Cloning and Characterization of Dynamitin, the 50 kDa Subunit of Dynactin: A Study of Dynactin and Cytoplasmic Dynein Function in Vertebrates

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CLONING AND CHARACTERIZATION OF DYNAMITIN, THE 50 kDa SUBUNIT OF DYNACTIN: A STUDY OF DYNACTIN AND CYTOPLASMIC DYNEIN FUNCTION IN VERTEBRATES

A Dissertation Presented

By

Christophe de Jésus Echeverri

Submitted to the Faculty of the
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Chromosome Alignment and Spindle Organization during Mitosis.

Dynamitin (p50) Subunit of the Dynactin Complex Disrupts Dynein-Dependent
CLONING AND CHARACTERIZATION OF DYNAMITIN, 
THE 50 kDa SUBUNIT OF DYNACTIN: 
A STUDY OF DYNACTIN AND CYTOPLASMIC DYNEIN 
FUNCTION IN VERTEBRATES

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January 1998
Pour Mamie et Dominique,
(Ne t’en fais pas Mamie...) 

"Qui serait assez insensé pour mourir sans avoir fait au moins le tour de sa prison?"

-Marguerite Yourcenar
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-Chris/Putuf/Tonton Christophe/Sarraud/SDR.
ABSTRACT

Dynactin is a multi-subunit complex which was initially identified in 1991 as an activator of cytoplasmic dynein-driven microtubule-based organelle motility in vitro. Although genetic studies also supported the involvement of both complexes in the same functional pathways in yeast, filamentous fungi, and Drosophila, none of these findings yielded significant insights into dynactin’s mechanism of action. The full range of cytoplasmic dynein functions in vertebrate cells has also remained poorly understood, due, in large part, to the lack of a specific method of inhibition. The present thesis work was designed to investigate these issues through a study of the 50 kDa subunit of dynactin.

As a first step (Chapter 1), I cloned mammalian p50 and characterized its expression at the tissue and subcellular levels. Rat and human cDNA clones revealed p50 to be a novel α-helix-rich protein containing several highly-conserved structural features including one predicted coiled-coil domain. Immunofluorescence staining of p50, as well as other dynactin and cytoplasmic dynein components in cultured vertebrate cells showed that both complexes are recruited to kinetochores during prometaphase and concentrate near spindle poles thereafter. These findings represented the first evidence for dynactin and cytoplasmic dynein co-localization within cells, and for the presence of dynactin at kinetochores.

The second major phase of the thesis (Chapter 2) was focused on investigating dynactin and cytoplasmic dynein function in cultured cells in vivo using a dominant negative inhibition approach based on transient transfections of p50 constructs. Overexpression of wild type human p50 in cultured cells resulted in a dramatic fragmentation and dispersal of the Golgi apparatus. Time-lapse fluorescence microscopy analysis of p50-overexpressing cells revealed that microtubule-based vesicle transport from the

vi
endoplasmic reticulum to the Golgi was inhibited. Also, the interphase microtubule organizing center was found to be less well-focused in some but not all transfected cells. Overexpression of p50 also disrupted mitosis, causing cells to accumulate in a prometaphase-like state. Chromosomes were condensed but unaligned, and spindles, while still generally bipolar, were dramatically distorted. Sedimentation analysis revealed the dynactin complex to be dissociated in the transfected cultures. Furthermore, both dynactin and cytoplasmic dynein staining at prometaphase kinetochores was markedly diminished in cells expressing high levels of p50. These findings provided the first \textit{in vivo} evidence for the role of dynactin in cytoplasmic dynein function, i.e. mediating the motor’s binding to at least one “cargo” organelle, the kinetochore, and probably also to others such as vesicles destined for the Golgi complex. These data also strongly implicated both dynactin and dynein in Golgi organization during interphase, and chromosome alignment and spindle organization during mitosis. Based on the remarkable disruptive phenotypic effects associated with overexpressing of p50, the name of dynamtin was proposed for this polypeptide.

In the third and last phase of the thesis (Chapter 3), two issues were addressed: first, the dynamtin-induced mitotic arrest phenotype was studied in greater detail to better understand the exact sites of dynactin and cytoplasmic dynein activity throughout mitosis. Second, a domain analysis of dynamtin was performed to gain insight into its function within the dynamtin complex. A time-lapse fluorescence microscopy study of mitosis in living dynamtin-overexpressing COS-7 cells strongly suggested specific defects in interactions of astral microtubules with the cell cortex, and in both spindle pole assembly and maintenance. Analysis of the mitotic arrest phenotype in a second cell line revealed a second arrest point at metaphase, and a clear effect of dynamtin overexpression on spindle axis orientation, again consistent with defects in interactions between microtubules and the cell cortex. Refined analyses of kinetochore and spindle pole components also confirmed specific defects in kinetochore function and spindle pole organization. Taken together, these findings support three main sites of dynactin and cytoplasmic dynein activity during vertebrate mitosis: prometaphase kinetochores, spindle poles, and the cell cortex. Finally, the domain analysis revealed dynamtin to be capable of self-association through at least two separate
interaction domains, consistent with models of the mechanism underlying dynamitin-induced dynactin dissociation, and therefore, yielding important new insights into dynactin assembly. This study also indicated that a third region within dynamitin, residues 105 to 154, is essential for dynamitin and dynactin function. An independent study confirmed this finding, implicating this region in binding to ZW10, an upstream kinetochore protein. Dynamitin has therefore been revealed to be the kinetochore-targeting subunit of dynactin, and indirectly, cytoplasmic dynein. Through the body of this thesis work, dynamitin has also emerged as a powerful new tool for studying vertebrate dynactin and cytoplasmic dynein function in vivo and in vitro.
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GENERAL INTRODUCTION: 
BRIEF HISTORICAL OVERVIEW

Early hints as to the presence of an elaborate skeletal system organizing the cytoplasm of eukaryotic cells were noted at the turn of the century, through observations of the spindle apparatus of dividing cells (Wilson, 1911). It wasn’t until the development of electron microscopy techniques during the 1950’s and early 60’s, however, that researchers were at last afforded their first direct glimpses at just how complex this cytoskeleton was. The same spindle fibers detected for more than sixty years were identified as tubular structures ubiquitously found throughout the cytoplasm of eukaryotic cells, and named "microtubules" (Porter, 1955; Slatterback, 1963). With the subsequent identification of the tubulin α/β heterodimer as their primary building block (Borisy and Taylor, 1967a; Borisy and Taylor, 1967b), the next three decades saw an explosion of new research in this area. During that time, microtubules have emerged as key players in many basic cellular processes including cell division, morphogenesis, intracellular organelle transport, and cell motility. The capacity of microtubules to effect such a wide range of functions derives in part from their inherent physical and chemical properties. Most notable among these are the polar structure, defined by the tandem head-to-tail organization of the α/β dimers to form the parallel protofilaments - 13 in most cases - which make up the tube, and the dynamic growth and shrinkage behavior of the two ends of the polymer (Mitchison and Kirschner, 1984). Of course, most of these functions have also been found to require other associated proteins.

