Brain Cytoplasmic and Flagellar Outer Arm Dyneins Share a Highly Conserved M, 8,000 Light Chain*

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Stephen M. King†§, Elisa Barbarese†, James F. Dillman III†‡, Ramila S. Patel-King†, John H. Carson‡, and K. Kevin Pfister**

From the Departments of †Biochemistry and ‡Neurology and the iCenter for Biomedical Imaging Technology, University of Connecticut Health Center, Farmington, Connecticut 06032-3305 and the **Department of Cell Biology, University of Virginia Health Science Center, Charlottesville, Virginia 22908-0439

Sequence comparisons with the M, 8,000 light chain from Chlamydomonas outer arm dynein revealed the presence of highly conserved homologues (up to 90% identity) in the expressed sequence tag data base (King, S. M. & Patel-King, R. S. (1995a)) J. Biol. Chem. 270, 11445–11452). Several of these homologous sequences were derived from organisms and/or tissues that lack motile cilia/flagella, suggesting that these proteins may function in the cytoplasm. In Drosophila, lack of the homologous protein results in embryonic lethality (Dick, T., Ray, K., Salz, H. K. & Chia, W. (1996) Mol. Cell. Biol., 16, 1966–1977). Fractionation of mammalian brain homogenates reveals three distinct cytosolic pools of the homologous protein, one of which specifically copurifies with cytoplasmic dynein following both ATP-sensitive microtubule affinity/sucrose density gradient centrifugation and immunoprecipitation with a monoclonal antibody specific for the 74-kD intermediate chain (IC74). Quantitative densitometry indicates that there is one copy of the M, 8,000 polypeptide per IC74. Dual channel confocal immunofluorescent microscopy revealed that the M, 8,000 protein is significantly colocalized with cytoplasmic dynein but not with kinesin in punctate structures (many of which are associated with microtubules) within mammalian oligodendrocytes. Thus, it appears that flagellar outer arm and brain cytoplasmic dyneins share a highly conserved light chain polypeptide that, at least in Drosophila, is essential for viability.

Dyneins are microtubule-based molecular motors that function in both the cytoplasm and flagellum. Within the flagellum, these enzymes provide the force required for interdoublet microtubule sliding, which forms the basis for flagellar bending (for reviews see various chapters in Warner et al. (1989)). The roles of the cytoplasmic isozymes remain to be fully elucidated. However, to date, known or suspected functions for cytoplasmic dynein include retrograde transport in axons, movement of endosomes and lysosomes, subcellular organization of the Golgi apparatus, nuclear migration, and positioning of the mitotic spindle (Corfhey-Theulaz et al., 1992; Li et al., 1993; Paschal & Vallee, 1987; Schroer et al., 1989; Xiang et al., 1994). There are also several studies supporting a role for dynein during anaphase (Pfarr et al., 1990; Saunders et al., 1995; Steuer et al., 1990).

Recent molecular data demonstrate unequivocally that flagellar and cytoplasmic dyneins are related. Each dynein contains two to three heavy chains (DHCs) of ~520 kDa that contain the motor domains and sites of ATP hydrolysis (see Holzbaur et al. (1994) and Witman et al. (1994) for reviews). However, except in the vicinity of the ATP binding loops, the degree of conservation between DHCs is rather low; e.g. the Chlamydomonas flagellar gamma DHC (Wilkinson et al., 1994) and rat brain cytoplasmic DHC (Mikami et al., 1993) are only ~26% identical overall. In addition, the cytoplasmic and flagellar outer arm isoforms contain related intermediate chains (ICs) of 70–80 kDa (Mitchell & Kang, 1991; Ogawa et al., 1995; Paschal et al., 1992; Wilkerson et al., 1995) that are located at the base of the soluble dynein particle (King & Witman, 1990; Sale et al., 1985). All these IC polypeptides contain multiple WD repeats that likely are involved in protein-protein interactions (Neer et al., 1994; Wilkerson et al., 1995). Within the Chlamydomonas flagellum, ICs are essential for outer arm assembly (Mitchell & Kang, 1991; Wilkerson et al., 1995), and one (IC78) has been shown to mediate attachment of the outer arm motor to its cargo (King et al., 1993, 1995). Multiple forms of the cytoplasmic dynein IC generated by alternative splicing and phosphorylation have been described (Dillman & Pfister, 1994; Paschal et al., 1992; Pfister et al., 1996a, 1996b), and it has been hypothesized by analogy with the flagellar ICs that these are involved in the targeting of cytoplasmic dynein to various intracellular cargoes. Indeed, IC74 has recently been shown to mediate the interaction of cytoplasmic dynein with dynactin (Karki & Holzbaur, 1995; Vaughan & Vallee, 1995). Again, however, the conservation between cytoplasmic and flagellar components is low (~25% identity between Chlamydomonas IC78 and rat brain IC74).

