

Dynactin, a Conserved, Ubiquitously Expressed Component of an Activator of Vesicle Motility Mediated by Cytoplasmic Dynein

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Abstract. Although cytoplasmic dynein is known to attach to microtubules and translocate toward their minus ends, dynein's ability to serve in vitro as a minus end-directed transporter of membranous organelles depends on additional soluble factors. We show here that a ~20S polypeptide complex (referred to as Activator I; Schroer, T. A., and M. P. Sheetz. 1991a. *J. Cell Biol.* 115:1309-1318.) stimulates dynein-mediated vesicle transport. A major component of the activator complex is a doublet of 150-kD polypeptides for which we propose the name dynactin (for *dynein activator*). The 20S dynactin complex is required for in vitro vesicle motility since depletion of it with a mAb to dynactin eliminates vesicle movement. Cloning of a brain specific isoform of dynactin from

chicken reveals a 1,053 amino acid polypeptide composed of two coiled-coil α -helical domains interrupted by a spacer. Both this structural motif and the underlying primary sequence are highly conserved in vertebrates with 85% sequence identity within a central 1,000-residue domain of the chicken and rat proteins. As abundant as dynein, dynactin is ubiquitously expressed and appears to be encoded by a single gene that yields at least three alternative isoforms. The probable homologue in *Drosophila* is the gene *Glued*, whose protein product shares 50% sequence identity with vertebrate dynactin and whose function is essential for viability of most (and perhaps all) cells in the organism.

CYTOPLASMIC dynein is a microtubule-based, mechanochemical ATPase found in virtually all animal cells (Lye et al., 1987; Paschal et al., 1987; Euteneuer et al., 1988; Neely and Boekelheide, 1988; Amos, 1989; Collins and Vallee, 1989; Gilbert and Sloboda, 1989; Schnapp and Reese, 1989; Schroer et al., 1989; Koonce and McIntosh, 1990), which is capable of translocating toward the minus end of a microtubule. Cytoplasmic dynein was initially identified in *Caenorhabditis elegans* (Lye et al., 1987) and bovine brain (Paschal et al., 1987) to be a high molecular weight, microtubule-dependent ATPase that when immobilized on glass could power microtubule gliding. Based on the direction of microtubule translocation (Paschal and Vallee, 1987), it was inferred that soluble cytoplasmic dynein moved toward the minus end of microtubules, a prediction confirmed by observing transport of dynein-coated plastic beads along microtubules of known orientation (Schnapp and Reese, 1989; Steuer, E. R., and M. P. Sheetz, unpublished observations).

Cytoplasmic dynein has now been isolated from several sources and contains two heavy (~400 kD), three or four intermediate (~70 kD), and four light (~50 kD) chain subunits, along with a variable number of substoichiometric polypeptides, prominent ones of which are 150-170 kD

(Neely and Boekelheide, 1988; Collins and Vallee, 1989; Schnapp and Reese, 1989; Hirokawa et al., 1990; Schroer and Sheetz, 1991a). The functional properties, if any, conferred by these minor components remain unknown, mirroring the uncertainty as to the in vivo role of cytoplasmic dynein itself. Unlike axonemal dynein, whose role is to drive sliding of adjacent microtubule doublets, thereby powering ciliary and flagellar motility, cytoplasmic dynein has been implicated in several processes: it may represent the translocator responsible for retrograde transport of vesicles within nerve axons, for basolateral-to-apical membrane transport within polarized epithelial cells, and for delivery from endosomes to lysosomes (e.g., see Schroer and Sheetz, 1991b, for review). In addition to its potential role as a vesicle motor, at mitosis dynein localizes to the kinetochore domain of chromosomes as well as to microtubules of the mitotic spindle (Pfarr et al., 1990; Steuer et al., 1990; Wordeman et al., 1991), where it may drive poleward movement of chromosomes during prometaphase and anaphase.

Although cytoplasmic dynein purified from several sources has microtubule-stimulated ATPase activity that powers movement of microtubules on glass coverslips and plastic beads on microtubules (Lye et al., 1987; Paschal et al., 1987; Euteneuer et al., 1988), these activities do not necessarily

reflect the behavior of the enzyme in a biological context. A better indicator of dynein's direct role in vesicle transport came from an *in vitro* system composed of microtubules, salt-washed vesicles, and cytosol-containing dynein (Schroer et al., 1989). Minus end-directed vesicle movement was seen and the movement was inhibited by conditions that inactivate dynein (vanadate and ultraviolet irradiation), indicating that dynein is the transporter responsible. However, while addition of purified dynein to inactivated cytosol restored vesicle motility, dynein alone supported vesicle movement to a markedly reduced extent, suggesting a requirement for additional cytosolic factors. Two such activator fractions (named activators I and II) have been identified (Schroer and Sheetz, 1991a), although the specific polypeptides necessary for function of either activator are not yet known.

We use this *in vitro* system here to characterize further one activator of dynein-mediated vesicle motility. The activator is a large complex (sedimenting at $\sim 20S$) composed of several polypeptides, a prominent component of which is a doublet of 150-kD proteins for which we propose the name dynactin (for *dynein activator*). We show that dynactin is present as a substoichiometric component of 20S dynein, and using a specific mAb (mAb 150.1; Steuer et al., 1990) to these polypeptides, we demonstrate that immunodepletion of the dynactin complex eliminates vesicle motility. We find that dynactin is ubiquitously expressed, with at least three isoforms encoded by a single gene. The cloning and sequencing of a brain-specific isoform of chicken dynactin predicts a structural motif composed of a pair of long α -helical domains. Comparison with the sequence of a rat protein (Holzbaur et al., 1991) identified by antibodies to a 150-kD polypeptide found in some, but not all (Collins and Vallee, 1989), mammalian dynein preparations, reveals this protein to be the rat homologue of chicken dynactin. Highly conserved in sequence and predicted structure, dynactin also shows 50% sequence identity throughout most of its length to the *Drosophila* gene *Glued*, which encodes an essential protein that had earlier been implicated in several events, including a requirement for viability of individual cells in general (Harte and Kankel, 1982) and, more specifically, in eye organization and development of neurons in the optic lobe (Meyerowitz and Kankel, 1978; Harte and Kankel, 1982; Garen et al., 1984).

Materials and Methods

Dynein and Dynactin Purification

Details of the purification of dynein and dynactin and a thorough study of their effects on vesicle motility are presented elsewhere (Schroer and Sheetz, 1991a). Briefly, dynein from a high speed supernatant of chick embryo brain was bound to bovine microtubules in AMP-PNP and eluted with ATP (Vale et al., 1985a). The ATP release (or purified activator I complex) was subjected to velocity sedimentation in a 5–20% sucrose gradient. 20S dynein was applied to a MonoQ column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with KCl to separate dynein and dynactin.

Dynactin Immunoabsorption

Purified anti-dynactin IgG (mAb 150.1; Steuer et al., 1990) and mouse IgG (Sigma Chemical Co., St. Louis, MO) were coupled to CNBr-activated Sepharose CL-4B (Pharmacia Fine Chemicals) at a concentration of 5 mg IgG/ml resin. Dynactin from the MonoQ column was diluted 1:1 with 35 mM K-Pipes, pH 7.2, 5 mM MgSO₄, 5 mM EGTA, 0.5 mM EDTA, mixed with adsorbent, and incubated at 4°C for 4 h on an end-over-end rotator. The final supernatants were processed and assayed for motility as described below.

Vesicle Motility Assay

Vesicles were isolated from chick embryo fibroblasts and extracted with KI as described (Schroer et al., 1989). Phosphocellulose purified bovine microtubules (Williams and Lee, 1982) were sedimented onto glass coverslips whose surfaces were then coated with carrier protein to prevent microtubule gliding (Schroer and Sheetz, 1991a). Dynein and dynactin samples were desalted and concentrated by ultrafiltration to a final concentration of 0.02–0.10 mg/ml protein. Vesicle motor activity and directionality were determined as described (Schroer et al., 1988, 1989; Schroer and Sheetz, 1991a).

