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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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0006-291X/

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

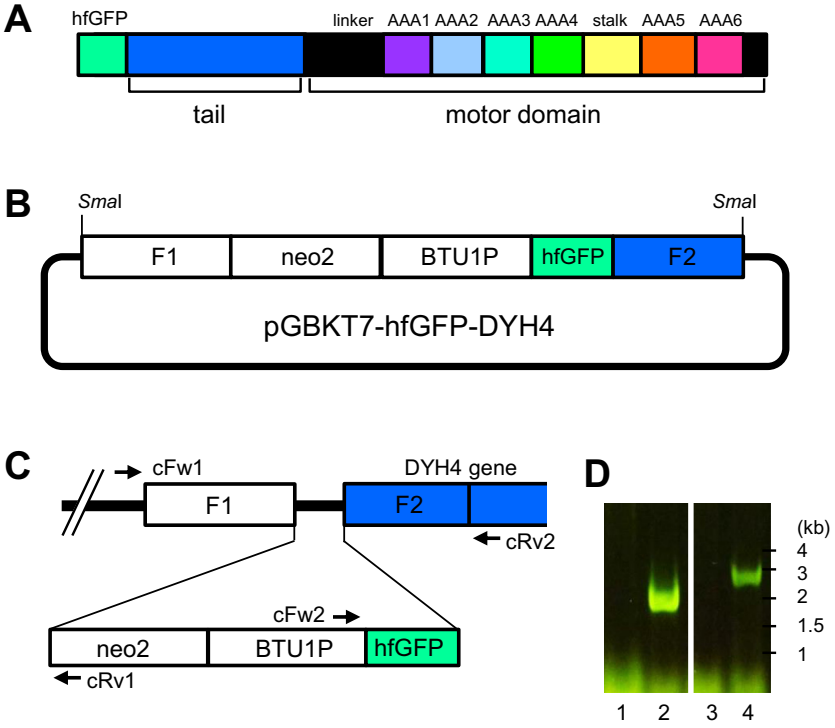

Fig. 1. Construction of the expression cassette and PCR analysis. (A) Schematic representation of DYH4. (B) Construction of the expression cassette. F1: 1.8-kb fragment of an untranslated region of the DYH4 gene; neo2: neomycin-resistance gene cassette [22]; BTU1P: constitutive beta-tubulin 1 promoter; 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.