

# Characterization of the p22 Subunit of Dynactin Reveals the Localization of Cytoplasmic Dynein and Dynactin to the Midbody of Dividing Cells

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**Abstract.** Dynactin, a multisubunit complex that binds to the microtubule motor cytoplasmic dynein, may provide a link between dynein and its cargo. Many subunits of dynactin have been characterized, elucidating the multifunctional nature of this complex. Using a dynein affinity column, p22, the smallest dynactin subunit, was isolated and microsequenced. The peptide sequences were used to clone a full-length human cDNA. Database searches with the predicted amino acid sequence of p22 indicate that this polypeptide is novel. We have characterized p22 as an integral component of dynactin by biochemical and immunocytochemical methods. Affinity chromatography experiments indicate that p22 binds directly to the p150<sup>Glued</sup> subunit of dynactin. Immunocytochemistry with antibodies to p22

demonstrates that this polypeptide localizes to punctate cytoplasmic structures and to the centrosome during interphase, and to kinetochores and to spindle poles throughout mitosis. Antibodies to p22, as well as to other dynactin subunits, also revealed a novel localization for dynactin to the cleavage furrow and to the midbodies of dividing cells; cytoplasmic dynein was also localized to these structures. We therefore propose that dynein/dynactin complexes may have a novel function during cytokinesis.

**Key words:** microtubules • motor proteins • microtubule-associated proteins (MAPs) • cell division • cytokinesis

**D**YNACTIN is a macromolecular complex that is a required activator for cytoplasmic dynein-mediated vesicular transport (Gill et al., 1991; Waterman-Storer et al., 1997). Dynactin consists of at least seven polypeptides ranging in size from 22 to 150 kD. Genetic evidence from *Saccharomyces cerevisiae*, *Neurospora crassa*, *Aspergillus nidulans*, and *Drosophila* suggest that cytoplasmic dynein and dynactin are involved in the same cellular pathways (Eshel et al., 1993; Li et al., 1993; Clark and Meyer, 1994; Muhua et al., 1994; Plamann et al., 1994; McGrail et al., 1995; Tinsley et al., 1996). Cytoplasmic dynein is a minus end-directed microtubule motor (Paschal et al., 1987) with an established role in retrograde axonal transport (Schnapp and Reese, 1989; Hirokawa et al., 1990). However, accumulating data suggest that dynein and dynactin are involved in a diverse array of cellular functions (for reviews see Sweeney and Holzbaur, 1996; Vallee and Sheetz, 1996). For example, dynein and dynactin are thought to drive ER-to-Golgi transport (Presley et al., 1997; Harada et al., 1998), the centripetal movement of

lysosomes and endosomes (Aniento et al., 1993; Lin et al., 1994), spindle formation (Vaisberg et al., 1993; Gaglio et al., 1996; Merdes et al., 1996), chromosome movement (Saunders et al., 1995), nuclear positioning (Eshel et al., 1993; Li et al., 1993), and axonogenesis (Phillis et al., 1996; Reddy et al., 1997).

While the heavy chains of cytoplasmic dynein contain the microtubule-binding site and the ATPase site, the 74-kD dynein intermediate chain has been shown to interact directly with the p150<sup>Glued</sup> subunit of dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), suggesting that the intracellular targeting of dynein is modulated by dynactin. Support for this idea comes from recent studies in which the perturbation of dynactin function resulted in the disruption of dynein-mediated processes (Echeverri et al., 1996; Burkhardt et al., 1997; Presley et al., 1997; Waterman-Storer et al., 1997) and the loss of dynein from membranous vesicles (Waterman-Storer et al., 1997).

To understand how dynactin functions in conjunction with cytoplasmic dynein, subunits of dynactin have been cloned, sequenced, and characterized. The first dynactin subunit to be characterized was the 150-kD polypeptide from rat (Holzbaur et al., 1991). The sequence of this polypeptide had significant homology to the *Drosophila* gene *Glued*, and hence was named p150<sup>Glued</sup> (Swaroop and Garen, 1987; Holzbaur et al., 1991). More recent studies

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have shown that these two polypeptides are functional homologues (McGrail et al., 1995; Waterman-Storer and Holzbaaur, 1996). The original *Glued* (*Gl*) mutation (Plough and Ives, 1935) caused a dominant rough eye phenotype in the heterozygote. Further analysis showed that the homozygous form of this mutation was embryonic lethal (Harte and Kankel, 1982). Mutations in the dynein heavy chain gene in *Drosophila* produce a eye phenotype similar to that caused by the *Gl* mutation (McGrail et al., 1995). This genetic data supports biochemical binding assays that have demonstrated a direct interaction between p150<sup>Glued</sup> and the dynein intermediate chain (Karki and Holzbaaur, 1995; Vaughan and Vallee, 1995). p150<sup>Glued</sup> also binds directly to microtubules via an NH<sub>2</sub>-terminal CAP-Gly motif (Waterman-Storer et al., 1995). A brain-specific 135-kD isoform of p150<sup>Glued</sup> has been described that lacks this microtubule-binding domain (Tokito et al., 1996). Human (Tokito et al., 1996), mouse (Jang et al., 1997), chick (Gill et al., 1991), and fungal (Tinsley et al., 1996) homologues of p150<sup>Glued</sup> have also been cloned and characterized.

Another well-characterized dynactin subunit is the 45-kD actin-related protein (Arp1<sup>1</sup>; Clark and Meyer, 1992; Lees-Miller et al., 1992), which is also called centractin because of its prominent localization to the centrosome (Clark and Meyer, 1992). Centractin is the most abundant polypeptide in dynactin (Paschal et al., 1993; Schafer et al., 1994), with a stoichiometry of 8–13 molecules per complex. Centractin was found to form a minifilament within dynactin that is similar in morphology to actin filament as visualized by electron microscopy (Schafer et al., 1994). Overexpression of centractin results in the formation of novel filaments that distort the morphology of the Golgi apparatus, suggesting an interaction between dynactin and this organelle (Holleran et al., 1996).

The 50-kD subunit of dynactin (also known as dynamitin) was characterized by Echeverri et al. (1996). Transient transfection of cultured mammalian cells with cDNA encoding p50 suggested a role for dynactin in mitosis because a significant number of transfected cells were arrested in a prometaphase-like state and displayed an irregular spindle morphology (Echeverri et al., 1996). Biochemical analysis of lysates prepared from transfected cell cultures revealed that excess p50 disrupted the association of the p150<sup>Glued</sup> sidearm with the remainder of the dynactin complex (Echeverri et al., 1996). Further studies have shown that the overexpression of dynamitin disrupts ER-to-Golgi transport and the centrosomal localization of the Golgi apparatus (Burkhardt et al., 1997; Presley et al., 1997), a result consistent with a role for dynactin in anchoring the motor dynein to pre-Golgi or Golgi membranes (for review see Holleran and Holzbaaur, 1998).

Although most of the larger subunits of dynactin have been cloned and characterized, the molecular and biochemical properties of the smaller subunits, namely the 27- and the 22-kD polypeptides, are not known. As the characterization of individual components has provided insights into novel functions for dynactin in dynein–dynac-

tin pathway, we initiated studies to clone and characterize p22, the smallest dynactin subunit. p22 was isolated as a band on SDS-PAGE of affinity-purified dynactin (Karki and Holzbaaur, 1995; Karki et al., 1997) and microsequenced. A full-length human cDNA encoding this polypeptide was cloned and sequenced from a human cDNA library. Database comparisons of the predicted amino acid sequence suggest that p22 is a novel protein. Immunocytochemistry of cultured mammalian cells with polyclonal antibodies against p22 revealed that p22 has a prominent localization to the midbodies of dividing cells. Our subsequent analysis has confirmed the localization of dynactin as well as cytoplasmic dynein to midbodies, raising the possibility that dynein may be recruited to the cleavage furrow by dynactin, and suggesting a role for dynein and dynactin both in spindle assembly (Vaisberg et al., 1993; Gaglio et al., 1996; Merdes et al., 1996) and cytokinesis.

## Materials and Methods

### Isolation of Dynactin

Recombinant dynein intermediate chain was cross-linked to activated CH-Sepharose 4B beads at a concentration of ~2 mg ligand/ml of drained beads. To isolate dynactin, 10 ml of brain cytosol was loaded onto a 2-ml dynein affinity column, and the column was washed with 50 vol of PHEM (50 mM Na-Hepes, 50 mM Na-Pipes, 1 mM EDTA, 2 mM MgCl<sub>2</sub>). The column was eluted with PHEM containing 1 M NaCl and dialyzed against PHEM containing 0.2 mM DTT. The dialyzed sample was concentrated and resolved on a 5–20% sucrose gradient. Fractions corresponding to the 20-S peak were pooled and methanol precipitated.

### Peptide Sequencing and cDNA Cloning

Affinity-purified dynactin was methanol precipitated, resolved by SDS-PAGE, transferred to Immobilon-P, and briefly stained with Ponceau S. A polypeptide band corresponding to p22 was excised and subjected to *in situ* proteolysis and microsequencing by Dr. J. Leszyk of the Worcester Foundation for Biomedical Research. Two nonoverlapping peptide sequences were obtained: WVYGGGGAR and YLDPEYIDR.