The first microtubule-associated proteins, or MAPs, were identified biochemically in the mid-70’s by virtue of their ability to co-purify with brain tubulin through multiple cycles of microtubule assembly and disassembly (Borisy, et al., 1975; Sloboda, et al., 1975). Two broad
classes of MAPs have since emerged (Holzbaur and Vallee, 1994; Schoenfeld and Obar, 1994; Moore and Endow, 1996). The structural MAPs, also known as fibrous MAPs, bind to microtubules in a static, nucleotide-independent manner, and have been shown to alter microtubule dynamics, most often through stabilization of the polymer (MAP1, MAP2, tau: Sloboda, et al., 1976, reviewed by Schoenfeld and Obar, 1994). Proposed functions for structural MAPs have also included the regulation of inter-microtubule spacing, and mediating interactions between microtubules and other cytoskeletal proteins or organelles (e.g. CLIP170, Pierre, et al., 1992).

The second class of MAPs is characterized by nucleotide-dependent microtubule binding and the ability to hydrolyze ATP and convert this chemical energy into mechanical force, thus permitting translocation along the microtubule lattice. Axonemal dynein was the first such “motor MAP” to be identified (Gibbons and Rowe, 1965). As the protein responsible for the sliding of axonemal microtubules along one another, dynein was shown to provide the force which underlies ciliary and flagellar movements. However, axonemal dynein could not account for the numerous cytoplasmic motility events which were observed in many systems - particularly evident during mitosis and axonal transport - and it would take another 20 years for researchers to identify the cytoplasmic motor MAPs responsible.

During the characterization of high-molecular weight MAPs from brain, one of these, originally known as MAP1C, was identified as the long-sought cytoplasmic form of dynein (Paschal, et al., 1987; Vallee, et al., 1988). Through in vitro motility assays, cytoplasmic dynein was shown to move towards the minus end of microtubules, in contrast to the other cytoplasmic motor MAP, the much smaller plus end-directed kinesin, also found in the mid-80's (Vale, et al., 1985). Since then, a plethora of kinesin-like proteins have emerged, defining a new superfamily which includes both plus- and minus-end directed motors (Moore and Endow, 1996). Both motors are composed of two large heavy chains (~110-140 kDa for kinesin, ~530 kDa for dynein) containing the ATPase and microtubule-binding activities, associated with either a pair of light
chains for conventional kinesin, or a more complex assembly of intermediate (~74 kDa), light intermediate (53-59 kDa) and light (~8-20 kDa) chains for dynein (reviewed in Holzbaur, et al., 1995). Electron microscopy studies have revealed both motors to appear as two-headed molecules, joined by a long fibrous tail for kinesin, and a short “stem” ending in a dense base region for dynein (Vallee, et al., 1988; see Chapter 1, Fig. 1). These studies also revealed the imposing size of cytoplasmic dynein (~1.2 MDa; Vallee, et al., 1988), another feature which distinguishes it from its much smaller anterograde counterpart.

While kinesin-like proteins exhibit variations in the domain structure of the heavy chain, as well as differences in associated light chains, oligomerization properties and even direction of motility (Hoyt, 1994; Moore and Endow, 1996), the dynein heavy chain family, on the other hand, has shown much less diversity. Although three cytoplasmic dynein heavy chain genes have been proposed in vertebrates to date (Vaisberg, et al., 1996), only one of these appears to be expressed ubiquitously at high levels, and its product appears to be the only one which associates with intermediate chains to form the complete complex (Vaisberg, et al., 1997). These intermediate chains, found at the base of the motor (Steffen, et al., 1996; Chapter 1, Fig. 1), have long been thought to mediate the interaction of cytoplasmic dynein with cellular cargo structures, consistent with the axonemal homologue’s role in anchoring that motor within the flagellum (King, et al., 1991; King and Witman, 1990). Little else has been found, however, to elucidate the functions of this and other dynein chains, and, therefore, much work has focused since the late 1980’s on the molecular cloning of the genes involved, and on developing new functional assays to address these issues in vitro.

The use of functional assays, combined with new biochemical purification procedures, also permitted the testing of whether or not purified motors alone were fully capable of mediating organelle motility. Results from in vitro motility assays indicated that while mono Q-purified
cytoplasmic dynein was capable of supporting microtubule gliding on coverslips, accessory cytosolic factors were required to permit *in vitro* motility of organelles on purified microtubules (Schroer and Sheetz, 1991). One such factor was identified as a large complex (~1.1 MDa) of approximately 9 subunits, and termed dynactin (Gill, *et al*., 1991), for *dynein-activating* complex.

In late 1992, when the present thesis work was initiated, the study of dynactin was a nascent field which held the promise of yielding important new insights into cytoplasmic dynein function. To date, the field of dynein research had been hampered by the lack of an efficient, specific method for inhibiting the motor's activity in living vertebrate systems, and the full range of its functions, therefore, remained largely speculative. Since early on, several groups had used UV-vanadate photocleavage of the dynein heavy chains (Gibbons, *et al*., 1978; Gibbons, *et al*., 1987) but this reaction cannot be performed in intact cells, and specificity remained difficult to prove. Several anti-dynein antibodies have also been produced and injected into cells to attempt inhibition studies more recently. Although some provocative results were reported, these have been subject to similar criticism, i.e., worries about specificity of the effects, especially considering the high concentrations of antibodies used (e.g. Vaisberg, *et al*., 1993).

The goals of this thesis were therefore defined in this context. I set out to advance the characterization of the dynactin complex by cloning one of its major subunits, the 50 kDa component (Chapter 1). Then, by targeting this subunit using transfection-based methods in cultured cell systems to achieve dominant negative inhibition phenotypes (Chapter 2, 3), I sought to gain new insights into dynactin and cytoplasmic dynein function in vertebrates cells *in vivo*. In the process, this work has defined a new, powerful tool to inhibit dynactin function *in vivo* and *in vitro* (Chapter 2), and has implicated dynactin in all forms of dynein-based motility tested to date. This has resulted in the first direct evidence regarding the specific role played by dynactin in dynein-based motility, i.e. to mediate the interaction of the motor with its cargo structures (Chapter 2).
Furthermore, this work has also yielded important advances in our understanding of the roles of dynactin and cytoplasmic dynein in Golgi organization and cell division, identifying three major sites of activity of the two complexes in mitotic cells: prometaphase kinetochores, spindle poles and the cell cortex (Chapter 2, 3). Finally, domain analysis of the 50 kDa subunit (Chapter 3), combined with findings from an independent study, give the first clues regarding this subunit's own function within the dynactin complex: to mediate the interaction of dynactin - and, indirectly, cytoplasmic dynein - with at least one cargo structure, the kinetochore, and possibly all other structures as well.
CHAPTER 1

CLONING OF DYNAMITIN, THE 50 kDa SUBUNIT OF DYNACTIN, AND CHARACTERIZATION OF EXPRESSION AT TISSUE AND SUBCELLULAR LEVELS

Introduction

Dynactin was initially identified as a cytosolic complex which stimulated cytoplasmic dynein-based organelle motility in vitro (Gill, et al., 1991; Schroer and Sheetz, 1991). Biochemical purification from chicken embryos (Gill, et al., 1991; Schafer, et al., 1994) and immunoprecipitation from bovine brain (Paschal, et al., 1993) revealed dynactin to contain polypeptides of 160, 150, 62, 50, 45, 36, 32, 27, and 24 kDa, with approximate stoichiometries of 1, 1, 1, 4, 9, 1, 1, 1, 1, respectively (Fig. 1). The major 45 kDa component was cloned and identified as the actin-related protein Arp1 (Clark and Meyer, 1992; Lees-Miller, et al., 1992; Paschal, et al., 1993), which assembles into a 37 nm-long F-actin-like filament to form the structural core of the complex (Schafer, et al., 1994)(Fig. 1). This filament was found to be capped at one end, the barbed end, by a capping protein heterodimer corresponding to the 32 and 36 kDa subunits of the complex (Schafer, et al., 1994). A monoclonal antibody specific for the 62 kDa polypeptide was found to decorate the other end of the Arp1 filament (Schafer, et al., 1994). The 150-160 kDa dimer was identified as p150Glued, the vertebrate homologue of the Drosophila Glued gene product (Holzbaur, et al., 1991; Swaroop, et al., 1987). Mutations in this protein have long been known to cause pleiotropic effects during early development of heterozygous flies, including severe eye and CNS axonal guidance defects (Harte and Kanel, 1982; Plough and Ives, 1935). The p150Glued dimer forms a thin, 10 nm-long “arm” domain extending laterally outward at varying angles from a variable, shoulder-like region along the wall of the Arp1 filament.
Figure 1: Structure and subunit composition of cytoplasmic dynein and dynactin.

