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The functional expression and motile properties of recombinant outer arm dynein from *Tetrahymena*

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ABSTRACT

Cilia and flagella are motile organelles that play various roles in eukaryotic cells. Ciliary movement is driven by axonemal dyneins (outer arm and inner arm dyneins) that bind to peripheral microtubule doublets. Elucidating the molecular mechanism of ciliary movement requires the genetic engineering of axonemal dyneins; however, no expression system for axonemal dyneins has been previously established. This study is the first to purify recombinant axonemal dynein with motile activity. In the ciliated protozoan *Tetrahymena*, recombinant outer arm dynein purified from ciliary extract was able to slide microtubules in a gliding assay. Furthermore, the recombinant dynein moved processively along microtubules in a single-molecule motility assay. This expression system will be useful for investigating the unique properties of diverse axonemal dyneins and will enable future molecular studies on ciliary movement.

1. Introduction

In motile cilia and flagella, nine peripheral microtubule doublets surround the central pair of microtubules (9 + 2 structure) and are associated with axonemal dyneins, which produce the force needed for ciliary and flagellar motility. Impaired motility of cilia causes human diseases, such as primary ciliary dyskinesia (PCD) [1–3].

Dynein is a large microtubule-based motor protein that belongs to the AAA+ (ATPases associated with diverse cellular activities) superfamily (for review, see [4]). The heavy chain (HC) of dynein is approximately 500 kDa and is divided into the motor domain and the tail. The motor domain is composed of six AAA modules that fold into a ring-like structure containing four P-loops, which are implicated in ATP binding and hydrolysis [5–7].

Dyneins are classified into two groups, axonemal and cytoplasmic dyneins, which function in ciliary motility and intracellular transport, respectively (for a review, see [8]). Axonemal dynein is considered to have distinct properties from cytoplasmic dynein. In ciliary beating, to generate a ciliary waveform, axonemal dyneins must be locally activated on certain doublet microtubules and coordinated so that a wave of activity passes along the axoneme. Another difference is that the motile activities of axonemal dyneins are enhanced by ADP [9,10], which is not observed in cytoplasmic dynein.

Axonemal dyneins are divided into outer arm and inner arm dyneins based on their position in the axoneme. The outer arm dynein consists of two or three HCs, whereas the inner arm dynein consists of one or two HCs (for a review, see [11]). In *Tetrahymena*, there are 23 axonemal dynein HC (DYH3–DYH25) genes [12]. DYH3–DYH5 are outer arm dynein HCs, and DYH6–DYH25 are inner arm dynein HCs. DYH3, DYH4 and DYH5 correspond to the γ, β and α HCs of *Chlamydomonas*, respectively, which form the three-headed structure of the outer arm dynein [13]. Mutation analyses in *Chlamydomonas* revealed that the outer and inner arm dyneins are involved in the flagellar beat frequency and waveform, respectively [11,14]; functional communications between the outer arm and inner arm dyneins have also been reported [15]. Additionally, recent advances in cryo-electron tomography have produced 3D maps of the axonemal repeat unit containing outer and inner arm dyneins [16–21].

To perform further structural and functional analyses on axonemal dyneins, the genetic engineering of axonemal dynein HCs is necessary. However, no previous studies have reported recombinant axonemal dynein HC with motile activity, probably because of the complexity and large size of the complex.

Functional recombinant axonemal dynein was demonstrated for the first time in this study by purifying the complex from a

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Table 1
PCR primers used in this study.

Primer name	Primer sequence (5′ to 3′)
F1-Fw	GGGCTCGAGCCCGGGCCTAATGCTCCTTAATCACC
F1-Rv	GGGGGATCCGTCGACGCAACTACCAAAAATCAATGC
neo2-Fw	GGGCTCGAGATCTTCAAAGTATGGATTAATTATTTC
neo2-Rv	GGGTCGACTGCATTTTTCCAGTAAAAATTTGA
BTU1P-Fw	GGGCTCGAGCTTATTCGCTTTTGCACTTTTG
BTU1P-Rv	GCCGGATCCGTCGACTCTCATTTTTAATTGCTTAAAGG
hfGFP-Fw	GGGCTCGAGCACCACCACCATCATCATGATTATAA GGATGATGATGATAAGATGAGTAAAGGAGAAGAAC
hfGFP-Rv	GGGTCGACTTTGTATAGTTCATCCATGC
F2-Fw	GGGCTCGAGATGGGTGATCATAGTCAAAAAG
F2-Rv	GGGGGATCCGTCGACCCCGGGGAGAGGTAACATTGTTTTAC
cFw1	TTTAGTAGCTTCTGATCATATGAAAAGCT
cRv1	CTAATAATTTGAAATAATTAATCCATACTTTGAAG
cFw2	CAAAATCACTCCTTTAAGCAATTAAAAATG
cRv2	CTTTATCAGGAGTAGTATCAGAGGAAGTAAG

