTS

Transiently expressed CLIP-170 binds to microtubules in vivo

To analyse the functional domains of CLIP-170 in vivo we transiently (over-) expressed intact or mutant protein in HeLa and Vero cells using a eukaryotic expression vector (pcDNA-1) placed under the control of the cytomegalovirus (CMV) early promotor (Ruben et al., 1989). CLIP-170 and its mutants were tagged with either the Myc epitope (EQQKLISEEDL; M-CLIP-170) derived from the human c-Myc sequence (Evan et al., 1985) at the N terminus, or the VSV-G(ctl) epitope (TDIEMRNL; G-CLIP-170) derived from the cytoplasmic C terminus of the glycoprotein of vesicular stomatitis virus (Gallione and Rose, 1985) at the C terminus. These epitope tags enabled us to visualize the expressed proteins by immunofluorescence separately from the endogenous protein using the corresponding epitope-specific affinity-purified antibodies.

Micronjection

Glass capillary microinjection of the 7A3 antibody into cells was performed with an automated microinjection system (Zeiss AIS; Ansorge and Pepperkok, 1988). All microinjection experiments were performed in 3 cm Petri dishes containing 4 ml of Hanks’ medium as described elsewhere (Pepperkok et al., 1993).

Other methods

Restriction enzymes and other molecular biology reagents were purchased from Boehringer Mannheim unless stated otherwise. All radioactive chemicals were obtained from Amersham International, Zürich, Switzerland. Gel electrophoresis of proteins and immunoblotting were performed as described (Rickard and Kreis, 1990). All molecular biology manipulations were performed as described (Sambrook et al., 1989).
9E10 (Evan et al., 1985) and anti-P4 (Kreis, 1986). No significant difference in the distribution of exogenous polypeptides was observed between cells transfected with the native (no tag) or the tagged forms of CLIP-170 (data not shown). Furthermore, no significant difference was observed when the tag was coupled to the C or N terminus of the mutants (M-/G-CΔ1240, data not shown).

In HeLa cells the distribution of CLIP-170 changes with increasing levels of expression (Fig. 1A). At early time-points of expression (up to ~16 hours after transfection) the pattern of M-CLIP-170 is virtually identical to the normal distribution of the endogenous protein (Fig. 1A; see also Rickard and Kreis, 1990). M-CLIP-170 appears in numerous small patches that are associated with peripheral microtubule ends. Occasionally, larger patches of expressed protein can be seen (arrows in Fig. 1A); these are always at the cell periphery and appear to be associated with numerous microtubule ends. With increasing levels of expression of the protein (at ~24 hours after transfection), CLIP-170 is aligned along segments of microtubules (arrowheads in Fig. 1C) and associated with large patches that overlap with apparently bundled microtubules (arrows in Fig. 1C). At late time points of transfection (~36 hours), the microtubule network is dramatically reorganized and intensely labelled for M-CLIP-170 (Fig. 1E,F). Thick ‘bundles’ of rings of microtubules containing the expressed protein can be detected in these cells. These rings of microtubules appear similar to those observed in cells transfected with neuronal microtubule-associated proteins (MAPs) (MAP-2 and tau); Lewis et al., 1989; Lee and Rook, 1992).

The distribution and effects on microtubules of expressed M-CLIP-170 are similar in Vero and HeLa cells. However, the formation of the larger peripheral patches and the aberrant rings of bundled microtubules appear much quicker in Vero than in HeLa cells, and the association of expressed protein with the ‘early, small’ patches is more difficult to observe (data not shown). Taken together, these experiments show that transfected CLIP-170 initially behaves like the endogenous protein; it associates in distinct patches with the peripheral plus-ends of microtubules. With increasing levels of expressed protein, these patches appear to grow from the cell periphery towards the cell centre and induce aggregation or bundling of the microtubules.

