

Homology of the 74-kD Cytoplasmic Dynein Subunit with a Flagellar Dynein Polypeptide Suggests an Intracellular Targeting Function

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Abstract. In previous work we found cytoplasmic dynein to be a complex of two catalytic heavy chains and at least seven co-purifying polypeptides of unknown function. The most prominent of these is a 74-kD electrophoretic species which can be resolved as two to three bands by SDS-PAGE. We have now selected a series of overlapping rat brain cDNAs encoding the 74-kD species. The deduced sequence of a full-length cDNA predicts a 72,753 D polypeptide which includes the amino acid sequences of nine peptides determined by NH₂-terminal microsequencing. PCR performed on first strand rat brain cDNA together with the sequence of a partially matching tryptic peptide indicated the existence of at least three isoforms of the 74-kD cytoplasmic dynein subunit. Comparison with known sequences revealed that the carboxyl-terminal half of the polypeptide is 26.4%

identical and 47.7% similar to the product of the *Chlamydomonas ODA6* gene, a 70-kD intermediate chain of flagellar outer arm dynein. Immunoblot analysis with a monoclonal antibody to the 74-kD species indicated a widespread tissue distribution, as expected for a cytoplasmic dynein subunit. Nonetheless, the antibody recognized a 67-kD species in ram sperm flagella and pig tracheal cilia, supporting the existence of distinct but related cytoplasmic and axonemal polypeptides in mammals. In view of evidence for a role for the *ODA6* gene product in anchoring flagellar dynein to the A subfiber microtubule in the axoneme, we predict an analogous role for the 74-kD polypeptide, perhaps in mediating the interaction of cytoplasmic dynein with membranous organelles and kinetochores.

CYTOPLASMIC dynein is a multisubunit enzyme complex which couples ATP hydrolysis with force production towards the microtubule minus end (Paschal et al., 1987; Paschal and Vallee, 1987). It is thought to be responsible for retrograde axonal transport (Paschal and Vallee, 1987; Schnapp and Reese, 1989; Schroer et al., 1989; Hirokawa et al., 1990; Lacy and Haimo, 1992) as well as minus end-directed movements of organelles such as lysosomes (Lin and Collins, 1992) and chromosomes (Reider and Alexander, 1990; Pfarr et al., 1990; Steuer et al., 1990; Hyman and Mitchison, 1991).

Biochemical and structural studies of cytoplasmic dynein have revealed several common properties with its axonemal counterparts, but significant differences as well (Paschal et al., 1987; Shpetner et al., 1988; Vallee et al., 1988). Among the conserved features are the high mol wt heavy chains,

responsible for ATP hydrolysis and force production. Both cytoplasmic and axonemal dyneins also contain a collection of accessory subunits, known in the case of axonemal dyneins as intermediate and light chains. However, the composition of accessory subunits is strikingly different between axonemal and cytoplasmic forms of the enzyme, suggesting that these polypeptides may specify distinct functions. Cytoplasmic dynein from mammalian brain, testis, and liver all contain co-purifying polypeptides of 150, 74, 59, 57, 55, 53, and 40–50 kD (Paschal et al., 1987; Neely and Boekelheide, 1988; Collins and Vallee, 1989). The intermediate and light chains of axonemal dyneins range in size from 69–120 and 14–20 kD depending on the species examined (for simplicity, the 68–70- and 78–80-kD intermediate chains of *Chlamydomonas* outer arm dynein are referred to as 70 and 80 kD, respectively) (Pfister et al., 1982; Piperno and Luck, 1979; Tang et al., 1982; reviewed by Johnson, 1985).

Relatively little is known about the function of the accessory subunits, but recent work has suggested that several of these polypeptides could be involved in subcellular targeting. The most extensively studied axonemal dynein accessory subunits are those of *Chlamydomonas* flagellar outer arm dynein. Direct evidence for a role for these proteins in

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For simplicity, the 68–70- and 78–80-kD intermediate chains of *Chlamydomonas* outer arm dynein are referred to as 70 and 80 kD, respectively.

The RDF2 program (Lipman and Pearson, 1985) calculates the z value according to the equation $z = (\text{similarity score} - \text{mean of random scores}) / \text{SD of random scores}$. A z value >10 is considered significant.

targeting has come from cross-linking studies in detergent permeabilized axonemes, which revealed direct binding between the 80-kD intermediate chain and alpha tubulin (King et al., 1991). The 80-kD subunit can also be dissociated from the dynein holoenzyme in a complex with the 70-kD intermediate chain (Mitchell and Rosenbaum, 1986), the latter of which has been localized to the base of the holoenzyme by immunoelectron microscopy (King and Witman, 1990). The 70-kD subunit has been identified as the product of the *ODA6* gene in *Chlamydomonas* (Mitchell and Kang, 1991) and mutations in this locus result in defective flagellar motility (Kamiya, 1988). Whether the accessory subunits of cytoplasmic dynein are similarly localized and play comparable roles in organelle or kinetochore targeting remain unanswered questions of considerable importance.

To address the role of the cytoplasmic dynein accessory subunits, we have set out to determine their primary structures by cDNA cloning. In an earlier study we described the molecular cloning and sequencing of a 150-kD cytoplasmic dynein-associated polypeptide. We found this polypeptide to be the apparent mammalian homologue of a similar-sized *Drosophila* protein, the product of the *Glued* locus (Holzbaur et al., 1991). p150^{Glued} is present in most (Collins and Vallee, 1989; Gill et al., 1991), but not all cytoplasmic dynein preparations (Paschal et al., 1987). It also is a component of a biochemical fraction reported to stimulate in vitro vesicle motility (Schroer and Sheetz, 1991), though a specific role for the p150^{Glued} in the active fraction has not been resolved.

The present study regards the molecular cloning of the 74-kD subunit of cytoplasmic dynein. This polypeptide has been found to co-purify with the brain cytoplasmic dynein complex at a constant stoichiometry of $3.1 \pm 0.2:1$ by sucrose gradient centrifugation, gel filtration, ion exchange, and hydroxylapatite chromatography (Paschal et al., 1987). It has also been found at comparable levels in cytoplasmic dynein purified from rat liver and testis (Collins and Vallee, 1989; Neely and Boekelheide, 1988), and what appears to be the same polypeptide has been described in HeLa cells (Pfarr et al., 1990) and chick brain (Steuer et al., 1990).

We report here the primary structure of the 74-kD subunit along with evidence for the existence of at least three isoforms. We find that the 74-kD species contains significant sequence identity with the product of the *ODA6* locus in *Chlamydomonas* (Mitchell and Kang, 1991), the 70-kD intermediate chain of outer arm dynein (Mitchell and Kang, 1991). This suggests that the cytoplasmic and axonemal intermediate chains may carry out related functions within their respective holoenzymes.

Materials and Methods

Protein Chemistry

Cytoplasmic dynein was purified from calf brain white matter cytosol as previously described (Paschal et al., 1991). For peptide sequencing, the 20S sucrose gradient fractions were pooled and centrifuged at 45,000 rpm for 16 h in a 50Ti rotor (Beckman Instruments, Palo Alto, CA) to concentrate the cytoplasmic dynein. The resulting small, clear pellet was resuspended in 0.5 ml of SDS-PAGE sample buffer (Laemmli, 1970) modified to contain 10% SDS, and boiled for 10 min. The sample, containing ~250 µg of total protein, was applied to a 9% polyacrylamide minigel (Bio-Rad Laboratories, Richmond, CA) and electrophoresed at 25 mA constant current. The gel was transferred to nitrocellulose (0.45-µm pore size; Schleicher and

Schuell, Keene, NH) in a methanol-containing buffer (Towbin et al., 1979) at 100 V constant voltage for 1 h. The nitrocellulose filter was stained with 0.1% Ponceau S in 1% acetic acid for 1 min to visualize protein. The filter was destained for 1–2 min in 1% acetic acid. The 74-kD band was excised and further destained in 0.2 mM NaOH for 1–2 min, washed in distilled water, and stored wet at –20°C until use. Trypsin digestion of the nitrocellulose-immobilized protein (in situ digestion), elution of the peptides, and HPLC using a C₁₈ reverse phase column were carried out essentially as described (Aebersold, 1989). The amino acid sequences of seven HPLC-purified peptides were determined on a sequenator (model 470A; Applied Biosystems, Foster City, CA) at the Worcester Foundation for Experimental Biology Protein Chemistry Facility (Shrewsbury, MA).

