

Differential Phosphorylation In Vivo of Cytoplasmic Dynein Associated with Anterogradely Moving Organelles

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Abstract. Two microtubule-stimulated ATPases, cytoplasmic dynein, and kinesin, are believed to be responsible for the intracellular movement of membrane-bound organelles in opposite directions along microtubules. An unresolved component of this model is the mechanism by which cells regulate these two motors to direct various membrane-bound organelles to their proper locations. To determine if phosphorylation may play a role in the regulation of cytoplasmic dynein, the *in vivo* phosphorylation state of cytoplasmic dynein from two cellular pools was examined. The entire cellular pool of brain cytoplasmic dynein was metabolically labeled by the infusion of [³²P]orthophosphate into the cerebrospinal fluid of rat brain ventricles. To characterize the phosphorylation of dynein associated with anterograde membrane-bound organelles, the optic nerve fast axonal transport system was used. Using a monoclonal antibody to the

74-kD polypeptide of brain cytoplasmic dynein, the native dynein complex was immunoprecipitated from the radiolabeled tissue extracts. Autoradiographs of one and two dimensional gels showed labeling of nearly all of the polypeptide isoforms of cytoplasmic dynein from rat brain. These polypeptides are phosphorylated on serine residues. Comparison of the amount of ³²P incorporated into the dynein polypeptides revealed differences in the phosphorylation of dynein polypeptides from the anterograde and the cellular pools. Most interestingly, the 530-kD heavy chain of dynein appears to be phosphorylated to a lesser extent in the anterograde pool than in the cellular pool. Since the anterograde pool contains inactive dynein, while the entire cellular pool contains both inactive and active dynein, these results are consistent with the hypothesis that phosphorylation regulates the functional activity of cytoplasmic dynein.

THE directed movement of membrane-bound organelles along microtubules is important for various cellular functions including membrane transport, secretion, and axonal transport (38, 58). Data from a variety of experimental approaches support the model that two motor proteins, kinesin and dynein, move membrane-bound organelles in opposite directions along microtubules (for review see references 1, 7, 60, 68). Kinesin moves organelles in the anterograde direction, toward the plus ends of microtubules, while cytoplasmic dynein moves organelles in the retrograde direction toward the minus ends of microtubules (4, 17, 56, 57, 58). One unexplained component of this model is the mechanism by which cells coordinate the activity of these two motor proteins to target specific organelles to their proper locations (1, 7, 38, 58). The movement of membrane-bound organelles in neuronal axons, termed fast axonal transport, provides a unique model system to address this question *in vivo*. In the neuron, the protein synthetic machinery is localized to the somatodendritic compartment (20). Therefore, newly synthesized dynein must be moved down the axon to

the synaptic terminal by anterograde transport before it can function as the motor for retrograde axonal transport.

Immunocytochemical localization studies of ligated nerves have demonstrated that dynein is present on both anterogradely and retrogradely moving organelles (25, Pfister, K., J. Li, S. Brady, and A. Dahlstrom, manuscript in preparation). Biochemical studies indicate that dynein is associated with membrane-bound organelles, including highly purified synaptic vesicles, a class of anterogradely moving organelles (31, 77). Observations such as these led to the proposal that neurons regulate the motor activity of cytoplasmic dynein; that is, inactive dynein is transported on anterogradely moving membrane-bound organelles to synaptic terminals where it is activated to become the motor for retrograde transport (1, 24, 25, 60). The additional observations that cytoplasmic dynein and kinesin colocalize to some of the same organelles both in cultured cells and in ligated nerves (Lin, S. X. H., K. K. Pfister, and C. A. Collins. 1991. *J. Cell Biol.* 115: 168a; Pfister, K., J. Li, S. Brady, and A. Dahlstrom, manuscript in preparation) support the proposition that inactive cytoplasmic dynein is transported on anterograde organelles by kinesin.

One candidate mechanism for regulating cytoplasmic dynein is phosphorylation (1, 25, 38, 60). Protein phos-

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phorylation regulates a wide range of cellular processes (71) and experimental evidence implicates phosphorylation in the regulation of numerous microtubule-based motile systems (6, 14, 22, 29, 35, 54, 63, 65). Considerable evidence indicates that phosphorylation regulates the movement of pigment granules along microtubules in fish pigment cells (34, 35, 53, 54, 65). It has recently been shown that the anterograde motor, kinesin, is phosphorylated *in vivo*, and *in vitro* experiments suggest that phosphorylation may regulate kinesin function (26, 37, 55). Phosphorylation of axonemal dynein polypeptides has been implicated in the regulation of flagellar function (for reviews see references 64, 72). Brain cytoplasmic dynein is a protein complex with a proposed structure consisting of two 530-kD heavy chains, three 74-kD polypeptides, and four polypeptides of 53, 55, 57, and 59 kD (referred to hereafter as the 53–59 kD set of polypeptides), for a total molecular mass of $1.2\text{--}1.5 \times 10^6$ D (39, 45, 69). If phosphorylation regulates cytoplasmic dynein motor activity, then differences in the level of phosphorylation *in vivo* of one or more of these polypeptides in various cellular pools of dynein would be expected.

To test this hypothesis, we examined two functionally distinct pools of cytoplasmic dynein that were labeled *in vivo* with ^{32}P . In both experiments, [^{32}P]orthophosphate was provided to mature neural tissue with minimal perturbation of the physiological environment of the cells, thus avoiding phosphorylation artifacts which are often associated with cultured cells and other *in vitro* systems. (a) To characterize the phosphorylation of a mixture of both active and inactive dynein, the entire cellular pool of brain cytoplasmic dynein was metabolically labeled by infusion of [^{32}P]orthophosphate into the cerebrospinal fluid of rat brain ventricles (13). This method provides ^{32}P , which is metabolized into ATP 32 , to all cells and cellular compartments in the brain, including axons, dendrites, and glial and neuronal cell bodies. ^{32}P is also provided to the numerous synaptic terminals where dynein is presumably converted from an inactive protein to an active, functional motor for retrograde transport. (b) The rat optic nerve fast axonal transport system, which exploits the spatial separation of dynein synthesis and function in axons, was used to examine the phosphorylation state of dynein associated with small tubulo-vesicular anterogradely moving organelles (8, 20). Dynein has no known function in the movement of these organelles, which are believed to be transported down the axon by kinesin; therefore dynein associated with these organelles is likely to be in an inactive state. [^{32}P]Orthophosphate was injected into the vitreous of the eye and radiolabeled cytoplasmic dynein associated with organelles moving with the anterograde fast axonal transport component of the optic nerve was analyzed.

The optic nerve system takes advantage of the fact that proteins, synthesized exclusively in the retinal ganglion cell bodies, are transported into their axons, which comprise the optic nerve, at different rates (7, 8, 20). When the retinal ganglion cells are provided radioactive precursors for protein synthesis by intravitreal injection and the optic nerve is removed within 2–5 h after injection, the proteins associated with small tubulo-vesicular organelles in the fast anterograde transport compartment of the nerve are radiolabeled (7, 8, 20). Longer time intervals between injection and analysis are required to detect labeling in the slower moving mitochondria, as well as the components of slow transport a, slow

transport b, and retrograde transport (7, 8, 20). In addition to radiolabeled amino acids, a variety of radiolabeled metabolites, including ^{32}P , have been successfully used to study the transport of posttranslationally modified proteins and other membrane components in anterograde transport model systems (8, 20, 62). Snyder et al. (62) showed that the transport of protein and other macromolecules labeled by ^{32}P from the cell bodies into nerves parallels that of [^{35}S]methionine-labeled protein.