The N-terminal domain mediates binding of CLIP-170 to microtubules in vivo
A tripartite structure was predicted from the amino acid sequence of CLIP-170, and we have shown that the basic N-terminal domain of 350 amino acids containing a conserved tandem repeat of 57 amino acids shares homology with a sequence present both in p150Glued and BIK1p, is essential for co-sedimentation of the protein with polymerized tubulin in vitro (Pierre et al., 1992). We have further studied the role of this tandem repeat by expressing mutant polypeptides containing specific point mutations in this region of the protein, which we have previously shown affect their interaction with microtubules in vitro. Results obtained with Vero cells are shown in Fig. 4; essentially the same effects were found in HeLa cells (not shown). For this analysis, we used the mutant form of CLIP-170 that lacks the C-terminal domain (CA1240) to avoid the formation of patches that complicate the analysis of effects solely due to microtubule binding. A dramatic reorganization of the microtubule network is observed in 80% (24 hours) and 100% (36 hours) of the cells transfected with G-CA1240, leading to the formation of prominent microtubule ‘bundles’ (Fig. 4A,B). The same results were obtained with the mutant M-CA1240 (data not shown). Interestingly, these aggregated microtubules appear to be still nucleated by the microtubule organizing center (MTOC, see arrow in Fig. 4A,B). Both polypeptides with point mutations in the first or second repeat (G-R1*CA1240 and G-R2*CA1240) bind to microtubules in vivo (Fig. 4C,E); they do not significantly perturb the arrangement of the microtubule network and no microtubule bundles can be detected. Similarly, a mutant of CLIP-170 lacking the entire first repeat (CAR1) still binds to microtubules when transiently expressed in cells (data not shown). These data indicate that in the dimer one of the two repeats is sufficient for microtubule binding of CLIP-170 in vivo. Point mutations in both repeats lead to a significantly decreased labelling of microtubules (Fig. 4G). These observations in vivo are all consistent with the data obtained for binding of CLIP-170 to microtubules in vitro (Pierre et al., 1992).

The C-terminal domain targets CLIP-170 to peripheral patches
CLIP-170 is normally associated with numerous patches
Fig. 1. Transient expression of CLIP-170 in HeLa cells. CLIP-170 tagged with the Myc epitope was transfected into HeLa cells. Cells were fixed with paraformaldehyde 16 (A,B), 24 (C,D) or 36 (E,F) hours after transfection and stained with murine mAb 9E10 against the Myc epitope (A,C,E) and rabbit anti-T13 against tubulin (B,D,F). Expressed CLIP-170 is in numerous patches at early time-points of transfection (arrows in A), which appear to co-localize with peripheral ends of microtubules. These patches increase in size at later time points (arrows in C) and CLIP-170 is now also associated in tubular segments, along microtubules (arrowheads in C). The expressed protein induces reorganization of microtubules into bundles at later times after transfection (E,F). Bar, 10 μm.
underlying the plasma membrane colocalized with the peripheral plus-ends of microtubules. After treatment of cells with nocodazole and complete microtubule depolymerization, this patchy pattern remains, although it appears now more random, suggesting that CLIP-170 is able to associate with other intracellular structures as well as microtubules (Rickard and Kreis, 1990). Interestingly, removal of the C-terminal 152 amino acids of CLIP-170 completely abolished its association with such patches (Fig. 3C,D), implicating this C-terminal domain of the protein with its targeting to and the anchoring at the cell periphery. To further analyze the role of this domain in targeting of CLIP-170 to subcellular structures, cells transfected with the intact protein (M-CLIP-170) or with the form lacking the C terminus (M-CA1240) were treated with nocodazole to depolymerize the microtubule network (Fig. 5). Intact CLIP-170 appears in large, spherical aggregates containing tubulin (Fig. 5A,B). Such aggregates are not observed with CA1240 (Fig. 5C), confirming a specific role for this domain of CLIP-170. The precise nature of these clusters remains to be determined. It is possible that they contain membranes, since we have previously shown partial co-localization of CLIP-170 with transferrin receptor (Pierre et al., 1992).