Cytoplasmic Dynein

- Heavy Chains (HCs)
- Intermediate Chains (ICs)
- Light Intermed. Chains (LICs)
- Light Chains (LCs)

Dynactin Complex

- p150\textsuperscript{GLUED}
- p62
- p50 (Dynamitin)
- Arp1
- Capping protein
- p27
- p24
This arm domain ends distally with a pair of globular heads which contain the microtubule-binding domain of p150Glued (Waterman-Storer, et al., 1995). The location of the remaining subunits, including the second most abundant 50kDa protein, was not determined in these early studies.

Early studies of dynactin subunits showed a ubiquitous distribution in all major tissues throughout development, consistent with a role in basic cellular processes such as organelle transport and mitosis. Characterization by northern and western blotting revealed detectable p150Glued expression in all chicken tissues, including embryonic chick brains (Gill, et al., 1991). Interestingly, additional brain-specific isoforms were detected both at the mRNA and protein levels (Gill, et al., 1991), as seen also for cytoplasmic dynein intermediate chains (ICs) (Vaughan and Vallee, 1995). Three distinct isoforms of Arp1 have also been reported, only two of which, originally named α and β-centractin, are present in dynactin at a constant ratio of 15:1, and are ubiquitously-expressed in all major human tissues (Clark and Meyer, 1992; Clark, et al., 1994).

At the subcellular level, Arp1 was initially localized to the centrosome in cultured cells (thus, the original name of centractin, Clark and Meyer, 1992). In addition to this pattern, p150Glued was also found to exhibit a dimer pattern of detergent-soluble fine punctate staining throughout the cytoplasm and was absent from the nucleus (Gill, et al., 1991; Paschal, et al., 1993). Although the generalized subcellular distribution of the Glued gene product was found to mirror that of cytoplasmic dynein throughout oogenesis in Drosophila (McGrail, et al., 1995), co-localization of the two complexes on specific sub-cellular structures was not reported beyond the similarity in dense fine dispersed cytoplasmic staining seen in both cases. Cytoplasmic dynein itself had been localized by some (Pfarr, et al., 1990; Steuer, et al., 1990), but not all (Lin and Collins, 1992), to kinetochores in prometaphase cells. In contrast, anti-p150Glued staining was reported to be absent from kinetochores (Gill, et al., 1991). Thus, while the subcellular distribution of dynactin was initially expected to shed new light on its functions, these early results
caused some confusion in the field, as it was unclear how dynactin and cytoplasmic dynein could participate in the same pathways without showing clear co-localization within cells.

In the first part of this thesis work, I set out to advance the molecular characterization of dynactin by cloning and characterizing its 50 kDa component. Then, using a monoclonal antibody to this subunit (Paschal, et al., 1993), I characterized its expression levels in adult rat tissues, and its localization patterns throughout the cell cycle in cultured cells. Using a battery of antibodies to dynactin and cytoplasmic dynein subunits, I was able to show the first clear evidence of co-localization of both complexes on a specific subcellular structure, i.e. on prometaphase kinetochores.
Materials and Methods

Peptide sequencing

The dynactin complex was immunoprecipitated out of calf brain cytosol as previously described (Paschal, et al., 1993), separated by SDS-PAGE (9% minigel, Bio Rad Laboratories, Richmond, CA), and electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). After Ponceau S staining and destaining, the p50 band was excised, subjected to in situ trypsin digestion, and the eluted peptides were separated by C18 reverse-phase HPLC as previously described (Abersold, 1989). N-terminal amino acid sequence from six peptides was determined on an Applied Biosystems 477A Sequenator (Applied Biosystems, Foster City, CA), with a 120A phenylthiohydantoin analyzer by Dr. J. Leszyk of the Worcester Foundation for Biomedical Research Protein Chemistry Facility (Shrewsbury, MA).

Cloning of p50

The peptide #21 sequence was used to design an inosine-containing, partially degenerate oligonucleotide probe, composed of equal amounts of two oligonucleotides: (5'-AAT-GA[G,A]-CCI-GAT-GTI-TAT-GA[G,A]-ACI-AG[C,T]-GA[C,T]-TT[G,A]-CC[G,T,A]-GA-3' and 5'-AAT-GA[G,A]-CCI-GAT-GTI-TAT-GA[G,A]-ACI-TCI-GA[C,T]-CTI-CC[G,T,A]-GA-3'). This probe was [32P] end-labelled, and used for hybridization screening of a bovine brain λgt10 cDNA library (#BL1027a, Clontech Laboratories, Palo Alto, CA), in tetramethylammonium chloride (TMAC) solution (Jacobs, et al., 1988) containing 3 M TMAC, 0.1 M NaPO₄ (pH 6.8), 1 mM EDTA (pH 8.0), 5x Denhardt's solution, 0.6% SDS, 100 µg/ml denatured salmon sperm DNA, at 53°C for 18 hrs. Filters were then washed in 3 M TMAC 50 mM Tris-HCl (pH 8.0), 0.2% SDS at room temperature for 15 min, followed by 53°C for 1 hr. The TMAC was removed from the filters by three 10 min washes in 2x SSC, 0.1% SDS at room temperature. Bacteriophage from one positive plaque (B14A) were picked, purified by two more rounds of screening, and the insert DNA was sequenced (Sequenase version 2.0, US Biochemical, Cleveland, OH).
The B14A insert was EcoRI-excised, labelled with α[^32]P]-dCTP by random-priming (Boehringer Mannheim Biochemicals, Indianapolis, IN), and used for hybridization screening of a rat brain λZAPII cDNA library (#936515, Stratagene, La Jolla, CA). Hybridization was performed overnight at 65°C, in Rapid-Hyb solution (Amersham Corp., Arlington Heights, IL), and washes were according to the supplier's protocols, except that SSPE was used instead of SSC. The insert from one partial clone (R4A) was EcoRI-excised and used to re-screen the same library as described above, yielding 12 positive clones. Two of these were found to be full-length (R11C, R11D).

A human expressed sequence tag cDNA clone (EST06385)(Adams, et al., 1993), identified using the B14A insert sequence in a BLAST search (Altschul, et al., 1990) of the National Center for Biotechnology Information (NCBI, Bethesda, MD) databases, was obtained from ATCC (renamed H50A), and fully-sequenced. All DNA and protein sequence was assembled and analyzed using the GCG analysis programs, including MOTIFS and BESTFIT. The NCBI databases were also scanned using FASTA (Lipman and Pearson, 1988). The statistical significance of sequence alignments was determined using the RDF program (Lipman and Pearson, 1985), and additional homology was examined using the BLOCKS email server (database version 7.01, Henikoff and Henikoff, 1994).

