

# Cytoplasmic Dynein Binds Dynactin through a Direct Interaction between the Intermediate Chains and p150<sup>Glued</sup>

Kevin T. Vaughan and Richard B. Vallee

Cell Biology Group, Worcester Foundation for Biomedical Research, Shrewsbury, Massachusetts 01545

**Abstract.** Cytoplasmic dynein is a retrograde microtubule motor thought to participate in organelle transport and some aspects of minus end-directed chromosome movement. The mechanism of binding to organelles and kinetochores is unknown. Based on homology with the *Chlamydomonas* flagellar outer arm dynein intermediate chains (ICs), we proposed a role for the cytoplasmic dynein ICs in linking the motor protein to organelles and kinetochores. In this study two different IC isoforms were used in blot overlay and immunoprecipitation assays to identify IC-binding partners. In overlays of complex protein samples, the ICs bound specifically to polypeptides of 150 and 135 kD, identified as the p150<sup>Glued</sup> doublet of the dynactin complex. In

reciprocal overlay assays, p150<sup>Glued</sup> specifically recognized the ICs. Immunoprecipitations from total Rat2 cell extracts, rat brain cytosol, and rat brain membranes further identified the dynactin complex as a specific target for IC binding. Using truncation mutants, the sites of interaction were mapped to amino acids 1-123 of IC-1A and amino acids 200-811 of p150<sup>Glued</sup>. While cytoplasmic dynein and dynactin have been implicated in a common pathway by genetic analysis, our findings identify a direct interaction between two specific component polypeptides and support a role for dynactin as a dynein "receptor". Our data also suggest, however, that this interaction must be highly regulated.

CYTOPLASMIC dynein is a minus end-directed microtubule motor (Paschal and Vallee, 1987) thought to participate in retrograde axonal transport, centripetal organelle movement, and some aspects of chromosome segregation (for a review see Holzbaur and Vallee, 1994). It has been found to be associated with a variety of membranous organelles including endocytic vesicles (Lin and Collins, 1992; Aniento et al., 1993), lysosomes (Lin and Collins, 1992), and components of the Golgi apparatus (Corthezy-Theulaz et al., 1992; Fath et al., 1994). Cytoplasmic dynein has also been implicated in the formation of endoplasmic reticulum networks in *Xenopus* interphase extracts (Allan, 1995), and has been found to bind to microtubules in vitro (Yu et al., 1992). Cytoplasmic dynein may also function in minus end-directed chromosome movement. Antibodies to subunits of the complex have been found to label kinetochores during mitosis (Pfarr et al., 1990; Steuer et al., 1990). Furthermore, analysis of chromosome movement in vitro (Hyman and Mitchison, 1991) and in vivo (Rieder and Alexander, 1990) has suggested a role for cytoplasmic dynein in this process during prometaphase. Additional roles for cytoplasmic dynein in

spindle organization in cultured mammalian cells (Vaisberg et al., 1993), nuclear positioning in yeast (Li et al., 1993; Eshel et al., 1993), and nuclear migration in filamentous fungi (Xiang et al., 1994; Plamann et al., 1994) have been reported.

Cytoplasmic dynein is a large multisubunit complex (Paschal et al., 1987) composed of two catalytic heavy chains (HCs<sup>1</sup>, 532 kD) and accessory subunits including several intermediate chains (ICs, 74 kD) and light intermediate chains (LICs, 53-59 kD). The cytoplasmic dynein ICs (Paschal et al., 1992) share homology with the two *Chlamydomonas* flagellar dynein ICs (Mitchell and Kang, 1991; Wilkerson et al., 1995), which have been localized to the base of the dynein complex (King and Witman, 1990) and bind directly to the A-microtubule of the flagellar outer doublet (King et al., 1991). The homology between the ICs has led us to predict that the cytoplasmic forms play the analogous role of linking cytoplasmic dynein to membranous organelles and kinetochores (Paschal et al., 1992).

Dynactin is another large multisubunit complex which has been proposed to stimulate cytoplasmic dynein-mediated vesicle movements in an in vitro motility assay (Schroer and Sheetz, 1991; Gill et al., 1991). It is composed of: p150<sup>Glued</sup>, which exhibits extensive sequence homol-

Address all correspondence to Dr. Richard B. Vallee, Cell Biology Group, Worcester Foundation for Biomedical Research, 222 Maple Avenue, Shrewsbury, MA 01545. Tel.: (508) 842-8921 x 265. Fax: (508) 842-3915.

1. Abbreviations used in this paper: HC, heavy chain; IC, intermediate chain; LIC, light intermediate chain.

ogy with the product of the *Glued* gene in *Drosophila* (Holzbaur et al., 1991; Gill et al., 1991); Arp1, a novel actin-related protein (Lees-Miller et al., 1992; Paschal et al., 1993); p50 (Paschal et al., 1993; Echeverri et al., 1996); actin-capping protein (Schafer et al., 1994); and several uncharacterized subunits. Like cytoplasmic dynein, dynactin localizes to small, punctate, detergent-extractable structures distributed throughout the cytoplasm (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993). During mitosis, the two complexes have recently been found to colocalize to kinetochores (Echeverri et al., 1996). Dynein and dynactin have also been deduced to function in a common pathway by genetic analysis in *Saccharomyces cerevisiae* (Eshel et al., 1993; Li et al., 1993; Clark and Meyer, 1994; Muhua et al., 1994), *Neurospora crassa* (Plamann et al., 1994), *Aspergillus nidulans* (Xiang et al., 1994), and *Drosophila melanogaster* (McGrail et al., 1995). Furthermore, the two complexes substantially copurify through microtubule sedimentation, ATP extraction, and sucrose density gradient sedimentation (Collins and Vallee, 1989; Schroer and Sheetz, 1991; Gill et al., 1991; Paschal et al., 1993), though they can largely be separated by ion-exchange chromatography (Schroer and Sheetz, 1991; Gill et al., 1991). Substantial coimmunoprecipitation from cytosolic extracts was not observed using antisera to the ICs or p50 (Paschal et al., 1993; Clark et al., 1994).

This study was initiated as a search for cytoplasmic dynein receptors, based on the premise that the ICs should mediate interactions between cytoplasmic dynein and the surface of organelles and kinetochores. We report that a previously cloned and a novel IC isoform both interact with the p150<sup>Glued</sup> component of the dynactin complex. These data represent the first evidence for a direct interaction between dynein and dynactin. In addition, in view of the known properties of p150<sup>Glued</sup>, they suggest that the interaction of cytoplasmic dynein with its subcellular targets is likely to be complex.