The sequence of peptide 8 was derived from an SDS-PAGE-purified 40-kD tryptic fragment of 20S dynein, the details of which will be described elsewhere. Peptide 9 was generated by *Staphylococcus aureus* V8 protease digestion of SDS-PAGE-purified 74-kD protein (Gooderham, 1984). The fragments resulting from the V8 digest were separated on a 9% polyacrylamide gel, transferred to Immobilon (Millipore Continental Water Systems, Medford, MA), visualized by Coomassie blue staining, and excised for NH₂-terminal microsequence analysis (Matsudaira, 1987).

cDNA Cloning

The 57-base sense strand oligonucleotide, 5'AAGCCTGTGGCTGTGACAGGCATGGCCTCCCCACAGGGGATGTGAACAACCTTTGTG-3', was a "guessmer" whose design was based on the sequence of peptide 6 (Lathe, 1985). Subsequent cDNA sequence analysis revealed this oligonucleotide to be 87.7% identical with the actual 74-kD cDNA. An oligo(dT)_{12–18} primed λgt10 library prepared from adult rat brain poly(A) RNA was plated and screened using standard methods (Sambrook et al., 1989). Hybridization of the ³²P end-labeled 57 mer (8×10^6 cpm/pmol oligonucleotide) was carried out in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 100 µg/ml sheared salmon sperm DNA, and 0.05 M sodium phosphate, pH 6.8, at 52°C overnight. The final wash condition, determined empirically, was 1× SSC/0.1% SDS at 56°C for 25 min. The positive clones were plaque purified by two additional rounds of screening. The inserts of four clones ranging in size from 1.4 to 2.6 kbp were amplified by polymerase chain reaction (PCR)¹ using λgt10 forward and reverse primers, restriction digested with the enzymes AluI, HaeIII, and HinfI (New England Biolabs, Beverly, MA), repaired with Klenow, and electrophoresed on a 2% agarose gel. The gel was photographed and processed for Southern blotting according to standard methods (Sambrook et al., 1989) using the membrane Duralon-UV (Stratagene, La Jolla, CA). The blot was probed with the 57 mer as described above, and the final wash condition was 0.2× SSC/0.1% SDS at 60°C for 30 min. An AluI fragment of ~100 bp was found to hybridize to the 57mer under these conditions.

The ~100-bp AluI fragment was purified by adsorption to glass beads (GeneClean, Bio 101, La Jolla, CA) and subcloned into EcoRV and alkaline phosphatase-treated pBluescript SK(-) vector. Double-stranded DNA sequencing with Sequenase (US Biochemical, Cleveland, OH) was performed according to the supplier's recommendations, except that primer annealing was at 60°C for 30 min.

The λ DNA was prepared from polyethylene glycol-precipitated phage (Sambrook et al., 1989) and subcloned into EcoRI and alkaline phosphatase-treated pBluescript SK(-). The 2.6-kbp insert of p74 contains an internal EcoRI site, and was, therefore, subcloned as two fragments. The orientation of the two fragments was unambiguously established by comparison with the sequence of peptide 9 which spans the internal EcoRI restriction site. Subclones of the p74 cDNA were sequenced on both strands as described above.

Analysis of Transcripts Encoding 74-kD Isoforms

Northern blotting was carried out by standard methods (Sambrook et al., 1989) using the Duralon-UV membrane. The blot was probed with the 839-bp 5' EcoRI fragment of p74 labeled with ³²P by nick translation. The probe for β-actin message, designed from the highly conserved second exon, was the antisense oligonucleotide 5'CCAAATCTTCTCCATATC-GTCCCAGTTGGT-3' (Nudel et al., 1983).

Transcript analysis by PCR was performed on first strand cDNA prepared using rat brain poly(A) RNA, AMV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD), and oligo(dT)_{12–18} primers. 28 cycles of PCR were carried out using Taq polymerase (Perkin Elmer, Nor-

1. *Abbreviation used in this paper:* PCR, polymerase chain reaction.

walk, CT) and standard nucleotide, primer, and buffer concentrations (Innis et al., 1990). The initial denaturation was at 95°C for 2 min. Denaturation during cycling was at 94°C for 30 s, primer annealing was at 52°C for 30 s, and extension was at 72°C for 30 s. The PCR products were repaired with Klenow, analyzed on a 4% NuSieve GTG agarose gel (FMC Bio-Products, Rockland, ME), and subcloned into pBluescript for sequencing. The primer pairs are given below, with the corresponding region amplified in parentheses. (a) 5'-GCAAATATGTCTGACAAGAGCG-3' and 5'-GGA-GTGCTCACTGATTTCCGAAG-3' (153-475); (b) 5'-TCTTCGAAATCAGT-GAGCACTC-3' and 5'-CCAGTCTCTGGCCGCTGTAGTC-3' (453-933); (c) 5'-CCTCCAAGAGAGCTGACGGAAG-3' and 5'-TGTTCCAAACCA-AGGCCACTCC-3' (786-1128); (d) 5'-GCTGATGGTTGCTTCTTATAGC-3' and 5'-CCATTCCTGTAACAGCCACAGG-3' (1055-1488); (e) 5'-TCA-ACAACCTTCGTGGTCGGCAG-3' and 5'-TATTGGCGCGGATCTCCAC-CAG-3' (1507-2046); (f) 5'-GACCTCTGGAACCTCAACAGTG-3' and 5'-GACACCGATACGGAAGGACA-3' (1824-2195).

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli (1970), with the acrylamide concentrations noted in the legends. The gel in Fig. 6 was a 5-10% polyacrylamide gradient with dimensions 160 × 140 × 1.5 mm, and was electrophoresed at 30 mA constant current. The method of Oakley et al. (1980) was used for silver staining. For Western blots, post-transfer nitrocellulose filters were blocked overnight in a solution containing 5% nonfat dry milk, 0.1% Tween-20, and 1 mM sodium azide in TBS. The anti-74-kD mAb was used as ascites fluid in TBS containing 0.1% Tween-20 and 2% BSA. The dilutions were 1:1,000 or 1:10,000 for 1- or 16-h incubations, respectively. Washes (3 × 20 min each) were in TBS containing 0.1% Tween-20. The secondary antibody was an affinity-purified peroxidase-conjugated sheep anti-mouse diluted 1:10,000 in TBS containing 0.1% Tween-20 and 2% BSA. Detection was achieved with Enhanced Chemiluminescence (Amersham Corp., Arlington Heights, IL), using exposure times of 2-5 s.

Preparation of Samples for Expression Analysis

Tissue samples were taken from adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). Tissues were homogenized with a motor-driven teflon pestle unit at 2,000 rpm in 2 vol of buffer containing 100 mM Pipes, 1 mM MgSO₄, 0.25 M sucrose, and 1 mM PMSF. The homogenate was centrifuged for 30 s at half speed in a tabletop centrifuge to sediment debris and unbroken cells. Brain samples at different stages of development were prepared in a similar manner, except that sucrose was omitted and homogenization was performed using a Dounce tissue grinder. Aliquots for SDS-PAGE and protein determination (Bicinchoninic acid method; Pierce, Rockford, IL) were frozen in liquid nitrogen and stored at -80°C until use.