Cytoplasmic dynein also contains several polypeptides (light intermediate chains (LICs)) of ~50–60 kDa that are related to the ABC transporter family of ATPases (Gill et al., 1994; Hughes et al., 1995). No flagellar homologues of the LICs have yet been positively identified, although the trout sperm outer arm dynein does contain several polypeptides of appropriate mass (Gatti et al., 1989).

Both inner and outer arm flagellar dyneins contain one or more polypeptides of 8–30 kDa that have not been reported as components of cytoplasmic dynein. For example, the outer arm from Chlamydomonas flagellum contains eight such light chain

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§ To whom correspondence should be addressed: Dept. of Biochemistry, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06032-3305. Tel.: 860-679-3347; Fax: 860-679-3408; E-mail: king@panda.uchc.edu.

1 The abbreviations used are: DHC, dynein heavy chain; IC, intermediate chain; LC, light chain; LIC, light intermediate chain; TBS, Tris-buffered saline.
polypeptides (LCs), several of which are present in multiple copies (Pfister et al., 1982; Piperno & Luck, 1979). Biochemical and molecular studies of LCs from several outer arm systems have implicated a number of these polypeptides in the cAMP and Ca\(^{2+}\)-mediated regulation of dynein motor function (Barkalow et al., 1994; King & Patel-King, 1995b; Stephens & Prior, 1992).

Recently, we described the molecular cloning of the M, 8,000 and 11,000 LCs from the outer arm of Chlamydomonas flagella (King & Patel-King, 1995a). These polypeptides are thought to associate with the ICs at the base of the soluble dynein particle (Mitchell & Rosenbaum, 1986). Examination of the sequence data bases revealed a number of LC homologues of unknown function that share up to ~90% amino acid sequence identity with the Chlamydomonas M, 8,000 protein. Unexpectedly, several of these homologous sequences were obtained from CDNA libraries derived from organisms and/or tissues that do not contain motile cilia/flagella. For example, CDNAs for M, 8,000 LC homologues have now been identified in nematodes, higher plants, and a number of human tissue-specific libraries including one derived from white blood cells. Identification of these homologues from nonciliated/flagellated systems suggested that these proteins may function in the cytoplasm and raised the intriguing possibility that they represent previously unrecognized components of cytoplasmic dynein (King & Patel-King, 1995a).

Partial and total loss-of-function mutants for a Drosophila M, 8,000 LC homologue have now been isolated by Dick et al. (1996). Partial loss of protein function gave rise to severe pleiotropic morphogenetic deficiencies in bristle and wing development and also caused defects in oogenesis resulting in female sterility. Total loss of function of this protein resulted in mass lethality. Thus, it has become of considerable interest to identify the subcellular location of these LC homologues to determine whether these proteins are in fact components of cytoplasmic dynein and if, as a consequence, the severe phenotypes observed in the Drosophila mutants might be due to the dys-function of that enzyme. In this report, we demonstrate that the mammalian homologue of the M, 8,000 protein is indeed a component of brain cytoplasmic dynein.

MATERIALS AND METHODS

Computational Methods—Searches of the GenBank\(^{30}\) and Expressed Sequence Tag data bases maintained at NCBI were performed using BLAST. Sequence comparisons were generated by the GCG programs PILEUP and GAP using the default parameters for the creation and extension of gaps in the sequences (Devereux et al., 1984).

Anti-M, 8,000 LC Antibody Preparation—A rabbit polyclonal anti-serum was prepared against the Chlamydomonas M, 8,000 LC expressed as a C-terminal fusion with maltose binding protein (R4058; King & Patel-King (1995a)). For some experiments, we obtained a highly purified anti-M, 8,000 LC antibody fraction by blot purification (Olmedo, 1986). In this case, whole bovine serum protein or the factor Xa-digested recombinant protein was separated in a 5–15% acrylamide gradient gel and blotted to nitrocellulose. The appropriate molecular weight regions of the axoneme and recombinant LC blots were excised after 1–2-min incubation with a small volume of 0.2 M glycine, pH 2.16. Eluted antibody was immediately neutralized by the addition of 1.5 M TrisCl, pH 8.8, and stored at ~20 °C until use.

Preparation of Axonemes and Dynein—Wild type axonemes were prepared from Chlamydomonas reinhardtii strain cc124 using standard methods (Witman, 1988). Outer arm dynein was extracted with 0.6 M NaCl and purified by sucrose density gradient centrifugation as described in King et al. (1986).

