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Characterization of DLC-A and DLC-B, Two Families of Cytoplasmic Dynein Light Chain Subunits

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Cytoplasmic dynein is a minus-end–directed, microtubule-dependent motor composed of two heavy chains (~530 kDa), three intermediate chains (~74 kDa), and a family of ~52–61 kDa light chains. Although the ~530 kDa subunit contains the motor and microtubule binding domains of the complex, the functions of the smaller subunits are not known. Using two-dimensional gel electrophoresis and proteolytic mapping, we show here that the light chains are composed of two major families, a higher M_r family (58, 59, 61 kDa; dynein light chain group A [DLC-A]) and lower M_r family (52, 53, 55, 56 kDa; dynein light chain group B [DLC-B]). Dissociation of the cytoplasmic dynein complex with potassium iodide reveals that all light chain polypeptides are tightly associated with the ~530 kDa heavy chain, whereas the ~74 kDa intermediate chain polypeptides are more readily extracted. Treatment with alkaline phosphatase alters the mobility of four of the light chain polypeptides, indicating that these subunits are phosphorylated. Sequencing of a cDNA clone encoding one member of the DLC-A family reveals a predicted globular structure that is not homologous to any known protein but does contain numerous potential phosphorylation sites and a consensus nucleotide-binding motif.

INTRODUCTION

Cytoplasmic dynein is a microtubule-based, minus-end–directed motor found in a wide variety of tissues (McIntosh and Porter, 1989; Vallee and Shpetner, 1990). In axons, cytoplasmic dynein functions as the motor driving fast retrograde vesicle transport (Schnapp and Reese, 1989; Vallee *et al.*, 1989; Hirokawa *et al.*, 1990), whereas in nonneuronal cells, cytoplasmic dynein is implicated in the movement of membrane-bounded organelles toward the centrosome (Schroer *et al.*, 1989; Lacey and Haimo, 1992; Lin and Collins, 1992). The enzyme appears to function in determining the cell-cycle dependent distribution of the Golgi complex around the microtubule organizing center (Corthesy-Theulaz *et al.*, 1992) and is required for transport from early to late endosomes (Aniento *et al.*, 1993). Immunolocalization of cytoplasmic dynein to spindle microtubules and to the kinetochores of mitotic chromosomes (Pfarr *et al.*, 1990; Steuer *et al.*, 1990) suggests that dynein plays a role in chromosome movements. Finally, mitotic spindle formation is disrupted in cells microinjected with anti-dynein antibodies, suggesting a role for the motor in spindle assembly (Vaisberg *et al.*, 1993).

Cytoplasmic dynein was first isolated as a high molecular weight, multi-subunit complex from *Caenorhabditis elegans* (Lye *et al.*, 1987) and bovine brain (Paschal and Vallee, 1987). The complex typically contains a pair of heavy chains (~530 kDa), three intermediate chains (~74 kDa), and a family of light chains (~53–59 kDa in bovine brain and ~52–61 kDa in chick embryo brain) (Neely and Boekelheide, 1988; Collins and Vallee, 1989; Schnapp and Reese, 1989; Hirokawa *et al.*, 1990; Schroer and Sheetz, 1991). The ~530-kDa subunit has been cloned and sequenced from several species, including *Dictyostelium discoideum* (Koonce *et al.*, 1992), *Rattus norvegicus* (Mikami *et al.*, 1993; Zhang *et al.*, 1993), and *Saccharomyces cerevisiae* (Eshel *et al.*, 1993; Li *et al.*, 1993). Analysis of the primary sequences predicts that the cytoplasmic dynein heavy chains contain four putative nucleotide-binding sites, or P-loops (Walker *et al.*, 1982; Saraste *et al.*, 1990), in a central domain that is highly conserved in all cytoplasmic dyneins (~42% identity and ~65% similarity between

the *S. cerevisiae, D. discoideum,* and *R. norvegicus* polypeptides). Disruption of the dynein gene in *S. cerevisiae* has demonstrated that one function of dynein in this organism is to position the mitotic spindle between mother and budding daughter cells, presumably by exerting force on cytoplasmic microtubules (Eshel *et al.*, 1993; Li *et al.*, 1993).

Cloning and sequencing of the cytoplasmic dynein ~74-kDa intermediate chain subunit from rat (Paschal *et al.*, 1993) has revealed that it is similar to the product of the *Chlamydomonas* oda6 gene, a 69-kDa intermediate chain of flagellar outer arm dynein. The similarity between the two polypeptides is greatest in the C-terminal domains that are 26.4% identical. Immuno-EM with anti-69 kDa monoclonal antibodies (mAbs) has shown that the oda6 polypeptide is positioned at the base of the flagellar dynein complex (King and Witman, 1990) where it is closely linked to the flagellar dynein cargo, the A subfiber microtubule in the axoneme (Goodenough and Heuser, 1984). Paschal and coworkers (1993) have proposed that the ~74-kDa cytoplasmic dynein subunit plays a similar role to 69-kDa flagellar dynein subunit in attaching cytoplasmic dynein to its cargo, either a membranous organelle or the kinetochore plate of a chromosome.

Little is known about the function of the light chains or their location within the cytoplasmic dynein molecule. Two possible functions include: 1) linking targeted organelles and/or chromosomes to the ~530 kDa heavy chain motor domain and 2) regulating the mechanochemical cycle of the motor. In the work described here, we use a combination of biochemical and molecular biological methods as a first step in addressing these possibilities. We show that cytoplasmic dynein isolated from chick embryo brain contains several ~52–61-kDa subunits that are tightly associated with the dynein ~530-kDa heavy subunit. Based on two-dimensional (2-D) gel electrophoresis and peptide mapping, the light chains can be divided into two distinct groups: a high M_r (58–61 kDa) group, dynein light chain family A (DLC-A)[1] and a low M_r (52–56 kDa) group, dynein light chain family B (DLC-B). Alkaline phosphatase treatment alters the mobility of four of the light chains to generate two predominant species, indicating that phosphorylation contributes to the diversity of isoforms observed. The primary structure of one DLC-A subunit, determined by DNA sequencing of a full length cDNA clone, predicts a protein of M_r 55,890 with a globular tertiary structure. Analysis of the primary amino acid sequence revealed numerous potential phosphorylation sites and a putative nucleotide binding domain, GEDGAGKT, near the N-terminus. A search of the protein databases uncovered no polypeptides with significant similarity.

[1] Abbreviations used: DLC-A, dynein light chain group A; DLC-B, dynein light chain group B; KI, potassium iodide.

