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A Single-headed Recombinant Fragment of *Dictyostelium* Cytoplasmic Dynein Can Drive the Robust Sliding of Microtubules*

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A cytoplasmic dynein is a microtubule-based motor protein involved in diverse cellular functions, such as organelle transport and chromosome segregation. The dynein has two ring-shaped heads that contain six repeats of the AAA domain responsible for ATP hydrolysis. It has been proposed that the ATPase-dependent swing of a stalk and a stem emerging from each of the heads generates the power stroke (Burgess, S.A. (2003) *Nature* 421, 715–718). To understand the molecular mechanism of the dynein power stroke, it is essential to establish an easy and reproducible method to express and purify the recombinant dynein with full motor activities. Here we report the expression and purification of the C-terminal 380-kDa fragment of the *Dictyostelium* cytoplasmic dynein heavy-chain fused with an affinity tag and green fluorescent protein. The purified single-headed recombinant protein drove the robust minus-end-directed sliding of microtubules at a velocity of 1.2 μm/s. This recombinant protein had a high basal ATPase activity ($\sim$4 s^{-1}), which was further activated by >15-fold on the addition of 40 μM microtubules. These results show that the 380-kDa recombinant fragment retains all the structures required for motor functions, *i.e.* the ATPase activity highly stimulated by microtubules and the robust motility.

The cytoplasmic dynein, which is an enormous complex composed of two identical heavy-chains (molecular mass, >500 kDa) as well as several intermediate-, light intermediate-, and light-chains, plays an essential role as the molecular motor for cellular transports and chromosomal segregation (1, 2). The heavy-chain folds into a ring-like head composed of six AAA domains, a stem, and a stalk emerging from the head (3–5). The stalk is the microtubule-binding domain with a long coiled-coil structure (6–8). The swing of the stem and the stalk induced by ATP hydrolysis has been proposed to be responsible for the force-producing power stroke (9). To elucidate further the molecular details of this force production by dynein, it is essential to construct an easy and reproducible system for expressing and purifying recombinant dynein with motile activity. Although it was reported that the purified full-length heavy-chain of rat cytoplasmic dynein expressed in insect cells drove the microtubule sliding (10), it remains to be clarified how the endogenous insect dynein present in the rat dynein preparation contributed to the observed microtubule sliding. Considering the fact that the cytoplasmic dynein is a processive motor and drives the microtubule sliding even at a very low concentration (11, 12), *in vitro* motility assays must be designed to eliminate the possibility that contaminating proteins rather than the recombinant dyneins drive the microtubule sliding.

The 380-kDa fragment of the *Dictyostelium* dynein heavy-chain has been successfully expressed in *Dictyostelium* cells (13). Electron microscopic studies on the fragment have shown that it has a single ring-shaped head and a stalk protruding from it (14). This fragment binds to microtubules in an ATP-sensitive fashion and is susceptible to VO_4-mediated photocleavage (13), an indication that the fragment may have ATPase activity. However, it remains to be shown whether this recombinant dynein fragment is an active motor with microtubule-activated ATPase activity and motility. Here, we have established an expression and purification system of the single-headed *Dictyostelium* 380-kDa dynein fragment fused with the N-terminal His tag and GFP[1] (designated HG380) and have shown that it can drive the robust sliding of microtubules and has high microtubule-activated ATPase activity. Thus, this heavy-chain fragment is an active motor that contains all structures required for force generation.

MATERIALS AND METHODS

Plasmid Constructs and Expression of Cytoplasmic Dynein Fragments—A 14-kbp fragment of the *Dictyostelium* Ax2 genome encoding the full-length cytoplasmic dynein heavy-chain (13, 15) was cloned by genomic PCR. A fragment of the cloned DNA that spans the KpnI site (4,146 bp from the start codon) and the stop codon (14,175 bp) was manipulated so that the His_6 tag and enhanced GFP were fused at the N terminus of the 380-kDa fragment, as shown in Fig. 1. A linker was also inserted so that -Gly-Gly-Gly- would be inserted between the C terminus of GFP and the N terminus of the truncated dynein heavy-chain. This HG380 construct was cloned into an extrachromosomal vector for tetracycline-regulated expression in *Dictyostelium* cells (16). A deletion construct lacking the microtubule-binding site at the end of the stalk (deletion from 10099 to 10476 bp) was also constructed. The latter was designated HG380ΔMT.