Ram testes, prepared from tissue which had been stored at -80°C, was homogenized with a teflon pestle and processed as described above. Pig tracheal cilia, a kind gift of Dr. Annette Hastie (Thomas Jefferson University School of Medicine, Philadelphia, PA) were prepared according to published methods (Hastie, 1991). Ram sperm tails were generously provided by Drs.

Jovi San Agustin and George Witman (Worcester Foundation), and were isolated essentially as described by Calvin (1976).

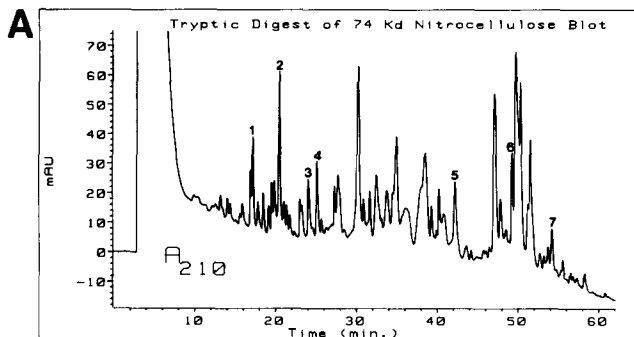
Results

Isolation of cDNAs Encoding the 74-kD Polypeptide

Tryptic peptides prepared from the nitrocellulose-immobilized 74-kD electrophoretic species by in situ digestion were purified by HPLC on a C₁₈ column (Fig. 1). The sequences of seven peptides as determined by pulse liquid phase microsequencing are shown in Fig. 1. Two additional peptide sequences were obtained from tryptic digestion of dynein in solution (peptide 8) and from *Staphylococcus aureus* protease digestion of SDS-PAGE-purified 74-kD (peptide 9).

Using the amino acid sequence of peptide 6, a 57 nucleotide "guessmer" was designed taking into account codon bias and the selective deficiency of the dinucleotide pair 5' C-G 3' in higher eukaryotes (Lathé, 1985). An adult rat brain λgt10 cDNA library was screened and four clones containing inserts of 1.5 to 2.6 kb were isolated. The inserts were amplified by PCR using λgt10 library primers, and examined by restriction enzyme analysis and Southern blotting with the 57-mer probe. The fragment patterns generated with AluI, HaeIII, and HinfI revealed extensive overlap between the four clones. A short (~100 bp) AluI fragment which hybridized to the 57-mer probe was sequenced, and the deduced amino acid sequence was found to match exactly with that of tryptic peptide 6. The longest cDNA, designated p74, was amplified in *Escherichia coli* and subcloned into pBluescript for DNA sequencing.

The first 158 bp of the p74 cDNA was found to be closed in all reading frames (Fig. 2). Translation initiation is predicted to begin with the first in-frame AUG at base 159, which is preceded by a purine (A) in the -3 position (Kozak, 1991). The open reading frame encodes a 643-amino acid polypeptide with an isoelectric point of 4.9 and a mol wt of 72,753 D, in good agreement with our estimate of 74,000 D by SDS-PAGE. The open reading frame terminates with the stop codon UAA, and is followed by 568 bp of 3'-untranslated sequence including the polyadenylation signal AAUAAA and a terminal poly (A) tract. The amino acid sequences of all nine peptides were identified within the single open read-



- B**
- 1 TPVQR
 - 2 WAQGGK
 - 3 EVVSYK
 - 4 SVSTPSEAGSQDSGDGAVG
 - 5 ITQV
 - 6 SKPVAVTGMAFPTGDVNNFV
 - 7 LDLWNLNNDT
 - 8 KTTPEYVFHXQ
 - 9 VKEAPPRELTEEEKQQLHSEEFLLIFFXRTIDVI

Figure 1. In situ tryptic digestion of the 74-kD intermediate chain of brain cytoplasmic dynein. (A) HPLC elution profile of tryptic peptides chromatographed on a C₁₈ reverse phase column. Sequence was obtained from the seven peptides indicated. (B) NH₂-terminal amino acid sequences of peptides generated by in situ tryptic digestion (1-7), by tryptic digestion in solution (8), and by *S. aureus* V8 protease digestion (9).