MATERIALS AND METHODS

Materials

Endoproteinase-Glu-C (V8 protease), chymotrypsin, and alkaline phosphatase were purchased from Calbiochem (La Jolla, CA). Immobilon-P polyvinylidene difuoride (PVDF) membrane was obtained from Millipore (Danvers, MA). Ponceau S was purchased from Sigma (St. Louis, MO).

Purification of Chick Embryo Brain and Bovine Brain Cytoplasmic Dynein

High speed supernatant (S2) and Mono-Q-purified cytoplasmic dynein were prepared from 13 d old chick embryo brains and from bovine brain as previously described (Schroer and Sheetz, 1991).

Potassium Iodide (KI) Dissociation of Dynein

Mono-Q-purified dynein was subjected to gel filtration chromatography on a Superose 6 column (Pharmacia Fine Chemicals, Piscataway, NJ) in 10 mM tris(hydroxymethyl) aminomethane (Tris)-HCl pH 7.4, 1 mM EDTA with or without 0.6 M KI. Dynein (1 ml) was either left untreated (control) or brought to 0.6 M KI (KI-extracted), then incubated for 5 min on ice before being injected onto the column. The column was run at 0.5 ml/min, and samples were concentrated by trichloroacetic acid (TCA) precipitation and analyzed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel.

Peptide Mapping

Mono-Q-purified dynein was run on a 0.75-mm, 7.5% SDS-polyacrylamide gel, lightly stained with Coomassie brilliant blue; then individual light chain bands were excised with a razor blade and equilibrated as described (Cleveland, 1983). The bands were then loaded into the sample wells of a 15% gel and overlaid with either 100 ng endoproteinase Glu-C or 125 ng chymotrypsin. Multiple bands were loaded in some lanes to equalize the amounts of the different light chain isoforms.

Peptide Microsequencing

The 59-kDa DLC-A and 56-kDa DLC-B light chain subunits were excised from a Coomassie-stained gel and subjected to digestion with endoproteinase-Glu-C (Endo-Glu-C or V8 protease) (Cleveland, 1983). The peptides were then transferred to PVDF membrane as described (LeGendre and Matsudaira, 1989). Individual bands, visualized by transillumination or by staining with Ponceau S, were excised and subjected to microsequencing on an Applied Biosystems sequencer (Foster City, CA) at the Protein/Peptide Laboratory, The Johns Hopkins School of Medicine.

Alkaline Phosphatase Treatment of Cytoplasmic Dynein

Using spin column chromatography (Neal and Florini, 1973) through Sephadex G-25, Mono-Q purified chick embryo brain cytoplasmic dynein was equilibrated into a buffer containing 50 mM Tris-HCl pH 8.3, 50 mM NaCl, 0.6 mM dithiothreitol, 0.6 mM phenylmethylsulfonyl fluoride, 0.06 mM $ZnCl_2$. Alkaline phosphatase was added to a final concentration of 100 U/ml, and the reaction was incubated at 37°C for 2 h. The dephosphorylation control reaction was supplemented with phosphate buffer pH 8.3 to a final concentration of 80 mM. The extent of dephosphorylation was determined by a SDS-polyacrylamide gel and immunoblotting with the antibody, pAbDLC.

Gel Electrophoresis and Blotting

Proteins were analyzed on 6 or 7.5% SDS polyacrylamide gels as described (Laemmli, 1970). Gels were stained with Coomassie brilliant blue or with silver according to Merril *et al.* (1981). Protein concentrations were determined using either the bicinchoninic acid assay (Smith *et al.*, 1985) or the Bradford assay (Bradford, 1976). SDS-polyacrylamide gels were blotted to Immobilon-P PVDF membrane and probed with primary antibody (pAbDLC), followed by secondary antibody (alkaline-phosphatase–conjugated goat anti-rabbit IgG); immunoreactivity was assayed by the Western Light enhanced chemiluminescence system (Tropix, Bedford, MA).

Antibodies

Affinity Purification of Anti-Dynein Antisera. Two rabbits and one goat (Pelfreeze, Rogers, AR) were immunized repeatedly with 20S chick brain dynein; the goat and one rabbit mounted good responses. An affinity matrix for the purification of anti-dynein antibodies from serum was prepared by coupling Mono-Q-purified dynein (1 mg) to 0.5 ml CNBr-activated Sepharose CL-4B (Pharmacia Fine Chemicals). Serum (1 ml) was diluted threefold in column buffer (CB) of 50 mM Tris-Cl pH 7.5, precleared 15 min at 12 000 × *g*, and applied to the dynein-Sepharose column. The column was washed with 10 vol of CB, 20 vol of CB containing 0.5 M KCl, and 10 more vol of CB. Antibodies were eluted with 1 M KCl, 100 mM diethanolamine containing 2.5 mg/ml bovine albumin and immediately dialyzed against CB and then 50 mM NaH_2PO_4, 1 mM EDTA. Both the goat and rabbit affinity-purified antibodies (pAbD1 and pAbD2, respectively) were monospecific for dynein, recognizing only the dynein heavy, intermediate, and light chains on immunoblots of chicken brain extracts.
Production of Antibody to the Bacterially Expressed Dynein Light Chain. The initial cDNA clone, cDNA 58.1, was cloned into the bacterial expression vector, pMALcI (New England Biolabs, Beverly, MA) for expression of a maltose-binding protein-dynein light chain fusion protein. The fusion protein was purified to homogeneity on a maltose-binding protein affinity column, mixed with RIBI adjuvant (Hamilton, MT) and used to repeatedly immunize two rabbits (Spring Valley Labs, Woodbine, MD). Both animals mounted good responses, and the serum (pAbDLC) was monospecific for the dynein light chains with stronger reactivity to the higher M_r forms (DLC-A). Whole rabbit antiserum (pAbDLC) was used in the experiments in Figure 4, A and C.

Preparation and Hybridization of RNA

Total RNA was prepared from 13-d chick embryo brains as previously described (Gill *et al.*, 1991). Selection for polyA+ mRNA was done with Dynabead oligodT-linked magnetic beads (Dynal, Oslo, Norway). Denaturing RNA gels were prepared, blotted, and hybridized essentially as previously described (Gill *et al.*, 1991). The ^{32}P-labeled hybridization probe was prepared from the entire 2358 basepairs (bp) of dynein light chain cDNA 58.1 by random priming.

