Functionally Distinct Isoforms of Dynactin Are Expressed in Human Neurons

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p150^{Glued} is the largest subunit of dynactin, which binds to cytoplasmic dynein and activates vesicle transport along microtubules. We have isolated human cDNAs encoding p150^{Glued} as well as a 135-kDa isoform; these isoforms are expressed in human brain by alternative mRNA splicing of the human *DCTN1* gene. The p135 isoform lacks the consensus microtubule-binding motif shared by members of the p150^{Glued}/Glued/CLIP-170/BIK1 family of microtubule-associated proteins and, therefore, is predicted not to bind directly to microtubules. We used transient transfection assays and in vitro microtubule-binding assays to demonstrate that the p150 isoform binds to microtubules, but the p135 isoform does not. However, both isoforms bind to cytoplasmic dynein, and both partition similarly into cytosolic and membrane cellular fractions. Sequential immunoprecipitations with an isoform-specific antibody for p150 followed by a pan-isoform antibody revealed that, in brain, these polypeptides assemble to form distinct complexes, each of which sediments at ~20 S. On the basis of these observations, we hypothesize that there is a conserved neuronal function for a distinct form of the dynactin complex that cannot bind directly to cellular microtubules.

INTRODUCTION

Retrograde transport of vesicles and organelles along axonal microtubules is driven by the molecular-motor cytoplasmic dynein (Paschal and Vallee, 1987; Hirokawa *et al.*, 1990; reviewed, Holzbaur and Vallee, 1994; Sweeney and Holzbaur, 1996). Purified cytoplasmic dynein is capable of driving microtubule translocation in vitro, but additional factors seem to be required to reconstitute vesicular transport along microtubules. Dynactin, an oligomeric 20 S complex, may be required to mediate or activate the dynein-driven minus-end-directed motility of vesicles along microtubules (Gill *et al.*, 1991).

Components of the dynactin complex originally were identified as polypeptides which copurified along with cytoplasmic dynein in preparations from rat liver and testis (Collins and Vallee, 1989) and have since been identified in a range of cell and tissue types and species (Steuer *et al.*, 1990; Clark and Meyer, 1994; Muhua *et al.*, 1994; Plamann *et al.*, 1994; Melloni *et al.*, 1995). Subsequently, dynactin was determined to be a heteromeric complex composed of polypeptides of 150, 135, 62, 50, 45, 37, 32, 27, and 24 kDa (Gill *et al.*, 1991; Paschal *et al.*, 1993; Schafer *et al.*, 1994). The first of these polypeptides to be characterized at the molecular level was p150^{Glued}; the cloning and sequencing of rat cDNAs encoding the 150-kDa polypeptide of the dynactin complex revealed significant homology with the product of the *Drosophila* gene *Glued* (Holzbaur *et al.*, 1991). The *Glued* gene is essential in *Drosophila*; null mutations at this locus are lethal early in

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embryogenesis (Plough and Ives, 1935; Harte and Kankel, 1982).

Molecular analysis of the 45-kDa polypeptide of the dynactin complex led to its identification as an actin-related protein (Clark and Meyer, 1992; Lees-Miller *et al.*, 1992; Paschal *et al.*, 1993). The predicted amino acid sequence of centractin, or Arp1, shares ~50% sequence identity with that of conventional actins. Both the stoichiometry of the dynactin complex and its appearance in electron micrographs suggest that centractin may form short filaments (Schafer *et al.*, 1994). Other subunits of dynactin that have been characterized are the 37- and 32-kDa polypeptides, identified as the α and β subunits of capping protein (CapZ) (Schafer *et al.*, 1994), and a novel 50-kDa polypeptide (Echeverri *et al.*, 1996).

Both genetic and biochemical analyses have now established that dynactin interacts directly with cytoplasmic dynein. Genetic evidence from yeast and *Neurospora* suggested a direct interaction *in vivo*; in both of these organisms mutations in polypeptides of either the dynein or dynactin complexes produce similar phenotypes of aberrant nuclear migration (Eshel *et al.*, 1993; Li *et al.*, 1993; Clark and Meyer, 1994; Muhua *et al.*, 1994; Plamann *et al.*, 1994). Recent evidence also indicates that a mutation in the cytoplasmic dynein heavy-chain gene of *Drosophila* yields a phenotype very similar to that originally described for the *Gl¹* allele of *Glued* (McGrail *et al.*, 1995). We have determined by affinity chromatography that the p150^{Glued} component of dynactin binds directly to the intermediate chain of cytoplasmic dynein, thereby mediating the interaction of the two complexes (Karki and Holzbaur, 1995). A similar observation of direct binding was made by Vaughan and Vallee (1995) with a blot overlay technique.

Cellular transfection studies as well as *in vitro* binding assays have revealed a microtubule-binding motif within the sequence of p150^{Glued} (Waterman-Storer *et al.*, 1995). This motif allows the direct association of the dynactin complex with cellular microtubules, independent of the association of dynactin with the microtubule-based motor cytoplasmic dynein. The microtubule-binding motif identified in p150^{Glued} shares significant homology with the microtubule-binding motif present as a tandem repeat within the sequence of the microtubule-endosome linker polypeptide CLIP-170 (Pierre *et al.*, 1992). We have also determined that p150^{Glued} binds to centractin, in an interaction potentially mediated by a charged motif near the C terminus of the polypeptide (Waterman-Storer *et al.*, 1995). Taken together, these data suggest that p150^{Glued} is a multifunctional polypeptide with at least three interacting domains that mediate binding to cytoplasmic dynein, to the microtubule, and to the centractin filament of the dynactin complex. However, the mechanism by which the dynactin complex may

activate or potentiate dynein-mediated vesicular transport is not yet clear.

Retrograde axonal transport catalyzed by cytoplasmic dynein and potentially activated by dynactin is essential for normal neuronal development and function (Kristensson and Olsson, 1971; Hendry *et al.*, 1974; Hendry, 1980). A specific role for dynactin in neuronal cells is suggested by the observations of disrupted neuronal architecture in the compound eye and optic lobe of *Drosophila* expressing the dominant *Gl¹* mutation (Meyerowitz and Kankel, 1978). The cellular morphology of the individual neurons was observed to be grossly altered in the mutant flies (Garen and Kankel, 1983). In vertebrates, also, there are data suggesting a neuron-specific role for dynactin, because *in situ* hybridization studies in developing and adult rat have indicated that the expression of mRNA encoding p150^{Glued} is highly enriched in neuronal cells at all stages of development (Melloni *et al.*, 1995).

Analysis of the expression of p150^{Glued} in rat tissues also revealed the expression of multiple tissue-specific isoforms (Melloni *et al.*, 1995). Therefore, the purpose of the current study was to further investigate the expression of the p150^{Glued} component of the dynactin complex in neurons. We examined this expression in brain and in neurons with the NTera2/c1.D1 model system, a human teratocarcinoma cell line that can be induced to differentiate into a neuronal phenotype (NT2-N) by treatment with retinoic acid (Andrews, 1984; Pleasure *et al.*, 1992; Pleasure and Lee, 1993). We have determined that distinct isoforms of p150^{Glued} are expressed both in human brain and in NT2-N cells, and that these forms are generated by alternative mRNA splicing. These isoforms have distinct functional domains and lead to the assembly of two distinct forms of dynactin that differ in their ability to bind microtubules. Because the expression of these two functionally distinct isoforms seems to be conserved among evolutionarily diverse species and because tissue surveys have suggested that the isoform that cannot bind microtubules is expressed only in brain, we propose that there is a neuron-specific function for a form of dynactin that cannot bind independently to microtubules, such as the regulation of dynein motor function in axonal transport.