The *Dictyostelium* cells used for the tetracycline-regulated expression system were derived from Ax2 as described (16). These cells can be negatively regulated by tetracycline and have antibiotic resistance for G418. For the expression of HG380 or HG380ΔMT, the extrachromosomal plasmids carrying the corresponding genes were introduced into

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[1] The abbreviations and trivial names used are: GFP, green fluorescent protein; HG380, the 380-kDa fragment of dynein heavy chain fused with His tag and GFP; GST·GFP, GFP fused with glutathione *S*-transferase.

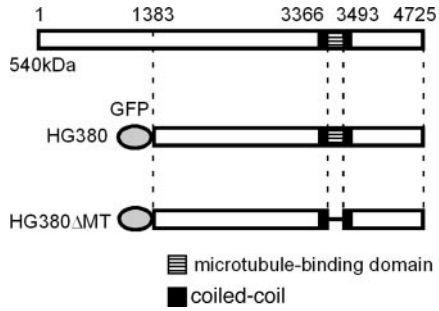

FIG. 1. **Schematic diagram of the expression constructs.** The amino acid residue numbers of the *Dictyostelium* dynein heavy-chain are shown on the bar representing its primary sequence (*top*). At the N terminus of each construct, the His_6 tag and enhanced GFP are fused.

these *Dictyostelium* cells by electroporation as described (17). The transformed cells were grown as described (18) in an axenic medium supplemented with 10 μg/ml blasticidin S, 10 μg/ml G418, and 10 μg/ml tetracycline to inhibit expression of the recombinant proteins. When cells grew to 5×10^6/ml, the medium was replaced with one without tetracycline to start the induction of these recombinant proteins.

Protein Purification—All procedures were carried out at 4 °C or on ice unless otherwise stated. Twenty-four hours after induction, *Dictyostelium* cells expressing HG380 or HG380ΔMT were harvested and resuspended in an equal volume of PMG buffer (100 mM PIPES-KOH, 4 mM $MgCl_2$, 0.1 mM EGTA, 0.9 M glycerol, 10 mM imidazole, 1 mM β-melcaptoethanol, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 50 μg/ml leupeptin, 500 μM phenylmethylsulfonyl fluoride, and 0.1 mM ATP, pH 7.0). The cells were homogenized by sonication and centrifuged at 24,000 $\times$ *g* for 20 min and then at 187,000 $\times$ *g* for 60 min. The high speed supernatant was mixed with Ni-nitrilotriacetic acid-agarose beads and incubated for 60 min. After precipitation and washing, the adsorbed proteins were eluted from the beads four times with PMG buffer supplemented with 250 mM imidazole. The four eluted fractions were mixed and loaded onto a PD-10 desalting column (Amersham Biosciences) to replace the solvent to a PMEG buffer (100 mM PIPES-KOH, 4 mM $MgCl_2$, 5 mM EGTA, 0.1 mM EDTA, 0.9 M glycerol, 1 mM dithiothreitol, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 50 μg/ml leupeptin, 500 μM phenylmethylsulfonyl fluoride, and 0.1 mM ATP, pH 7.0) (19). The eluted fraction from PD-10 was mixed with microtubules prepared from porcine brain (20), paclitaxel, and adenosine 5′-(β,γ-imido) triphosphate so that their final concentrations would be 1 mg/ml, 10 μM, and 0.5 mM, respectively. The mixture was then incubated at room temperature for 30 min. The HG380 molecules complexed with microtubules were pelleted at 46,200 $\times$ *g* for 20 min at 25 °C through a PMEG cushion containing 25% sucrose without ATP. The pellet was suspended in a PMEG buffer containing 10 mM MgATP and 10 μM paclitaxel. The microtubules were pelleted again at 356,000 $\times$ *g* for 10 min at 25 °C to release HG380. The supernatant contained HG380. Glycerol and ATP were included throughout the HG380 preparation because we observed that the protein was unstable without them. The purified proteins were stored on ice and used within 2 days.

