

## Microtubule-binding properties of dynactin p150 expedient for dynein motility

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### Abstract

Dynactin is a hetero-oligomeric protein complex that has an important role in dynein-based intracellular transport. The expressed N-terminal fragments of dynactin p150 bound to microtubules in the ratio of one to one tubulin dimer, independent from the binding of dynein stalk head. Single molecule observation revealed that these fragments moved around on microtubules by Brownian motion. When the dynein–dynactin complex moves on microtubules, p150 can support dynein to maintain contact with microtubules and does not interfere with the motility of dynein, and thus, the dynein–dynactin complex can efficiently achieve long-distance carriage of the cargo.

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The cytoplasmic dynein–dynactin complex is involved in organelle positioning, vesicle transport [1,2], and cell division [3]. In the centrosome positioning, it is known that dynactin contributes to centrosome anchoring on microtubules [4]. To carry the cargo, dynein binds the dynactin complex at its intermediate chain [5] and the dynactin complex binds the cargo. The dynactin complex is also required for bidirectional organelle transport together with dynein and kinesin [6].

The dynactin complex consists of Arp1, dynamitin, p150, and other proteins [7,8]. Dynactin p150 has regions of microtubule binding, dynein binding, and Arp1 binding [9] (Fig. 1A). p150 has two coiled-coil regions and forms homodimers [4,10].

In the dynein-coated bead motility assay, the beads coated with dynein–dynactin complex traveled for longer distance than beads coated with only dynein, although p150 did not affect dynein ATPase activity [11]. These

results suggest that the interaction between dynactin p150 and microtubules is important for the processive movement of dynein molecules. However, the molecular mechanism of the binding of p150 to microtubules has not been elucidated. Two regions of p150, the CAP-Gly domain [12] and the K-rich region, are involved in microtubule binding [8]. The CAP-Gly domain is reported to bind to microtubules [13], and the K-rich region is a positively charged region often found in microtubule-binding proteins [14]. To characterize the binding of p150 to microtubules, we prepared fragments of the p150 microtubule-binding region. N145 has both these regions but N105 has just the CAP-Gly domain. Using these fragments, the microtubule-binding mechanism of p150 was investigated by microtubule coprecipitation and the single-molecule observation. Our results show that the CAP-Gly domain is essential for the specific binding to microtubules and the K-rich region supports this binding. These results suggest that together these regions assist the processive movement of dynein.

### Materials and methods

*Expression and purification of p150 microtubule-binding fragments.* N105 and N145 were consisted of N-terminal 1–105 and 1–145 a.a., respectively, of p150. The genes of these fragments were obtained using

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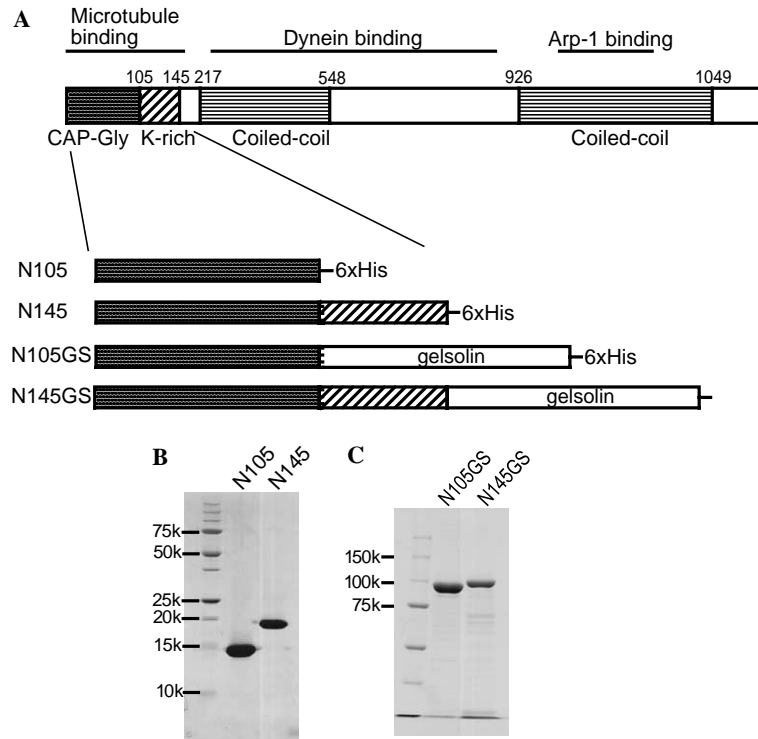


Fig. 1. Microtubule-binding fragments of p150. (A) Schematic diagram of p150 and its fragments. Expressed and purified fragments were analyzed by SDS-PAGE. (B) N145 and N105 (15% acrylamide gel), (C) N145GS and N105GS (7.5% acrylamide gel).

