Overexpression of Cytoplasmic Dynein's Globular Head Causes a Collapse of the Interphase Microtubule Network in *Dictyostelium*

Michael P. Koonce^{*†‡} and Montserrat Samsó^{*}

^{*}Division of Molecular Medicine, Wadsworth Center, Empire State Plaza, Albany, New York 12201-0509; and [†]Department of Biomedical Sciences, State University of New York, Albany, New York 12201-0509

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Cytoplasmic dynein is a minus-end directed microtubule-based motor. Using a molecular genetic approach, we have begun to dissect structure-function relationships of dynein in the cellular slime mold *Dictyostelium*. Expression of a carboxy-terminal 380-kDa fragment of the heavy chain produces a protein that approximates the size and shape of the globular, mechanochemical head of dynein. This polypeptide cosediments with microtubules in an ATP-sensitive fashion and undergoes a UV-vanadate cleavage reaction. The deleted amino-terminal region appears to participate in dimerization of the native protein and in binding the intermediate and light chains. Overexpression of the 380-kDa carboxy-terminal construct in *Dictyostelium* produces a distinct phenotype in which the interphase radial microtubule array appears collapsed. In many cells, the microtubules form loose bundles that are whorled around the nucleus. Similar expression of a central 107-kDa fragment of the heavy chain does not produce this result. The data presented here suggest that dynein may participate in maintaining the spatial pattern of the interphase microtubule network.

INTRODUCTION

In many eukaryotic cells, the interphase microtubule cytoskeleton originates from a centralized organizing center (MTOC) and extends in a radial array throughout the cytoplasm. This spatial organization is maintained as a cell moves and changes shape, suggesting that there are forces responsible for its order (Eute-neuer and Schliwa, 1992; Kellogg *et al.*, 1994). Two obvious means of generating force that could move the MTOC and thus affect the microtubule array, could be derived from assembly/disassembly reactions of the microtubule polymer (Kirschner and Mitchison, 1986; Inoue and Salmon, 1995) and from coupling the microtubules to force-generating molecules such as dyneins or kinesins (Kellogg *et al.*, 1994). These are not mutually exclusive categories and both could work in tandem (for example, see Lombillo *et al.*, 1995).

Several recent studies suggest a role for cytoplasmic dynein as part of a force-generating system for cytoplasmic microtubules. Gene knockouts of the dynein heavy chain in *Aspergillus* (Xiang *et al.*, 1994) and *Neurospora* (Plamann *et al.*, 1994) eliminate the migration of nuclei into the growing germ tubes. Dynein presumably acts via the microtubules to pull the MTOC and attached nucleus into the tube. Knockouts of the heavy chain gene in *S. cerevisiae* perturb mitotic spindle positioning (Eshel *et al.*, 1993; Li *et al.*, 1993). In these studies, a pulling force on the astral microtubules appears necessary to position the spindle in the neck region between the mother and daughter cell. Injection of antibodies that inhibit dynein's ability to move microtubules in vitro block bipolar spindle assembly during prophase in PtK₂ cells (Vaisberg *et al.*, 1993), suggesting a role for dynein in the separation of spindle poles. Pulling forces on astral microtubules in a number of experimental systems have a long and rich history in studies on cell division (Waters *et al.*, 1993; reviewed in Ault and Rieder, 1994).

[‡] Corresponding author: Wadsworth Center, Empire State Plaza, PO Box 509, Albany, NY 12201-0509.

Our work seeks to characterize the function(s) of cytoplasmic dynein in the cellular slime mold *Dictyostelium*. *Dictyostelium* dynein is a high molecular mass complex primarily composed of a 540-kDa heavy chain (DHC), an 80- to 85-kDa intermediate chain, and at least two light chains of approximately 58 and 53 kDa (Koonce *et al.*, 1994). Attempts to disrupt heavy-chain synthesis, either by homologous recombination or by expression of antisense RNA, suggest that this gene is essential for viability in *Dictyostelium* (Koonce and Knecht, unpublished data). As a second approach to understanding dynein function, and one that more directly addresses structure-function relationships, we have sought to express and characterize portions of the coding sequence in the native organism. We demonstrate here that the carboxy-terminal 380 kDa of the DHC primarily encodes the globular head domain, the region of the dynein molecule shown previously to interact with and generate force along the microtubule (reviewed in Vallee, 1993; Holzbauer and Vallee, 1994; Schroer, 1994). In turn, the amino-terminal 158 kDa of the DHC is essential for forming the native dimeric structure and for binding the intermediate and at least one light chain to the intact protein. Furthermore, overexpression of the 380-kDa construct in *Dictyostelium* results in a collapse of the radial interphase microtubule network, suggesting that dynein may play a role in the machinery that keeps the MTOC in the center of the cell and maintains the astral microtubule pattern.

MATERIALS AND METHODS

Plasmid Construction

Cloning of the DHC gene and promoter sequences have been previously described (Koonce *et al.*, 1992, 1994). The entire 14.7-kb promoter/coding sequence was assembled onto a single plasmid. For the constructs described here, portions of the coding sequence and promoter were removed and ligated into a modified plasmid containing a G418 selectable marker and an Actin 8 transcriptional terminator (pBORP; Ostrow *et al.*, 1994). Plasmid DNA was purified using an alkaline lysis/PEG precipitation method (Sambrook *et al.*, 1989) and used for transformation.

For the 380-kDa construct, a 4112-bp fragment between a *Spe*I restriction enzyme site (34 bp downstream from the the predicted translational start) and a *Kpn*I site at 4146 bp was removed, and the two ends were ligated together with a linker. Clones were sequenced using Sequenase 2.0 (United States Biochemicals, Cleveland, OH) to confirm preservation of the open reading frame. This manipulation produced a promoter-driven construct that encoded the initial 12 aa of the amino-terminal end of the DHC, 3 aa due to linker addition, and 3342 aa of the carboxy terminal end (aa 1384 to 4725). This 10.5-kb construct was excised from the plasmid and ligated into pBORP.