We report here that nearly all of the cytoplasmic dynein subunits from the entire cellular pool of brain cytoplasmic dynein are phosphorylated *in vivo*. These polypeptides are phosphorylated on serine residues. We also find that the incorporation of ^{32}P into dynein polypeptides associated with the anterograde pool of cytoplasmic dynein differs from the incorporation of ^{32}P into dynein polypeptides from the entire cellular dynein pool. Most interestingly, our quantitative results indicate that there is less radiolabel incorporated into the 530-kD heavy chain of dynein from the anterograde pool than into the 530-kD heavy chain of dynein from the entire cellular pool. This is direct evidence that cytoplasmic dynein polypeptides are differentially phosphorylated in neural tissue *in vivo* and is consistent with the hypothesis that phosphorylation regulates the motor activity of dynein.

Materials and Methods

Purification of Cytoplasmic Dynein

Bovine brains were procured from Dinner Bell Meats (Lynchburg, VA) and cytoplasmic dynein was isolated to ~85% purity essentially as described by Paschal et al. (45) using a microtubule affinity step followed by ATP release and sucrose density gradient centrifugation. Dynein injected into mice for monoclonal antibody production was further purified by hydroxyapatite column chromatography.

Production of Monoclonal Antibodies

Monoclonal antibodies against cytoplasmic dynein were produced using the procedure of Brodsky (10) as modified by Pfister et al. (48), except that 13 injections of hydroxyapatite column purified bovine brain cytoplasmic dynein (30–90 $\mu\text{g}/\text{injection}$) were used.

Phosphorylation of Cytoplasmic Dynein *In Vivo*

Adult male Sprague-Dawley rats (225–250 g) were obtained from Hilltop (Scottsdale, PA) or Harlan (Indianapolis, IN). All surgery was done under anesthesia using ketamine (80 mg/kg, IM) and xylazine (8 mg/kg, IM).

Stereotaxic intraventricular injections were performed with a 10- μl Hamilton syringe. Right lateral ventricles were injected at coordinates 0.0 mm anterior to bregma, 1.5 mm lateral to the midline, 3.0 mm ventral to the dural surface (13, 46) with 2 mCi of [^{32}P]orthophosphate (New England Nuclear, Boston, MA; 8500–9120 Ci/mmol) diluted to 8–10 μl with PBS and administered by infusion over 8–10 min. The animal remained anesthetized during the 1–3-h labeling period. The animal was then sacrificed by decapitation and the brain removed.

Intravitreal injections were performed essentially as described by Brady and Lasek (8). In each experiment five anesthetized animals were injected with 1 mCi [^{32}P]orthophosphate in 4 μl PBS per eye for a total of 10 mCi of ^{32}P in 10 rat eyes. After 4 h the animals were anesthetized and sacrificed by decapitation. The brains were removed and segments of optic nerve (from ~2 mm posterior to the retina to ~2 mm posterior to the optic chiasm) were collected in liquid nitrogen.

Immunoprecipitation

Dynein was immunoprecipitated using a modification of the method developed by Elluru et al. (14a) described briefly here. All operations were per-

formed at 4°C unless otherwise noted. For most experiments, antibody 74.1 (from ascites fluid) was bound to protein A-Sepharose beads (Zymed Labs Inc., San Francisco, CA). In the immunoprecipitation used in the experiment presented in Fig. 3, the antibody was first purified by protein A affinity chromatography, and then covalently coupled to cyanogen bromide activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ). Rat brains or rat optic nerves were homogenized in Triton X-100 lysis buffer (25 mM Tris-Cl, pH 8.1, 50 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM benzamidine, 2 mM EDTA, 3.3 U/ml apyrase, 20 nM microcystin-LR, 20 nM calyculin A, 200 nM staurosporine). The homogenate was spun at 27,000 g (SS-34 rotor; Sorvall Instruments Div., Dupont Co., Newton, CT) for 15 min. The supernatant was recovered, respun, and then precleared using fixed *Staphylococcus aureus*. The precleared supernatant was incubated with the antibody-linked beads in the presence of NET gel (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100, 0.02% NaN₃) and protease inhibitors (1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM benzamidine) for 3 h. The beads were washed with 0.5% Triton X-100, 0.05% deoxycholate, 0.01% SDS, 0.02% NaN₃, in PBS, pH 7.4, followed by washes with 125 mM Tris-Cl, pH 8.1, 500 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 0.02% NaN₃, and finally with water. The protein was eluted from the beads with electrophoresis sample buffer. Immunoblots analyzed by ECL (Amersham Corp., Arlington Heights, IL) were used to demonstrate that >95% of the dynein was removed from homogenates and supernates by this immunoprecipitation procedure. Immunoprecipitation after lysis of tissue in 2% SDS lysis buffer was performed after the method of Halpain and Greengard (21).

Electrophoretic Procedures

The method of Laemmli (32) was used for one dimensional SDS-PAGE, except that piperazine diacrylamide (27) was substituted for bis-acrylamide. To resolve the 530-kD heavy chain of dynein, the separating gel was 4% acrylamide, 8 M urea, and 1.5 mm thick; to resolve all the polypeptides of the dynein complex, a gradient from 4% to 16% acrylamide, and 0–6 M urea was used.

Two dimensional electrophoresis was modified from the methods of Brady et al. (9) and O'Farrell (43). Isoelectric focusing gel solution (3.6% acrylamide, 0.09% bis-acrylamide, 7.8 M urea, 1.9% Triton X-100, 1.9% pH 5–7 ampholines, 1.9% pH 6–8 ampholines, 2.8% pH 3–10 ampholines) was poured to a height of 12 cm in glass tubes (I.D. of 0.3 cm). Gels were polymerized for 2 h and used the same day. After prefocusing the gels, samples were loaded and electrophoresis was carried out for 7,100 V·h.

To reduce contamination due to airborne keratin (42), the extruded isoelectric focusing gels were washed with equilibration buffer (2.3% SDS, 10% glycerol, 0.625 M Tris-Cl, pH 6.8) containing 0.1 M dithiothreitol for 5 min, washed briefly in equilibration buffer alone, and incubated for 15 min in equilibration buffer containing 0.3 M iodoacetamide (19). The IEF gel was loaded on an 8% acrylamide, 1.5-mm thick slab gel and electrophoresed at 8 W.

Protein in the gels was visualized with Coomassie blue or by the silver staining method of Wray et al. (75). Western blotting and immunostaining were carried out according to the methods of Towbin et al. (66) and Bloom and Vallee (5), with horseradish peroxidase-conjugated secondary antibodies and either 4-chloro-1-naphthol, or enhanced chemiluminescence (ECL, Amersham) was used to detect the antibody.

One and two dimensional gels from the same experiment were analyzed by autoradiography. The two dried gels were exposed either to the same sheet of x-ray film (Kodak XAR-5) with one intensifying screen or to the same storage phosphor screen (Molecular Dynamics, Sunnyvale, CA). A single photographic negative was made of the exposed x-ray film and it was printed to ensure that the photographic reproduction of each of the exposed gels was identical. The storage phosphor screen was analyzed with a Molecular Dynamics PhosphorImager, and dye sublimation prints of each gel were made using identical gray scale settings to provide comparable reproductions of the gels.