The two peptide sequences were used as probes to search the National Center for Biotechnology Information (NCBI; Bethesda, MD) databases in a BLAST search (Altschul et al., 1990). Two overlapping expressed sequence tags (ESTs; Adams et al., 1993) were identified (clones 176551 and 121348) and were made available to us by the Washington University–Merck EST project. The two ESTs were subcloned, fully sequenced, and found to encode either one (clone 176551) or both (clone 121348) of the sequenced peptides. All cDNA sequencing was performed using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH) according to manufacturer's instructions. An EcoRI/SphI fragment (~400 bp) of clone 121348 was used as a probe to screen a human fetal brain cDNA library (catalog #936206; Stratagene, La Jolla, CA). The hybridization probe was made using the Prime-It II Random Primer Kit (Stratagene). The library was screened according to the manufacturer's protocol (Stratagene), and the phage plaques were isolated by three rounds of purification. Three separate clones were obtained; however, sequencing of the 5'-ends revealed that none of them contained the complete open reading frame. To obtain sequences more 5' to EST clone 121348, we designed a primer closer to the 5'-end region of EST 121348 (5'-GCCACCTGCACCTTGACCAG-3') and used this and a T3 primer to amplify a mature hNT neuron cDNA library (catalog #939233; Stratagene) using the ELONGASE Amplification System (Life Technologies, Rockville, MD). Major bands from the amplification were cloned into PCR2.1 vector using the TA Cloning Kit (Invitrogen, Carlsbad, CA). The DNA corresponding to each clone was subsequently analyzed by Southern blot and DNA sequence analysis.

### Northern Blot Analysis

The hybridization probe was made using a 640-bp-long EcoRI/ApaI frag-

1. Abbreviations used in this paper: Arp1, 45-kD actin-related protein; DIC, dynein intermediate chain; EST, expressed sequence tag.

ment of EST clone 121348 and the Prime-It II Random Primer Kit as described above. To determine the approximate size and tissue distribution of p22 transcript, a Multiple Tissue Northern Blot was probed with the cDNA probe using ExpressHyb hybridization solution (CLONTECH Laboratories, Palo Alto, CA) and manufacturer's protocols.

### **Production of Antibody to p22 and the Cytoplasmic Dynein Heavy Chain**

An EcoRI/NotI fragment of EST clone 121348 was subcloned into pET15b expression vector (Novagen Inc., Madison, WI) and expressed in *Escherichia coli* by IPTG induction. The inclusion bodies were purified after lysis of the bacterial pellet with lysozyme treatment and sonication and removal of the soluble fraction. The inclusion bodies were solubilized in 0.5% SDS and purified on a Ni<sup>2+</sup> affinity column according to manufacturer's protocols (Novagen Inc.). The Ni<sup>2+</sup>-purified p22 was used as an immunogen to inject guinea pig or rabbit, and the antibodies were purified on a p22 affinity column. Initially, the affinity-purified anti-p22 antibodies were not found to be specific as judged by cross-reactivity to several other polypeptides on a Western blot as well as the decoration of stress fibers in cultured mammalian cells. The p22-containing antiserum was thus subjected to adsorption by non-20-S fractions from a sucrose gradient of total rat brain cytosol before loading the serum on to a p22 affinity column followed by adsorption on F-actin preadsorbed with F-actin-binding proteins. These two additional steps completely abolished any cross-reactivity because only one band from rat brain cytosol (corresponding to p22) reacted with this antibody.

To raise rabbit polyclonal antibody against the dynein heavy chain, NheI (filled)/ClaI fragment from a pET5b containing a 2.1-kb BglII fragment of cDNA encoding *Dictyostelium* dynein heavy chain (generously provided by Dr. M. Koonce, Wadsworth Center, Albany, NY) was subcloned into pET15b expression vector (Novagen Inc.) at XhoI (filled)/ClaI site and expressed in *E. coli* by IPTG induction. The recombinant protein was purified on a Ni<sup>2+</sup> column and used as immunogen to inject rabbits. The polyclonal antidynein antibody was affinity purified on a recombinant dynein heavy chain affinity column. The monoclonal antidynein intermediate chain antibody was generously provided by Dr. W. Steffen of the University of Vienna, Austria, (Steffen et al., 1996).

### **Partial Disruption of Dynactin by Recombinant p50**

A human cDNA clone encoding full-length p50 was obtained from American Type Tissue Culture Collection (Rockville, MD; clone 85018) and subcloned into the pET15b expression vector. The p50-pET15b construct was transformed into BL21(DE3) strain of *E. coli* and grown in LB containing ampicillin. The bacterial culture was induced with 0.4 mM IPTG for 2 h. The purification of urea-solubilized inclusion bodies using Ni<sup>2+</sup> affinity column was essentially the same as described in Karki and Holzbaur (1995) for purification of dynein intermediate chain and p150<sup>Glued</sup>.

p50 purified as described above was concentrated and equilibrated in PBS. A fraction enriched in dynactin was obtained by extracting microtubules, polymerized from rat brain cytosol using 10 μM taxol, with 10 mM MgATP, as previously described (Karki and Holzbaur, 1995). The ATP extract was divided equally into two 250-μl volumes and mixed with either 200 μl of recombinant p50 (0.4 μg/μl) or 200 μl of PBS. The samples were mixed by gentle rocking at room temperature for 2 h followed by 10 min at 37°C. Samples were then loaded on a 5–20% sucrose gradient and spun at 32K rpm for 18 h (model SW41.Ti rotor; Beckman Instruments, Fullerton, CA). Fractions of ~0.9 ml were collected, of which 25 μl were loaded in each lane and analyzed by SDS-PAGE followed by Western blotting using antibodies to p150<sup>Glued</sup>, Arp1, p50, and p22. A 5-μl aliquot of ATP extract was also loaded as a positive control.

### **Affinity Chromatography**

For blocking experiments, dynein intermediate chain columns were blocked either with a 10-fold molar excess of a bacterially expressed fragment of p150<sup>Glued</sup> or with BSA before loading cytosol prepared from rat brain. The columns were extensively washed as described above and eluted with 1 M NaCl. The 1-M NaCl eluates were TCA-precipitated and analyzed by Western blot probed with rabbit polyclonal anti-p22 antibody and mouse monoclonal anti-p150<sup>Glued</sup> antibody.

To probe for a direct binding interaction between p150<sup>Glued</sup> and p22, a recombinant polypeptide corresponding to the COOH-terminal 1,286 amino acid residues of rat p150<sup>Glued</sup>, fused to an NH<sub>2</sub>-terminal histidine

tag was expressed and purified from *E. coli* as described (Waterman-Storer et al., 1995). The purified recombinant p150<sup>Glued</sup> was covalently linked to activated CH-Sepharose as described (Karki and Holzbaur, 1995); a control column was generated by coupling an equivalent amount of BSA to the matrix. p22 labeled with [<sup>35</sup>S]methionine (in a total of 200 μl reaction volume) was produced in an in vitro transcription/translation assay (Promega Corp., Madison, WI), diluted fivefold (to 1 ml) with HEM buffer (50 mM Na-Hepes, 1 mM EDTA, 2 mM MgCl<sub>2</sub>, pH 7.0), divided into two and loaded onto either the p150<sup>Glued</sup> column or the BSA column. The columns were washed extensively with HEM buffer and then eluted with 500 μl of 1 M NaCl in HEM buffer. The load and flow-through fractions (3 μl) were loaded at 1/10th the volume of the wash and eluate fractions (30 μl). The samples were fractionated by SDS-PAGE using a 12% resolving gel versus prestained molecular weight markers. The gels were dried and processed for fluorography, and the resulting autorads were scanned.

### **Cell Culture, Transient Transfections, and Immunocytochemistry**

Mammalian cells (PtK2, REF52, and Rat2 as noted) were maintained in EME or DME supplemented with 2 mM glutamine, 10% heat-treated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin and were split twice weekly using trypsin-EDTA (Life Technologies). For overexpression studies, cells were transiently transfected by the calcium phosphate precipitation method (Sambrook et al., 1989) with the construct p22-FLAG, in which the human cDNA encoding p22 was subcloned into the pFLAG-CMV-2 vector (Eastman Kodak Corp., Rochester, NY). Untransfected or transfected cells were fixed 24 h after washout using -20°C MeOH with 1 mM EGTA for 5 min. The coverslips were rinsed in PBS and blocked in 5% goat serum, 1% BSA, and 0.05% sodium azide in PBS, pH 7.4.

The fixed cells were assayed by immunofluorescence with primary antibodies as noted, either at 4°C overnight or 1 h at room temperature. Fluorescein-labeled goat anti-rabbit and anti-mouse and/or Texas red-labeled anti-mouse or anti-rat secondary antibodies were used to detect the primary antibodies. Some samples were processed for DNA visualization after the secondary antibody treatment using Hoechst 33342 (bis-benzimide; Molecular Probes, Eugene, OR) at 1 μg/ml in PBS for 15 min. Coverslips were mounted using ProLong Antifade Kit (Molecular Probes), viewed using an epifluorescence microscope (model DMRB; Leica, Deerfield, IL) fitted with a 100× objective (1.4 NA) and appropriate dichromatic filters, and photographed using a Leica photoautomat system.