1 CCAAGCTCAGGAGAGAGGAAGTGATCGCTCCGGGAGGAGCTAGCCTAGCCGCGCAGTGAGACAGCACATCCTGCTGGCGCGGGCCCGCTG 90
91 CTCCCCGCTCTCCCGCGCTCTCCACCACCTCAGTGAGCCTCCGCAGACAGCATCCAGGAAGCAAATATGTCTGACAAGAGCGACCTAA 180
M S D K S D L K 8
181 AAGCTGAGCTGGAGCGCAAAAAGCAGCGCTTAGCGCAGATAAGAGAGGAGAAGAAACGGAAGGAAGAGGAGAGGAAAAAGAAAGAGGCAG 270
9 A E L E R K K Q R L A Q I R E E K K R K E E E R K K K E A D 38
271 ATATGCAGCAGAAGAAAGAGCCCGTTCCAGATGACTCCGATCTGGACCGCAAACGACGAGAGACAGAAGCTTTGCTTCAGAGCATCGGCA 360
39 M Q Q K K E P V P D D S D L D R K R R E T E A L L Q S I G I 68
361 TATCGCCGGAGCCCCCTCTAGTGCAGCGCTGCATTTTTTAACATGGGATACCTGTATTTCATTATTTAGTCCCAACCCCTATGTCTC 450
69 S P E P P L V Q P L H F L T W D T C Y F H Y L V P T P M S P 98
451 CCTCTTCGAAATCAGTGAGCACTCCCAGTGAAGCTGGAAGCCAAGACGATCTGGGGCCATTAAACAAGACCCTGCAGTGGGACACAGACC 540
99 S S K S V S T P S E A G S O D D L G P L T R T L Q W D T D P 128
541 CCTCAGTGTCCAGCTGCAGTGCAGTGCAGACTCAGAACTGGGAGAAGACTGAACAAGCTGGGCGTGTCAAAGGTTACCCAGGTGGATTTCTCGC 630
129 S V L Q L Q S D S E L G R R L N K L G V S K V T Q V D F L P 158
631 CTAGGGAGGTGGTATCTACTCCAAGGAGACACAGACTCCTCTTGAACCTCATCAGTCAGAAGAGGATGAGGAAGATGAGGAGATGGTTG 720
159 R E V V S Y S K E T Q T P L A T H Q S E E D E E D E E M V E 188
721 AGCCCAAAGTTGGTCAATGATTCTGAAC TGGAAAATCAAGACAAAAGCAGGAGACAAAGGAAGCCCTCCAAGAGAGCTGACGGAAGAAG 810
189 P K V G H D S E L E N Q D K K Q E T K E A P P R E L T E E E 218
811 AAAACAGCAGATCCTGCAGTGCAGAGGAATCCTCATCTCTTTGACCGAACGATCCGAGTGATTGAACGAGCTCTGGCTGAGGACTCTG 900
219 K O O I L H S E E F L I F F D R T I R V I E R A L A E D S D 248
901 ACATCTTTTTGACTACAGCGCGGAGAGCTGGAGGAGAAGATGGGGATGTACAGGCTGGAGCCAACCTTTCTTTCAACCGTCAGTTCT 990
249 I F F D Y S G R E L E E K D G D V Q A G A N L S F N R Q F Y 278
991 ATGATGAGCATTGGTCTAAGCACCGGGTGGTCACATGTATGGACTGGTCTCTCCAGTACCCGAGCTGATGGTTGCTTCTTATAGCAACA 1080
279 D E H W S K H R V V T C M D W S L Q Y P E L M V A S Y S N N 308
1081 ATGAAGATGCTCCCATGAACCAGATGGAGTGGCCTTGGTTTGGAAATGAAGTTCAAGAAAACCACACCAGAATATGCTTCCACTGTC 1170
309 E D A P H E P D G V A L V W N M K F K K T T P E Y V F H C Q 338
1171 AGTCTCTGTGATGTCAGTCTGCTTTGCCCGTTCCATCCTAATTTGGTGGTTGGTGGCACCTATTCCAGCCAGATTGCTCTCTGGGACA 1260
339 S S V M S V C F A R F H P N L V V G G T Y S G Q I V L W D N 368
1261 ATCGCAGTCAATGAAGAACCCTGTCCAGAGGACCCCTGTGCAGCCGCTGCACACACATCCTGTGTAATGTTGTTGGGA 1350
369 R S H R R T P V O R T P L S A A A H T H P V Y C V N V V G T 398
1351 CCCAGAATGCTCACAACTGATCACTGTCTTACCAGTGGCAAAATGTGTTCCCTGGAGCCTGGACATGCTCTCAACCCACAGGAGAGCA 1440
399 Q N A H N L I T V S T D G K M C S W S L D M L S T P Q E S M 428
1441 TGGAGCTGGTGTACAACAAGTCCAAGCCTGTGGCTGTTACAGGAATGGCCTTCCCGACGGGAGATGTCAACAACCTTCGTGGTCCGGCAGTG 1530
429 E L V Y N K S K P V A V T G M A F P T G D V N N F V V G S E 458
1531 AGGAAGGTACCGTCTACACAGCTTGTCCGTCATGGAAGCAAAGCAGGCATTGGTGAGGTCTTTGAAGGTCACCAAGGGCCAGTGACAGGCA 1620
459 E G T V Y T A C R H G S K A G I G E V F E G H Q G P V T G I 488
1621 TTAAGTCCACATGGCAGTGGTCCCATCGACTTTTCTCATCTGTTGTGACATCATCATTGACTGGACTGTCAAACCTGTGGACCACAA 1710
489 N C H M A V G P I D F S H L F V T S S F D W T V K L W T T K 518
1711 AGCACAACAAGCCGCTCTACTCCTTTGAAGACAATGCAGACTATGTGTACGATGTATGTTGCTCCCGCTGCATCCCGGCTCTTTGCT 1800
519 H N K P L Y S F E D N A D Y V Y D V M W S P V H P A L F A C 548
1801 GCGTGGACGGGATGGGGCGCTTGGACCTCTGGAACCTCAACAGTGACACCGAGGTGCCAACAGCAAGTGTGGCTATTGAGGGGGCTTATG 1890
549 V D G M G R L D L W N L N S D T E V P T A S V A I E G A Y A 578
1891 CCCTAAACCGGGTTCGTTGGGCACAAGGTGGTAAAGGTTGCAGTAGGTGACTCAGAAGCCGCATCTGGATCTACGATGTTGGAGAGC 1980
579 L N R V R W A O G G K E V A V G D S E G R I W I Y D V G E L 608
1981 TTGCGTTCCCCACAATGATGAATGGACGCGATTGCCCCGACCCTGGTGGAGATCCGCGCAATAGGGCTGACAGCGAGGAGGAAGGTG 2070
609 A V P H N D E W T R F A R T L V E I R A N R A D S E E E G A 638
2071 CGGTTGAGTTAGCTGCCTAAATGTGAGCCCAGTCTGCACGCTGTGGCTCCCATCCTTCCATTCTAGAATCAGTCTGCAGCCAAGGCCCC 2160
639 V E L A A *
2161 TGTGCACTGTTGGTGTCTTTCCGTATCGGTGTCCCGTGGCAACTTGGGTGCCATATTGTGCACTGTTTCCCCATATCTCCCATGT 2250
2251 ACCCTACTCACCCGAAATCCACCACAGCATTCTACCTTTGTGTCCCTGTCTCAAGAAACAAAATGGGGGAGGGAACCCCTAGGGGTTA 2340
2341 GTTGTGCCCTTTAACTACTTGGTAGCTGATTTAATAGCTGGAACCTCTGGTTGAATTTGTCTTACATGTAATCTGGCGTAGTCT 2430
2431 ATTAATCCATATGAAGCCAGATATGATTGGGTGCAGATGTGGCGTGCCTCGTGATACGGTGCAGGGCCAAAGACTTGAGATGTGGTGT 2520
2521 TTACATGGTGATTACATTATGAATGGATTACTACAAGAACATTGCTGCTCTTATTTATTGTAACGTGCTGCATGGAACATATTTATT 2610
2611 TGAAGCATTAAATAAACGATCTTAGAGCAAAAAAAAAAAAAAAAAA

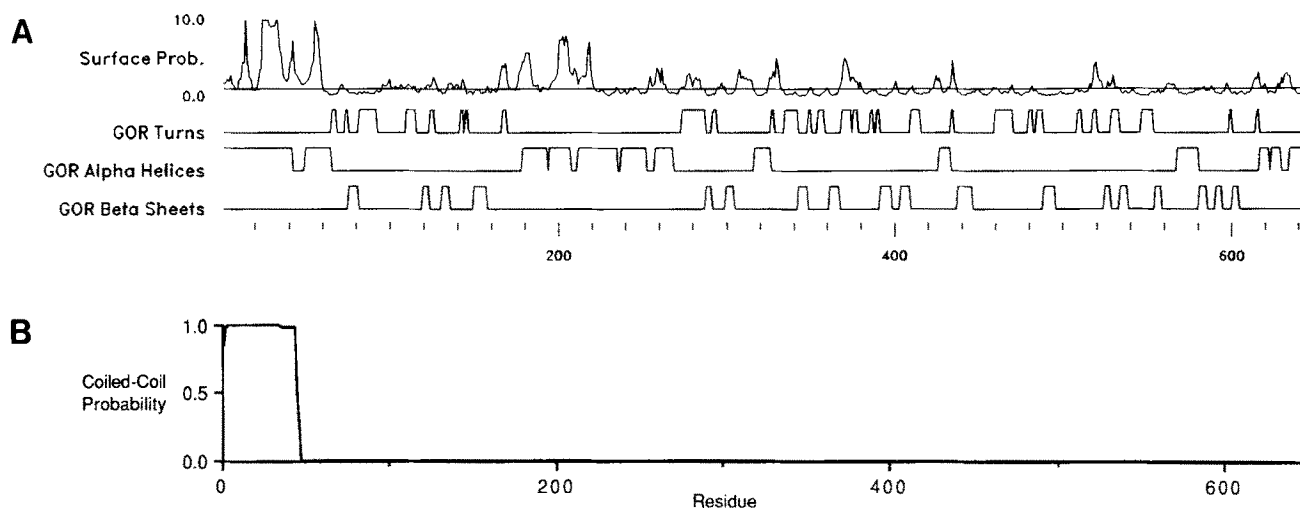


Figure 3. Structural features of the 74-kD polypeptide. (A) The surface probability was predicted using the method of Emini et al. (1985). Secondary structure predictions are based on the method of Garnier et al. (1978). The graphic representation was generated with the GCG Programs PEPTIDESTRUCTURE and PLOTSTRUCTURE on a Vax 11/750 computer. In the uppermost plot, regions predicted to reside on the surface of the polypeptide are indicated by positive values. In the secondary structure analysis, regions which are predicted to be β turn, α helix, and β sheet are indicated when the line in the respective graph is in the up position. Amino acid coordinates are shown along the bottom. (B) Probability of forming a coiled-coil structure. The coiled-coil prediction algorithm analyzes the relative occurrence of amino acids at the seven positions of the heptad repeat using the sequences of the coiled-coil domains of myosins, tropomyosins, and keratins as a database (Lupas et al., 1991).

ing frame. Single amino acid substitutions were observed in peptide 5 (valine for isoleucine), peptide 7 (serine for asparagine), and peptide 9 (threonine for valine and arginine for aspartic acid). Surprisingly, despite an exact match over the first 12 residues, peptide 4 diverged completely from the deduced amino acid sequence after the first aspartic acid. This issue will be addressed further below.