Quantitative Analyses

Quantitation of the ³²P incorporated into the polypeptides of cytoplasmic dynein resolved by one and two dimensional electrophoresis was performed using a PhosphorImager supported by ImageQuant software. Each pair of gels was exposed to the same storage phosphor screen. The digitized autoradiographic images were quantified by volume integration of the background corrected pixel values assigned to each polypeptide region. PhosphorImager exposures were calibrated to give a sum pixel volume for all

dynein polypeptides equal to 100,000 per experiment. The protein concentration of the individual immunoprecipitated dynein polypeptides resolved by gel electrophoresis was determined by quantitative densitometry of Coomassie blue-stained gels with a Molecular Dynamics Personal Densitometer and the data analyzed with ImageQuant. Student's *t* test was used to statistically compare the data. TCA precipitable counts per minute (cpm) in immunoprecipitated dynein were determined after the method of Mans and Novelli (36).

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed after the method of Hemmings et al. (23). Briefly, polypeptides were excised from gels and the gel pieces were treated with thermolysin (0.15 mg/ml in 20 mM NH₄HCO₃) for 12–20 h. The digest was dried and resuspended in 6 N HCl; the tube was then evacuated with N₂ and capped. The samples were incubated at 105°C for 90 min, transferred to fresh tubes, and dried. They were then resuspended in 2% formic acid, 8% acetic acid, pH 1.9, and spotted onto thin layer chromatography plates (Kodak 13255 cellulose) and electrophoresed in the same buffer at 500 V until the dye front traveled 1/3 the length of the plate. The plates were then transferred to 1% pyridine, 10% acetic acid, pH 3.5, and electrophoresed at 350 V until the dye front was 3 cm from the end of the plate.

Biochemical Reagents

Calyculin A and microcystin-LR were obtained from GIBCO-BRL (Gaithersburg, MD). Acrylamide and ammonium persulfate were obtained from Polysciences, Inc. (Warrington, PA). Piperazine diacrylamide (PDA), was obtained from Bio-Rad Labs. (Hercules, CA). Ampholines were obtained from Pharmacia LKB Biotechnology. Thermolysin was from Boehringer Mannheim Corp. (Indianapolis, IN). All other reagents were obtained from Sigma Chem. Co. (St. Louis, MO).

Results

Characterization of Monoclonal Antibody 74.1

The hybridoma cell line which secretes monoclonal antibody 74.1 was generated from a fusion of NS-1 myeloma cells and lymphocytes from mice injected with highly purified bovine brain cytoplasmic dynein. Tissue culture supernatants from the hybridomas were screened on Western blots of sucrose density gradient purified dynein. Antibody 74.1 demonstrated cross-reactivity with two isoforms of the 74-kD polypeptide of cytoplasmic dynein (Fig. 1 A). High specificity for the 74-kD dynein polypeptide was seen when a crude rat brain extract was screened (Fig. 1 B). This monoclonal antibody has affinity for all the isoforms of the 74-kD polypeptide of purified bovine brain cytoplasmic dynein resolved by two dimensional gel electrophoresis, and cross-reacts with cytoplasmic dynein from various tissues of a number of other vertebrates, including fish, mouse, and sheep.

Phosphorylation of Rat Brain Cytoplasmic Dynein In Vivo

To characterize the phosphorylation of cytoplasmic dynein polypeptides in vivo, dynein was metabolically labeled by infusion of [³²P]orthophosphate into rat brain ventricles. [³²P]Orthophosphate infused into the cerebrospinal fluid of brain ventricles is circulated throughout the brain. Therefore, the ³²P may be used by protein kinases in cell bodies, axons, and synaptic terminals. One hour after injection of [³²P]orthophosphate into the ventricles of the brain, cytoplasmic dynein was immunoprecipitated and equal amounts of protein were resolved by one and two dimensional gel electrophoresis and stained. The phosphorylated polypeptides were visualized by autoradiography of the dried gels.

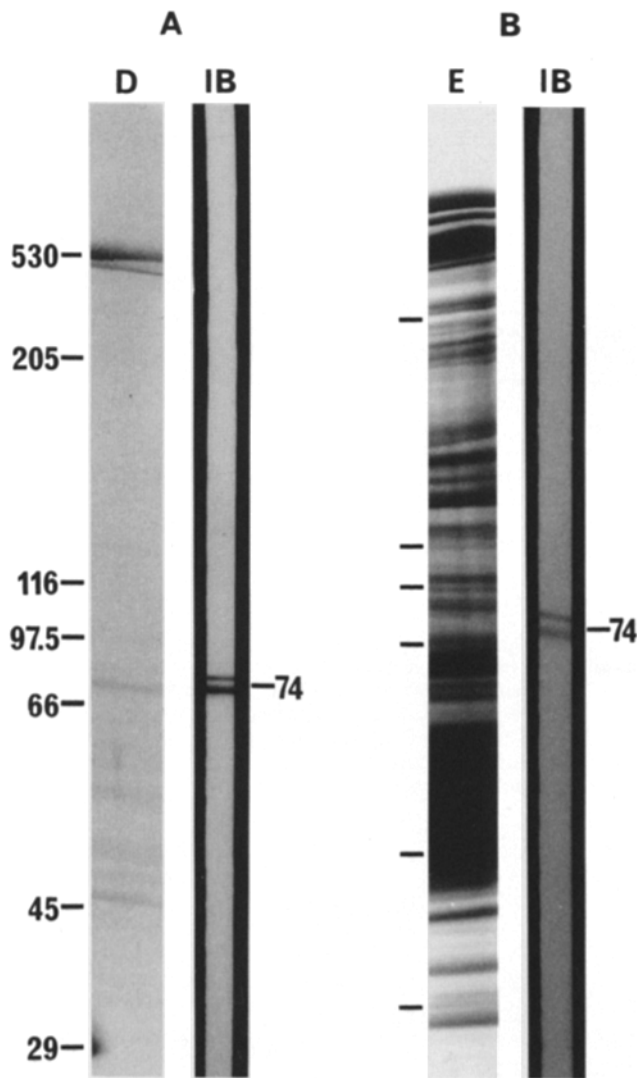


Figure 1. Characterization of monoclonal antibody 74.1 against the 74-kD polypeptide of cytoplasmic dynein. (A) Sucrose density gradient purified cytoplasmic dynein polypeptides were resolved by SDS-PAGE, a portion of the curtain gel was stained with Coomassie blue and the remainder of the gel was transferred to nitrocellulose. Strips of nitrocellulose were probed with tissue culture supernatants from cloned hybridoma colonies. Cell line 74.1 produced an IgG2b which cross-reacts with two isoforms of the 74-kD cytoplasmic dynein polypeptide. (Lane D) Sucrose density gradient purified cytoplasmic dynein; (lane IB) corresponding immunoblot. (B) A crude rat brain extract was resolved by SDS-PAGE, a portion of the gel was stained for total protein with Coomassie blue, and the rest of the gel was transferred to nitrocellulose. When the nitrocellulose strip was probed with monoclonal antibody 74.1, two isoforms of the 74-kD polypeptide were detected. (Lane E) Crude rat brain extract stained for protein; (lane IB) corresponding immunoblot. Molecular mass standards are 205 kD, rabbit skeletal muscle myosin; 116 kD, β -galactosidase; 97.4 kD, phosphorylase B; 66 kD, bovine serum albumin; 45 kD, egg albumin; 29 kD, carbonic anhydrase. The positions of the 530-kD heavy chain and the isoforms of the 74-kD polypeptide are also indicated.