Dictyostelium Transformation

AX-2 cells were maintained in liquid culture with HL-5 medium (Franke and Kessin, 1977). Freshly prepared spores were plated onto 10-cm plastic petri dishes and used just before cells reached confluency, at about 10^7 cells/dish. Plasmids were introduced into

AX-2 cells by $\text{Ca}^{2+}\text{PO}_4$ -mediated transformation according to described methods (Knecht *et al.*, 1990; Egelhoff *et al.*, 1991).

Following an overnight recovery in HL-5/Bis Tris, the medium was replaced with HL-5 containing 10 $\mu\text{g}/\text{ml}$ G418 (Sigma Chemical, St. Louis, MO). Culture medium was changed every 3 days. Colonies were detected after 3–5 days of drug selection. Individual colonies were picked with a pipette and transferred to a 24-well plate.

Biochemical Methods

Cells were maintained in petri dishes or in shaking liquid cultures. For protein purification, cells were harvested by low speed centrifugation, suspended in PMEG (100 mM piperazine-*N,N'*-bis-(ethansulfonic acid) [PIPES], 4 mM MgCl_2 , 5 mM EGTA, 0.1 mM EDTA, 0.9 M glycerol) containing 10 $\mu\text{g}/\text{ml}$ leupeptin, 0.1 mg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. Cells were sonicated and centrifuged as described by Koonce and McIntosh (1990), to generate a high speed supernatant (HSS). Following ATP depletion with apyrase, taxol-stabilized, purified bovine microtubules were incubated with the HSS at room temperature for 30 min. The microtubules were pelleted, resuspended in PMEG, and pelleted again through a 20% sucrose cushion. This second pellet was extracted with PMEG containing 10 mM MgATP and recentrifuged. The final supernatant (the ATP extract) was removed and the microtubules were resuspended in the same volume of PMEG (the microtubule pellet). Sucrose density gradient centrifugation and electrophoresis were performed as described by Koonce and McIntosh (1990).

For immunoblot analysis, proteins were transferred to nitrocellulose following electrophoresis. Blots were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20. Primary antibodies and alkaline phosphatase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) were incubated for 1 h at room temperature.

For the initial part of this work (Figure 1), an affinity-purified DHC antibody was kindly supplied by Drs. Eugeni Vaisberg and J. Richard McIntosh (Vaisberg *et al.*, 1993). For subsequent work (e.g. Figure 3), we raised and purified a similar antibody, using the same 78-kDa portion of the dynein heavy chain described in the paper above. A high titer rat serum that recognizes the dynein intermediate chain was kindly supplied by Drs. Leda Triviños-Lagos and Rex Chisholm (Northwestern University Medical School, Chicago, IL).

Electron Microscopy

Samples taken from the sucrose gradient fractions were applied to 300 mesh copper grids using a modification of the double carbon layer technique (Stöffler-Meilike and Stöffler, 1988). The grids had a thin carbon layer over a thicker, holey layer, and were activated with UV light for 30 min. Five microliters of the dynein solution were applied to the grid. After 1 min, several drops of 0.5% uranyl acetate were flowed over the inclined grid. The grid was then immersed in a small container filled with the staining solution onto which a small piece of carbon film had been floated. The grid was centered under the carbon film (sample side up) and pulled up out of the solution. Excess liquid was blotted from the side. Microscopy was performed on a Phillips EM420 operated at 100 kV and at a magnification of 52,000 $\times$ (calibrated using TMV as a reference). Images were recorded using Kodak S0163 film (Rochester, NY).

Immunofluorescence and Other Microscopy

For fluorescence microscopy, cells were fixed with 2.5% formaldehyde in 15 mM PIPES, 1 mM EGTA for at least 10 min at room temperature, then extracted for 5 min with methanol and 1% formaldehyde at -20°C . Subsequent rinses and incubations were with PBS. Primary antibodies were incubated for 1 h at room temperature. Texas Red or FITC-conjugated secondary antibodies (Jackson

Laboratories, West Grove, PA) were incubated for 45 min at room temperature. Following rinses, coverslips were mounted in 5% *n*-propyl gallate, 50% glycerol in PBS, and sealed with nail polish. Images were recorded with a Nikon Microphot microscope on Kodak T-Max 400 film.

A monoclonal antibody raised against *Drosophila* tubulin (Piperno and Fuller, 1985) was used to visualize the microtubules. Hoechst 33342 (Sigma Chemical) was used at 10 μ g/ml in the final rinse to visualize the nuclei. Texas Red-conjugated wheat germ agglutinin (Molecular Probes, Eugene, OR) was used to visualize the Golgi. Cells were incubated with 0.1 μ g/ml in PBS for 15-min followed by 3×5 min washes before the primary tubulin antibody (Zhu *et al.*, 1993).

RESULTS

Biochemical Characterization of the Truncated Dynein Heavy Chains

Expression constructs containing the native DHC promoter and four regions of the coding sequence (Figure

1A) were assembled onto plasmids containing a selectable marker and a transcriptional termination sequence. These constructs were introduced into *Dictyostelium* and clones resistant to the antibiotic G418 were isolated. Cells derived from individual clones were harvested and used to prepare HSS of soluble protein for biochemical analysis. Two of these constructs (380 and 107 kDa) abundantly expressed polypeptides of predicted molecular mass (Figure 1B). Dishes transformed with the full length and the amino-terminal 158-kDa constructs produced significantly fewer colonies (Table 1). Furthermore, none of these colonies showed a marked increase in the full length polypeptide or a detectable expression of a new 158-kDa polypeptide. This reduced transformation efficiency and failure to obtain the predicted products could suggest that overexpression of these constructs is le-