Protein Concentrations—Protein concentrations of HG380 were routinely determined by the Bradford method (21) by using bovine serum albumin as a standard. These concentrations were calibrated by using the absorption of GFP as follows. First, we determined the concentration of purified GST·GFP expressed in *Escherichia coli* by using the molecular extinction coefficient of GFP (55,900 at 488 nm; Ref. 22). Then we determined the concentration of HG380 by comparing the fluorescence intensity of GST·GFP at 510 nm (excited at 488 nm) to that of HG380. We did not directly use the molar extinction coefficient of GFP to determine the concentration of HG380 because the GFP absorbance of purified HG380 at 488 nm was not high enough for accurate measurements. The results showed that the Bradford method gave almost the same concentrations as those obtained by the absorption of GFP at 488 nm (within an error of 20%). The concentration of tubulin was also determined by the Bradford method as described (23).

Measurements of Microtubule-activated ATPase Activity—Just before ATPase measurements, the amount of ATP in the purified HG380 was depleted to 0.1 mM by a NAP-5 desalting column (Amersham Biosciences). The ATPase assay was performed using the EnzChek phosphate assay kit (Molecular Probes, Eugene, OR). The assay conditions were as follows: assay buffer, 10 mM PIPES-KOH, 50 mM potassium acetate, 4 mM $MgSO_4$, 1 mM EGTA, 10 μM paclitaxel, and 1 mM dithiothreitol, pH 7.0; HG380, 5 μg/ml (12.5 nM); microtubules, 0 ~40 μM; temperature, 25 °C. The reaction was followed by continuously monitoring the absorbance at 360 nm. For each set of measurements of the microtubule-activated ATPase activity, we checked the background phosphate-release rate of microtubules alone. The average background rate of steady-state increase of absorption at 360 nm in the presence of microtubules without HG380 was ~0.2 nM/s/μM microtubule. Thus, the free phosphate ions present in tubulin preparation or released from the intrinsic GTPase activity of microtubules rarely contributed to the ATPase activities of HG380 even at the highest microtubule concentration used here (40 μM).

The observed specific activity k_{obs} is a sum of the basal ATPase activity k_B and the maximally activated ATPase activity k_{CAT} as shown in Equation 1, where [MT] is the microtubule concentration and K_{MT} is the microtubule concentration at the half saturation of microtubule-activated ATPase activity.

$$k_{obs} = k_{CAT}\{[MT]/(K_{MT} + [MT])\} + k_B\{1 - [MT]/(K_{MT} + [MT])\}$$
$$= (k_{CAT} - k_B)\{[MT]/(K_{MT} + [MT])\} + k_B \quad \text{(Eq. 1)}$$

Thus, (k_{obs}-k_B) values were fitted to the Michaelis-Menten equation to obtain the k_{CAT} and K_{MT} values.

In Vitro Motility Assays—Assays were performed at 25 °C in the ATPase buffer without dithiothreitol. This buffer was used for every step of the motility assay unless otherwise stated. The assay chamber was coated sequentially with streptavidin (1 mg/ml), biotinyl protein G (1 mg/ml), and anti-GFP monoclonal antibody (Qbiogene, Carlsbad, CA) diluted to 100 μg/ml by the assay buffer containing 1 mg/ml bovine serum albumin. The chamber surface was finally blocked with 10 mg/ml bovine serum albumin. The purified HG380 diluted to 50 μg/ml was introduced into the chamber twice at 5-min intervals. After 5 min, 30 μg/ml of paclitaxel-stabilized microtubules was introduced into the chamber. After another 5 min, the assay chamber was washed, and then the assay buffer containing 1 mM ATP was introduced. The control IgG2a (Zymed Laboratories Inc., South San Francisco, CA), which belongs to the same subclass as that of the anti-GFP antibody but does not cross-react with GFP, was diluted to 100 μg/ml and used for control experiments (Fig. 3*b*). Microtubules were observed using dark-field illumination with a ×40 objective lens and recorded with video tape recorder. The recordings were digitized and analyzed. All microtubules longer than 5 μm in randomly selected microscopic fields were chosen for measurements. The sliding velocity of microtubules that translocated in a continuous manner for at least 4 s was measured. Some of the microtubules translocated only occasionally and for only a short period of time. They were counted as non-motile microtubules. For the experiments with the control IgG2a, the motion of microtubules was followed for more than 20 s.