MATERIALS AND METHODS

Generation and Characterization of Antibodies

Three rabbit polyclonal antibodies to p150^{Glued} and one rabbit polyclonal antibody to centractin were generated. Antiserum UP173 was raised against the peptide MKKEKFEETMDALQA (corresponding to amino acids 1006–1020 of the sequence for p150^{Glued} from rat) coupled to bovine serum albumin (BSA). Antiserum UP236 was raised against a fusion protein corresponding to amino acids 152–810 of p150^{Glued} from rat, and UP235 was raised against a fusion protein corresponding to amino acids 40–1280, close to the full-length rat polypeptide. An antibody to centractin, UP454, was gen-

erated to fusion protein produced from an 0.8-kbp fragment corresponding to the carboxyl-terminal half of centractin (EST00370 clone, obtained from the American Type Culture Collection). The subcloned DNA fragments were expressed in *Escherichia coli*, strain BL21 (DE3), using the pET15b vector (Novagen, Madison, WI). After induction of fusion protein expression with IPTG, the cells were lysed, and the fusion proteins were purified by chromatography on the basis of the binding affinity of an amino-terminal tag of six histidine residues for an Ni²⁺ column.

Antisera resulting from the inoculation of rabbits with these antigens were purified by ammonium sulfate fractionation, followed by affinity chromatography against the appropriate immunogen immobilized on CH-activated Sepharose 4B (Pharmacia, Piscataway, NJ). The purified antibodies were eluted with 100 mM triethylamine and equilibrated in phosphate-buffered saline (50 mM sodium phosphate, pH 7.4, and 150 mM NaCl) with 1% BSA by rapid gel filtration.

An antibody specific for the microtubule-binding domain of p150^{Glued} (anti-MTB) was generated by two rounds of purification of rabbit antisera UP235 against a purified, bacterially expressed polypeptide corresponding to residues 40–136 of the rat cDNA sequence for p150^{Glued}.

A monoclonal antibody to the 50-kDa subunit of dynactin was the generous gift of C. Echeverri and R. Vallee (Worcester Foundation for Biomedical Research, Shrewsbury, MA; Echeverri *et al.*, 1996), and a polyclonal antibody to cytoplasmic dynein heavy chain was generously provided by E. Vaisberg and J. R. McIntosh (University of Colorado; Vaisberg *et al.*, 1993).

Isolation of Microtubules and Microtubule-associated Proteins

NTera2/c1.D1 (NT2) cells, a human teratocarcinoma cell line, were induced to differentiate to a neuronal phenotype (NT2-N) by treatment with retinoic acid (Andrews, 1984; Andrews *et al.*, 1984; Lee and Andrews, 1986; Pleasure *et al.*, 1992; Pleasure and Lee, 1993). Microtubules and microtubule-associated proteins were isolated from the retinoic acid-treated NT2-N cells via a modification of the taxol-based method (Vallee, 1982). NT2-N cells were scraped into a small volume of PHEM buffer (50 mM PIPES, 50 mM HEPES, pH 7.0, 1 mM EGTA, and 2 mM MgSO₄) with added protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, and 10 µg/ml tosyl-arginine methyl ester. The cells were pelleted by centrifugation at low speed and then resuspended in the same buffer containing 0.2% Triton X-100 at a ratio of 1 ml of buffer per gram of cells. The cells were lysed by homogenization with a motor-driven Teflon pestle, and the resulting cell extracts were clarified by centrifugation at 38,000 × g for 10 min followed by centrifugation at 100,000 × g for 60 min at 4°C.

Polymerization of the tubulin in the cytosolic extract was induced by the addition of 200 µM taxol (generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD). The cell extracts were incubated with taxol for 15 min at 37°C, and the microtubules and associated proteins were isolated by centrifugation at 38,000 × g for 30 min at 25–30°C. The supernatant fractions were removed, and the microtubule pellets were resuspended in an equal volume of PHEM buffer containing 0.2% Triton X-100. Supernatant and pellet fractions were analyzed by SDS-PAGE and immunoblot. A parallel experiment was also performed from rat brain cytosol, prepared as described previously (Karki and Holzbaur, 1995).

Isolation and Characterization of cDNA Clones Encoding Human p150^{Glued}

A λZAPII human fetal brain cDNA library (Stratagene, La Jolla, CA) was screened by standard methods (Obar and Holzbaur, 1993) with the affinity-purified antibody UP173. Sixty-eight clones were iso-

lated and purified by additional rounds of screening. The cDNA inserts of 36 of these clones were excised into pBluescript by the ExAssist/SOLR protocol (Stratagene) and characterized by double-stranded DNA sequencing with either the Sequenase protocol (USB, Cleveland, OH) or the Ladderman system (Takara Shuzo, Kyoto, Japan). Partial cDNA clones encoding p150^{Glued} were identified by their homology to the previously characterized nucleotide sequence from rat (Holzbaur *et al.*, 1991). Extended sequences for these partial clones were obtained through rescreeing the library by hybridization or by 5'-rapid amplification of cDNA ends (RACE) (Life Technologies, Gaithersburg, MD), with the use of RNA isolated from NT2-N cells as a template for cDNA synthesis. The clones of interest were sequenced on both strands with nested deletion constructs generated by exonuclease III digestion. The sequence data were analyzed by the LaserGene (DNASar, Madison, WI) and Genetic Computer Group (Devereux *et al.*, 1984) sequence analysis software packages. Database searches were performed by the Blast network service to screen the National Center for Biotechnology Information database (Altschul *et al.*, 1990). Human cDNA sequences for the p150 and p135 isoforms of the Glued polypeptide have been deposited in the European Molecular Biology Laboratory (EMBL) database.

RNA Isolation and RNase Protection Assays

Total cytoplasmic RNA was isolated from NT2 and differentiated NT2-N cells as described by Sambrook *et al.* (1989) or, for some experiments, with the RNazol B protocol (Cinna/Biotech Laboratories, Houston, TX). Messenger RNA was purified with reagents from the MicroFastTrack kit (Invitrogen, San Diego, CA). Both RNA and mRNA were analyzed by Northern blot as described (Melloni *et al.*, 1995), with a probe generated by random primed labeling of a 3.7-kb fragment from cDNA clone MT4–6.

For RNase protection assays, a 199-bp *ApaI*/*BsaI* restriction fragment from cDNA clone MTIII4–1 (Figure 4A) was subcloned into Bluescript SK(–) (Stratagene) at the *SmaI* and *ApaI* sites in the polylinker. The fragment was then excised with *Bam*HI and *KpnI* and subcloned into pGem-7Zf(+) (Promega, Madison, WI), resulting in plasmid pG199. The pG199 plasmid was linearized 5' to the 199-bp cDNA inset with *XbaI* and used as a template for *in vitro* transcription with SP6 RNA polymerase (Promega) and [α -³²P]-UTP (400 Ci/mmol; Amersham, Arlington Heights, IL), as described by the vendor (Promega). Labeled antisense probe was used for RNase protection with the method described for the RPAII kit (Ambion, Austin, TX). RNA isolated either from NT2 or NT2-N cells (5 µg) was annealed with antisense probe for 16 h at 45°C, followed by digestion with RNase A at a concentration of 0.5 U per ml and RNase T1 at 100 U per ml for 30 min at 37°C. Protected bands, separated by electrophoresis on an 8% polyacrylamide/8 M urea gel, were visualized by autoradiography.