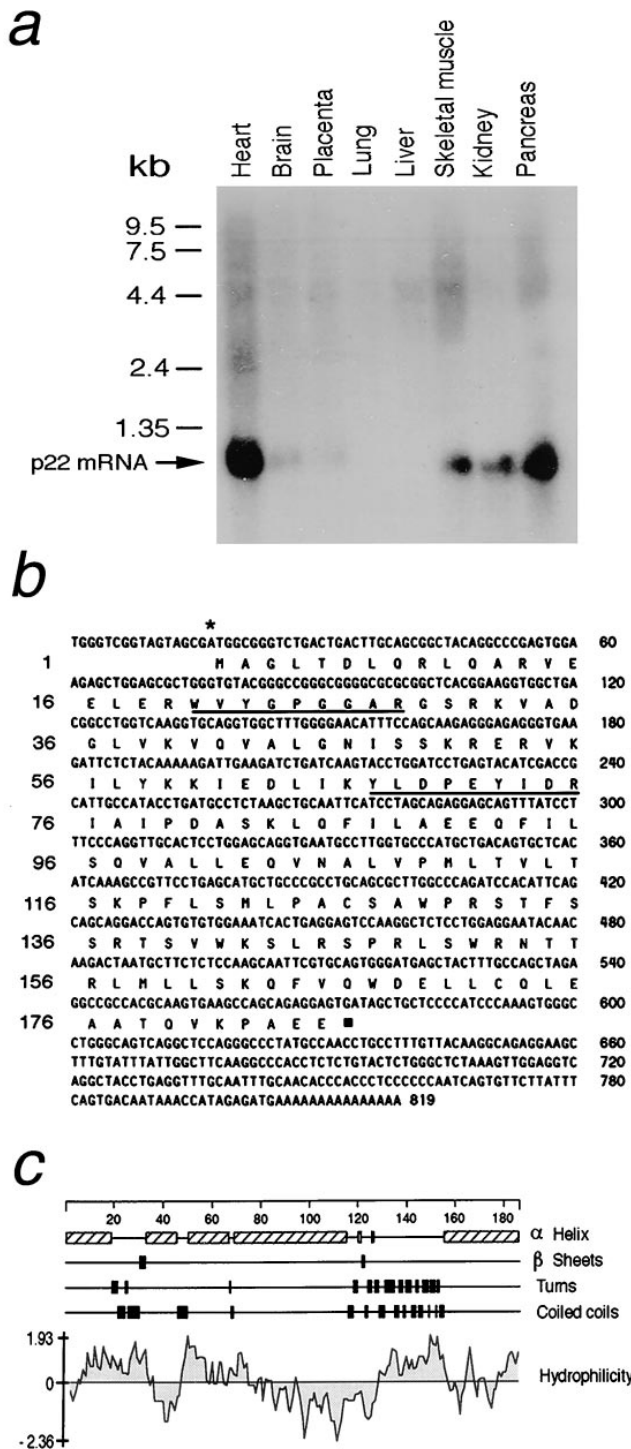
## **Results**

### **Cloning and Sequencing of the p22 cDNA**

The smallest dynactin subunit, p22, was isolated from dynactin purified by its affinity for cytoplasmic dynein (described in Karki et al., 1997). Two tryptic peptides from the p22 polypeptide were obtained. The two peptides were then used as probes in a BLAST search (Altschul et al., 1990) of the NCBI databases. Two EST clones were identified and used to isolate a full-length cDNA clone encoding p22.

A multiple tissue mRNA blot as shown in Fig. 1 *a* was probed with a cDNA encoding p22 to determine the approximate size and tissue distribution of p22-encoding mRNA. Full-length p22 mRNA is ~1 kb in length. While mRNAs encoding p22 are apparently ubiquitously expressed, expression levels are highest in muscle and pancreas, while lower levels were detected in brain. mRNAs encoding other dynactin subunits (Gill et al., 1991; Clark and Meyer, 1992; Echeverri et al., 1996) are also enriched in both cardiac and skeletal muscles, in addition to brain tissue. The significance of dynactin transcript enrichment in muscle tissues is not known.

The complete human cDNA encoding p22 is shown in Fig. 1 *b*. The predicted initiation codon is marked by an as-



**Figure 1.** Molecular characterization of p22. (a) Northern blot analysis of rat tissues. A 640-bp EcoRI/ApaI fragment of EST clone 121348 was used to probe a multiple tissue Northern blot (CLONTECH Laboratories). The position of the p22 mRNA transcript, which corresponds approximately to 1 kb, is shown. Note the higher levels of mRNA present in heart and pancreas compared with the levels in the brain. (b) A human cDNA encoding p22 was fully sequenced from both directions. The resulting DNA and the predicted amino acid sequences are shown. This clone has a complete open reading frame, the asterisk shows the predicted translational start, and the filled square marks the stop codon. The two underlined segments correspond to the two

terisk, and the termination codon is marked by a filled square. This open reading frame encodes a polypeptide of predicted molecular mass 21,034 D, slightly smaller than 22 kD, a value obtained from SDS-PAGE. While this polypeptide has been previously described as a 24-kD polypeptide in dynactin preparations from chick (Schafer et al., 1994), more thorough analysis of p22 from rat brain reveals that this polypeptide runs as a 22-kD band on 10% SDS-PAGE. Secondary structure analysis predicts that p22 is primarily an  $\alpha$ -helical protein with no significant coiled coil regions (Fig. 1 c). There is a relatively hydrophobic region between amino acids 81 and 128. BLAST and FASTA homology searches using the human p22 sequence yielded no related sequences, including any clearly identifiable homologues in the yeast database.

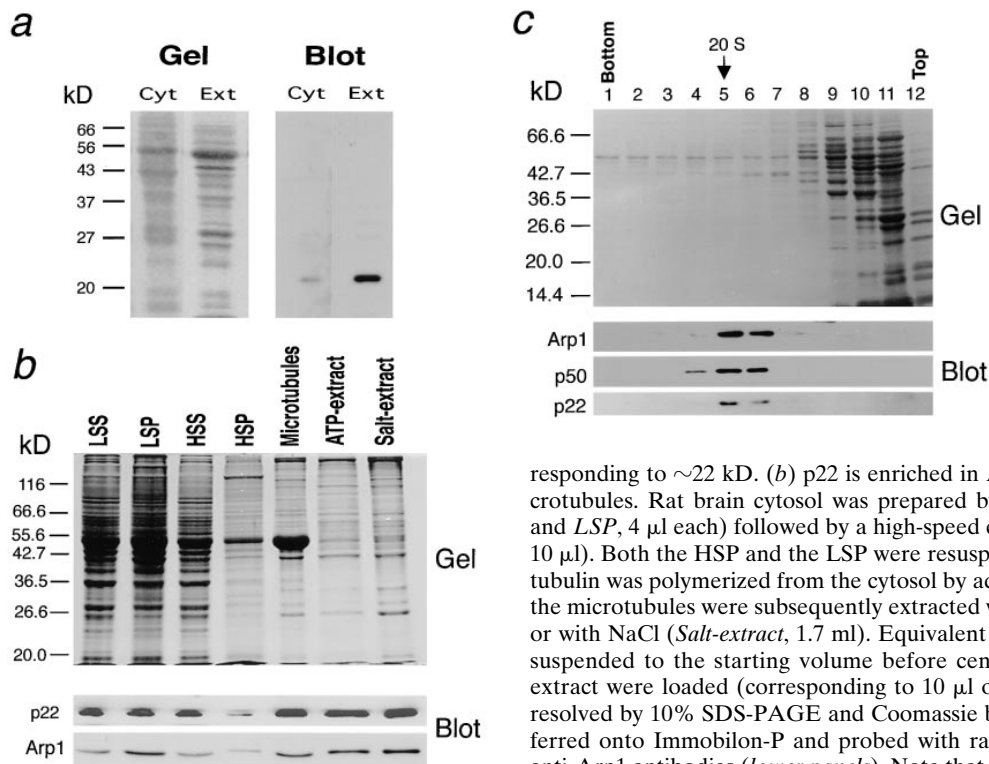
### *p22 Is Associated with Microtubules and Is Found Exclusively as a 20-S Dynactin Subunit*

We confirmed the specificity of the affinity-purified polyclonal antibodies we have raised against p22 on a Western blot of rat brain cytosol (Fig. 2 a). As judged by a highly sensitive chemiluminescence detection system, anti-p22 antibody reacted with only a single band corresponding to an  $\sim$ 22-kD polypeptide.

Since dynactin binds to microtubules and releases in the presence of salt or ATP, we tested whether p22 behaved in a similar fashion. Cytosol was prepared from rat brain by a low-speed centrifugation (Fig. 2 b, *LSS* and *LSP*) of the brain homogenate followed by a high-speed centrifugation (*HSS* and *HSP*). The high-speed supernatant was then warmed to 37°C in the presence of 20  $\mu$ M taxol to polymerize tubulin (*Microtubules*). The 20 mM Mg-ATP and 400 mM NaCl microtubule extracts (Fig. 2 b, *ATP-extract* and *Salt-extract*) were enriched in p22 as judged by Western blotting with anti-p22 antibody (Fig. 2 b, *Blot*). The blot was also reacted with antibodies to Arp1 as a positive control for dynactin. This indicates that p22, like other dynactin subunits, associates with taxol-stabilized microtubules in an ATP- and salt-dependent manner.