PCR from a human cDNA template. N105 has only the CAP-Gly domain of p150 while N145 has the CAP-Gly domain and the following K-rich region (Fig. 1A). These fragments were added with the 6 × His at the C-terminals. Further, we produced fragments fused with C-terminal truncated gelsolin [15,16] (N105GS and N145GS) to visualize single molecules using fluorescent actin filaments (Fig. 1A). These gene fragments were expressed in a pET expression system.

**Estimation of molecular mass.** The molecular masses of N105 and N145 were estimated by gel-filtration chromatography which was performed in the assay buffer [50 mM Tris-HCl pH 7.0, and 100 mM NaCl] using Superose12 column (Amersham Biosciences). BSA, carbonic anhydrase, and cytochrome *c* were used as molecular markers.

**Preparation of tubulin and microtubules.** Tubulin was purified from porcine brain as described by Weingarten et al. [17]. Tubulin was incubated for 30 min at 37 °C with 1 mM GTP and 5 mM MgSO<sub>4</sub> to polymerize. After the incubation, taxol was added to a final concentration of 40 μM. Microtubule concentrations were expressed as molar concentrations of tubulin heterodimer.

To remove the C-terminal acidic region (E-hook) from β-tubulin, microtubules were incubated with subtilisin at the ratio of 10:1 (w/w of tubulin:subtilisin) at 37 °C for 60 min, and the enzymatic reaction was stopped by the addition of 1 mM PMSF [18,19].

**Microtubule co-precipitation assay.** Microtubule co-precipitation with N105 or N145 was performed in the assay buffer at 25 °C. Various concentrations of N105 or N145 were added with the taxol-stabilized microtubules (final concentration 2 μM) in a volume of 50 μL. The final concentration of NaCl in the mixture was 100 mM. The samples were gently mixed and centrifuged at 244,000g for 10 min at 25 °C (Beckman TLA centrifuge). Pellets were resuspended in SDS-PAGE sample buffer and analyzed by SDS-PAGE on a 15% polyacrylamide gel.

Dynein stalk heads were expressed and purified as described by Mizuno et al. [20].

**Single-molecule observation on microtubule using gelsolin.** To observe the behavior of N105GS and N145GS on microtubules, N105GS and N145GS were visualized as described for kinesin-gelsolin by Yajima et al. [21].

## Results

### Characterization of the p150 fragments

N105, N145, N105GS, and N145GS were purified as a single band on SDS-PAGE (Figs. 1B and C). These fragments did not precipitate by ultracentrifugation without microtubules, suggesting that these fragments did not aggregate and were sufficiently soluble for co-precipitation experiments.

The molecular masses of N105 and N145 were estimated by gel filtration chromatography. These fragments eluted as single peaks. Molecular masses and oligomeric state of these fragments are described in Table 1.

### p150 fragments bound to microtubules

To investigate the binding affinity and the stoichiometries of N105 and N145 to microtubules, the fragments were co-sedimented with microtubules. The results of the assays were expressed in Scatchard plots (Figs. 2A and B). N105 and N145 bound to tubulin dimers on microtubules in the ratio of 1:1, and the dissociation constants of N105 and N145 to the microtubules were 5.09 and 3.25 μM, respectively (Table 2). Furthermore, the fragment of the K-rich region (106–145 a.a.) did not bind to microtubules (data not shown). These results indicate that the CAP-Gly domain is essential for the binding to microtubules. When N105 was assayed at higher pH (8.0), the binding affinity was lower ( $K_d = 11.2 \mu\text{M}$ ), suggesting that