Cellular Transfections

Human cDNAs encoding the p150 and the p135 isoforms were subcloned into the pcDNA3 vector (Invitrogen), in which expression is driven by the human CMV promoter. REF52 cells (a generous gift of Drs. Joe and Jean Sanger of the University of Pennsylvania School of Medicine, Philadelphia, PA) were maintained as monolayer cultures in Eagle's minimal essential medium with 10% fetal calf serum (FCS) with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) and were plated on coverslips for transfection experiments. Transfections were performed by calcium phosphate precipitation (Sambrook *et al.*, 1989) with the p150 and p135 constructs, as well as with the pcDNA3 vector only as a control. At 48 h after transfection, cells were fixed in –20°C methanol with 1 mM EGTA for 10 min, rinsed in phosphate-buffered saline, and then blocked in 5% goat serum and 1% BSA. Immunocytochemistry was performed with an affinity-purified rabbit polyclonal antibody to p150^{Glued} (UP236) and a monoclonal antibody to tubulin (YL1/2; Serotec, Oxford, UK),

followed by secondary antibodies labeled with fluorescein and Texas red (Jackson ImmunoResearch, West Grove, PA). Photomicroscopy was performed with a Leitz DMRB with a photoautomat system.

Immunoprecipitations

A frozen slice of 0.5 g of human frontal cortex was homogenized in 6 ml of 20 mM Tris, pH 7.4, buffer with 2 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged 10 min at $38,000 \times g$ to remove unlysed cells. The supernatant from this step was then centrifuged for 1 h at $100,000 \times g$ to separate the soluble and membrane fractions. The resulting membrane pellet fraction was washed with 6 ml of homogenization buffer, followed by centrifugation at $100,000 \times g$ for 30 min. Membrane-associated proteins were then solubilized by sonication of the pellet fraction in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 4 mM EDTA, 1% Triton X-100, and 0.1% SDS), followed by centrifugation for 1 h at $100,000 \times g$. The supernatant from this step was retained, and immunoprecipitations were performed in parallel on both the Tris-solubilized and RIPA-solubilized fractions with 10 μ l of affinity-purified antibody UP235 and 30 μ l of pansorbin (Calbiochem, La Jolla, CA). The precipitates were washed three times with RIPA buffer containing 350 mM NaCl and once with 20 mM Tris, pH 7.4, containing 150 mM NaCl and were then resuspended in SDS gel sample buffer. The samples were heated to 90°C to release bound protein, and the pansorbin was removed by low-speed centrifugation. The resulting supernatant fractions were analyzed by SDS-PAGE, followed by immunoblot.

Sequential immunoprecipitations were performed from cytosol prepared by homogenization of rat brains (Pel-Freezer) in PHEM buffer (50 mM sodium Pipes, 50 mM HEPES, 2 mM $MgCl_2$, and 1 mM EDTA, pH 7.0) supplemented with tosylarginine methyl ester, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride, and 0.2 mM dithiothreitol, at a 1:1 (wt/vol) ratio. The homogenate was clarified by centrifugation at 4°C at $39,000 \times g$ for 30 min, followed by centrifugation at $110,000 \times g$ for 60 min. The cytosol was preadsorbed with protein A-agarose beads (Life Technologies) for 30 min at 4°C, and the beads were removed by low-speed centrifugation. The clarified cytosol was then mixed with anti-MTB antibody bound to protein A-agarose beads. After a 3 h incubation at 4°C, the beads were isolated by centrifugation and washed five times in RIPA buffer without SDS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and 0.5% deoxycholate), and the final pellet was taken up in gel sample buffer for SDS-PAGE analysis. After the separation of the anti-MTB-coated beads, the supernatant was then mixed with protein A-agarose beads with the affinity-purified UP235 antibody bound. After a 3 h incubation, the beads were then isolated by centrifugation and washed and eluted as above. The samples were then analyzed by SDS-PAGE and immunoblot.

Rat brain cytosol was also analyzed by sucrose-density gradient centrifugation after immunodepletion with the anti-MTB antibody. One-half of a milliliter of rat brain cytosol was incubated for 3 h with anti-MTB antibody bound to protein A-agarose beads. The beads were removed by centrifugation, and the resulting supernatant fraction, immunodepleted of the p150 isoform, was layered onto a gradient of 5–20% sucrose in PHEM buffer. An equal volume of untreated cytosol was loaded on a parallel gradient. The gradients were centrifuged overnight at $110,000 \times g$ at 4°C and then fractionated. Gel samples of each fraction were analyzed by SDS-PAGE and immunoblotting.

Gel Electrophoresis and Immunoblotting

Protein samples were resolved by electrophoresis through 7 or 10% SDS-polyacrylamide gels, as noted, followed by electroblotting onto Immobilon-P membrane (Millipore, Bedford, MA). The resulting blots were probed with affinity-purified antibodies raised to either

p150^{Glued} (UP235) or contractin (UP454) or with a polyclonal antibody raised against the cytoplasmic dynein heavy chain, generously provided by Vaisberg and McIntosh, followed by the secondary antibodies donkey anti-rabbit IgG and rabbit peroxidase anti-peroxidase (both from Jackson ImmunoResearch), or with a monoclonal antibody to the 50-kDa polypeptide of the dynactin complex, provided by Echeverri and Vallee of the Worcester Foundation for Biomedical Research, followed by the secondary antibodies goat anti-mouse IgG and then by mouse peroxidase anti-peroxidase. Blots were developed with the Renaissance chemiluminescence reagent (DuPont NEN, Boston, MA).

RESULTS

Isoforms of the Glued Polypeptide Expressed in Human Neuronal Cells

Analysis of rat tissues both by in situ hybridization and by immunoblotting indicated that p150^{Glued} is most highly expressed in the brain (Melloni *et al.*, 1995). Immunoblot analysis with affinity-purified antibodies raised to p150^{Glued} also revealed the brain-specific expression of an immunoreactive polypeptide of 135 kDa. Because p150^{Glued} is encoded by a single gene in rat (Holzbaur *et al.*, 1991) and *Drosophila* (Swaroop *et al.*, 1986) and in human as determined by both Southern blotting and fluorescent in situ hybridization (Holzbaur and Tokito, 1996), the brain-specific 135-kDa polypeptide might result from alternative splicing of mRNA transcripts of the gene.

As a first step toward the characterization of p150^{Glued} isoforms expressed in human neurons, we prepared extracts from NT2-N cells and probed immunoblots with affinity-purified antibodies we have raised to bacterially expressed rat cDNAs encoding p150^{Glued}. We found that in this human neuronal cell line there are two major resolvable immunoreactive proteins, with molecular weights of 150 and 135 kDa (Figure 1A, lane 4).

