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\textsuperscript{Glued} 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

Dynactin 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 \textit{Saccharomyces cerevisiae}, \textit{Neurospora crassa}, \textit{Aspergillus nidulans}, and \textit{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\textsuperscript{Glued} 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 \textit{Drosophila} gene \textit{Glued}, and hence was named p150\textsuperscript{Glued} (Swaroop and Garen, 1987; Holzbaur et al., 1991). More recent studies...
have shown that these two polypeptides are functional homologues (McGrail et al., 1995; Waterman-Storer and Holzbaur, 1996). The original Glued (Gf) 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 Gf mutation (McGrai et al., 1995). This genetic data supports biochemical binding assays that have demonstrated a direct interaction between p150Glued and the dynein intermediate chain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). p150Glued also binds directly to microtubules via an NH2-terminal CAP-Gly motif (Waterman-Storer et al., 1995). A brain-specific 135-kD isoform of p150Glued has been described that lacks this microtubule-binding domain (Tokito et al., 1996). Human (Tokito et al., 1996), mouse (Jang et al., 1997), chicken (Gill et al., 1991), and fungal (Tinsley et al., 1996) homologues of p150Glued have also been cloned and characterized.

Another well-characterized dynactin subunit is the 45-kD actin-related protein (Arp1; Clark and Meyer, 1992; Lees-Miller et al., 1992), which is also called centrinactin because of its prominent localization to the centrosome (Clark and Meyer, 1992). Centrinactin is the most abundant polypeptide in dynactin (Paschal et al., 1993; Schafer et al., 1994), with a stoichiometry of 8–13 molecules per complex. Centrinactin 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 centrinactin 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 dynamin) 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 p150Glued sidearm with the remainder of the dynactin complex (Echeverri et al., 1996). Further studies have shown that the overexpression of dynamin 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 Holzbaur, 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–dynein 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 Holzbaur, 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 MgCl2). 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 Poncexus S. A polypeptide band corresponding to p22 was excised and subjected to in situ proteolysis and microsequencing by Dr. J. Leszysz of the Worcester Foundation for Biomedical Research. Two nonoverlapping peptide sequences were obtained: WVYGGPGAR 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 Center for Biotechnology Information (NCBI; Bethesda, MD) databases. Database comparisons of the predicted amino acid sequence for p22 revealed that the sequence of clone 121348 (~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'-GCCACCTGACCTTGACCGCAG-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 PCRII 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-
ment of EST clone 121348 and the Prime-It II Random Primer Kit as de-
scribed 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 Cytosplastic
Dynein Heavy Chain

An EcoRI/NcoI 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 af-
ter 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 Ni²⁺ affinity column according to manufac-
turer’s protocols (Novagen Inc.). The Ni²⁺-purified p22 was used as an im-
munogen to inject mice 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 antisera was thus subject-
ted to adsorption by non–20-3 fractions from a sucrose gradient of total rat brain cytosol before loading the serum on to a p22 affinity column fol-
lowed by adsorption on F-actin preadsorbed with F-actin–binding pro-
tein. These two additional steps completely abolished any cross-reactivity because only one band from rat brain cytosol (corresponding to p22) re-
acted 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 frag-
ment 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 Ni²⁺ column and used as an immunogen to inject rats. The polyclonal antidynein antibody was affinity purified on a re-
combinant dynein heavy chain affinity column. The monoclonal antidy-
nein intermediate chain antibody was generously provided by Dr. W. Stef-
fen 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 Ameri-
can Type Tissue Culture Collection (Rockville, MD; clone 58018) and subcloned into the pET15b expression vector. The p50/pET15b construct
was transformed into BL21(DE3) strain of E. coli and grown in LB con-
taining ampicillin. The bacterial culture was induced with 0.4 mM IPTG
for 2 h. The purification of urea-solubilized inclusion bodies using Ni²⁺ af-
finity column was essentially the same as described in Karki and Holzbaur
(1995) for purification of dynein intermediate chain and p50/pET15b
p50 purified as described above was concentrated and equilibrated in PBS.
A fraction enriched in dynactin was obtained by extracting microtub-
bules, 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, Full-
ton, 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 p50/pET15b, Arp1, p30, 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 p150glued or with BSA before loading cytosol prepared from rat brain. Two 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-p150glued antibody.

To probe for a direct binding interaction between p150glued and p22, a recombinant polypeptide corresponding to the COOH-terminal 1,286 amino acid residues of rat p150glued, fused to an NHE1-terminal histidine
tag was expressed and purified from E. coli as described (Waterman-
Storer et al., 1995). The purified recombinant p150glued 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 [35S]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 MgCl₂, pH 7.0), divided into two and loaded onto either the p150glued 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 frac-
tions (3 µl) were loaded at 1/10th the volume of the wash and eluate frac-
tions (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 autoradios were scanned.

