Structural basis for tubulin recognition by cytoplasmic linker protein 170 and its autoinhibition

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Cytoplasmic linker protein 170 (CLIP-170) is a prototype of the plus end-tracking proteins that regulate microtubule dynamics, but it is obscure how CLIP-170 recognizes the microtubule plus end and contributes to polymerization rescue. Crystallographic, NMR, and mutation studies of two tandem cytoskeleton-associated protein glycine-rich (CAP-Gly) domains of CLIP-170, CAP-Gly-1 and CAP-Gly-2, revealed positively charged basic grooves of both CAP-Gly domains for tubulin binding, whereas the CAP-Gly-2 domain possesses a more basic groove and directly binds the EExEEY/F motif of the C-terminal acidic-tail ends of α-tubulin. Notably, the p150^{Glued} CAP-Gly domain that is furnished with a less positively charged surface only weakly interacts with the α-tubulin acidic tail. Mutation studies showed that this acidic sextette motif is the minimum region for CAP-Gly binding. The C-terminal zinc knuckle domains of CLIP-170 bind the basic groove to inhibit the binding to the acidic tails. These results provide a structural basis for the proposed CLIP-170 copolymerization with tubulin on the microtubule plus end. CLIP-170 strongly binds the acidic tails of EB1 as well as those of α-tubulins, indicating that EB1 localized at the plus end contributes to CLIP-170 recruitment to the plus end. We suggest that CLIP-170 stimulates microtubule polymerization and/or nucleation by neutralizing the negative charges of tubulins with the highly positive charges of the CLIP-170 CAP-Gly domains. Once CLIP-170 binds microtubule, the released zinc knuckle domain may serve to recruit dynein to the plus end by interacting with p150^{Glued} and LIS1. Thus, our structures provide the structural basis for the specific dynein loading on the microtubule plus end.

plus end-tracking protein | cytoskeleton-associated protein glycine-rich | cytoskeleton | EB1 | microtubule

The microtubule (MT) network is one of the major cytoskeletal systems of all eukaryotic cells of plants, animals, and fungi. Dynamic MTs are hollow tubes made of α,β-tubulin dimers that can disassemble and reassemble at two ends, referred to as the slow-growing (minus) and fast-growing (plus) ends. The plus end explores the cell periphery and shows dynamic instability, switching rapidly between the two phases of growth and shrinkage. More than two decades have passed since microtubule dynamics, which is extremely important in all fields in cell biology, was first discussed. It is now clear that the plus end-tracking proteins (+TIPs) control microtubule dynamics (1–3). However, it is still unknown how these +TIPs are accumulated at the MT plus end and contribute to polymerization rescue.

Cytoplasmic linker protein 170 (CLIP-170) was initially identified in HeLa cells as the prototype of +TIPs localized preferentially to elongating MT plus ends, and is thought to mediate interactions of endocytotic organelles and chromosomes with MTs (4–7). CLIP-170 is composed of two functional regions at N and C termini, separated by a 950-residue-long coiled-coil region (Fig. 1*a*). The N-terminal region has two conserved cytoskeleton-associated protein glycine-rich (CAP-Gly) domains that mediate targeting of CLIP-170 to MT. The CAP-Gly domains are highly conserved in vertebrate CLIP-170 homologues, suggesting their essential role in MT targeting. The C-terminal region contains two predicted metal-binding motifs designated as zinc knuckles, which interfere with MT binding by interacting with the N-terminal region (8). The dynactin subunit p150^{Glued}, LIS1, and CLIP-associating proteins (CLASPs) are known to interact with CLIP-170 (9–12). Among them, p150^{Glued} and LIS1 compete with the CLIP-170 CAP-Gly domains for binding to the CLIP-170 C-terminal zinc knuckle domains (8). A recent study revealed the direct binding of CLIP-170 to IQGAP1, an actin binding protein that binds Rac1 and Cdc42, which resulted in the formation of the ternary complex CLIP-170–IQGAP1–Rac1/Cdc42 (13, 14). Thus, CLIP-170 may function as a linker between MT plus ends and the cortical actin meshwork, which would lead to cell polarization.

Despite structural studies of the CAP-Gly domains (15, 16), structural basis of MT or tubulin recognition by CLIP-170 remains elusive. Moreover, two different controversial modes of interaction between the p150^{Glued} CAP-Gly domain and the EB1 C-terminal fragment have been proposed: Hayashi *et al.* (17) put forward the observed contacts with the EB1 residues 249–255 as the biologically relevant contacts, whereas Honnappa *et al.* (18) claimed contacts with the EB1 tail end residues 266–268 as the essential interactions. In this study, we report on the crystal structures of the CAP-Gly domains of human CLIP-170 and the solution structure of the second CAP-Gly domain in complex with the C-terminal acidic peptide of human α-tubulin. We revealed the highly positively charged groove of the CAP-Gly domain binds the acidic tail end. This basic groove is inconspicuous in the other CAP-Gly domains such as p150^{Glued}. The minimum region of the α-tubulin acidic tail for CLIP-170 binding is the EExEEY/F motif, in contrast to the EEY/F motif suggested based on the p150^{Glued}/EB1 complex (18). We also revealed that both the inhibitory zinc knuckle domain and α-tubulin share the same binding surface on the CAP-Gly domain, presenting structural evidence for the CLIP-170 autoinhibition. Our results provide insights into the CAP-Gly-MT/tubulin interactions and regulation.