Inspection of the deduced amino acid sequence reveals a striking density of charged residues in the first 220 amino acids including two predicted alpha helical regions, the amino (NH_2 -) terminal 60 amino acids and another 90 amino acids centered about residue 240 (Fig. 3 A). The highly charged NH_2 -terminal region, which shows a significant degree of surface probability (Fig. 3 A), includes the sequences $^{13}\text{RK}(\text{X})_{10}\text{KKRK}$ and $^{42}\text{KK}(\text{X})_{10}\text{RKRR}$ (one letter code, (X)₁₀ denotes a ten-residue spacer). These sequences are similar to the bipartite nuclear targeting signal of *Xenopus* nucleoplasmin, $\text{KR}(\text{X})_{10}\text{KKKK}$ (Robbins et al., 1991).

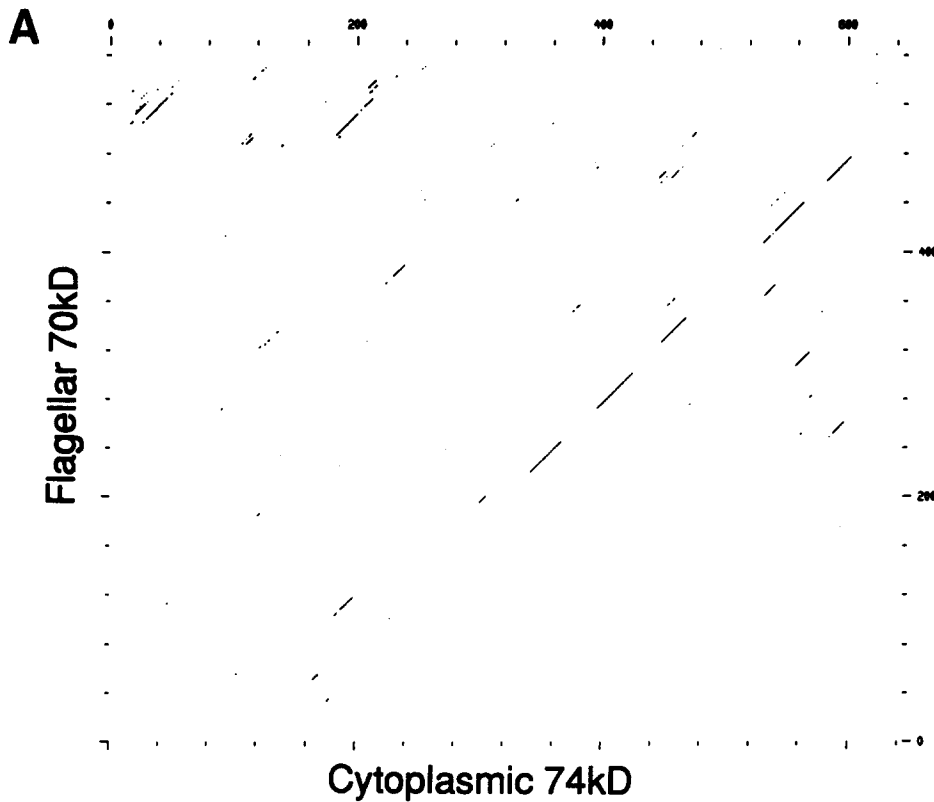
We analyzed the 74-kD sequence for the presence of heptad repeats with the potential to form coiled-coil structures using the algorithm developed by Lupas et al. (1991). Residues 2–44 show a high probability (>0.99) of forming a coiled-coil structure which contains six heptad repeats (Fig. 3 B). Since this number of repeats is close to the minimum number (four to five) required to form a stable coiled-coil structure, it may be involved in interchain rather than intrachain interactions.

Relationships to Other Dynein Polypeptides

We searched the GenBank, EMBL, and SwissProt data bases with the complete 74-kD coding sequence and found no significant relatedness to other proteins. However, a direct comparison of 74- with a 70-kD subunit of *Chlamydomonas reinhardtii* flagellar dynein (Mitchell and Yang, 1991) revealed that the two polypeptides are 23.9% identical and 47.5% similar. A dotplot comparison of the aligned sequences is shown in Fig. 4 A. The COOH-terminal halves of the cytoplasmic and flagellar polypeptides are 26.4% identical and 47.7% similar. The optimal alignment of this region (Fig. 4 B) obtained with the GCG Program BESTFIT required only four minor gaps in the cytoplasmic sequence and five minor gaps in the flagellar sequence. The region of highest identity (28.5%) spans amino acids 330–598 and 208–473 of the cytoplasmic and flagellar polypeptides, respectively.

To evaluate the statistical significance of the extent of sequence identity between the cytoplasmic and flagellar polypeptides, we compared the authentic alignment score with the mean of the alignment scores of the 74-kD sequence and 1,000 randomized 70-kD sequences using the RDF2 Program (Lipman and Pearson, 1985). The mean score of the authentic alignment of the full-length comparisons was 34.74 SD (z value) above the mean score of the randomized sets, indicating that the match between the cytoplasmic and flagel-

Figure 2. Nucleotide and predicted amino acid sequences of the full-length rat brain cDNA (p74) encoding the 74-kD intermediate chain of brain cytoplasmic dynein. The sequences of nine peptides derived from the bovine 74-kD subunit are underlined. Translation from the predicted translation initiation site AUG (159) to the termination codon UAA (2088) would produce a polypeptide of mol wt 72,753, in good agreement with our estimate of 74 kD from SDS gels (Paschal et al., 1987). The 3'untranslated sequence includes the polyadenylation signal AAUAAA (2623) and a terminal poly (A) tract. The GCG program MOTIFS revealed a total of 27 potential phosphorylation sites, two for cAMP dependent kinase (R/KXXS/T), seven for protein kinase C (S/TXR/K), and 18 for casein kinase II (S/TXXD/E). These sequence data are available from EMBL/GenBank/DBJ under accession number X66845.



B

cyto	TPEYVFHCQSSVMSVCFARFHPNLVVGTTYSGQIVLWDNR	369
flag	TPEYEMVPTSQICCAKFNLDNNLVGAGQYNGQLAYFDVR	247
cyto	SHRRTPVQRTPLSAAAHTHPVYCVNVVGTQNAHNLIIVST	409
flag	KG.NGPVEATPI.DISHRDP.IYDFAWLQSKTGTECMTVST	285
cyto	DGKMCSWSLDMLSTPQESMELVYNKSK.PVAVTGMAFPT.	448
flag	DGNVLWWDLRKMNECVENMPLKEKNSETTVGGVCLEYDTN	325
cyto	GDVNNFVVGSEEGTVYTACRHGSKAG..IGEVEFGHQGPV	486
flag	AGPTNFMVGTQQQIFSCNRKAKNPVDRVKYVLSGHHGPI	365
cyto	TGINCHMAVGPIDFSLFVTSSFDWTVKLWTTKHNKPLYS	526
flag	YG....LRRNPFNSKYFLSIGDWTARVWVEDTAVKTPILT	401
cyto	FEDNADYVYDVMWSPVHPALFACVDGMGRDLWNLNSDTE	566
flag	TKYHPTYLTGGTWSPSRPGVFFTIKMDGAMDVWDLYYKHN	441
cyto	VPTASVAIEGAYALNRVRWAQGGKEVAVDSEGRWIYDV	606
flag	EPTLTVQV.SDLALTAFAVQESGGTVAVGTSVDGCTSVLQL	480
cyto	G.ELAVPHNDEWTRFARTLVEIRANRADSEEEGAVELAA*	644
flag	STGLSEASPAEKANIN.AMFERETTREKNLEKAIKEAKVK	519
flag	ARKEQGRRDEVKDNVTEEQLKALEDEFFKTTDPAVGGGYG	559
flag	AGEGAAAE*	567