## Materials and Methods

### Intermediate Chain cDNA Cloning

Screening for additional IC cDNAs was performed by RT-PCR using primers in the 5'-end of the coding sequence (cf. Paschal et al., 1992) and by DNA hybridization to a  $\lambda$ -ZAPII rat brain cDNA library (Stratagene, La Jolla, CA) using a divergent human cytoplasmic dynein IC cDNA for which limited sequences had been reported. 26 novel cDNAs were identified by the latter method and were characterized after three rounds of plaque purification and excision by helper phage. Individual clones were end-sequenced and the longest representative of each of three splicing variants was sequenced to completion using a combination of convenient restriction sites and custom oligonucleotides.

cDNA fragments representing the 3' untranslated regions of IC-1 (562 bp) and IC-2 (553 bp) were isolated by restriction digestion, agarose gel electrophoresis, and gel purification. These fragments were used as hybridization probes after random priming and removal of unincorporated nucleotides by spin-column chromatography (Boehringer-Mannheim, Indianapolis, IN). A rat multi-tissue Northern blot (MTN, Clontech Laboratories, Palo Alto, CA) was probed with both the IC-1-specific and IC-2-specific probes using Rapid-Hyb (Amersham Corp., Arlington Heights, IL), at 58°C, followed by high stringency washing in 0.2 X SSPE (2 mM NaPO<sub>4</sub>, pH 7.0, 36 mM NaCl, 0.2 mM sodium EDTA) at 60°C.

### Sequence Analysis

Coiled-coiled domains were identified using the NEWCOILS program

(Lupas et al., 1991). Sequence assembly, comparisons, and alignments were performed using the GELASSEMBLE, COMPARE, and PILEUP programs of GCG (GCG sequence analysis package). Statistical significance of comparisons was assessed using RDF2 (Lipman and Pearson, 1988).

### Expression of IC Isoforms in *E. coli*

The coding regions of IC-1A and IC-2B were subcloned into the pET-14b expression vector (Novagene, Madison, WI) after PCR mutagenesis and standard cloning techniques. After transformation into the expression strain BL21 (DE3) and induction with 0.4 mM IPTG, bacteria were lysed using a French press and expressed proteins were purified using nickel-affinity chromatography. The NH<sub>2</sub>-terminal his-finger was removed by thrombin cleavage followed by dialysis, and cleavage was confirmed by SDS-PAGE. A *c-myc* epitope-tag was added by introducing a NcoI restriction site in place of the termination codons of IC-1A and IC-2B by PCR mutagenesis followed by subcloning into a pBluescript vector containing the *myc* epitope-encoding sequence.

Truncation mutants were prepared in pET-14b using internal restriction sites in the IC-1A cDNA as well as PCR mutagenesis. Expressed polypeptides were purified as described above. p150<sup>Glued</sup> truncation mutants expressing residues 39-1325, 150-811, and 811-1325 have been previously described (Waterman-Storer et al., 1995). An NH<sub>2</sub>-terminal p150<sup>Glued</sup> mutant containing residues 1-200 was prepared by PCR and standard cloning techniques.

### Preparation of Antisera

Antibody against IC-1 was prepared by immunizing New Zealand White rabbits with the purified recombinant IC-1A polypeptide and was affinity-purified against the same polypeptide. An IC-2 antiserum was prepared against the COOH-terminal 19 residues of the IC-2 protein (Research Genetics, Huntington, AL). A previously described p150<sup>Glued</sup> antibody was used for immunoblotting (Waterman-Storer et al., 1995). For immunoprecipitation a rabbit polyclonal antiserum was generated against the NH<sub>2</sub>-terminal 200 amino acids of p150<sup>Glued</sup>, and shown to bind p150<sup>Glued</sup> specifically in both immunoblotting (not shown) and immunoprecipitation assays (see Fig. 9 A). Hybridomas producing monoclonal antibodies to *c-myc* were obtained from Amer. Type Culture Collection (Rockville, MD) (Evan et al., 1985). Polyclonal anti-*myc* antiserum was generously provided by Melissa Gee.

### Blot Overlay Assays

Protein samples were fractionated by SDS-PAGE and transferred to PVDF (polyvinylidene difluoride) membranes electrophoretically. After overnight incubation at 4°C in PBS (8 mM NaPO<sub>4</sub>, 1 mM KPO<sub>4</sub>, pH 7.4 containing 140 mM NaCl and 3 mM KCl) containing 0.05% Tween-20 and 6% nonfat dry milk, the membrane strips were incubated in PBS/Tween-20/milk containing the recombinant protein probe for 1 h at room temperature. Binding in the overlay assay improved with extended preincubation of the membrane strips in PBS/Tween-20/milk at 4°C. Recombinant ICs with an intact HIS-tag did not work in our assay. Reaction intensity was dependent on recombinant protein concentration over the range of 0.1  $\mu$ g/ml to 10  $\mu$ g/ml and concentrations from 0.1  $\mu$ g/ml to 1  $\mu$ g/ml were typically used to minimize background. To visualize the recombinant protein, strips were washed 4  $\times$  15 min in PBS/Tween-20, incubated with primary antibody in PBS/Tween-20/milk for 1 h at room temperature, washed 4  $\times$  15 min in PBS/Tween-20, incubated in secondary antisera (HRP-conjugated donkey anti-mouse, Jackson Immunoresearch, West Chester, PA; HRP-conjugated goat anti-rabbit, Pierce, Rockford, IL), and processed for ECL (Amersham Corp.).

### Immunoprecipitation

Rat brain cytosol was prepared as described (Paschal et al., 1993). The crude membrane pellet fraction recovered from 150,000 g centrifugation of the postnuclear supernatant was washed five times in PEM buffer and solubilized in 1% NP-40. Goat anti-rabbit agarose beads (American Qualex, LaMirada, CA) were preloaded with rabbit anti-IC antibody or rabbit anti-*myc* antiserum plus the appropriate recombinant IC. Immunoprecipitates were analyzed by immunoblotting. Blots were probed with rabbit antibodies recognizing p150<sup>Glued</sup> (Waterman-Storer et al., 1995), a mouse monoclonal antibody against p50 (Paschal et al., 1993) and a mouse mono-

clonal antibody against Arp-1 (Clark, S., and D. Meyer, personal communication) by standard methods.

Alternatively, subconfluent Rat2 cells grown in DMEM containing 10% FCS were incubated in Met/Cys-deficient DMEM containing 5% dialyzed FCS for 1 h. Cells were labeled in Met/Cys-deficient DMEM containing 5% dialyzed FCS and 80  $\mu$ Ci/ml Tran<sup>35</sup>SLabel (ICN Biomedicals, Costa Mesa, CA) for 4 h, and chased for 1 h in normal DMEM containing 10% FCS. Cells were scraped into RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.05% Tween-20, 100  $\mu$ g/ml PMSF, 1  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml TPCK, 10  $\mu$ g/ml TAME, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin A), followed by 10 passes in a tight fitting Dounce homogenizer. The extract was centrifuged at 3,000 g for 20 min at 4°C, followed by 150,000 g for 1 h at 4°C, preadsorbed against Protein A-Sepharose beads (Pharmacia, Piscataway, NJ), incubated with immune beads overnight at 4°C, and eluted with 4% acetic acid. Samples were subjected to SDS-PAGE, and the gel was incubated in Enlightening (Dupont, New England Nuclear, Boston, MA) and analyzed by autoradiography.