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The authors declare no conflict of interest.

Abbreviations: MT, microtubule; CLIP-170, cytoplasmic linker protein 170; CAP-Gly, cytoskeleton-associated protein glycine-rich; SPR, surface plasmon resonance.

Data deposition: The atomic coordinates for the crystal structures of the CAP-Gly1 domain and the CAP-Gly2 domain and solution structure of the CAP-Gly2 domain in complex with α-tubulin peptide have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2E3I, 2E3H, and 2E4H, respectively).

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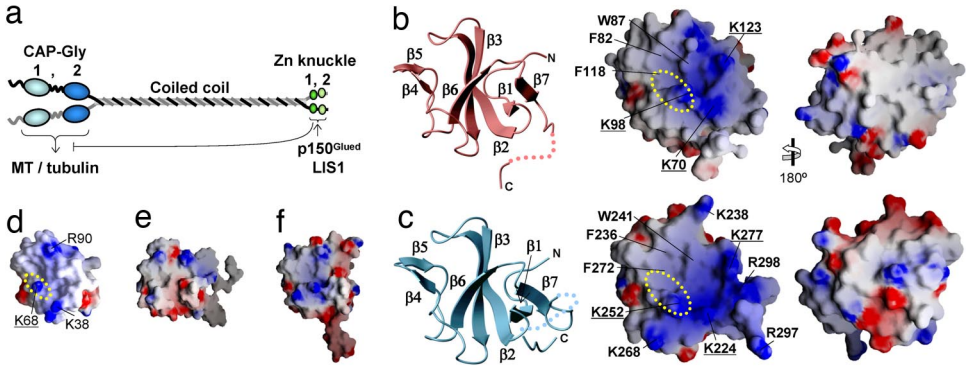

Fig. 1. Crystal structures of the CAP-Gly-1 and CAP-Gly-2 domains. (*a*) Domain organization of the CLIP-170 dimer. The N-terminal CAP-Gly domains directly bind MT/tubulin. This binding is autoinhibited by the C-terminal zinc knuckle domains, which also serve to recruit dynein by interacting with p150^{Glued} and LIS1. (*b*) Ribbon drawings of the crystal structure of the CLIP-170 CAP-Gly-1 domain (*Left*), electrostatic potentials on the front molecular surface in the same molecular orientation (*Center*), and a 180° rotated image (*Right*). The region corresponding to the GKNDG motif is circled with yellow broken lines. (*c*) Same figures as in *b* but for the CLIP-170 CAP-Gly-2 domain. Electrostatic potentials of the front surfaces of the CAP-Gly domains of p150^{Glued} (PDB ID, 2HL3) (*d*), CYLD (1IXD) (*e*), and F53.43 (1LPL) (*f*).

Results

The CAP-Gly Domains Possess Basic Grooves. The N-terminal 300-aa residues of CLIP-170 form the MT-binding domain (CAP-Gly-12) that consists of tandem repeated CAP-Gly-1 (residues 56–141) and CAP-Gly-2 (203–300) domains connected by a long 60-residue linker (5). To establish the structural details of the domains, we determined the crystal structures of the CAP-Gly-1 and CAP-Gly-2 domains (Fig. 1 *b* and *c*). The CAP-Gly-2 structure was refined to a resolution of 1.45 Å [supporting information (SI) Table 4]. The structure of the CAP-Gly-1 domain was determined by molecular replacement using the coordinates of the CAP-Gly-2 domain, and was refined to a resolution of 2.0 Å. The CAP-Gly-1 and CAP-Gly-2 domains display similar folds consisting of two layers of antiparallel β-sheets (Fig. 1 *b* and *c Left*), as with the previously reported *Caenorhabditis elegans* CAP-Gly domain (15). The mainframe of the fold is a larger twisted β-sheet composed of β1–β3, β6, and β7 with an additional smaller β-sheet (β4–β5). The larger β-sheet forms aromatic and aliphatic hydrophobic cores on both sides of the sheet (SI Fig. 5).

We found positively charged shallow grooves on the molecular surfaces of both the CAP-Gly domains (Fig. 1 *b* and *c Center*). Each groove contains three conserved lysines: Lys-70, Lys-98, Lys-123 (CAP-Gly-1) and Lys-224, Lys-252, Lys-277 (CAP-Gly-2). Lys-98 and Lys-252 are members of the conserved GKNDG motif that is highly conserved among CAP-Gly domains (SI Fig. 6). In addition to the conserved lysines, the CAP-Gly-2 domain possesses additional basic residues (Lys-238, Lys-268, R297, and R298), which make the groove more positively charged. An aromatic hydrophobic patch represents part of the groove: Phe-82, Trp-87, and Phe-118 (CAP-Gly-1) and Phe-236, Trp-241, and Phe-272 (CAP-Gly-2). Structural studies showed that MTs/tubulins possess the negatively charged molecular surfaces with their flexible acidic tails (19, 20). Because of the electrostatic complementarity, the basic groove is an attractive candidate responsible for MT/tubulin binding. It should be noted that the existence of the highly basic groove is not necessarily a common feature of all CAP-Gly domains because basic grooves are not apparent in CAP-Gly domains of p150^{Glued} (17), CYLD (16), and F50F5.3 (15) (Fig. 1 *d–f*, respectively). Interestingly, p150^{Glued} loses five basic residues of seven found in the CLIP-170 CAP-Gly-2 domain (see SI Fig. 6). These differences are important for the CAP-Gly function, especially interactions with MT/tubulin, as discussed later.