Table 1  
Oligomeric state of N-terminal fragments of dynein p150

	Mw (k)		Ratio (b/a)	Oligomeric state
	Theoretical (a)	Experimental (b)		
N105	11.7	10.4	0.89	Monomer
N145	17.6	23.5	1.34	Monomer

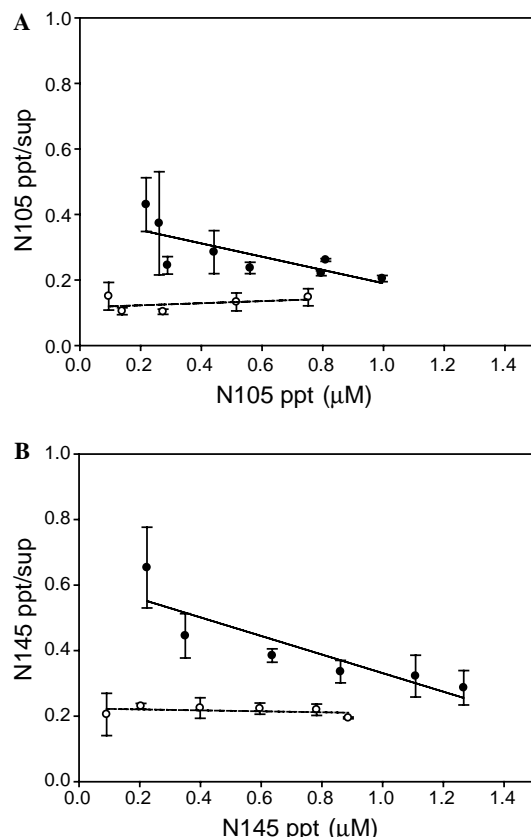


Fig. 2. Cosedimentation analysis of N-terminal fragments of p150. Binding isotherms for 0.7–5.8  $\mu\text{M}$  N105 (A) and 0.5–5.7  $\mu\text{M}$  N145 (B) to 2  $\mu\text{M}$  microtubules. Data are shown in Scatchard plots. Linear fit of intact microtubules (solid line) and subtilisin-treated microtubules (dashed line). Dissociation constants and binding stoichiometries are summarized in Table 2.

Table 2  
Dissociation constant ( $K_d$ ) and stoichiometry

	$K_d$ ( $\mu\text{M}$ )		Stoichiometry	
	Intact	E-hook removed	Intact	E-hook removed
N105	5.09	17.2	1.03	-0.55
N145	3.25	8.95	0.92	0.13
N105 (+ DSH)	6.25	n.d.	0.92	n.d.
N145 (+ DSH)	3.58	n.d.	0.68	n.d.

n.d. is no data.

basic amino acid residues of N105 were an important factor for binding to microtubules.

In the conditions of subtilisin treatment of microtubules used in our experiments, the E-hook of  $\beta$ -tubulin was

removed leaving most E-hooks of  $\alpha$ -tubulin intact. N105 and N145 bound to subtilisin-treated microtubules with very low affinity (Figs. 2A and B), indicating that the E-hook of  $\beta$ -tubulin is important for microtubule binding of p150.

*The N105 binding was not affected by dynein stalk head binding*

To investigate the binding affinity and stoichiometries of N105 to microtubules in the presence of the dynein stalk head (DSH), these fragments were co-sedimented with microtubules. N105 bound to microtubules unaffected by DSH binding (Fig. 3A), and the affinity of N105 did not change (Table 2). The amount of DSH binding to microtubules was independent of the input concentrations of N105 (Fig. 3B). These results indicate that N105 and DSH bind to different regions on microtubules and did not affect the binding properties of each other. The effect of DSH on microtubule binding of N145 was almost the same as that of N105 (Figs. 3C and D).

*Single molecules of p150 fragments showed Brownian movement on microtubules*

When N105GS was mixed with actin filaments labeled with rhodamine-phalloidin, the actin filaments were severed and visualized as fluorescent spots in the microscope. This binding is attributed to the N105 part of the N105GS fusion protein, because the gelsolin molecules severed the fluorescent actin filaments and the fluorescent spots did not bind to the microtubules in the control experiments.