## Results

### Cytoplasmic Dynein Intermediate Chain Isoform Complexity

The ICs of purified cytoplasmic dynein migrate by 1-D (Paschal et al., 1987, 1992) and 2-D gel electrophoresis (Dillman and Pfister, 1994) as a heterogeneous group of bands of approximately 74 kD. As a first step in our attempt to identify IC-binding partners, we sought to resolve to what extent the observed electrophoretic complexity reflects isoform diversity. RT-PCR analysis of rat brain mRNA had revealed the existence of an apparent alternative splicing site near the 5'-end of the IC sequence (Paschal et al., 1992). In addition, homologous but partially divergent amino acid sequence was obtained from a bovine tryptic peptide from the same region of the molecule, suggesting further polypeptide diversity.

Further PCR based screening of rat brain mRNA failed to reveal additional IC isoforms. However, a report of limited sequence from two partial cDNA clones supported the possibility of further mRNA complexity (Adams et al., 1993a,b). Complete sequencing of human clone EST 04626 revealed 1,058 bp of IC-related coding sequence, exhibiting 75% identity to our previously reported IC, and corresponding to an internal fragment from aa 76 to 456. Because this sequence contained the divergent sequence observed in our bovine peptide fragment (see above; Paschal et al., 1992), we speculated that the human cDNA might encode a novel IC isoform. To evaluate this possibility, we probed a rat brain cDNA library using the human clone and selected 26 additional IC cDNAs. These clones were distinct in sequence from that of the initially cloned rat cytoplasmic dynein IC (Paschal et al., 1992). They exhibited extensive regions of amino acid sequence identity with our initial IC clone, and showed two regions of variability near the 5'-end of the coding sequence (Fig. 1, A and B). Seven of sixteen peptide fragments sequenced for the initial IC cloning (Paschal et al., 1992; and unpublished data) appear to have been derived from this new IC polypeptide.

Together, the sequence data suggest the existence of two IC genes, which we term IC-1 and IC-2, each encoding multiple isoforms as the result of a probable alternative splicing mechanism. The existence of two genes is supported by genomic mapping data, which place IC-1 and IC-2 at distinct loci within the mouse genome (K. T.

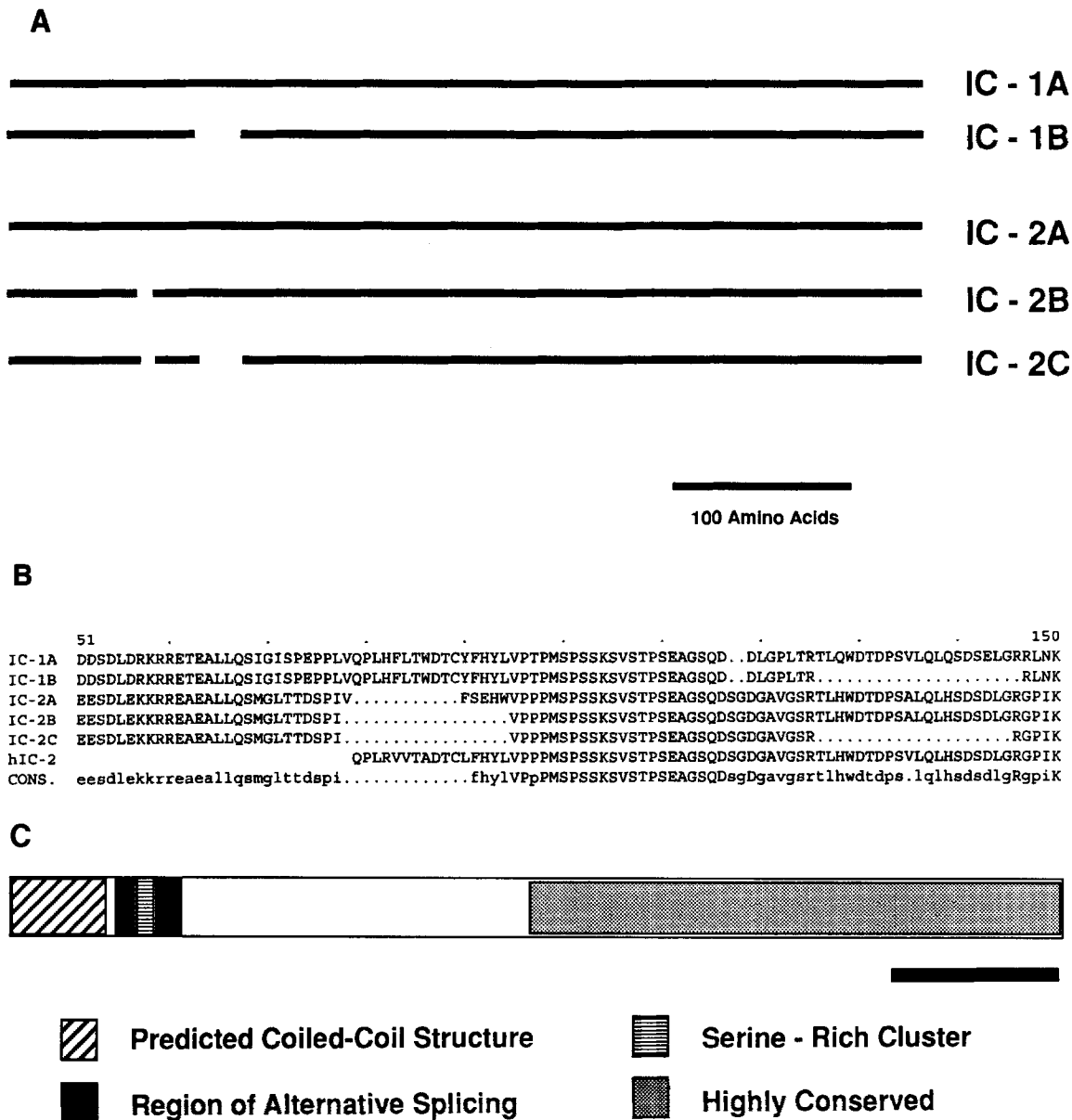
Vaughan, A. Mikami, B. M. Paschal, E. L. F. Holzbaur, S. M. Hughes, C. J. Echeverri, K. J. Moore, D. J. Gilbert, N. G. Copeland, N. A. Jenkins, and R. B. Vallee. 1995. Identification of multiple genomic loci involved in dynein-based motility. *Mol. Biol. Cell* 6:154a). Apparent alternative splicing occurs at at least two sites within IC-2. The second site is at the same position as a comparable site of alternative splicing in IC-1, and the alternate sequence at this site is closely related between IC-1 and IC-2 (Fig. 1 b). Overall sequence identity between IC-1 and IC-2 is highest within the COOH-terminal half, but regions of identity were also observed in the NH<sub>2</sub>-terminal half. This consistent pattern among all known cytoplasmic and axonemal ICs (Paschal et al., 1992; Mitchell and Kang, 1991; Wilkerson et al., 1995) suggests a common function associated with the COOH-terminal half, such as binding to the dynein HCs, and distinct functions associated with the NH<sub>2</sub>-terminal half. Pairwise comparisons show that each of the rat brain ICs is more closely related to the IC78 of *Chlamydomonas* flagellar dynein than to IC70 (data not shown). Common structural features of the rat cytoplasmic dynein ICs (Fig. 1 C) are an NH<sub>2</sub>-terminal predicted coiled-coil domain including a region rich in amino acids of mixed charge, and a serine-rich cluster comparable to a putative site of phosphorylation in CLIP-170 (Pierre et al., 1992). Phosphorylation in this region of CLIP-170 has been implicated in the regulation of microtubule binding (Rickard et al., 1990). Each of the IC isoforms contains a series of "WD" repeats (Neer et al., 1994), a proposed protein-protein interaction motif also identified in the *Chlamydomonas* flagellar outer arm dynein ICs (Wilkerson et al., 1995).