We have recently determined that there is a microtubule-binding motif in the amino-terminus of p150^{Glued} that shares sequence homology with a similar motif in the microtubule-endosome linker protein CLIP-170 and the yeast microtubule associated protein BIK1 (Berlin *et al.*, 1990; Pierre *et al.*, 1991; Waterman-Storer *et al.*, 1995). Therefore, we compared the association of the two immunoreactive polypeptides with microtubules. We isolated microtubule-associated proteins from NT2-N cells by cosedimentation with taxol-stabilized endogenous microtubules (Figure 1A, lane 6). Comparison of the supernatant and pellet fractions by immunoblot with affinity-purified antibodies (Figure 1A, lanes 5 and 6) indicated that only the 150-kDa polypeptide was observed to cosediment with microtubules in this assay. The 135-kDa polypeptide remained in the supernatant fraction (lane 5), as did a significant fraction of the 150-kDa polypeptide. For comparison, we performed a similar experiment

Figure 1. Microtubule association of the p150^{Glued} isoforms expressed in NT2-N cells and in rat brain. (A) Cytosolic extract from NT2-N cells (lanes 1 and 4), supernatant fraction after polymerization of endogenous microtubules with taxol followed by sedimentation of the microtubules (lanes 2 and 5) and associated proteins, and the corresponding pellet fraction (lanes 3 and 6) were resolved on a 7% gel and transferred to Immobilon. Lanes 1–3 were stained for total protein with Coomassie blue. Lanes 4–6 are the corresponding immunoblot reacted with an affinity-purified polyclonal antibody raised against p150^{Glued}, which recognizes multiple isoforms in these cells. Only the p150 isoform was observed to cosediment with microtubules in this assay. (B) Shown are the cytosolic extract from rat brain (lanes 1 and 4), supernatant fraction after polymerization of endogenous microtubules with taxol followed by sedimentation of the microtubules and associated proteins (lanes 2 and 5), and the corresponding pellet fraction (lanes 3 and 6). Lanes 1–3 were stained for total protein; lanes 4–6 are the corresponding immunoblot reacted with an antibody raised to p150^{Glued}. In rat brain, as in NT2-N cells, only the p150 isoform cosedimented with microtubules.

from rat brain cytosol. Microtubule-associated and supernatant fractions from rat were fractionated by SDS-PAGE (Figure 1B, lanes 1–3) and analyzed by immunoblot with affinity-purified antibodies (Figure 1B, lanes 4–6). In rat brain, as was observed in human neurons, two major immunoreactive polypeptides were observed of 150 and 135 kDa. The 150-kDa polypeptide was found to partition between the supernatant and pellet fractions, whereas the 135-kDa polypeptide was not observed to associate with microtubules (Figure 1B, lanes 5 and 6).

Immunoblots of NT2-N cell extract revealed an additional, higher molecular weight polypeptide of ~180 kDa, which bound the affinity-purified antisera we have raised against the p150^{Glued} polypeptide (Figure 1A, lane 5). Corresponding immunoblots from rat brain did not reveal a similar-sized immunoreactive polypeptide; instead, a slightly smaller immunoreactive band was observed. Neither of these immunore-

active polypeptides was observed to cosediment with microtubules. Both of these polypeptides also were recognized by two independent antisera to the Glued polypeptide.

Sequence Diversity in the Expression of the Glued Polypeptide in Human Brain

To determine the sequence for the human homologue of p150^{Glued}, as well as to characterize the molecular basis for the apparent isoform diversity of this protein, we screened a human fetal brain cDNA expression library with an anti-peptide antibody UP173. This antibody was raised against an epitope that is highly conserved between p150^{Glued} from rat and the Glued polypeptide from *Drosophila*.

More than 100 clones were initially identified by immunoscreening the library. Of the 36 clones chosen for further characterization, 19 were identified as encoding the human homologue of p150^{Glued}, on the basis of the similarity of the encoded sequences to the previously characterized rat clone. Sequences for partial clones were extended by both rescreeing of the library by hybridization and by 5'-RACE, with RNA purified from NT2 cells.

The predicted amino acid sequence encoded by the overlapping human cDNA clones encoding p150 is highly homologous to that predicted from the previously characterized rat cDNA. Overall, the two polypeptide sequences are predicted to be 95% identical. Both the cDNA and predicted protein sequence for human p150^{Glued} have been deposited in the EMBL database (accession #X98801). Comparison of the predicted protein sequences for p150^{Glued} from human, rat, *Drosophila*, and *Neurospora* reveal several highly conserved motifs (Figure 2C). At the amino terminus, residues 55–106 (numbering is relative to the previously published rat sequence throughout) are highly conserved. The microtubule-binding domain of p150^{Glued} has been mapped to this region (Waterman-Storer *et al.*, 1995); a homologous motif has also been found in the microtubule-associated proteins CLIP-170 and BIK1 (Berlin *et al.*, 1990; Pierre *et al.*, 1991). A highly charged motif in p150^{Glued}, which has been implicated in the direct binding of the polypeptide to centractin (Waterman-Storer *et al.*, 1995), is also conserved from fungi to humans (residues 1005–1008). Several other sequence motifs are particularly well conserved. Although these may represent key structural or folding domains, they might also be involved in the binding of p150^{Glued} to the cytoplasmic dynein intermediate chain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; Waterman-Storer and Holzbaur, 1996); we are currently mapping the site of this interaction.

Of the eight cDNA clones characterized by extended sequence analysis, six were found to vary consider-

20 and 13 amino acids, respectively, in an otherwise highly homologous region near amino acid 131 (Figure 2B). Preliminary analysis of human genomic

clones encoding p150^{Glued} has revealed that this is a complex region with multiple short exons as small as 21 bp in length (Tokito and Holzbaur, unpublished observations). It seems that this region of the gene may have a complex expression pattern, with alternative splicing leading to significant microheterogeneity.

However, a more extensively variant cDNA clone was also identified in this region. cDNA clone III4-1 was found to encode the amino acids MMRQ immediately upstream of a putative splice site (Figure 2B). This four-residue sequence was not found in any of the other variant clones. Upstream of the two adjacent methionine codons in cDNA clone III4-1 are stop codons in all three frames. These data indicate that clone III4-1 encodes a variant splice form, which lacks the amino-terminal domain of p150^{Glued}. The predicted molecular weight for a polypeptide initiating at this point is 135 kDa, which is the observed size of the smaller immunoreactive isoform of p150^{Glued} as expressed in human NT2-N cells (Figure 1) and human brain (Figure 6) and in rat brain (Figure 1; also see Melloni *et al.*, 1995).

Two Isoforms of p150^{Glued} Are Expressed in Neurons by Alternative Splicing

Analysis of Northern blots of RNA isolated from NT2-N cells hybridized with a cDNA probes encoding p150^{Glued} revealed a single broad band of ~4–4.4 kb (Figure 3). Northern blot analysis of RNA from rat brain yielded a similar result (Holzbaur *et al.*, 1991; Melloni *et al.*, 1995). Because the resolution of similarly sized messages of this length by Northern blot analysis is limited and may be complicated by differential polyadenylation of the transcripts, we used RNase protection assays to determine whether human neuronal cells express an RNA transcript that corresponds to the sequence encoded by clone III4-1 and that would encode the p135 isoform.

We constructed a probe that spanned the 5'-UTR and the predicted translation initiation site of clone III4-1. We hybridized the radiolabeled antisense RNA probe to total cytoplasmic RNA isolated from the NT2-N neuronal cell line and then digested single-stranded RNA with RNases. Under these conditions, only when the antisense probe hybridizes exactly to expressed RNA will there be a protected fragment, the size of which can be determined from a high-resolution polyacrylamide gel. If a message corresponding to clone III4-1 is expressed in NT2-N cells, then we would predict the protection of a 199-base fragment of the probe (Figure 4A). Hybridization of the same probe to message encoding the full-length p150 isoform would be predicted to protect only a 92-base fragment of the probe because of an inability to hybridize to a contiguous sequence, including the 5'-UTR and the four amino acid MMRQ sequence unique

Figure 3. Northern blot of RNA purified from NT2-N cells, hybridized with a cDNA probe that spans the sequence shared by clones III3-3, II4-7, and III4-1, as illustrated in Figure 2B. A single broad band was observed on the autoradiograph. Sizes were determined in comparison to RNA size markers (Life Technologies).

to the p135 isoform. Because of the stringency of RNase digestion conditions, only the exact probe sequence in common between the two isoforms would be protected by a message encoding p150^{Glued}, predicting a band of 92 bases (see schematic in Figure 4A). As shown in Figure 4B, protected fragments of 199 bases and 92 bases were detected in RNA isolated from NT2-N neurons.