Fig. 2 A shows a portion of a two dimensional gel containing the 74-kD and 53–59-kD set of polypeptides of cytoplasmic dynein immunoprecipitated from a rat brain homogenized in Triton X-100 lysis buffer. Multiple isoforms

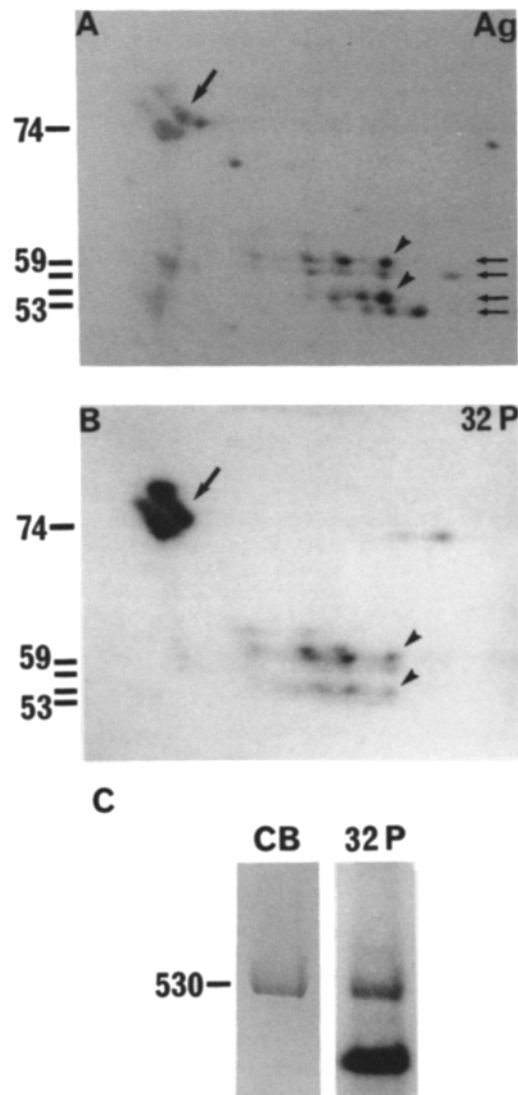


Figure 2. Phosphorylation of cytoplasmic dynein from the entire cellular pool of rat brain. Cytoplasmic dynein was immunoprecipitated from rat brain radiolabeled by intraventricular infusion of [32 P]orthophosphate and equal amounts were analyzed by one and two dimensional gel electrophoresis; $\sim 3 \mu\text{g}$ and $\sim 320 \text{ cpm}$ of TCA precipitable protein were loaded onto each gel. (A) Portion of a silver stained two dimensional gel revealing cytoplasmic dynein polypeptides immunoprecipitated from the crude rat brain extract. A large diagonal arrow points out the isoforms of the 74-kD polypeptide on the gel (focusing between pH 4.1 and 4.9). The isoforms of the 53–59-kD set of polypeptides (focusing between pH 5.9 and 6.3) are indicated with small horizontal arrows. The molecular weights of the dynein polypeptides are shown on the left side of the gel. (B) Portion of an autoradiograph of a gel from the same experiment as A, revealing phosphorylated rat brain cytoplasmic dynein polypeptides. The large diagonal arrow in both A and B point to the B¹ isoform of the 74-kD polypeptide. Small arrowheads point to the most basic isoforms of the 55- and 59-kD polypeptides. The most basic, the B, isoform of the 74-kD polypeptide was not labeled with ^{32}P . The most basic isoforms of the 53- and 57-kD polypeptides were also not labeled with ^{32}P . To more clearly show the 53–59-kD set of polypeptides the isoforms of the 74-kD polypeptide are slightly overexposed. (C) One dimensional SDS-PAGE (4% acrylamide, 8 M urea) revealing phosphorylation of the 530-kD cytoplasmic dynein heavy chain. Lane CB is the Coomassie blue-stained gel lane; lane ^{32}P , autoradiograph of the same lane.