Figure 1. (A) Schematic representation of the expression constructs. The 380- and 107-kDa constructs encode the first 12 aa at the amino-terminus, a linker encoding 3 aa, and aa 1384 to aa 4725 (380 kDa) and to aa 2303 (107 kDa). The native heavy chain contains all 4725 aa of the DHC open reading frame, and the C-terminal truncation encodes aa 1 to 1384. The positions of the translational start and stop sequences are indicated. The closed boxes labeled P-1 through P-4 mark the positions of the four P-loop motifs that form part of consensus sequences for ATP-binding domains. (B) Characterization of 380-kDa and 107-kDa DHC expression. Lanes 1–3 show a Coomassie blue-stained 7.5% acrylamide gel of high speed supernatants (HSS) from wild-type AX-2 cells (lane 1), cells expressing the 380-kDa (lane 2), and the 107-kDa polypeptides (lane 3). Lanes 4–6 show aliquots from the same samples transferred to nitrocellulose and probed with a DHC antibody. The arrow on the left of lane 1 denotes the position of the native heavy chain, and the arrowheads on the right of lane 3 mark the positions of the 380-kDa and 107-kDa polypeptides. Molecular weight standards are marked to the left of lane 1.

Table 1. Quantitation of transformation and expression efficiency

Construct	#Colonies/ #dishes	#Expressing/ #cloned
380-kDa C-terminal domain	8/8	4/6
107-kDa Central Domain	17/3	9/9
538-kDa Full length	6/20	0/6
158-kDa N-terminal domain	4/12	0/4
pBORP (vector control)	15/3	—

The first column of numbers represent the total number of colonies found on the dishes used for transformation after 2 wk of G418 selection. The second column represents the number of colonies expressing the predicted product per colonies that were cloned.

thal in *Dictyostelium*. Low levels of the 158-kDa polypeptide can be detected if the discoidin promoter is used to drive transcription of the same 4.2-kb region of coding sequence, suggesting the basic plasmid design and detection methods are functional (Koonce and Tikhonenko, unpublished data).

Characterization of the 380-kDa and 107-kDa cells is shown in Figure 1B. Similar results were obtained from several clones. The amount of native HC product relative to other cellular proteins does not appear to be substantially altered by transformation. Gel densitometry scans reveal that both the 380-kDa and 107-kDa fragments are expressed at a molar level roughly nine- to tenfold greater than the native DHC. Plasmid integration in *Dictyostelium* often occurs in tandem arrays with 5–200 inserted copies (Nellen *et al.*, 1984; Knecht *et al.*, 1986). Densitometry scans of Southern blots reveal that approximately 15–20 copies of the plasmid integrated in each clone. This likely accounts for the increased level of expression.

The 380-kDa polypeptide copellets with microtubules in an ATP-sensitive manner and undergoes a UV-vanadate cleavage reaction (Figure 2); both are diagnostic features of a dynein molecule (Warner *et al.*, 1989). The molecular mass of the heavy UV cleavage fragment appears to be the same as the native DHC (Koonce and McIntosh, 1990). However, a novel, approximately 60-kDa, light UV cleavage fragment can be detected. This corresponds to the size predicted from the amino-terminal truncation (Figure 2, lane 6).

Although not detectable by Coomassie staining, immunoblot analysis reveals that the 107-kDa polypeptide does show a weak ability to copellet with microtubules, and partitions about equally between the supernatant and microtubule pellet following ATP extraction (Figure 3). UV-vanadate irradiation of the 107-kDa HSS causes a robust cleavage of the native DHC, but no appreciable cleavage of the 107-kDa fragment can be detected.

Figure 4 shows the density gradient sedimentation profile of the ATP extract from the 380-kDa cells. The

Figure 2. Microtubule affinity and UV cleavage of the 380-kDa polypeptide. Coomassie blue-stained gel showing high speed supernatants from wild-type AX-2 cells and 380-kDa cells (lanes 1 and 2, respectively). Lane 3 shows the microtubule pellet following incubation in the 380-kDa supernatant and ATP extraction. Lane 4 shows the ATP extract. The 380-kDa polypeptide as well as the native heavy chain are substantially enriched in this sample. Lanes 5 and 6 demonstrate UV cleavage of a similar ATP extract. Lane 5 shows the unirradiated control, lane 6 shows an aliquot from the same tube following 60 min of UV irradiation on ice with a 365 nm lamp in the presence of 1 mM Na orthovanadate. The two arrowheads (h and l) near the top right of lane 6 demark the positions of the native UV-cleavage fragments, huv and luv (see Koonce and McIntosh, 1990). A substantial amount of the huv fragment is present (derived from both the native and truncated heavy chain), but there is only a small amount of the native luv (derived only from the full length chain). The arrowhead (l') on the right side of lane 6 just below the 66-kDa marker shows a novel luv fragment at the molecular weight predicted for the truncated heavy chain.

native dynein sediments as a 20 S particle and has a profile peak near the bottom in fractions 2 and 3. The 380-kDa fragment sediments as a 10–12 S particle, peaking in fractions 6 and 7. These separate profiles suggest that the native and truncated forms of the DHC do not physically interact with one another.

In the lower panel of Figure 4, the *Dictyostelium* dynein intermediate (IC) and light chains (LC) are visible at the 97-kDa and just below the 66-kDa molecular weight marker positions. As in gradients of native dynein alone, the intermediate chain and lower molecular weight light chain appear to closely follow the sedimentation profile of the native DHC. Immunoblot analysis of a similar gradient using a serum that recognizes the intermediate chain shows the same sedimentation profile as seen in Figure 4. On the other hand, the higher molecular weight light chain sediments with a less defined peak, and appears to smear into the fractions containing the 380-kDa polypeptide. This could suggest some affinity between the 58-kDa light chain and the 380-kDa polypeptide.