Interactions Between the CAP-Gly Basic Groove and MT/α,β-Tubulin. We first performed a GST pull-down assay by using purified GST-fusion proteins of CLIP-170 CAP-Gly domains. We found that the CAP-Gly-2 domain binds tubulin more tightly than the CAP-Gly-1 domain (Fig. 2*a*). This observation is consistent with a mutation study that showed the greater dominant role in tubulin binding of the GAP-Gly-2 domain (5). Similarly, the

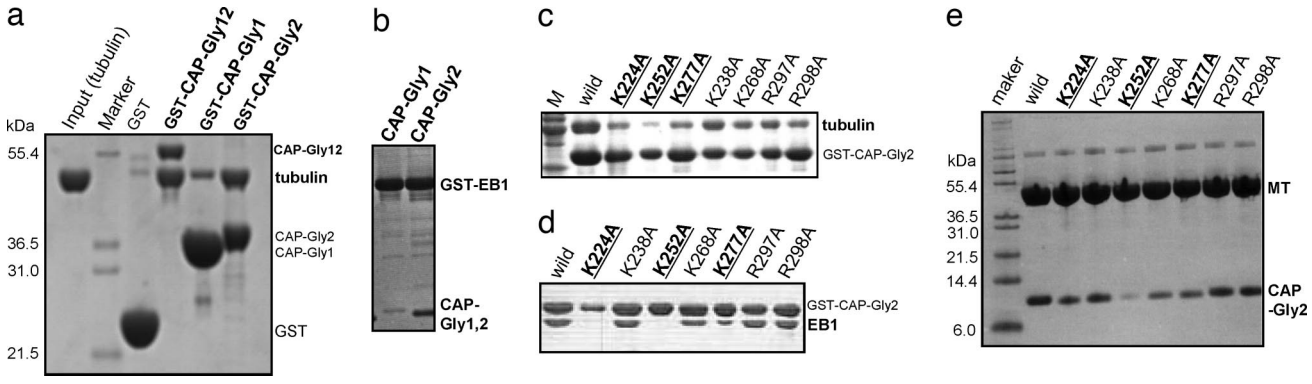

Fig. 2. Interactions of CLIP-170 CAP-Gly domains with MT/tubulin and EB1. (*a*) Pull-down assays of tubulin with GST-CAP-Gly domains. To show clear visible tubulin bands, the applied amounts of proteins on the gel were 1:4:2 for CAP-Gly-12:CAP-Gly-1:CAP-Gly-2. (*b*) Pull-down assays of CAP-Gly domains with GST-EB1. (*c*) Pull-down assays of tubulin with GST–CAP-Gly-2 mutants. Wild-type and mutant GST–CAP-Gly-2 domains were immobilized on the resin and tubulin was used as an input. Labels for conserved lysines are bold and underlined. (*d*) Pull-down assays of EB1 with GST–CAP-Gly-2 mutants. (*e*) Cosedimentation experiments of CAP-Gly-2 domains with MT.

Table 1. Binding of CAP-Gly2 domain to the acidic tails

Peptide	Sequence	K_d, μM
α2-tubulin	ALEKDYEEVGVDSVEAEA-EEGEEY	3.79 ± 0.01
α3-tubulin	ALEKDYEEVGVDSVEGEGEEEGEEY	3.27 ± 0.02
α8-tubulin	ALEKDYEEVGTDSF-EEENEGEEF	4.95 ± 0.03
β2-tubulin	QQYQDATAEEEEDF-GEEAEEEA	80.1 ± 1.1
β1-tubulin	DAKAVLEEDEEVTEEAEMEPEDKGH	194 ± 2
β3-tubulin	DATAEEEGEMYEDDEEESEAQGPK	113 ± 2
α3-tubulin-ΔY	ALEKDYEEVGVDSVEGEGEEEGEE	205 ± 6
	αααααααααα~~~~~~~~~~~~~~~~~*	
EB1	DILYATDEGFVIPDEGGP--QEEQEEY	3.54 ± 0.02
EB3	GILYATEEGFAPPEDDEIEEHQQEDQDEY	2.65 ± 0.01
	ααααα~~~~~~~~~~~~~~~~~~~~~~~~~*	

*The last α-helix (α) and the acidic tail (~).