ciliary extract of *Tetrahymena*. The recombinant dynein slid microtubules and moved processively along microtubules in *in vitro* motility assays. This expression system will be useful for the preparation and characterization of diverse axonemal dyneins.

2. Materials and methods

2.1. Strains and culture conditions

Wild-type *Tetrahymena thermophila* strains B2086.1 and CU428.1 were obtained from the Stock Center for *T. thermophila* at Cornell University (Ithaca, NY, USA). *Tetrahymena* cells were grown in SPP medium (1% protease peptone, 0.1% yeast extract, 0.2% glucose and 0.003% Fe-EDTA) at 30 °C.

2.2. Construction of the expression cassette

The genomic sequence of the DYH4 gene (gene ID: TTHERM_00499300) was obtained from the *Tetrahymena* Genome Database (TGD) Wiki (http://ciliate.org/index.php/home/welcome). The components of the expression cassette were cloned into the pBluescript vector (Agilent Technologies; CA, USA), sequenced and sequentially introduced into the pGBKT7 vector (Clontech; Santa Clara, CA, USA). The PCR primers used in this study are listed in (Table 1).

2.3. Transformation of Tetrahymena cells

Transformation was performed according to [22]. The expression cassette was released from the pGBKT7 vector using *Sma* I and purified by phenol/chloroform extraction and ethanol precipitation. Electroporation was performed by using a BTX model ECM 630 (BTX Inc.; Holliston, MA, USA) according to [22].

2.4. PCR analysis

Genomic DNA was isolated according to [23]. The primers for PCR analysis are listed in (Table 1). The PCR products were analyzed on an agarose gel and visualized by GR green (LabSupply; Dunedin, New Zeeland). Homologous recombination was also confirmed by sequencing the corresponding region.

2.5. Purification of recombinant outer arm dynein

The transformant was grown to stationary phase in SPP medium containing 40 μg/ml paromomycin at 30 °C, with agitation. The cilia were isolated according to [24] and treated with 0.5% Nonidet-P40 and 100 mM NaCl in buffer A (10 mM Hepes pH 7.4, 4 mM $MgCl_2$, 1 mM EGTA and 0.2 mM PMSF) for 30 min on ice. After the axoneme