For bacterial expression of p50, the H50A coding region was subcloned into the pET-14b expression vector (Novogene, Madison, WI) using PCR mutagenesis and standard cloning techniques. After transformation into the E. coli expression strain BL21(DE3) and induction with 0.4mM IPTG, bacteria were lysed in SDS-PAGE sample buffer, and analysed by western blotting as described below.
**Northern blot analysis**

The R11C cDNA, which contains the entire rat p50 coding region with 5' and 3' untranslated sequences, was EcoRI-excised, labelled with α[^32P]-dCTP by random-priming (Boehringer Mannheim Biochemicals), and used to probe a multiple tissue northern (MTN) blot of adult rat poly(A) RNA (Clontech Laboratories). Hybridization was overnight at 65°C, in Rapid Hyb solution, followed by washes according to supplier's instructions, except that SSPE was used instead of SSC. Results were detected on a Molecular Dynamics PhosphorImager SF.

**Preparation of tissue samples**

Calf brain and tissue samples from adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were homogenised as previously described (Paschal, *et al.*, 1992), except that the buffer was 80 mM PIPES, 5 mM EGTA, 1 mM MgCl₂, 0.25 M sucrose, 2 mM EDTA, 2 μg/ml leupeptin, 100 μg/ml PMSF, 100 μg/ml TPCK, 2 μg/ml aprotinin, 1 μg/ml pepstatin A. Cytosolic extracts were prepared by centrifugation at 100,000g for 1 hr at 4°C. Protein concentrations were determined using the BCA method (Pierce, Rockford, IL). Samples were separated by SDS-PAGE, and either stained with Coomassie blue, or processed for western blotting as described below.

**Cell Culture**

Cell cultures (Rat2, HeLa, COS-7, and PtK1, all from American Type Culture Collection, Rockville, MD) were maintained as subconfluent monolayers in "growth medium": DMEM (Gibco/BRL, Life Technologies, Grand Island, NY) + 10% fetal calf serum (Gibco/BRL) + 100U/ml penicillin + 100 μg/ml streptomycin (Sigma Chemicals, StLouis, MO). For immunofluorescence staining experiments, cell were trypsinized, and seeded onto sterile 18 mm² glass coverslips in 6-well dishes (Corning Glass Works, Corning, NY) to reach 70-80% confluence.
after 48 hours. Nocodazole (Sigma) was kept as a 1 mg/ml stock in DMSO, and used at 10 μM for 3-4 hours prior to fixation.

**Immunohistochemical methods**

Antibodies used in this study were: "50-1" monoclonal anti-p50 (Paschal, *et al.*, 1993), "RA1/10" (Clark and Meyer, 1992) and "A27" affinity-purified rabbit polyclonal anti-Arp1 antisera (gifts from Drs. D. Meyer and S. Clark, UCLA, Los Angeles, CA), "UP235" (for western blotting) and "UP236" (for immunofluorescence) affinity-purified rabbit polyclonal anti-p150(γtub) antisera (Vaughan and Vallee, 1995; Waterman-Storer, *et al.*, 1995)(gifts from Dr. E. Holzbaur, Univ. of Pennsylvania, Philadelphia, PA), "L5" affinity-purified rabbit polyclonal anti-cytoplasmic dynein intermediate chain (Vaughan and Vallee, 1995), "74.1" monoclonal anti-cytoplasmic dynein intermediate chain (Dillman III and Pfister, 1994)(gift from Dr. K. Pfister, Univ. of Virginia, Charlottesville, VA), rabbit antiserum against the myc epitope tag (courtesy of Dr. M. A. Gee, Worcester Foundation for Biomedical Research), "SH" human CREST autoimmune antiserum (Simerly, *et al.*, 1990), rabbit polyclonal anti-CENP-E antiserum (Lombillo, *et al.*, 1995)("pAb-1.6", gift from B. Schaar and Dr. T. Yen, Fox Chase Cancer Center, Philadelphia, PA), monoclonal anti-tubulin (DM1A, Amersham), rabbit polyclonal anti-tubulin (gift from Dr. J.C. Bulinski, Columbia University, NY), and monoclonal (Boehringer Mannheim Biochemicals) and rabbit polyclonal (5 Prime - 3 Prime Inc., Boulder, CO) anti-β-galactosidase.

For western blotting, samples were separated by SDS-PAGE, and electrophoretically-transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA). After blocking in 5% nonfat dry milk in TBS + 0.1% Tween-20 (TBST) at 4°C overnight, the blot was incubated with primary antibody diluted in TBST + 1% milk for 1 hr, washed (3x 10 min in TBST+ 1% milk), then incubated for 40 min in HRP-conjugated donkey anti-mouse or anti-rabbit IgG diluted 1:10,000 in TBST + 1% milk. After final washing (3x 10 min in TBST), signal detection was achieved by ECL (Amersham).
For immunofluorescence staining, cells grown on glass coverslips were briefly rinsed with D-PBS, and processed according to one of the following protocols. Some samples were pre-extracted for 1 min in 0.5% Triton X-100 (Pierce) in PEMG buffer [80 mM PIPES (pH 6.8), 5mM EGTA, 1 mM MgCl$_2$, 4 M glycerol], followed by either 10 min in 100% methanol at -20°C, or 15 min in 4% formaldehyde (from 16% EM grade, Electron Microscopy Sciences, Ft. Washington, PA) in PEMG. Some samples ("methanol fixation") were simply incubated for 10 min in 100% methanol at -20°C. Other samples were fixed in 4% formaldehyde in D-PBS for 15 min, followed by either incubation in 0.5% Triton X-100 in D-PBS for 2 min, or 10 min in 100% methanol at -20°C. Samples destined for anti-tubulin staining were simultaneously fixed and extracted ("FGE method") in 4% formaldehyde + 0.25% glutaraldehyde (from 8% EM grade, Polysciences Inc., Warrington, PA) + 0.5% Triton X-100 in PEMG for 15 min, rinsed in PBS (3x 5min), and incubated in 0.5 mg/ml sodium borohydride in PBS (3x 5min) to reduce free aldehyde groups.

All samples were then rinsed in PBS (3x 5min), incubated in primary antibody solution for 30-45 min, rinsed again in PBS (3x 5min), and incubated in secondary antibody solution for 30-40 min. All antibodies were diluted in PBS + 1% normal donkey serum (Jackson Immunoresearch Labs., West Grove, PA). All secondary antibodies were made in donkey, conjugated to DTAFT, Texas Red, Cy3, or Cy5, and made species-specific by cross-adsorption ("ML" series, Jackson Immunoresearch Labs). Labeling of chromosomal DNA was achieved with a brief incubation in Hoechst dye #33258 (Pierce). Samples were mounted in 0.1% p-phenylenediamine in PBS + 50% glycerol.