Identification of 58-kDa Light Chain Dynein cDNA Clones by Expression Library Screening

A λgt11 chick embryo library (obtained from B. Vennstrom, European Molecular Biology Laboratory, Heidelberg, Germany) was screened using an affinity-purified goat polyclonal antibody (pAbD1) that reacts with all cytoplasmic dynein subunits. Bound primary antibody was detected with ^{125}I-labeled sheep anti-goat IgG (ICN, Costa Mesa, CA). Clones were plaque purified and DNA isolated as previously described (Lopata *et al.*, 1983). The DNA from all the clones was digested with *Eco*RI and ligated into the *Eco*RI site of pBluescript KS II+ for subsequent manipulations.

Isolation of the Full-length cDNA

RACE-PCR (rapid amplification of cDNA ends-polymerase chain reaction) (Frohman, 1988) was used to obtain the putative ATG start codon of the cDNA. A gene specific primer, 50 RT: 5′-CTCGTTTGGTCATCTCGATCTTCATCATGC-3′ (nucleotides [nt] 320–349) (Figure 5A) was used to prime synthesis of first strand cDNA using poly A+ mRNA from 13-d chick embryo brains. The reverse transcription was done at 70°C with *Tth* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). After dA tailing, the PCR reactions were performed essentially as described (Frohman, 1988). A primary PCR reaction was performed with the gene specific primer 50-1, 5′-GCCGGATCCATTCAAATACAAATACTCCATGCC-3′ (nt 294–318 with a *Bam*HI site at the 5′ end) (Figure 5A), and a primer matching the synthesized dA tail 5′ AMP, 5′-GACTCGAGTCGACATCGATTTTTTTTTTTT-TTTTT-3′. The PCR products from the primary reaction were reamplified with a nested gene specific primer reaction 50-2, 5′-GCCGGATCCATTCCTCGATTCCTTGAATTTTTCC-3′ (nt 255–279 with a *Bam*HI site at the 5′ end) (Figure 5A) and 5′ AMP. All PCR reactions were 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min using AmpliTaq (Perkin Elmer Cetus, Norwalk, CT) in a PCR buffer supplemented with 2.5% formamide. The final 279-bp PCR product was gel purified and ligated into the TA cloning vector (Invitrogen, San Diego, CA) for sequence analysis.

DNA Sequencing

A series of nested deletions were made into both ends of cDNA 58.1 using ExoIII (Boehringer Mannheim, Indianapolis, IN), and the deleted fragments were cloned into M13 for single stranded sequencing. Additional clones and PCR-generated products were sequenced using a double-stranded method. Sequenase 2.0 (United States Biochemical, Cleveland, OH) was used for all sequencing reactions. The DNA sequence from both strands was assembled using the VAX/Wisconsin GCG DNA analysis program.

In Vitro Transcription/Translation of cDNA

To construct a full length cDNA (cDNA 58.2) for in vitro protein expression, two PCR primers, 50-10 and 50-2, were used to amplify a DNA segment containing the ATG start codon embedded within a *Nde* I restriction site. The sequence of the 50-10 primer is 5′-GGCACATATGGCGGCGGTGGGGAGAGCC-3′ (the underlined sequence corresponds to nt 33–53 [the ATG start codon begins at nt 33] with a *Nde* I site at the 5′ end) (Figure 5A). The DNA substrate for this PCR reaction was the 279-bp RACE-PCR clone that extended beyond the putative ATG start codon. To assemble a full length construct, the original cDNA 58.1 clone was first cloned as a 2358-bp *Eco*RI fragment 3′ to the T7 promoter in the pVEX expression vector (pVEX was obtained from Sankar Adhya, Lab of Molecular Biology, National Cancer Institute, National Institutes of Health [NIH]). The amplified DNA fragment containing the ATG start codon was then inserted using *Nde* I and *Ava* I sites (*Ava* I is an internal site near the 5′ end of cDNA 58.1), resulting in the final full length cDNA, cDNA 58.2. T7 RNA polymerase (ProMega Biotech, Madison, WI) was used to synthesize the mRNA encoding the full length cDNA. After phenol/chloroform extraction and ethanol precipitation, the mRNA was translated using a rabbit reticulocyte translation mix (GIBCO/BRL, Gaithersburg, MD) containing ^{35}S-methionine and analyzed by SDS-polyacrylamide gels.

RESULTS

The Light Chain Isoforms in Chicken Cytoplasmic Dynein fall into Two Distinct Families

To characterize the complexity of the light chain isoforms in chick embryo brain cytoplasmic dynein, Mono-Q-purified dynein was analyzed by SDS-polyacrylamide gel electrophoresis (Figure 1A). Several distinct isoforms of the light chains (52, 53, 55, 56, 58, 59, 61 kDa) could be resolved by this method. A similar pattern

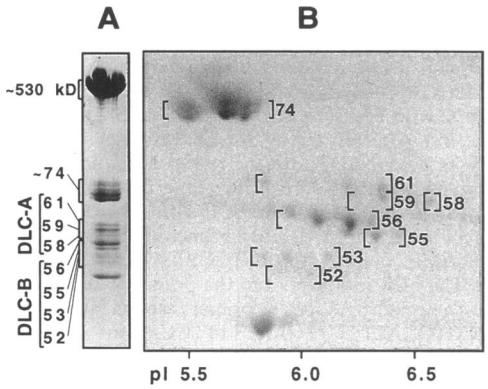

Figure 1. The different light chains of chick embryo brain cytoplasmic dynein have different but overlapping isoelectric points. Mono-Q-purified cytoplasmic dynein was electrophoresed on 1-D 7.5% SDS-polyacrylamide (A) and 2-D polyacrylamide (B) gels and stained with Coomassie blue. The positions of DLC-A (61, 59, and 58 kDa) and DLC-B (56, 55, 53, and 52 kDa) polypeptides are marked on both gels. The band below DLC-B and the spot at pI $\approx$ 5.8 near the bottom of the 2-D gel panel is actin, which is present in trace amounts in dynein purified on the Mono-Q column (Schafer *et al.*, 1994).

was seen in every chicken cytoplasmic dynein preparation, making it unlikely that the multiple bands are products of in vitro proteolysis. When the light chain isoforms were further resolved by 2-D electrophoresis (Figure 1B), all except the 58-kDa subunit were found to be comprised of several pI isoforms.