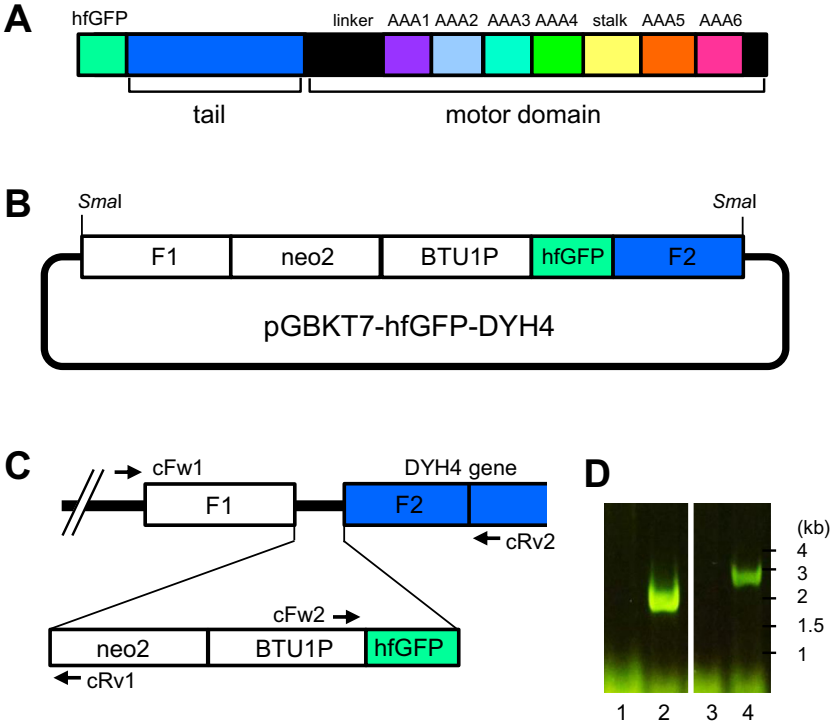

Fig. 1. Construction of the expression cassette and PCR analysis. (A) Schematic representation of DYH4. (B) Construction of the expression cassette. F1: 1.8-kb fragment of an untranslated region of the DYH4 gene; neo2: neomycin-resistance gene cassette [22]; BTU1P: constitutive beta-tubulin 1 promoter; hfGFP: His6- and FLAG-tagged GFP gene; F2: 1.6-kb fragment of a coding sequence of the DYH4 gene. (C) Integration of the expression cassette into the DYH4 locus. The 0.3 kb region upstream of the DYH4 gene was replaced with the expression cassette. The arrows represent the position of the primers used in the PCR analysis. (D) PCR analysis. Lanes 1 and 2: PCR using cFw1 and cRv1 primers; lanes 3 and 4: PCR using cFw2 and cRv2 primers. Lanes 1 and 3: wild-type; lanes 2 and 4: transformant.