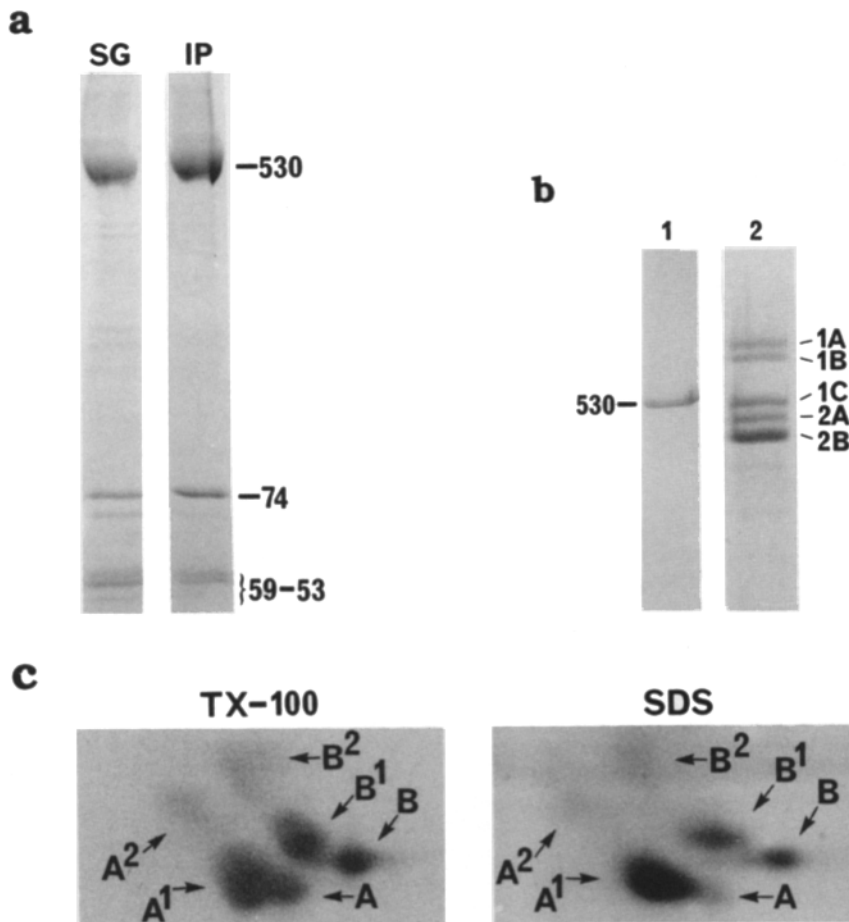


Figure 3. Monoclonal antibody 74.1 specifically and stoichiometrically immunoprecipitates the dynein complex. (a) Portion of an SDS-PAGE gel showing cytoplasmic dynein which was partially purified by microtubule affinity and sucrose density gradient centrifugation (SG) (the major contaminant is tubulin), and dynein which was immunoprecipitated from the sample using monoclonal antibody 74.1 (IP). (b) The 530-kD heavy chain of cytoplasmic dynein immunoprecipitated from rat brain was resolved by SDS-PAGE using a 4% acrylamide, 8 M urea gel, lane 1. To confirm the identity of the immunoprecipitated high molecular mass polypeptide, the high molecular weight MAPS from a 0.6 M NaCl extract of a rat brain microtubule pellet were also analyzed, lane 2. The immunoprecipitated high molecular mass polypeptide is the 530-kD heavy chain of the dynein complex (also known as MAP 1C [45]). Furthermore, no contaminating MAPS are seen in the immunoprecipitate. (c) Enlargement of the 74-kD polypeptide region from two dimensional gels of cytoplasmic dynein immunoprecipitated from rat brain homogenized in either 0.5% TX-100 (TX-100), or 2% boiling SDS (SDS), showing the isoforms of the 74-kD polypeptide. In both immunoprecipitates, 6 isoforms of the 74-kD polypeptide are resolved. For purposes of discussion the isoforms have been labeled A, A¹, A² and B, B¹, B². The positions of the isoforms of the 74-kD polypeptide are indicated with arrows. The TX-100 gel panel is an enlargement of the gel shown in Fig. 2 A. Fig. 3, a and b were stained with Coomassie blue; 3 c was silver stained.

of the 74-kD polypeptide and the 53–59-kD set of polypeptides were resolved. In particular, at least six isoforms of the 74-kD polypeptide that differ by charge and apparent molecular mass are consistently resolved in dynein from rat brain. Evidence for at least three different sequences of the 74-kD polypeptide has been previously reported (44). The left panel of Fig. 3 c shows an enlargement of the 74-kD polypeptide region from the two dimensional gel shown in Fig. 2 A. For purposes of identification and discussion, the isoforms are labeled A, A¹, A² and B, B¹, B². The isoelectric point of the B isoform is pH 4.9, which is in agreement with that predicted from the deduced protein sequence (44). In the course of two dimensional gel analyses, occasionally one or another of the various dynein polypeptide isoforms appeared to be resolved into two spots. Furthermore, the A isoform is a relatively minor species and is often obscured by A¹. The A² and B² isoforms are not as well resolved as the other isoforms with this gel system. Unfortunately, it was found that modifications to the gel system which improve the resolution of these two isoforms decrease the resolution of the other dynein polypeptide isoforms. Most of the remaining spots, seen in Fig. 2 A, including the two spots which are directly below the 74-kD polypeptides and to the left of the

53–59-kD set of polypeptides, are seen in “mock” immunoprecipitates of lysis buffer.

The autoradiograph (Fig. 2 B) of the two dimensional gel shown in Fig. 2 A reveals label incorporated into all the isoforms of the 74-kD polypeptide except for the most basic isoform (the B isoform), though the labeling of the A isoform is very faint. All the isoforms of the 53–59-kD set of polypeptides appear to be labeled to some extent, with the exception of the most basic 53-kD isoform and the most basic 57-kD isoform.

The immunoprecipitated 530-kD dynein heavy chain was analyzed by SDS-PAGE, and the autoradiograph showed that the 530-kD heavy chain is also phosphorylated in rat brain (Fig. 2 C). ³²P is also incorporated into a minor contaminant of the immunoprecipitate with greater relative mobility than either the 530-kD heavy chain of cytoplasmic dynein or MAP 2.

Several methods were used to verify that the immunoprecipitated dynein and its phosphorylation accurately reflected the state of the dynein complex in vivo. To demonstrate that the immunoprecipitation procedure does not dissociate the dynein complex, dynein which was partially purified by sucrose gradient centrifugation was immunoprecipitated and

analyzed by SDS-PAGE (Fig. 3 a). Consistent with the previous study of purified cytoplasmic dynein (58), quantitative densitometry indicated that the molar ratios of the immunoprecipitated cytoplasmic dynein polypeptides were two heavy chains, three 74-kD polypeptides, and four 53–59-kD polypeptides. It can therefore be concluded that under these conditions neither Triton X-100 nor 0.5 M NaCl releases subunits from the immunoprecipitated dynein complex.

As the 530-kD heavy chain of dynein is not well resolved by two dimensional gel electrophoresis, one dimensional gels (4% acrylamide) were used to analyze this polypeptide. Fig. 3 b shows that the high molecular mass polypeptide immunoprecipitated from rat brain by antibody 74.1 comigrates with MAP 1C, which is the 530-kD heavy chain of cytoplasmic dynein (36). Furthermore, the immunoprecipitate is not contaminated with any of several microtubule-associated proteins (MAPs)¹ of similar electrophoretic mobility.

Two approaches were used to ensure that the phosphorylation state of the cytoplasmic dynein analyzed by gel electrophoresis accurately reflected the phosphorylation state of dynein found in vivo. First, inhibitors of protein kinases and protein phosphatases were included in the lysis buffer (Materials and Methods). In the absence of these inhibitors, different dynein phosphorylation patterns were occasionally observed. Second, the effectiveness of the inhibitors at preventing charge modifications during lysis was further confirmed by replacing the Triton X-100 lysis buffer with a 2% SDS lysis buffer. SDS denatures cellular proteins during lysis and homogenization (21). Though treatment with SDS interfered slightly with the resolution of the polypeptide spots, the isoform pattern of the 74-kD polypeptide of dynein immunoprecipitated from rat brain homogenized in SDS lysis buffer was the same as that of dynein immunoprecipitated from brain homogenized in the Triton X-100 lysis buffer with the inhibitors (Fig. 3 c). However, when SDS lysis buffer was used, the rest of the polypeptides which comprise the native dynein complex were not immunoprecipitated. This is presumably due to the fact that SDS dissociated the dynein complex and antibody 74.1 is specific for the 74-kD polypeptide.

Phosphoamino Acid Analysis

To determine the phosphorylated amino acid residues in each of the polypeptides in vivo, the cytoplasmic dynein immunoprecipitated from metabolically labeled rat brain was fractionated by gel electrophoresis. Phosphorylated dynein polypeptides were excised from wet gels and subjected to phosphoamino acid analysis as described in Materials and Methods. The ³²P incorporated into the various cytoplasmic dynein polypeptides was exclusively associated with serine (Fig. 4).