Figure 3. Microtubule affinity and UV cleavage of the 107-kDa polypeptide. Lanes 1–4 show a Coomassie blue-stained gel of samples prepared as in Figure 2, except from cells expressing the 107-kDa polypeptide. Lanes 5–8 show an immunoblot replica of lanes 1–4, probed with a DHC antibody. Although not readily detectable by Coomassie staining, the immunoblot shows that a small amount of 107-kDa polypeptide does cosediment with the microtubules (lane 7) and is partially released into the supernatant following ATP extraction (lane 8). The prominent band visible by Coomassie staining in lane 4, near the 97-kDa marker is probably the 105-kDa kinesin heavy chain (McCaffrey and Vale, 1989). Lanes 9 and 10 show an immunoblot of HSS samples equivalent to lane 2. The sample in lane 10 has undergone a 60-min UV-vanadate cleavage reaction. The antibody recognizes epitopes located on the huv fragment, marked to the right of lane 10. There does not appear to be a prominent cleavage of the 107-kDa polypeptide.

Single-headed Structures Are Seen by Electron Microscopy

Figure 5 shows negative-stained images of gradient-purified native dynein and the 380-kDa polypeptide. The top three micrographs show the typical native structure of two globular heads joined at a common base through thin, flexible stalks. Gradient fractions of the 380-kDa heavy chain predominantly contain 10- to 15-nm diameter globular head-like structures. Unlike native dynein, these are single particles. There are no obvious tail-like structures, and there is nothing that appears to connect closely opposed heads into dimers.

The truncated globular heads look slightly larger than their wild-type counterparts. It is not clear whether the heads have stalk-like projections, but these may not necessarily be obvious in this type of imaging. Some orientations of the native dynein show a prominent accumulation of stain in the center of the head structure, suggesting a cavity or depression in this region. This structural feature is also preserved in some views of the truncated protein.

Figure 4. Sucrose density gradient centrifugation of an ATP-extract from 380-kDa cells. The upper panel shows a Coomassie-stained 7.5% gel containing fractions of the entire gradient. Lane 1 represents the bottom of the gradient, and lane 13 represents the top. The native dynein sediments with peaks in fractions 2 and 3, and the truncated heavy chain sediments with peak fractions in lanes 6 and 7. The dynein intermediate chain is seen at the 97-kDa marker position in fractions 2 and 3. The similar molecular weight band in fractions 9 and 10 represents the major kinesin isoform. The lower panel shows the central portion of a silver-stained 10% acrylamide gel, loaded with aliquots of the same samples. The dynein intermediate and the two light chains are visible in this gel, running at the 97-kDa and slightly below the 66-kDa molecular weight markers, respectively.

Spatial Patterns of the Microtubule Cytoskeleton Are Altered

Initial examination of the 380-kDa cells suggests they are either flatter or slightly larger than wild-type cells (Figure 6). These cells are capable of completing a development cycle, and produce viable spores that express the 380-kDa heavy chain (data not shown). However, their rate of growth is significantly slower than wild-type AX-2 cells and the 107-kDa cells (Figure 7).

Immunolocalization of dynein in *Dictyostelium* shows a punctate cytoplasmic staining with some accumulation around the MTOC (Koonce and McIntosh, 1990). Using antibodies that recognize the amino-terminal region of the heavy chain, and thus bind only the native dynein, this pattern is not dramatically altered in the 380- or 107-kDa cells. Antibodies that recognize both the native and truncated heavy chains

Figure 5. Electron microscope characterization of sucrose gradient-purified dynein. The top three panels show representative negative-stained images of native dynein. The two globular heads are oriented to the top of the figure, the tail is oriented toward the bottom. The middle three panels show what appear to be single heads from the peak gradient fraction containing the 380-kDa heavy chain. Even though some particles lie close to one another, structures connecting them cannot be detected. The two arrowheads highlight globular domains that show some stain accumulation in their central region. Scale bar in the six upper micrographs, 25 nm. The bottom panel shows a lower magnification field view of the 380-kDa-containing fraction. Scale bar, 50 nm.

(either the 380 kDa or the 107 kDa) show a large increase in signal intensity, but otherwise the same pattern seen in wild-type cells.

One obvious difference between the 380-kDa cells and either the 107-kDa or wild-type cells lies in the organization of the microtubule array. Figure 8, A–C and D–F, shows microtubule patterns typical of wild-type AX-2 cells and 107-kDa cells, respectively. A single, centrally located MTOC serves as a focus for a radial microtubule array. Figure 9, A–I, shows examples of typical microtubule patterns in the 380-kDa cells. A single organizing center remains the focal point for microtubule nucleation, but the microtubules no longer form a complete radial array. Instead, they form loose whorled bundles that frequently appear to enwrap the nucleus. Eighty to ninety percent of the cells on the coverslip have patterns similar to these

shown here. The remaining 10–20% of the cells look fairly conventional. All stages of mitotic figures appear the same as in wild-type cells (see, for example Figure 9G).

In *Dictyostelium*, the MTOC is tightly attached to the nucleus. This attachment does not appear altered in the 380-kDa cells. Figure 10 shows pairs of cells double-labeled for microtubules (A, C, and E) and DNA (B, D, and F). The MTOC is closely opposed to the nucleus but, unlike wild-type cells, it is usually found adjacent to the plasma membrane. Thus not only is the microtubule pattern altered, but the positioning of the MTOC is aberrant. For additional comparison with wild-type AX-2 cells, see Figure 3 in Zhu *et al.*, 1993.

It is unlikely that this phenotype results from selective disruption of a single gene unrelated to the dynein heavy chain. All four isolated 380-kDa clones express this polypeptide to approximately the same level and all four show the same phenotype. Analysis by Southern blots demonstrates that the plasmid integrated at its homologous site in two clones (but did not disrupt the wild-type gene) and at two different sites in the other clones.