Northern blot analysis using 3'-untranslated region-specific probes indicated differential expression of these two isoform families (Fig. 2). While the IC-2 specific probe detected mRNAs in each tissue tested, the IC-1 specific probe only hybridized to transcripts expressed in brain. A weak IC-1 hybridization signal was also detected in testis at long exposures (data not shown).

### Identification of IC-binding Proteins

To identify proteins that ICs were capable of binding, we expressed NH<sub>2</sub>-terminal his-tagged IC-1A and IC-2B with and without a COOH-terminal myc-tag, in *E. coli*. A substantial fraction of the recombinant protein was soluble in each case, and could be purified using nickel-affinity chromatography (Fig. 3) along with a variable level of premature-termination products. Test protein samples were fractionated by SDS-PAGE, electroblotted, and incubated with recombinant ICs, which were visualized using anti-IC or anti-myc antibodies.

In control samples of whole Rat2 cell lysate incubated in anti-IC antibody, endogenous ICs were detected (Fig. 4 A, lane 3). After incubation with recombinant IC-1A, the anti-IC antibody detected an additional prominent doublet at 150 and 135 kD (Fig. 4 A, lane 2), as well as other minor bands. The protein doublet appeared to comigrate with the p150<sup>Glued</sup> doublet (Fig. 4, B and C, lane 3). A comparable pattern of IC reactivity was observed using rat brain cytosol (see below, Fig. 8 A), bovine brain microtubules (Fig. 4 B) and sucrose density gradient-purified cyto-

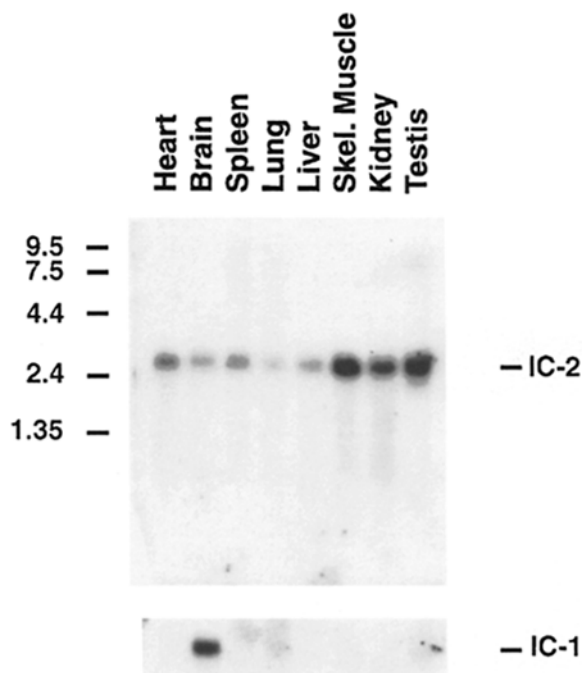


**Figure 1.** Cloning of novel cytoplasmic dynein intermediate chain isoforms. (A) Line diagram depicting protein sequence alignment of rat cytoplasmic dynein intermediate chain family members. Isoforms IC-1A and IC-1B have been described previously (Paschal et al., 1992). The longest representative cDNA of each IC-2 variant is presented. IC-1 and IC-2 exhibit 76% identity overall, with the COOH-terminal halves showing 82% identity and the NH<sub>2</sub>-terminal halves showing 72% identity. The sequences of IC-2A, IC-2B, IC-2C, and the complete sequence of the human cDNA EST04626 can be obtained from Genbank through Accession numbers U39044, U39045, U39046, and U39575. (B) Alignment of cytoplasmic dynein IC family members within NH<sub>2</sub>-terminal variable region. Gaps were inserted by PILEUP (GCG sequence analysis package) to optimize alignment. The human sequence is 93.5% identical to the rat IC-2 isoforms and is referred to as hIC-2. (C) Summary of structural features identified through comparison of IC isoforms. The NH<sub>2</sub>-terminal coiled-coil domain is highly conserved, as is a serine cluster nested between the two regions of alternative sequence. Scale bar represents 100 amino acids.

plasmic dynein (not shown). Myc-tagged IC-1A followed by anti-myc antibody also showed prominent staining of the 150/135-kD doublet. Staining of endogenous ICs was not observed (Fig. 4 B, lane 2). Blots were also probed with recombinant IC-2B, which again bound specifically to the 150/135-kD doublet (Fig. 4 C, lane 1). Similar results were obtained with myc-tagged IC-2B (Fig. 4 C, lane 2).

#### Identification of IC-binding Polypeptides as p150<sup>Glued</sup>

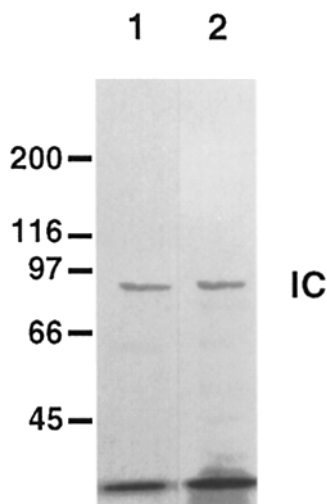
The electrophoretic mobility of the 150/135 kD doublet was reminiscent of that of p150<sup>Glued</sup> (Holzbaur et al., 1991). Furthermore, the reactive bands were observed in the same fractions in which p150<sup>Glued</sup> has been found to be enriched (Collins and Vallee, 1989; Holzbaur et al., 1991; Gill et al., 1991). Therefore, we assayed purified recombinant



**Figure 2.** Northern blot analysis of rat cytoplasmic dynein intermediate chains. Isoform-specific hybridization probes were used to test expression of IC-2 (upper panel) and IC-1 (lower panel) in various rat tissues (see Materials and Methods). RNA molecular weight standards are shown at left. The relatively broad bands of hybridization may reflect small variations in mRNA size, consistent with the short segments of alternative sequence observed among IC isoforms.

p150<sup>Glued</sup> for IC reactivity. Because full-length p150<sup>Glued</sup> could not be expressed intact, fragments of the protein were used. Recombinant polypeptides consisting of residues 39-1325 and 150-811 showed strong IC-1 and IC-2 reactivity (Fig. 5, A and B, lanes 1 and 2), in contrast to a fragment consisting of residues 811-1325 which was negative in the assay (Fig. 5, A and B, lanes 3).