Table 2. The CAP-Gly binding to the α2-tubulin peptide (the C-terminal α2-tubulin peptide shown in Table 1)

Protein	Domain	Residues*	K_d, μM
		Wild	
p150^{Glued}	CAP-Gly		403 ± 42
CLIP-170	CAP-Gly12		0.513 ± 0.005
	CAP-Gly1		70.1 ± 0.5
	CAP-Gly2		3.79 ± 0.01
		Mutants	
CLIP-170	CAP-Gly2	**K224A**	53.7 ± 0.4
		K238A	7.01 ± 0.03
		K252A	124 ± 6
		K268A	6.86 ± 0.05
		K277A	26.0 ± 0.1
		R297A	8.9 ± 0.1
		R298A	7.7 ± 0.1

*Conserved basic residues are shown in bold with underlining.

CAP-Gly-2 domain binds EB1 with a higher affinity than the CAP-Gly-1 domain (Fig. 2*b*). This inequality in binding affinity could be explained by the existence of additional basic residues on the CAP-Gly-2 groove as described above.

The CAP-Gly-2 domain was then subjected to site-directed mutagenesis in an effort to determine the individual contributions of each residue to MT/tubulin binding. As expected, each alanine mutation resulted in a reduction of the tubulin binding affinity in pull-down experiments, and significant reductions were recorded for mutations of the three conserved lysines (Lys-224, Lys-252, and Lys-277) (Fig. 2*c*). Furthermore, we observed a similar reduction of binding to EB1, which has a similar C-terminal acidic tail, suggesting that the same residues of the CAP-Gly-2 domain participate in both tubulin and EB1 binding (Fig. 2*d*). We then performed cosedimentation experiments with MT (Fig. 2*e*). Again, we observed a reduction of binding to MT with the mutations, which indicate importance of the basic residues. Particularly, the Lys-252 mutation effectively abolishes MT binding.

The C-Terminal Acidic Tails of α-Tubulin and EB1 Are Responsible for CLIP-170 Binding. Tubulin and EB1 proteins possess ≈15- and ≈25-residue C-terminal acidic tails, respectively. To verify the acidic tail binding to CLIP-170 CAP-Gly domains, we have performed quantitative binding assays by using surface plasmon resonance (SPR) measurements (Table 1). Our measurements showed that the CAP-Gly-2 domain tightly binds to EB1 and EB3 peptides, which is consistent with a previous *in vitro* binding study showing an important role for the EB1 C-terminal acidic tail in CLIP-170 binding (21). Similarly, the CAP-Gly-2 domain exhibited a significant binding affinity (K_d values of 3.27–4.95 μM) to α-tubulin peptides, but a weak binding to the β-tubulin peptides. The C-terminal tyrosine-deleted α3-tubulin (α3ΔY) significantly reduced the binding affinity, which is consistent with recent reports showing that tyrosination of the α-tubulin C-terminal end by tubulin tyrosine ligase is associated with CLIP-170 localization and loading to MTs, but not to detyrosinated MTs (22, 23).

The CAP-Gly-12 domain exhibits a strong binding to the α2-tubulin peptide with a submicromolar K_d value. The major contribution to this strong affinity is attributable to that of the CAP-Gly-2 domain (Table 2). Contrary, the CAP-Gly-1 domain alone exhibits a weak binding affinity to the peptide. Essentially the same results were obtained by using the α3-tubulin peptide (data not shown). Quantitative analysis using mutant CAP-Gly-2 domains revealed a ≈34-fold (for Lys-252), 10-fold (for Lys-224 and Lys-277), and 2-fold (for each nonconservative Lys/Arg residue) reduction of the binding affinity (Table 2) (also see SI Fig. 7*a*), indicating that Lys-252 and the two other conserved lysines play a major role in binding to the α-tubulin acidic tail, but that nonconserved basic residues also contribute to the binding. Indeed, a 12-residue truncation of the CAP-Gly-2 C-terminal region, lacking two nonconserved arginines (Arg-297 and Arg-298), resulted in a ≈20-fold reduction of the binding affinity (SI Fig. 7*b*).

The results obtained with the α-tubulin peptide are consistent with the results of the binding assay by using MT/tubulin. We therefore concluded that CAP-Gly-2 binding to the C-terminal acidic tails represents the major intermolecular interaction concerning CLIP-170 binding to α-tubulin and EB1. Moreover, we found that the α-tubulin peptide effectively inhibits pull-down of soluble α,β-tubulin, which presumably contains dimers and oligomers, by GST–CAP-Gly-2. This result reinforced our conclusion concerning the essential role of the C-terminal tail in tubulin binding (SI Fig. 7*c*).

Structure of the CAP-Gly-2–Peptide Complex. The 3D structure of the complex between the CAP-Gly-2 domain and the C-terminal α3-tubulin peptide (residues 416–451) was determined by heteronuclear multidimensional NMR spectroscopy. The ensemble of 20 structures was in excellent agreement with a large body of experimental data (SI Table 5). The rms deviations of backbone and all heavy atoms over residues 212–281 of the CAP-Gly-2 domain and residues 448–451 of the peptide are 0.13 and 0.51 Å, respectively. The rest of the peptide residues are disordered even in complex with the CAP-Gly-2 domain. The overall structure of the CAP-Gly-2 domain of the complex is essentially identical with that of the free form with an atomic rms difference of 0.96 Å when backbone atoms of residues 212–281 are superimposed (SI Fig. 8).