Not all dynactin subunits exist solely as components of the dynactin complex. The  $\alpha$  and  $\beta$  subunits of capping protein (CapZ) are found as stoichiometric subunits of dynactin, and are also found free in the cytosol and in association with the actin cytoskeleton (Schafer et al., 1994). To investigate whether p22 exists exclusively as a dynactin component or is also found free in the cytosol, rat brain cy-

nine-residue peptides that were microsequenced from the band corresponding to the 22-kD polypeptide isolated from affinity-purified dynactin. Numbers to the right reflect the positions of nucleotides, while the numbers to the left refer to the corresponding amino acid residues. (c) The predicted amino acid sequence was analyzed for secondary structure using the DNA-STAR™ sequence analysis package. The analysis reveals that p22 is primarily an  $\alpha$ -helical protein with very little predicted coiled coil. Most of the predicted turns of the protein occur between amino acid 120 and 155. The hydrophilicity plot predicts that p22 is mostly hydrophilic except for a central 45-amino acid residue stretch. These sequence data are available from GenBank/EMBL/DBJ under accession number AF082513.



**Figure 2.** (a) Specificity of the anti-p22 antibody. Rabbit polyclonal antibodies raised against human p22 were affinity purified with additional steps to ensure specificity (see Materials and Methods for details). The antibodies were then tested for specificity using total cytosol (Cyt, 5  $\mu$ l of 1:1 brain cytosol) or salt-extract of microtubules from rat brain (Ext, 20  $\mu$ l of a 2 ml extract from 10 rat brains). The Western blot on the right panel shows that the p22 antibody recognizes only one band corresponding to ~22 kD. (b) p22 is enriched in ATP- and salt-extracts of brain microtubules. Rat brain cytosol was prepared by a low-speed centrifugation (LSS and LSP, 4  $\mu$ l each) followed by a high-speed centrifugation (HSS, 4  $\mu$ l and HSP, 10  $\mu$ l). Both the HSP and the LSP were resuspended to the original volumes. The tubulin was polymerized from the cytosol by addition of taxol (Microtubules), and the microtubules were subsequently extracted with Mg-ATP (ATP-extract, 1.7 ml) or with NaCl (Salt-extract, 1.7 ml). Equivalent amounts of total microtubules (resuspended to the starting volume before centrifugation), ATP-extract, or salt-extract were loaded (corresponding to 10  $\mu$ l of ATP-extract). The samples were resolved by 10% SDS-PAGE and Coomassie blue stained (upper panel) or transferred onto Immobilon-P and probed with rabbit polyclonal anti-p22 as well as anti-Arp1 antibodies (lower panels). Note that p22, like other dynactin subunits, is associated with microtubules in an ATP- and salt-sensitive manner. (c) p22 exists

as a part of the 20-S dynactin complex. A total of 800  $\mu$ l rat brain cytosol (prepared at 1:1, wt/vol) was layered on top of a 5–20% linear sucrose density gradient and centrifuged for 18 h at 32K rpm at 4°C using a Beckman SW41.Ti rotor. 1-ml fractions were collected from the bottom, and 12  $\mu$ l of each fraction was analyzed by SDS-PAGE and Western blot using antibodies to p22, p50 (dynamitin), and Arp1 (centractin). The p22 peak corresponds to Arp1 and p50 peak, indicating that p22 exists exclusively as a component of the 20-S dynactin complex. LSS, low-speed supernatant; LSP, low-speed pellet; HSS, high-speed supernatant; HSP, high-speed pellet.

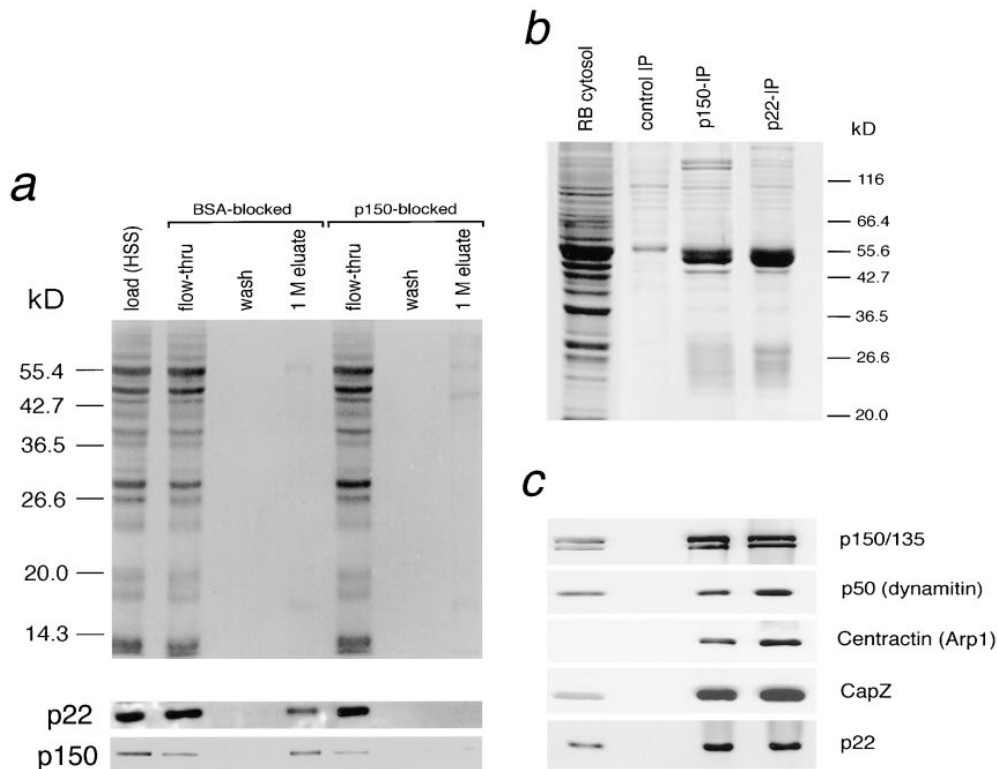
tosol was fractionated on a linear sucrose density gradient; the resulting fractions were analyzed by SDS-PAGE followed by immunoblotting with antibodies to p22, Arp1, and p50. Results in Fig. 2 c demonstrate that p22 fractionates with the 20-S dynactin peak suggesting that p22, like most other dynactin subunits, exists only as a part of the 20-S dynactin complex.

To further characterize the p22 polypeptide, we performed a column blocking experiment as well as immunoprecipitations and immunocytochemistry. We have previously demonstrated that a dynein intermediate chain affinity column retains the dynactin complex (Karki and Holzbaur, 1995) and that this binding can be specifically blocked by pretreating the column with exogenous p150<sup>Glued</sup> (Karki et al., 1997). We used this property of the dynein intermediate chain column to evaluate whether p22 behaved as a bona fide dynactin subunit. Fig. 3 a shows that p22 from brain cytosol binds to a dynein intermediate chain (DIC) column in a salt-dependent fashion as does p150<sup>Glued</sup>. This binding, however, is blocked by pretreating the column with a recombinant fragment corresponding to the NH<sub>2</sub>-terminal half of p150<sup>Glued</sup>. We have demonstrated that this blocking is specific since other DIC-binding proteins such as CKII are not blocked from binding to the affinity column by pretreatment with excess p150<sup>Glued</sup> (Karki et al., 1997).

We also performed immunoprecipitations from brain cytosol using an affinity-purified polyclonal anti-p150<sup>Glued</sup> antibody (Fig. 3, b and c, lane 3) and a polyclonal anti-p22 antibody (Fig. 3, b and c, lane 4). After extensive washing, the immunoprecipitates were resolved by SDS-PAGE, transferred onto Immobilon-P, stained with Coomassie brilliant blue (Fig. 3 b), and subsequently probed with a panel of antibodies directed against the dynactin subunits: p150<sup>Glued</sup>, p50 (dynamitin), centractin (Arp1), CapZ, and p22 (Fig. 3 c). The results show that immunoprecipitation with either anti-p150<sup>Glued</sup> or anti-p22 antibody coprecipitates the known subunits of dynactin, demonstrating that p22 is an integral component of dynactin.