Phosphorylation of Anterograde Cytoplasmic Dynein In Vivo

The rat optic nerve axonal transport model system was used to examine the phosphorylation state of dynein associated with organelles moving in the anterograde fast axonal transport pool. A preliminary experiment was performed to establish that cytoplasmic dynein is associated with the anterogradely moving membrane-bound organelles in the optic

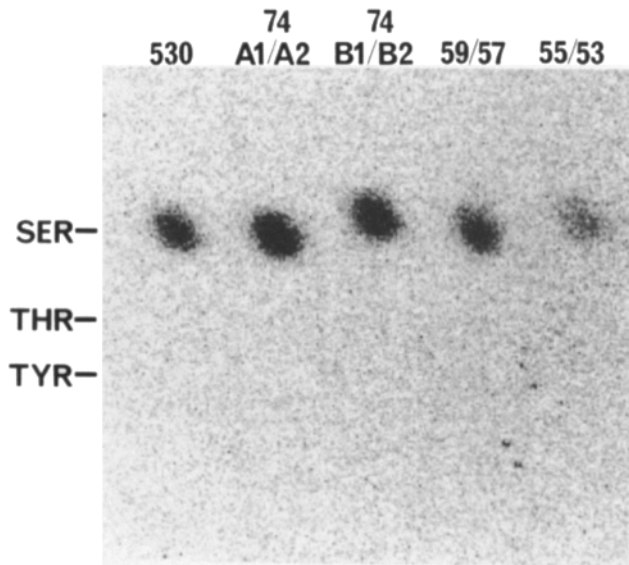


Figure 4. Phosphoamino acid analysis of cytoplasmic dynein polypeptides reveals phosphoserine. Cytoplasmic dynein was immunoprecipitated from rat brain labeled with [³²P]orthophosphate and resolved by gel electrophoresis. Phosphorylated polypeptides were excised from wet gels and subjected to phosphoamino acid analysis. 530, phosphoamino acid analysis of the 530-kD dynein heavy chain; 74 A1/A2, analysis of the A isoforms of the 74-kD polypeptide; 74 B1/B2, analysis of the B¹ and B² isoforms of the 74-kD polypeptide; 59/57, analysis of the 59-kD and 57-kD polypeptides; 55/53, analysis of the 55-kD and 53-kD polypeptides. The 530-kD heavy chain was excised from a one dimensional gel, the other polypeptides were excised from two dimensional gels. The [³²P]phosphoamino acids were visualized with a PhosphorImager. The positions of the standards, phosphoserine (SER), phosphothreonine (THR), and phosphotyrosine (TYR) are shown. Cytoplasmic dynein polypeptides were found to contain exclusively phosphoserine.

nerve. [³⁵S]Methionine was injected into the vitreous of rat eyes. To sample the fast anterograde transport compartment, the rat was sacrificed four hours later and the optic nerve was removed. Cytoplasmic dynein was immunoprecipitated from the nerve and analyzed by two dimensional gel electrophoresis. Fluorography of the dried gel demonstrated that the polypeptides of cytoplasmic dynein were labeled with ³⁵S, the result expected of a protein moving with fast anterograde transport.

Dynein associated with organelles moving with anterograde fast axonal transport in rat optic nerve was then labeled by intravitreal injection of [³²P]orthophosphate. Four hours after injection the optic nerves were removed and the dynein was immunoprecipitated. Equal amounts of the immunoprecipitated dynein were resolved by one and two dimensional gel electrophoresis. Fig. 5 A is a portion of a two dimensional gel stained for protein. The stained gel reveals dynein polypeptides associated with anterograde as well as retrograde axonal transport. The phosphorylated dynein polypeptides associated with anterogradely moving organelles were visualized using a PhosphorImager. Fig. 5 B, an autoradiographic image of the gel in Fig. 5 A, shows ³²P incorporated into isoforms of the 74-kD polypeptide and the 53–59-kD set of polypeptides of anterograde dynein. The 530-kD heavy chain of dynein immunoprecipitated from optic nerve was resolved by SDS-PAGE as described above and incorporation

1. Abbreviation used in this paper: MAP, microtubule-associated protein.

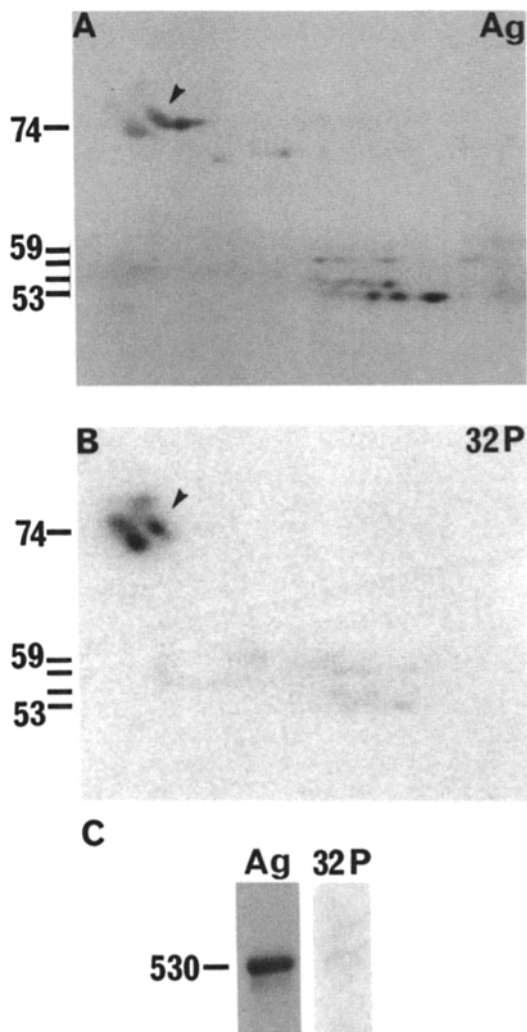


Figure 5. Phosphorylation of cytoplasmic dynein associated with anterograde fast axonal transport. Cytoplasmic dynein was immunoprecipitated from rat optic nerves radiolabeled by intravitreal injection of [^{32}P]orthophosphate and analyzed by one and two dimensional gel electrophoresis; $\sim 0.5 \mu\text{g}$ and $\sim 144 \text{cpm}$ of TCA precipitable protein were loaded onto each gel. (A) Portion of a silver stained two dimensional gel revealing cytoplasmic dynein polypeptides immunoprecipitated from rat optic nerve extracts. The immunoprecipitated polypeptides shown in this panel represent the total pool of optic nerve dynein. (B) Portion of an autoradiographic image of the same gel as A, revealing the phosphorylated anterograde optic nerve cytoplasmic dynein polypeptides. The arrows in A and B mark the position of the B 1 isoform of 74 kD polypeptide. (C) One dimensional SDS-PAGE (4% acrylamide, 8 M urea) revealing barely detectable phosphorylation of the 530-kD cytoplasmic dynein heavy chain. Lane Ag, silver stained gel lane; lane ^{32}P , autoradiographic image of the same lane.

of ^{32}P into the heavy chain was visualized with a PhosphorImager (Fig. 5 C). Extremely faint labeling of the 530-kD heavy chain of anterograde dynein is observed.

Comparison of the Incorporation of ^{32}P into Anterograde and Cellular Cytoplasmic Dynein Polypeptides

The incorporation of ^{32}P into each of the dynein polypep-

tides from the two sets of experiments was quantified and is shown in Table I. There was almost 40-fold less ^{32}P incorporated into the 530-kD heavy chain than the 74-kD polypeptide of cytoplasmic dynein from the anterograde pool, while in cytoplasmic dynein from brain, the 530-kD heavy chain and the 74-kD polypeptide incorporated nearly equal amounts of label. To rule out the possibility that this difference in the labeling could be accounted for by differences in the rate of phosphate turnover on the two polypeptides, ^{32}P was injected into the ventricles of a rat brain and the labeling time was extended to three hours, and the incorporation of ^{32}P into the immunoprecipitated cytoplasmic dynein polypeptides was determined (Table I). No significant difference in the relative incorporation of ^{32}P into the 530-kD heavy chain, the 74-kD polypeptide, or the 53–59-kD polypeptides of brain cytoplasmic dynein was seen when the results from the two brain labeling times were compared.