**CLIP-170 over-expression stabilizes microtubules in vivo**

The effect of CLIP-170 on the dynamic organization of microtubules was further investigated by over-expression of normal and mutated CLIP-170 in cells treated with nocodazole. Treatment of Vero cells for one hour with 3.3 µM nocodazole depolymerizes the interphase microtubule network. Endogenous CLIP-170 is present in randomly scattered patches and on the few fragments of remaining microtubules (Fig. 5E). In cells expressing either the G-CA1240 (Fig. 5F), the G-R1*CA1240 (not shown), or the G-R2*CA1240 (not shown) mutant, consistently more microtubules resist the action of the microtubule depolymerizing drug. However, most of the microtubules are depolymerized by the drug, and increased incubation time or higher concentration of nocodazole leads to complete depolymerization of the microtubule network. In contrast, in cells over-expressing MAP2 (Lewis et al., 1989) or E-MAP115 (Masson and Kreis, 1993), the bulk of the stabilized microtubules remains unaffected by nocodazole.

We have also examined the potential effects of over-expressed CLIP-170 on microtubule repolymerization after removal of nocodazole (Fig. 6). In control cells, removal of nocodazole leads to the rapid repolymerization of microtubules off the MTOC, and endogenous CLIP-170 labels the initial short microtubule segments (Fig. 6A,B). In HeLa and Vero cells (data not shown) transfected with intact M-CLIP-170, numerous sites nucleating microtubule repolymerization can be detected early (5 minutes) after nocodazole removal (Fig. 6C); most likely, these sites are the aggregates that form when cells are treated with the drug (see Fig. 5A). In Vero cells transfected with M-CA1240, 5 minutes after nocodazole removal the protein is observed on short microtubules, many of which are not MTOC-nucleated (Fig. 6D). At later time points (30 minutes) longer bundles of microtubules are present in these cells throughout the cytoplasm, and the cells can easily be distinguished from the non-transfected cells, which have most of their microtubules nucleated by the MTOC (Fig. 6E,F). Microtubule repolymerization appears to be normal in cells transfected with CLIP-170 mutated in the second repeat (Fig. 6G,H). These data suggest that CLIP-170 may promote random microtubule aster formation, perhaps by stabilization of microtubule plus ends.

**CLIP-170 is not associated with vimentin filaments**

It has been proposed that ‘restin’, a protein identical in
sequence to CLIP-170 except for a 35 amino acid long insert in the rod domain (at amino acids 457), is an intermediate filament-associated protein (Bilbe et al., 1992). We have further analysed the localization of transiently expressed CLIP-170 in HeLa and Vero cells and find that its distribution is independent of the network of intermediate filaments. Overexpression of the wild-type protein has no significant effect on the organization and distribution of vimentin filaments in HeLa (not shown) and Vero cells (Fig. 7A,B). No vimentin labelling is observed in the nocodazole-induced aggregates (data not shown).

Fig. 3. Transient expression of CLIP-170 mutants in HeLa cells. CLIP-170 deletion mutants tagged with the Myc or VSV-G(ct) epitope were transfected into HeLa cells. Cells were fixed 24 hours after transfection with paraformaldehyde (A,B,G,H) or methanol (C-F) and double-stained for transfected protein with 9E10 (A,G) or anti-P4 (C,E) and for tubulin with anti-T13 (B,H) or 1A2 (D,F). Cells were transfected with M-CLIP-170 (A,B); G-CAΔ1240 (C,D) lacking the C-terminal 20 kDa of the protein; G-H CLIP-170 (E,F), the head domain of CLIP-170; or M-CAH (G,H), with the head domain deleted. Clearly, the N-terminal head domain of CLIP-170 is essential for the microtubule binding of the protein in vivo (e.g. arrow in E,F), since the mutant lacking this domain does not associate with microtubules. The patches (arrows in A) observed with wild-type CLIP-170 (A) are absent in the cells transfected with C-terminal deletion mutants (C,E). Bar, 10 µm.
showed). Furthermore, microinjection of the monoclonal antibody 7A3, which leads to the complete collapse of the intermediate filament network within 5-6 hours of microinjection into cells (Matteoni and Kreis, 1987), does not lead to an obvious redistribution of endogenous CLIP-170 (Fig. 7E,F). These data clearly demonstrate that CLIP-170 is not a vimentin intermediate filament-associated protein.