Identification of Dynactin cDNA Clones by Expression Library Screening

A λ gt11 chick embryo library (obtained from B. Vennstrom, EMBL, Heidelberg, Germany) was screened using mAb 150.1 (Steuer et al., 1990) that binds to the 150-kD doublet of dynein associated proteins. Bound primary antibody was detected with ¹²⁵I-labeled sheep anti-mouse IgG (ICN Biomedicals, Inc., Costa Mesa, CA). Antibody binding was done at room temperature in 50 mM Tris-Cl (pH 8.0), 300 mM NaCl, 0.3% Tween 20, and 4% bovine albumin. Washes were done in the same buffer, but without albumin. Clones were plaque purified and DNA was isolated as previously described (Lopata et al., 1983). The 3.5-kb cDNA sequences were liberated by digestion with EcoRI and subcloned into the EcoRI site of pBluescript KS II+ to produce plasmid p150A. This construct was subsequently used for both DNA sequencing and expression of the protein encoded by the cDNA.

DNA Sequencing

DNA from plasmid p150A was randomly sheared by sonication into fragments ranging from 300 bp to 1.0 kb. The ends of these fragments were repaired with Klenow and subcloned into M13-mp8. Single-stranded M13 DNA was prepared as previously described (Sanger et al., 1977) and sequenced using the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH). The sequence was assembled and analyzed using the Wisconsin GCG sequence analysis package.

Expression of Bacterial cDNA Protein

The cDNA clone in pBluescript (p150A) was transformed into XL-1 blue, a protease-deficient *Escherichia coli* strain. Expression of the cDNA-encoded protein was induced by growing the bacterial culture to an OD₆₀₀ of 0.5 and then adding isopropylthiogalactoside to a final concentration of 20 mM. The culture was grown for an additional 1 h and bacteria were pelleted by centrifugation, resuspended in 0.5% SDS, 50 mM Tris-Cl (pH 6.8), and lysed by sonication. After mixing with an equal volume of SDS sample buffer (Laemmli, 1970), this extract was used on SDS-PAGE. The lysate was stored at -20°C .

Generation of a Polyclonal Antibody to Bacterially Expressed Dynactin

The protein obtained by induction of p150A was partially purified by excising the appropriate gel band from an SDS polyacrylamide gel followed by electroelution. 20 μg of purified protein were mixed with Freund's adjuvant and injected intraperitoneally into BALB/c mice at 10-d intervals. Serum was obtained by either tail bleed or (for a final bleed) by heart puncture.

Preparation of DNA and RNA

Genomic DNA was isolated from chicken, human, mouse, and fly tissue as previously described (Maniatis et al., 1982). Briefly, the tissue was frozen in liquid nitrogen, ground into small particles, suspended in 10 mM Tris-Cl (pH 8.0), 0.1 M EDTA, 0.5% SDS, and 20 $\mu\text{g}/\text{ml}$ RNase A, and incubated with 100 $\mu\text{g}/\text{ml}$ proteinase K at 50°C overnight. The DNA was extracted several times with phenol:chloroform, ethanol precipitated, and resuspended in 10 mM Tris (pH 7.0) and 1 mM EDTA. Total RNA was prepared using the guanidine thiocyanate/CsCl method of Chirgwin et al. (1979). Tissues (or cultured cells) were frozen in liquid nitrogen and then disrupted in 4 M guanidinium isothiocyanate using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). RNA was pelleted through a CsCl cushion. After resuspension, poly A+ mRNA was selected by chromatography on oligo(dT) cellulose as described earlier (Cleveland et al., 1981).

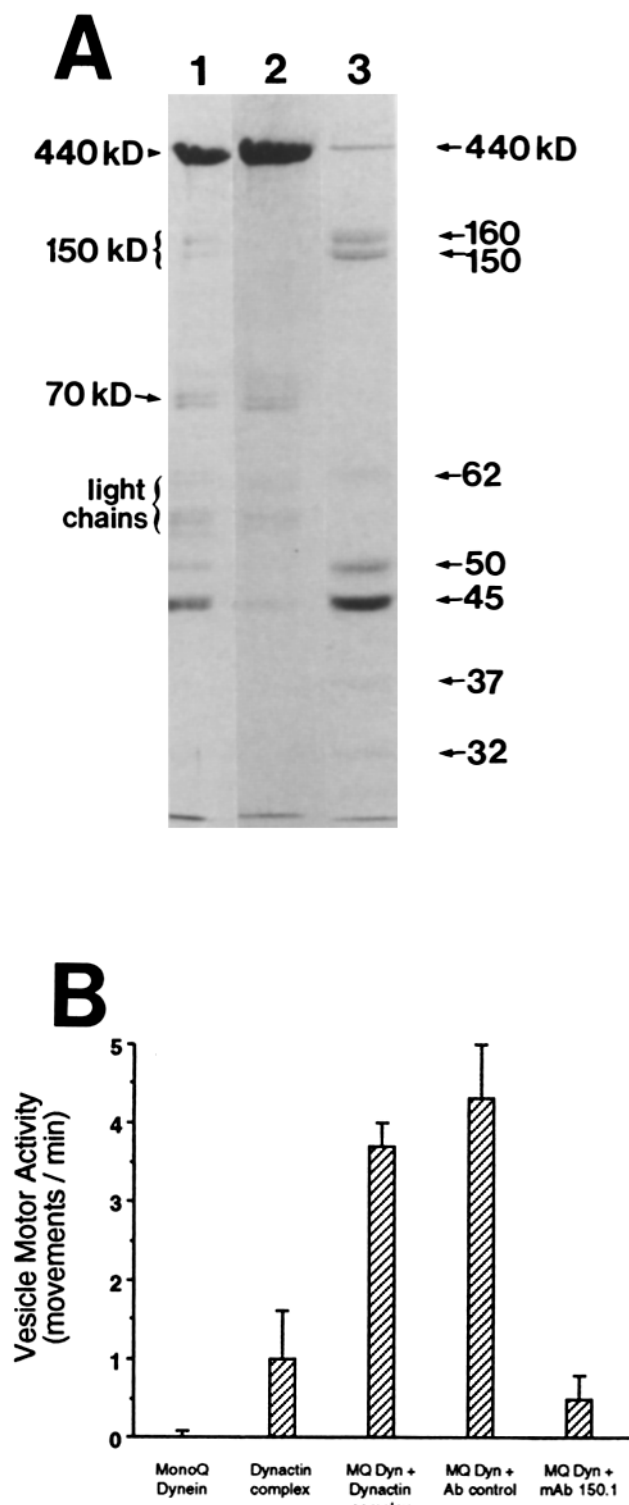


Figure 1. Vesicle motility mediated by monoQ purified dynein requires the 150-kD dynein associated proteins. (A) MonoQ ion exchange chromatography was used to fractionate 20S dynein and fractions were analyzed by SDS-PAGE. Shown are Coomassie blue-stained gels of 20S dynein (lane 1), monoQ purified dynein (lane 2), and the monoQ fraction enriched in the 150-kD polypeptides (lane 3). Positions of the 440-kD dynein heavy chain, 150-kD associated protein, 70-kD intermediate chains, and light chains are marked at the left. Molecular weights of the polypeptides in the motility activating fraction are marked at the right. (B) In vitro vesicle motility mediated by various dynein fractions: the ability of monoQ

Preparation of Tissue and Cell Extracts

Total cell protein was extracted from chicken tissues and from cultured MSB cells using a Polytron homogenizer to disrupt the tissue (or cells) in 50 mM Tris (pH 8.0) and 1% SDS. The samples were then boiled for 10 min and clarified by a brief centrifugation to remove insoluble debris. In some experiments, chick embryo brain extract was prepared by Dounce homogenization as described (Schroer et al., 1989; Schroer and Sheetz, 1991a). Preparations of soluble and cytoskeletal fractions from cultured cells were performed exactly as described previously (Lopata and Cleveland, 1987). Briefly, the soluble component was extracted by lysis in 1 ml of a microtubule stabilizing buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, 4% polyethylene glycol, and 0.5% Triton X-100). The remaining cytoskeletal portion was solubilized directly in Laemmli gel sample buffer. Equal proportions of soluble and cytoskeletal samples were then analyzed by gel electrophoresis and immunoblotting.