As the specific activity of the pool of ATP 32 was different in brain and eye, the relative labeling of the individual dynein polypeptides from the two experiments, was compared by graphing the incorporation of ^{32}P into each polypeptide as the percent of the total ^{32}P incorporated into the immunoprecipitated dynein (Fig. 6). In optic nerve, the ^{32}P incorporated into the 74-kD polypeptide accounts for 56% of the total label, while the heavy chain has $\sim 1.5\%$ of the total label. In brain, at both 1 and 3 h, the incorporation of ^{32}P into both the 74-kD and the heavy chain is $\sim 23\%$ of the total label. There is no significant difference in the labeling of the 53–59-kD set of polypeptides.

The optic nerve is found on the ventral surface of the brain, and the synaptic terminals of the optic nerve are in the lateral geniculate nucleus and superior colliculus. Unlike the retinal ganglion in the eye, the morphology of these regions is not suitable for the concentrated application of ^{32}P necessary to identify retrograde specific phosphorylation of dynein. However, the phosphorylation of the entire cellular pool of cytoplasmic dynein, including active/retrograde dynein was characterized using rat brain. Labeling rat brain with ^{32}P has the advantage of labeling all dynein in the cell,

Table I. Quantitation of ^{32}P Incorporation into Cytoplasmic Dynein Polypeptides from Anterograde Optic Nerve and the Entire Cellular Pool of Rat Brain

Poly-peptide (kD)	^{32}P Incorporation (U/pmol polypeptide)		
	Anterograde organelles from optic nerve	Brain	
		1 h	3 h
530	1,900 (± 938)	2,370 (± 288)	2,330 (± 365)
74	73,900 ($\pm 2,250$)	2,660 (± 190)	2,600 (± 345)
59	27,600 ($\pm 1,750$)	2,860 (± 560)	1,890 (± 440)
57	11,800 ($\pm 2,040$)	1,080 (± 342)	1,620 ($\pm 1,050$)
55	10,300 ($\pm 3,650$)	698 (± 149)	1,760 (± 630)
53	5,410 (± 250)	539 (± 55)	1,040 (± 203)

As described in Materials and Methods, [^{32}P]orthophosphate was injected into either the brain ventricles or vitreous of the eye of anesthetized rats and the immunoprecipitated dynein polypeptides from each experiment were resolved by one and two dimensional gel electrophoresis. The ^{32}P incorporated into the polypeptides was quantified using a PhosphorImager. The concentration of the individual dynein polypeptides was determined by quantitative densitometry. The incorporation of ^{32}P into each polypeptide (from duplicate analyses with standard deviations) is expressed in units of sum pixel volume/pmol polypeptide.

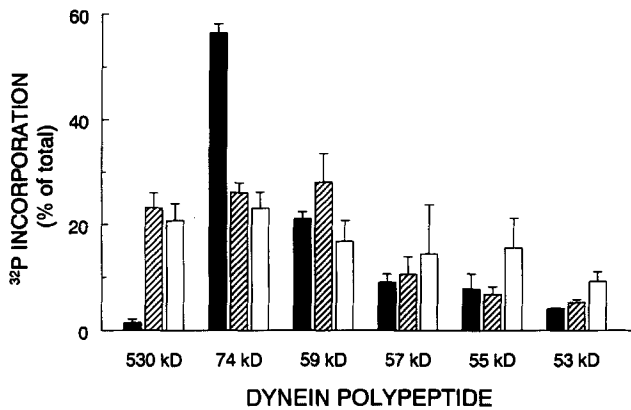


Figure 6. Comparison of the incorporation of ^{32}P into polypeptides of cytoplasmic dynein from the anterograde pool of optic nerve with cytoplasmic dynein from the entire cellular pool of rat brain. The relative incorporation of ^{32}P into cytoplasmic dynein polypeptides immunoprecipitated from the anterograde and cellular pools are graphed as the percent of ^{32}P incorporated into a given polypeptide relative to the labeling of the entire dynein molecule. (Black bar) Cytoplasmic dynein from the anterograde optic nerve pool; (hatched bar) cytoplasmic dynein immunoprecipitated from rat brain labeled for 1 h; (white bar) cytoplasmic dynein immunoprecipitated from rat brain labeled for 3 h.

including cell bodies, processes, and synaptic terminals. Like the optic nerve, these cells are in their native environment. Rat brain is, however, composed of a heterogeneous cell population including neurons and glia. Cultured NIE-115 neuroblastoma cells, which can be induced to form adrenergic axon-like processes, were also used to examine the steady state phosphorylation of cytoplasmic dynein (2). All the ^{32}P incorporated into cytoplasmic dynein in this homogeneous cell type is also on serine residues. Most importantly, in NIE cells as in brain, the incorporation of ^{32}P into the 530-kD heavy chain and 74-kD polypeptide was almost equal ($23.8 \pm 3.1\%$ of total for the heavy chain and $27.6 \pm 6.0\%$ of total for the 74-kD polypeptide).

Discussion

The mechanism by which cells regulate the activity of cytoplasmic microtubule-based motor proteins in order to target specific organelles to their proper locations is a crucial, though poorly understood, element of organelle transport. One model proposes that change in a posttranslational modification, in particular phosphorylation, is the basis for the regulation of organelle motility (1, 25, 38, 60). The prediction of this model is that if phosphorylation regulates the function of dynein, then dynein from different functional pools will differ in their extent of phosphorylation. To test this, we identified differences in the *in vivo* phosphorylation of dynein polypeptides in two functionally distinct cellular pools. (a) Dynein associated with membrane-bound organelles moved by kinesin toward the plus ends of microtubules was radiolabeled with ^{32}P using the optic nerve fast axonal transport system. (b) The entire cellular pool of cytoplasmic dynein was metabolically labeled by infusion of [^{32}P]orthophosphate into the cerebrospinal fluid of rat brain ventricles.

The optic nerve system has valuable properties which make it useful for the identification of the phosphorylation state of dynein polypeptides associated with anterograde organelles. First, the cell bodies of the optic nerve are in the eye, a compartment which is physically distinct from their axons and synapses in the brain. Although amino acids are incorporated into proteins only in the cell body, phosphate can be incorporated into proteins in the axon. Nevertheless, the physical separation of eye and nerve means that while ^{32}P injected into the vitreous of the eye will be in the fluid surrounding the cell bodies of the optic nerve, it will not be in the fluid surrounding the axons and synapses. Significant labeling of axonal proteins by ^{32}P or ATP^{32} which may have diffused from the cell body into the axon is ruled out by the work of Snyder et al. (62). In a careful study, they demonstrated that the transport of ^{32}P -labeled material from the cell bodies into nerves parallels that of the [^{35}S]methionine-labeled protein. Up to 95% of the ^{32}P label in the nerve was TCA precipitable, and the transport of the ^{32}P -labeled material was inhibited by the microtubule poison vinblastine. Second, because of the different velocities of fast and slow anterograde transport and the length of the axons of the optic nerve, the radiolabeled small tubulo-vesicular organelles moving in fast anterograde transport were physically separated from other radiolabeled components within the cell body. Therefore, when the dynein in the nerve axons was immunoprecipitated 4 h after injection of ^{32}P , only the phosphorylation state of cytoplasmic dynein associated with small tubulo-vesicular anterogradely moving organelles was determined. In the brain-labeling experiments, ^{32}P in the cerebrospinal fluid could be metabolized into ATP^{32} for use not only by kinases in the cell bodies, but also by kinases in axons, dendrites, and synaptic terminals. Similarly in the cultured neuroblastoma experiments, ATP^{32} is available to kinases throughout the cell. Therefore the phosphorylation state of the entire cellular pool of dynein, both the active and inactive motor protein, was determined.