The polarity-marked microtubules were prepared as described (24). The motility assay using polarity-marked microtubules was performed in the assay buffer containing 20 μM paclitaxel.

RESULTS AND DISCUSSION

The single-headed cytoplasmic dynein fragment HG380 was expressed in *Dictyostelium* cells by using the tetracycline-dependent promoter (16). When tetracycline was depleted from the medium to activate the transcription, the recombinant dynein was expressed transiently (Fig. 2*a*, *HSS*). The expressed HG380 was purified first by nickel-nitrilotriacetic acid-agarose beads (Fig. 2*a*, *elution-1–4*) and then by co-precipitation with microtubules. Finally, HG380 was released from the precipitated microtubules by MgATP. When checked by SDS-PAGE, this final HG380 preparation did not show any visible band corresponding to the endogenous dynein heavy-chain (Fig. 2*a*, *+ATP sup*).

We first examined whether HG380 retains motile activity. To eliminate the possibility that the endogenous *Dictyostelium* dynein or kinesin that might be present in the HG380 preparation or the brain dynein that might be present in the tubulin preparation could drive the microtubule sliding, we constructed the following assay system (Fig. 3*a*). A slide glass was covered sequentially with streptavidin, biotinyl protein G, and monoclonal anti-GFP antibody. The HG380 was fixed on the glass surface by this antibody through the GFP moiety, though some of the recombinant protein might have bound to the glass

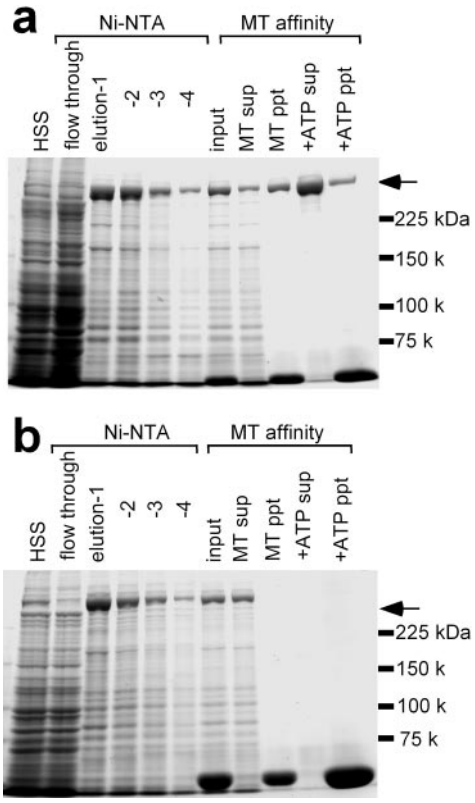

FIG. 2. *a*, purification steps of HG380. A 5.0% acrylamide gel shows the sequential steps of purification of HG380. *HSS*, high-speed supernatant of the cell extract. *Elution-1–4*, four fractions eluted from the nickel-nitrilotriacetic acid beads with 250 mM imidazole. *Input*, mixture of the eluted fractions and microtubules. *MT sup* and *MT ppt*, supernatant and pellet fractions of the microtubule co-precipitation, respectively. *+ATP sup* and *+ATP ppt*, supernatant and pellet fractions after centrifugation of MT ppt resuspended in the presence of 10 mM MgATP, respectively (see "Materials and Methods"). The purified HG380 fraction (*+ATP sup*) was used for ATPase activity measurements and motility assays. The *arrow* indicates the position of HG380. *b*, purification steps of HG380ΔMT. A 5.0% acrylamide gel shows the sequential steps of purification of HG380ΔMT. Most of the HG380ΔMT did not co-precipitate with microtubules (*MT ppt*). The supernatant of the final step (*+ATP sup*) was used for motility assays. The *arrow* indicates the position of HG380ΔMT.