Gel Electrophoresis and Blotting

SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with Coomassie blue or with silver (using the silver nitrate protocol of Merrill et al., 1981). Protein concentrations were determined using the bicinchoninic acid assay (Smith et al., 1985). SDS-polyacrylamide gels were blotted to nitrocellulose probed with primary (mAb 150.1) and secondary antibodies (¹²⁵I-labeled sheep anti-mouse IgG) using conditions identical to that in the expression library screen.

Gel electrophoresis of RNA and DNA was as previously described (Lopata et al., 1983; Havercroft and Cleveland, 1984). Briefly, denaturing RNA gels were prepared in 1% agarose containing 2.2 M formaldehyde and DNA gels were prepared in 0.9% agarose. Both RNA and DNA gels were blotted to Gene Screen Plus (DuPont/New England Nuclear, Boston, MA). For both RNA and DNA blots, a ³²P-labeled hybridization probe was prepared from the entire 3,514 bp of cDNA from p150A using a random priming method. DNA blots were hybridized at 42°C for 16 h in a solution containing 50% formamide, 5× SSC (1× SSC is 15 mM sodium citrate, 150 mM NaCl), 0.01% albumin, 0.01% Ficoll 400, 0.01% polyvinylpyrrolidone, 1% SDS, 10% dextran sulfate, 50 mM sodium phosphate, and 100 μg/ml denatured herring sperm DNA. RNA blots were hybridized at 60°C for 16 h in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/ml denatured herring sperm DNA. Both DNA and RNA blots were washed twice for 15 min at room temperature in 2× SSC, 0.1% SDS, followed by two 30-min washes at 65°C in 0.2× SSC, 0.1% SDS.

Immunofluorescence Localization

Secondary chick embryo fibroblasts were grown on glass coverslips 24–36 h before use. For cells fixed before extraction, coverslips were put directly into -20°C methanol for 20 min. Coverslips were rehydrated in TTBS (0.3 M NaCl, 20 mM Tris, pH 7.5, 0.05% Tween 20) containing 2% albumin for 15 min at room temperature. For cells extracted before fixation, coverslips were incubated for 1 min at 37°C in microtubule stabilizing buffer (0.1 M Pipes, pH 6.9, 1 mM EGTA, 2 mM EDTA, and 4 M glycerol; Solomon et al., 1979) and then incubated for 1 min at 37°C in stabilization buffer containing 0.5% Triton X-100. Coverslips were then rinsed for 1 min in stabilization buffer at 37°C and plunged into methanol at -20°C for 20 min. The mAbs 150.1 and 70.1 (Steuer et al., 1990) and a tubulin polyclonal antibody (Cleveland et al., 1981) were diluted in buffered saline containing 2% albumin, added to each coverslip and incubated in a humidified cham-

purified dynein to support vesicle movement in vitro analyzed using video enhanced light microscopy (*monoQ dynein*); vesicle motility mediated by the 150-kD monoQ fraction only (*dynactin complex*); vesicle motility assayed after supplementing monoQ dynein with monoQ fractions enriched in the 150-kD associated proteins (*MQDyn + dynactin complex*); the 150-kD monoQ fraction was incubated with a nonspecific antibody covalently linked to Sepharose beads, then added to monoQ dynein and assayed for activation of vesicle motility (*MQDyn + Ab control*); vesicle motility after antibody depletion of the dynactin complex (*MQDyn + mAb 150.1*). The 150-kD monoQ fraction was immunoabsorbed with Sepharose-bound mAb 150.1, then added to monoQ dynein and assayed for vesicle motility.

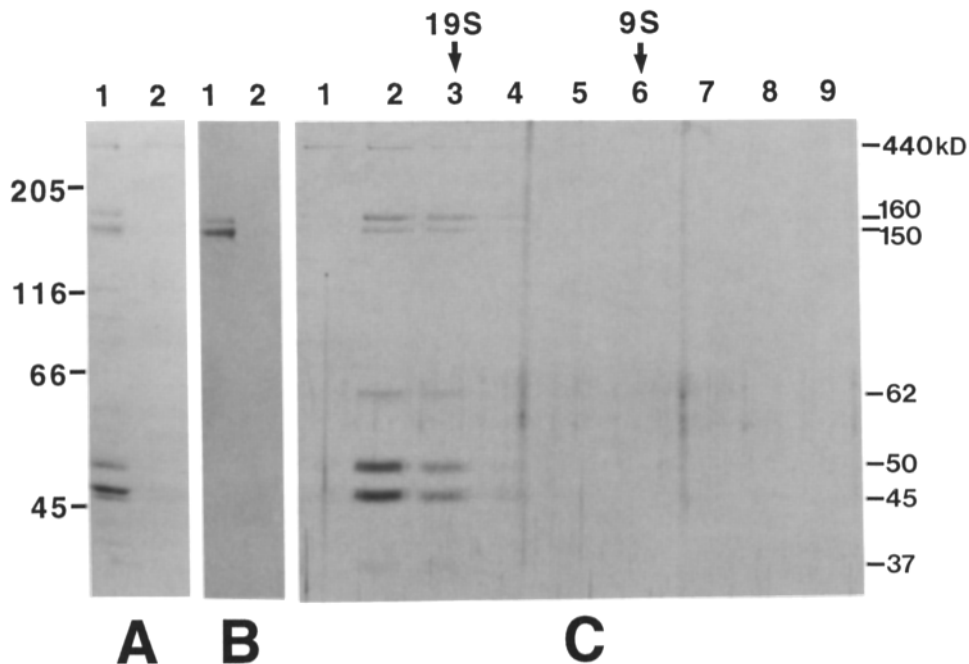


Figure 2. An activator of vesicle motility is a complex of proteins, a prominent component of which is a ~150-kD doublet. (A and B) Immunodepletion of the 150-kD monoQ fraction with Sepharose-bound mAb 150.1. (A) Coomassie blue-stained gel and (B) corresponding immunoblot with mAb 150.1 of the 150-kD monoQ fraction after incubation with a control, nonspecific antibody (lane 1) and after immunodepletion with mAb 150.1 (lane 2). Positions of molecular weight markers (in kilodaltons) are marked at the left of A. (C) Cosedimentation of the proteins eluting with the 150-kD polypeptides from the monoQ column. The material shown in Fig. 1 A, lane 3, was sedimented into a 5–20% sucrose gradient and fractions analyzed by gel electrophoresis. Positions of purified proteins (thyroglobulin [19S] and amylase [9S]) sedimented in a parallel gradient are noted by arrows.

ber for 30 min at room temperature. Coverslips were then washed two times for 5 min in TTBS. The murine antibodies were detected using biotin-conjugated horse anti-mouse antibody (1:250) and Texas red-conjugated streptavidin (1:1,000; both from Vector Laboratories, Inc., Burlingame, CA). The rabbit antibodies were detected using a fluorescein-conjugated goat anti-rabbit antibody (1:30; Vector Laboratories, Inc.).