As a further means to assay for IC-p150<sup>Glued</sup> binding, the aa 150-811 fragment was used to probe blots of total Rat2 cell extract (Fig. 6 A) and bovine brain microtubules (Fig. 6 B). In control samples incubated in anti-p150<sup>Glued</sup> anti-



**Figure 3.** Expression of cytoplasmic dynein intermediate chains in *E. coli*. Coomassie brilliant blue staining of (lane 1) purified recombinant IC-1A and (lane 2) purified recombinant IC-2B.

body, only the p150<sup>Glued</sup> doublet was observed to react, reflecting detection of endogenous p150<sup>Glued</sup> (Fig. 6, A and B, lanes 3). After incubation with recombinant p150<sup>Glued</sup>, a single additional prominent band comigrating with the ICs was observed (Fig. 6, A and B, lanes 1).

We used a series of truncation mutant constructs in overlays of partially purified microtubules, to gain further insight into the region of p150<sup>Glued</sup> responsible for IC-binding (Fig. 7, Table I). Truncated forms of p150<sup>Glued</sup> containing residues 39-1325 (Fig. 7, lane 3) and 150-811 (Fig. 7, lane 4) bound the ICs, whereas mutants containing residues 811-1325 (Fig. 7, lane 5) and 1-200 (Fig. 7, lane 6) did not. We note that truncation mutants containing residues 39-1325 (Fig. 7, lane 3) and 1-200 (Fig. 7, lane 6) also bound tubulin in our assays.

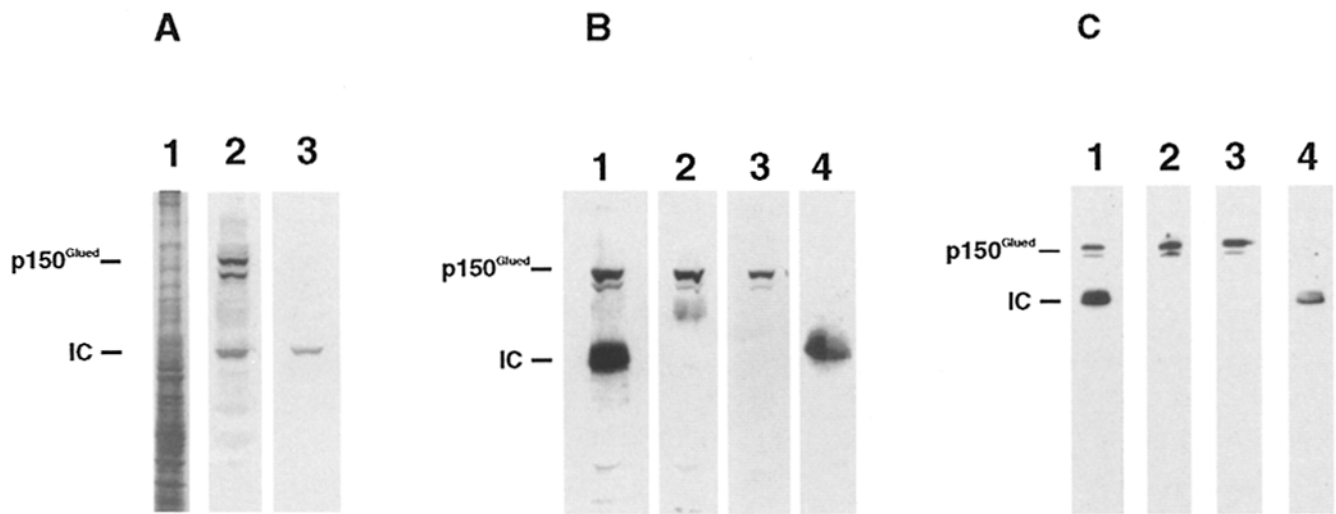
### IC-binding Site for p150<sup>Glued</sup>

To identify the region of the ICs involved in binding to the p150/135 doublet, a series of truncation mutants of IC-1A were expressed in *E. coli* and used to probe rat brain cytosol (Fig. 8 A, Table I). The anti-IC antibody detected endogenous ICs in each case. In addition, reaction was observed with p150<sup>Glued</sup> in lanes incubated with COOH-terminal IC truncation mutant polypeptides as short as 123 amino acids (IC-1A residues 1-123, Fig. 8 A, lane 4). This portion of the ICs contains the predicted coiled-coil domain, the serine cluster, and the region of apparent alternative splicing. No reaction with p150<sup>Glued</sup> was observed using a smaller COOH-terminal truncation fragment (aa 1-61, Fig. 8 A, lane 5), or NH<sub>2</sub>-terminal truncation fragments lacking either the first 61 (Fig. 8 A, lane 6) or 123 amino acids (Fig. 8 A, lane 7).

To confirm the identification of the p150<sup>Glued</sup>-binding region, the IC fragments were blotted to PVDF membranes, and probed with a recombinant p150<sup>Glued</sup> fragment (aa 150-811)(Fig. 8 B, Table I). Binding was again detected with COOH-terminal truncation fragments as small as aa 1-123 (Fig. 8 B, lane 4), but was undetectable with the smaller COOH-terminal fragment (aa 1-61, Fig. 8 B, lane 5) and with an NH<sub>2</sub>-terminal truncation fragment (aa 61-643, Fig. 8 B, lane 6).

### Solution Assays for an IC-Dynactin Interaction

To assess the ability of the ICs to interact with components of the dynactin complex which had not been subjected to denaturing conditions, we performed immunoprecipitations from metabolically labeled cultured Rat2 cells (Fig. 9 A), or rat brain cytosol and membranes (Fig. 9, B and C). As observed previously using a monoclonal anti-IC antibody (Paschal et al., 1993), our polyclonal anti-IC antibodies did not immunoprecipitate dynactin efficiently (Fig. 9 A, lane 2; Fig. 9, B and C, lanes 2). However, after supplementing extracts with purified ICs, we observed substantial, specific coimmunoprecipitation of the p150<sup>Glued</sup>, p50, and Arp1 components of the dynactin complex (Fig. 9 A, lanes 3 and 5; Fig. 9, B and C, lanes 3, 5, 7, and 8). Comparable results were obtained using IC-1A (Fig. 9 A, lane 3; Fig. 9 B, lane 3), IC-1A-myc (Fig. 9 A, lane 5; Fig. 9 B, lane 7), IC-2B (Fig. 9 B, lane 5), or IC-2B-myc (Fig. 9 B, lane 8). Similar levels of p150<sup>Glued</sup> were observed in immunoprecipitates from rat cytosol and membranes (Fig. 9 C). In a separate series of experiments, both p150<sup>Glued</sup> and p50