### p22 Binds Directly to p150<sup>Glued</sup>

To better define the relative position of p22 in the context of the dynactin complex, we disrupted the dynactin complex using excess recombinant p50 (dynamitin). p50 has been previously shown to dissociate the p150<sup>Glued</sup> sidearm from the centractin filament at the base of dynactin when overexpressed in cultured mammalian cells (Echeverri et al., 1996). We incubated excess recombinant p50 with a sample (ATP-extract) enriched in dynactin, and then sedimented the reaction through a linear sucrose gradient. Gradient fractions were analyzed by Western blotting with



**Figure 3.** (a) p22 binding to the DIC column is blocked by p150<sup>Glued</sup>. Two identical DIC affinity columns were constructed. One column was blocked with excess p150<sup>Glued</sup> (fifth through seventh lanes), whereas the other was blocked with BSA as a control (second through fourth lanes). Rat brain cytosol (1 ml) was then loaded (first lane), and the 1 M NaCl eluates (fourth and seventh lanes) were analyzed by Western blotting. Amounts of sample loaded in load and flow through lanes are equivalent (4  $\mu$ l each), as are wash and 1 M eluate lanes (20  $\mu$ l each from a total of 50  $\mu$ l TCA precipitate). The results show that, like other dynactin subunits, p22 does not bind to a DIC column that is pre-blocked with p150<sup>Glued</sup>. (b and c) p22 antibody immunoprecipitates the dynactin complex. Immunoprecipitations were carried out using antibodies to p150<sup>Glued</sup> (1.5 ml),

p22 (0.7 ml), and beads only (control) on rat brain cytosol (1 ml each). After thorough washing with RIPA buffer, the precipitates were eluted with 100  $\mu$ l 1 $\times$  Laemmli sample buffer. 1  $\mu$ l each of control and anti-p150<sup>Glued</sup> precipitate and 2  $\mu$ l of anti-p22 precipitate were loaded and analyzed by SDS-PAGE (b) followed by Western blotting (c). A panel of dynactin subunit was used. The results demonstrate that p22 antibody coprecipitates the same subunits coprecipitated by anti-p150<sup>Glued</sup> antibody.

antibodies to dynactin subunits. In control reactions, dynactin subunits sedimented as a single peak near 20 S (Fig. 4 a). After incubation with excess p50, dynactin was partially disrupted (Fig. 4 b) as compared with the control (compare p150 in a and b). When the fractions were probed with an antibody to p22, in addition to the peak at 20 S we observed a peak in p22 immunoreactivity in fractions 9–11 (Fig. 4 b). Immunoblot analysis of these same fractions with antibodies to p150<sup>Glued</sup> and to p50 indicated that each of these polypeptides comigrated with p22 after disruption of the dynactin complex with excess recombinant dynamitin.

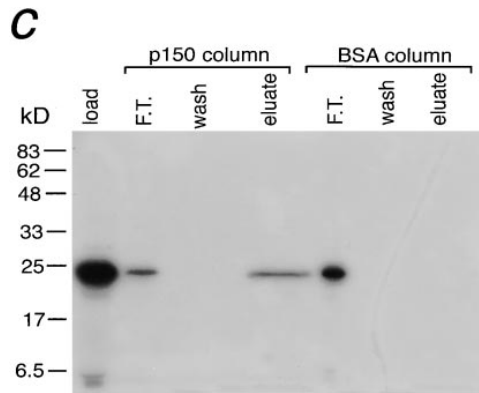
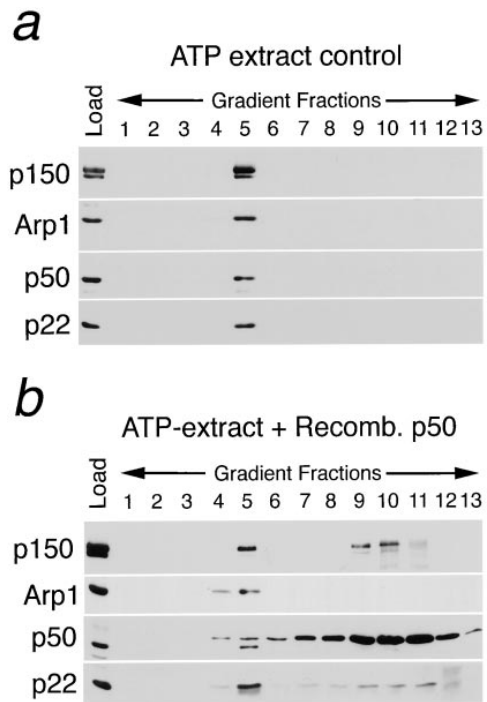
These observations suggested that p22 might bind directly to either the p150<sup>Glued</sup> or p50 subunits of dynactin. We tested for a direct interaction between p150<sup>Glued</sup> and p22 by affinity chromatography. An affinity column of recombinant p150<sup>Glued</sup> and a control BSA column were constructed and loaded with <sup>35</sup>S-labeled p22 translated in vitro. The columns were extensively washed and eluted with 1 M NaCl. The load, flow-through, wash, and eluate samples were analyzed by SDS-PAGE followed by autoradiography. The results shown in Fig. 4 c indicate that p22 was retained on the p150<sup>Glued</sup> affinity column but not on the BSA control column. These data suggest that p22 binds directly to p150<sup>Glued</sup>, as has been previously shown for Arp1 (Waterman-Storer et al., 1995). Parallel experiments were performed to probe for a direct binding inter-

action between p50 and p22 by affinity chromatography and by gel overlay; no evidence for a direct interaction between these two dynactin subunits was observed (data not shown).

### *Dynein and Dynactin Localize to the Midbodies of Dividing Cells*

Immunocytochemistry with antibodies to the dynactin subunits p150<sup>Glued</sup>, Arp1, and p50 has shown that dynactin has a prominent perinuclear distribution. Immunostaining is concentrated at the centrosome, although a more diffuse punctate distribution is found throughout the cytoplasm. Immunolocalization studies using anti-p22 antibodies to label interphase PtK2 cells also results in perinuclear, centrosomal, and punctate cytoplasmic distributions, the latter indicative of a vesicular association (Fig. 5, a and b). Furthermore, as has been previously shown for the p50 (dynamitin) subunit of dynactin, p22 also localizes to the kinetochores of dividing cells (Figs. 5, c–e, and 6). However, this localization of p22 is visible early in metaphase and is persistent until late anaphase (Fig. 6), in contrast to the staining previously described for dynamitin, which was found at the kinetochores only up to the alignment of chromosomes at the metaphase plate (Echeverri et al., 1996).

During our immunolocalization studies on PtK2 cells us-



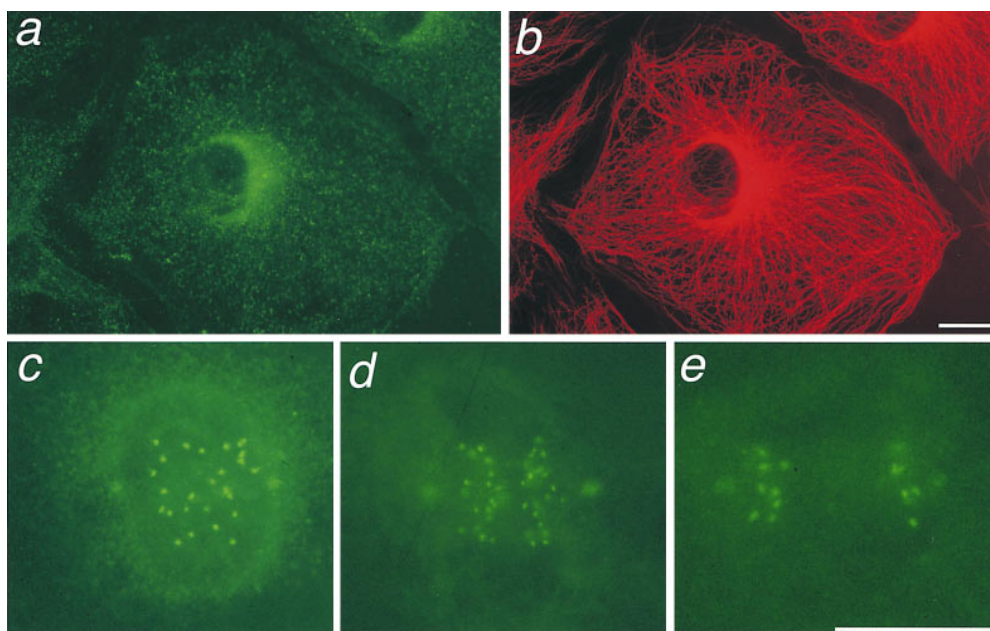
**Figure 4.** p22 binds to the p150<sup>Glued</sup> subunit of dynactin. ATP-extract (500  $\mu$ l) prepared from five rat brains was incubated with PBS alone (control) or with recombinant p50 (dynamitin) and subjected to linear density sucrose gradient (5–20%) for 18 h at 4°C in a Beckman SW41.Ti rotor at 32K rpm. Approximately 0.9-ml fractions were collected, and the fractions were analyzed by SDS-PAGE followed by Western blotting using anti-

bodies to p150<sup>Glued</sup>, Arp1, p50, and p22. In *a*, all subunits probed peak exclusively at fraction 5, corresponding to the 20-S peak. However, incubation of ATP-extract with recombinant p50 partially disrupts the dynactin complex as indicated by the presence of p150<sup>Glued</sup> at fractions 9–11 (*b*). Interestingly, p22 is also found at the second peak at fraction 10. Note that the heavy p50 staining in *b* is due to excess recombinant p50 used for dynactin disruption. (*c*) A p150<sup>Glued</sup> affinity column and a BSA control column were constructed and loaded with in vitro-translated and radio-labeled recombinant p22. The columns were extensively washed and eluted with 1 M NaCl. The loaded material (*load*), flow-through (*F.T.*), wash, and the eluate samples were analyzed by SDS-PAGE followed by autoradiography.