The observation of two protected fragments that correspond exactly in size to the predicted products in this experiment indicates that two distinct transcripts encoding Glued polypeptides are expressed in the human neuronal cell line NT2-N. The demonstrated expression of an mRNA that hybridizes exactly to cDNA III4-1, which encodes contiguous untranslated and translated sequence, suggests that the 135-kDa polypeptide arises from a distinct transcript produced by alternative splicing of the human *DCTN1* gene.

RNA from uninduced NT2 cells was also examined in this assay, but, as shown in Figure 4B, the signal was below the limit of detection. It is likely that during differentiation of neuronal cells the expression of p150^{Glued} is upregulated, a hypothesis that is consistent with our previous results examining the distribution of p150^{Glued} mRNA by *in situ* hybridization in developing rat (Melloni *et al.*, 1995).

The 135-kDa Isoform Lacks the Microtubule-binding Domain

The predicted protein sequence for the 135-kDa isoform does not include the consensus microtubule-binding domain found in the first 106 amino acids of the 150-kDa isoform. This observation suggests that p135 should not bind to microtubules, a prediction consistent with the initial observations shown in Figure 1 and that the immunoreactive p135

Figure 4. RNase protection assay to analyze the expression of mRNA encoding multiple isoforms of the Glued polypeptide in human NT2-N cells. (A) Schematic of the protection assay, in which an antisense RNA probe that spans both the 5'-UTR and the putative translation start site was constructed from the human cDNA clone III4-1 and was hybridized to RNA isolated from NT2-N cells. Hybridization to mRNA encoding the p135 isoform should lead to the protection of a 199-base fragment of the probe. Hybridization to mRNA that encodes the p150 isoform would be predicted to protect only a shorter fragment of 92 bases, because this mRNA should lack the 5'-untranslated region that initiates clone III4-1. (B) Products of the protection assay were resolved by polyacrylamide electrophoresis vs. molecular weight standards. The full-length ³²P-labeled antisense probe is shown in lane 1 (probe). Lane 2 (tRNA) is a control reaction for possible incomplete digestion of the probe by RNase after incubation with nonspecific, nonhybridizing tRNA. Lane 3 (NT2) shows the results of a protection experiment in which the probe was hybridized to RNA isolated from uninduced NT2 cells. RNA encoding p150^{Glued} is expressed at a significantly lower level before induction to a neuronal phenotype. Lane 4 (NT2-N) shows the results obtained from hybridizing the probe with RNA isolated from NT2-N cells, followed by digestion with RNase. Two major protected fragments were observed of 199 and 92 bases, which correspond exactly to the predicted products from transcripts encoding the p135 and p150 isoforms, respectively. Two exposures of the marker lane (MW) are shown to unambiguously and exactly determine the sizes of the protected fragments, because several of the marker bands are labeled to a lesser extent.

polypeptide does not bind to microtubules polymerized from either NTera2 cell extract or from rat brain.

To further establish the functional difference between isoforms of the Glued polypeptide expressed in human brain, we performed transient transfection assays with human cDNAs encoding the p150 and p135 isoforms, subcloned into the pcDNA3 expression vector. As previously observed in transfection assays with rat cDNAs encoding p150^{Glued}, overexpression of a human cDNA encoding p150 in REF52 cells led to the decoration of the microtubule cytoskeleton with the Glued polypeptide (Figure 5A). In contrast, transfection of REF52 cells with the human cDNA encoding the p135 isoform produced no detectable decoration of cytoplasmic microtubules (Figure 5C). Instead, the overexpressed protein was distributed throughout the cytoplasm in a punctate pattern. Although this distribution resembled wild-type distribution patterns (Paschal *et al.*, 1993), transfected cells were easily distinguished from nontransfected cells in the population on the basis of the levels of overexpression that were observed. Transfected cells were also examined for

any alteration in the distribution of cytoplasmic dynein. No significant perturbations were detected in cells transfected with cDNAs encoding either the p150 or p135 isoforms.

Cellular Distribution of the p150 and p135 Isoforms

Although p150^{Glued} was initially identified as a soluble polypeptide in cytosol (Collins and Vallee, 1989; Gill *et al.*, 1991; Holzbaur *et al.*, 1991), immunocytochemistry with antibodies to p150^{Glued} revealed a punctate, apparent vesicular distribution in cultured cells (Gill *et al.*, 1991; Paschal *et al.*, 1993). To determine whether either the p150 or p135 isoforms preferentially localize to a distinct cellular compartment, we isolated both cytosolic and membrane fractions from human brain. Immunoprecipitations were performed from both the cytosolic and solubilized membrane fraction under denaturing conditions with an antibody that recognizes both isoforms (UP235). As shown in Figure 6, both isoforms were observed to partition similarly between the soluble and membrane-associated fractions in human brain.

Figure 5. Overexpression of human cDNAs encoding p150 and p135 in REF52 cells by transient transfection assays. REF52 cells were transfected with cDNAs encoding either p150 (A and B) or p135 (C and D). The transfected cells were analyzed by immunocytochemistry with an affinity-purified polyclonal antibody to p150^{Glued} (A and C) and a monoclonal antibody to tubulin (B and D). The overexpressed p150 isoform was found to decorate cytoplasmic microtubules, whereas the p135 isoform was found in a punctate distribution throughout the cytoplasm.

p150^{Glued} and p135^{Glued} Assemble into Distinct Complexes in Brain

The stoichiometry of the dynactin complex indicates that there are 2 mol of p150/p135 per 20 S complex (Paschal *et al.*, 1993; Schafer *et al.*, 1994). Because both the p150 and p135 isoforms are expressed in neurons, it is possible that one mol of each polypeptide is found in dynactin isolated from brain. Alternatively, each of these isoforms could self-associate. Either of the homodimers could bind to the centractin filament that forms the base of the dynactin complex (Schafer *et al.*, 1994), because both the p150 and the p135 isoforms are predicted to encode the highly charged motif implicated in the association of the Glued polypeptide side-arm of dynactin with centractin (Waterman-Storer *et al.*, 1995). Self-association would, therefore, allow the assembly of distinct forms of dynactin that would differ in their ability to bind directly to microtubules.

To address the question of whether the p150 and p135 isoforms are found in a single complex, we generated an antibody specific for the microtubule-binding domain (see MATERIALS AND METHODS). As expected, this antibody anti-MTB reacted specifically with the p150 isoform (Figure 7). We were not able to generate a p135-specific antibody because the shorter isoform has only four amino acid residues at its amino terminus which are distinct from the p150 sequence at that point. The isoform-specific anti-MTB antibody, as well as an antibody that recognizes both isoforms, was then used to perform sequential immunoprecipitations under nondenaturing conditions that should favor the continued association of dynactin polypeptides.