The N105GS spots were observed to attach to microtubules, move along the microtubules, and then detach from them (Supple Fig. 1). Motion vertical to microtubules was not detected within the accuracy of our observation. The trajectory of the movement along the microtubule axis showed that movement was diffusive and not unidirectional (Fig. 4A). The spread of distribution of displacement increased with passage of time (Fig. 4B). The mean square distance showed linear increase (Fig. 4C). These results revealed Brownian motion. The difference of the slopes of the mean square distance may reflect the diffusion coefficients of different lengths of actin filaments. Biased movement as reported for KIF1A [22] was not observed. Mean lifetime of the duration of N105GS on microtubules was 2.86 s (Fig. 4D), and the maximum displacement was observed to be about 2  $\mu\text{m}$ .

*The K-rich region increased the duration of the interaction*

N145GS exhibited Brownian motion on microtubules similar to N105GS (Figs. 5A–C, Supple Fig. 2). The fraction of N145GS spots that stayed on microtubules for more

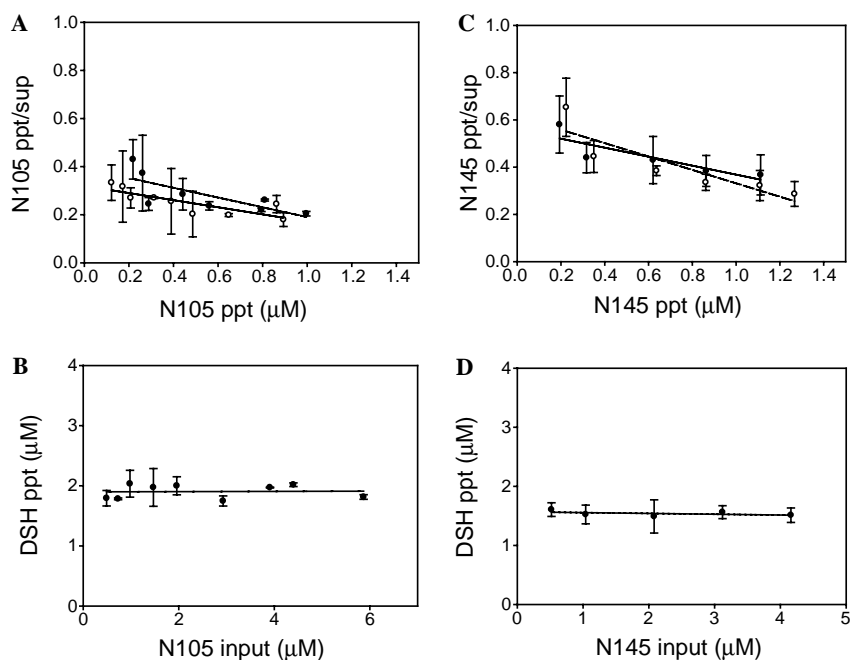


Fig. 3. Cosedimentation analysis of N105 or N145 in the presence of DSH. Binding isotherms for 0.5–5.7 μM N105 (A) or 0.5–4.2 μM N145 (C) to 2 μM microtubules, in the presence of 4.5 μM DSH. Data are shown in Scatchard plots. Linear fit of absence (solid line) and presence (dashed line) of DSH. Dissociation constants and binding stoichiometries are summarized in Table 2. The data are replotted in amount of DSH bound to microtubules vs. input N105 (B) or N145 (D).