Electrophoretic analysis and autoradiography revealed phosphorylation of nearly all the polypeptide subunits of cytoplasmic dynein from the cellular and anterograde dynein pools. This phosphorylation was coupled to serine residues. Quantitation of the incorporation of ^{32}P into the entire cellular pool of cytoplasmic dynein from brain and neuroblastoma cells, revealed approximately equal amounts of phosphate were incorporated into the 74-kD polypeptide and the 530-kD heavy chain. In contrast, quantitation of ^{32}P incorporation into cytoplasmic dynein associated with anterogradely moving organelles revealed that ~ 40 -fold more phosphate was incorporated into the 74-kD polypeptide than the 530-kD heavy chain. In brain and neuroblastoma cells, the labeling of the 530-kD heavy chain represented $\sim 25\%$ of the total label while only 1.5% of the label was incorporated into the heavy chain of dynein associated with anterogradely moving organelles. There was also an approximately twofold difference in the percent labeling of the 74-kD polypeptides from the entire cellular pool and the anterograde pool.

These results demonstrate differences in the phosphorylation of cytoplasmic dynein from different cellular pools and support models for the regulation of cytoplasmic dynein by phosphorylation. As cytoplasmic dynein has no known role in anterograde (plus end directed) transport (1, 7, 24, 25, 60,

69), and the entire cellular pool of dynein from brain includes dynein which is functioning as a motor in retrograde transport, we hypothesize that the difference in the phosphorylation of dynein from the cellular pool as compared to the anterograde pool is associated with activation of dynein for retrograde transport. Since the 530-kD heavy chain contains the sites of ATP hydrolysis and microtubule binding (16, 39, 44, 47), either or both of these functional activities may be regulated by phosphorylation. Alternatively, phosphorylation may be involved in the interaction of dynein with membrane proteins on specific organelles which facilitate its motor function (18) or other steps involved in the shift of dynein from the anterograde to retrograde pathways. Changes in the phosphorylation of the 74-kD polypeptide, may be important for its proposed role in mediating the interaction of dynein with membrane-bound organelles (38, 44). There are numerous protein kinases present in the synapse which could be involved in phosphorylating dynein for activation for retrograde transport (71).

Several observations support the hypothesis that the heavy chain of dynein contains a site important for the regulation of organelle transport. Lin et al. (33a) find that the protein phosphatase inhibitor okadaic acid induces a redistribution of cytoplasmic dynein in cultured cells, and a corresponding increase in the phosphorylation of the 530-kD dynein heavy chain. Flagellar axonemes have 5–10 different dyneins which function to slide microtubules past one another, producing the force necessary to generate flagellar bending (16, 52, 73). Unlike the cytoplasmic dyneins (16, 41, Dillman, J. F., and K. K. Pfister, unpublished observations), many of the axonemal dyneins are heteromers composed of two or more different heavy chains identified as the α , β , and γ heavy chains (74). Several sets of proteins in the axoneme, including the radial spoke proteins, participate in the regulation of axonemal dynein (50, 61). Huang et al. (28) identified a mutation of the β heavy chain of the outer arm dynein of *Chlamydomonas* which restored the motility of paralyzed radial spoke deficient mutants. This mutation had no effect on the assembly or enzymatic properties of the outer dynein arm, but it does bypass the regulatory activity of the radial spoke-central pair complex. Recently Porter et al. (51) characterized this mutation as a deletion of seven amino acids and mapped it to an α helical region near the presumptive nucleotide binding P loops. This region is conserved in all flagellar and cytoplasmic dyneins identified to date, suggesting that it has an important function in both families of dynein. While the β heavy chain of the outer arm dynein is not phosphorylated in vivo, the α heavy chain of the outer arm dynein of *Chlamydomonas* axonemes is phosphorylated in vivo (30, 49). It has also been reported that phosphorylation of the α dynein heavy chain in cilia of marine invertebrates is correlated with activation of flagellar beating (63). Interestingly, several of the heavy chains of the inner arm dyneins of *Chlamydomonas* are also phosphorylated (49).

Studies on axonemal dynein also provide evidence for a role of other polypeptides regulating dynein function. Phosphorylation of a polypeptide of 29 kD activates axonemal dynein in *Paramecium* (22, 72), however, no analogous protein has yet been found associated with cytoplasmic dynein. The 70-kD polypeptide of the outer arm dynein of *Chlamydomonas* is 47.5%, similar to the 74-kD polypeptide of mammalian cytoplasmic dynein (44). Mutations of this

polypeptide which do not effect the assembly or structure of the outer arms but do alter flagellar motility have been identified (40). These mutations indicate that this polypeptide plays a role in the function of the dynein arm independent of its structural (cargo-binding) role (38, 44). Interestingly, this polypeptide is not phosphorylated in vivo in *Chlamydomonas* (30). Several polypeptides, including species of 76 kD and 78 kD associated with dynein from *Tetrahymena* are phosphorylated (11). Removal of these phosphates has effects on the ATPase activity of this dynein. In fish melanophores and xanthophores, pharmacological and other studies on pigment granule transport demonstrate that the movement of the pigment granules along microtubules between aggregated and dispersed states is regulated by phosphorylation (34, 35, 53, 65). In particular, protein dephosphorylation is necessary for MT-minus end directed (retrograde) motility (34, 35, 53, 65).

The experiments described in this report clearly demonstrate that cytoplasmic dynein polypeptides are phosphorylated in vivo, and that there are differences in the phosphorylation of dynein from the anterograde pool compared to the entire cellular pool. This supports models for the regulation of motor function by phosphorylation. These results suggest that one step in the transition of dynein from a passive association with anterogradely moving organelles to a functioning motor for retrograde transport is the phosphorylation of the heavy chains. Evidence has previously been presented that cytoplasmic dynein has a functional role in nuclear migration (76), spindle orientation (15, 33, 67), Golgi localization (12), and endocytic transport (3). The results presented here, therefore, have implications for the regulation of these processes as well as that of axonal transport. As previous in vitro data had not demonstrated phosphorylation of the cytoplasmic dynein heavy chain (70), the results presented here emphasize the importance of in vivo approaches for the study of dynein phosphorylation.

We wish to thank Drs. R. Elluru and M. Fisher, and S. Halpain for teaching J. F. Dillman the intravitreal injection, the stereotaxic injection, and phosphoamino acid analyses, respectively, N. Swygert for technical assistance, Dr. C. Collins for sharing unpublished data, and members of the Cell Motility and Cytoskeletal Group (Drs. R. Bloodgood, G. Gorbsky, S. Halpain, J. Lye, and C. Otey) and S. Parsons for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (NIH), the Jeffress Memorial Trust, and The University of Virginia Cancer Center to K. K. Pfister, and a National Institutes of Health Biomedical Research Support grant to the University of Virginia School of Medicine. J. F. Dillman has been supported by a NIH Biotechnology Training grant.

Received for publication 24 November 1993 and in revised form 17 August 1994.

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