Results

The Dynactin Complex: an Activator of Dynein-mediated Vesicle Motility

Standard dynein preparations rely on cosedimentation of microtubules and tightly bound dynein in the absence of ATP, release of dynein by addition of ATP, and a final sedimentation step to separate the rapidly sedimenting (20S) dynein complex from more slowly sedimenting contaminants. As shown in Fig. 1 A (lane 1), the final 20S fraction (from embryonic chick brain) is composed primarily of heavy (440 kD), intermediate (70 kD), and light chain (50–55 kD) dynein subunits, as well as a few other components, the most prominent of which is a doublet of 150-, 50-, and 45-kD polypeptides. When assayed for motor activity using video-enhanced microscopy to visualize microtubule movement mediated by immobilized dynein, this 20S dynein promotes microtubule gliding at 0.5 $\mu\text{m/s}$ as reported previously (Lye et al., 1987; Paschal et al., 1987; Schroer et al., 1989), and supports low levels of vesicle motility in an *in vitro* system composed of microtubules, dynein, and salt-washed chick fibroblast vesicles (Schroer and Sheetz, 1991a).

A core complex of dynein subunits can be further purified from 20S dynein by monoQ ion exchange chromatography (Fig. 1 A, lane 2). MonoQ dynein is composed of the 440-, 70-, and 50-kD polypeptides and retains full gliding activity

(Schroer and Sheetz, 1991a). However, it is incapable of supporting vesicle movement (Fig. 1 B). Unlike this monoQ dynein fraction, a small amount of motility (Fig. 1 B, dynactin complex bar) is seen in a monoQ fraction (referred to as activator I; Schroer and Sheetz, 1991a) containing only a trace amount of dynein and high levels of a 150-kD doublet as well as 62-, 50-, 45-, 37-, and 32-kD species (Fig. 1 A, lane 3). (The level of vesicle motility displayed by this fraction alone is always low, but is highly variable between different preparations, probably the result of the activator stimulating a variable amount of dynein that trails into this monoQ fraction [Fig. 1 A, lane 3].) Full vesicle motor activity is restored by readdition to monoQ dynein of this Activator I fraction (Fig. 1 B, MQDyn+Dynactin complex bar). A ratio of activator/dynein approximating that found in 20S dynein is required to restore a comparable level of vesicle motility, although the variable amount of residual activity in the activator I fraction alone makes precise analysis difficult.

Using a mAb that binds to the 150-kD doublet of polypeptides (mAb 150.1; Steuer et al., 1990), the requirement of the 150-kD component for activating dynein-mediated vesicle motility was tested by immunoadsorbing the activator I monoQ fraction before the motility assay. This procedure effectively removed the 150-kD components as judged by SDS-polyacrylamide gels (Fig. 2 A, lane 2) or by immunoblotting (Fig. 2 B, lane 2). However, most polypeptides in the initial monoQ fraction (including the 62-, 50-, 45-, 37-, and 32-kD species) were also quantitatively removed by the anti-150-kD antibody, strongly suggesting that all exist in a single complex. This possibility was supported by the consistent coelution of these polypeptides from the monoQ column and by copurification on a mAb 150.1 affinity column (not shown). Further confirmation was obtained by velocity sedimentation. As shown in Fig. 2 C, sucrose gra-

dient centrifugation revealed that the 150-kD doublet cosediments with the 62-, 50-, 45-, 37-, and 32-kD subunits (at a position similar to that of 20S or core dynein; not shown). Densitometry of gel lanes such as in Fig. 1 A, lane 3, revealed an apparent molar stoichiometry of 1:1:1:3:8:1:1, respectively, for the 160-, 150-, 62-, 50-, 45-, 37-, and 32-kD components. Immunoblotting has revealed that at least a portion of the abundant 45-kD component is actin (Schroer and Sheetz, 1991a).

In any event, the immunodepleted material no longer activated dynein-based vesicle movements (Fig. 1 B), while mock adsorbed controls were unaffected. This finding demonstrates that in this *in vitro* assay the 150-kD doublet (along with the associated polypeptides) plays a key role in dynein-based vesicle motility. We propose the name dynactin (for *dynein activator*) for these immunologically related 150-kD subunits, and the name dynactin complex for the polypeptide complex in Activator I.

Identification of a cDNA Clone Encoding Dynactin

As a first step in examining how the dynactin complex enhances vesicle motility, we sought to determine the primary structure of one dynactin polypeptide. To do this, we used mAb 150.1 to screen a λ gt11 expression library constructed from chicken embryo mRNA. Immunopositive clones were plaque purified and one was found to contain 3.5 kb of cDNA. DNA sequencing of the ends of the cDNA showed that the cDNA extended to the poly A tail, but was out of frame with β galactosidase. However, immunoblotting revealed that an immunoreactive polypeptide of \sim 150 kD was accumulated after induction (not shown). This putative dynactin cDNA was subcloned adjacent to the lacZ promoter to produce plasmid p150A. Induction of expression again produced a 150-kD protein (along with a series of degradation products) that was immunoreactive not only with mAb 150.1 (Fig. 3, lane 8) but also with a polyclonal antibody affinity purified against authentic dynactin (not shown). The bacterially produced protein displayed an electrophoretic mobility indistinguishable from that of the smaller dynactin polypeptide (compare lanes 7 and 8 in Fig. 3). These data indicate that the cDNA contains an open reading frame encoding a protein of \sim 150 kD and that translation initiates from a site within the cDNA.

Immunologic methods were used to verify that the clone represented authentic dynactin sequences. As shown above, the bacterial expression product of p150A was recognized both by mAb 150.1 (Fig. 3, lane 8) and by an affinity-purified polyclonal antibody specific for the 150-kD subunits in 20S dynein. Further, using either the polyclonal antibody or the original mAb, the immunoreactivity of the cDNA-encoded protein was as strong as immunoreactivity against the authentic 150-kD polypeptides, as would be anticipated if all share a common set of epitopes. Second, a polyclonal antibody (pAb 150) raised against the bacterially expressed protein recognized authentic dynactin polypeptides in a total chick brain extract (Fig. 3, lane 2) and in 20S cytoplasmic dynein (lane 5). (pAb 150 also recognizes a 53-kD polypeptide [Fig. 3, lanes 2 and 5]; whether this is a proteolytic fragment or a related protein is not established.) Taken together with the sequence similarity to a 150-kD dynein-associated protein from rat (see below), we conclude that cDNA clone p150A encodes chicken dynactin.