Bovine and rat brain cytoplasmic dyneins were prepared using a minor modification of the standard ATP-sensitive microtubule affinity procedure followed by sucrose density gradient centrifugation (Paschal et al., 1991). Briefly, a high speed supernatant of homogenized bovine or rat brain was incubated at 37 °C in the presence of taxol to allow microtubule polymerization. Following centrifugation, the microtubule pellet was washed with buffer and then sequentially eluted with 5 mM GTP, 5 mM ATP, and 1.0 M NaCl. The dynein in the ATP eluate then was further purified by centrifugation through 5–20% sucrose density gradients.

Cytoplasmic dynein, kinesin, and dynactin also were isolated directly from rat brain homogenate by immunoprecipitation with antibodies 74-1 (dynein; Dillman & Pfister (1994)), H-2 (kinesin; Pfister et al. (1989)) and 50-1 (dynactin; Paschal et al. (1993)) using the methodology described in Dillman & Pfister (1994). A mock immunoprecipitation containing beads but no primary antibody also was included as a negative control.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Fractions of cytoplasmic and flagellar dyneins at various stages of purification were electrophoresed in 5–15% or 4–16% acrylamide gels, as described in King and Patel-King (1994). These systems ensure that proteins as small as the M, 8,000 LC are separated from the dye front. Polyacrylamide gels were stained first with Coomassie Blue and in some cases, subsequently with silver (Merril et al., 1981). Quantitation of Coomassie Blue-stained gels was performed using an IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA) or a Molecular Dynamics personal densitometer and ImageQuan software. Alternatively, gels were electrophorotized to nitrocellulose (BA-83; Schleicher & Schuell, Keene, NH) in 10 mM NaHCO\(_3\), 3 mM Na\(_2\)CO\(_3\), 0.01% SDS, 20% methanol. Blots were incubated with 5% dried milk, 0.1% Tween 20 in TBS and then probed with primary antibody diluted at least 1:500 in the same buffer. Following several washes, blots were incubated with a peroxidase-conjugated secondary antibody (diluted 1:3000) and washed several more times in 0.1% Tween 20 in TBS and once in 0.5% Triton X-100 in TBS. Antibody reactivity was detected using an enhanced chemiluminescent system (Amersham Corp.) and Fuji RX film. Following immunodetection, blots were stained with Amido Black to reveal total protein.

Peptide Purification and Sequencing—Sucrose gradient-purified bovine brain cytoplasmic dynein was concentrated in a Centricon 30 ultrafiltration unit (Amicon, Danvers, MA) that had previously been incubated with 5% Tween 20 in TBS overnight to reduce nonspecific protein binding. The concentrated sample was then electrophoresed in a 5–15% acrylamide gradient gel and blotted to polyvinylidene difluoride membrane (Immobilon P\(^{\text{TM}}\), Millipore, Woburn, MA) using the conditions described above. The M, 8,000 band was excised from the blot and digested with trypsin. Peptides eluting from the membrane were purified by reverse phase chromatography on an Aquapore RP-300 (C\(_18\)) column. Peptides were sequenced using an Applied Biosystems model 492A sequencer in the protein chemistry facility at the Worcester Foundation for Biomedical Research (Shrewsbury, MA).

Cell Culture and Immunofluorescence Microscopy—Mouse and rat brain oligodendrocytes were cultured, fixed, and treated for immunocytochemistry as described previously (Ainger et al., 1993; Barbarese et al., 1995). Samples were imaged using dual channel confocal fluorescence microscopy. Ratiometric analysis of single particles in the dual channel images was performed as described in Barbarese et al. (1995). Briefly, the pixel coordinates for well resolved particles in the green channel were determined from the intensity in the green channel divided by the sum of the intensities in both channels. Particles labeled in the red channel with little or no label in the green channel have low ratio values (near 0) and appear red. Conversely, particles primarily labeled in the green channel have high values (near 1.0) and are green. Those particles, which are labeled in both channels, are yellow and have intermediate ratio values. Intensity ratios were calculated from several hundred particles for each combination of fluorescently tagged antibodies.

RESULTS

The outer dynein arm from Chlamydomonas flagella contains eight distinct LCs (Pfister et al., 1982; Piperno & Luck (1979); for review see Witman et al. (1994)). Molecular cloning of the M, 8,000 and 11,000 LCs identified a novel protein family including several highly conserved homologues derived from organisms or tissues lacking cilia and flagella (King & Patel-King, 1995a). Recently, several additional mammalian and higher plant homologues of the M, 8,000 flagellar LC have been entered into the Expressed Sequence Tag data base, a related Saccharomyces cerevisiae protein (DYN2) has been revealed by sequence comparison.
the genome sequencing project and a highly conserved homologue from Drosophila also has been described (Dick et al., 1996). Together, these sequences define two distinct subsets within this protein family. The first group comprises the Chlamydomonas flagellar M, 8,000 LC and mammalian, insect, and nematode homologues, all of which share ~90% sequence identity with the Chlamydomonas protein. A comparison between these polypeptides generated by the GCG program PILEUP is shown in Fig. 1. The second group is more diverse (sharing 25–40% identity with the Chlamydomonas M, 8,000 protein) and contains both higher plant and yeast homologues as well as the Chlamydomonas flagellar M, 11,000 LC (not shown).