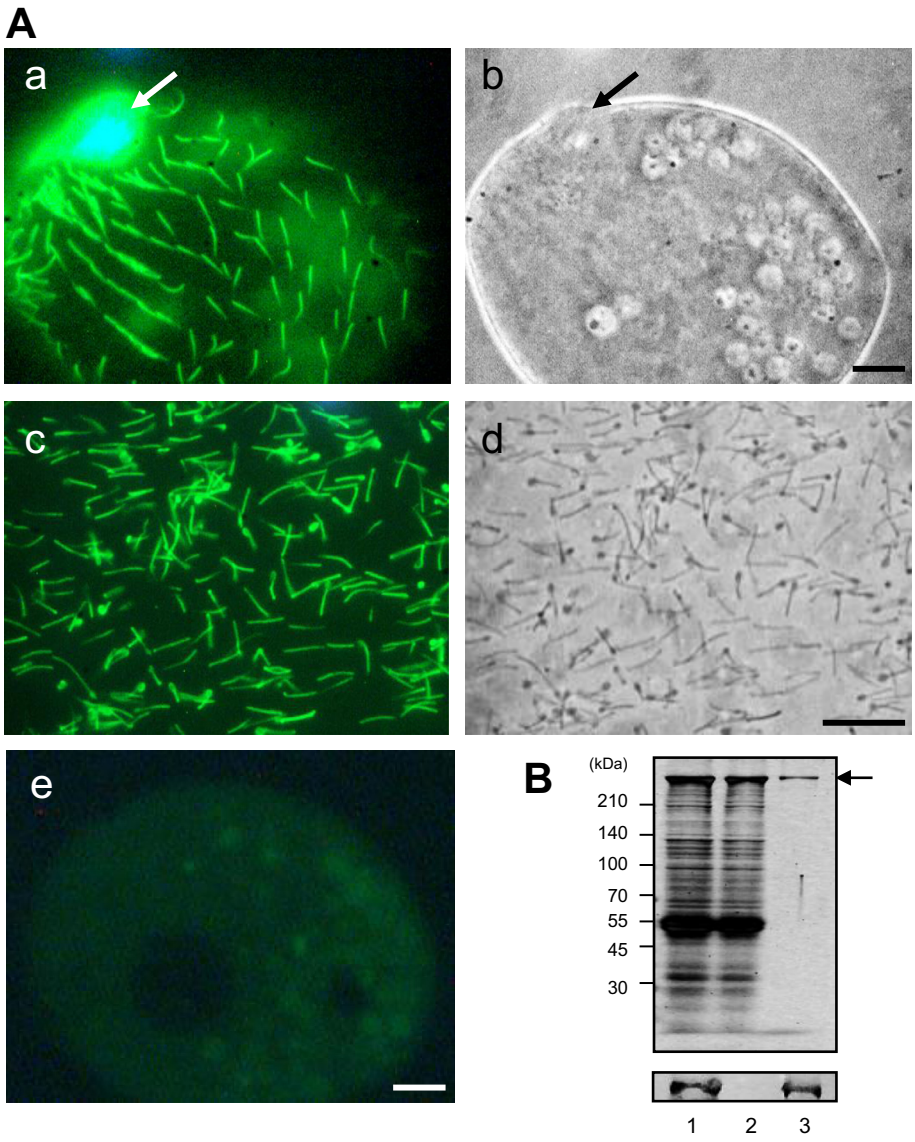

Fig. 2. Localization and purification of the recombinant outer arm dynein. (A) Localization of the recombinant outer arm dynein in *Tetrahymena* cells. (a and b) Recombinant cell; (c and d) isolated cilia; (e) control cell; (a, c and e): fluorescence images; (b and d) phase-contrast images. The arrow represents the oral apparatus. Scale bar represents 5 μm. (B) Purification of the recombinant dynein. Lane 1: axoneme of transformant; lane 2: axoneme of wild-type; lane 3: purified protein; (upper panel) SDS–PAGE; (lower panel) Western blot. The arrow represents the purified dynein.

was washed with buffer A containing 100 mM NaCl, the recombinant dynein was extracted with buffer A containing 600 mM NaCl for 30 min on ice, and the mixture was centrifuged at 25,000*g* for 10 min. The extract was diluted with four volumes of buffer A containing 0.1% Nonidet-P40 and incubated with anti-FLAG M2 affinity gel (Sigma–Aldrich; Tokyo, Japan) for 2 h at 4 °C on a rotator. The gel was washed several times using buffer A containing 0.1% Nonidet-P40 and 100 mM NaCl, and the recombinant dynein was then eluted with 0.5 mg/ml 3× FLAG peptide (Sigma–Aldrich) for 30 min on ice.

2.6. Western blot analysis

The proteins were separated using SDS–PAGE, transferred to a nitrocellulose membrane and detected using anti-GFP monoclonal antibody (Clontech). The secondary antibody was a sheep anti-mouse IgG antibody coupled to horseradish peroxidase (GE Healthcare; Tokyo, Japan).

2.7. Preparation of tubulin and polarity-marked microtubules

Tubulin was purified from porcine brain [25]. Polarity-marked microtubules labeled with X-rhodamine succinimidyl ester (Life Technologies) were prepared according to [26]. The Cy5 (GE Healthcare) labeling of tubulin was performed according to [27].

2.8. 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, 0.2 mM ATP and 10 μM paclitaxel) under a dark-field microscope or fluorescence microscope according to [27]. The flow chamber was sequentially coated with anti-GFP antibody (Life Technologies), BSA (Sigma–Aldrich), casein (Sigma–Aldrich) and tubulin to specifically immobilize the recombinant dynein. The gliding velocity was measured with a dark-field microscope using unlabeled microtubules. Polarity-marked fluorescent microtubules