The CAP-Gly-2 domain forms a 1:1 complex with the α-tubulin peptide that positions its C-terminal six residues at the basic groove (Fig. 3*a* and SI Fig. 9*a*). The α-tubulin peptide anchors its C-terminal end residue Tyr-451 to the hydrophobic patch (Phe-236, Trp-241, Phe-272, and Val-257) of the basic groove. In fact, the resonance of the ε-H of the Phe-236 aromatic ring exhibits significant up-field shifts (4.65 ppm) probably because of a ring current shift caused by CH-π stacking with Tyr-451.

The backbone carboxyl group of Tyr-451 is engaged with a small cavity by forming a hydrogen bond with the side chain of Asn-253 from the GKNDG motif (Fig. 3*b* and SI Fig. 9*a*). Mutations of Asn-253 significantly reduced the binding ability (Fig. 3*c*). The side chain conformation of Asn-253 is stabilized by a hydrogen bond to the Gly-255 main chain. Its well-ordered conformation is demonstrated by significantly separated ^{1}H signals of the side chain amide (SI Fig. 10 *a* and *b*): one of the Asn-253 NH_2 signals and the Gly-255 main-chain amide signal show remarkable down-field shifts (SI Fig. 10*c*), which indicate their participation in proper hydrogen bonds. The other NH_2

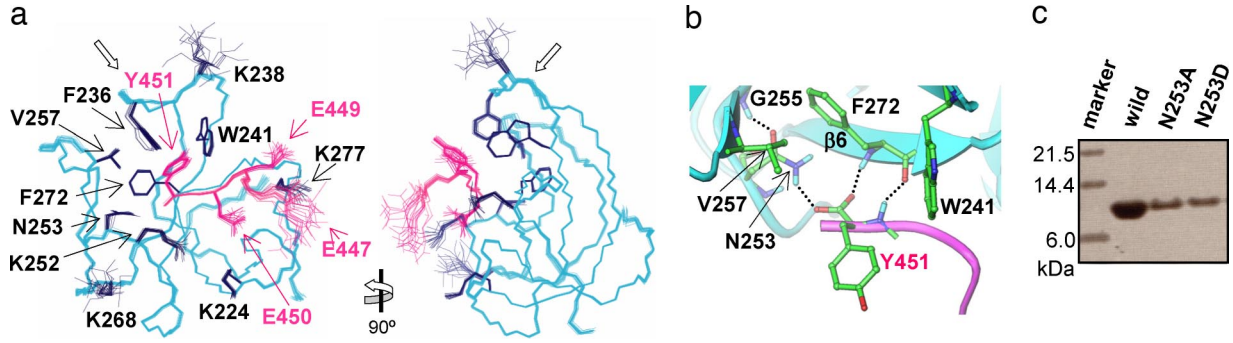

Fig. 3. Solution structure of the CAP-Gly-2–peptide complex. (*a*) A best-fit superimposition of the final 20 simulated annealing structures of the CLIP-170 CAP-Gly-2 domain (residues 212–281) bound to the α3-tubulin peptide (447–451) with the lowest energies is displayed. The CAP-Gly-2 domain backbone (cyan) and selected side chains (blue) are depicted with the peptide (magenta). (*b*) Close-up view of the hydrogen bonds (dotted lines) formed in the complex along the arrow in *a* (*Right*). The side chain of Phe-236 is omitted for clarity. (*c*) Pull-down assay of mutated CAP-Gly-2 domains with the α3-tubulin peptide suggests a critical role for Asn-253 in peptide binding.

signals of Asn-253 show an up-field shift possibly because of the close proximity of the Phe-272 aromatic ring.

The main chain of Tyr-451 makes antiparallel β-sheet-like interactions with strand β6 by forming hydrogen bonds with the main chain of Phe-272 (Fig. 3*b*), which is suggested by the significant down-field shifts of the amide ^{1}Hs of Phe-272 and Tyr-451 (SI Fig. 10 *c* and *d*). Phe-272 is one of the conserved triplet GLF (residues 270–272) motifs on strand β6 (SI Fig. 6). Gly-270 makes contact with the GKNDG loop to position Lys-252 and Asn-253 appropriately for interactions with the α-tubulin peptide. Thus, the GLF motif should be one of the distinct profiles of the CAP-Gly domain for peptide recognition.

Both the conserved and additional basic residues of the CAP-Gly-2 domain contributed to peptide binding through electrostatic interactions with the four negatively charged glutamates of the C-terminal hexapeptide region, EEGEEY (residues 446–451). The side chain carboxylate of Glu-450 is stabilized by Lys-252 and Lys-224. Glu-449 projects the side chain carboxylate toward Lys-277 to form a salt bridge (Fig. 3*a*). Glu-447 is more mobile than the terminal tripeptide EEY, but the negatively charged side chain stays in the vicinity of Lys-244, Arg-298, and Arg-297 for possible electrostatic interactions (SI Fig. 9). Glu-446 is much more mobile than Glu-447, but is within a space between Lys-277 and Arg-298. Lys-238 and Lys-268 may contribute to the negatively charged peptide binding by long-range electrostatic interactions while occupying remote positions from the glutamate binding sites.