Altered Microtubule Patterns Are Not Due to Bundling

To address whether the whorled patterns result from a general microtubule bundling, the interphase networks were depolymerized with nocodazole, and their recovery following drug removal was monitored by immunofluorescence. Figure 11 shows a time series of microtubule regrowth in the 380-kDa cells. At the zero time point (either after 45 min or 2 h in 10 μ M nocodazole), short microtubules remain at the MTOC. These patterns were indistinguishable from wild-type cells. Furthermore, the MTOC was randomly located in the cytoplasm. Following 2 min of recovery in culture media (Figure 11B), radial asters of microtubules were seen. These grew outward over the next few minutes (Figure 11C) but, by 10 min, whorled microtubule patterns began to form (Figure 11D). This suggests that in the 380-kDa cells, the microtubule/MTOC complex can form a conventional radial array, but this array cannot be maintained once formed.

High resolution video microscopy of *Dictyostelium* reveals that a substantial amount of particle motility occurs in the cytoplasm (Roos *et al.*, 1987). The 380-kDa cells continue to show prominent saltatory movements. However, by video microscopy alone, it is not clear to what extent these movements are microtubule or microfilament based. The structure and position of the Golgi apparatus is largely organized by the microtubule cytoskeleton in eukaryotic cells, as well as in *Dictyostelium*. Localization of the Golgi by labeled wheat germ agglutinin shows a dense clustering of

Figure 6. DIC micrographs of wild-type AX-2 cells (A) and of the cells transformed with the 380-kDa expression construct (B). The cells in B look slightly larger and flatter than their wild-type counterparts. Scale bar, 10 μ m.

structures near the MTOC in both wild-type and 380-kDa cells. In the 380-kDa cells this clustering occurred regardless of whether the MTOC was positioned in the cell center or was against the plasma membrane.

Figure 7. Growth curves of wild-type AX-2 cells, 107-kDa cells, and two clones (#7 and #2) expressing the 380-kDa fragment. The wild-type and 107-kDa cells grow in a similar exponential fashion. Over the same time period, the two 380-kDa cell clones grow at rates similar to each other, but overall much slower than the AX-2 or 107-kDa cells.

DISCUSSION

Using comparative sequence information between cytoplasmic and axonemal dyneins as a guide, we have begun to develop a series of expression constructs to produce large size fragments for structure-function mapping of the dynein heavy chain. In this report, we describe the expression of two such fragments: a 380-kDa carboxy-terminal domain and a 107-kDa portion centered over the primary P-loop involved with ATP hydrolysis. We also describe similar attempts to express the full length DHC and the 158-kDa amino-terminal region missing from the carboxy-terminal constructs.

Structural Characterization of the Truncated Heavy Chains

The 380-kDa carboxy terminal region is generally well conserved among dyneins. Overall it shows a 31.5% identity with sea urchin axonemal β -dynein, as opposed to 19.1% identity in the deleted amino-terminal domain (Gibbons *et al.*, 1991; Oogawa, 1991). Because of the similarity, this region has been proposed to represent the essential structural and mechanochemical features that are conserved within the dynein family (Koonce *et al.*, 1992; Vallee, 1993; Schroer, 1994). The 380-kDa polypeptide shows features characteristic of native dynein: ATP-sensitive microtubule-binding, UV-vanadate-mediated cleavage, and antibody cross-reactivity. It sediments slower than native dynein in a sucrose density gradient, more so than can be accounted for by polypeptide loss due to the truncation. Consis-

Figure 8. (A–C) Three representative examples of wild-type AX-2 cells stained with a monoclonal antibody to tubulin. Similar to most eukaryotic cells, the MTOC serves as a central focus for the microtubule array, which extends in a radial pattern throughout the cytoplasm. (D–F) Representative cells expressing the 107-kDa heavy chain polypeptide. The microtubule arrays in these cells are not dramatically different than wild-type cells. Scale bar, 5 μ m.

tent with this, the 380-kDa polypeptide appears by electron microscopy to be a single particle that approximates the size and shape of the native dynein head. STEM analyses of dyneins have previously estimated the mass of the head domain to be 327 ($\pm$ 50) kDa for rat cytoplasmic and 375- to 400-kDa for two axonemal dyneins (Witman, 1992; Vallee, 1993). The 380-kDa fragment described here generally agrees with these measurements. The resolution of the negative-stained images does not permit a detailed analysis of whether the stalk or a small portion of the tail remains attached to the truncated polypeptide, but this work defines a starting point for a more focused characterization.

Using antibodies, proteolysis, and mutational analyses, the amino-terminal region of the axonemal

dynein heavy chain has been shown to constitute much of this molecule's tail (Mocz and Gibbons, 1993; Sakakibara *et al.*, 1993). Localization of the intermediate chains by immunoelectron microscopy and chemical cross-linking have further shown that these accessory polypeptides bind to the tail region where they may function to link the axonemal heavy chain to its cargo, the A-subfiber microtubule (King and Witman, 1990; King *et al.*, 1991). Using a molecular genetic approach, the data presented here suggest that the amino-terminal domain of the cytoplasmic DHC has a similar role in binding accessory chains. In addition to the intermediate chain, the 158-kDa amino-terminus appears to bind at least one of the major dynein light chains (53 kDa) and to play a primary role in heavy chain dimer formation.

Figure 9. (A–I) Representative images of three different cell clones expressing the 380-kDa polypeptide. All four clones isolated contain similar patterns. An MTOC remains the primary organizing center, but the microtubule pattern forms loose cytoplasmic “whorls.” Furthermore, the microtubule array occupies a much smaller fraction of cellular space than the native arrays (see also Figure 10). Panel G shows a cell in late anaphase. The mitotic figures in these cells do not look different from wild-type cells. Scale bar, 5 μ m.

Density gradient sedimentation suggests some, albeit weak, affinity of the 58-kDa light chain for the 380-kDa fragment. This could suggest that the binding site for this chain is located at or near the site of truncation. Given the general lack of sequence identity in the amino terminal region, it seems surprising that a similar tail structure is found in both axonemal and cytoplasmic dyneins by electron microscopy. On the other hand, the few short conserved amino acid regions might be good candidates to look for functional motifs.