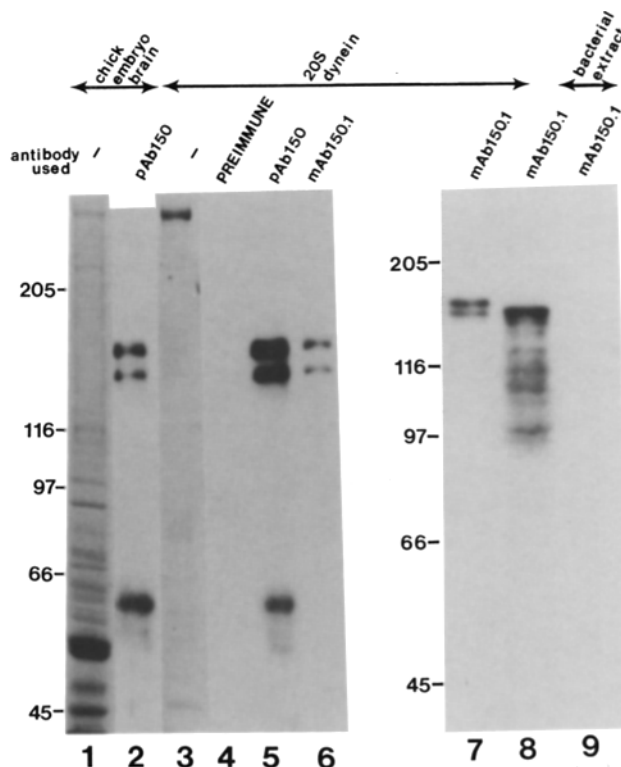


Figure 3. Verification that the cDNA clone, p150A, encodes an authentic 150-kD dynactin. Polyclonal antibodies (pAb150) raised against the protein encoded by cDNA p150A bind specifically to the authentic 150-kD dynactin doublet and a mAb to authentic dynactin (mAb 150.1) recognizes the protein encoded by p150A. Preparations of total chick brain extract, chick 20S dynein, and bacterial protein extracts with and without the p150A encoded protein were electrophoresed on SDS polyacrylamide gels and then either stained with Coomassie blue (lanes 1 and 3) or blotted to nitrocellulose (lanes 2 and 4–9). Lanes 1 and 2, chick embryo brain extract; lanes 3–7, 20S dynein; lane 8, bacterial extract after induction of expression of the p150 encoded protein; lane 9, control bacterial extract. Blotted proteins were probed with antibody pAb150 (lanes 2 and 5), preimmune serum from the mice used to produce pAb150 (lane 4), mAb 150.1 (used to isolate the p150A cDNA clone) (lanes 6–9).

The Primary Structure of One Dynactin Isoform Reveals Two Long, Coiled-Coil α -helical Domains

The cDNA clone p150A was completely sequenced to determine the primary structure of the encoded dynactin polypeptide. This revealed a putative 5' untranslated region of 69 bases, a 1,053 amino acid open reading frame, a 135 base 3' untranslated region, and a terminal poly A tract (the nucleotide sequence has been deposited in EMBL; copies are available upon request). Several lines of evidence suggest that the 5'-most ATG in the sequence represents the authentic translation initiation site of this dynactin isoform. First, RNA blotting demonstrated that one of two dynactin mRNAs in chick brain is \sim 3.6 kb, essentially the size of the 3,514-bp cDNA (Fig. 6 A, lane 1). Second, when this site is used for translation initiation in bacteria, the product is indistinguishable in size from the smaller dynactin isoform. Third, the sequences surrounding the ATG lie in a reasonable context for eukaryotic translation initiation (Kozak, 1987). Still, no in-

Chick M-----ynrtpsqrmsteasarpagrlpcggdwehgrgtvayvgatlfatqkvvgwildeakgndgtvqrkyftcdeghgiivrqsqivfcdgadt----- 103
Rat Magskrh----- 120
Glued Msvrsvlespslswshttrqtqrerdvrekpesgrpradrqsaahgclrgdddlrvqgvvvldepkgkngsikqgqyfqdcengmfvptqirllleaapgrsrsiedvsg

Chick t-----sssepstpaqtP-----Iva-----PvipsP 22
Rat t-----sssepstpaqtP-----Iva-----PvipsP 197
Glued t-----sssepstpaqtP-----Iva-----PvipsP 240

Chick s-----sssepstpaqtP-----Iva-----PvipsP 141
Rat s-----sssepstpaqtP-----Iva-----PvipsP 317
Glued s-----sssepstpaqtP-----Iva-----PvipsP 360

Chick s-----sssepstpaqtP-----Iva-----PvipsP 250
Rat s-----sssepstpaqtP-----Iva-----PvipsP 424
Glued s-----sssepstpaqtP-----Iva-----PvipsP 480

Chick s-----sssepstpaqtP-----Iva-----PvipsP 370
Rat s-----sssepstpaqtP-----Iva-----PvipsP 546
Glued s-----sssepstpaqtP-----Iva-----PvipsP 600

Chick s-----sssepstpaqtP-----Iva-----PvipsP 485
Rat s-----sssepstpaqtP-----Iva-----PvipsP 661
Glued s-----sssepstpaqtP-----Iva-----PvipsP 720

Chick s-----sssepstpaqtP-----Iva-----PvipsP 604
Rat s-----sssepstpaqtP-----Iva-----PvipsP 780
Glued s-----sssepstpaqtP-----Iva-----PvipsP 840

Chick s-----sssepstpaqtP-----Iva-----PvipsP 718
Rat s-----sssepstpaqtP-----Iva-----PvipsP 895
Glued s-----sssepstpaqtP-----Iva-----PvipsP 957

Chick s-----sssepstpaqtP-----Iva-----PvipsP 828
Rat s-----sssepstpaqtP-----Iva-----PvipsP 1004
Glued s-----sssepstpaqtP-----Iva-----PvipsP 1076

Chick s-----sssepstpaqtP-----Iva-----PvipsP 948
Rat s-----sssepstpaqtP-----Iva-----PvipsP 1124
Glued s-----sssepstpaqtP-----Iva-----PvipsP 1188

Chick s-----sssepstpaqtP-----Iva-----PvipsP 1028
Rat s-----sssepstpaqtP-----Iva-----PvipsP 1244
Glued s-----sssepstpaqtP-----Iva-----PvipsP 1257

Chick s-----sssepstpaqtP-----Iva-----PvipsP 1053
Rat s-----sssepstpaqtP-----Iva-----PvipsP 1325
Glued s-----sssepstpaqtP-----Iva-----PvipsP 1319

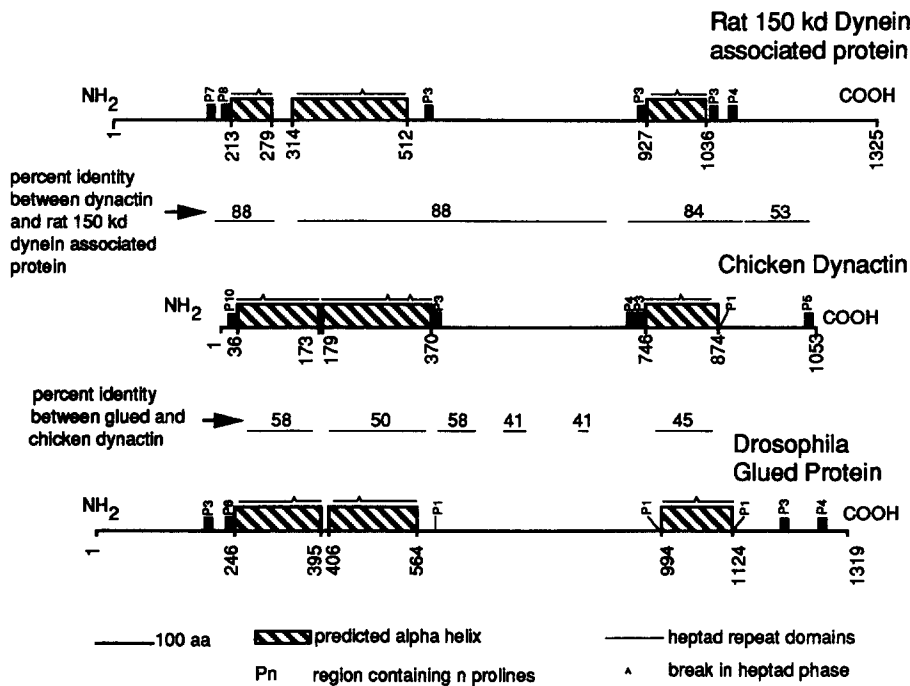


Figure 5. Predicted structure of dynactin and its similarity to a rat dynein associated protein and to the *Drosophila* Glued protein. The predicted secondary structure of dynactin, a rat dynein associated protein, and the *Drosophila* Glued protein were determined by combining the methods of Chou and Fasman (1974) and Garnier et al. (1978). The schematic aligns the three proteins to highlight their similarity in structure as well as domains of high sequence similarity. The percent of identical amino acids shared is shown for the domains of highest similarity.

frame translation terminating codons are found in the 69 bases of 5'-most sequence and attempts to identify longer cDNA clones and to use primer extension to determine additional 5' sequence have been unsuccessful. Hence, although we cannot rigorously exclude the possibility that an ATG lying upstream may represent the true translation initiation site, the 5'-most ATG is likely to be the correct site.