The Sextette EEGEEY/F Acidic Motif. Our NMR structure suggests that the C-terminal EEGEEY motif participates in interactions with the basic groove of the CAP-Gly-2 domain. This suggestion is well supported by our SPR measurements by using shorter α-tubulin peptides (Table 3). The C-terminal octapeptide GEEEGEEY exhibited a strong binding affinity comparable with longer peptides. More importantly, an alanine mutation of the first three glutamates, **AAAA**GEEY, resulted in a significant reduction of binding affinity that was approximately >100-fold weaker than that of GEEEGEEY, whereas **AA**EEGEEY had no significant effect on the binding affinity. Indeed, **AAAA**GEEY showed no detectable band in our pull-down assay (data not shown). Thus, Glu-446 and Glu-447 (GE**EE**GEEY) clearly participate in binding to the CAP-Gly-2 domain. Consistently, these glutamates are highly conserved among human α-tubulins, suggesting their importance in CLIP-170 binding.

The negatively charged backbone carboxylate of C-terminal Tyr-451 plays a key role in binding to the CAP-Gly-2 domain: amidation ($-NH_2$) or peptidation ($-$AA) of the carboxylate caused a significant reduction of the binding affinity (Table 3). As found in α8-tubulin, this tyrosine residue is replaceable with phenylalanine, but not alanine. Taken together with our NMR structure, we conclude that the C-terminal sextette EExEEY/F motif is a prerequisite for CLIP-170 CAP-Gly-2 binding.

Table 3. Binding affinity (K_d in micromolar concentration) of C-terminal α-tubulin peptides to CAP-Gly domains

Peptide	CAP-Gly1	CAP-Gly2	CAL-Gly12
GEEEGEEY	64.2 ± 0.5	3.12 ± 0.13	0.587 ± 0.13
AAEEGEEY		2.64 ± 0.01	
AAAAGEEY		Weak*	
GEEEGEEY-NH_2		114.0 ± 0.8	
GEEEGEEYAA		73.2 ± 0.4	
GEEEGEEF		6.4 ± 1.1	
GEEEGEEA		Weak*	

*Data could not be subjected to rigorous analysis, although SPR changes suggested K_d values in the millimolar range.

Autoinhibition. The first zinc knuckle domain of CLIP-170 is suggested to dominantly autoinhibit MT binding by direct binding to the CAP-Gly-2 domain (8). We performed a chemical shift perturbation experiment to verify this masking mechanism and survey the molecular surface on the CAP-Gly-2 domain for binding to the C-terminal peptide containing the knuckle domains (SI Fig. 11). Our experiments revealed perturbed residues confined to the molecular surface covering the basic groove for α-tubulin binding (Fig. 4*a*). This result is consistent with a folded-back structure of masked inactive CLIP-170 that has been suggested by scanning force microscopy and fluorescence resonance energy transfer (8). Interestingly, the key residue for α-tubulin binding, conserved Lys-252, was not strongly affected in the perturbation experiment when using the C-terminal peptide. In addition, our pull-down assay using CAP-Gly-2 mutants to inhibit tubulin binding did not exhibit any drastic reduction of binding affinity to the C-terminal region (SI Fig. 12). These results might support the appealing hypothesis that the knuckle domain binds the CAP-Gly-2 domain with a binding mode that is somewhat different from that of the α-tubulin acidic tails.

Discussion

Copolymerization. Several hypotheses have been proposed for plus end-tracking mechanisms, such as physical translocation and end loading with copolymerization (7, 24–27). Our study suggests that CLIP-170 binds both tubulin dimers and MTs. This is consistent with the fact that both tubulin dimers/oligomers and polymerized MTs project the C-terminal flexible acidic tails