**Microscopy Techniques**

Conventional immunofluorescence microscopy was carried out on a Zeiss Axiophot photomicroscope (Carl Zeiss Inc., Thornwood, NY) equipped for epifluorescence, and micrographs were taken on Kodak TMAX-400 film. Images were digitized by scanning the negative with a
Nikon Coolscan Scanner (Nikon Inc., Melville, NY). Confocal microscopy was carried out on a Bio Rad MRC1000 system (Bio Rad Microscience, Hercules, CA) equipped with Kr/Ar laser, mounted on a Nikon Diaphot 200 microscope. All digitized images (from conventional and confocal microscopy) were cropped using Adobe Photoshop (v. 3.0, Adobe Systems Inc., Mountain View, CA), and imported into CorelDraw (v.5.0, Corel Corp., Ottawa, Canada) for figure assembly. Figures were printed on a Kodak Colorease PS printer (Eastman Kodak Co., Rochester, NY).
Results

Molecular characterization of mammalian p50

In order to isolate cDNA clones encoding mammalian p50, amino acid sequences of six tryptic peptides from immunoprecipitated calf brain p50 were analyzed (courtesy of Dr. B. Paschal and Dr. J. Leszyk, Worcester Foundation). One of these (peptide 21, Fig. 2a,b) was used to design a mixed oligonucleotide probe (16411, 38mer) prepared for hybridization screening of a bovine brain cDNA library. This yielded a single 0.4 kb positive clone (B14A) which was found to contain the full peptide 21 sequence (Fig. 2a,b). The B14A insert was then used as a hybridization probe to isolate additional p50 clones from a rat brain cDNA library. Of a total of 13 positive clones isolated, two independent rat cDNAs (R11C, R11D) were found to share the same, complete 1221 bp open reading frame (ORF) encoding 407 residues (Fig. 2a). A homology search of nucleic acid databases also identified a partially-characterized human cDNA clone (EST06385, Adams, et al., 1993, renamed H50A) which shared extensive identity with B14A. Complete sequencing of clone H50A revealed the same complete ORF found in the rat clones (Fig. 2a,b). The deduced full-length rat and human polypeptide sequences are 96% identical (97% similar), and contain all six calf brain p50 tryptic peptide sequences.

The H50A ORF was expressed in bacteria, and the resulting whole cell lysates analyzed by SDS-PAGE and western blotting using a p50-specific monoclonal antibody (Paschal, et al., 1993)(see Fig.5a). A single immunoreactive band in these extracts (lane 2) was found to migrate slightly above calf brain p50 (lane 1). We conclude that the ORFs contained in clones H50A, R11C and R11D constitute the full-length coding regions for the 50kDa component of human and rat dynactin complex. The genomic locus for the p50 gene was identified by interspecies backcross analysis, and localized to mouse chromosome 10, which is syntenic with human chromosome region 12q13, suggesting the existence of a single p50 gene in mammals (Vaughan, et al., 1996).
Figure 2: Identification of mammalian p50-encoding cDNA clones

(a) Alignment diagram of bovine (B14A), and full-length human (H50A) and rat (R11C, R11D) p50 cDNA clones, showing relative positions of six calf brain p50 tryptic peptide sequences characterized in this study. The star indicates the position of the hexanucleotide polyadenylation signal sequence (ATTAAA in H50A, and AATAAA in R11C), and the hatched box shows the position of the poly(A) stretch (26mer) in H50A. The open boxes represent the open reading frames (ORF).

(b) Nucleotide and amino acid sequences of human full-length p50 clone H50A. Note the Kozak consensus box (Kozak, 1991) at the predicted translational initiation site (AUG in bold, at 79), which was preceded by no other in-frame methionine codons in this or any other clones characterized in this study. A hexanucleotide polyadenylation signal (AATAAA in bold, at 1657) and a poly(A) stretch (26mer starting at 1678) are both found downstream from the termination codon (TGA in bold, at 1297).
Rat and human p50 have predicted molecular weights of 44,736 and 44,819 Da, respectively, both somewhat lower than the value of 50 kD estimated from SDS-PAGE. The predicted isoelectric points are 4.92 and 4.96, respectively, both consistent with the observed mobility on two-dimensional gel analyses of bovine brain 20S dynein preparations (S. Hughes, and R. Vallee, unpublished observation). Secondary structure predictions using the PHDsec neural network algorithms (Rost and Sander, 1994) indicate an α-helix-rich profile (Fig. 3a). Using the COILS program, residues 105 to 135 are strongly predicted to form a coiled-coil domain (99% probability with and without α/δ weighting Lupas, et al., 1991)(Fig. 3b). Although two other regions also showed high coiled coil potential using the original COILS analysis, i.e. residues 219 to 251 (69% probability) and 281 to 308 (72%), a more refined analysis (COILS version 2.2, see Appendix) using an improved scoring matrix and 2.5-fold weighting for the α and δ heptad positions produced only 24% and 21% probabilities respectively, indicating that these are most likely not coiled coils, but rather, highly-charged α-helices (Fig. 3b). Residues 358 to 404 show low COILS scores, the significance of which is unclear.

A GCG/MOTIF analysis revealed that residues 129 to 154 match the consensus sequence for the LysR subfamily of helix-turn-helix (HTH) domains (Fig.3c), indicative of a putative DNA-binding capability. This match, however, was not detected using a BLOCKS analysis (Henikoff and Henikoff, 1994). Furthermore, all members of this HTH subfamily are prokaryotic transcription factors, clearly inconsistent with our current understanding of p50, which has no nuclear localization signal and exhibits no interphase nuclear staining (see below).

BLAST and FASTA homology searches of nucleotide databases using human and rat p50 have identified many partially-characterized human EST clones containing p50 sequences, as well as similar clones from Drosophila, mouse, rat and C. elegans. However, as of December 1997, no significant domain similarities with other non-p50 proteins have been detected. Nevertheless,
Figure 3: Sequence analysis of mammalian p50: secondary structure predictions and motifs

(a) Secondary structure predictions using PHDsec analysis (Rost and Sander, 1994);
(b) Coiled coil predictions for human p50 using non-weighted vs a/d-weighted COILS analyses (28 residue window)(see Appendix);
(c) Summary diagram of p50 showing predicted coiled coil domain (blue), putative highly-charged α-helices (grey), and location of HTH-like region.
a.

MTK matrix
no weighting

b.

window=28
MTK matrix
no weighting

C.

NH₂
(99%)

Coil

HTH

COOH
Figure 4: Analysis of inter-species conservation of p50

Multiple sequence alignment of p50 sequences from human, Drosophila, C. elegans, and S. cerevisiae. Alignment was made using GCG PILEUP program, and output was produced using BOXSHADE program on ISREC web server. Drosophila p50 sequence was obtained from Dan Starr (Goldberg laboratory, Cornell Univ., Ithaca, NY). It should be noted that the C. elegans p50 sequence is derived from automated sequencing of an EST clone, and therefore, may contain errors.
inter-species comparisons have revealed high primary sequence conservation within small blocks, particularly in the N-terminal half of the protein (Fig. 4). Beyond this, the two major features which remain conserved across species lines are the predicted low isoelectric points, all between 4.5 and 5.0, and the predicted α-helix-rich secondary structure profiles. Based on these, as well as phenotypic criteria, I proposed that the product of the Jm1 gene may represent the p50 homologue in S. cerevisiae. Jm1p, like cytoplasmic dynein, is required for proper nuclear migration during mitosis (McMillan and Tatchell, 1994). It contains 373 residues, has a predicted molecular weight of 43,620 Da, an estimated pI of 4.65, and 3 predicted coiled coil domains with sizes and distributions similar to those initially found in p50 (McMillan and Tatchell, 1994). However, a GCG/BESTFIT comparison of the amino acid sequences shows only 22% identity (47% similarity), spanning the entire sequence (as seen also by GCG/COMPARE and DOTPLOT analyses). Statistical analysis of this comparison using the RDF program (Lipman and Pearson, 1985) yielded a mean score for the optimal p50-Jm1p alignment of only 4.2 standard deviations (z value) above that of 1000 randomized sequence sets, indicating this relation to be of negligible significance.