To determine to what extent the individual light chains were related, each polypeptide resolved on the one-dimensional (1-D) gel was subjected to proteolytic mapping. The individual subunits (52, 53, 55, 56, 58, 59, and 61 kDa) were isolated from a SDS-polyacrylamide gel, digested with either chymotrypsin or endoproteinase-Glu-C (endo-Glu-C), and the products were displayed on a second SDS-polyacrylamide gel (Figure 2). The 58-, 59-, and 61-kDa subunits yield similar proteolytic patterns when digested with either protease (endo-Glu-C, lanes 1–3; chymotrypsin, lanes 8–10). A similar result was seen with light chain subunits 52, 53, 55, 56 kDa (endo-Glu-C, lanes 4–7; chymotrypsin, lanes 11–14). Based upon the peptide mapping data and the M_r we have assigned the isoforms to two groups, the higher M_r group (58, 59, 61 kDa) that we name dynein light chain group A (DLC-A) and the lower M_r group (52, 53, 55, 56 kDa) that we name dynein light chain group B (DLC-B).

The DLC-A and DLC-B Polypeptides Are Tightly Associated with the Cytoplasmic Dynein Heavy Chain

To examine the molecular interactions between the subunits of chick embryo brain cytoplasmic dynein, the enzyme was treated with the chaotropic salt, KI, and then resolved by gel filtration chromatography on a Superose 6 fast-performance liquid chromatography (FPLC) column. Whereas treatment of Mono-Q-purified dynein with 0.6 M KI quantitatively removed the ~74 kDa dynein intermediate chain polypeptides (Figure 3, lanes 8–13) from the ~530 kDa heavy chains, the dynein light chain polypeptides remained tightly associated (Figure 3, lanes 2–6). Only at significantly higher KI concentration (0.9 M) are the light chain subunits extracted from the ~530 kDa heavy subunits (unpublished observations). The stepwise dissociation of the ~74 kDa intermediate chain and light chain subunits indicates that the light chains are more tightly associated with the ~530 kDa heavy chain and suggests that intermediate and light chains are not tightly associated with each other in the dynein molecule.

To examine whether intermediate or light chains were necessary for dynein-mediated motility, the ability of cytoplasmic dynein stripped of the ~74 kDa subunits to promote microtubule gliding on glass coverslips was examined. Microtubules bound to the coverslip in a dynein-dependent manner but did not translocate across the surface, indicating that the ~74 kDa subunit was necessary for motor activity. Addition of the isolated

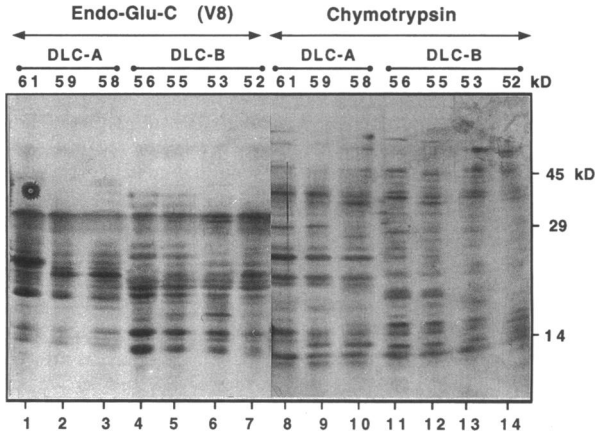

Figure 2. Proteolytic digestion of the light chains from chick embryo brain cytoplasmic dynein demonstrates that DLC-A and DLC-B form two families. Mono-Q-purified cytoplasmic dynein was electrophoresed on a 7.5% SDS-polyacrylamide gel; the seven light chain polypeptides (52–61 kDa) were individually excised and digested with Endoproteinase-Glu-C (endo-Glu-C) (lanes 1–7) or chymotrypsin (lanes 8–14), and the proteolytic fragments were resolved on a 15% SDS-polyacrylamide gel and visualized by silver staining. To equalize the amount of protein in the different lanes, one gel band was loaded for the 59- and 56-kDa isoforms, two gel bands were loaded for each of the 61- and 55-kDa isoforms, and three gel bands were loaded for each of the remaining three isoforms. Digests of the 61-kDa protein are in lanes 1 and 8, 59 kDa in lanes 2 and 9, 58 kDa in 3 and 10, 56 kDa in 4 and 11, 55 kDa in 5 and 12, 53 kDa in 6 and 13, and 52 kDa in lanes 7 and 14. Molecular weight markers are on the right of the gel. When the DLC-A family (61, 59, and 58 kDa) is digested with endo-Glu-C (lanes 1–3) or chymotrypsin (lanes 8–10), the patterns of proteolytic fragments are similar and generally distinct from the patterns yielded by the DLC-B family (56, 55, 53, and 52 kDa) (endo-Glu-C, lanes 4–7; chymotrypsin, lanes 11–14).

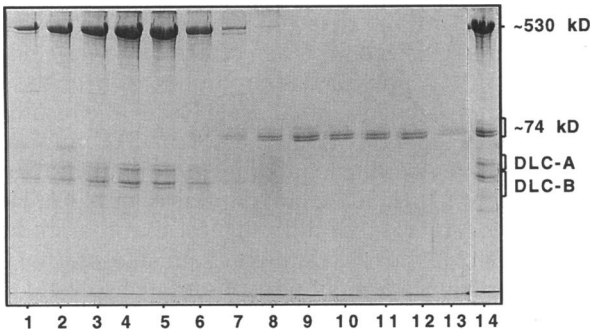

Figure 3. The DLC-A and DLC-B light chains are tightly associated with the cytoplasmic dynein heavy chain. Mono-Q-purified chick embryo brain cytoplasmic dynein was treated with 0.6 M KI and injected onto a Superose 6 FPLC column. Thirteen fractions (lanes 1–13, fraction 1 is in lane 1, etc.) were collected after the column void volume, TCA precipitated, electrophoresed on a 7.5% SDS-polyacrylamide gel, and stained with Coomassie blue. Lane 14 contains a sample of the extracted cytoplasmic dynein that was loaded onto the column. After KI extraction, the ~530-kDa dynein heavy chain and ~52–61–kDa light chains remain associated through column fractionation (lanes 1–6), whereas the ~74-kDa chain is dissociated (lanes 8–13). The molecular weight standards thyroglobulin (670 kDa) and ferritin (440 kDa) eluted in fractions 9 and 12, respectively.

~74 kDa subunit back to dynein heavy chain-light chain complexes did not restore microtubule gliding activity.