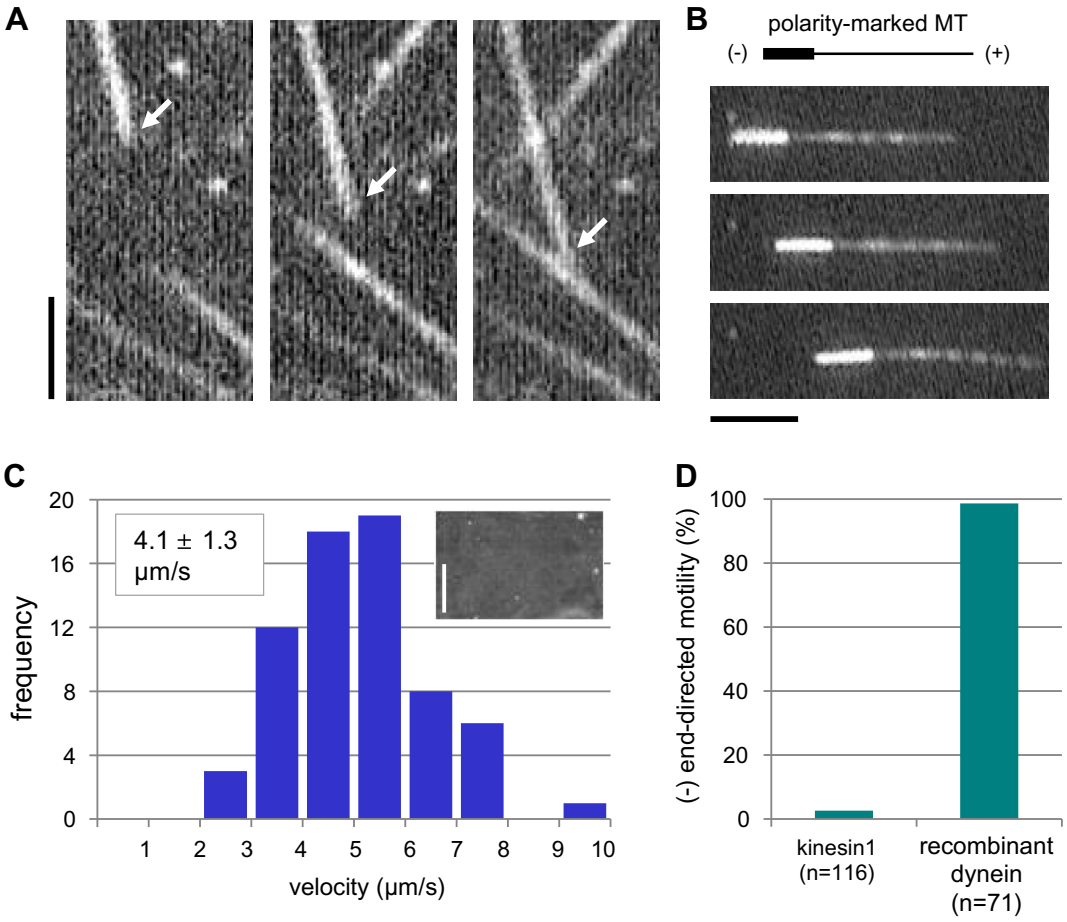

Fig. 3. Gliding assay. (A) Gliding of microtubules. Each frame shows 0 s (left panel), 0.5 s (middle panel) and 1.0 s (right panel) after recording was initiated. The arrows represent a gliding microtubule. Scale bar represents 3 μm. (B) Movement of a polarity-marked fluorescent microtubule. The frames show 0 s (upper panel), 2 s (middle panel) and 4 s (lower panel) after recording was initiated. Scale bar represents 3 μm. (C) Histogram of gliding velocity. Average velocity and standard deviation are shown. (Inset) control experiment without the anti-GFP antibody. Scale bar represents 15 μm. (D) Bar graph of motile direction in the gliding assay. The percentages of the total microtubules moving in the minus-end direction are shown.

were used to determine the directionality of the dynein. For control experiments, rat kinesin 1 (amino acids 1–430) fused to GFP at the C-terminus was used.

In the single-molecule motility assay using a total internal reflection fluorescence (TIRF) microscope, the movement of the recombinant dynein was recorded according to [27]. The flow chamber was coated with anti-FLAG M2 antibody (Sigma–Aldrich) followed by casein; the microtubules were then introduced into the chamber, and the dynein was bound to the microtubules without ATP. Finally, the flow chamber was filled with assay buffer B [10 mM Tris pH 8.0, 0.5 mM $MgSO_4$, 0.5 mM EGTA, 1 mM DTT, 3 μM ATP, 10 μM paclitaxel, 0.1% NP-40, 1 mg/ml casein, 0.1% (w/w) glucose, 43 U/ml glucose oxidase (Sigma–Aldrich), 650 U/ml catalase (Sigma–Aldrich), 50 μg/ml pyruvate kinase (Roche), 1 mM phosphoenolpyruvate (Roche) and 0.1% glucose].

3. Results

In this study, full-length axonemal dynein HC was expressed and purified from ciliary extract of *Tetrahymena*. DYH4, also known as outer arm dynein beta, was used for expression. The genomic sequence of the DYH4 gene was obtained from the TGD Wiki Database and is predicted to encode 4595 amino acids (Fig. 1A).

The expression cassette is shown in (Fig. 1B). The neomycin resistance gene was used as a selective marker [22], flanked on the 5′end by a 1.8-kb untranslated region of DYH4 and on the 3′ end by the beta-tubulin 1 promoter, GFP gene and a 1.6-kb coding region of DYH4. The GFP gene was optimized for *Tetrahymena* codon usage at six positions (Gly24, Arg80, Gly104, Leu201, Ser208 and Gly228) through site-directed mutagenesis, and the gene was amplified using PCR primers containing the sequences for His6- and FLAG-tags (Table 1).