**Tissue and subcellular distribution of p50**

Northern blot analysis of adult rat poly(A)RNA revealed a single major 1.7 kb mRNA species in all tissues tested (Fig.5c). Immunoblotting of adult rat tissue cytosolic extracts (Fig.5b) also revealed a ubiquitous distribution for p50, consistent with expression patterns observed for other dynactin components (Clark and Meyer, 1992; Gill, et al., 1991). The prominent 38kD doublet detected in liver was not observed in anti-p50 immunoprecipitates (not shown), nor were there any indications of a smaller transcript encoding this species (Fig.5c).

Immunofluorescence microscopy was used to examine the subcellular distribution of p50 in several cultured mammalian cell lines, including HeLa, PtK1, Cos-7, and Rat2, all of which yielded equivalent patterns. Fine punctate staining densely filled the entire cytoplasm throughout
Figure 5: Confirmation of p50 clone and characterization of p50 expression in adult rat tissues. (a) Anti-p50 immunoblot (using 50-1 monoclonal antibody) of SDS-PAGE-separated (10% gel) whole cell lysates of E. coli strain BL21(DE3) transformed with pET14b-driven H50A ORF (lane 2), compared with calf p50 from brain cytosolic extract (lane 1) and whole cell lysate from untransformed BL21(DE3) culture (lane 3). The single H50A-encoded protein expressed in bacteria is clearly immunoreactive, and migrates slightly above calf brain p50, but well-within the range of variability seen in rat tissues (panel b). (b) Anti-p50 immunoblots of adult rat tissue cytosolic extracts (25 μg protein/lane) separated by SDS-PAGE (5-18% gradient gel) also reveal a ubiquitous distribution for p50. Variability in electromobility of p50 bands as seen in (a) and (b) was not consistently observed, and is likely due to distortion effects from large bands migrating just above (such as tubulin) or below (such as actin) the p50 bands. (c) Northern blot of adult rat tissue poly(A) RNA probed with rat full-length p50 clone R11C insert, revealing a single 1.7kb mRNA species in all tissues tested. Abbreviations: H: heart, B: brain, S: spleen, Ln: lung, Lv: liver, M: skeletal muscle, K: kidney, T: testis, P: pancreas.
the cell cycle, and was excluded from the nucleus during interphase (Fig. 6a). Prominent 
centrosomal staining (Fig. 6a,b) was also observed, appearing as a closely spaced group of 2-4 
brighter spots (inset in Fig. 6a). These spots were often obscured from late prometaphase to mid-
anaphase by an accumulation of the fine punctate staining along spindle microtubules, particularly 
toward the spindle poles (Fig. 6c, g-k). Detergent extraction of the cells prior to fixation abolished 
most of the fine punctate staining, while the centrosomal spots remained prominent (Fig. 6b).
These patterns are consistent with previous reports of anti-Arl (Clark and Meyer, 1992), and 

Staining of mitotic cells with anti-p50 also showed a strong apparent kinetochore pattern 
beginning after nuclear envelope breakdown (NEB)(Fig. 6g), and disappearing from each 
chromosome upon its alignment at the metaphase plate (Fig. 6i). The intensity of the staining was 
consistent with a time-dependent accumulation of the antigen, from a dim signal just after NEB, to 
very bright levels in kinetochores of non-aligned metaphase chromosomes (Fig. 6c).

Because kinetochore staining was not observed previously in cells stained with anti-
150Glued antibodies (Gill, et al., 1991), I examined the effects of multiple fixation conditions on 
mitotic staining obtained with a battery of monoclonal and polyclonal antibodies to dynactin 
components (Table 1, Fig. 7). Seven independent antibodies including monoclonals and affinity-
purified polyclonal antisera, directed against 150Glued (UP236, DART, PORT, ARA, and mp150) 
and Arp1 (RA1/10 and A27) gave kinetochore staining patterns comparable to that seen with the 
anti-p50 monoclonal antibody (Table 1, Fig. 7). Both a monoclonal (74.1", Table 1) and an 
affinity-purified polyclonal anti-cytoplasmic dynein IC antibody (Vaughan and Vallee, 1995) also 
produced the same kinetochore pattern (Table 1, Fig. 7e). Importantly, all antibodies consistently 
showed this pattern in cells fixed without detergent pre-extraction, thus minimizing the risk of 
artifactual redistribution of cytosolic antigens (Table 1, Fig. 7). It should also be noted that the 
increased clarity of kinetochore staining obtained in detergent pre-extracted samples (Table 1)
Figure 6: Subcellular distribution of p50 throughout the cell cycle in cultured mammalian cells. (a, b, c, e, g, i, k, m) anti-p50 staining, (d, f, h, j, l, n) Hoechst DNA staining. (a, b) interphase HeLa cells, (c, d) late prometaphase COS-7, (e, f) prophase Rat2, (g, h) early prometaphase Rat2, (i, j) metaphase Rat2, (k, l) anaphase Rat2, (m, n) telophase Rat2. (a) Interphase cells fixed in formaldehyde without detergent pre-extraction show fine punctate staining densely filling the cytoplasm, but excluded from the nucleus. Prominent centrosomal staining is also seen, appearing as 2-4 closely-spaced spots at high magnification (inset). (b) Cells fixed in formaldehyde after detergent pre-extraction. Centrosomal staining persists prominently, but most of the fine punctate staining is lost. Centrosomal spots are visible during prophase (e, f), but become obscured by an accumulation of fine punctate staining along spindle microtubules, particularly near the poles, from prometaphase (c, d, g, h) to anaphase (k, l). Spindle pole staining is often notably dimmer in telophase cells (m, n). Kinetochore staining was also observed in prometaphase cells (c, d, g, h), found most prominent in late-attaching chromosomes before their alignment at the metaphase plate (arrows in c, d). Cells in (e-n) were fixed in formaldehyde followed by detergent extraction. Kinetochores in panels c and g would be scored as “+++” and “++”, respectively, in Table 1.
was largely due to the concomitant decrease in fine punctate cytoplasmic staining observed with this method (see Fig.6a,b). The kinetochore localization of anti-dynactin staining was confirmed by double-labeling with a CREST human autoimmune antiserum (Fig.7g-i). Nocodazole-induced pseudo-prometaphase cells showed enhanced staining of all kinetochores with all anti-dynactin and anti-dynein antibodies. The kinetochores often appeared as paired crescents (Fig.7f, Figs.9, 10) clearly reminiscent of previous electron microscopic observations made under similar microtubule-depolymerizing conditions (Rieder, 1982). This increased size and curvature of kinetochores is thought to result from the continued growth of the fibrous corona through recruitment of cytosolic components, combined with the continued condensation of the underlying chromatin, thus yielding a "wet photograph" effect. We also noted that chromosomes isolated from vinblastine-arrested CHO cells, known to contain little or no tubulin bound to kinetochores (Mitchison and Kirschner, 1985), showed strong anti-p50 staining of the primary constriction (B. Paschal, L. Wordeman, and R. Vallee, unpublished observations).

Table 1: Effect of Fixation Methods on Prometaphase Kinetochore Staining with anti-Dynactin and anti-Cytoplasmic Dynein Antibodies in Cultured Vertebrate Cells.