Cytoplasmic Dynein Light Chain Polypeptides Are Phosphorylated

To determine if the heterogeneity of the cytoplasmic dynein light chains was at least in part because of phosphorylation, chick embryo brain cytoplasmic dynein was treated with alkaline phosphatase, and the individual subunits were resolved on SDS-polyacrylamide gels. Light chain subunits were detected by immunoblotting with a polyclonal antibody, pAbDLC, that recognizes both DLC-A and DLC-B (albeit unequally, see below). Phosphatase treatment resulted in a disappearance of the 59- and 52-kDa light chain subunits and parallel increase of the 58-kDa subunit and a band at ~51 kDa (Figure 4A, lanes 1 and 2). The 56- and 55-kDa subunits were also diminished after phosphatase treatment, whereas the 53-kDa subunit did not appear to be affected. On this particular immunoblot, cytoplasmic dynein was loaded so as to allow optimal resolution of the individual light chains. As pAbDLC recognizes the minor 61-kDa subunit weakly, it was not detected in this analysis so we could not evaluate its response to phosphatase treatment. Addition of phosphate buffer to the reaction prevented all mobility changes indicating that the observed results were because of dephosphorylation and not proteolysis. Our results are similar to those reported by Hughes *et al.* (1993) who found that phosphatase treatment of bovine brain cytoplasmic dynein shifted the 59, 57, 55, and 53 kDa light chain subunits to bands of 57 and 53 kD.

Identification of a cDNA Clone Encoding a DLC-A Subunit

To determine the primary structure of individual light chain subunits, an affinity-purified anti-cytoplasmic dynein antibody, pAbD1 (see MATERIALS AND METHODS) was used to screen a λgt11 chick embryo cDNA library. The initial screen with pAbD1 yielded 11 positive phage, one of which was recognized by a second affinity-purified antibody to cytoplasmic dynein (pAbD2) (see MATERIALS AND METHODS). Digestion of the phage DNA with *Eco*RI released a ~2.3-kilobase (kb) cDNA fragment (cDNA 58.1) that was subcloned into both Bluescript KSII (for sequencing) and pMALcI (for production of a maltose-binding fusion protein).

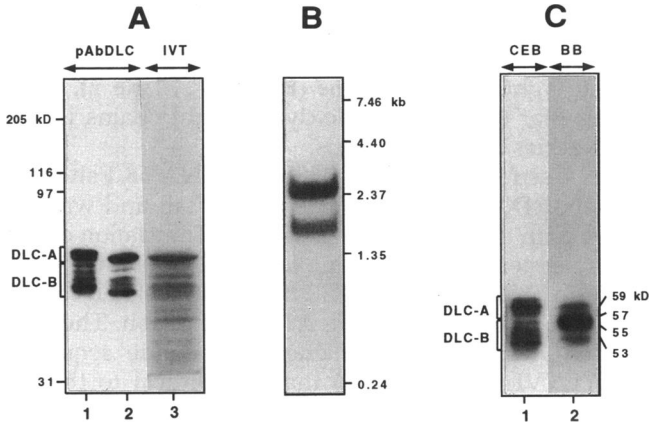

Figure 4. The DLC-A family: phosphorylation and mRNA. (A) The cDNA 58.2 clone encodes an in vitro translation product of the same M_r as the dephosphorylated 58-kDa subunit of the DLC-A family. Chick embryo brain cytoplasmic dynein was treated with alkaline phosphatase. The cDNA 58.2 clone was transcribed with T7 RNA polymerase and translated in vitro (^{35}S-methionine was included in the translation mix). Alkaline phosphatase-treated dynein (lane 2), untreated cytoplasmic dynein (lane 1), and the in vitro translation product (lane 3) were electrophoresed on the same 7.5% SDS-polyacrylamide gel and blotted to Immobilon-P PVDF membrane. Lane 3, containing the in vitro translation product, was excised and exposed to film separately. The remaining lanes were probed with pAbDLC (lanes 1 and 2). After developing the immunoblots, the entire blot was reconstructed to directly compare the M_r of the dephosphorylated dynein and the in vitro translation product. Alkaline phosphatase treatment shifts the 59- and 58-kDa DLC-A isoforms to a M_r of 58 kDa and the DLC-B family to M_rs of 55, 53, and 51 kDa. (B) Northern blot of chick embryo brain poly(A) RNA (5 μg) probed with the 2358 bp cDNA 58.1 clone. An abundant ~2.4-kb message and a less abundant ~1.7-kb message was detected. (C) The light chain dynein subunits of chick embryo and bovine brain are homologues. Mono-Q-purified cytoplasmic dynein from chick embryo (lane 1) and bovine brain (lane 2) was electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with pAbDLC. The antibody recognizes all light chain isoforms of chick (lane 1) and bovine (lane 2) dyneins.

As a first step to determine which dynein subunit was encoded by the cDNA, the rabbit polyclonal antiserum pAbDLC was raised to the maltose-binding protein-cDNA fusion protein. This antiserum recognized the DLC-A and DLC-B dynein polypeptides exclusively (Figure 4A, lane 1 and 4C, lane 1), suggesting that the cDNA encoded a dynein light chain subunit. On the basis of its unequal binding to the different light chain subunits (compare Figure 4, A and C, lanes 1 with Figure 1A that shows the same bands visualized by Coomassie blue staining), pAbDLC appeared to be slightly more immunoreactive with the DLC-A family than the DLC-B family. The cross-reactivity of the antiserum with both light chain families suggests that cDNA 58.1 encodes one of the DLC-A dynein polypeptides but that certain pAbDLC epitopes are also found in the DLC-B polypeptides.

Bovine cytoplasmic dynein also contains multiple light chain subunits (Paschal *et al.*, 1987). To investigate the relationship between these polypeptides and the DLC-A and DLC-B subunits in chicken, immunoblots of Mono-Q-purified dynein from the two species were probed with pAbDLC. The antibody binds to all of the bovine light chain isoforms (Figure 4C, lane 2), indicating that the cytoplasmic dynein light chains in the two species are homologous.

DNA sequencing of the 2358 bp cDNA 58.1 showed that the cDNA extended to a poly A tail and was in-frame with the λgt11 β-galactosidase. Translation of the DNA sequence revealed one long open reading frame (ORF) of 1528 bp with a 830 bp 3′ untranslated region (Figure 5A) but no putative ATG start codon. The predicted ORF contains the encoded peptide sequence EYLYLNVHD (Figure 5A) that is identical to the N-terminal sequence of an internal peptide derived from the 59-kDa DLC-A subunit; a sequence corresponding to the N-terminal 10 amino acids of a peptide derived from the 56-kDa DLC-B subunit was not found in the predicted peptide sequence. Overall, the immunoreactivity of the pAbDLC antiserum against the bacterially expressed fusion protein and the peptide sequencing results suggest that the cDNA 58.1 clone encodes a member of the DLC-A family of polypeptides.