The expression cassette was released from the pGBKT7 vector using *Sma* I and introduced into *Tetrahymena* cells through electroporation. PCR analysis using specific primers to confirm the correct recombination showed 1.8 kb and 2.3 kb bands only in the genomic DNA of the transformant (Fig. 1C and D). This result indicated that the expression cassette was replaced with the target DNA through homologous recombination.

Next, the localization of the recombinant dynein was examined. The GFP fluorescence was clearly observed in the cilia (Fig. 2A). The fluorescence was prominent around the oral apparatus, a location known to contain many cilia. No fluorescence was observed in the control cells (Fig. 2A). These findings indicate that the recombinant dynein was expressed and transferred into the cilia.

The recombinant outer arm dynein was purified from ciliary extract. Anti-FLAG gel selectively bound the recombinant dynein in the high-salt extract of cilia, and 3x FLAG peptide was used to elute the protein from the gel. The purified protein was detected using an anti-GFP antibody and confirmed to be recombinant (Fig. 2B).

The motile activity of the recombinant outer arm dynein was examined using *in vitro* motility assays. In the gliding assay, to

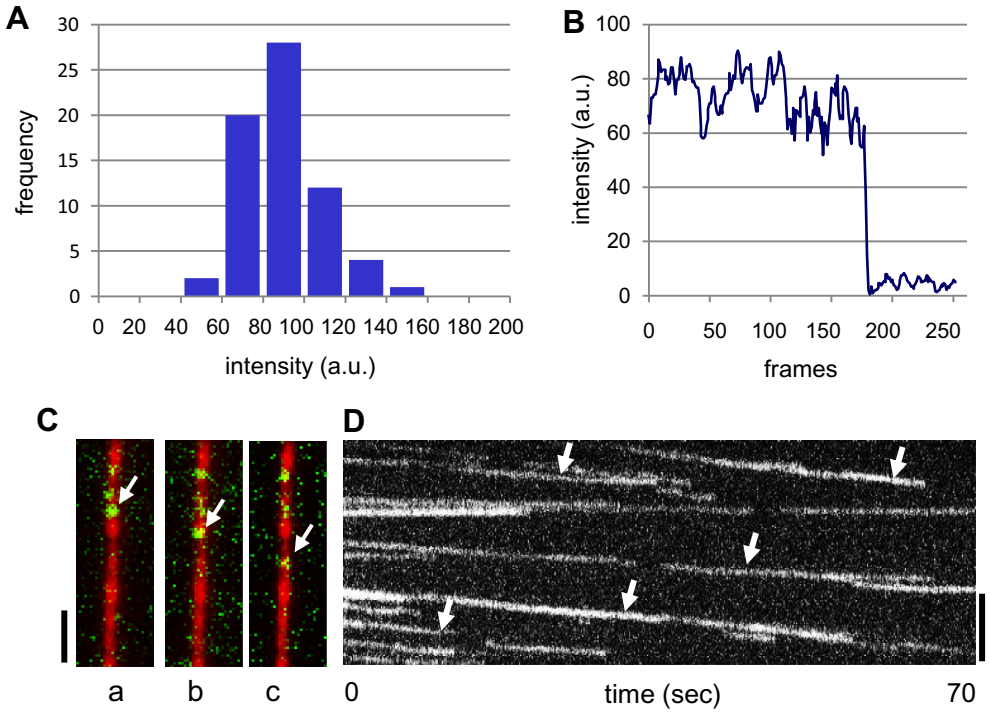

Fig. 4. Single-molecule motility assay. (A) Histogram of fluorescent intensity of a moving spot on microtubules. (B) Photobleaching of the fluorescent spot. (C) Movement of the recombinant dynein in the single-molecule motility assay. Spots of the dynein are colored green, and the microtubule is colored red. The frames represent 0 s (a), 14 s (b) and 28 s (c) after recording was initiated. Scale bar represents 0.5 μm. The arrows represent a moving spot of the dynein. (D) Kymograph in the single-molecule motility assay. The arrows represent the spots moving processively along a microtubule. Scale bars represent 2 μm.

specifically bind the N-terminus of the GFP construct to a glass slide, a glass slide was coated with anti-GFP antibody, and the dynein was then introduced into the flow chamber. The recombinant dynein slid microtubules at a speed of 4.1 ± 1.3 μm/s (average velocity ± standard deviation) (Fig. 3A and C), and also moved polarity-marked microtubules with their minus-end lagging (Fig. 3B and D), which was indicative of minus-end-directed motility. In a control experiment without the anti-GFP antibody, the microtubules did not bind to the flow chamber (Fig. 3C), which indicated that the sliding of microtubules was driven by the recombinant dynein.