Figure 4. Alignment of the deduced polypeptide sequences of rat cytoplasmic 74-kD and *Chlamydomonas* flagellar 70-kD intermediate chains. (A) Comparison of the full-length polypeptides generated using the GCG Programs COMPARE and DOTPLOT with a stringency of 15 in a window size of 30. Overall the two polypeptides show 23.9% identity and 47.5% similarity. (B) Comparison of the COOH-terminal regions of the cytoplasmic (*cyto*) and flagellar (*flag*) subunits using the GCG Program BEST-FIT with the default settings. Beginning at amino acids 330 and 208 of the cytoplasmic 74-kD and flagellar 70-kD sequences, respectively, the polypeptides are 26.4% identical and 47.7% similar. Comparisons greater than or equal to similarity threshold of 1.5 are indicated by vertical bars, and represent identities. Comparisons greater than or equal to the similarity threshold of 0.5 are indicated by colons, and those greater than or equal to 0.1 are indicated by single dots.

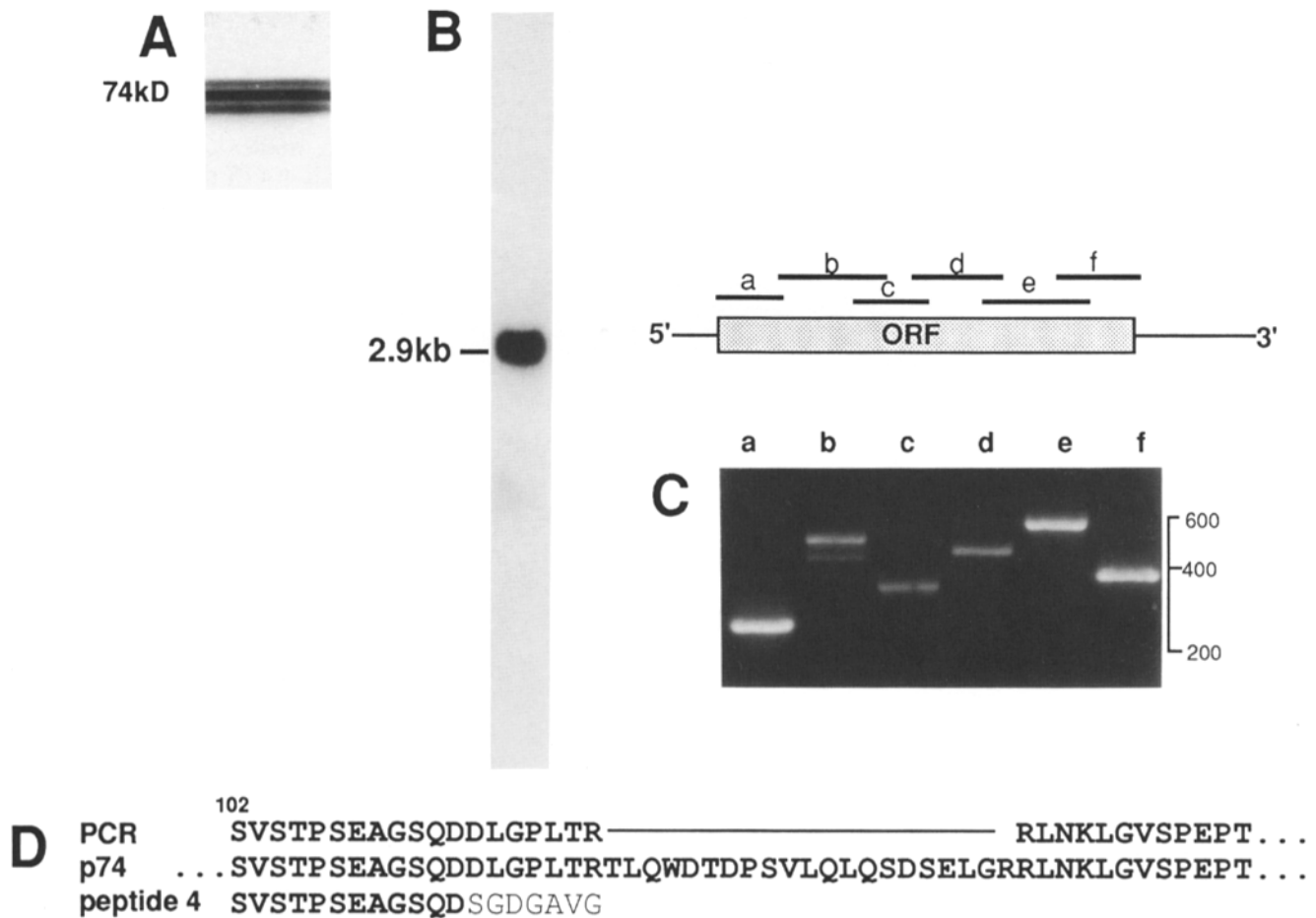


Figure 5. Identification of multiple 74-kD isoforms and mRNAs. (A) SDS-PAGE showing heterogeneity of the 74-kD polypeptides. A linear gradient of 5–10% acrylamide was used, and the gel was stained with silver by the method of Oakley et al. (1980). (B) Northern blot of adult rat brain poly(A) RNA (10 μ g) probed with an 839-bp EcoRI fragment of p74. An abundant 2.9-kb message was detected in brain, and in testes as well (not shown). (C) PCR analysis of transcripts encoding portions of the 74-kD subunit. The diagram illustrates the full-length cDNA and the six overlapping regions (a–f) of the open reading frame (ORF) amplified by PCR. Also shown is an ethidium bromide-stained agarose gel (4% NuSieve) of the PCR products resulting from amplification of first strand brain cDNA. Two products were generated using primers corresponding to region b, which were subcloned into pBluescript for dideoxy sequencing. (D) Comparison of the amino acid sequence predicted from a subclone of the smaller PCR product amplified from region b with the full-length cDNA (p74) and tryptic peptide 4. The 21-amino acid deletion, deduced from the 63-bp deletion in the smaller PCR product, is indicated by the horizontal bar. Amino acid identities in the three isoforms are shown in boldface type. Peptide 4 was identical to the sequence encoded by the cDNA at 12 contiguous residues, but then diverged completely after the first aspartic acid (regular type). This suggests that it represents a third isoform, though it is possible that the divergent residues in the peptide are due to species-specific sequence differences.

lar polypeptides is indeed significant. (The RDF2 Program [Lipman and Pearson, 1985] calculates the z value according to the equation $z = [\text{similarity score} - \text{mean of random scores}] / [\text{SD of random scores}]$. A z value >10 is considered significant). This analysis applied to the COOH-terminal comparison (26.4% identity) gave a z value of 34.88. In contrast, a comparison of the NH₂-terminal halves of the two polypeptides (20.1% identity) revealed a z value of -0.33 , indicating that a statistically significant relationship is restricted to the COOH-terminal region.

Our earliest attempts to isolate cDNAs encoding the 74-kD polypeptide using a mAb had led to the selection of nine clones encoding the 150-kD dynein-associated polypeptide (Holzbaur et al., 1991). This result, and our demonstration of cross-reactivity by immunoblotting (op. cit.) suggested a potential structural relationship between the two species. Comparison of their primary sequences revealed that the two

polypeptides are 21.7% identical. However, analysis using the RDF2 Program indicates the authentic alignment score is only 1.49 SD above the mean score of the randomized sets, suggesting that this degree of relatedness is probably not significant. Alignments generated by the GCG programs BESTFIT and GAP did not reveal any common domains in the 74- and 150-kD species, though the sequence LKAE is found in the NH₂-terminal region of both polypeptides. Since the mAb recognizes bacterially expressed 150 kD, the epitope is probably not defined by a posttranslational modification. It is more likely that the epitope resides in secondary structure conserved between the two polypeptides.