In order to identify highly conserved M, 8,000 LC homologues, the Chlamydomonas LC was expressed as a C-terminal fusion with maltose binding protein, and the resulting preparation was used to obtain a high affinity polyclonal antiserum (R4058; King & Patel-King (1995a)). This antibody is highly specific for the M, 8,000 LC. When used to probe Chlamydomonas flagellar axonemes or purified outer arm dynein, it reacts solely with M, 8,000 LC and does not react with other dynein or axonomal components, including the homologous M, 11,000 LC with which it shares 42% identity (Fig. 2a). Similar blots were stained with the preimmune serum at a dilution of 1:50. These blots showed no reactive bands, indicating that the preimmune serum does not contain antibodies that recognize Chlamydomonas axonomal proteins. To further assess the specificity of this antibody, it was used to probe rat oligodendrocyte protein on immunoblots. Only a single immunoreactive band migrating at M, ~8,000 was observed (Fig. 2b). Oligodendrocytes lack cilia and flagella, thus suggesting that the rat M, 8,000 LC homologue is a cytosolic protein.

To investigate the hypothesis that the cytosolic M, 8,000 protein is associated with cytoplasmic dynein (King & Patel-King, 1995a), successive fractions from a standard cytoplasmic dynein purification scheme were probed with the R4058 antibody and with antibody 74-1 (Dillman & Pfister, 1994), which reacts specifically with IC74 from cytoplasmic dynein (Fig. 3). Approximately 40% of the M, 8,000 protein and ~90% of IC74 were found in the first microtubule pellet. Thus, there appears to be a significant cytoplasmic pool of the M, 8,000 protein in whole brain that does not associate with microtubules at least under the homogenization/polymerization conditions used here. However, nearly all the M, 8,000 protein found in the first microtubule pellet remained microtubule-bound following sequential incubations with taxol, buffer, 5 mM GTP, 5 mM ATP, and 1.0 M NaCl are shown. Similar samples (lower panels) were blotted to nitrocellulose and probed with antibodies 74-1 and R4058 to reveal IC74 of cytoplasmic dynein and the M, 8,000 protein, respectively.
polyclonal antibody made against the M, 8,000 protein of Chlamydomonas was immunoprecipitated from a rat brain homogenate supernatant with antibodies 74-1 (dynein), H-2 (kinesin), and 50-1 (dynactin). These samples and a bead control containing no antibody were electrophoresed in a 5-15% acrylamide gradient gel and blotted to nitrocellulose. The blot was first probed sequentially with the R4058 and 74-1 antibodies (lower strips) and subsequently stained with Amido Black to reveal total protein (upper panel). The heavy bands migrating at approximately M, 50,000 and 30,000 are due to antibody heavy and light chains. Although a high background was observed in the dynactin lane when the blot was reprobed with the 74-1 antibody, no discrete band corresponding to IC74 was evident. Thus, both the M, 8,000 protein and IC74 were found exclusively in the cytoplasmic dynein fraction.

The R4058 antibody was also used to probe electrophoretic gels of the crude cytoplasmic dynein preparation. A band corresponding to the M, 8,000 protein was seen in both the crude cytoplasmic dynein fraction and in the supernatant of the rat brain homogenate. The M, 8,000 protein was found to be specifically precipitated by the R4058 antibody.

To further investigate the association of cytoplasmic dynein with the M, 8,000 protein, cytoplasmic dynein, kinesin, and dynactin were each purified directly from a rat brain homogenate by immunoprecipitation with specific antibodies, i.e. by a methodology distinct from that used above. An Amido Black-stained blot of proteins in the resulting precipitates and in the bead control is shown in Fig. 5. In each immunoprecipitate, only the cognate complex was observed on the stained blot. When these samples were probed with monoclonal antibody 74-1 and with the R4058 antibody, both IC74 and the M, 8,000 protein were found exclusively in the cytoplasmic dynein sample; no detectable R4058 or 74-1 immunoreactivity was found in either the bead control, kinesin, or dynactin samples. These results strongly support the hypothesis that the M, 8,000 protein is a component of cytoplasmic dynein and further demonstrate that it is not associated with either kinesin or dynactin.