The anti-MTB antibody was used to precipitate quantitatively the p150 isoform from rat brain cytosol. As shown in lane 2 of Figure 8, after immunopre-

Figure 6. Distribution of the p150 and p135 isoforms between soluble and membrane fractions from human brain. Human brain cytosol (lane 1) and immunoprecipitated fractions obtained with the pan-isoform antibody UP235 from soluble (lanes 2 and 4) and membrane fractions (lanes 3 and 5) were fractionated by SDS-PAGE on a 7% gel, transferred to Immobilon, and stained with Coomassie blue for total protein (lanes 1–3). The blot was then destained and probed with an affinity-purified antibody to p150^{Glued}, which recognizes both isoforms (UP235), to visualize more clearly the Glued polypeptides (lanes 4 and 5). The darkly staining bands near the bottom of the blots correspond to the immunoglobulin heavy chain of the precipitating antibody.

precipitation with the anti-MTB antibody no detectable p150 polypeptide remained in the cytosolic extract. However, the p135 remained in the cytosol. Subsequent immunoprecipitation of the p150-cleared cytosolic extract with a pan-isoform antibody resulted in the complete removal of p135 from this fraction (Figure 8, lane 3).

The selective immunoprecipitation of each of these polypeptides from rat brain cytosol with the antibodies described supports the hypothesis that p150 and p135 are present in distinct dynactin complexes. To verify that each isoform is associated with other dynactin components, we resolved the fractions obtained by sequential immunoprecipitation by SDS-PAGE and electroblotted and probed with antibodies specific for two other dynactin polypeptides, centractin and p50. As shown in Figure 8, C and D, both p50 and centractin were found to be present in extracts selectively immunoprecipitated as described. Therefore, each of the Glued isoforms selectively precipitates with other dynactin subunits.

To further compare the two populations of dynactin, we used the anti-MTB antibody to immunodeplete rat

Figure 7. Specificity of the anti-MTB antibody for the p150^{Glued} isoform. A rat brain fraction enriched in dynactin was resolved by SDS-PAGE on a 7% gel, transferred to Immobilon, and stained for total protein with Coomassie blue (lane 1) or probed with antibodies recognizing both isoforms (UP235, lane 2) or with an antibody specific for the microtubule-binding domain (anti-MTB, lane 3).

Figure 8. Sequential immunoprecipitations of the p150 and p135 isoforms from rat brain. Sequential immunoprecipitations were performed under nondenaturing conditions from rat brain cytosol. Immunoprecipitation with the anti-MTB antibody was followed by immunoprecipitation with an antibody that recognizes both isoforms (UP235). Lane 1, total rat brain cytosol; lane 2, cytosolic extract after immunoprecipitation with the anti-MTB antibody; lane 3, cytosolic extract after a subsequent immunoprecipitation with the pan-isoform antibody UP235; lane 4, fraction immunoprecipitated with the anti-MTB antibody; lane 5, fraction immunoprecipitated with the pan-isoform antibody; lane 6, rat brain sample enriched in dynactin for comparison. (A) The fractions were resolved by SDS-PAGE on a 7% gel, transferred to Immobilon, and stained with Coomassie blue for total protein. (B) The blot was destained and then probed with an affinity-purified antibody to p150^{Glued}, which recognizes both isoforms. (C) The same blot was reprobed with a monoclonal antibody to the p50 subunit of dynactin. (D) The same fractions were resolved by SDS-PAGE on a 10% gel, electroblotted, and probed with an affinity-purified antibody to centractin. Examination of the cytosol after reaction with anti-MTB indicates that all of the p150 isoform was removed, leaving only the p135 isoform, which was then immunoprecipitated with an antibody that recognizes both isoforms. Immunoblots of the sequential immunoprecipitates with antibodies specific for other subunits of dynactin, p50 and centractin, reveal that both the p150 and p135 isoforms are associated with other dynactin components.

brain cytosol, thus selectively removing p150 and associated subunits (compare lanes 1 and 2 in Figure 9B). Then we fractionated the depleted cytosol by centrifugation through a sucrose-density gradient (Figure 9A). Immunoblotting of the resulting fractions with an antibody that recognizes both isoforms revealed that the p135 subunit of dynactin was found exclusively in a fraction sedimenting near 20 S (Figure 9B). The immunoblot was also reacted with an antibody specific to centractin, indicating that this dynactin subunit

Figure 9. Dynactin with the p135 subunit sediments near 20 S. Rat brain cytosol (lane 1) was immunodepleted of the p150 isoform of Glued by using the anti-MTB antibody (lane 2). The cytosol was then fractionated over a 5–20% sucrose-density gradient (lanes 3–14). (A) The fractions were resolved through a 7% gel, electroblotted to Immobilon, and stained for total protein with Coomassie blue. (B) The blot was reacted with an affinity-purified antibody that recognizes both the p150 and p135 isoforms of Glued. The p135 isoform was found in a single peak near 20 S. (C) The immunoblot was also reacted with an affinity-purified antibody to centractin. A single peak was observed, which comigrated with the p135 peak. (D) The immunoblot was then reacted with an antibody to the cytoplasmic dynein heavy chain (Vaisberg *et al.*, 1993). Note a displacement of approximately half a fraction between the peak for dynein relative to dynactin; a similar shift was also noted by Echeverri *et al.* (1996). Gradients were calibrated with internal markers.

cosedimented with p135, again indicating that, under the conditions used to separate p150 and p135, the dynactin complex remained intact. For comparison, the immunoblot was also probed with an antibody to the heavy chain of cytoplasmic dynein (a generous gift of Drs. Vaisberg and McIntosh). The shift of approximately half a fraction between the peaks of cytoplasmic dynein and dynactin was also observed by Echeverri *et al.* (1996). Control fractionation of untreated cytosol revealed that the p150 and p135 isoforms peak in the same fraction on sucrose gradients, as has been shown previously (Paschal *et al.*, 1993; Echeverri *et al.*, 1996).

Together, these experiments demonstrate that two isoforms of the Glued polypeptide are expressed in human neurons. These isoforms correspond in size to

the predicted products of two human brain cDNAs we have isolated and characterized. One of these cDNAs, clone III4–1, encodes an open reading frame, preceded by stop codons in all three frames, which is predicted to encode a polypeptide of 135 kDa. This polypeptide is predicted to lack the microtubule-binding motif; binding studies in cell lysates and in transfected cells confirm that both the p135 polypeptide and the expressed product of the cDNA lack the microtubule-binding domain. Analysis of RNA purified from human neuronal cells by Northern blot and higher resolution mapping indicates that at least two distinct transcripts are expressed, one of which corresponds exactly to the 5' end of cDNA clone III4–1. In brain cytosol the p150 and p135 isoforms of dynactin assemble into distinct forms of dynactin. Both of these isoforms are associated with other dynactin polypeptides, both are present in both the soluble and membrane fractions, and, as shown in previously published studies, both can bind to cytoplasmic dynein (Karki and Holzbaur, 1995). However, only dynactin complex, which contains the p150 isoform, is capable of a direct association with microtubules.

DISCUSSION

p150^{Glued} is an integral component of the dynactin complex. This complex seems to function as a potentiator of intracellular organelle transport driven by the microtubule-based motor cytoplasmic dynein. In an *in vitro* motility assay, dynactin was found to be required to reconstitute cytoplasmic dynein-mediated vesicular trafficking along microtubules (Gill *et al.*, 1991). However, the mechanism for its role as an activator or mediator for dynein-driven vesicular transport is currently unknown.