ing antibodies against p22, we also noted a prominent localization of p22 to the midbodies of dividing cells (Fig. 7, *e* and *f*). This was a novel localization for a dynactin subunit. The localization of p22 to the cleavage furrow appears to occur early in cytokinesis (Figs. 7, *a–d*, and 6 *q*) and is reminiscent of the localization of actin to the contractile ring. To investigate whether other dynactin subunits also localized to the midbody, we analyzed cells stained with antibodies to the p150<sup>Glued</sup> subunit of dynac-

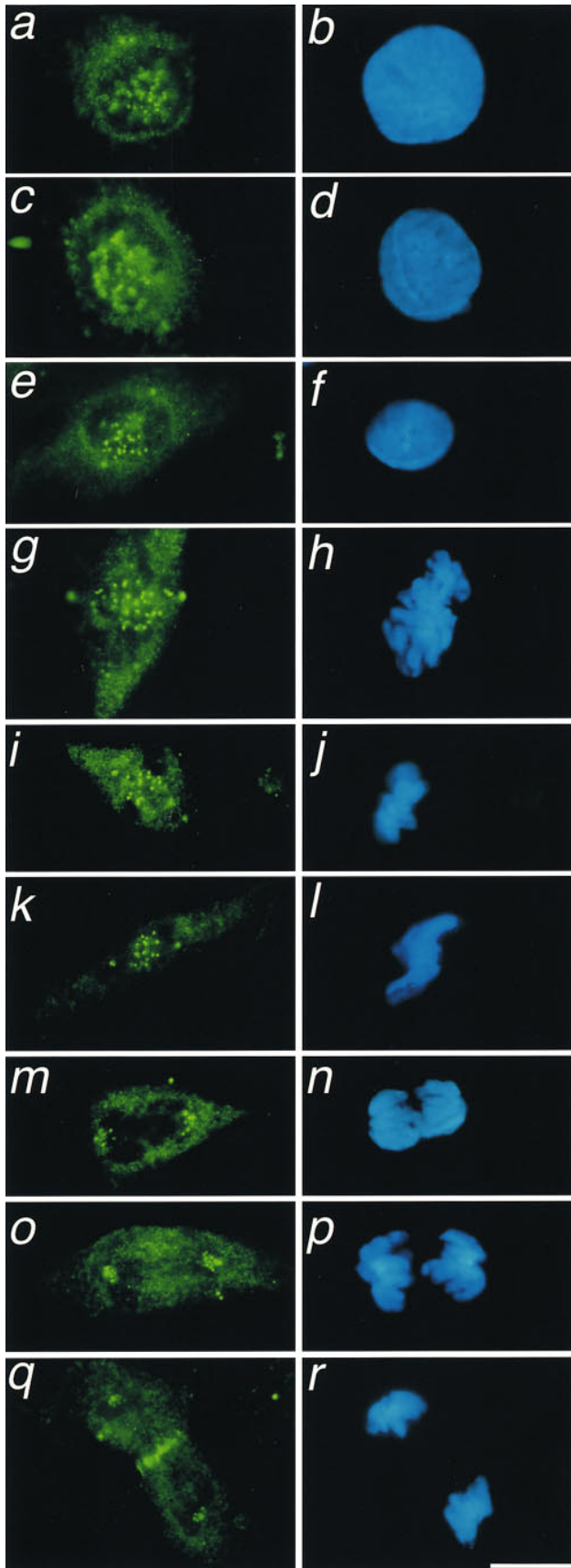
tin and found that this polypeptide also was localized to this structure (Fig. 7, *g* and *h*). Occasionally, striking images were observed in which p150<sup>Glued</sup> formed what appears to be a ring around the midbody (Fig. 7 *h*, *inset*, and *k*). The dynactin rings that form around the midbody are persistent as evidenced by their continued presence even after cell division has been completed (Fig. 7 *k*).

The presence of dynactin at the midbody prompted us to investigate whether cytoplasmic dynein is localized to



**Figure 5.** Epifluorescence micrographs of cultured Ptk2 cells stained with the p22-specific antibody UP831 (*a* and *c–e*) and antitubulin antibody (*b*). Ptk2 cells were seeded on 18 mm  $\times$  18 mm glass coverslips and grown to 75% confluency. The cells were then rapidly fixed in  $-20^{\circ}\text{C}$  100% methanol and 1 mm EGTA for 5 min and processed for immunocytochemistry. (*a*) An interphase Ptk2 cell stained with rabbit polyclonal anti-p22 antibody. (*b*) The same cell in *a* stained with anti- $\alpha$ -tubulin antibody (Sigma Chemical Co., St. Louis, MO) to reveal microtubules. Note the prominent localization of p22 at the perinuclear and centrosomal regions. Prominent

localization of p22 was also observed at the kinetochores at various stages of mitosis (*c*, prometaphase; *d*, anaphase A; *e*, late anaphase). Bar in *b* is for *a* and *b*; bar in *e* is for *c–e*. Bars, 5  $\mu$ m.



**Figure 6.** Dynamics of p22 localization through mitosis. Ptk2 cells were grown to 75% confluency and methanol fixed as in Fig. 5 and subsequently stained with anti-p22 polyclonal antibodies.

this structure as well. When polyclonal antibodies directed against dynein heavy chain were used to immunostain Ptk2 cells, we observed the colocalization of dynein at the midbody (Fig. 7, *i* and *j*). This staining was also confirmed using monoclonal antibodies directed against the dynein intermediate chain (not shown; monoclonal antibodies were a generous gift of Dr. Walter Steffen). We have also localized dynein perpendicular to the plane of the cleavage furrow of dividing Rat2 cells, consistent with an association with microtubules.

Potentially, dynactin may localize to the cleavage furrow and subsequently to the midbody of dividing cells by its association with an actomyosin-based network, a microtubule-based network, or a direct association with the membrane-rich cortex. As a first step toward discriminating between these possibilities, we performed double immunocytochemistry on Ptk2 cells using anti-p150<sup>Glued</sup> and anti-myosin II (Fig. 8, *a-c*) or anti-actin and anti-p22 (Fig. 8, *d-f*) antibodies. The results show that at the midbody where prominent localization of dynactin subunits is observed, neither myosin II nor actin was colocalized at the level of resolution of immunofluorescence microscopy. In contrast, microtubules are localized at the midbody (see Fig. 7, *e-j*), supporting the possibility that dynactin is associated with the microtubular network and not the actomyosin network. The nature of dynactin's association with the membrane cortex at the cleavage furrow and the midbody still remains to be determined. Potentially, dynactin may be associated with short actin filaments at the cellular cortex that were not resolved in these studies.

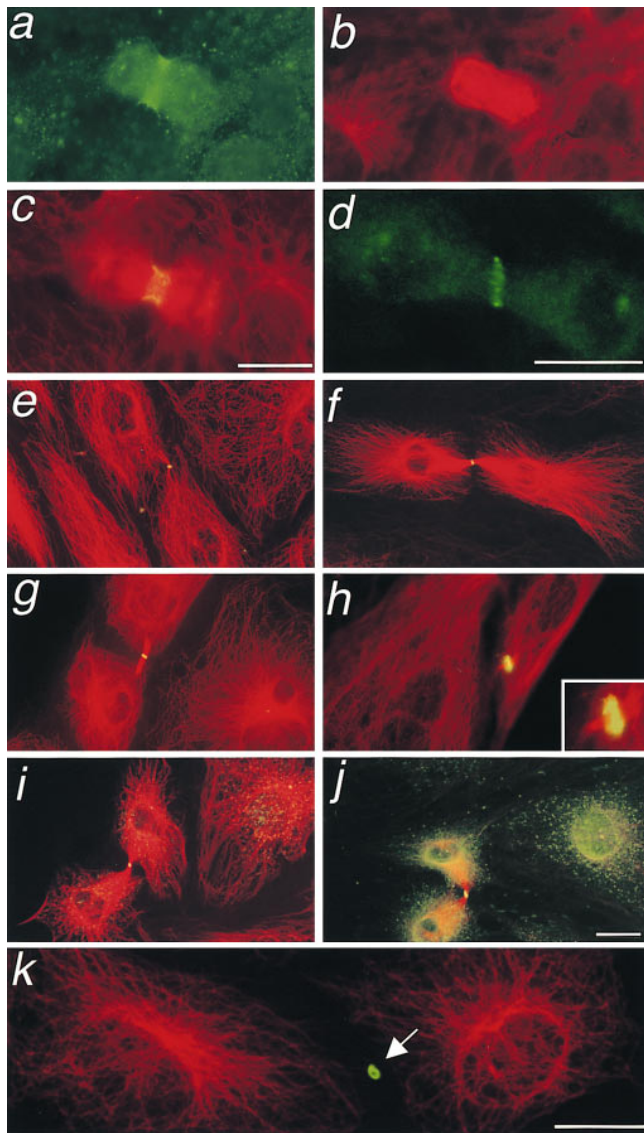
We have previously noted an interaction between dynactin and spectrin (Holleran et al., 1996). Since the cleavage furrow and the midbody are both enriched in an electron-dense vesicular matrix and since dynactin may associate with cellular membranes through spectrin (Holleran et al., 1996), we investigated whether spectrin had a similar distribution in dividing cells. However, we failed to detect a specific localization of this protein to the cleavage furrow or to the midbody.