No components of less than ~50 kDa have previously been described associated with cytoplasmic dynein. However, in view of the high degree of sequence identity between LC homologues and our identification of an R4058-immunoreactive protein within cytoplasmic dynein, this complex was examined for the presence of LC components. The bovine brain enzyme purified by ATP-sensitive microtubule affinity, and sucrose density gradient centrifugation was subject to electrophoretic conditions known to resolve the light chains of flagellar dynein both from each other and from the dye front. Upon Coomassie Blue staining (Fig. 6a), the DHC, IC74, and both sets of LICs were readily detected in the cytoplasmic dynein sample. This same gel subsequently was silver-stained (Fig. 6b), which revealed additional discrete bands of M, 22,000, 14,000, and 8,000 in the cytoplasmic dynein sample. Comparison of these low molecular weight components of brain cytoplasmic dynein with the LCs of Chlamydomonas outer arm dynein is shown in Fig. 6c. Bands of similar M, also were observed in immunoprecipitated rat brain cytoplasmic dynein following Coomassie Blue staining (Fig. 6d).

The results discussed above rely solely on antibody reactivity to identify the dynein-associated M, 8,000 protein. In order to further demonstrate the identity of the cytoplasmic dynein-
associated M, 8,000 protein, we isolated bovine cytoplasmic dynein by microtubule affinity and sucrose density gradient centrifugation. Following electrophoresis and transfer to polyvinylidene difluoride membrane, the M, 8,000 protein band was excised and digested with trypsin, and the resulting peptides were purified by reverse phase chromatography. Two peptides were sequenced and yielded a total of 19/19 unambiguous residue assignments (Table I). One peptide is 100% identical with a portion of the human and rat M, 8,000 proteins (cf. Fig. 1). The second bovine peptide is 100% identical to sections of the Chlamydomonas, nematode, and Drosophila proteins; both human and rat sequences show a single conservative substitution. Thus, the peptide sequencing unambiguously confirms the identity of the R4058-immunoreactive M, 8,000 protein associated with cytoplasmic dynein as a highly conserved homologue of the Chlamydomonas flagellar M, 8,000 protein.

Determination of the stoichiometry of the M, 8,000 LC within the dynein particle might provide significant clues as to its function. Previously reported quantitative densitometry of Coomassie Blue-stained gels suggested a very high stoichiometry for the M, 8,000 LC within flagellar dynein, with perhaps as many as ten copies per Chlamydomonas outer arm dynein particle (data quoted in King & Witman (1989)). The composition, polypeptide associations, and stoichiometry of the Chlamydomonas outer arm dynein arm are tabulated in Table II. To determine the stoichiometry of the M, 8,000 protein within cytoplasmic dynein, quantitative densitometry of Coomassie Blue-stained gels of dynein purified both by microtubule affinity/sucrose density gradient centrifugation and by immunoprecipitation was performed (Table III). The data indicate that there is approximately one copy of the M, 8,000 protein per copy of IC74. Thus this protein is indeed a stoichiometric component of cytoplasmic dynein.

Double label immunofluorescence confocal microscopy was used to identify the M, 8,000 protein within mouse brain oligodendrocytes in culture. This preparation is particularly useful for immunolocalization experiments because the highly flattened morphology of the oligodendrocyte disperses cytoplasmic contents in a plane, thereby minimizing interference from overlapping structures above or below the confocal image plane. Merged dual channel images of oligodendrocytes stained to reveal the M, 8,000 LC (green channel) and cytoplasmic dynein, kinesin, or microtubules (red channels in panels a–c, respectively) are shown in Fig. 7. A large number of discrete punctate structures were revealed by the R4058 antibody. These were found in the cell body and aligned along microtubules in the cell processes and apparently single microtubules at the periphery. In addition, many of these particles were present in the thin membranous sheets between processes in regions apparently devoid of microtubules (Fig. 7c). Many but certainly not all of the M, 8,000 LC-containing particles colocalized with structures stained with antibody against cytoplasmic dynein (this is evident as yellow in the merged color images). In contrast, the M, 8,000 protein and kinesin (which was present almost exclusively in the cell body and proximal region of the processes) showed essentially no overlap except in areas of the cell sufficiently thick and crowded with particles to allow superimposition of the two signals.

To quantify the relative distribution of the M, 8,000 protein, IC74, and kinesin in individual particles within oligodendrocytes, we employed single particle ratiometric analysis (Fig. 8). This methodology requires that the particles be well resolved. Thus in oligodendrocytes, ratiometric analysis is limited to structures in the cell periphery and excludes those in the perikaryon where the increased cell thickness results in the overlap of multiple particles. In cells stained to reveal the M, 8,000 protein and IC74, most particles have intermediate ratios (of 0.3–0.9), indicating that they contain both components (Fig. 8a) with approximately uniform relative stoichiometries. The absolute stoichiometries cannot be determined from this analysis because the labeling intensities of the two components depends on a variety of unrelated experimental variables (e.g. antibody concentration, gain, black level adjustments, etc.). There also appears to be a distinct minor population (with a ratio of 0.9/1.0) that contains the M, 8,000 protein but little or no IC74. Importantly, no significant population is revealed that contains only IC74. In contrast, many of the particles that contain the M, 8,000 protein do not contain kinesin and have ratios >0.9 (Fig. 8b).