**DISCUSSION**

The CLIPs constitute a new family of cytoplasmic linker proteins mediating interaction of membrane-bounded organelles with microtubules; CLIP-170 is the member of this family that links endosomes to microtubules (Pierre et al., 1992). Three distinct domains for CLIP-170 have been

**Fig. 4.** Transient expression of CLIP-170 mutants in Vero cells. CLIP-170 mutants were transfected into Vero cells fixed in methanol after 24 (A,B) or 36 (C-H) hours and immunolabelled as described for Fig. 3 with anti-P4 (A,C,E,G) and 1A2 (B,D,F,H). G-CΔ1240 is shown in (A), G-R1*CΔ1240 in (C), G-R2*CΔ1240 in (E) and G-R1*/R2*CΔ1240 in (G). CLIP-170 is associated with microtubules in cells transfected with the constructs having only one of the two repeat units mutated (compare C-F with A,B). The protein with both motifs mutated G-R1*/R2*CΔ1240 binds only very weakly to microtubules (G,H). Bar, 10 µm.
predicted from its amino acid sequence, two presumably globular domains at the N and C termini of the protein, separated by a long $\alpha$-helical rod (Pierre et al., 1992). In this study we have extended the characterization of the role of the two terminal domains of CLIP-170 and analysed their behaviour in primate cells in vivo using transient expression of epitope-tagged intact and mutant forms of the protein. From these data we conclude that the acidic C-terminal domain of the protein is required for the targeting of CLIP-170 to cytoplasmic structures that interact with the peripheral microtubule plus-ends, and that the tandem repeat in the basic N terminus is essential for binding of the protein to microtubules. At higher levels of expression, CLIP-170 induces dramatic reorganization of the microtubule network. Furthermore, we demonstrate here that CLIP-170 is clearly not an intermediate filament-associated protein as has been proposed recently for restin, a protein that is identical to CLIP-170 except for a 35 amino acid insert in the rod domain (Bilbe et al., 1992).

The affinity of CLIP-170 for microtubules is regulated in vitro (Pierre et al., 1992) and in vivo (this study) by a tandem repeat of 57 amino acids located in the N-terminal basic domain of the protein. The presence of only one of these repeat units is sufficient for binding of CLIP-170 to microtubules. Interestingly, one copy of this repeat is also present in yeast BIK1p, Drosophila Glued protein and rat p150. None of these proteins (including also restin) has so far been shown to interact directly with microtubules, although it is tempting to speculate that the presence of one of these microtubule binding units should enable them to bind to microtubules. It is remarkable in this context that mutation of only one of the repeats reduces the affinity for microtubules of CLIP-170 in vivo, as does removal of the rod domain that is presumably essential for interaction with microtubules.