Cell Culture, Transient Transfections, and Immunocytochemistry

Mammalian cells (PK2, 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 overexpres-
sion 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 pCMV-2 vector (Eastern Kodak Corp., Rochester, NY). Un-
transfected or transfected cells were fixed 24 h after washout using ~3°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.

The fixed cells were assayed by immunofluorescence with primary anti-
bodies as noted, either at 4°C overnight or 1 h at room temperature. Fluoro-
rescein-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 af-
ter 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 photomation system.

Results

Cloning and Sequencing of the p22 cDNA

The smallest dynactin subunit, p22, was isolated from dyna-
tin purified by its affinity for cytoplasmic dynein (de-
scribed 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 iden-
tified and used to isolate a full-length cDNA clone encoding p22.

A multiple tissue mRNA blot as shown in Fig. 1a 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 ex-
pressed, 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. 1b. The predicted initiation codon is marked by an as-

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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 mM 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-

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**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 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/DDBJ 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 μl of 1:1 brain cytosol) or salt-extract from rat brain (Ext, 20 μl 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 μl each) followed by a high-speed centrifugation (HSS, 4 μl and HSP, 10 μl). Both the HSP and the LSP were resuspended to the original volumes. The tubulin was polymerized from the cytosol by addition of taxol (Microtubes), 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 μl of ATP-extract). The samples were resolved by 10% SDS-PAGE and Coomassie blue stained (upper panel) or transferred onto Immobilon-P, stained with Coomassie brilliant blue (Fig. 3b), and subsequently probed with a panel of antibodies directed against the dynactin subunits: p150Gal, 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 dynein complex. LSS, low-speed supernatant; LSP, low-speed pellet; HSS, high-speed supernatant; HSP, high-speed pellet.

Localization of Dynein/Dynactin to the Midbody

We also performed immunoprecipitations from brain cytosol using an affinity-purified polyclonal anti-p150Gal 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. 3b), and subsequently probed with a panel of antibodies directed against the dynactin subunits: p150Gal, p50 (dynamitin), centractin (Arp1), CapZ, and p22 (Fig. 3c). The results show that immunoprecipitation with either anti-p150Gal or anti-p22 antibody coprecipitates the known subunits of dynactin, demonstrating that p22 is an integral component of dynactin.

p22 Binds Directly to p150Gal

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 p150Gal 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

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. 2c 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 the exogenous p150Gal (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. 3a shows that p22 from brain cytosol binds to a dynein intermediate chain (DIC) column in a salt-dependent fashion as does p150Gal. This binding, however, is blocked by pretreating the column with a recombinant fragment corresponding to the NH2-terminal half of p150Gal. 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 p150Gal (Karki et al., 1997).
antibodies to dynactin subunits. In control reactions, dynactin subunits sedimented as a single peak near 20 S (Fig. 4a). After incubation with excess p50, dynactin was partially disrupted (Fig. 4b) as compared with the control (compare p150 in a and b). When the fractions were pruned 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. 4b). Immunoblot analysis of these same fractions with antibodies to p150 Glued 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 Glued or p50 subunits of dynactin. We tested for a direct interaction between p150 Glued and p22 by affinity chromatography. An affinity column of recombinant p150 Glued and a control BSA column were constructed and loaded with 35S-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. 4c indicate that p22 was retained on the p150 Glued affinity column but not on the BSA control column. These data suggest that p22 binds directly to p150 Glued, as has been previously shown for Arp1 (Waterman-Storer et al., 1995). Parallel experiments were performed to probe for a direct binding interaction 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 Glued, 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).
Using 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 g) 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 p150Glued subunit of dynactin and found that this polypeptide also was localized to the midbody (Fig. 7, g and h). Occasionally, striking images were observed in which p150 Glued 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 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. 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 μm.
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\textsubscript{Glued} (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 (Hollerman 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\textsubscript{Glued}, Arp1, and p50 (dynamitin) subunits of dynactin have provided important insights into how this complex may function within the cell. p150\textsubscript{Glued} 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 (Hollerman et al., 1996). Overexpressed Arp1 was found to associate with spectrin, suggesting a possible linking mechanism for dynactin with membranous organelles (Hollerman 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-
viously characterized proteins or structural or functional domains. Secondary structural analysis using the DNA-STABLE software package predicts that p22 is highly α-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. 1a). 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 p150Glued pretreatment of the column. Fourth, anti-p22 antibody precipitates the same dynactin subunits that are coprecipitated by an anti-p150Glued 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 p150Glued. These data characterize p22 as a tightly associated dynactin subunit, as has been previously shown for p150Glued, dynamitin, and Arp1. This behavior is in contrast to the observation that the α and β 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 p150Glued 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-

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**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 p150Glued (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-p150Glued 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 μm.

**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 NH2 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-p150Glued (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 p150Glued (a and b), dynein (c and d), or the Golgi apparatus (e and f) were observed in p22-overexpressing cells. Bar, 10 μm.
region 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; Sellitto 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, tвинstar, diaphанous, 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 ports indicating localization of other kinesin-like proteins 1995). Besides KLP3A, there have been a number of re-

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