DISCUSSION

In this report, we demonstrate that a highly conserved homologue of the Chlamydomonas flagellar outer arm M, 8,000 LC is a previously unreported component of purified mammalian brain cytoplasmic dynein. Several lines of evidence support this contention. First, homologues of the flagellar dynein protein that exhibit ~90% sequence identity have been identified from a number of organisms and/or tissues that completely lack cilia and flagella (e.g. white blood cells and nematodes). Second, double label immunofluorescence has established that in mammalian oligodendrocytes (which lack cilia and flagella), this polypeptide shows significant colocalization with cytoplasmic dynein (but not with kinesin) in punctate structures, many of which are associated with microtubules. Third, biochemical analysis revealed several small polypeptides that copurified with cytoplasmic dynein; one of these was specifically recognized by an antibody raised against the Chlamydomonas flagellar protein. Fourth, the identity of this protein as a flagel-

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3 K. K. Pfister and G. B. Witman, unpublished observations.
Peptide sequences obtained from the electrophoretically-purified M, 8,000 protein associated with bovine brain cytoplasmic dynein

| Peptide Sequence                  | Identity (cf. Fig. 1) |
|----------------------------------|-----------------------|
| NADMSEMQQDS                     | 100% with residues 10-21 of the human (T34147) and rat (R47168) M, 8,000 proteins. |
| DIAAYIK                          | 100% with Chlamydomonas (residues 39-45; U19490), nematode (residues 37-43; T26A5-9), and Drosophila (residues 37-43; ddc1) proteins. Both human and rat sequences contain an H instead of a Y. This is scored as a conservative replacement by BLAST. |

A M, 8,000 Cytoplasmic Dynein Light Chain

Table I

Peptide sequences obtained from the electrophoretically-purified M, 8,000 protein associated with bovine brain cytoplasmic dynein

| Peptide | Associations | Stoichiometry | Known Function/Activity |
|---------|--------------|---------------|-------------------------|
| α DHC   | M, 16,000 LC | 1             | ATPase/motor            |
| β DHC   | M, 19,000 LC + IC/LC complex | 1          | ATPase/motor            |
| γ DHC   | M, 22,000 and 18,000 LCs | 1           | ATPase/motor            |
| IC78    | IC/LC complex | 1             | Regulation              |
| IC69    | IC/LC complex | 1             | Regulation              |
| M, 22,000 LC | γ DHC | 2            | NK                      |
| M, 20,000 LC | IC/LC complex | 1          | NK                      |
| M, 19,000 LC | β DHC | 1            | NK                      |
| M, 18,000 LC | γ DHC | 1            | Ca2+ binding           |
| M, 16,000 LC | α DHC | 2            | Sulphhydril oxidoreductase |
| M, 14,000 LC | IC/LC complex | 1       | Sulphhydril oxidoreductase |
| M, 11,000 LC | IC/LC complex | 1       | NK                      |
| M, 8,000 LC | IC/LC complex | 10     | Essential for cytoplasmic dynein activity |

α King et al. (1991), Mitchell & Rosenbaum (1986), Pfister & Witman (1984), Pfister et al. (1982).  
β Based on quantitative densitometry of Coomassie-blue stained gels.  
γ Data quoted in King & Witman (1989).  
δ See Mitchell (1995) and Witman et al. (1994) for reviews.  
ε King et al. (1995).  
η Mitchell & Kang (1993).  
ι NK, not known.  
κ King & Patel-King (1995b).  
λ Patel-King et al. (1996).  
μ Dick et al. (1996).  
ν Dick et al. (1996).

Table II

Polypeptides within Chlamydomonas outer arm dynein

| Polypeptide         | Associations | Stoichiometry | Known Function/Activity |
|---------------------|--------------|---------------|-------------------------|
| AM r 8000 Cytoplasmic Dynein Light Chain |                        |               |                        |
| TABLE III           |               |               |                        |

Stoichiometry of the M, 8,000 protein in cytoplasmic dynein

| Source | Cytoplasmic dynein purification method | IC74 | M, 8,000 protein |
|--------|---------------------------------------|------|-----------------|
| bovine brain | microtubule-affinity and sucrose density gradient centrifugation | 1.0 | 0.8 (1)* |
| rat brain | immunoprecipitation                   | 1.0 | 0.7, 1.2 (1) * |

* The actual values obtained are shown with the most likely number of M, 8,000 proteins per IC74 in parentheses.