In contrast to the 380-kDa fragment, the 107-kDa portion of coding sequence shows a weak affinity for microtubules and does not undergo a UV-vanadate cleavage reaction detectable by our assay. Either this fragment lacks a complete microtubule-binding motif and/or ATP-catalytic site or additional sequence is necessary for the proper folding and activation of these two domains. Synthesis of the 107-kDa fragment provides a useful expression control for the 380-kDa cells. Since the 107-kDa cells display a microtubule pattern typical of wild-type cells, the phenotype seen

Figure 10. (A, C, and E) 380-kDa cells stained with a tubulin antibody. (B, D, and F) The same respective cells stained with the DNA dye, Hoechst 33342. (G and H) A microtubule and phase contrast image pair. For each image, the approximate position of the MTOC is marked with an arrow. The cell shown in panels E and F is binucleate. Not only has the microtubule pattern lost its radial character, but, in most instances, the MTOC appears to be pushed up against the plasma membrane. For further comparison with wild-type AX-2 cells, see Figure 3 in Zhu *et al.* (1993). Scale bar, 5 μ m.

in the 380-kDa cells is not a general effect due to peptide overexpression.

We failed to obtain clones that overexpress the amino-terminal region or the full length heavy chain.

Figure 11. Microtubule regrowth following nocodazole treatment. Tubulin-stained images of 380-kDa cells 0 min (A), 2 min (B), 5 min (C), and 10 min (D) following nocodazole removal. Within the first few minutes of recovery, the microtubules grow radially from the MTOC in a manner indistinguishable from wild-type cells.

Since the work described above indicates that the amino-terminal region binds the intermediate and light chains, increased expression of this region would likely compete for these polypeptides. Low amounts of the amino-terminal fragment can be expressed from a less active promoter and some of this polypeptide dimerizes with the full length heavy chain (Koonce and Tikonenko, unpublished data). The likely result of this association would be a single-headed dynein with a functional tail, which may or may not be motile. Since previous work suggests that the heavy chain is essential in *Dictyostelium* (Koonce and Knecht, unpublished data), it seems reasonable to conclude that such gross perturbations in the dynein protein pool would also be lethal. A similar argument can be made for the full length sequence: functional dynein is a complex of several gene products that likely interact with multiple cellular targets. Overexpression of one of these components without a concomitant increase in the others would lead to aberrant structural and functional forms of the molecule.

Phenotypic Analysis of the 380-kDa DHC Overexpression

Overexpression of the 380-kDa DHC in *Dictyostelium* results in a morphologic change in the spatial pattern of the interphase microtubule cytoskeleton. The radial array focused at the MTOC is altered to a pattern that in most cells is "whorled" around the nucleus. In addition, the MTOC itself is displaced from the usually central position found in these cells. Microtubules in the 380-kDa cells regrow from the MTOC in a radial pattern following nocodazole-induced disruption, and only after the pattern is established, does it become altered. Thus, we do not feel that a general microtubule-bundling phenomenon is playing a major role in this effect. Rather, we favor the interpretation that the microtubule network has lost a cellular connection necessary to maintain the radial array and thus is "free-floating" in the cytoplasm. As these cells move, the microtubules are compressed into loose bundles. Because the MTOC is physically connected to the nucleus, nuclear movement and rotation probably accounts for the bulk of this compressive force. Given sufficient time for microtubule turnover, nonmotile cells should have arrays similar to wild-type cells. Indeed 10–20% of the 380-kDa cells do have this appearance.

In *Dictyostelium* (Rubino *et al.*, 1984) and other highly motile cells (e.g. Koonce *et al.*, 1984), and in quiescent cells stimulated to move (e.g. Euteneuer and

(Figure 11 cont.) Only once the microtubule array has been established are there noticeable differences between the wild-type and 380-kDa cells. Scale bar, 5 μ m.

Schliwa, 1992), the spatial organization of the microtubules is maintained as cells crawl and change shape. Isolated fragments of cytoplasm can rapidly establish and maintain a radial microtubule array (McNiven and Porter, 1988; Maniotis and Schliwa, 1991). One formative component of this array is the dynamic property of microtubules. The rapid assembly/disassembly reactions ensure a constant supply of microtubule polymer extending radially from the MTOC, which could provide pushing or pulling forces to position the interphase array. The phenotype we observe here suggests that, in *Dictyostelium*, there is at least one additional component involved with maintaining the interphase microtubule array.

A number of studies have previously suggested a minus-end directed force-generating molecule anchored at or near the cell cortex that interacts with microtubules (for example see Geiger *et al.*, 1982; Lutz *et al.*, 1988; Hyman, 1989; Schliwa and Höner, 1993). Studying microtubule reorganization during directed cell movements, Euteneuer and Schliwa (1992) found that the microtubule array must extend to the cell cortex if the MTOC was to reposition itself in response to a motility stimulus. These studies, together with the molecular genetic data described in the introduction provide a strong suggestion that cytoplasmic dynein may serve as a link between the cytoplasm/cell cortex and the microtubule array. The data described in this paper is consistent with this idea.

Since the 380-kDa head domain does not interact directly with the native dynein, then how might this phenotype result? At present there are several possibilities. First, overexpression of the 380-kDa protein could have a direct effect on the microtubule polymer, affecting its dynamics or the motility it supports. Although there is no evidence that a single dynein head can make processive movements along a microtubule, simple on/off reactions could impair kinesin or dynein-mediated traffic. However, given the level of saltatory particle transport seen along linear tracks in living 380-kDa cells, this seems unlikely. If the 380-kDa fragment was excessively binding to microtubules, one might expect to see a microtubule pattern in fixed cells stained for the truncated dynein, and this is not the case.