The 1,053 amino acid sequence encoded by p150A, shown in Fig. 4, yields a calculated molecular mass of 117 kD with a predicted pI of 5.3. This agrees with the measured pI of the smaller dynactin isoform (Toyoshima, I., and M. Sheetz, unpublished observations). Although the calculated molecular weight is smaller than that estimated for dynactin from mobility on SDS-polyacrylamide gels, this is not uncommon for large proteins. Most importantly, the 117-kD polypeptide comigrates with the smaller authentic dynactin isoform (Fig. 3, lanes 7 and 8).

The secondary structure of dynactin was deduced from the primary amino acid sequence using the methods of Chou and Fasman (1974) and Garnier et al. (1978). Both methods predict dynactin to be composed of two long α -helical domains of ~ 380 and ~ 130 amino acids that lie near the amino and carboxy termini, respectively (Fig. 5). Within each of these predicted helical domains runs a prominent heptad repeat (marked with asterisks above the amino acid sequence in Fig. 4) in which the first and fourth residues are usually hy-

drophobic. This motif, widely found in structural proteins, facilitates homo- or hetero-dimerization promoted by hydrophobic interactions between parallel α -helices. A search for other motifs within the primary sequence did not reveal putative nucleotide binding domains or other known motifs, except for 37 potential phosphorylation sites.

Dynactin Sequence and Structure Are Highly Conserved in Vertebrates and Show High Homology to Glued, an Essential Drosophila Protein

Search of currently available protein sequences revealed high sequence homology of dynactin to only two proteins: a 150-kD rat protein (Holzbaur et al., 1991) identified by its immunological relatedness to a ~ 150 -kD polypeptide present in some (Collins and Vallee, 1989), but not all (Paschal et al., 1987; Pfarr et al., 1990), mammalian dynein preparations and the *Drosophila* Glued protein (the aligned sequences are presented in Fig. 4 and summarized schematically in Fig. 5). Throughout a central 1,000 amino acid domain, chicken dynactin is 85% identical to the 150-kD rat protein. Both the chicken and rat proteins are predicted to contain similar coiled-coil domains separated by a spacer region that is conserved in sequence. The rat protein is longer at both amino and carboxy termini and the extreme carboxy termini show no similarity. While we conclude that these two

Figure 4. Primary sequence of chicken dynactin deduced from cDNA clone p150A. (*Top line*) The amino acid sequence of chick dynactin was deduced from the 3,514 nucleotide sequence of p150A. All regions were sequenced multiple times on both strands. Amino acids predicted to be in a helical conformation are overlined; within those domains, amino acids comprising a hydrophobic heptad repeat are marked with asterisks, while charged residues in positions 1 and 4 of the repeat are marked with (-). A denotes the positions of breaks in the heptad phase. (*Middle line*) The deduced amino acid sequence of a rat 150-kD protein found in some dynein preparations (Holzbaur et al., 1991) aligned with chick dynactin (using the algorithm of Lipman and Pearson, 1985). (*Bottom line*) The deduced amino acid sequence of the *Drosophila* Glued protein (Swaroop et al., 1987) aligned with chick dynactin. Boldface capital letters denote positions of sequence identity in all three sequences. These sequence data are available from EMBL/GenBank/DBJ under accession number X62773.

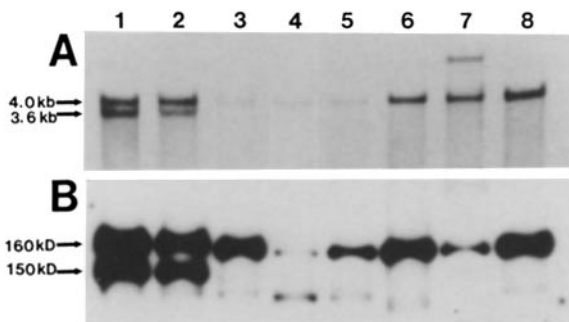


Figure 6. Three mRNAs encode at least three dynactin isoforms in various chicken tissues. (A) RNA blot analysis of total RNA isolated from various chicken tissues probed with the cloned dynactin cDNA from p150A. (B) Immunoblot analysis of dynactin isoforms in chicken tissue extracts probed with mAb 150.1. Lane 1, 11-d embryonic chick brain; lane 2, adult brain; lane 3, cardiac muscle; lane 4, liver; lane 5, kidney; lane 6, smooth muscle; lane 7, skeletal muscle; lane 8, MSB cells, a Marek's virus transformed lymphoblastoid line. Sizes are shown at the left in kilobases or kilodaltons, respectively, for A and B.

proteins both represent dynactins, the terminal sequence differences suggest that they represent different isoforms of this conserved polypeptide family (see below).

Both chicken and rat dynactins also display multiple domains of strong sequence identity (between 58 and 41%) to the *Drosophila* Glued protein (see Fig. 5). Such sequence similarity is highly significant and is reflected not only in the primary sequence, but also the secondary structure features. All are predicted to have two major coiled-coil helical domains of similar lengths and relative locations within the polypeptide backbone. These helical domains and the non-helical sequences just adjacent (particularly for the amino-terminal helix) represent the regions of highest sequence identity. This is surprising since only a helical domain with a repeating heptad motif, rather than strongly conserved primary sequence, is required to retain a common coiled-coil structure. Also conserved is a short segment predicted to disrupt the amino-terminal helix, splitting it into two parts. Not conserved are the positions of disruptions within the heptad phase (four of which occur in dynactin, compared with three in Glued; see Fig. 5). Substantial sequence similarity con-

tinues as well in portions of the segment that separates the two major helices, demonstrating selective constraints beyond the helical domains. The most amino-terminal and carboxy-terminal sequences show little conservation.

From the strong sequence and structural similarity along most of their lengths, it is likely that dynactin is the vertebrate homologue of *Glued*.

Dynactin Is an Abundant Protein Composed of at Least Three Isoforms Encoded by a Single Gene

Mutations in the *Drosophila* *Glued* gene have shown that *Glued* function is required for proper early development, suggesting a general role in many, perhaps all, cells (Harte and Kankel, 1982). To determine the corresponding distribution and abundance of dynactin, we used RNA (Fig. 6 A) and protein (Fig. 6 B) blots of samples from selected chicken tissues. Parallel immunoblots were probed for dynein heavy and light chains (not shown), as were additional immunoblots of various fractions from a dynein purification (Table I). Several relevant points emerge from this analysis. First, dynactin is found ubiquitously. Second, using known amounts of purified dynactin or dynein as quantitation standards, we find in these extracts that dynactin is present in stoichiometric amounts with dynein heavy chain and represents ~0.1% of total soluble protein (except in liver where it is ~10-fold less abundant). Third, three electrophoretically distinct isoforms of dynactin are present in different tissues: two are found in brain (Fig. 6 B, lanes 1 and 2), while in all other tissues examined a third form of intermediate mobility is detected (Fig. 6 B). Fourth, in addition to a ubiquitous 4.0-kb RNA (Fig. 6 A, lanes 1-8) and a 3.6-kb brain-specific mRNA (Fig. 6 A, lanes 1 and 2), a much larger (7 kb) dynactin mRNA is present in skeletal muscle (Fig. 6 A, lane 7), raising the possibility of a fourth dynactin isoform, although no corresponding dynactin of unique mobility was observed (Fig. 6 B, lane 7). Fifth, using immunoblotting to follow dynactin and dynein recovery throughout dynein purification from chick brain (Table I), only half as much dynactin sediments with microtubules as does dynein, and only about one-fifth of that elutes from the microtubules with ATP. This results in 20S dynein containing only approximately one-tenth the initial molar level of dynactin, consistent with the reduced ability of this fraction to support vesicle motility

Table I. Fractionation of Dynactin during Dynein Purification

	Soluble brain homogenate	Sedimentation with microtubules	ATP release	20S dynein
Percentage of total protein comprised by dynein heavy chain	0.6	2.5	20	80
Percentage of total protein comprised by dynactin	0.2	0.4	0.6	2.4
Percentage of initial dynein heavy chain recovered in this step	100	25	25	20
Percentage of initial dynactin recovered in this step	100	12	2.5	1.8
Molar ratio of dynactin:dynein heavy chain	1	0.5	0.09	0.09

Abundance of dynein heavy chain and dynactin in each fraction was measured by immunoblotting. Quantitation standards were provided by parallel immunoblots of a series of dilutions of known amounts of dynein heavy chain or dynactin.