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<th>Fixation Method</th>
<th>Anti-p50 (mAb &quot;50-1&quot;)</th>
<th>Anti-p150 (pAb &quot;UP236&quot;)</th>
<th>Anti-p150* (pAb &quot;DART&quot;)</th>
<th>Anti-p150* (pAb &quot;PORT&quot;)</th>
<th>Anti-p150* (pAb &quot;ARA&quot;)</th>
<th>Anti-p150* (mAb)</th>
<th>Anti-Arp1 (pAb &quot;RA1/10&quot;)</th>
<th>Anti-Arp1 (pAb &quot;A27&quot;)</th>
<th>Anti-dynein IC (pAb &quot;L5&quot;)</th>
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+++; prominent staining; ++; clear staining; +; dim staining; +/-; variable dim staining; -; no detectable staining; nd, not done. See Materials and Methods for detailed protocols. Abbreviations: form., formaldehyde; glut., glutaraldehyde; simult., simultaneous.
Figure 7: Dynactin and cytoplasmic dynein components localize to kinetochores.

Prometaphase COS-7 cells show clear kinetochore staining with anti-p50 (a), anti-p150Glued (c), anti-Arp1 ("A27") (g), and anti-cytoplasmic dynein IC (e). (b, d, f) are DNA staining patterns corresponding to (a, c, e), respectively. Double-labeling using anti-Arp1 (g, f) and human CREST autoimmune serum (i, l) confirms kinetochore localization of dynactin. The dynactin pattern is restricted to non-aligned kinetochores in control cells (g-i), but appears on all kinetochores in nocodazole-treated (10μm, 3hrs at 37°C) cells (j-l). (h) and (k) show superimposition of (g+i) and (j+l), respectively, showing signal co-localization in yellow. (a-f) are conventional fluorescence micrographs, and (g-l) are through-focus maximal projections of complete x/y optical section stacks (22 sections, 0.4 μm step) acquired by confocal microscopy. All cells were fixed in formaldehyde followed by detergent extraction, except (e, f) which was fixed directly in methanol. All kinetochores in this figure would be scored as “++” in Table 1, except for panel j which would be scored “+++".
Discussion

Molecular characterization of p50

During this first phase of my thesis work, I found that p50 is a novel protein, encoded as a single transcript in all tissues tested, apparently from a single gene locus in mammals. Sequence analysis suggested an \( \alpha \)-helix-rich secondary structure, including one strongly-predicted coiled coil domain and two highly-charged \( \alpha \)-helices. Although one motif search suggested the presence of an HTH domain within mammalian p50, the same motif was not found using a second, more powerful search program, nor was it found in any other non-mammalian p50 sequences analyzed to date. In addition, all confirmed members of this HTH subfamily are prokaryotic in origin, and the consensus sequence in question has been known to yield false positives (pers. comm., Dr. S. Henikoff). Thus, it appears doubtful that p50 truly contains this structure or its associated DNA-binding activity. Interestingly however, the tentative identification of Jnm1p as the yeast p50 homologue was recently confirmed by the finding that Jnm1p indeed forms a dynactin-like complex with ACT3/5 and YDG1, the yeast Arp1 and p150\(^{\text{Glued}}\) homologues, respectively (Kahana, et al., 1996). Along with the recent identification of Drosophila (pers. comm., D. Starr, Cornell Univ., Ithaca, NY) and C. elegans p50 sequences, these findings offer the promise that future progress from studies in these genetically-tractable systems may become more directly interpretable with respect to vertebrate p50 and dynactin.

Subcellular distribution of dynactin and cytoplasmic dynein during mitosis

Early reports of the staining patterns obtained with anti-cytoplasmic dynein and anti-dynactin antibodies yielded conflicting results. Dynein was found by some laboratories to associate with kinetochores during prometaphase, and subsequently, to appear in a more diffuse pattern toward the spindle poles (Pfarr, et al., 1990; Steuer, et al., 1990). However, this pattern was relatively difficult to see in unextracted cells, and was not observed universally (Lin and Collins, 1992). Furthermore, despite an earlier claim to the contrary (Steuer, et al., 1990), a comparable kinetochore pattern was not observed in cells stained with an antibody against...
p150Glued (Gill, et al., 1991). Thus, in order to alleviate this confusion, I undertook an exhaustive study of the subcellular distribution of dynactin and cytoplasmic dynein antigens under a wide range of preservation conditions (Table 1). Several epitopes showed some variability in immunoreactivity, particularly evident with anti-cytoplasmic dynein IC staining, which was most sensitive to fixation conditions, perhaps in part reflecting the differences in dynein staining reported among other labs. Despite these caveats, I was able to demonstrate conclusively that both complexes do indeed co-localize on prometaphase kinetochores.

As seen for cytoplasmic dynein, I found dynactin immunoreactivity to appear at the kinetochore just after NEB. Kinetochore staining intensity increased during early prometaphase, consistent with the reported maturation of these structures during this phase of mitosis (Rieder, 1982). Indeed, once exposed to the cytoplasm, vertebrate kinetochores develop to form characteristic trilaminar plate structures located over the constricted centromeric regions, partly through recruitment of several cytosolic factors including the kinesin-like proteins CENP-E (Yen, et al., 1991) and MCAK (Wordeman and Mitchison, 1995), CLIP-170 (Dujardin, et al., 1996), ZW10 (Starr, et al., 1997), and now also dynactin and cytoplasmic dynein. This maturation process also involves the formation of a variably-sized, fibrous layer extending out from the cytosolic face of the outer plate, known as the fibrous corona (Rieder, 1982). Cytoplasmic dynein and CENP-E have both been localized to this region (Wordeman, et al., 1991; Yao, et al., 1997), and preliminary experiments have also revealed p50 staining in the same region (unpub. results, Drs. B. Paschal and L. Wordeman).

Consistent with electron microscopy observations of fibrous corona material (Rieder, 1982), I also found that cytoplasmic dynein and dynactin disappeared from kinetochores upon chromosome alignment at the metaphase plate, and that both appeared enlarged and much brighter in kinetochores of nocodazole-arrested pseudo-prometaphase cells. The loss of kinetochore staining in late prometaphase cells was delayed in non-attached chromosomes (Fig. 6c,d and
Fig. 7g-i), thus indicating that control of dynactin and cytoplasmic dynein release from kinetochores depends on the specific behavior of individual chromosomes with respect to their interactions with spindle microtubules. While the decrease in kinetochore staining by metaphase could also reflect an antibody accessibility problem, this interpretation is disputed by the multiplicity of independent dynactin and cytoplasmic dynein antibodies which have shown the same pattern in this and previous studies (Pfarr, et al., 1990; Steuer, et al., 1990).

The results of this first part of my thesis work therefore finally offered the subcellular co-localization data to support the involvement of dynactin in cytoplasmic dynein function, and suggested a role for both complexes in kinetochore function during prometaphase. Although the initial analysis of p50 sequences did not yield direct insights into its function within dynactin, the cDNA clones now permitted the next phase of thesis work to begin: using transient transfections of p50 constructs for a dominant negative inhibition study of dynactin and, indirectly, cytoplasmic dynein functions in vivo within cultured cells.
CHAPTER 2

OVEREXPRESSION OF WILD TYPE DYNAMITIN (p50) CAUSES INHIBITION OF DYNACTIN AND CYTOPLASMIC DYNEIN FUNCTIONS

Introduction

After initial advances in the characterization of dynactin structure and composition, the central question of the complex's function still remained open. Although biochemical co-purification data (Collins and Vallee, 1989; Holzbaur, et al., 1991; Steuer, et al., 1990), in vitro motility data (Gill, et al., 1991; Schroer and Sheetz, 1991), and genetic data (McGrail, et al., 1995; Muhua, et al., 1994; Plaman, et al., 1994; Xiang, et al., 1994) clearly supported the involvement of dynactin in dynein-based transport, these studies yielded few direct insights into the specific role of dynactin in this process.

