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Bidirectional motility of the fission yeast kinesin-5, Cut7

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ABSTRACT

Kinesin-5 is a homotetrameric motor with its motor domain at the N-terminus. Kinesin-5 crosslinks microtubules and functions in separating spindle poles during mitosis. In this study, the motile properties of Cut7, fission yeast kinesin-5, were examined for the first time. In *in vitro* motility assays, full-length Cut7 moved toward minus-end of microtubules, but the N-terminal half of Cut7 moved toward the opposite direction. Furthermore, additional truncated constructs lacking the N-terminal or C-terminal regions, but still contained the motor domain, did not switch the motile direction. These indicated that Cut7 was a bidirectional motor, and microtubule binding regions at the N-terminus and C-terminus were not involved in its directionality.

1. Introduction

Kinesins are molecular motors that move along microtubules using chemical energy derived from the hydrolysis of ATP. The kinesin superfamily is divided into 14 subfamilies on the basis of molecular structure [1]. The subfamilies are involved in different processes that include intracellular transport, mitosis, meiosis, and axonal transport in the nervous system (for reviews see [2,3]). In the kinesin superfamily, most members have a conserved motor domain at the N-terminus and show plus-end-directed motility. Minus-end-directed motility is unique to the kinesin-14 subfamily, in which, the motor domain is at the C-terminus, thereby making these kinesins structurally distinct from other kinesin subfamilies (for reviews see [2,3]).

The plus-end-directed motility of kinesin-5 is necessary for its function in mitosis. Kinesin-5 functions as a bipolar homotetramer, which enables kinesin-5 to crosslink antiparallel microtubules emanating from the opposite poles of the mitotic spindle and then induce sliding of the microtubules relative to one another via plus-end-directed motility [6]. Recently, however, budding yeast kinesin-5 displayed bidirectional motility [4,5] and thus the directionalities of other kinesin-5 motors were of current interest.

Cut7, fission yeast kinesin-5, was identified by Hagan and Yanagida [7]. Similar to other kinesin-5 variants, Cut7 is involved in the separation of spindle pole bodies during mitosis. The temperature-sensitive mutant of Cut7 displayed an abnormal V-shaped spindle under restrictive temperature and was unable to complete mitosis [7]. Considering the intracellular function of Cut7, it is predicted that Cut7 moves toward the plus-end along microtubules, but the motile properties of this protein have not been investigated.

In this study, the motility of Cut7 was examined for the first time. Cut7 was a minus-end-directed N-terminal kinesin, which also showed plus-end directionality. The N-terminal and C-terminal regions, which are known to be microtubule binding sites in kinesin-5, were not involved in switching the directionality of Cut7.

2. Materials and methods

2.1. Construction and preparation of recombinant proteins

For the constructs in the gliding assay, biotin carboxyl carrier protein (BCCP) and his6 tags were introduced into pGEX-2T (GE Healthcare Japan; Tokyo, Japan) and pCold (Takarabio; Shiga, Japan) vectors. Cut7H (amino acids 1–506) and Cut7ΔN (amino acids 70–506) were amplified by PCR and cloned into the pGEX-BCCP vector. Cut7F (amino acids 1–1085) and Cut7FΔC (amino acid 1–956) were amplified by PCR and cloned into the pCold-BCCP vector. For the constructs of the total internal reflection fluorescence (TIRF) assay, green fluorescent protein (GFP) and his6 tags were inserted into the pCold vector. Cut7F and Cut7FΔC were cloned into the pCold-GFP vector. For control experiments, rat conventional kinesin (amino acids 1–430) fused to AviTag (Cosmobio; Tokyo, Japan) for biotinylation or GFP at the C-terminus was generated.

These constructs were expressed in *Escherichia coli* BL21-CodonPlus (DE3) RIL (Agilent Technologies; Tokyo, Japan) by

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adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h at 25 °C (for pGEX vector) or for 20 h at 15 °C (for pCold vector). The proteins were purified essentially as described [8]. Cells were lysed by sonication in lysis buffer [10 mM Na-phosphate, pH 7.5, 250 mM NaCl, 10 mM imidazole, 1 mM $MgSO_4$, 50 nM ATP, and 0.2 mM phenylmethanesulfonylfluoride (PMSF)]. The soluble proteins were purified using Ni-IMAC resin (Bio-Rad, Tokyo, Japan) followed by gel-filtration chromatography and nucleotide-dependent microtubule-affinity purification. The purified proteins were obtained in elution buffer [10 mM Tris, pH 8.0, 400 mM NaCl, 10 mM $MgSO_4$, 1 mM EGTA, 10 mM ATP, 1 mM dithiothreitol (DTT), 0.1% NP-40, 10% sucrose, and 20 μM paclitaxel].

2.2. Preparation of tubulin and polarity-marked microtubules

Tubulin was purified from porcine brain [9]. Polarity-marked microtubules labeled with X-rhodamine succinimidyl ester (Life Technologies Japan; Tokyo, Japan) were prepared according to [10]. Cy5 (GE Healthcare Japan) labeling of tubulin was performed according to [8].