Fig. 5. Distribution of expressed CLIP-170 in cells treated with nocodazole. HeLa (A-D) or Vero (E,F) cells were transfected with M-CLIP-170 (A,B), M-CA1240 (C,D) or G-CA1240 (F) and treated for one hour with 10 $\mu$M (A-D) or 3.3 $\mu$M (E,F) nocodazole, 23 hours after transfection. Cells were then fixed with paraformaldehyde (A-D) or methanol (E,F), CLIP-170 was labelled with anti-55 (A,C,E) or anti-P4 (F) and tubulin with 1A2 (B,D). In the cells transfected with intact CLIP-170, big patches positive for both CLIP-170 and tubulin are observed (arrowheads in A,B); these patches are absent in the cells expressing the mutant CA1240 lacking the C-terminal domain of the protein (C,D). Although most microtubules are depolymerized in the nontransfected control cells (E), more microtubules resist the drug treatment in cells expressing the CLIP-170 mutant (F; see arrows in C,E,F). Bar, 10 $\mu$m.
for coiled coil formation and dimerization of the protein. No significant effect on the microtubule affinity of these mutants could be detected in the in vitro co-sedimentation assays (Pierre et al., 1992), suggesting that, in contrast to living cells, the large excess of tubulin polymer over the in vitro translated protein masked the weaker microtubule-binding of these mutant forms of the protein. Thus, four intact repeat units may be required for the efficient binding of CLIP-170 to microtubules in vivo, and since BIK1p, Glued and p150 have only two (in the predicted dimeric forms of these proteins), their binding to microtubules may appear significantly weaker.

Over-expression of CLIP-170 in cells leads to a significant reorganization and apparent stabilization of microtubules. Taking into account the predominant localization of endogenous CLIP-170 at peripheral microtubule plus-ends, we consider it most likely that it may lead to stabilization of these microtubules...

Fig. 6. Microtubule repolymerization after nocodazole wash-out in cells transfected with CLIP-170. Cells were treated for one hour with 10 µM nocodazole 23 hours after transfection with M-CLIP-170 (C), M-CA1240 (D-F) or G-R2*CA1240 (G,H); (A,B) show control cells. After removal of the drug HeLa (C) and Vero (A,B,D-H) cells were incubated in normal culture medium for 5 (A-D) or 30 (E,H) minutes, fixed with methanol and labelled with anti-55 (A), 9E10 (C-E), anti-P4 (G), 1A2 (B,H) or anti-T13 (F). Numerous asters can be seen in the cells expressing the full-length CLIP-170 (C); many non-MTOC nucleated microtubules are also seen in the cells transfected with CLIP-170 lacking the C terminus (D-F). Bar, 10 µm.
microtubule ends when present in excess. Interestingly, at late time points of transfection, microtubules (still MTOR-nucleated to a large extent) appear in bundles, often forming circumferential rings in the cells, reminiscent of the marginal bands in erythrocytes. We assume that, as a consequence of this plus-end stabilization, microtubules grow extensively and coil in a bundle-like pattern around the perimeter of the cells (see also Lee and Brandt, 1992). We propose that these microtubules appear more resistant to the activity of nocodazole, simply because it takes more time for a longer microtubule to depolymerize than it would take for microtubules with normal length. Obviously, alternative explanations for these observations are possible, and in particular we cannot exclude the possibility that the CLIP-170 dimer with four potential microtubule-binding sites could crosslink microtubules in vivo and lead to the formation of genuine microtubule bundles. Interestingly, mutation or removal of one of the repeat units, or removal of the rod and putative dimerization domain, completely abolishes the formation of microtubule bundles. It should be emphasized, however, that microtubule-associated proteins like MAP2, tau and E-MAP-115, stabilize microtubules more permanently (Lewis et al., 1989; Lee and Rook, 1992; Masson and Kreis, 1993), perhaps by laterally cross-linking two or more tubulin subunits, in contrast to the microtubule-binding proteins CLIP-170 or kinesin (Navone et al., 1992).

Although initially associated with patchy structures that coincide with the peripheral ends of microtubules, overexpressed CLIP-170 binds along the entire length of the microtubules and leads to the formation of extensive rings of microtubules at the perimeter of transfected cells. The distribution of CLIP-170 thus changes from one corresponding to a protein probably preferentially associated with subcellular structures (e.g. endosomes) that are linked for limited periods of time via CLIP-170 to microtubule plus ends, to one typical for MAPs that bind all along the length of microtubules. We therefore assume that the interaction of CLIP-170 with microtubules is under tight control. In fact, we have previously shown that

![Image](https://example.com/fig7.png)