**Figure 4.** Detection of IC-binding partners by blot overlay. Rat-2 cell lysate (*A*), and an ATP-extracted microtubule pellet fraction (*B* and *C*: P5 microtubule pellet, Paschal et al., 1987) were probed with recombinant ICs. (*A*) Coomassie brilliant blue staining of Rat-2 cell lysate (lane 1), Rat-2 cell lysate incubated with IC-1A followed by anti-IC antiserum (lane 2), and Rat-2 cell lysate incubated with anti-IC antiserum (lane 3). (*B*) ATP-extracted microtubule samples incubated with: IC-1A followed by anti-IC antiserum (lane 1), myc-tagged IC-1A followed by anti-myc antibody (lane 2), anti-p150<sup>Glued</sup> antiserum (lane 3), and anti-IC antiserum (lane 4). Note that, in addition to p150<sup>Glued</sup>, a band of ~120 kD was weakly detected by the myc-tagged IC-1A construct. (*C*) ATP-extracted microtubule samples incubated with IC-2B followed by anti-IC2 antiserum (lane 1), myc-tagged IC-2B followed by anti-myc antibody (lane 2), anti-p150<sup>Glued</sup> antiserum (lane 3), and anti-IC antiserum (lane 4). In addition to p150<sup>Glued</sup>, an ~65-kD band was inconsistently detected by IC-2.

were also found to bind to recombinant IC-1A and IC-2B affinity matrices, while no binding was observed using BSA or a control IC construct consisting of IC-1A residues 61-643 (data not shown).

### Discussion

Through our search for cytoplasmic dynein-binding partners, we have discovered a direct interaction between the ICs and p150<sup>Glued</sup>. These data represent the first compelling evidence for an interaction between cytoplasmic dynein and dynactin, and indicate that binding occurs specifically through the ICs and p150<sup>Glued</sup>. In view of the likely role for the ICs in subcellular targeting, our data shed new light on the potential function of dynactin in cytoplasmic dynein-mediated motility.

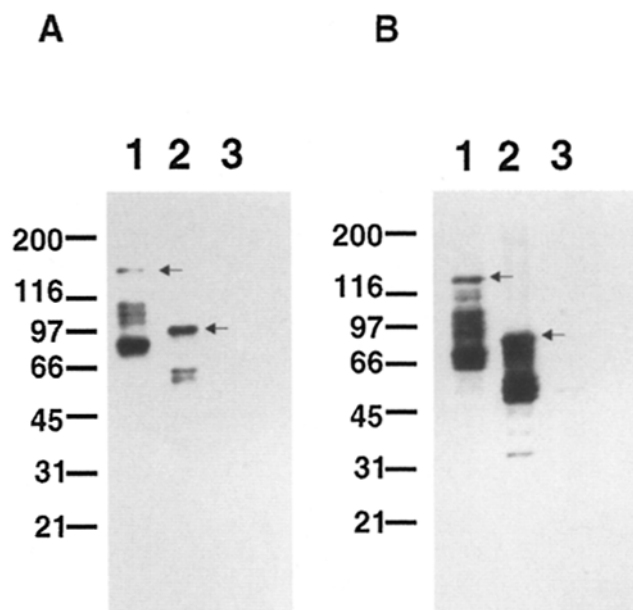
### Primary Structure of the Intermediate Chains

Our current analysis of numerous additional cDNAs has indicated the existence of at least five IC isoforms in rat, apparently products of an alternative splicing mechanism involving two genes. The expression patterns of IC-1 and IC-2 were found to be very different. We note that a monoclonal anti-IC (74.1) antibody used in our earlier study (Paschal et al., 1992) detects both IC-1 and IC-2 (data not shown), explaining the widespread IC expression pattern observed by immunoblotting in that study.

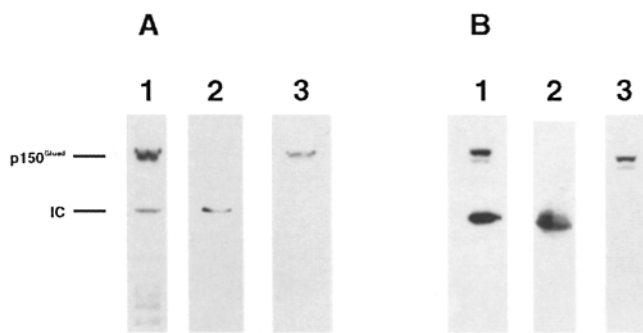
Apparent alternative splicing was revealed at two positions within the NH<sub>2</sub>-terminal half of the cytoplasmic ICs, one of which coincides between the IC-1s and IC-2s. The pattern of alternative splicing observed for the ICs is similar to that observed for the kinesin light chains, where two sites of splicing generate at least three isoforms (Cyr et al., 1991). This degree of IC isoform complexity suggests that different populations of cytoplasmic dynein are likely to exist in the cell, each with a different IC complement.

### Interaction between Intermediate Chains and p150<sup>Glued</sup>

The interaction of the cytoplasmic dynein ICs with p150<sup>Glued</sup> was observed using both solid-phase and solution-phase assays. Unmodified and epitope-tagged versions of two IC



**Figure 5.** Blot overlay of recombinant p150<sup>Glued</sup> with cytoplasmic dynein ICs. Truncated recombinant p150<sup>Glued</sup> polypeptides consisting of residues 39-1325 (lane 1), 150-811 (lane 2), or 811-1325 (lane 3) were subjected to electrophoresis and electroblotting and incubated with recombinant IC-1A followed by anti-IC antibody (*A*), or recombinant IC-2B followed by anti-IC2 antiserum (*B*). Arrows indicate the SDS-PAGE mobility of the complete product of each expression construct. Lower molecular weight bands represent premature termination products resulting from differing codon bias between mammals and bacteria.

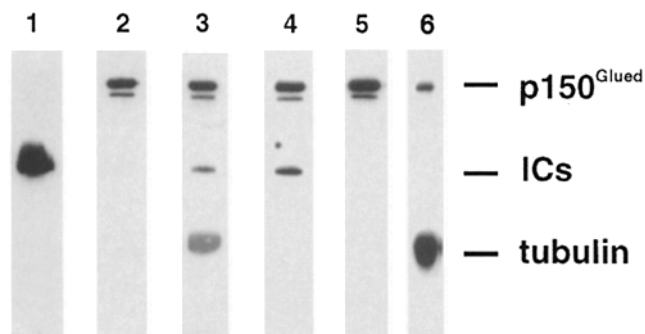


**Figure 6.** Detection of p150<sup>Glued</sup>-binding partners by blot overlay. Rat-2 cell lysate (A) and ATP-extracted microtubule samples as described in Fig. 4 (B) were incubated with an aa 150-811 p150<sup>Glued</sup> fragment followed by anti-p150<sup>Glued</sup> (lane 1), anti-IC alone (lane 2), or anti-p150<sup>Glued</sup> alone (lane 3).