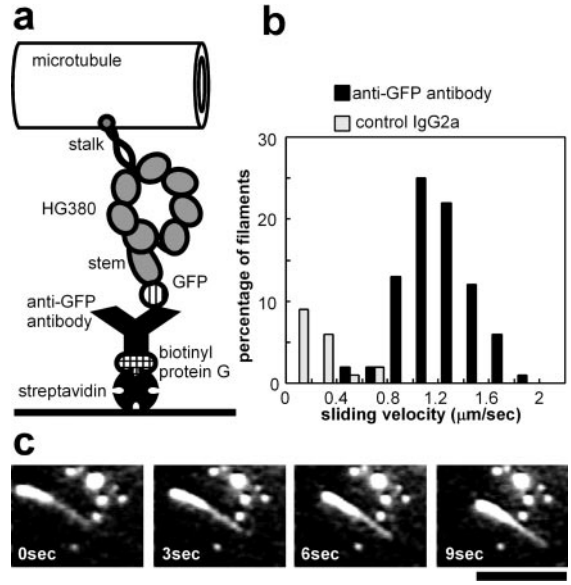

FIG. 3. *a*, schematic drawing of the motility assay system. Monoclonal anti-GFP antibodies were fixed on the glass surface to selectively bind the HG380 molecules through their GFP moiety. *b*, distribution of the sliding velocities of microtubules driven by HG380. The majority (83%) of microtubules smoothly slid on a glass surface covered with anti-GFP antibodies (*filled bars*) with an average velocity of 1.2 ± 0.3 μm/s (n = 83). When control IgG2a was used in place of the anti-GFP antibody (*open bars*), only 18% of the microtubules slid at a much lower rate (average velocity; 0.3 ± 0.2 μm/s (n = 18)). For both cases, about 100 microtubules were counted. The microtubules that did not slide or slid only occasionally for a short period of time were counted as non-motile microtubules and are not displayed on this histogram. *c*, sequential images of a polarity-marked microtubule driven by HG380. The *thick and curved end* marks the minus-end. *Bar*, 10 μm.

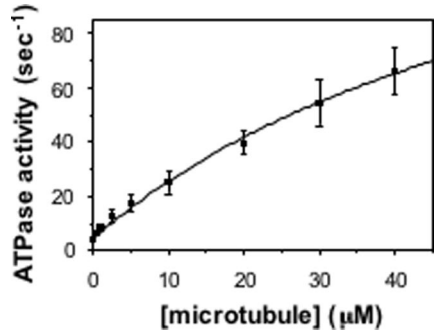

FIG. 4. **Microtubule activation of ATPase activity of HG380.** The solid curve represents a hyperbola with the calculated k_{cat} (160 s^{-1}) and K_{MT} (68 μM). For the calculation, basal activity was subtracted from the measured activities. *Error bars*, S.D. Assay conditions were as follows: assay buffer, 10 mM PIPES-KOH, 50 mM potassium acetate, 4 mM $MgSO_4$, 1 mM EGTA, 10 μM paclitaxel, and 1 mM dithiothreitol, pH 7.0; HG380, 5 μg/ml; microtubules, 0 ~40 μM; temperature, 25 °C.

surface nonspecifically. When the motility assays were carried out in this system, most of the microtubules (83%) slid smoothly with an average velocity of 1.2 μm/s (Fig. 3*b*), which is comparable with the velocity of the brain cytoplasmic dynein (23, 25). In the assays using polarity-marked microtubules, most of these sliding filaments (>98%) moved so that their modified minus-ends were back (Fig. 3*c*), indicating that the minus-end-directed motors drove the robust sliding of these microtubules (24). Thus, it is very likely that the sliding was driven by dynein, not by the plus-end-directed kinesin that might be present in the HG380 preparation as contamination. Some microtubules (17%) did not move, possibly because they bound on the HG380 molecules nonspecifically absorbed on the glass surface.