...results that the M, 8,000 LC may be essential for cytoplasmic dynein function. However, this conclusion must be tempered by the apparent association of this same protein with some other (as yet unknown) cytosolic component(s) that does not coexist with microtubules (see below). Even so, given a very high sequence conservation, it is likely that this protein plays a generic role in dynein function and consequently implies an important activity for this molecule within the flagellum as well as the cytoplasm.

Only a single gene for the M, 8,000 LC has been reported in Chlamydomonas (King & Patel-King, 1995a) and Drosophila (Dick et al., 1996), although in the latter case two distinct transcripts (one of which is developmentally regulated) are obtained. This suggests that the same M, 8,000 LC isoform may function in both the flagellum and cytoplasm. Furthermore, there are currently 10 human expressed sequence tags in the data base that are sufficiently long to encode most or all of the LC. These derive from a variety of tissue-specific cDNA libraries including brain, testis, white blood cell, liver, adrenal gland, and whole embryo. Sequence analysis indicates that all encode identical proteins, and with one exception all have the same nucleotide sequence in both the coding and 5′-untranslated regions. In one case, derived from whole embryo, there is a 14-base pair insertion within the 5′-untranslated region. However, the remainder of the sequence (including the other 80 base pairs of 5′-untranslated region) is identical to that found for the other cDNAs, suggesting that this small insertion may represent either a splice variant or possibly a cloning artifact. Certainly, this one minor exception provides no evidence for an additional M, 8,000 LC gene.

Chemical dissection of the Chlamydomonas outer dynein arm suggests that the M, 8,000 LC interacts with the intermediate chains (IC78 and IC69) located at the base of the soluble particle (Mitchell & Rosenbaum, 1986) and thus forms part of...
the intermediate chain-light chain complex (see Witman et al., 1991 for review). Both ICs are required for stable assembly of the arm (Mitchell & Kang, 1991; Wilkerson et al., 1995). IC78 is known to mediate the ATP-insensitive or cargo binding association of the outer arm with the doublet microtubule (King et al., 1995), and IC69 apparently has a role in regulating arm activity (Mitchell & Kang, 1993). By analogy, it seems most likely that the cytoplasmic Mr 8,000 LC associates with the ICs (i.e., IC74) of that complex. These proteins are related to their flagellar counterparts (Paschal et al., 1992), contain multiple copies of the WD repeat motif in the C-terminal half of the molecule (Wilkerson et al., 1995), and have recently been shown to be involved in the interaction of cytoplasmic dynein and dynactin (Karki & Holzbaur, 1995; Vaughan & Vallee, 1995).

Given that the Mr 8,000 LC has been extraordinarily well conserved during evolution, it would seem likely a priori that it plays an important role in dynein function; a point supported by the phenotype of the Drosophila mutants. However, at present the nature of that role is obscure. We noted previously that this protein contains a highly amphiphilic α-helical segment (King & Patel-King, 1995a), which may be involved in protein-protein interactions perhaps between the multiple copies of the LC. One possibility is that this protein mediates intradynein associations either between ICs or between ICs and DHCs and/or other LCs.

Fig. 7. Immunolocalization of the Mr 8,000 LC homologue in mammalian cells. Dual channel confocal immunofluorescent localization of the Mr 8,000 LC homologue in oligodendrocytes from mouse brain. Oligodendrocytes were double labeled with antibody R4058 and antibodies against cytoplasmic dynein (a), kinesin (b), and tubulin (c). In each merged dual channel image, the LC is shown in green, and the other component is in red. Thus, the image appears yellow when the two components coincide. In each panel, an enlarged image corresponding to the small white rectangle is shown at bottom left. The scale bars are 10 μm.
Fractionation of whole brain homogenates revealed that 
−40% of the M\(_r\) 8,000 protein sedimented with microtubules 
and of that only −30% was tightly associated with cytoplasmic 
dynein. Of the remainder, some is likely associated with those 
small fractions of cytoplasmic dynein that did not cosediment 
with or did not release from microtubules; some also may 
derive from the many cilia lining the ependyma of the brain as 
any extracted ciliary dynein could attach to microtubules via 
an ATP-insensitive interaction. However, it is unlikely that the 
above could account for all the unbound M\(_r\) 8,000 protein 
remaining in the microtubule-depleted supernatant. Thus, it is 
probable that a significant amount of this protein either is 
bound to some other cytosolic component or is much less tightly 
associated with cytoplasmic dynein such that it dissociates 
during the initial stages of purification. In the latter case, the 
soluble M\(_r\) 8,000 protein pool does appear more than sufficient 
to account for the observed differences in the stoichiometry of 
this component between flagellar and cytoplasmic dyneins.