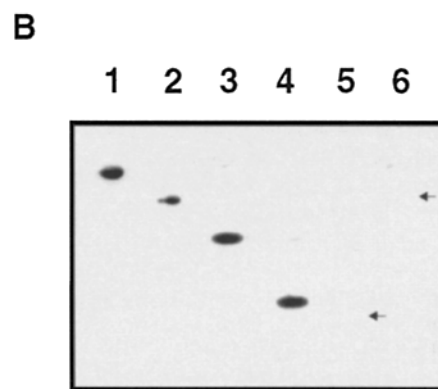
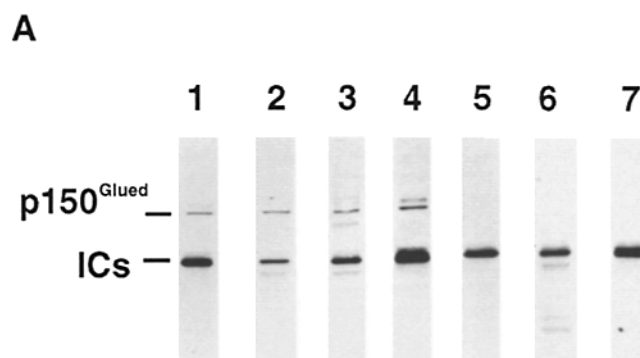
isoforms were used to test binding specificity. The interaction was detected using either the ICs or p150<sup>Glued</sup> as the probe molecule, and its specificity was evident using complex protein samples (Figs. 4, 6, 7, and 9).

The interaction was also specific to limited regions of both the IC and p150<sup>Glued</sup> polypeptides (Figs. 5, 7, and 8; Table I). We note that the binding site within the ICs for p150<sup>Glued</sup> resides near the NH<sub>2</sub> terminus, in a segment of the IC which includes the predicted coiled-coil domain and the serine-rich cluster. Both regions appear to be essential, in that the coiled-coil segment alone failed to recognize p150<sup>Glued</sup> (Fig. 8), while removal of this region was sufficient to abolish binding (Fig. 8). The involvement of the serine cluster suggests that the IC-p150<sup>Glued</sup> interaction might be regulated by phosphorylation. The coiled-coil domain could interact directly with the coiled-coil portion of p150<sup>Glued</sup> (see below). However, it is also possible that this region is responsible for IC self-association. In this view, removal of the domain might result in dissociation of an active IC dimer into inactive IC monomers.

Our data indicate that the IC-binding domain of p150<sup>Glued</sup> resides between residues 200 and 811. This region includes a large predicted coiled-coil segment lying between residues ~210 and ~550, but lacks the previously identified NH<sub>2</sub>-terminal microtubule-binding and COOH-terminal



**Figure 7.** Identification of cytoplasmic dynein intermediate chain-binding site within p150<sup>Glued</sup>. Membrane strips containing ATP-extracted bovine brain microtubule samples as described in Fig. 4 were incubated with anti-IC (lane 1), anti-p150<sup>Glued</sup> (lane 2), or p150<sup>Glued</sup> fragments followed by anti-p150<sup>Glued</sup> (lanes 3–6). p150<sup>Glued</sup> fragments used consisted of aa residues 39-1325 (lane 3), 150-811 (lane 4), 811-1325 (lane 5), and 1-200 (lane 6).

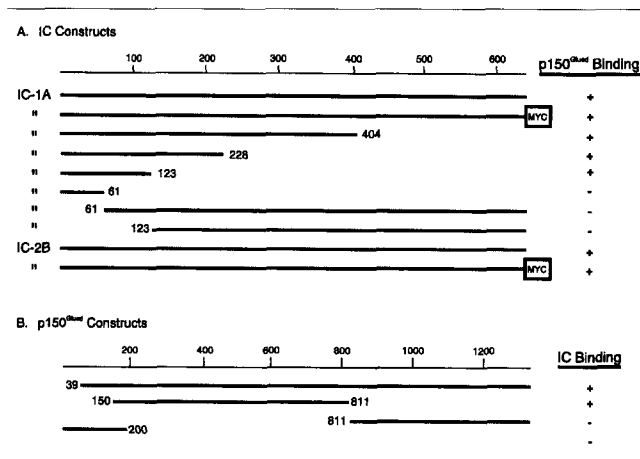


**Figure 8.** Identification of p150<sup>Glued</sup>-binding site within cytoplasmic dynein ICs. (A) Membrane strips containing rat brain cytosol were incubated with recombinant IC fragments which were detected using anti-IC antibody. Fragments containing IC-1 residues 1-643 (lane 1), 1-404 (lane 2), 1-228 (lane 3), and 1-123 (lane 4) bound to p150<sup>Glued</sup>, whereas fragments containing residues 1-61 (lane 5), 61-643 (lane 6), and 123-643 (lane 7) did not. (B) Recombinant fragments containing residues 1-643 (lane 1), 1-404 (lane 2), 1-228 (lane 3), and 1-123 (lane 4), 1-61 (lane 5), and 61-643 (lane 6) were incubated with the recombinant p150<sup>Glued</sup> fragment consisting of residues 150-811 and detected with anti-p150<sup>Glued</sup> antiserum. Arrowheads indicate SDS-PAGE mobility of IC truncation mutants containing residues 1-61 (lane 5) and 61-643 (lane 6). Samples were separated on a 5–18% acrylamide gel, and the region of the blot containing the IC mutant polypeptides is shown.

Arp-1-binding domains (Waterman-Storer et al., 1995). We conclude, therefore, that the IC-binding domain must represent a distinct functional region within the polypeptide. Conceivably, the ICs may interact with p150<sup>Glued</sup> via a coiled-coil interaction, but further work will be needed to define the interaction sites more precisely.

We also note that the original *Glued* mutation, *Gl<sup>I</sup>* (Plough and Ives, 1935), is known to result from a transposon insertion causing deletion of the COOH-terminal ~300 residues (Swaroop et al., 1987). Based on our findings, we predict that the mutant product of the *Gl<sup>I</sup>* allele should retain the IC-binding domain, leading us to speculate that the defect lies in the inability of the mutant p150<sup>Glued</sup> to interact with other dynactin components. In particular,

Table I. Summary of IC and p150<sup>Glued</sup> Interacting Domains



Identification of IC-binding domain for p150<sup>Glued</sup> and p150<sup>Glued</sup>-binding domain for the ICs. (A) Line diagram depicting the results of binding experiments using full-length and truncated forms of the ICs. The p150<sup>Glued</sup> interaction site resides within the first 123 residues of IC-1A and requires both the coiled-coil domain, and part of the variable region. Line scale indicated on the top line in amino acid residues. (B) Line diagram depicting regions of p150<sup>Glued</sup> responsible for IC binding. The IC-binding site resides between residues 200 and 811, in a region containing extensive coiled-coil structure and distinct from microtubule-binding and Arp-1-binding domains (Waterman-Storer et al., 1995). Line scale indicated on top line in amino acid residues.

p150<sup>Glued</sup> in *G<sup>l</sup>* should lack the Arp-1-binding domain (Waterman-Storer et al., 1995).