Second, we cannot rule out the possibility that the overexpression is causing either the dynein head or the native dynein to function in a novel capacity, and is indirectly perturbing some component(s) involved in microtubule organization. Nonetheless, dissecting the pathway by which this occurs should shed light on this interesting process.

Third, because cytoplasmic dynein is believed to be multifunctional, its activities are likely to be regulated (Dillman and Pfister, 1994; Lin *et al.*, 1994). As is the case for axonemal dyneins, there may be a family of "intermediate" light chains (50–60 kDa; Holzbaur and

Vallee, 1994; Schroer *et al.*, 1994) and a number of "true" light chains of much lower molecular mass that are not yet well characterized (for example, see King and Patel-King, 1995). The light chains of other motor molecules are known to regulate motor activities; some of these are bound to or near the globular head (Endow and Titus, 1992). It is possible that the dynein head domain binds one or more accessory light chains (e.g. Witman, 1992). Overexpression of the head would deplete the cellular pool of these molecules, causing the native dynein to be deficient in these capacities. In support of this idea, the *nudG* locus in *Aspergillus nidulans* encodes the 8-kDa dynein light chain first described by King and Patel-King in 1995 (Beckwith and Morris, 1995). A mutation at this locus abolishes the localization of cytoplasmic dynein at the tip of the growing mycelium, suggesting that the low molecular mass chain has some targeting function of the molecule. Further characterization of the 380-kDa polypeptide should identify whether there are low molecular mass dynein-associated polypeptides attached to this molecule.

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REFERENCES

- Ault, J.G., and Rieder, C.L. (1994). Centrosome and kinetochore movement during mitosis. *Curr. Opin. Cell Biol.* 6, 41–49.
- Beckwith, S.M., and Morris, N.R. (1995). Cytoplasmic dynein is missing at the tip in *nudG*, a nuclear migration mutant in *Aspergillus nidulans*. *Mol. Biol. Cell.* 6 (suppl), 5a.
- Dillman, J.F., and Pfister, K.K. (1994). Differential phosphorylation in vivo of cytoplasmic dynein associated with anterogradely moving organelles. *J. Cell Biol.* 127, 1671–1681.
- Egelhoff, T.T., Titus, M.A., Manstein, D.J., Ruppel, K.M., and Spudich, J.A. (1991). Molecular genetic tools for the study of the cytoskeleton in *Dictyostelium*. *Methods Enzymol.* 196, 319–334.
- Endow, S.A., and Titus, M.A. (1992). Genetic approaches to molecular motors. *Annu. Rev. Cell Biol.* 8, 29–66.
- Eshel, D., Urrestarazu, L.A., Vissers, S., Jauniaux, J.-C., van Vliet-Reedijk, J.C., Planta, R.J., and Gibbons, I.R. (1993). Cytoplasmic dynein is required for normal nuclear segregation in yeast. *Proc. Natl. Acad. Sci. USA* 90, 11172–11176.
- Euteneuer, U., and Schliwa, M. (1992). Mechanism of centrosome positioning during wound response in BSC-1 cells. *J. Cell Biol.* 116, 1157–1166.

