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Recombinant human cytoplasmic dynein heavy chain 1 and 2: Observation of dynein-2 motor activity in vitro

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ABSTRACT

Cytoplasmic dynein is a microtubule (MT) motor protein comprising two classes: dynein-1 and dynein-2. We purified recombinant human dynein-1 and dynein-2 from HEK-293 cells by expressing the streptavidin-binding peptide-tagged human cytoplasmic dynein-1 and dynein-2 heavy chains (HCs), respectively. Electron microscopy of the purified molecules revealed a two-headed structure composed of characteristic dynein motor domains. In an in vitro MT gliding assay, both dynein-1 and dynein-2 showed minus-end-directed motor activities. This is the first demonstration of dynein-2 motor activity, which supports the retrograde intraflagellar transport role of dynein-2. Our expression system of dynein HCs provides a useful means to investigate dynein functions.

Structured summary of protein interactions:
DYNC1H1 (Homo sapiens) binds to **DYNC1H1 (Homo sapiens)** by electron microscopy (View interaction)
DYNC1H1 (Sus scrofa) binds to **DYNC1H1 (Sus scrofa)** by electron microscopy (View interaction)
DYNC2H1 (Homo sapiens) binds to **DYNC2H1 (Homo sapiens)** by electron microscopy (View interaction)

1. Introduction

Cytoplasmic dynein is a microtubule (MT) motor protein that is involved in various activities within eukaryotic cells. Cytoplasmic dynein comprises two classes: dynein-1 and dynein-2 [1]. Cytoplasmic dynein-1 is a minus-end-directed MT motor protein that moves by the energy derived from ATP hydrolysis [2], and is involved in cell division, cargo transport, and neural development [3,4]. The structure of dynein-1 and its motile properties have gradually been ascertained via electron microscopy, recombinant protein expression, motility assays, and X-ray crystallography [5–8]. In contrast, cytoplasmic dynein-2 is reported to be involved in intraflagellar transport (IFT), which is a bidirectional transport of particles along axonemes in cilia and flagella. Dynein-2 is a putative retrograde IFT motor, which transports IFT particles from the ciliary tip toward the cell body [9]. Recent studies have shown that deficits of dynein-2 heavy chain (HC) cause some human ciliopathies [10]. However, the molecular structure and motile properties of cytoplasmic dynein-2 are yet to be elucidated. In previous studies, the information on dynein-2 motility was limited to in situ data obtained from *Chlamydomonas* and *Caenorhabditis elegans* [11,12].

To date, the expression system of functional cytoplasmic dynein HC has been limited to lower eukaryotes such as *Dictyostelium* and yeast [6,7]. In this report, we purified recombinant human cytoplasmic dynein-1 and dynein-2 from HEK-293 cells by expressing the streptavidin-binding peptide (SBP)-tagged human cytoplasmic dynein-1 HC (DYNC1H1, ~530 kDa) and cytoplasmic dynein-2 HC (DYNC2H1, ~490 kDa). Electron microscopy (EM) showed that both purified dynein-1 and dynein-2 molecules had a two-headed structure composed of two motor domains. Both purified dynein-1 and dynein-2 possessed the minus-end-directed motor activities as demonstrated by an in vitro MT gliding assay. This is the first report to describe the structure and motility of dynein-2 in vitro.

2. Materials and methods

2.1. Generation of cDNA construct and transfection

Full lengths of DYNC1H1 cDNA or DYNC2H1 cDNA were inserted into pcDNA5/FRT/TO vector (Invitrogen). SBP-tag (for purification) and SNAP-tag (for analysis) were inserted at N-termini of both DYNC1H1 and DYNC2H1 (further details are provided in the Supplementary methods).

Abbreviations: IFT, intraflagellar transport; SBP, streptavidin-binding peptide

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HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamine. To generate the SBP–SNAP–dynein-1 HC and SBP–SNAP–dynein-2 HC stable cell lines, the vectors were transfected into the HEK-293 cells using a Lipofectamine reagent and Plus reagent (Invitrogen) according to manufacturer's instructions. The transfectants were screened by hygromycin (100 μg/ml) resistance and Western blots targeting the SNAP-tag.

2.2. Preparation of proteins

After inducing protein expression with 2 μg/ml doxycycline, the HEK-293 cells expressing the recombinant dynein-1 HC or recombinant dynein-2 HC were homogenized and centrifuged in buffer A (50 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 10% sucrose, 5 mM $MgSO_4$, 0.1 mM ATP, 1 mM DTT) containing 0.05% Triton X-100 and complete mini protease inhibitor cocktail (Roche). The lysates were applied to a StrepTrap HP column (GE Healthcare) or a Streptactin-column (IBA) pre-equilibrated with buffer A. The bound proteins were eluted with buffer A containing 2.5 mM desthiobiotin.