(Schroer et al., 1989). Sixth, since the dynactin with the smallest apparent size and a corresponding 3.6-kb brain-specific mRNA are seen only in brain tissue, it is likely that this isoform arises from this shorter mRNA. In view of the co-migration of our cloned dynactin protein with the smaller isoform, it seems reasonable to suggest that p150A encodes this smaller, brain-specific dynactin.

Whether the multiple RNAs and isoforms are products of a single gene or a gene family (as is the case, for example, for the kinesin-like group of microtubule motors; e.g., see Endow and Hatsumi, 1990) was examined by genomic DNA blotting. Moderate stringency hybridizations revealed a pattern consistent with a single gene in the chicken, mouse, or human genomes (not shown).

Dynactin Localizes to Punctate Cytoplasmic Spots and to Interphase and Mitotic Centrosomes

To determine the intracellular distribution of dynactin, double immunofluorescence microscopy was used to localize dynactin and tubulin within the same cells. In cells fixed without prior extraction dynactin was found in a punctate cytoplasmic pattern (Fig. 7 A). The punctate pattern was similar to that found for the 70-kD subunit of dynein (Fig. 7 E). (Unfortunately, double immunofluorescence could not be performed to visualize dynein and dynactin simultaneously as all our dynactin antibodies and antibodies monospecific for dynein are mouse antibodies.) Although many spots were aligned in a pattern consistent with an association with microtubules (Fig. 7 B), given the large number of spots this may only be coincidental. If soluble components were extracted by detergent lysis in a solution that stabilizes microtubules, most dynactin staining in interphase and mitotic cells was eliminated (Fig. 7 C), although the microtubule array remained intact as expected (Fig. 7 D). The most prominent dynactin localization remaining in such cytoskeleton preparations was at the centrosomes of both interphase (Fig. 7 C, main panel) and mitotic (Fig. 7 C, inset) cells, a localization not found for dynein itself (Steuer et al., 1990; not shown). Staining of mitotic spindles was also found, although whether this reflects true association or nonspecific trapping in the dense mitotic arrays is not certain. In all cases, similar distributions were found following alternative fixation procedures (paraformaldehyde with or without prior extraction) or after extraction with 1% saponin, which permeabilizes membranes while leaving the phospholipids largely intact (not shown).

That little dynactin is tightly associated with the cytoskeleton was further examined by immunoblotting soluble and cytoskeletal fractions. As shown in Fig. 7 F (top, lanes 1 and 2), about two-thirds of cell tubulin was in polymer form under normal culture conditions, while only ~10% of dynactin was in the cytoskeletal fraction (Fig. 7 F, middle). Cytoskeleton-associated dynactin was not affected by colchicine-induced microtubule disassembly (lanes 3 and 4) or complete tubulin polymerization stimulated by taxol (lanes 5 and 6), mirroring the behavior of dynein (Fig. 7 F, bottom). Despite the association of a small amount of dynactin with microtubules during dynein purification (Table I), we conclude that *in vivo* most dynactin is not tightly bound to microtubules and is either soluble or attached to vesicles. Further, the potential binding of the purified dynactin complex to mi-

cro-tubules was examined *in vitro*. As assayed by copelleting with taxol-stabilized microtubules, monoQ dynactin complex failed to bind to microtubules either in the presence or absence of ATP (5 mM), while monoQ dynein bound in the absence of ATP as expected (not shown).

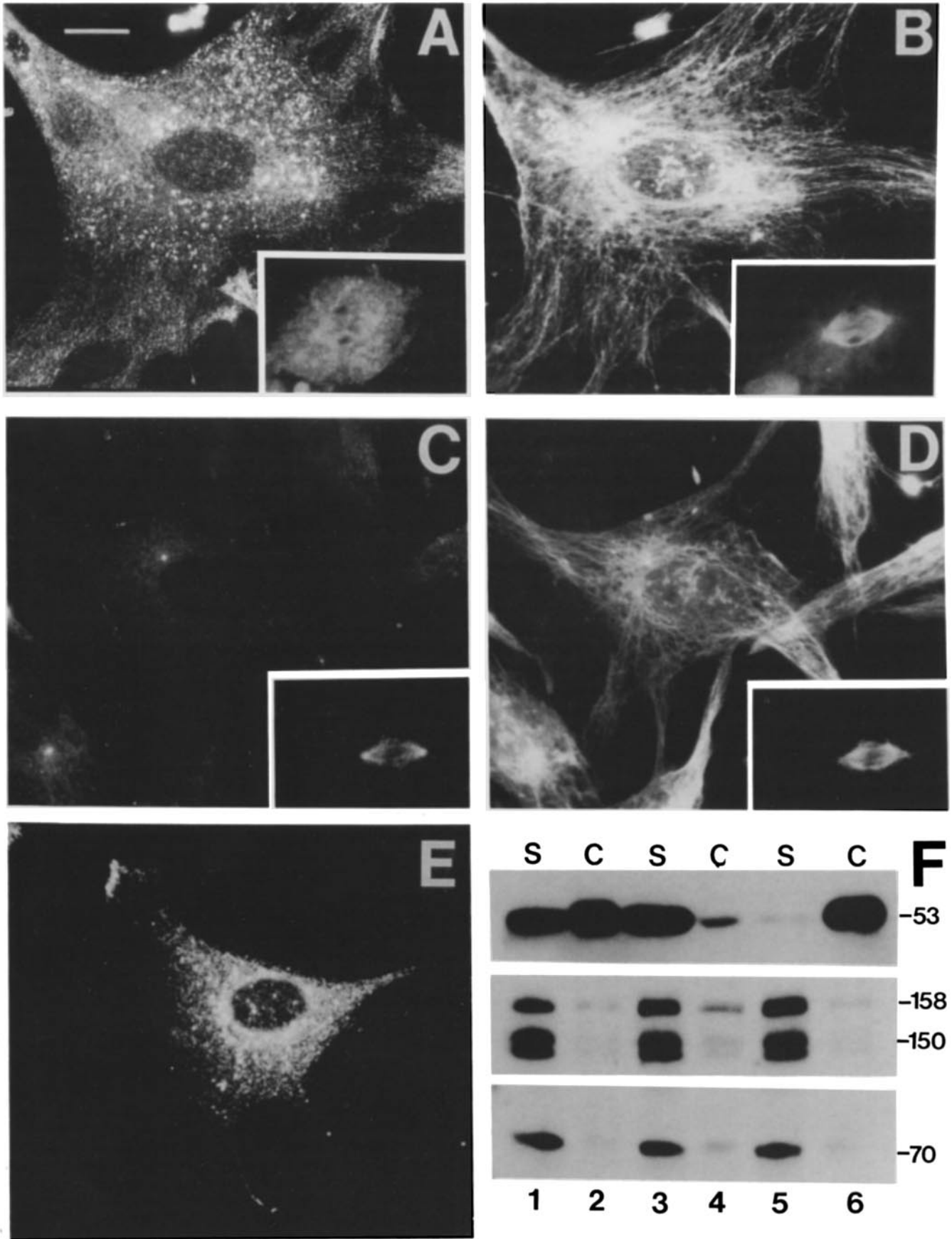
No binding of dynactin antibodies to kinetochores was apparent at any phase of the cell cycle, although weak staining of mAb 150.1 has been reported on isolated chromosomes (Steuer et al., 1990). Since immunofluorescence analyses are inevitably subject to caveats concerning artifacts of fixation and nonspecific antibody binding, interpretation of such weak immunofluorescent signals cannot be made unambiguously. What is very clear from our analysis here is that little, if any, dynactin is tightly bound to microtubules, and even less to chromosomes.