The p150^{Glued} component of the dynactin complex has multiple binding interactions. We have mapped the domains of the polypeptide that bind to the microtubule (Waterman-Storer *et al.*, 1995) to cytoplasmic dynein (Karki and Holzbaur, 1995; Waterman-Storer and Holzbaur, 1996; see also Vaughan and Vallee, 1995), and to the actin-related protein centractin (Arp1), which is also an integral component of the dynactin complex (Waterman-Storer *et al.*, 1995). Although p150^{Glued} appears as a characteristic doublet on immunoblots of both cell extracts and purified dynactin complex, only a single gene encoding this protein has been detected from rat (Holzbaur *et al.*, 1991) and human (*DCTN1*; Holzbaur and Tokito, 1996). Therefore, it is not surprising that the observed isoform diversity is due to alternative splicing, as we have demonstrated. However, it is of particular interest to note that the shorter isoform we have cloned and characterized is functionally distinct. The predicted amino acid sequence of the p135 isoform does not encode the microtubule-binding motif identified

within p150^{Glued} and also in the microtubule–endosome linker protein CLIP-170 (Pierre *et al.*, 1991) and the yeast microtubule-associated protein BIK1 (Berlin *et al.*, 1990). Binding assays described above support the hypothesis that the p135 isoform cannot associate directly with microtubules. Together with our previous studies, these data confirm that the microtubule-binding motif of p150^{Glued} is found within the first 139 residues; sequence comparisons among evolutionarily divergent sequences for p150^{Glued} (Figure 2C), as well as comparisons with the predicted protein sequences of CLIP-170 and BIK1, suggest that the critical motif involves amino acid residues 55–106.

Analysis of the predicted protein sequence for p135 suggests that this polypeptide will bind centractin via the C-terminal KKEK motif we have previously characterized (Waterman-Storer *et al.*, 1995). It has been previously determined that in bovine brain both p150 and p135 are found in cytosol exclusively as components of the 20 S dynactin complex (Paschal *et al.*, 1993). Data from the sequential immunoprecipitation experiments shown here indicate that both the p150 and the p135 polypeptides are associated with other dynactin subunits. However, these immunoprecipitation data also indicate that, in brain cytosol, p150 and p135 do not coassemble into the same dynactin complex. Previously reported stoichiometries for the complex suggest that, on average, 1 mol of each is present within dynactin. However, it is more likely that either 2 mol of p150 or 2 mol of p135 dimerize, potentially via the extended coiled coil motifs distributed through the length of the polypeptide, and that these “homodimers” form the arm of the dynactin complex visualized by electron microscopy (Schafer *et al.*, 1994). Thus, the alternative splicing observed in neurons leads to the formation of functionally distinct forms of dynactin, one capable of binding to microtubules via two binding domains and the other incapable of a direct association with microtubules.

However, it should be noted that, when examined in whole-cell extracts, a significant fraction of the p150 isoform does not bind to microtubules (Figure 1). This differential affinity may reflect differences in the post-translational modification of the dynactin complex. The binding of the related polypeptide CLIP-170 to microtubules has been shown to be modulated by its phosphorylation state (Rickard and Kreis, 1991); p150 may be regulated in a similar manner. We have determined that p150^{Glued} is a phosphoprotein (Farshori and Holzbaur, unpublished data), although the effects of phosphorylation on the affinity of p150^{Glued} for microtubules has yet to be determined.

A mechanism for the role of dynactin in potentiating dynein-mediated cell motility has yet to be established. It may be that the functionally distinct isoforms of p150 and p135 that we have characterized are involved in adapting the conserved cytoplasmic dynein

motor protein to a range of disparate intracellular functions. Immunoblot analysis has shown that in rat the expression of the p135 isoform is brain specific (Melloni *et al.*, 1995); p135 is also expressed in NT2-N neurons. Therefore, the p135 isoform may have a specialized role in the cell biology of neurons. One possibility may be a role for dynactin in slow axonal transport. Although the mechanism of fast-retrograde, fast-axonal transport in neurons may be similar to that observed for more generalized retrograde vesicular transport in non-neuronal cells, it has been proposed that cytoplasmic dynein (Dillman *et al.*, 1996) and dynactin (Dillman *et al.*, 1995) may have a specialized, neuron-specific role in the slow-anterograde transport of microtubules along the axon. It is tempting to speculate that the p135 isoform, whose expression is brain-specific in the rat, has a particular role in this type of motility that would not be required in cells with less extended processes. Alternatively, dynactin that contains the p135 isoform exclusively may, instead, function as a membrane linker or receptor for dynein. In contrast, dynactin complex that contains the p150 isoform may bind directly to microtubules, independent of the association of the complex with cytoplasmic dynein. Under these circumstances, dynactin may be capable of mediating the formation of an initial ternary complex of cytoplasmic dynein, the organelle, and the microtubule. In this case dynactin, and specifically p150^{Glued}, may share a similar mode of action to CLIP-170, which mediates the association of endosomes with the microtubule cytoskeleton (Pierre *et al.*, 1991).

It is clear that further research will be required to discriminate among potential models for the function of the dynactin complex. However, because at least two distinct isoforms of p150^{Glued} seem to be expressed in both vertebrates such as human (this study), rat (Melloni *et al.*, 1995), and chick (Gill *et al.*, 1991), and invertebrates such as squid (Waterman-Storer *et al.*, unpublished data) and *Drosophila* (McGrail *et al.*, 1995; Waterman-Storer and Holzbaur, 1996) and because the p150 and p135 isoforms differ significantly in their functional properties, it is likely that these isoforms may be involved in distinct cellular roles that remain to be determined.