Porcine brain dynein-1 and tubulin was prepared as described in [13].

2.3. SDS–PAGE, Western blotting

The proteins were separated by SDS–PAGE with 10% polyacrylamide gels, and stained with Coomassie brilliant blue (CBB). For Western blotting, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using standard techniques. Polyclonal antibodies against SNAP-tag (GenScript, A00684) and DYNC2H1 (a kind gift from Dr. Kazuo Ogawa, NIBB) were both used at a dilution of 1:1000. The polyclonal IC74 (Abcam, ab6304) antibody was used at a 1:2000 dilution and the polyclonal LIC3 (ProteinTech, 15949-1-AP) antibody was diluted 1:500. Positive bands were detected using a BCIP/NBT system (KPL).

2.4. Electron microscopy

The purified proteins (15–30 μg/ml) were applied to carbon grids pre-hydrophilized as described elsewhere [14] and negatively stained with 1.5% uranyl acetate. The specimens were examined in an H-7500 electron microscope (Hitachi) at 15 000× or 40 000× magnification operating at 80 kV. The distance between dynein motor domains was measured using ImageJ software (NIH).

2.5. In vitro MT gliding assay

The in vitro MT gliding assay was observed under a dark-field microscope and analyzed basically as described elsewhere [15]. The motility assay for dynein-1 was carried out in buffer B (10 mM PIPES–KOH, 4 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 10 μM paclitaxel, 1 mM ATP, pH 7.0) containing 50 mM K-acetate. The motility assay for dynein-2 was carried out in buffer B without K-acetate. The MTs that moved continuously at least 2 μm were analyzed to measure gliding velocity.

Polarity marked MTs were prepared as described previously [16].

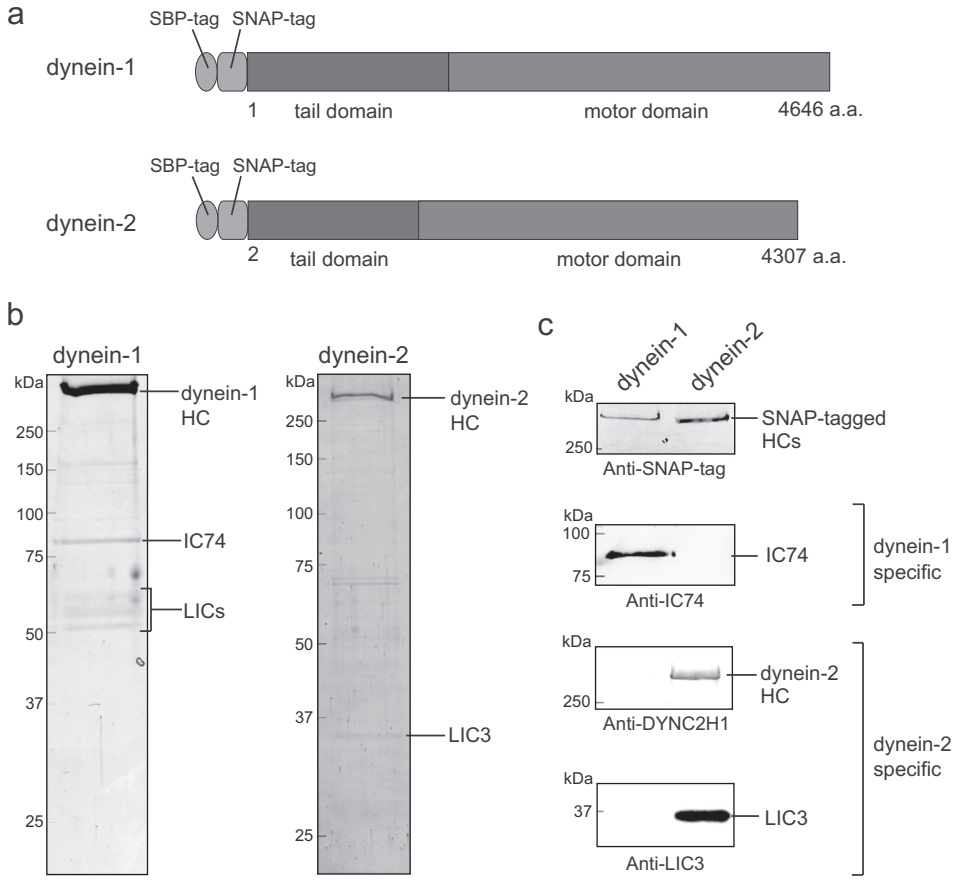

Fig. 1. (a) A schematic diagram of the constructs used in this study. (b) SDS–PAGE of purified dynein-1 and dynein-2 (CBB). (c) Western blot analysis of purified dynein-1 and dynein-2 with the indicated antibodies, showing no cross-contamination between the two dynein classes.