To obtain the 5′ end of the mRNA represented in clone 58.1, including an ATG start codon, RACE-PCR was performed using a thermostable reverse transcriptase (*Tth* DNA polymerase). This yielded a 279-bp PCR product that extended 52 additional nt beyond the original 5′ end of cDNA 58.1 and contained an in-frame ATG start in a reasonable context for eukaryotic translation initiation (Kozak, 1987). Although an in-frame stop codon was not found in the 32 bases upstream of this ATG, two pieces of evidence support its identification as the initiating methionine codon. First, the assembled full length cDNA containing the putative start codon (cDNA 58.2) (see MATERIALS AND METHODS for details) is 2410 bases long, the same size as the longest (~2.4 kb) mRNA detected by blotting of poly A^+ RNA (Figure 4C). Second, when cDNA 58.2 was cloned adjacent to a T7 polymerase promoter, transcribed in vitro, and translated in a reticulocyte lysate, the resulting ^{35}S-labeled polypeptide (Figure 4A, lane 3) comigrated with the 58-kDa product of alkaline phosphatase digestion of the 59-kDa DLC-A polypeptide (Figure 4A, lane 2). Taken together, these data suggest that the product of cDNA 58.2 is the 58 kDa light chain subunit that, in vivo, is posttranslationally phosphorylated to yield the 59-kDa (and likely the 61-kDa) light chain subunit(s).

The DLC-A Subunit Is a Globular Protein with a Predicted Nucleotide-Binding Site

The primary structure of the 58-kDa DLC-A polypeptide was deduced by DNA sequencing, revealing an untranslated 5′ region of 32 bases, an ORF that encodes a polypeptide with a predicted M_r of 55 890, a 830 bp 3′ untranslated region, and a terminal poly A tract (Figure 5A). The secondary structure of the polypeptide was predicted from the primary amino acid sequence using the methods of Chou and Fasman (1974) and Garnier and coworkers (1978). Both methods predict the protein to have a globular structure with short stretches of alpha-helix. (Figure 5B). Analysis with a coiled-coil prediction program (using window sizes of 21 and 28) (Lupas *et al.*, 1991) indicated that the 58-kDa polypeptide does not form a coiled-coil structure. A search for other motifs within the primary sequence revealed numerous potential phosphorylation sites, and a putative nucleotide-binding domain (GEDGAGKT, which fits the $GX_4GK(S/T)$ consensus) near the N-terminus of the protein (Figure 5, A and B).

Among five other cDNAs examined, all but one contained sequences identical to that shown in Figure 5A. The exception, a 1.6-kb cDNA (cDNA 58.3), was identical to cDNA 58.1 up to nt 1620 (Figure 5A) and contained the same ORF but diverged in the 3′ untranslated domain. It is likely that the ~1.7 kb mRNA detected on RNA blots probed with cDNA 58.1 (Figure 4C) corresponds to this ~1.6 kb cDNA and that the smaller mRNA encodes the same polypeptide as cDNA 58.2. Hybridization of chicken genomic DNA with the cDNA 58.1 clone indicates that there is one gene for the ~58 kDa dynein polypeptides (unpublished observations), suggesting that the ~2.4 kb and ~1.6 kb mRNAs may be the result of differential polyadenylation of a single transcript.

DISCUSSION

As a microtubule-based motor believed to participate in intracellular organization of membrane-bound organelles (Schnapp and Reese, 1989; Schroer *et al.*, 1989; Corthesy-Theulaz *et al.*, 1992; Lin and Collins, 1992; Aniento *et al.*, 1993), mitotic spindle assembly (Vaisberg

A

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                                                                                1 CGGCGGGGCGGGGAAGGGAGGAGGCAGCGAAG 32

 33 ATGGCGGCGGTGGGGAGAGCCGGTTCCTTCGGTTCCTCCTCGGCATCCGGCGCCGCGAACAACGCTAGCGCGGAGCTGCGGGCGGGCGGCGAGGAGGACGATGGGCAGAACCTCTGGTCC 152
  1 MetAlaAlaValGlyArgAlaGlySerPheGlySerSerSerAlaSerGlyAlaAlaAsnAsnAlaSerAlaGluLeuArgAlaGlyGlyGluGluAspAspGlyGlnAsnLeuTrpSer 40

153 TGCATCCTCAGCGAGGTGTCCACGCGCTCCCGCTCCAAGCTGCCCTCGGGGAAGAGCGTCCTGCTGCTGGGTGAGGATGGAGCAGGTAAAACTAGCTTAATAGGAAAAATTCAAGGAATC 272
 41 CysIleLeuSerGluValSerThrArgSerArgSerLysLeuProSerGlyLysSerValLeuLeuLeuGlyGluAspGlyAlaGlyLysThrSerLeuIleGlyLysIleGlnGlyIle 80

273 GAGGAATACAAAAAAGGAAGAGGCATGGAGTATTTGTATTTGAATGTGCATGATGAAGATCGAGATGACCAAACGAGATGCAATGTACGGATTTTGGATGGTGACCTGTATCACAAAGGT 392
 81 GluGluTyrLysLysGlyArgGlyMetGluTyrLeuTyrLeuAsnValHisAspGluAspArgAspAspGlnThrArgCysAsnValArgIleLeuAspGlyAspLeuTyrHisLysGly 120

393 CTTCTTAAATTTGCAATGGAGGCAAACTCATTAAAGGACACTCTAATTATGTTGGTAGTAGACATGTCAAGGCCTTGGACTGCAATGGATTCTTTGCAAAAATGGGCAAGTGTTGTAAGA 512
121 LeuLeuLysPheAlaMetGluAlaAsnSerLeuLysAspThrLeuIleMetLeuValValAspMetSerArgProTrpThrAlaMetAspSerLeuGlnLysTrpAlaSerValValArg 160

513 GAACACATTGACAAGTTAAAAATTCCTCCTGAAGAAATGAAAGAAATGGAACAAAAGTTGGTAAGAGACTTCCAGGAATATGTAGAACCAGGCGAGGATTTCCCAGCTTCTCCACAGAGA 632
161 GluHisIleAspLysLeuLysIleProProGluGluMetLysGluMetGluGlnLysLeuValArgAspPheGlnGluTyrValGluProGlyGluAspPheProAlaSerProGlnArg 200