**Fig. 7.** CLIP-170 is not associated with vimentin filaments. Vero cells were transfected with M-CLIP-170 for 24 hours (A,B), fixed with paraformaldehyde and double stained with anti-55 (A) and mAb V9 (B). Nontransfected Vero cells were also microinjected with mAb 7A3 (C,D), fixed 5 hours after injection, and endogenous CLIP-170 was visualized with anti-55 (C) together with injected 7A3 (D). No co-localization of overexpressed CLIP-170 with vimentin, or of endogenous CLIP-170 with the aggregated vimentin filaments, could be detected. Over-expressed CLIP-170 did not significantly affect the organization of the vimentin intermediate filaments, nor did the complete collapse of intermediate filaments affect the distribution of endogenous CLIP-170. Bar, 20 μm.
binding of CLIP-170 to microtubules in vitro is regulated by phosphorylation (Rickard and Kreis, 1991). Over-expression of CLIP-170 may disrupt this control; for example, by saturating the CLIP-170 kinase system, leading to abundant unphosphorylated protein, which then binds preferentially to the microtubules. Thus, the level of phosphorylation of CLIP-170 not only regulates whether or not the protein binds to microtubules (and whether these microtubules will grow), but may also regulate whether it binds to a microtubule end or along the length of the tubulin polymer. Interestingly, the mutant form of CLIP-170 lacking the C terminus (CA1240) also appears to associate with the peripheral microtubule ends at low expression levels. Clearly, more work is necessary to study these hypotheses further.

CLIP-170 expressed in Vero and HeLa cells is initially located in numerous patches co-localizing with peripheral microtubule ends. The precise nature and composition of the patches that CLIP-170 is normally associated with is still unclear. Under certain conditions (e.g. treatment of cells with brefeldin A) a significant co-localization of CLIP-170 with transferrin receptor can be shown (Pierre et al., 1992). Furthermore, CLIP-170 has been shown to mediate interaction of endosomes with microtubules in a functional in vitro assay (Scheel and Kreis, 1991; Pierre et al., 1992). Thus, these patches most likely represent membrane intermediates between early and late endosomal structures. It is also very likely that the interaction of CLIP-170 both with the endosomal membranes and with the microtubules is dynamic, and therefore rather short-lived and regulated. We have speculated that binding of CLIP-170 to a membrane receptor and thus targeting of CLIP-170 to these patches may lie in the C-terminal domain of the protein containing a consensus metal-binding motif. We demonstrate here that CLIP-170 is no longer associated with these patchy structures in transfected cells when the C terminus of the protein has been removed. We conclude therefore that the role of targeting of CLIP-170 to these subcellular structures resides in the last 20 kDa of the protein. Treatment of cells over-expressing CLIP-170, but not the mutant truncated at the C terminus (CA1240), with nocodazole, leads to the formation of large spherical aggregates. Unfortunately, we have not yet been able to characterize the precise nature of these aggregates (they are negative for known endocytic markers), but they might represent clusters of the putative endosomal membranes binding CLIP-170. Alternatively, these aggregates could be CLIP-170 precipitated due to electrostatic interactions of the basic N-termini (no longer microtubule-bound or hyperphosphorylated) with the acidic C-terminal domains of the protein.

Using transfection of primate cells with intact or mutant protein we have characterized two functional domains of CLIP-170 in vivo, the N-terminal microtubule-binding domain containing a conserved repeat motif and the C terminus targeting CLIP-170 to patchy subcellular structures, presumably endosomes associated with peripheral microtubule ends. These two domains are spaced by a long coiled-coil rod segment. The corroboration of these roles for the two widely spaced terminal domains thus supports the hypothesis that CLIP-170 is a linker protein mediating the interaction of cytoplasmic organelles with microtubules. Interestingly, the interaction of the N- and C-terminal domains with microtubules and peripheral patches is regulated. Further work will be necessary to characterize the interactive proteins on the endosomal membranes and the regulatory machinery that modulates the microtubule-binding activity of CLIP-170.

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