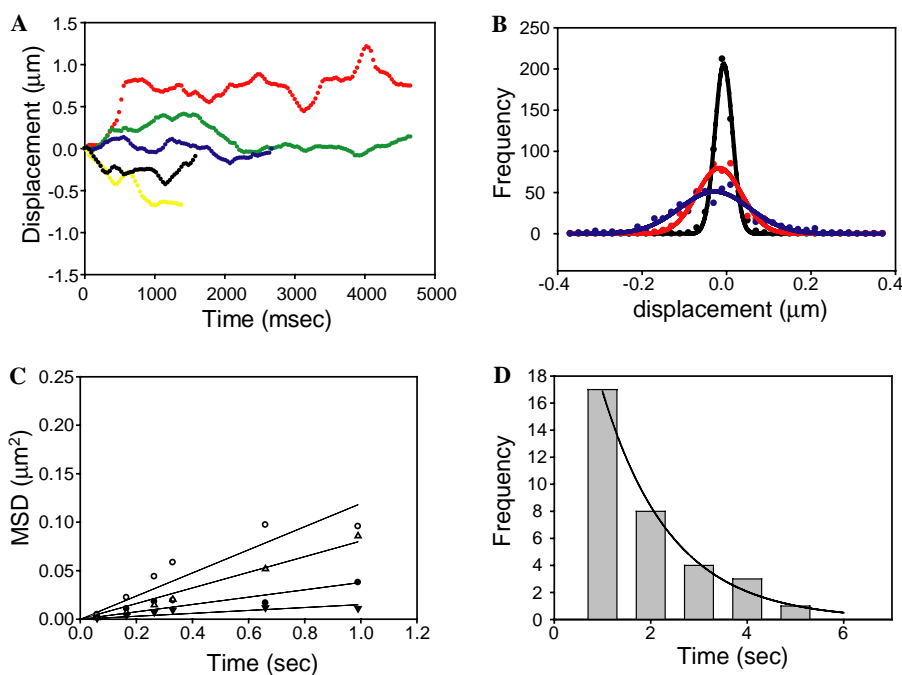


Fig. 4. Single molecule movement of N105GS on microtubules. (A) Trajectory of N105GS along microtubules. (B) Distribution of displacements of N105GS with intervals; 33 (black), 99 (red), and 165 ms (blue). The data are fitted by Gaussian curve. (C) Mean square displacement of N105GS. The data are fitted by linear regression. (D) Histogram of the duration of N105GS interacting with microtubules. The data are fitted by single exponential decay. Mean lifetime of the duration on microtubules was 2.86 s.

than 10 s was about 57%, suggesting that the mean duration must be more than 10 s. Since this fraction of N105GS was about 3.3%, the mean duration of N145GS

should be much longer than that of N105GS, suggesting that the K-rich region of N145GS supports the interaction between the CAP-Gly domain and microtubules.

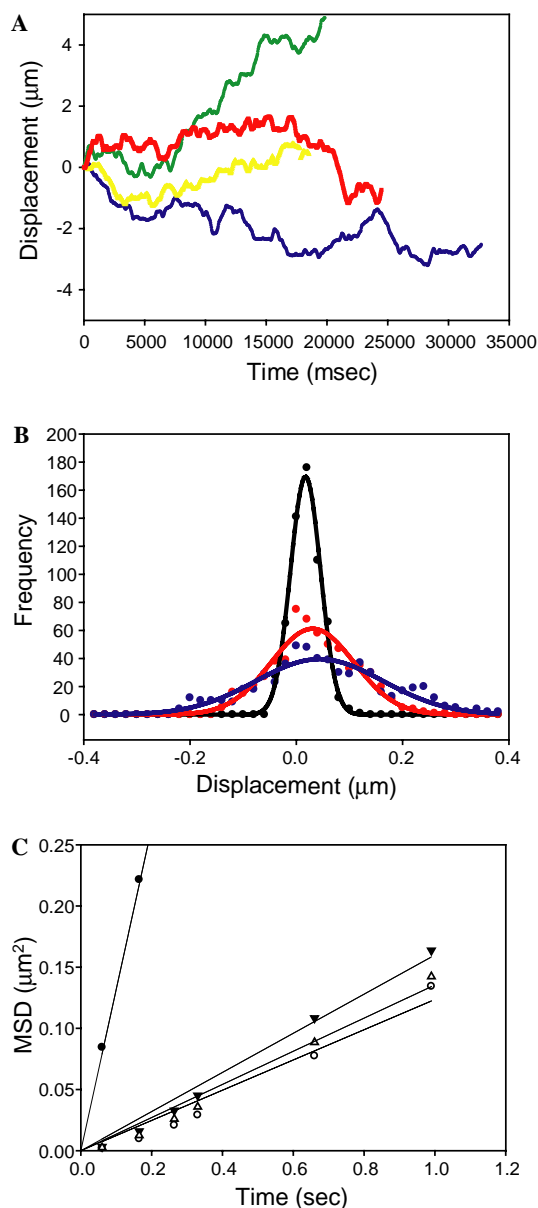


Fig. 5. Single molecule movement of N145GS on microtubules. (A) Trajectory of N145GS along microtubules. (B) Distribution of displacements of N145GS with intervals; 33 (black), 99 (red), and 165 ms (blue). The data are fitted by Gaussian curve. (C) Mean square displacements of N145GS. The data are fitted by linear regression.