### Cytoplasmic Dynein Interactions in the Cell

Dynactin was initially described as a factor capable of stimulating dynein-mediated vesicle motility in vitro (Schroer and Sheetz, 1991; Gill et al., 1991). However, the mechanism by which stimulation occurred was unclear. Our results identify dynactin as a subcellular target for cytoplasmic dynein binding. Could dynactin, therefore, represent a "receptor" for cytoplasmic dynein?

Dynactin has been localized to small punctate structures suggesting an organellar distribution (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993). Colocalization with cytoplasmic dynein on particular organelles has been difficult to demonstrate because of the large number of small dynein and dynactin positive structures in the interphase cell.

In vitro, efficient dynein-mediated movement of membranous organelles requires dynactin (Schroer and Sheetz, 1991; Gill et al., 1991), consistent with the assembly of these two complexes on organelles. In addition, recent work has revealed colocalization of dynactin with cytoplasmic dynein on the kinetochores of prometaphase chromosomes (Echeverri et al., 1996), where cytoplasmic dynein has already been detected (Pfarr et al., 1990; Steuer et al., 1990). Therefore, these two complexes seem likely to interact directly in the cell. Whether dynactin serves as a simple receptor on kinetochores and organelles is unclear, however. Dynactin can be purified as a soluble complex, and none of the dynactin components cloned to date contain a transmembrane domain. In these regards dynactin would represent an unconventional receptor. Conceivably it serves this role in a novel fashion, perhaps as a component of a more extensive organelle surface cytoskeleton analogous to the plasma membrane skeleton of erythrocytes. It is of interest that a Golgi-specific isoform of  $\beta$ -

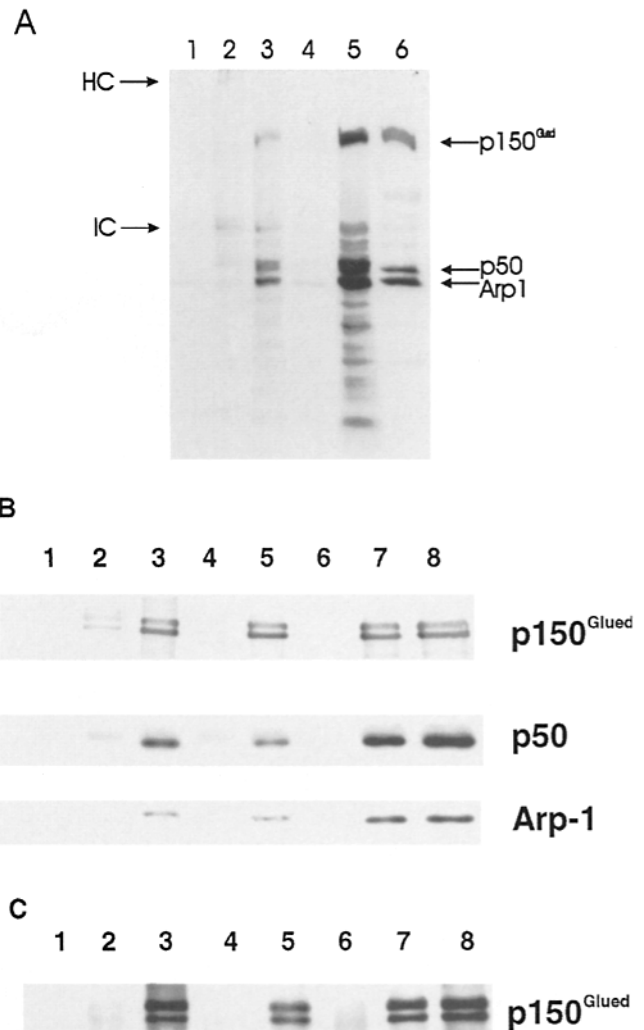


Figure 9. Coimmunoprecipitation of dynactin with cytoplasmic dynein ICs. (A) <sup>35</sup>S[Met/Cys]-labeled Rat2 cell extracts were immunoprecipitated with: (lane 1) beads alone; (lane 2) anti-IC; (lane 3) anti-IC plus recombinant IC-1A; (lane 4) anti-myc; (lane 5) anti-myc plus recombinant IC-1A-myc; and (lane 6) anti-p150<sup>Glued</sup>. The relative mobilities of p150<sup>Glued</sup>, p50, Arp-1, and the cytoplasmic dynein heavy chain (HC) and intermediate chain (IC) are indicated. Immunoprecipitations from rat brain cytosol (B) or rat brain membranes (C) were immunoblotted using anti-p150<sup>Glued</sup>, anti-p50, and anti-Arp-1 (B) or anti-p150<sup>Glued</sup> (C). Lanes represent: (lane 1) beads alone; (lane 2) anti-IC; (lane 3) anti-IC plus IC-1A; (lane 4) anti-IC-2; (lane 5) anti-IC-2 plus IC-2B; (lane 6) anti-myc; (lane 7) anti-myc plus IC-1A-myc; (lane 8) anti-myc plus IC-2B-myc. p150<sup>Glued</sup> is often observed as a doublet (p150/135) in brain preparations, but is sometimes observed as a single band in cultured cell extracts. This may indicate that p150<sup>Glued</sup> is subject to limited proteolysis during biochemical purification.

spectrin has recently been identified (Beck et al., 1994), which, conceivably, could participate with dynactin in construction of an organelle surface assembly.

However, such a simple model is problematic in view of the microtubule-binding activities of both cytoplasmic dynein and p150<sup>Glued</sup>. In a supercomplex of dynein and dynactin, the static microtubule-binding activity of p150<sup>Glued</sup> would be expected to counteract the force-producing activity of cytoplasmic dynein. This apparent contradiction strongly suggests regulation of the activities of the individ-



ual components of the complexes. In one view, dynactin would serve as an intermediary structure in the association of dynein with organelles and kinetochores; however, the microtubule-binding activity of p150<sup>Glued</sup> might be regulated to allow for organelle or chromosome translocation. Alternatively, the dynein-dynactin association might occur sequentially. For example, dynactin might bind to subcellular structures first, and serve in some manner to mediate dynein binding. Dynactin would subsequently be discarded in order to allow for force production to be realized. This mechanism is reminiscent of current models for vesicle targeting and fusion, and suggests that recognition of organelles and kinetochores by motor proteins could be a complex and highly regulated process. Understanding the regulation of the dynein-dynactin interaction and the role of the remaining cytoplasmic dynein and dynactin subunits promises to provide important new insight into this process.

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