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REFERENCES

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.
- Andrews, P.W. (1984). Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. *Dev. Biol.* 103, 285–293.
- Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C., and Fogh, J. (1984). Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. *Lab. Invest.* 50, 147–162.
- Berlin, V., Styles, C.A., and Fink, G.R. (1990). BIK1, a protein required for microtubule function during mating and mitosis in *Saccharomyces cerevisiae*, colocalizes with tubulin. *J. Cell Biol.* 111, 2573–2586.
- Clark, S.W., and Meyer, D.I. (1992). Centractin is an actin homologue associated with the centrosome. *Nature* 359, 246–250.
- Clark, S.W., and Meyer, D.I. (1994). ACT3: a putative centractin homologue in *S. cerevisiae* is required for proper orientation of the mitotic spindle. *J. Cell Biol.* 127, 129–138.
- Collins, C.A., and Vallee, R.B. (1989). Preparation of microtubules from rat liver and testis: cytoplasmic dynein is a major microtubule associated protein. *Cell Motil. Cytoskeleton* 14, 491–500.
- Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12, 387–395.
- Dillman, J.F., Dabney, L.P., and Pfister, K.K. (1995). The dynactin complex is associated primarily with slow axonal transport. *Mol. Biol. Cell* 6, 155a.
- Dillman, J.F., Dabney, L.P., and Pfister, K.K. (1996). Cytoplasmic dynein is associated with slow axonal transport. *Proc. Natl. Acad. Sci. USA* 93, 141–144.
- Echeverri, C.J., Paschal, B.M., Vaughan, K.T., and Vallee, R.B. (1996). Molecular characterization of 50-kDa subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J. Cell Biol.* 132, 617–633.
- Eshel, D., Urrestarazu, L.A., Vissers, S., Jauniaux, J.C., van Vliet-Reedijk, J.C., Planta, R.J., and Gibbons, I.R. (1993). Cytoplasmic dynein is required for normal nuclear segregation in yeast. *Proc. Natl. Acad. Sci. USA* 90, 11172–11176.
- Garen, S.H., and Kankel, D.R. (1983). Golgi and genetic mosaic analyses of visual system mutants in *Drosophila melanogaster*. *Dev. Biol.* 96, 445–466.
- Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P., and Cleveland, D.W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* 115, 1639–1650.
- Harte, P.J., and Kankel, D.R. (1982). Genetic analysis of mutations at the *Glued* locus and interacting loci in *Drosophila melanogaster*. *Genetics* 101, 477–501.
- Hendry, I.A. (1980). Retrograde axonal transport. In: *Proteins of the Nervous System*, 2nd ed., ed. R.A. Bradshaw and D.M. Schneider, New York: Raven Press, 183–211.
- Hendry, I.A., Stockel, K., Thoenen, H., and Iversen, L.L. (1974). The retrograde axonal transport of nerve growth factor. *Brain Res.* 68, 103–121.
- Hirokawa, N., Sato-Yoshitake, R., Yosida, T., and Kawashima, T. (1990). Brain dynein (MAP 1C) localizes on both anterogradely and retrogradely transported membranous organelles in vivo. *J. Cell Biol.* 111, 1027–1037.
- Holzbaumer, E.L.F., Hammarback, J.A., Paschal, B.M., Kravitz, N.G., Pfister, K.K., and Vallee, R.B. (1991). Homology of a 150K cytoplasmic dynein-associated polypeptide with the *Drosophila* gene *Glued*. *Nature* 351, 579–583.
- Holzbaumer, E.L.F., and Tokito, M.K. (1996). Localization of the *DCTN1* gene encoding p150^{Glued} to human chromosome 2p13 by fluorescence in situ hybridization. *Genomics* 31, 398–399.
- Holzbaumer, E.L.F., and Vallee, R.B. (1994). Dyneins: molecular structure and cellular function. *Annu. Rev. Cell Biol.* 10, 339–372.
- Karki, S., and Holzbaumer, E.L.F. (1995). Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. *J. Biol. Chem.* 270, 28806–28811.
- Kristensson, K., and Olsson, Y. (1971). Retrograde axonal transport of protein. *Brain Res.* 29, 363–365.
- Lee, V.M.-Y., and Andrews, P.W. (1986). Differentiation of NTera2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. *J. Neurosci.* 6, 514–521.
- Lees-Miller, J.P., Helfman, D.M., and Schroer, T.A. (1992). A vertebrate actin-related protein is a component of a multi-subunit complex involved in microtubule-based vesicle motility. *Nature* 359, 244–246.
- Li, Y.Y., Yeh, E., Hays, T., and Bloom, K. (1993). Disruption of mitotic spindle orientation in a yeast dynein mutant. *Proc. Natl. Acad. Sci. USA* 90, 10096–10100.
- McGrail, M., Gepner, J., Silvanovich, A., Ludmann, S., Serr, M., and Hays, T.S. (1995). Regulation of cytoplasmic dynein function in vivo by the *Drosophila*-*Glued* complex. *J. Cell Biol.* 131, 411–425.
- Melloni, R.H., Tokito, M.K., and Holzbaumer, E.L.F. (1995). Expression of the p150^{Glued} component of the dynactin complex in developing and adult rat brain. *J. Comp. Neurol.* 357, 15–24.
- Meyerowitz, E.M., and Kankel, D.R. (1978). A genetic analysis of visual system development in *Drosophila melanogaster*. *Dev. Biol.* 62, 112–142.
- Muhua, L., Karpova, T.S., and Cooper, J.A. (1994). A yeast actin-related protein homologous to that in vertebrate dynactin complex is important for spindle orientation and nuclear migration. *Cell* 78, 669–679.
- Obar, R.A., and Holzbaumer, E.L.F. (1993). Immunoselection of cDNA clones. In: *Methods in Cell Biology: Antibodies in Cell Biology*, vol. 37, ed. D. Asai, New York: Academic Press, 361–405.
- Paschal, B.M., Holzbaumer, E.L.F., Clark, S., Meyer, D., and Vallee, R.B. (1993). Characterization of a 50-kDa polypeptide in cytoplasmic dynein preparations reveals a complex with p150^{Glued} and a novel actin. *J. Biol. Chem.* 268, 15318–15323.
- Paschal, B.M., and Vallee, R.B. (1987). Retrograde transport by the microtubule-associated protein MAP 1C. *Nature* 330, 181–183.
- Pierre, P., Scheel, J., Rickard, J.E., and Kreis, T.E. (1992). CLIP-170 links endocytic vesicles to microtubules. *Cell* 70, 887–900.
- Plamann, M., Minke, P.F., Tinsley, J.H., and Bruno, K.S. (1994). Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. *J. Cell Biol.* 127, 139–149.
- Pleasure, S.J., and Lee, V.M.-Y. (1993). NTera2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. *J. Neurosci. Res.* 35, 585–602.

- Pleasure, S.J., Page, C., and Lee, V.M.-Y. (1992). Pure, postmitotic, polarized human neurons derived from NTera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.* 12, 1802–1815.
- Plough, H.H., and Ives, P.T. (1935). Induction of mutations by high temperature in *Drosophila*. *Genetics* 20, 42–69.
- Rickard, J.E., and Kreis, T.E. (1991). Binding of Pp 170 to microtubules is regulated by phosphorylation. *J. Biol. Chem.* 266, 17597–17605.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schafer, D.A., Gill, S.R., Cooper, J.A., Heuser, J.E., and Schroer, T.A. (1994). Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin. *J. Cell Biol.* 126, 403–412.
- Steuer, E., Wordeman, L., Schroer, T.A., and Sheetz, M.P. (1990). Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature* 345, 266–268.
- Swaroop, A., Sun, J.-W., Paco-Larson, M.L., and Garen, A. (1986). Molecular organization and expression of the genetic locus *Glued* in *Drosophila melanogaster*. *Mol. Cell. Biol.* 6, 833–841.
- Swaroop, A., Swaroop, M., and Garen, A. (1987). Sequence analysis of the complete cDNA and encoded polypeptide for the *Glued* gene of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, 84, 6501–6505.
- Sweeney, H.L., and Holzbaur, E.L.F. (1996). Mutational analysis of motor proteins. *Annu. Rev. Physiol.* 58, 751–792.
- Tinsley, J.H., Minke, P.F., Bruno, K.S., and Plamann, M. (1996). p150^{Glued}, the largest subunit of the dynactin complex, is nonessential in *Neurospora* but required for nuclear distribution. *Mol. Biol. Cell* 7, 731–742.
- Vaisberg, E.A., Koonce, M.P., and McIntosh, J.R. (1993). Cytoplasmic dynein plays a role in mammalian spindle formation. *J. Cell Biol.* 123, 849–858.
- Vallee, R.B. (1982). A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J. Cell Biol.* 92, 435–442.
- Vaughan, K.T., and Vallee, R.B. (1995). Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150^{Glued}. *J. Cell Biol.* 131, 1507–1516.
- Waterman-Storer, C.M., and Holzbaur, E.L.F. (1996). The product of the *Drosophila* gene *Glued* is the functional homologue of the p150^{Glued} component of the vertebrate dynactin complex. *J. Biol. Chem.* 271, 1153–1159.
- Waterman-Storer, C.M., Karki, S., and Holzbaur, E.L.F. (1995). The p150^{Glued} component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp1). *Proc. Natl. Acad. Sci. USA* 92, 1634–1638.