## Discussion

### *The role of the CAP-Gly domain and the K-rich region in p150*

The p150 microtubule-binding region consists of the CAP-Gly domain and the K-rich region. It is already known that the two CAP-Gly domains of CLIP-170 bind to microtubules [13]. The biochemical characteristics of the binding had not been clarified though binding to microtubules had been reported for p150 [9,23]. In this study, the one-to-one stoichiometry of N105 or N145 binding to microtubules demonstrated that the CAP-Gly domain rec-

ognizes a specific part of the tubulin-dimer and suggested that the binding sites are repeated at 8 nm along microtubules. The dissociation constants ( $K_d$ ) of N105 and N145 were 5.09 and 3.25  $\mu\text{M}$ , respectively, which were slightly higher than that of CLIP-170 ( $K_d = 0.5 \mu\text{M}$ ) [24]. Considering that CLIP-170 has two CAP-Gly domains, the  $K_d$  values of N105 and N145 appear to be reasonable. In another in vitro experiment, we observed that intermediate region (582–811 a.a.) of p150 bound strongly to microtubules (data not shown), which might explain the high affinity of some p150 fragments to microtubules reported so far [23]. The binding affinity was low when the E-hook of  $\beta$ -tubulin was removed, indicating that E-hook is important to bind to microtubules. Since the binding to the E-hook removed microtubules was observed to some extent, the target of the CAP-Gly domain of p150 does not seem to be the E-hook of  $\beta$ -tubulin.

That the duration of N145GS is longer than that of N105GS shows the K-rich region has an important role as a diffusional anchor [14]. Apparently, there seems to be discrepancy between a small difference of the dissociation constants and a considerable difference of the mean durations for N105 and N145. However, the observed mean duration does not directly mean the dissociation rate, but might depend on frequency of the re-binding to microtubules. The K-rich region might work as a counterpart to the highly negative charge of the E-hook, but the fragment of the K-rich region (106–145 a.a.) did not bind to microtubules (data not shown). Therefore, the specific binding is essentially caused by the CAP-Gly domain, while the K-rich region supports this binding.

### *The mechanism by which the dynein processive movement is supported by p150*

As p150 increased the processivity of dynein [11], p150 was thought to be an anchor for dynein on microtubules. Here, we revealed that N-terminal fragments of p150 bind the microtubules. Since N105 or N145 did not compete with DSH to bind to microtubules and did not accelerate the binding of each other, the binding properties of N105 or N145 and DSH are mutually independent. These results suggest that the p150 anchor is cast at a site on the microtubule that is different from the site for dynein stalk head binding on microtubules. Further, single molecules of N105GS and N145GS showed Brownian motion on microtubules, indicating that these fragments bind microtubules weakly and can slide on microtubules maintaining their contact with microtubules.

Dynactin p135 is an isoform of p150 and is expressed in brain cytosol at high levels [1,25]. Dynactin p135 lacks the CAP-Gly domain and the K-rich region, and forms a heterodimer with p150 [25]. Therefore, this heterodimer has only one CAP-Gly domain and one K-rich region. Because the dynactin p150 fragments used in this study were monomers and worked as diffusional anchors, p150 and p135 heterodimers would have this function and so must have

a physiological role. Physiological functions of p150 homodimer might be elucidated by future study using dimeric fragments.

The number of p150 molecules bound to microtubules in vivo is considered to be close to that of single molecules observed in vitro. Probably, p150 can move around on a microtubule in vivo while maintaining its contact with microtubule. When the dynein–dynactin complex moves on a microtubule, p150 can prevent the dynein detachment and does not disturb dynein movement for two reasons; p150 binds to a different site from which dynein binds to, and p150 binds weakly. As a result, the dynein–dynactin complex efficiently achieves long-distance carriage of the cargo.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2005.11.145](https://doi.org/10.1016/j.bbrc.2005.11.145).

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