Analysis of Transcripts Encoding 74-kD Isoforms

The mass analysis of cytoplasmic dynein by scanning trans-

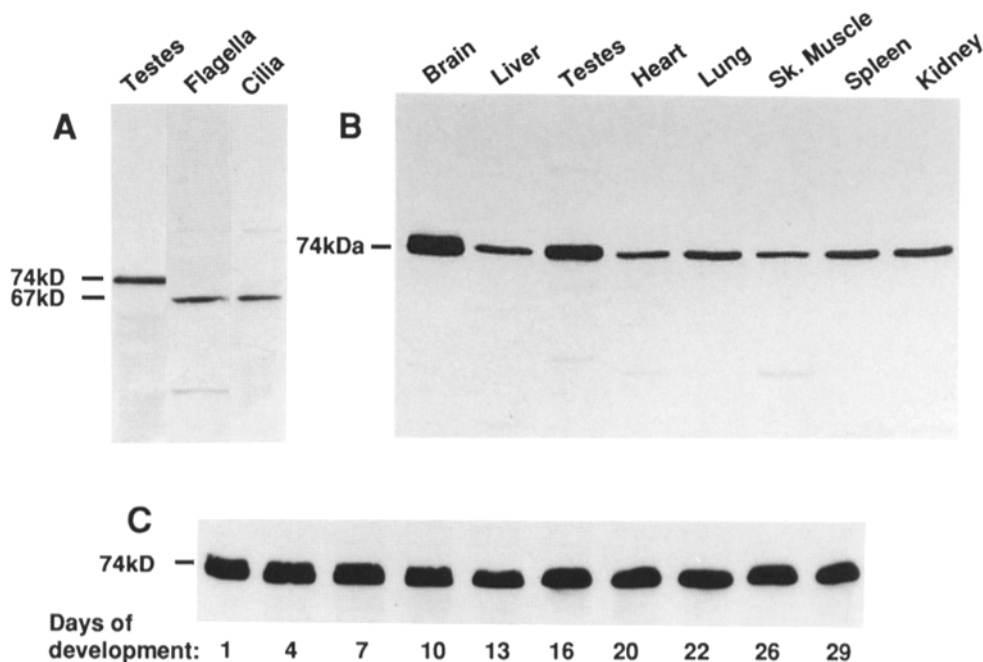


Figure 6. Tissue distribution and developmental expression of the 74-kD cytoplasmic dynein subunit, and identification of cross-reactive axonemal species. (A) Nitrocellulose blot of a 7% polyacrylamide gel of ram testes and flagella and pig cilia reacted with a mAb against the 74-kD cytoplasmic dynein subunit. A major immunoreactive product of 74,000- M_r was observed in sheep testes, while flagella and cilia showed only a 67,000- M_r species. (B) Nitrocellulose blot of an 8% polyacrylamide gel of adult rat tissues. 50 μ g of total protein was applied to each lane. Expression was highest in brain and testes and lowest in cardiac and skeletal muscle. The weak immunoreactivity with a 67,000- M_r species in rat and sheep testes may reflect the presence of spermatozoa. (C) Nitrocellulose blot of an 8% polyacrylamide gel of whole rat brain samples (50 μ g total protein per lane) prepared on day 1 through day 29 of development.

mission EM and the stoichiometry of the co-purifying subunits indicated that there are approximately three copies of the 74-kD subunit per complex (Paschal et al., 1987; Vallee et al., 1988). On well-resolving SDS-PAGE gels, two to three bands can be identified, each of which is recognized by an anti-74-kD mAb (Fig. 5 A, and unpublished data). To determine the basis for 74-kD heterogeneity, we probed northern blots of adult rat brain poly(A) RNA and observed a single, albeit broad, species at 2.9 kb (Fig. 5 B).

As a further means of testing for mRNA heterogeneity, overlapping regions of first strand rat brain cDNA were amplified by PCR (Fig. 5 C). Each of the primer pairs generated a product of the expected size. In addition, PCR amplification of the region corresponding to bases 453–933 of the cDNA generated a second, smaller product which migrated at about 430 bp. The larger PCR product yielded a sequence identical to that of p74. However, the sequence of the smaller PCR product revealed that it contained a 63-base deletion (Fig. 5 D). The corresponding polypeptide is predicted to have a 21 amino acid deletion and a total mol wt of 70,383 D.

Analysis of Expression 74 kD

Given the significant relatedness of the rat 74-kD cytoplasmic and *Chlamydomonas* 70-kD flagellar dynein polypeptides (Fig. 4, A and B), we tested whether mammalian cilia and flagella contain proteins which are immunologically related to the brain cytoplasmic dynein 74-kD species. Western blotting using our anti-74-kD mAb identified a major

immunoreactive species of 74-kD in sheep testes, consistent with the relative molecular weight of the subunit in rat, mouse, calf, and human (Fig. 6 B, and unpublished data). In contrast to this result, no reactivity was seen with a 74-kD species in isolated ram sperm flagella and pig tracheal cilia; however strong immunoreactivity was observed with a 67-kD polypeptide (Fig. 6). Since purified mammalian ciliary dynein contains a 67-kD intermediate chain (Hastie, 1991), our results suggest immunological relatedness between the axonemal and cytoplasmic dynein subunits.

Examination of other rat tissues revealed that expression of the 74-kD polypeptide was highest in brain and testes, consistent with the yield of cytoplasmic dynein from these sources (Paschal et al., 1987; Neely and Boekelheide, 1988; Collins and Vallee, 1989). The expression of 74 kD as a function of brain development was relatively constant from d 1 through d 29 (Fig. 6 C).

Discussion

We have determined the nucleotide and deduced polypeptide sequences of the 74-kD cytoplasmic dynein intermediate chain from mammalian brain. Significant relatedness was found with only one other polypeptide, the 70-kD intermediate chain of flagellar outer arm dynein from *Chlamydomonas* (Mitchell and Kang, 1991). This suggests that the genes for the intermediate chains for cytoplasmic and axonemal dyneins evolved from a common ancestral gene, and raises the

possibility of related functions for these polypeptides within the respective holoenzymes, as discussed below.

Functional Implications of 74-kD/70-kD Homology

Molecular genetic analysis of the *Chlamydomonas* 70-kD intermediate chain has revealed an essential role in flagellar dynein function (Kamiya, 1988; Mitchell and Kang, 1991). *oda6* mutants fail to assemble flagellar outer arms, resulting in a reduced beat frequency (Kamiya, 1988). It is unknown whether the dynein complex fails to assemble in these mutants, or whether it assembles but fails to attach to the A subfiber microtubule. Recently identified intragenic revertants of *oda6* have an apparently normal complement of outer arm subunits, but are still abnormal with regard to beat frequency (D. R. Mitchell, personal communication). This indicates that the force-transducing activity of the holoenzyme is closely linked with the function of the 70-kD intermediate chain.

EM with anti-70-kD mAbs has shown this polypeptide to be located at the base of the flagellar dynein complex (King and Witman, 1990), which is closely associated with the A subfiber microtubule in the axoneme (Goodenough and Heuser, 1984). The 70-kD polypeptide can be isolated as a heterodimer with a second intermediate chain of 80-kD (Mitchell and Rosenbaum, 1986), which can be chemically crosslinked to alpha tubulin in detergent permeabilized axonemes (King et al., 1991). Together, these data suggest a model whereby the 70–80-kD heterodimer constitutes a subcomplex which is responsible for structural binding of the flagellar outer arm dynein to the A subfiber microtubule (King and Witman, 1990).