- Geiger, B., Rosen, D., and Berke, G. (1982). Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T-lymphocytes and target cells. *J. Cell Biol.* 95, 137–143.
- Gibbons, I.R., Gibbons, B.H., Mocz, G., and Asai, D. (1991). Multiple nucleotide-binding sites in the sequence of dynein β -heavy chain. *Nature* 352, 640–643.
- Franke, J., and Kessin, R. (1977). A defined minimal medium for axenic strains of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* 74, 2157–2161.
- Holzbaur, E.L.F., and Vallee, R.B. (1994). Dyneins: molecular structure and cellular function. *Annu. Rev. Cell Biol.* 10, 339–372.
- Hyman, A.A. (1989). Centrosome movement in the early divisions of *Caenorhabditis elegans*: a cortical site determining centrosome position. *J. Cell Biol.* 109, 1185–1193.
- Inoue, S., and Salmon, E.D. (1995). Force generation by microtubule assembly/disassembly in mitosis and related movements. *Mol. Biol. Cell* 6, 1619–1640.
- Kellogg, D.R., Moritz, M., and Alberts, B.M. (1994). The centrosome and cellular organization. *Annu. Rev. Biochem.* 63, 639–674.
- King, S.M., and Patel-King, R.S. (1995). The M_r = 8,000 and 11,000 outer arm dynein light chains from *Chlamydomonas* flagella have cytoplasmic homologues. *J. Biol. Chem.* 270, 11445–11452.
- King, S.M., Wilkerson, C.G., and Witman, G.B. (1991). The M_r 78,000 intermediate chain of *Chlamydomonas* outer arm dynein interacts with α -tubulin in situ. *J. Biol. Chem.* 266, 8401–8407.
- King, S.M., and Witman, G.B. (1990). Localization of an intermediate chain of outer arm dynein by immunoelectron microscopy. *J. Biol. Chem.* 265, 19807–19811.
- Kirschner, M.W., and Mitchison, T. (1986). Beyond self assembly: from microtubules to morphogenesis. *Cell* 45, 329–342.
- Knecht, D.A., Cohen, S.M., Loomis, W.F., and Lodish, H.F. (1986). Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low copy and high copy transformation vectors. *Mol. Cell. Biol.* 6, 3977–3983.
- Knecht, D.A., Jung, J., and Matthews, L.R. (1990). Quantification of transformation efficiency using a new method for clonal growth and selection of axenic *Dictyostelium* cells. *Dev. Genet.* 11, 403–409.
- Koonce, M.P., Cloney, R.A., and Berns, M.W. (1984). Laser irradiation of centrosomes in newt eosinophiles: evidence of a centriole role in motility. *J. Cell Biol.* 98, 1999–2010.
- Koonce, M.P., Grissom, P.G., Lyon, M., Pope, T., and McIntosh, J.R. (1994). Molecular characterization of a cytoplasmic dynein from *Dictyostelium*. *J. Euk. Microbiol.* 41, 645–651.
- Koonce, M.P., Grissom, P.M., and McIntosh, J.R. (1992). Dynein from *Dictyostelium*: primary structure comparisons between a cytoplasmic motor enzyme and flagellar dynein. *J. Cell Biol.* 119, 1597–1604.
- Koonce, M.P., and McIntosh, J.R. (1990). Identification and immunolocalization of cytoplasmic dynein in *Dictyostelium*. *Cell Motil. Cytoskeleton* 15, 51–62.
- Li, Y.-Y., Yeh, E., Hays, T., and Bloom, K. (1993). Disruption of mitotic spindle orientation in a yeast dynein mutant. *Proc. Natl. Acad. Sci. USA* 90, 10096–10100.
- Lin, S.X.H., Ferro, K.L., and Collins, C.A. (1994). Cytoplasmic dynein undergoes intracellular redistribution concomitant with phosphorylation of the heavy chain in response to serum starvation and okadaic acid. *J. Cell Biol.* 127, 1009–1019.
- Lombillo, V., Nislow, C., Yen, T., Gelfand, V.I., and McIntosh, J.R. (1995). Antibodies to the kinesin motor domain and CENP-E inhibit depolymerization-dependent motion of chromosomes in vitro. *J. Cell Biol.* 128, 107–115.
- Lutz, D.A., Hamaguchi, Y., and Inoue, S. (1988). Micromanipulation studies of the asymmetric positioning of the maturation spindle in *Chaetopterus* sp. oocytes. I. Anchorage of the spindle to the cortex and migration of a displaced spindle. *Cell Motil. Cytoskeleton* 11, 83–96.
- Maniotis, A., and Schliwa, M. (1991). Microsurgical removal of centrosomes blocks cell reproduction and centriole generation in BSC 1 cells. *Cell* 67, 495–504.
- McCaffrey, G., and Vale, R.D. (1989). Identification of a kinesin-like microtubule-based motor protein in *Dictyostelium discoideum*. *EMBO J.* 8, 3229–3234.
- McNiven, M.A., and Porter, K.R. (1988). Organization of microtubules in centrosome free cytoplasm. *J. Cell Biol.* 106, 1593–1605.
- Mocz, G., and Gibbons, I.R. (1993). ATP-insensitive interaction of the amino-terminal region of the β heavy chain of dynein with microtubules. *Biochemistry* 32, 3456–3460.
- Nellen, W., Silan, C., and Firtel, R.A. (1984). DNA-mediated transformation in *Dictyostelium discoideum*: regulated expression of an actin gene fusion. *Mol. Cell. Biol.* 4, 2890–2898.
- Ogawa, K. (1991). Four ATP-binding sites in the midrange of the β -dynein heavy chain. *Nature* 352, 643–645.
- Ostrow, B.D., Chen, P., and Chisholm, R. (1994). Expression of a myosin regulatory light chain phosphorylation site mutant complements the cytokinesis and developmental defects of *Dictyostelium* RMLC null cells. *J. Cell Biol.* 127, 1945–1955.
- Piperno, G., and Fuller, M.T. (1985). Monoclonal antibodies specific for an acetylated form of α -tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101, 2085–2094.
- Plamann, M., Minke, P.F., Tinsley, J.H., and Bruno, K.S. (1994). Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. *J. Cell Biol.* 127, 139–149.
- Roos, U.-P., De Brabander, M., and Nuydens, R. (1987). Movements of intracellular particles in undifferentiated amoebae of *Dictyostelium discoideum*. *Cell Motil. Cytoskeleton* 7, 258–271.
- Rubino, S., Fighetti, M., Unger, E., and Cappuccinelli, P. (1984). Location of actin, myosin, and microtubular structures during directed locomotion of *Dictyostelium* amoebae. *J. Cell Biol.* 98, 382–390.
- Sakakibara, H., Takada, S., King, S.M., Witman, G.B., and Kamiya, R. (1993). A *Chlamydomonas* outer arm mutant with a truncated β -heavy chain. *J. Cell Biol.* 122, 653–661.
- Sambrook, E.J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Schliwa, M., and Höner, B. (1993). Microtubules, centrosomes, and intermediate filaments in directed cell movement. *Trends Cell Biol.* 3, 377–380.
- Schroer, T.A. (1994). Structure, function and regulation of cytoplasmic dynein. *Curr. Opin. Cell Biol.* 6, 69–73.
- Stöffler-Meilike, M., and Stöffler, G. (1988). Localization of ribosomal proteins on the surface of ribosomal subunits from *Escherichia coli* using immunoelectron microscopy. *Methods Enzymol.* 164, 503–520.
- Vaisberg, E.A., Koonce, M.P., and McIntosh, J.R. (1993). Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. *J. Cell Biol.* 123, 849–858.

- Vallee, R.B. (1993). Molecular analysis of the microtubule motor dynein. *Proc. Natl. Acad. Sci. USA* *90*, 8769–8772.
- Warner, F.D., Satir, P., and Gibbons, I.R. (eds.) (1989). *Cell Movement*. In: *The dynein ATPases*, vol. 1, New York: Alan R. Liss, 337.
- Waters, J.C., Cole, R.W., and Rieder, C.L. (1993). The force-producing mechanism for centrosome separation during spindle formation in vertebrates is intrinsic to each aster. *J. Cell Biol.* *122*, 361–372.
- Witman, G.B. (1992). Axonemal dyneins. *Curr. Opin. Cell Biol.* *4*, 74–79.
- Xiang, X., Beckwith, S.M., and Morris, N.R. (1994). Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. USA* *91*, 2100–2104.
- Zhu, Q., Liu, T., and Clarke, M. (1993). Calmodulin and the contractile vacuole complex in mitotic cells of *Dictyostelium discoideum*. *J. Cell Sci.* *104*, 1119–1127.