For TIRF microscopy, the glass surface was locally illuminated by excitation light, and the movement of fluorescent molecules on the microtubules was directly observed. This assay showed that individual spots of the recombinant dynein moved along the microtubules (Fig. 4C and D). The fluorescence intensities of the moving spots were distributed in a single-peak pattern (Fig. 4A), and the fluorescence was photobleached in a one-step manner (Fig. 4B). These results indicate that single molecules of the recombinant dynein moved processively along the microtubules.

4. Discussion

In this study, recombinant outer arm dynein with motile activity was purified for the first time. Outer arm dynein is implicated in ciliary movement, consistent with purification of the functional protein from ciliary extract. In preliminary experiments, functional motor domain of axonemal dynein was not obtained from cytoplasmic extract. Because it has hundreds of cilia and has been used for molecular genetics and biochemical studies, *Tetrahymena* is a suitable material for the preparation of recombinant dynein.

Dynein is a large molecular motor; it is not easy to construct its expression cassette. This homologous-recombination-based method is useful for generating large constructs because the full length of the dynein HC gene is not necessary and only relatively short DNA fragments are required for the cassette. In this study, a 1.8-kb untranslated region and a 1.6-kb coding region of DYH4 were cloned into the expression cassette; these regions were significantly smaller than the full length genomic DYH4 gene (>15,000 bp).

Although the recombinant dynein slid microtubules in the gliding assay, the gliding velocity was slightly slower than the previously reported values for native outer arm dynein [28]. In a gliding assay, the velocity is significantly influenced by the fixation method on a glass surface. In the previous paper, native dynein was non-specifically absorbed on a glass surface [28], whereas this study used an anti-GFP antibody for fixation, which may have influenced the gliding velocity.

In this study, single-molecule motility analysis was used for the recombinant outer arm dynein. The fluorescent spots of the recombinant dynein moved processively along microtubules. A previous report using optical tweezers showed that the native outer arm dynein of *Tetrahymena* moved processively at low ATP concentration [29], which appears to be consistent with this study.

There are 23 axonemal dynein HC genes in the *Tetrahymena* genome, all of which have been shown to be expressed in vegetatively growing cells [12]. Most of these dyneins have not been separated through ion-exchange chromatography and sucrose density gradient centrifugation. This expression system could be used for the purification of individual axonemal dyneins through the addition of purification tags and could also be applicable to other ciliary proteins, providing opportunities to investigate the molecular properties of these ciliary proteins.