The relationship reported here between the primary structure of the flagellar 70-kD and cytoplasmic 74-kD accessory subunits suggests that the latter is also likely to be located at the base of the cytoplasmic dynein complex. This is consistent with analysis by scanning transmission EM which revealed the base of the axonemal (Johnson and Wall, 1983; Witman et al., 1983) and cytoplasmic (Vallee et al., 1988) dynein complexes to have a similar mass, which could be accounted for by the combined masses of most of the non-catalytic subunits.

By analogy with the 70-kD flagellar subunit, the 74-kD cytoplasmic dynein subunit may also play a role in substrate attachment. In the case of cytoplasmic dynein there is no evidence for the nucleotide-insensitive, microtubule-binding site found in axonemal dynein (Paschal et al., 1987; Shpetner et al., 1988). Instead a growing body of evidence points to a specific interaction of cytoplasmic dynein with minus end directed organelles (Paschal and Vallee, 1987) such as lysosomes (Lin and Collins, 1992; Matteoni and Kreis, 1987), neuronal vesicles (Schnapp and Reese, 1989; Schroer et al., 1989; Hirokawa et al., 1990; Lacy and Haimo, 1992), chromosomes (Reider and Alexander, 1990; Pfarr et al., 1990; Steuer et al., 1990; Hyman and Mitchison, 1991), and possibly the Golgi apparatus (Rogalski and Singer, 1984). Thus, our finding of a structural relationship between the flagellar and cytoplasmic dynein subunits suggests a role for the 74-kD species in attachment to the organelle surface and to the kinetochore. Whether the 74-kD subunit links the cytoplasmic dynein holoenzyme to these structures directly or

indirectly will be an important issue to be resolved by further work.

Comparison of the Primary Structures of Cytoplasmic 74 kD and Flagellar 70 kD

Several features of the primary structures of the cytoplasmic 74-kD and flagellar 70-kD polypeptides are conserved. The two polypeptides are similar in total mass and have very acidic isoelectric points, 4.9 (this study) and 5.2 (Mitchell and Kang, 1991) for the cytoplasmic and flagellar subunits, respectively. The cytoplasmic and flagellar subunits contain a total of nine and eight cysteines, respectively. Seven of these are located in the homologous COOH-terminal region of each polypeptide, indicating that the number and relative distributions of cysteines have been conserved. Most conspicuous is the amino acid sequence homology within the COOH-terminal halves, which are 26.4% identical and 47.7% similar. It seems reasonable to assume that the COOH-terminal halves of the two polypeptides are involved in a conserved function such as binding to the dynein heavy chains. In the case of the axonemal polypeptide, extraction of the *Chlamydomonas* outer arm dynein complex with increasing levels of SDS revealed the 70-kD subunit to be the last polypeptide released from the β heavy chain, consistent with a direct interaction between these two species (Mitchell and Rosenbaum, 1986).

The NH₂-terminal regions of the 70- and 74-kD subunits appear to be unrelated, suggesting that this domain is specialized for functions unique to the flagellar and cytoplasmic enzymes, respectively. The amino acid composition of this region of the 74-kD cytoplasmic subunit is unusual, with lysine, arginine, glutamic, and aspartic acid comprising 65% of the NH₂-terminal 60 amino acids. Within this highly charged region are two nucleoplasmin-type bipartite nuclear targeting signals. There is, as yet, no evidence for localization of cytoplasmic dynein to the kinetochore before nuclear envelope breakdown. However, a search of the SwissProt data base revealed that the nucleoplasmin-type motif is conserved in nearly half of all nuclear proteins, and it occurs in <5% of non-nuclear proteins (Robbins et al., 1991). Thus, a potential role for this motif in nuclear targeting of cytoplasmic dynein warrants further investigation.

The charge clusters in the NH₂ terminus of the 74-kD polypeptide are also reminiscent of sequences which have been postulated to mediate dimerization in some eukaryotic transcription factors (reviewed by Brendel and Karlin, 1989). For example, the essential region of Fos which is necessary for Fos–Jun dimerization contains a mixed charge cluster (Turner and Tjian, 1989). In this regard, we note that the NH₂-terminal 44 amino acids are strongly predicted to participate in coiled-coil formation. We predict a role for this domain in subunit–subunit interactions or in the binding of hypothetical receptors on the surface of cytoplasmic organelles or kinetochores.

Multiplicity of Isoforms

In earlier work, approximately three copies of 74-kD polypeptide were deduced to co-purify with each cytoplasmic dynein holoenzyme. This conclusion was based on the relative content of the 74-kD species as determined by gel den-

sitometry (Paschal et al., 1987) and the mass of the holoenzyme as determined by scanning transmission EM (Vallee et al., 1988). SDS gels show some evidence of 74-kD heterogeneity, revealing from one to three bands depending on the gel system. In the present study, Northern blotting and PCR were used to test for possible transcript heterogeneity. This analysis and the peptide sequence data are consistent with the existence of three isoforms which differ near their NH₂ termini. The mol wt of the polypeptide calculated from the predicted amino acid sequence of the full-length cDNA is 72,753 D and the PCR analysis predicts a second isoform of 70,383 D, assuming there are no additional deletions or insertions. This is about the difference we estimate between the largest and smallest electrophoretic species from well-resolving gels. The sequence of peptide 4 indicates there is a third, distinct isoform which contains an insertion after residue 113. Together, the multiple isoforms suggested by the sequence data seem likely to account for the three similarly sized polypeptides which migrate at ~74-kD on SDS gels (Fig. 6, A and D).

The importance of multiple forms of 74-kD in cytoplasmic dynein function is unknown. They may account for the 3:1 stoichiometry of 74 kD/holoenzyme complex (Paschal et al., 1987; Vallee et al., 1988). However, it appears that the 74-kD isoforms are expressed unequally, both from examination of protein gels (Fig. 5 A) and from the relative intensity of PCR products generated across the region of transcript heterogeneity (Fig. 5 C). Such unequal expression could be better explained by differences in isoform composition between individual cytoplasmic dynein molecules with some forms predominating. In this case a role for isoform diversity in specifying function would seem a reasonable speculation, for example in targeting cytoplasmic dynein to the surface of different organelles or to the kinetochore.

While the multiple sequences may be generated by an alternative splicing mechanism, we note that preliminary genomic Southern blots are more complex than expected for a single copy gene. Further work will be needed to resolve this issue.

Evolutionary Considerations

One of the noteworthy differences between cytoplasmic, flagellar, and ciliary dyneins is in the composition of their accessory subunits. Nonetheless, some evidence for immunological relatedness of evolutionarily distant flagellar dynein intermediate chains has been obtained. King and coworkers (King et al., 1990) showed that a mAb directed against the 70-kD flagellar dynein subunit in *Chlamydomonas* recognized subunits of *Mt*, 73 and 76 kD in trout and sea urchin, respectively. A second mAb directed against the 80-kD subunit in *Chlamydomonas*, also recognizes the 73-kD subunit in trout. These data suggest some degree of conservation within the primary structure of metazoan and protozoan flagellar dynein subunits. Our observation of cross-reactive mammalian brain, flagellar, and ciliary polypeptides suggests that the cytoplasmic and axonemal intermediate chains in the same taxonomic class are related as well.

Our discovery of a relationship between mammalian cytoplasmic and algal axonemal dynein accessory subunits points to a common origin for the two genes. Assuming that cytoplasmic microtubules predate axonemal microtubules (though for a counterargument see Margulis, 1981), it seems reason-

able to speculate that the ancestral gene would have encoded a cytoplasmic dynein component. Molecular cloning of the mammalian axonemal subunit and comparison with the 74-kD polypeptide sequence should provide additional insight into their structural, functional, and evolutionary relationships.

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