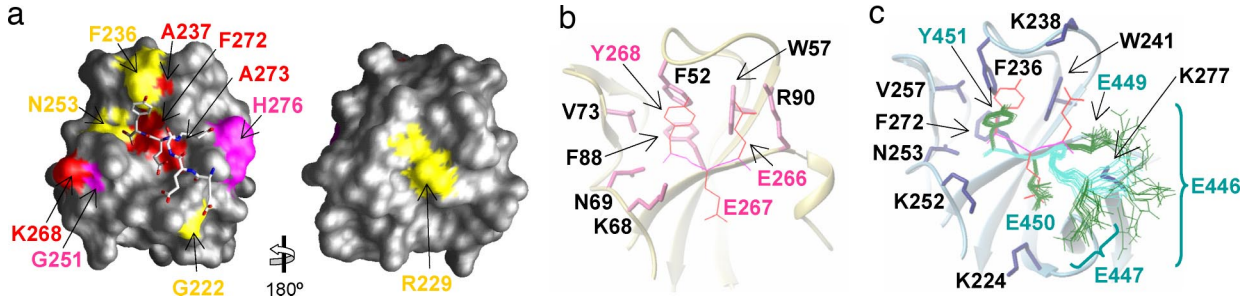

Fig. 4. Comparison of CAP-Gly-binding sites for zinc knuckle domains, the tubulin and EB1 tail peptides. (*a*) Zinc knuckle-induced chemical shift changes mapped on the molecular surface of the CAP-Gly-2 domain. The α-tubulin peptide is shown for comparison. Residues whose signal intensities were strongly reduced, such that $(I_{ref} - I_{per})/I_{ref} > 0.95$, are shown in red, >0.90 in yellow, and >0.85 in magenta, where I_{ref} and I_{per} represent the signal intensity of the reference and perturbed spectrum, respectively. The orientation of the molecule is identical with that of Fig. 1. (*b*) The EB1 peptide (magenta lines) bound to the p150^{Glued} CAP-Gly domain (gray ribbons with magenta side chains) in the crystal structure (2HL3). (*c*) The EB1 peptide bound to the p150^{Glued} CAP-Gly domain is compared with the NMR structure of the α-tubulin peptide (cyan/green) bound to the CLIP-170 CAP-Gly-2 domain (blue) with 20 ensemble structures.

toward solvent regions such that they are easily accessible for the CLIP-170 CAP-Gly domains (19, 20). This implies that CLIP-170-bound tubulin dimer or oligomer could participate in a copolymerization mechanism concerning plus end localization.

CLIP-170 binding to tubulin would contribute to neutralization of the negative charges in the tubulin acidic tails by using the positively charged CAP-Gly domains. This charge neutralization could be one of the possible mechanisms for stimulation of MT nucleation and polymerization by CLIP-170. The binding affinity of CLIP-170 to a tubulin dimer is reported to be higher than its affinity to MTs (26). However, EB1 concentrated at MT plus ends may assist in CLIP-170 recruitment by interacting with the CAP-Gly domains through the EB1 acidic tails. In fact, a recent study has shown that EB1 is required for the plus end localization of CLIP-170 in mammalian cells (28).

Tandem CAP-Gly Domains. It may be interesting to ask why CLIPs possess two tandem CAP-Gly domains. We speculate that the tandem arrangement of the CAP-Gly domains corresponds to two acidic tails of the tubulin dimer. In addition to the strong interaction between the CAP-Gly-2 and α-tubulin, *in vivo*, the basic surface of the CAP-Gly-1 domain may contribute to the binding affinity probably by electrostatically interacting with the acidic tail from β-tubulin. In fact, the affinity of the tandem CAP-Gly domain is ≈10-fold higher than that of the CAP-Gly-2 domain alone (Fig. 2*a* and Table 2). It should be noted that another CLIP target EB1 also forms a dimer that protrudes two acidic tails.

The p150^{Glued} CAP-Gly Domain Exhibits No Strong MT-Binding Activity. Our SPR measurements showed that the binding affinity of the p150^{Glued} CAP-Gly domain to the α-tubulin C-terminal tail is approximately >100- and 780-fold weaker than those of the CLIP-170 CAP-Gly-2 and CAP-Gly-12 domains, respectively (Table 2). We also observed an extremely weak binding to the EB1 C-terminal tail (data not shown). This seriously weak affinity for the C-terminal acidic tails could be caused by lack of basic residues on the basic grooves of the p150^{Glued} CAP-Gly domain as mentioned previously (Fig. 1*c*).

Recently, the crystal structure of the p150^{Glued} CAP-Gly domain bound to the EB1 acidic hexapeptide has been reported (PDB:2HL3; ref. 18). In the structure, however, only one EB1 peptide has been defined in one of the two crystallographically independent CAP-Gly domains, which may reflect a weak interaction between the p150^{Glued} CAP-Gly domain and the EB1 acidic tail. Moreover, the CAP-Gly domains form an artificially strand swapped dimer in the crystal, and the bound peptide model was limited to the three residues of the C terminus. The position and conformation of the bound three residues are shifted from those of our structure by participation in different interactions (Fig. 4*b*): the second C-terminal glutamate (Glu-266) of the EB1 peptide interacts with nonconserved Arg-90 of the p150^{Glued} CAP-Gly domain, but the corresponding residue of the CLIP-170 CAP-Gly-2 domain is proline (SI Fig. 6). Alternatively, the second C-terminal glutamate (Glu-449) of the α-tubulin peptide in our complex is recognized by conserved Lys-277 of the CAP-Gly-2 domain. Moreover, instead of CLIP-170 Lys-277, the p150^{Glued} CAP-Gly domain has glutamine that interacts with the first C-terminal glutamate (Glu-267) of the EB1 peptide. Based on the p150^{Glued}/EB1 complex, the EEY/F motif has been suggested as a CAP-Gly binding motif (18). This suggestion is inconsistent with our structural and mutational analyses showing that two glutamates at the fifth and sixth last C-terminal positions (**EE**xEEY/F) are essential for CLIP-170 CAP-Gly-2 binding.

It should be noted that the relatively strong binding (with K_d = 2.7 μM) of the p150^{Glued} CAP-Gly domain to the EB1 C-terminal fragment (residues 191–268) involves another contact site (17, 18), which is distinct from the EB1 recognition by CLIP-170, which grasps the C-terminal tail EExEEY/F motif.