2.3. In vitro motility assays

The gliding assay was performed in assay buffer A (10 mM PIPES, pH 7.0, 25 mM potassium acetate, 4 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 1 mM ATP and 10 μM paclitaxel) under a dark-field microscope or fluorescence microscope, according to [8]. The flow chamber was sequentially coated with biotinylated BSA (Sigma Aldrich Japan; Tokyo, Japan) and streptavidin (Wako; Osaka, Japan) to immobilize biotinylated Cut7 constructs. After microtubules were applied, the GST tag was digested by thrombin (Sigma Aldrich) in the flow chamber and washed away using assay buffer A. Gliding velocity was measured by dark-field microscope using unlabeled microtubules. In determining the directionality of Cut7, the movement of polarity-marked fluorescent microtubules was recorded at intervals of 1–2 min to minimize the bleaching of fluorescence.

In the TIRF assay, the movement of individual Cut7 molecules fused to GFP was recorded according to [8]. The flow chamber was coated with anti-FLAG antibody (Sigma Aldrich) followed by dephosphorylated casein (Sigma Aldrich), and microtubules were then introduced. The chamber was filled with assay buffer B [10 mM PIPES, pH 7.0, 150 mM potassium acetate, 4 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 1 mM ATP, 10 μM paclitaxel, 0.1% NP-40, 1 nM Cut7F-GFP, 1 mg/ml dephosphorylated casein, 0.1% (w/w) glucose, 43 U/ml glucose oxidase (Sigma Aldrich), 650 U/ml catalase (Roche; Tokyo, Japan)]. After the movement of Cut7F was recorded, the motile direction was determined by observing the direction of movement of rat conventional kinesin-GFP applied in excess.

3. Results and discussion

Kinesin-5 is a homotetrameric molecule that contains a conserved motor domain and a divergent stalk-tail region. The kinesin-5 in lower eukaryotes has a long extension at the N-terminus of the motor domain, which is a second microtubule binding site [11] (Fig. 1). The C-terminal region also binds to microtubules [12], and thus kinesin-5 interacts with microtubules in the three regions per polypeptide (N-terminal extension, motor domain and C-terminal region) in lower eukaryotes.

In gliding assays, a motor domain with a short coiled-coil region is usually used for analyzing the motile properties of kinesins. Initially, approximately half of the Cut7 motor, Cut7H (amino acids 1–506), was prepared and examined in a gliding assay. Because Cut7H that was non-specifically absorbed on glass slide did not induce gliding of microtubules, Cut7H was fused to a BCCP tag at the C-terminus and specifically bound to the glass surface using the BCCP tag (Fig. 1). By this method, gliding of microtubules occurred

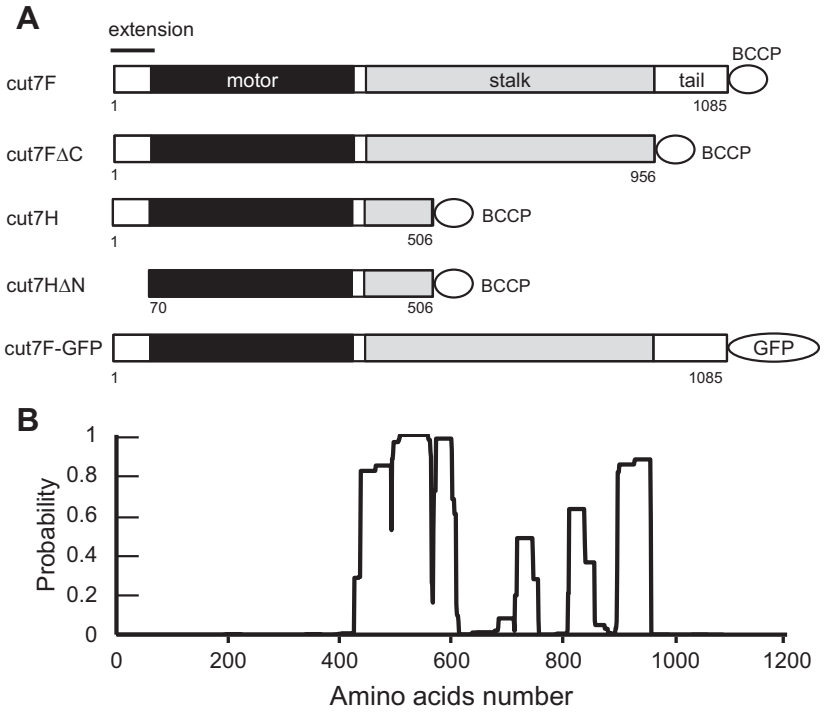

Fig. 1. Cut7 constructs used in this study. (A) Schematic representation of Cut7 constructs used in *in vitro* motility assays. (B) Probability of coiled-coil formation predicted by the COILS program [23].