633 AGAAATACTTCATTACAGGAAGACAAAGATGACAGTGTGATTTTACCCCTGGGTGCAGATACACTAACATGTAACTTAGGCATTCCAGTAGTAGTAGTTTGTACAAAGTGCGATGCCATC 752
201 ArgAsnThrSerLeuGlnGluAspLysAspAspSerValIleLeuProLeuGlyAlaAspThrLeuThrCysAsnLeuGlyIleProValValValValCysThrLysCysAspAlaIle 240

753 AGTGTTCTGGAAAAAGAGCATGACTACAGAGATGAACACTTCGACTTCATTCAGTCACATATCAGACGGTTTTGCTTACAGTATGGGGCTGCGCTTATTTACACTTCGGTAAAGGAGAAC 872
241 SerValLeuGluLysGluHisAspTyrArgAspGluHisPheAspPheIleGlnSerHisIleArgArgPheCysLeuGlnTyrGlyAlaAlaLeuIleTyrThrSerValLysGluAsn 280

873 AAAAACATTGATTTAGTCTATAAATATATAGTCCAGAAGTTGTACGGGTTTCCTTTCAATGTTCCAGCTGTTGTTGTGGAAAAAGATGCAGTATTTATTCCTGCAGGTTGGGATAACGAC 992
281 LysAsnIleAspLeuValTyrLysTyrIleValGlnLysLeuTyrGlyPheProPheAsnValProAlaValValValGluLysAspAlaValPheIleProAlaGlyTrpAspAsnAsp 320

993 AAGAAGATTGGCATCTTGCATGAGAACTTTCAAACACTAAAAGCAGAAGACAGTTTTGAAGACAGCATAAGAAAACCGCCAGTCAGAAAGTTTGTTCACGAGAAAGAAATTGTTGCAGAA 1112
321 LysLysIleGlyIleLeuHisGluAsnPheGlnThrLeuLysAlaGluAspSerPheGluAspSerIleArgLysProProValArgLysPheValHisGluLysGluIleValAlaGlu 360

1113 GATGACCAAGTGTTTCTTATGAAGCAGCAGTCACAATTGGCGAAGCAACCACCTACTGCTGCAGGAAGGCCAGTGGATGCCTCACCGAGAGTTCCTGGAGGATCTCCTAGGACACCAAAT 1232
 361 AspAspGlnValPheLeuMetLysGlnGlnSerGlnLeuAlaLysGlnProProThrAlaAlaGlyArgProValAspAlaSerProArgValProGlyGlySerProArgThrProAsn 400

1233 AGATCCGTAACATCCAACGTTGCCAGCGTTACACCTATCCCTGCTGGGTCCAAAAAAATTGATCCCAACATGAAAGCTGGAGCTACCAGTGAGGGAGTCCTGGCGAACTTCTTCAATAGT 1352
 401 ArgSerValThrSerAsnValAlaSerValThrProIleProAlaGlySerLysLysIleAspProAsnMetLysAlaGlyAlaThrSerGluGlyValLeuAlaAsnPhePheAsnSer 440

1353 CTGTTGAGTAAGAAAACTGGTTCCCCTGGTGGCCCTGGTGGTGTTGGTGGCAGTCCTGGCGGTGGCAGCGCTGGAGGTACTGGCAGCAATCTACCACCATCAGCAAAAAAGTCAGGTCAG 1472
 441 LeuLeuSerLysLysThrGlySerProGlyGlyProGlyGlyValGlyGlySerProGlyGlyGlySerAlaGlyGlyThrGlySerAsnLeuProProSerAlaLysLysSerGlyGln 480

1473 AAGCCAGTTTTAACAGATGTTCAGGCAGAATTGGACAGAATTTCACGAAAGCCTGAAATGGTTTCTCCTACATCACCTACGTCTCCCACAGAAGGTGAAGCATCTTGAAGACACCAAATA 1592
 481 LysProValLeuThrAspValGlnAlaGluLeuAspArgIleSerArgLysProGluMetValSerProThrSerProThrSerProThrGluGlyGluAlaSer 515

1593 AAACCAATTGTTGAGTTTTCTGGGGTAATTCAAACTTGCCTCTGCCTCTTCCTTCAGTGACTGGAACTAAAGAACTGAAACATCGTTTCAGAACACAAACAGTTTGTCTGTCTTTCTTTC 1712
     AAACCAATTGTTGAGTTTTCTGGGGTAAAAAAAAAAAAAAAAAAAAAAAAA  3'end of cDNA 58.3