Discussion

The discovery of motor proteins with the capacity to translocate along microtubules first came from examination of vesicle movement within axons. By exploiting new microscopic methods, extruded squid axoplasm was seen to support vesicle movement in two directions along individual microtubules (Allen et al., 1985; Schnapp et al., 1985). The mechanochemical enzyme kinesin was soon discovered to translocate in the plus end direction (Vale et al., 1985) and was initially reported to stimulate movement of impure organelles, although later efforts demonstrated that salt-washed vesicles did not move in the presence of kinesin alone (Schroer et al., 1988). Similar results are found for cytoplasmic dynein: depletion of dynein from a cell extract using UV photocleavage disrupts movement of salt-washed vesicles *in vitro*, yet 20S dynein alone has much reduced activity (Schroer et al., 1989) and purified cytoplasmic dynein has none.

Although, as expected, either motor is sufficient for ATP hydrolysis and translocation along microtubules (detected as microtubule gliding when the motors are immobilized), successful vesicle transport requires additional factors. We have now identified dynactin as a major component of one such factor. *In vitro*, the dynactin complex facilitates dynein-mediated transport of salt-washed vesicles. However, most dynactin fails to cofractionate stoichiometrically with dynein through microtubule binding and release steps, suggesting either that dynactin only transiently associates with dynein or microtubules, that it binds more tightly to other cell components (perhaps vesicles), or both. This is not too surprising, since binding to vesicles and activation of vesicle-bound motors represent properties not required for ATP hydrolysis or simple translocation. It must also be emphasized that the complex regulation of bidirectional transport mediated through multiple motors suggests the presence of additional soluble activators. Indeed, we have evidence for multiple dynein activators, one of which also facilitates kinesin-based motility (Schroer and Sheetz, 1991a).

With regard to the question of how the dynactin complex promotes vesicle movement, it does not seem to act exclusively through binding to dynein itself, even though the two are distributed similarly in the cytoplasm (Fig. 7). Initially equimolar with dynein heavy chain in brain extracts, only about a tenth of that level is recovered in 20S dynein. That ATP releases only ~20% of dynactin but most of dynein



bound to microtubules (Table I; see also Holzbauer et al., 1991) clearly indicates that dynactin and dynein bind microtubules independently. The dynactin-microtubule interaction is probably indirect, since (a) most dynactin is not cytoskeleton associated, (b) binding motifs common to known microtubule-associated proteins are not found in the dynactin sequence, and (c) monoQ purified dynactin does not rebind efficiently to microtubules in vitro. Nor is dynactin necessary to mediate dynein attachment to membranes since monoQ purified dynein alone is sufficient for vesicle-microtubule binding (Schroer and Sheetz, 1991a), although whether such binding represents a physiologically meaningful interaction is not established.

The most striking structural feature of dynactin is the presence of two long domains with all the hallmarks of forming coiled-coil α -helices. Such heptad repeat-containing domains are characteristic of filamentous structural proteins and it seems possible that dynactin forms homopolymeric or heteropolymeric filamentous structures. While dynactin shows some sequence similarity to several previously described α -helical, structural proteins (e.g., myosin), by far the highest similarities are to a rat brain dynein associated protein and to the *Drosophila* Glued protein. Since chicken dynactin, the rat protein, and Glued are homologous throughout most of their lengths (although the brain-specific dynactin isoform we have identified is shorter at both amino and carboxy termini) and since high sequence similarity extends outside the structurally conserved helical domains, it seems very likely that the three represent homologous proteins.

Previous genetic analyses of Glued offer persuasive, but as yet incomplete, views as to potential in vivo roles for dynactin. The first described mutation (Plough and Ives, 1935) was found to be a recessive lethal, indicating that the protein is probably essential. However, in the heterozygote the mutation produces a dominant phenotype in which normal development of the compound eye and of optic lobes innervated by retinal axons is disrupted (Meyerowitz and Kankel, 1978). Subsequent efforts have identified 10 additional Glued mutations, all of which are recessive embryonic lethals (Harte and Kankel, 1982). Further, because attempts to produce mosaic animals carrying clones of cells homozygous for these mutations were unsuccessful, it was concluded that Glued function is required for the viability of individual cells. As Harte and Kankel (1982) initially proposed, the requirement for Glued must lie in its effect on some fundamental cellular function, such as mitosis. The identification of Glued to be the homologue of a protein that mediates dynein-dependent vesicle transport and a small proportion of which is associated with centrosomes and mitotic spindles (Fig. 7C) clearly suggests that the essential function lies in some aspect of microtubule-based motility. Consistent with this is

the expression of dynactin or Glued in all chicken and fly tissues, respectively.

In addition to a general role in all cells, two lines of evidence suggest that dynactin may play a fundamental role in axonal transport within neurons. First, in chicken the major dynactin isoforms are of different mobility than dynactin in other tissues. Second, the dominant Glued mutation preferentially affects the axons growing from the photoreceptor cells of the developing eyes (Meyerowitz and Kankel, 1978). The dominance of this mutation (resulting from a transposon-like insertion near the 3' end of the Glued gene that results in a truncated RNA transcript [Swaroop et al., 1985, 1986]) suggests a special requirement in these neurons beyond that critical for basic cell function.

Remaining unresolved is the mechanism through which the dynactin complex stimulates dynein-mediated vesicle movement. Dynactin's ubiquity and size suggest a structural rather than catalytic mechanism and the coiled-coil motif clearly suggests assembly into higher order structures. Insight as to whether the interaction alters dynein ATPase or dynein's affinity for vesicles seems likely to emerge from the use of in vitro biochemical assays to extend our understanding of dynactin properties and the other components of the dynactin complex.

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Figure 7. Soluble dynactin is found in a punctate distribution throughout the cytoplasm and at centrosomes. Double immunofluorescence was used to localize dynactin and tubulin in cells fixed without prior extraction (A, B) or after detergent extraction of soluble components under conditions that stabilize microtubules (C, D). (A, C) Dynactin visualized with mAb 150.1; (B, D) tubulin localized in the same cells using a rabbit polyclonal antibody to tubulin. Insets show mitotic cells. (E) The 70-kD dynein subunit localized with mAb 70.1 in cells fixed without detergent extraction. Bar, 15 μ m. (F) Immunoblot analysis of tubulin (top), dynactin (middle), and the 70-kD dynein subunit (bottom) in soluble (S) or cytoskeletal (C) fractions of chick embryo fibroblasts. Lanes 1 and 2, soluble and cytoskeletal fractions from fibroblasts grown under normal conditions; lanes 3 and 4, soluble and cytoskeletal fractions from cells in which microtubules were disassembled by addition of 10 μ M colchicine for the final 5 h of culture; lanes 5 and 6, soluble and cytoskeletal fractions from cells treated for 5 h with 10 μ M taxol to force assembly of all cell tubulin. Molecular weights are shown at right.

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