The possibility of the M\(_r\) 8,000 protein also being a compo-
nent of some other cellular structure is supported by the dis-
tribution of this molecule within Chlamydomonas flagella. Al-
though the M\(_r\) 8,000 protein has been clearly demonstrated to 
be a component of the outer dynein arm by both biochemical 
and genetic criteria (King & Witman, 1989; Luck & Piperno, 
1989; Mitchell & Rosenbaum, 1986; Pfister et al., 1982; Piperno 
& Luck, 1979), axonemes derived from mutants lacking the 
outer dynein arm retain −50% of this polypeptide (Luck 
& Piperno, 1989). Similarly, treatment of wild type axonemes 
with 0.6 M NaCl extracts >90% of the outer dynein arms but 
only −50% of the M\(_r\) 8,000 protein.\(^4\) These data suggest that 
this polypeptide is also a component of some other axonemal 
structure that may be assembled in the absence of the outer 
dynein arm. Further detailed analysis of the distribution of this 
protein in both the axoneme and cytoplasm will be required to 
clarify this important point.

Because the sequence of the M\(_r\) 8,000 protein has been es-
established and because the R4058 antibody does not recognize 
any other dynein proteins, it is clear that this polypeptide is 
indeed a specific component of cytoplasmic dynein and not a 
proteolytic fragment of a higher molecular weight protein. 
However, we did observe two other proteins of 
\(M\(_r\)\) 8,000 in these cytoplasmic dynein samples. Both these 
additional polypeptides also appear to copurify with cytoplas-
mic dynein polypeptides both in sucrose density gradients and 
following immunoprecipitation. Until specific antibodies are 
generated against these molecules or until protein sequence 
data become available, we cannot be certain whether they 
represent bona fide components of cytoplasmic dynein or 
merely proteolytic fragments of, for example, the DHCs. How-
ever, these other small proteins are present in amounts com-
parable with the M\(_r\) 8,000 LC. Thus, because the DHCs, ICs, 
and LCs appear essentially intact in the samples examined, it is 
most probable that cytoplasmic dynein, like outer arm dy-
nein, indeed contains multiple LC components.

The evolutionary relationships between cytoplasmic and 
flagellar outer and inner arm dyneins remain to be fully eluci-
dated. However, recent data have suggested that outer arm 
and cytoplasmic dyneins may be more closely related to each 
other than they are to the inner arm dyneins. This idea is 
supported by several observations including, for example, the 
finding that cytoplasmic and outer arm dyneins contain related 
intermediate chains, whereas the inner arms do not (Mitchell 
& Kang, 1991; Paschal et al., 1992; Wilkerson et al., 1995). Our 
identification of a highly conserved LC found in both cytoplas-
mic and outer arm dyneins but not in inner arm dyneins (see Luck & Piperno (1989), and Witman et al. (1994)) lends further support to this hypothesis.

In oligodendrocytes, kinesin was predominantly found in the perikaryon and cell processes, whereas large numbers of cytoplasmic dynein-stained particles were found in the cell body, processes, and the membranous sheets at the cell periphery. At least at the periphery where these punctate structures were well resolved, it is clear that few, if any, of the cytoplasmic dynein-stained particles were found in the cell body, processes, and the membranous sheets at the cell periphery. At least at the periphery where these punctate structures were well resolved, it is clear that few, if any, of the cytoplasmic dynein-stained particles were found in the cell body, processes, and the membranous sheets at the cell periphery. At least at the periphery where these punctate structures were well resolved, it is clear that few, if any, of the cytoplasmic dynein-containing particles also contained kinesin. The punctate distribution of the M, 8000 protein and cytoplasmic dynein along microtubules in the peripheral regions of the oligodendrocyte also raises certain questions regarding motor function. These regions of the oligodendrocyte cytoplasm, which correspond to the myelin sheath produced by the cell in vivo, are extremely thin and contain few organelles. Granules containing myelin basic protein mRNA and various components of the protein synthetic machinery are present in these regions (Barbarese et al., 1995), and rapid movement of the myelin basic protein mRNA-containing granules has been observed in microinjected cells (Ainger et al., 1993). It is possible that the M, 8000 LC and cytoplasmic dynein are involved in the movement of mRNA-containing granules within the myelin sheath region of the oligodendrocyte.

In conclusion, we demonstrate here that a highly conserved homologue of the M, 8000 Chlamydomonas outer arm dynein LC is a previously unrecognized component of mammalian brain cytoplasmic dynein. Further detailed examination of this protein in both the cytoplasm and the flagellum will enable us to define the role it plays in dynein-mediated microtubule-based motility.

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