#### **Transient Transfection Assays to Overexpress p22 in Mammalian Cells**

To investigate the intracellular role of p22, we used transient transfection assays. Mammalian Ptk2 cells were transiently transfected with a cDNA construct encoding full-length human p22 or with a construct encoding FLAG-tagged human p22 under the control of CMV promoter. After fixation, the cells were processed for immunocytochemistry using antibodies against the FLAG epitope (M5; Eastman Kodak Corp.) and/or antibodies specific for p22. p22 was distributed throughout the cytoplasm in transfected cells expressing low to intermediate

The p22 localization was visualized by FITC-conjugated anti-rabbit antibodies (*left column*). The same cells were also stained with Hoechst 33258 (bis-benzimide) to facilitate chromosome visualization (*right column*). The kinetochore localization appears immediately after nuclear envelope breakdown in prophase (*a-f*), and persists through metaphase (*g-l*), anaphase (*m-p*), and cytokinesis (*q* and *r*). Note the dramatic localization of p22 at the cleavage furrow during cytokinesis (*q*). Bar, 5  $\mu$ m.





**Figure 7.** Epifluorescence micrographs demonstrating immunolocalization of dynein and dynactin to the cleavage furrow and to midbody of dividing cells. A Rat2 cell undergoing cytokinesis is stained for p22 (*a*) and tubulin (*b*). A separate Rat2 cell visualized for both p22 and tubulin staining (*c*). A Ptk2 cell undergoing cytokinesis stained for p22 shows the enrichment of this polypeptide at the cleavage furrow (*d*). Ptk2 cells were double-stained with antitubulin and anti-p22 (*e* and *f*), anti-p150<sup>Glued</sup> (*g* and *h*), or antidynein heavy chain (*i* and *j*). Note the prominent localization of both dynein and dynactin at the midbodies revealed by yellow spots. Occasionally, striking rings of p150<sup>Glued</sup> around the midbodies were observed (*h*, inset), which seemed to be persistent even after the completion of cytokinesis (*k*, arrow). Bar in *c* is for *a*–*c*; bar in *j* is for *e*–*j*. Bars, 5  $\mu$ m.

levels of the protein (Fig. 9, *a*, *c*, and *e*). In cells expressing very high levels of p22, the protein was found in aggregates throughout the cytoplasm (data not shown). We noted that overexpression of p22 in Ptk2 cells, as well as in REF52 and Rat2 cells, caused a significant number of transfected cells to detach from the coverslips after 24 h of transfection followed by 24 h of washout, suggesting that high levels of overexpression of p22 are lethal to the cell.

However, it is possible that this lethal effect might be a nonspecific result of protein aggregation.

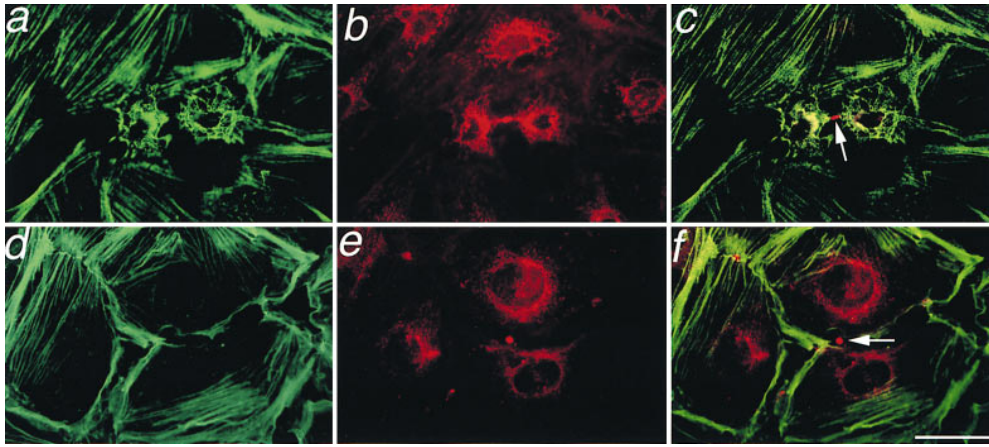
To further analyze the effects of p22 overexpression, Ptk2 cells were double immunostained with antibodies against the FLAG tag to visualize exogenous p22 as well as with antibodies to tubulin. We observed that while the microtubule organization of transfected cells with heavy punctate staining (observed at low to intermediate expression levels) was indistinguishable from those of untransfected cells, the microtubule organization in cells with large deposits of p22 appeared to be perturbed with multiple apparent foci (data not shown). However, costaining with an anti- $\gamma$ -tubulin antibody (a kind gift of H. Joshi, Emory University, Atlanta, GA) revealed that none of the p22-transfected cells displayed abnormal  $\gamma$ -tubulin localization.

We also examined the transfected cells for any perturbation in the distribution of p150<sup>Glued</sup> (Fig. 9, *a* and *b*) and cytoplasmic dynein (Fig. 9, *c* and *d*). No altered distributions were seen in transfected cells as compared with control cells. Finally, as it has been previously shown that the overexpression of the dynactin subunits centractin (Holleran et al., 1996) or dynamitin (Burkhardt et al., 1997) results in perturbations in Golgi structure, we examined transfected cells overexpressing p22 with antibodies to 58K, a resident Golgi protein, as well as with an antibody to p22. We did not observe any significant perturbation in Golgi structure induced by the overexpression of the 22-kD dynactin subunit (Fig. 9, *e* and *f*). Overexpression of dynactin has also been shown to lead to a mitotic block, and we noted that no transfected cell overexpressing p22 was observed to undergo mitosis, suggesting that p22 overexpression may interfere (directly or indirectly) with the cell cycle, very early in mitosis.

## Discussion

Dynactin, which is necessary for dynein-mediated vesicular transport, is a complex molecule consisting of at least seven polypeptides. Previous studies on the molecular characterization of the p150<sup>Glued</sup>, Arp1, and p50 (dynamitin) subunits of dynactin have provided important insights into how this complex may function within the cell. p150<sup>Glued</sup> was found to contain a microtubule-binding domain (Waterman-Storer et al., 1995) and a binding site for Arp1 (Waterman-Storer et al., 1995), as well as a cytoplasmic dynein intermediate chain-binding domain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Molecular analysis of Arp1 revealed that it is an actin-related protein (Clark and Meyer, 1992; Lees-Miller et al., 1992) that does not copolymerize with actin (Holleran et al., 1996). Overexpressed Arp1 was found to associate with spectrin, suggesting a possible linking mechanism for dynactin with membranous organelles (Holleran et al., 1996). Studies on p50 have demonstrated that disruption in dynactin function blocks mitosis (Echeverri et al., 1996) as well as ER-to-Golgi transport (Presley et al., 1997). However, little is known about the functions of other subunits of dynactin.

In this study, we isolated the previously uncharacterized 22-kD subunit of dynactin and obtained a human cDNA encoding this protein. We have characterized this polypeptide as a bona fide subunit of dynactin and have found that it is a novel protein, with no identifiable homology to pre-



**Figure 8.** p22 does not associate with actomyosin network at the midbody. Rapidly growing Ptk2 cells were methanol fixed as described previously and double immunostained for nonmuscle myosin II and for p150<sup>Glued</sup> (*a-c*) or for actin and for p22 (*d-f*). Anti-myosin II (mAb 1670; Chemicon International, Inc., Temecula, CA) and antiactin antibodies (C4; Boehringer Mannheim Corp., Indianapolis, IN) were mouse monoclonal and were visualized by FITC-conjugated anti-mouse secondaries (*a*

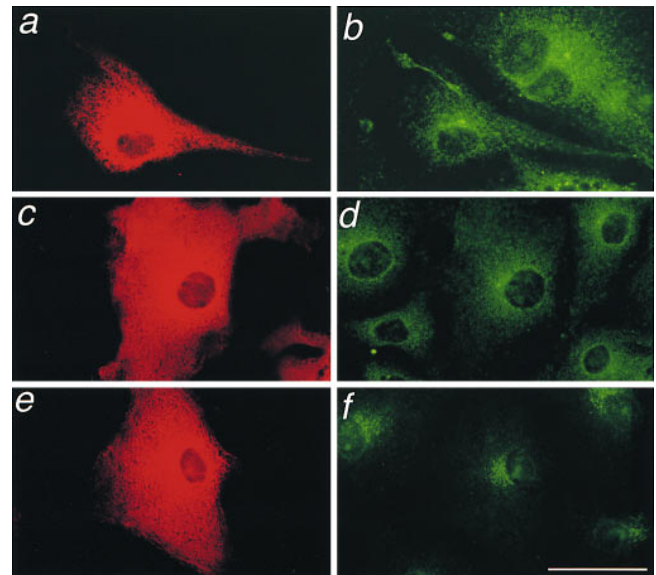
and *d*), whereas anti-p150<sup>Glued</sup> and anti-p22 antibodies were rabbit polyclonal and were visualized by Texas red-conjugated anti-rabbit secondaries (*b* and *e*). Superimposition of green and red channels shows that neither actin nor myosin II localize to the midbody where dynactin is prominently localized (*arrows, c* and *f*). Bar, 5  $\mu$ m.

viously characterized proteins or structural or functional domains. Secondary structural analysis using the DNA-STAR software package predicts that p22 is highly  $\alpha$ -helical with very little, if any, coiled coil domains. Northern blot analysis shows that the p22 transcript is enriched in muscle tissues and the pancreas as compared with brain or liver (Fig. 1 *a*). The significance of high levels of p22 transcript in these tissues is currently unknown.