When the control IgG2a that does not cross-react to GFP was used in place of the anti-GFP antibody, most of the microtubules did not slide on the addition of ATP, although some of them (18%) slid very slowly with an average velocity of 0.3 μm/s (Fig. 3*b*). These results showed that the HG380 molecules trapped with anti-GFP antibodies on the glass surface supported the smooth, robust sliding of microtubules.

To further confirm this notion, we performed mock experiments by using an HG380 derivative in which the microtubule-binding site at the end of the stalk (6, 26) was deleted (Fig. 1, *HG380ΔMT*). The deletion mutant was transiently expressed as in the case of HG380. The expression level of HG380ΔMT was very similar to that of HG380 (Fig. 2*b*, *HSS*). HG380ΔMT was first purified by nickel-nitrilotriacetic acid beads and then co-precipitated with microtubules. Unlike the case of HG380, however, HG380ΔMT did not co-precipitate with microtubules as expected, because it lacked the microtubule-binding site. Thus, only a small amount of proteins was released from the precipitated microtubules on the addition of ATP (Fig. 2*b*, *+ATP sup*). When this ATP-released protein mixture was used for the motility assay in place of HG380, virtually no microtubule was trapped on the glass surface. The result ensured that microtubules on the glass surface covered with HG380 were actually trapped there by binding to this recombinant protein.

This result eliminated the possibility that the endogenous *Dictyostelium* dynein that might be present in the HG380 preparation or the dynein that might be present in the brain tubulin preparation drove the microtubule sliding. Thus, based on these control experiments, we have established that the HG380 molecules were the motor proteins that drove the robust, minus-end-directed sliding of microtubules.

The HG380 protein had a high basal ATPase activity (4.4 s^{-1}), which was further stimulated >15-fold on the addition of 40 μM microtubules (Fig. 4). By fitting the microtubule-activated activities measured in the presence of various concentrations of microtubules with a hyperbola, the maximal ATPase activity stimulated by microtubules (k_{cat}) was calculated to be 160 s^{-1}. The microtubule concentration needed for the half-maximal stimulation (K_{MT}) was 68 μM. Although this K_{MT} value seems to be unusually high compared with other MT-based motor proteins, this value for HG380 was highly dependent on ionic strength, implying that MT and HG380 are held together in the presence of ATP by weak ionic interactions.

Both the basal and microtubule-activated ATPase activities of this single-headed dynein were much higher than those reported for the native two-headed cytoplasmic dyneins (23, 27). One reason for these high ATPase activities of HG380 could be that truncation of the stem tail leaves the single-headed motor domain in an uncontrolled state because of the loss of the other head, both of which could be kinetically coupled in the dimeric molecule during their ATPase cycle. In this context, it must be noticed that the recombinant heterodimeric complex of *Drosophila* cytoplasmic dynein containing only a single motor domain does not show the ATP-dependent MT-binding characteristic of the wild-type cytoplasmic dynein, suggesting that the functional coupling of two motor domains is lost by truncation of one of them (28). Because it has been shown that the conventional kinesin is in a folded and inhibited state with low microtubule-activated ATPase activity and is unfolded by truncation of the tail domain to exhibit a highly stimulated microtubule-activated ATPase activity (29), we may consider the possibility that the ATPase cycle of the motor domain of cytoplasmic dynein is released from the suppressed state by truncation of the stem tail.

In summary, we have established an easy and reproducible method for generating recombinant dyneins with full motor activities: the single-headed recombinant fragment HG380 retains the ability to drive the robust sliding of microtubules and exhibits high microtubule-activated ATPase activity.

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