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References

[1] P. Satir, S.T. Christensen, Structure and function of mammalian cilia, Histochem. Cell Biol. 129 (2008) 687–693.
[2] J.L. Badano, N. Mitsuma, P.L. Beales, N. Katsanis, The ciliopathies: an emerging class of human genetic disorders, Annu. Rev. Genomics Hum. Genet. 7 (2006) 125–148.
[3] W.F. Marshall, The cell biological basis of ciliary disease, J. Cell Biol. 180 (2008) 17–21.
[4] R.D. Vale, AAA proteins. Lords of the ring, J. Cell Biol. 150 (2000) F13–F19.
[5] M. Samso, M. Radermacher, J. Frank, M.P. Koonce, Structural characterization of a dynein motor domain, J. Mol. Biol. 276 (1998) 927–937.
[6] S.A. Burgess, M.L. Walker, H. Sakakibara, P.J. Knight, K. Oiwa, Dynein structure and power stroke, Nature 421 (2003) 715–718.
[7] N. Mizuno, A. Narita, T. Kon, K. Sutoh, M. Kikkawa, Three-dimensional structure of cytoplasmic dynein bound to microtubules, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 20832–20837.
[8] I.R. Gibbons, Dynein family of motor proteins: present status and future questions, Cell Motil. Cytoskeleton 32 (1995) 136–144.
[9] T. Yagi, ADP-dependent microtubule translocation by flagellar inner-arm dyneins, Cell Struct. Funct. 25 (2000) 263–267.
[10] K. Kikushima, T. Yagi, R. Kamiya, Slow ADP-dependent acceleration of microtubule translocation produced by an axonemal dynein, FEBS Lett. 563 (2004) 119–122.
[11] R. Kamiya, Functional diversity of axonemal dyneins as studied in *Chlamydomonas* mutants, Int. Rev. Cytol. 219 (2002) 115–155.
[12] D.E. Wilkes, H.E. Watson, D.R. Mitchell, D.J. Asai, Twenty-five dyneins in *Tetrahymena*: a re-examination of the multidynein hypothesis, Cell Motil. Cytoskeleton 65 (2008) 342–351.
[13] Y.Y. Toyoshima, Chymotryptic digestion of *Tetrahymena* 22S dynein. I. Decomposition of three-headed 22S dynein to one- and two-headed particles, J. Cell Biol. 105 (1987) 887–895.
[14] C.J. Brokaw, R. Kamiya, Bending patterns of *Chlamydomonas* flagella: IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function, Cell Motil. Cytoskeleton 8 (1987) 68–75.
[15] T. Oda, T. Yagi, H. Yanagisawa, M. Kikkawa, Identification of the outer-inner dynein linker as a hub controller for axonemal dynein activities, Curr. Biol. 23 (2013) 656–664.
[16] D. Nicastro, C. Schwartz, J. Pierson, R. Gaudette, M.E. Porter, J.R. McIntosh, The molecular architecture of axonemes revealed by cryoelectron tomography, Science 313 (2006) 944–948.
[17] K.H. Bui, H. Sakakibara, T. Movassagh, K. Oiwa, T. Ishikawa, Molecular architecture of inner dynein arms in situ in *Chlamydomonas* reinhardtii flagella, J. Cell Biol. 183 (2008) 923–932.
[18] K.H. Bui, H. Sakakibara, T. Movassagh, K. Oiwa, T. Ishikawa, Asymmetry of inner dynein arms and inter-doublet links in *Chlamydomonas* flagella, J. Cell Biol. 186 (2009) 437–446.
[19] T. Movassagh, K.H. Bui, H. Sakakibara, K. Oiwa, T. Ishikawa, Nucleotide-induced global conformational changes of flagellar dynein arms revealed by in situ analysis, Nat. Struct. Mol. Biol. 17 (2010) 761–767.
[20] K.H. Bui, T. Yagi, R. Yamamoto, R. Kamiya, T. Ishikawa, Polarity and asymmetry in the arrangement of dynein and related structures in the *Chlamydomonas* axoneme, J. Cell Biol. 198 (2012) 913–925.
[21] T. Heuser, M. Raytchev, J. Krell, M.E. Porter, D. Nicastro, The dynein regulatory complex is the nexin link and a major regulatory node in cilia and flagella, J. Cell Biol. 187 (2009) 921–933.
[22] J. Gaertig, L. Gu, B. Hai, M.A. Gorovsky, High frequency vector-mediated transformation and gene replacement in *Tetrahymena*, Nucleic Acids Res. 22 (1994) 5391–5398.
[23] J. Gaertig, T.H. Thatcher, L. Gu, M.A. Gorovsky, Electroporation-mediated replacement of a positively and negatively selectable beta-tubulin gene in *Tetrahymena thermophila*, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 4549–4553.
[24] T.M. Gibson, D.J. Asai, Isolation and characterization of 22S outer arm dynein from *Tetrahymena* cilia, Methods Cell Biol. 62 (2000) 433–440.
[25] M.D. Weingarten, A.H. Lockwood, S.Y. Hwo, M.W. Kirschner, A protein factor essential for microtubule assembly, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 1858–1862.
[26] A. Hyman, D. Drechsel, D. Kellogg, S. Salser, K. Sawin, P. Steffen, L. Wordeman, T. Mitchison, Preparation of modified tubulins, Methods Enzymol. 196 (1991) 478–485.
[27] K. Furuta, Y.Y. Toyoshima, Minus-end-directed motor Ncd exhibits processive movement that is enhanced by microtubule bundling in vitro, Curr. Biol. 18 (2008) 152–157.
[28] R.D. Vale, Y.Y. Toyoshima, Rotation and translocation of microtubules in vitro induced by dyneins from *Tetrahymena* cilia, Cell 52 (1988) 459–469.
[29] E. Hirakawa, H. Higuchi, Y.Y. Toyoshima, Processive movement of single 22S dynein molecules occurs only at low ATP concentrations, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 2533–2537.