We have characterized p22 as a tightly associated dynactin subunit by several different criteria. First, like other dynactin subunits, p22 is enriched in taxol-polymerized brain microtubules; the association of p22 with microtubules is sensitive to Mg-ATP and NaCl. Second, p22 exists exclusively as a part of the 20-S dynactin complex as determined by sedimentation through a linear sucrose gradient. Third, p22 from cytosol binds to a dynein intermediate chain column as a part of the dynactin complex; this binding can be specifically blocked by p150<sup>Glued</sup> pretreatment of the column. Fourth, anti-p22 antibody precipitates the same dynactin subunits that are coprecipitated by an anti-p150<sup>Glued</sup> antibody. Immunolocalization studies revealed a dense perinuclear and punctate cytoplasmic distribution for p22, consistent with the cellular localization of other dynactin subunits. Finally, we have demonstrated a direct binding interaction between p22 and p150<sup>Glued</sup>. These data characterize p22 as a tightly associated dynactin subunit, as has been previously shown for p150<sup>Glued</sup>, dynamitin, and Arp1. This behavior is in contrast to the observation that the  $\alpha$  and  $\beta$  subunits of capping protein are found as integral subunits of the dynactin complex, as well as in association with the cellular actin cytoskeleton.

More detailed immunolocalization studies revealed that p22 localizes to the cleavage furrow and the midbody of dividing cells. This localization was also seen with antibodies raised against p150<sup>Glued</sup> and cytoplasmic dynein, raising the possibility that dynactin may recruit dynein to these structures. The dynactin staining at the cleavage furrow early in cytokinesis is reminiscent of actin filaments at the contractile ring. Careful analysis of staining later in cytokinesis indicates that dynactin is associated with the cell cortex surrounding the intracellular bridge (see Fig. 7, *h* and *k*).

We have previously suggested that dynactin may associate with membrane-bound spectrin (Holleran et al., 1996). We therefore investigated whether spectrin was also present at the midbody and the cleavage furrow. Antibodies to fodrin (nonerythroid spectrin) and human spectrin did not localize spectrin to the cleavage furrow or at the midbody. It is possible that spectrin is localized to this re-



**Figure 9.** Epifluorescence micrographs of Ptk2 cells overexpressing p22. Ptk2 cells were grown to 75% confluency and transfected with cDNA encoding p22 fused to FLAG epitope at the NH<sub>2</sub> terminus. Cells were fixed in 1 mM EGTA in MeOH and processed for immunocytochemistry. The transfected cells were examined by epifluorescence microscopy using double-label immunocytochemistry with anti-FLAG and anti-p150<sup>Glued</sup> (*a* and *b*), anti-p22 and antidynein intermediate chain antibodies (*c* and *d*), and anti-p22 and anti-58K antibodies (*e* and *f*). No clear effects on the distribution of p150<sup>Glued</sup> (*a* and *b*), dynein (*c* and *d*), or the Golgi apparatus (*e* and *f*) were observed in p22-overexpressing cells. Bar, 10  $\mu$ m.

gion but is not accessible to antibodies. The midbody consists of interdigitating antiparallel microtubules surrounded by an amorphous, electron-dense matrix material (McIntosh and Landis, 1971; Mullins and Biesele, 1977). Microtubules at the midbody are inaccessible to antitubulin antibody, which results in a dark appearance when cells undergoing cytokinesis are stained with antitubulin antibody and visualized by immunofluorescence (Saxton and McIntosh, 1987; Sellito and Kuriyama, 1988). We have also noted that antibodies to the dynactin subunit Arp1 do not stain the midbody. Alternatively, the antispectrin antibodies we tested may not recognize the spectrin isoform involved, or the localization of dynein and dynactin to the cleavage furrow and later to the midbody and the persistent ring may be mediated by a mechanism independent of an association between dynactin and spectrin.

The localization of dynactin at the cleavage furrow and the midbody raises several interesting questions. First, why would dynein/dynactin be required at these structures? And second, what is the mechanism that targets dynein/dynactin there in a cell cycle-dependent manner? While the actomyosin-based contractile ring drives cytokinesis (for reviews see Schroeder, 1981; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995), it has been suggested that the interdigitating spindle microtubules (that eventually make up the microtubules of cleavage furrow and the midbody) are involved in the bipolar flow of surface receptors and in the organization of the cortical cytoskeleton to initiate the contractile ring (Fishkind et al., 1996). Cooperative interactions between the central spindle and the contractile ring have recently been observed by Giansanti et al. (1998) in their analysis of the *chickadee*, *twinstar*, *diaphanous*, and *KLP3A* mutations in *Drosophila*. Our observations that dynein and dynactin accumulate at the cleavage furrow and the midbody may suggest that dynein/dynactin activity is required for the flow of cytoskeletal elements or the interdigitation of polar microtubules, or for the apparent cross talk between the spindle and the contractile ring. Dynein/dynactin may potentially cross-link the interdigitating polar microtubules at the cleavage furrow and the midbody as both dynein and dynactin have microtubule-binding domains. Or, dynein and dynactin may link the interzonal microtubules to cortical actin at the cleavage furrow, in an interaction mediated by the actin-like filament that forms the base of the dynactin complex.

A kinesin-like protein in *Drosophila* (*KLP3A*) was observed to localize to the cleavage furrow and midbody and was suggested to produce signals to initiate cleavage furrow formation in eukaryotic cells (Williams et al., 1995). Mutations in the *KLP3A* gene were shown to disrupt the interdigitation of microtubules at the midzone of spindles, which resulted in a failure of cytokinesis (Williams et al., 1995). Besides *KLP3A*, there have been a number of reports indicating localization of other kinesin-like proteins at the cleavage furrow and/or the midbody: *Eg5* (Sawin and Mitchison, 1995), *KRP<sub>85/95</sub>* (Henson et al., 1995), *Xklp1* (Vernos et al., 1995), *CENP-E* (Brown et al., 1994; Yao et al., 1997), and *pavarotti* (Adams et al., 1998). It is of interest to note that the human homologue of *Eg5*, *HsEg5*, has recently been reported to associate with dynactin via an interaction with *p150<sup>Glued</sup>* (Blangy et al.,

1997). Together, these observations suggest that microtubule-based motor activity may be required for proper cytokinesis as well as for the mitotic events preceding cytokinesis; potentially oppositely directed microtubule motors may be required to supply counterbalancing forces at multiple stages during cell division. In the future, it will be critical to determine which dynactin subunit targets dynactin/dynein to the cleavage furrow, which cell cycle determinants may direct this localization, and what specific role or roles dynein and dynactin play in cytokinesis.

Overexpression of *p22* in transient transfection analysis indicates that a high cellular concentration of this protein is lethal, although this may be due to nonspecific effects of protein aggregation. More specifically, at moderate levels of overexpression we did not detect any perturbation induced by *p22* in the cellular distribution of *p150<sup>Glued</sup>* or cytoplasmic dynein, or in the morphology of the Golgi or the organization of the microtubule cytoskeleton. In contrast, in previous studies the overexpression of the dynactin subunits Arp1 and dynamitin have been shown to lead to the disruption of the Golgi (Holleran et al., 1996; Burkhardt et al., 1997). It is possible that high cellular levels of *p22* may induce a mitotic block because cells overexpressing this polypeptide did not undergo cell division; a mitotic block was previously observed upon overexpression of the dynamitin subunit of dynactin (Echeverri et al., 1996).

In summary, we have cloned and characterized the smallest subunit of dynactin, and studies on this subunit have revealed a novel localization for dynein/dynactin at the cleavage furrow and at the midbody. This novel localization suggests a possible new role for dynein and dynactin during cytokinesis. It will be of considerable interest to see how the microtubule-dependent motor system is involved in a cellular process where actomyosin is thought to provide the contractile force.

We would like to thank Dr. Harish Joshi and Dr. Walter Steffen for the gift of anti- $\gamma$ -tubulin and antidynein intermediate chain antibodies, respectively, and Dr. Michael Koonce for providing us the partial cDNA clone for *Dyctyostelium* dynein heavy chain. We would also like to thank Mariko K. Tokito and Elizabeth A. Holleran for their advice on transient transfection assays, immunoprecipitations, and immunocytochemistry as well as for critically reading the manuscript, and Gundula Gries for her contributions to the binding experiments as well as for her comments on the manuscript.

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