Dynein Recruitment to the Plus End. Unlike the CLIP-170 CAP-Gly domain, the p150^{Glued} CAP-Gly domain probably lack a sufficient affinity for MT binding by itself, but may play a role in targeting dynactin/dynein toward the MT plus ends by interacting with the C-terminal zinc knuckle domain of CLIP-170 (8, 10). In the free form of CLIP-170, the C-terminal knuckle domain binds the CAP-Gly domain. Once CLIP-170 binds MT, however, the released zinc knuckle domain may serve to recruit dynein to the plus end of MT by interacting with p150^{Glued} and LIS1. Alternatively, CLIP-170 bound to tubulin may also bind p150^{Glued} and recruit it to the plus ends in the copolymerization process. These pictures are consistent with the observed requirement of plus end localization of CLIP-170 for localization of p150^{Glued} to the plus ends (28).

Structural Comparison with PDZ Domains. The C-terminal short peptide recognition by the CAP-Gly domain is reminiscent of the canonical PSD95–Dgl–ZO1 (PDZ) domain that recognizes three or four C-terminal residues of target proteins with a hydrophobic C-terminal-end residue, such as a QTSV peptide by PSD95 (29) (SI Fig. 13). In both cases, ligand peptides bound to the domain expand its antiparallel β-sheet. Like the GLF motif of the CAP-Gly domain, PDZ domains conserve the RXXXGLGF motif that plays a key role in C-terminal recognition. The conserved phenylalanine of the GLGF (PDZ) and GLF (CAP-Gly) motifs forms β-sheet-like hydrogen bonds with the C-terminal end residue and traps the nonpolar side chain of this residue through hydrophobic interactions.

Conclusions

Our structural data show that CLIP-170 binds tubulin not by interacting with the globular domain of tubulin, but rather by the specific interaction between the CLIP-170 CAP-Gly-2 domain and the C-terminal EExEEY/F motif of the flexible tail end of α-tubulin. This result supports the notion that CLIP-170 is copolymerized with tubulin dimers or oligomers to the MT plus end. The highly positive charges of the tubulin-binding surface on the CLIP-170 CAP-Gly domains argues that CLIP-170 stimulates MT polymerization and/or nucleation by neutralizing the negative charges of tubulins. The tubulin/MT binding of CLIP-170 is autosuppressed by the C-terminal knuckle domains, which cover the α-tubulin binding surface of the CAP-Gly-2 domain. Once CLIP-170 binds MT, the released zinc knuckle domain may serve to recruit dynein to the plus end of MT by interacting with $p150^{Glued}$ and LIS1. Thus, our structures provide the structural basis for the specific dynein loading on the MT plus end. Our binding assay shows that CLIP-170 binds the acidic tails of EB1/EB3 as well as those of α-tubulins. This fact strongly suggests that EB1/EB3 that localized at the MT plus end contribute to the CLIP-170 recruitment to the plus end. Finally, we showed that the positively charged binding surface is not necessarily preserved in other CAP-Gly domains, suggesting that the CAP-Gly domain is a common structural module frequently found in cytoskeletal proteins but may participate in versatile protein–protein interactions. Notably, the $p150^{Glued}$ CAP-Gly domain that is furnished with a less positively charged surface only weakly interacts with the α-tubulin acidic tail.

Methods

Crystallographic Analyses of CAP-Gly Domains. Expression, purification, and crystallization of proteins are detailed in *SI Methods*. Intensity data sets were collected with a CCD detector (DIP 2040) at the synchrotron radiation source of the BL44XU station in SPring-8, Harima, Japan. The data were processed with HKL2000 and SCALEPACK (30). The structures were determined by molecular replacement. A summary of data collection and structure refinement statistics is given in SI Table 4. The crystal structure of the CAP-Gly-1 domain was solved by molecular replacement with the CAP-Gly-2 domain structure (SI Table 4). The current model of the CAP-Gly-2 domain includes residues 211–286 and 296–300, and the CAP-Gly-1 domain includes residues 56–130 and 139–141. There is no residue in the disallowed region as determined by PROCHECK (31).

NMR Spectroscopy and Structure Determination. Uniform-labeled protein samples with ^{15}N or ^{15}N and ^{13}C were prepared at $\approx$1 mM for measurements. NMR spectra were acquired at 30°C on a Bruker AVANCE 500 equipped with a cryogenic probe and a Bruker (Tsukuba, Japan) DRX800 NMR spectrometer. All spectra were processed with the NMRPipe package (32) and analyzed by using Sparky (33). The program CYANA, version 2.1, with the CANDID protocol was used for the purposes of structural restraint collection (34). Finally, an ensemble of 100 CAP-Gly-2–α-tubulin structures was calculated by using the program CNS, version 1.1, with residual $^1D_{NH}$ couplings by a simulated annealing protocol (35). The structural data and statistics are summarized in SI Table 5, and experimental details including chemical shift perturbation are given in *SI Methods*.

Binding Studies. Equilibrium SPR measurements were carried out with a BIAcore3000 (Biacore, Uppsala, Sweden) sensor. Biotinylated polypeptides of tubulins and EB1/EB3 were purchased from the TORAY Research Center (Tokyo, Japan). Each peptide was coupled via the N-terminal biotin moiety to a streptavidin-coated sensor chip (Biacore). The kinetic parameters were evaluated by using the BIA evaluation software. Experimental details including cosedimentation experiments are given in *SI Methods*.

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