1713 ATGTGTTGCCCAACTTTACAAAAGGGAGCTGTACAGGTGAAAAAAGGTATCACATCACGTAGGACTACTGTTCACAGTGCAAGAATAGTAGATAAATTTCTGGGTTTTGTTGATAAGTCA 1832
1833 GTGTAATTCTGTGAAACTGGCAAGTATGTCCACTTACAGATAAAGGTTGCTTAGAGCAGGAAGCTGATGCACACCACAGGCACTTAATTTATATTGATTTCCCAACTTATTAGTTTATTT 1952
1953 TAATACCCTCTTAAGTAGAATGTAGACCTCTAAAGGAGAGAACTACTACTAGTTGTTGGAAAATAAAATCACTTGCACAAAACTGTAACGACAAACTCCAAATATACTTGAATGCTTGAT 2072
2073 TTGGAGCATGTTTGCGAATCTGTTTGATCTCCCCTGAGATCTATACTGCATTCAAAAACCCATGCAAAAATTTCACTCCAGACTAAGACCTTAAATTACTGGCCGGTTTTTGGTTGTTTT 2192
2193 CTTTGATTAATTTCAGACACTGATAAAAGATGGTATAAAGAAAAAGCAGAGGTTAAATGTACAGTTATCAGTATCCAGATTTGTCTATATATATATGCTCAGCCTTAATGTCCCCCACTA 2312
2313 TGTTTCCCTATTAATTATGGTTTAAGATTGGTTCATCAAAACGTGAATCAGTTAATAAAGTGAACCTGCATTCAAAAAAAAAAAAAAAAAAAAAAAAAA 2410 3' end of cDNAs
                                                                                                                  58.1/58.2
```

B

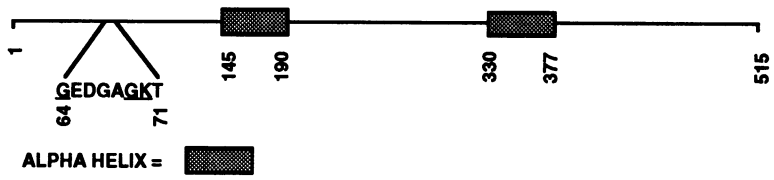

Figure 5. (A) nt and predicted amino acid sequences of the full-length cDNA (cDNA 58.2) encoding the 58-kDa light chain of chick embryo brain cytoplasmic dynein. The N-terminal sequence of a peptide derived from the DLC-A subunits is underlined. Translation from the predicted translation initiation site AUG (nt 33) to the termination codon UGA (nt 1578) would produce a polypeptide of M_r = 55 890. The 3′ untranslated sequence includes two polyadenylation signals AAUAAA (nt 1588 and 2013) and a terminal poly A tract. The MacVector prosite program (IBI, New Haven, CT) revealed a total of 36 potential phosphorylation sites, six for cAMP dependent kinase (R/KXXS/T), six for casein kinase II (S/TXXD/E), 20 sites for glycogen synthase kinase-3 (S/TPXXS), and four for mitogen-activated protein kinase (PXT/SP). A putative nt binding site is double underlined. The sequence data are available from EMBL/Genbank under accession number X79088. (B) Structural features of the 58-kDa polypeptide. Secondary structure predictions based on the methods of Chou and Fasman (1974) and Garnier *et al.* (1978) were generated using MacVector software (IBI). The positions of the predicted alpha-helix domains and the putative nt-binding site are shown in this schematic. The amino acid positions are indicated along the bottom.

et al., 1993), and movement of chromosomes toward the spindle poles (Pfarr *et al.*, 1990; Steuer *et al.*, 1990), cytoplasmic dynein is an important component of normal cellular function. The mechanisms that regulate cytoplasmic dynein-driven motility and its affinity for organelles and the kinetochore plate of chromosomes are not well understood. In the studies described here, we explore the role played by the cytoplasmic dynein light chains in the structure, function, and activity of the enzyme. We find the light chains to be tightly associated with the dynein heavy chains, the site of mechanochemical activity. On the basis of their sizes, peptide

maps and immunoreactivities, the cytoplasmic dynein light chain isoforms can be divided into two distinct, yet similar, classes (DLC-A and DLC-B). These data, in conjunction with molecular cloning studies, indicate that the DLC-A and DLC-B families are encoded by different, but related, genes.

We propose that the cDNA 58.2 is a full length cDNA encoding the smallest subunit (58 kDa) of the DLC-A cytoplasmic dynein family. The results of the alkaline phosphatase digestion experiment, in combination with the peptide mapping data, suggest that the 59- and 61-kDa DLC-A isoforms are the result of phosphorylation of a single 58-kDa gene protein product. Although it is formally possible that the different DLC-A isoforms are the products of related, but distinct mRNAs derived by alternative splicing, the scenario detailed above is a simpler explanation of our results.

The finding of multiple, related genes encoding a dynein subunit is not unprecedented. The large number of distinct heavy chain polypeptides found in axonemal dyneins are believed to be the products of a large multigene family (Gibbons *et al.*, 1994; Rasmusson *et al.*, 1994). The genes encoding the 78- and 69-kDa intermediate chains of *Chlamydomonas* flagellar dynein share homology with each other and with the 74-kDa intermediate chain of cytoplasmic dynein (Mitchell and Kang, 1991; Paschal *et al.*, 1993; King and Witman, personal communication).

Chicken cytoplasmic dynein contains seven light chain isoforms, as compared to four in the bovine molecule (Paschal and Vallec, 1987). This difference most likely reflects species-specific modification rather than a functional difference between the two enzymes. Indeed, we have shown that phosphorylation is largely responsible for the diversity of light chain isoforms observed in both the DLC-A and DLC-B families (Figure 4A). The relationship between the chicken light chains and the bovine 53, 55, 57, and 59 kDa subunits has not been investigated rigorously, but all four bovine light chain subunits are recognized by pAbDLC (Figure 4C) suggesting overall homology with the chicken light chains. An assessment of the relationship between the lower M_r (8–30 kDa) light chains of axonemal dyneins (Pfister *et al.*, 1982; King and Witman, 1989; Hamasaki *et al.*, 1991) and the cytoplasmic dynein light chains awaits further sequence information.

However they contribute to dynein function, the dynein light chains are tightly associated with the ~530 heavy chain and not with the ~74 kDa intermediate chain. This structural arrangement is similar to that observed in outer arm dynein from *Chlamydomonas*, where light chain subunits are tightly associated with gamma (Pfister *et al.*, 1982; Pfister and Witman, 1984) and beta (Mitchell and Rosenbaum, 1986) heavy chains. On the basis of molecular mass (STEM) analysis of bovine brain cytoplasmic dynein (Vallee *et al.*, 1988), the light chain subunits are proposed to lie at the base of the molecule, in the vicinity of the ~74-kDa intermediate chains, where they may play a role in binding dynein to membranous organelles or chromosomes. It is also possible that the light chains associate with the heavy chains in the dynein "head" where they might regulate enzyme activity, in analogy to the regulatory light chains of myosin. Because microtubule-based motor activity is lost after removal of the ~74-kDa intermediate chains, it is not possible to assess dynein function in vitro in the absence of the more tightly associated light chains. Although not rigorous proof, the motility data suggest that the ~74-kDa subunits are required for normal cytoplasmic dynein function. These results are similar to in vitro motility studies that indicate that the complex of β DHC/IC is active for microtubule gliding, whereas the naked α DHC is not (Sale and Fox, 1988). These results are consistent with molecular genetic evidence from *Chlamydomonas* that the force-transducing activity of the flagellar dynein is linked to the function of the flagellar dynein 70-kDa subunit (Mitchell and Kang, 1991).

A potential mechanism for regulation of cytoplasmic dynein activity is reversible phosphorylation, particularly by kinases whose activities are limited to certain cell types (e.g., neurons) or phases of the cell cycle (e.g., mitosis) (Hyman and Mitchison, 1991; Gorbsky and Ricketts, 1993). Using alkaline phosphatase digestion, we have determined that the 59-kDa DLC-A and the 56-, 55-, and 52-kDa DLC-B light chain subunits are phosphorylated. Cytoplasmic dynein heavy and intermediate chains have also been demonstrated to be subject to this form of posttranslational modification (Pfister, personal communication). Although phosphorylation of an axonemal dynein subunit (*Paramecium* outer arm dynein p29) has been correlated with increased enzyme activity (Hamasaki *et al.*, 1991), the effects of phosphorylation on the activity of the cytoplasmic enzyme have not yet been determined. Establishment of the relationships between specific phosphorylation events and cytoplasmic dynein activity is clearly of great importance to our overall understanding of dynein function.

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