An important first issue was to determine the nature of the interaction - if any - between dynactin and cytoplasmic dynein. This was achieved through biochemical analysis including western blot overlays, immunoprecipitation assays, and affinity chromatography techniques, all of which established that the two complexes can indeed interact through the intermediate chain (IC) of cytoplasmic dynein and the p150Glu subunit of dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995) (see Fig. 8). In view of the localization of the ICs to the cargo-facing base of the dynein complex (Steffen, et al., 1996, see Fig. 1), these results supported a "receptor model" of
dynactin function. According to this model, dynactin would mediate the interaction of cytoplasmic dynein with its cargo, as opposed to an alternative "activator model", whereby dynactin would otherwise regulate dynein motor activity. However, the sheer complexity of the dynactin complex, and the finding that p150Glu also contains a microtubule-binding domain of its own (Pierre, et al., 1992 Waterman-Storer, et al., 1995) lent doubt to the notion of dynactin as a simple receptor. The identification of Arp1 as the major subunit (Clark and Meyer, 1992; Lees-Miller, et al., 1992; Paschal, et al., 1993) also led some to suggest that dynactin may serve as the primary link between microtubule- and microfilament-based transport processes (Goldstein and Vale, 1992). Further work was clearly needed to test these models.

Throughout this time, two other fundamental questions remained unaddressed in this field: first, is dynactin required for all forms of dynein-based transport, or merely for a subset thereof? And second, is dynactin function restricted to retrograde transport, or is it also involved in anterograde transport? The full range of cytoplasmic dynein functions has long remained largely speculative due to the lack of a specific method of inhibition. Indirect data from numerous sources have supported roles in retrograde axonal transport (Hirokawa, et al., 1990; Paschal and Vallee, 1987; Schnapp and Reese, 1989), perinuclear localization of the Golgi complex (Corthésy-Theulaz, et al., 1992), centripetal movement of endosomes (Aniento, et al., 1993), organization of spindle-like radial arrays of microtubules in vitro (Verde, et al., 1991), poleward chromosome movements during prometaphase (Hyman and Mitchison, 1991; Reider and Alexander, 1990), and transport of MTs from the neuronal cell body into growing axons (Dillman III, et al., 1996), to name a few.

One antibody inhibition study did offer a more direct approach in principle, yielding an apparent inhibition of spindle pole separation during early mitosis, but specificity questions remained, especially in view of the high antibody concentrations needed to see these effects (Vaisberg, et al., 1993). More clear-cut physiological evidence emerged from genetic systems,
Figure 8: Schematic representation of mode of interaction between dynactin and cytoplasmic dynein.

Dynactin and cytoplasmic dynein have been shown to interact directly via the intermediate chains (Ics) of the latter and the p150Glu subunit of the former (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Based on previous ultrastructural analyses of the two complexes, the ICs are located at the base of the dynein molecule (Steffen, et al., 1996), and the IC-interaction domain of p150Glu is located near the base of dynactin’s arm domain, where it inserts into the "shoulder" region (Schafer, et al., 1994).

During the second phase of my thesis work, I worked to bridge this gap in the field by undertaking a study of p50 function in cultured vertebrate cells, hoping to gain new insights into dynactin and cytoplasmic dynein functions. I chose a dominant negative inhibition approach, transfecting cells to overexpress wild type and mutant p50 constructs and assay for interference with normal endogenous dynactin, and presumably, cytoplasmic dynein activities. I first examined two basic cellular processes which have long been proposed to involve dynein-based transport: perinuclear Golgi localization and mitosis. I found that overexpression of wild type p50 resulted in a dissociation of the dynactin complex, accompanied by multiple cytological defects consistent with inhibition of cytoplasmic dynein functions. This chapter therefore describes those findings using transfections of wild type p50 in cultured cells. Effects associated with overexpression of mutant p50 constructs will be described in Chapter 3.
Materials and Methods

Cell culture and transfections

Cell cultures (Rat2 and COS-7, both from ATCC) were maintained as described in Chapter 1. Briefly, all cultures were maintained as subconfluent monolayers in "growth medium": DMEM or F12K (Gibco/BRL) + 10% fetal calf serum (Gibco/BRL) + 100U/ml penicillin + 100 μg/ml streptomycin (Sigma). For immunofluorescence staining experiments, cells were trypsinized, and seeded onto sterile 18x18 mm glass coverslips in 6-well dishes (Corning) to reach 70-80% confluence after 48 hours.

For transient transfections, cells were seeded onto 18x18 mm glass coverslips in 6-well dishes at 1.5-2.5 x 10^5 cells per well, so that they reached ~70% confluence at the time of transfection (usually the next day). After 24 hours, growth medium was rinsed off with Ca^{++}- and Mg^{++}-free PBS (D-PBS), and replaced with transfection mixture (each well contained 1 μg plasmid DNA with 4 μl Lipofectamine reagent (Gibco/BRL) in 1 ml DMEM or F12K, prepared as per supplier’s instructions) for 6 hours before replacing again with growth medium. Transfected samples destined for biochemical analysis were plated at equivalent densities in 100mm or 150mm tissue culture dishes (Corning), and treated as above, except that 3 ml or 8 ml of transfection medium was used per dish, respectively. Transfected cultures were either fixed or harvested 24-45 hours later (typically 24 hours for SA498 and Rat2, and 32-35 hours for COS-7). Nocodazole (Sigma) was kept as a 1 mg/ml stock in DMSO, and used in all cases at 10 μM for 3-4 hours prior to fixation.

The plasmid used for p50 transfections was made by subcloning the full H50A coding region into the NotI sites of pCMVβ (Clontech), which itself, was used as is for β-galactosidase overexpression. The myc epitope tag (EQKLISEED-stop) (Evan et al., 1985) was inserted after the last p50 codon by PCR mutagenesis and subcloning through a shuttle vector (pARK2mycSTOP, courtesy of Dr. M. A. Gee, Worcester Foundation for Biomedical Research).
Subsequent optimization of this tag (to EQKLI SEEDLNG-stop) was carried out using the QuickChange mutagenesis kit (Stratagene). pCMV-GFP-LpA, encoding the full length green fluorescent protein under the control of the CMV promoter was a gift of Dr. H. Stunnenberg, EMBL, Heidelberg. pCNG2, encoding the GFP-, b1,2 N-acetylglucosaminyltransferase I (NAGT-I) fusion protein under control of the CMV promoter, was a gift of Drs. David Shima and G. Warren (Imperial Cancer Research Fund, London, UK).

Immmunochemical methods (as described in Chapter 1)

Antibodies used in this chapter were: "50-1A" ascites preparation of monoclonal anti-p50 (Paschal, et al., 1993), "A27" affinity-purified rabbit polyclonal anti-Arp1 antiserum (gift from Drs. D. Meyer and S. Clark, UCLA, Los Angeles, CA), "UP235" (for western blotting) and "UP236" (for immunofluorescence) affinity-purified rabbit polyclonal anti-p150<sub>Glue</sub> antiserum (Vaughan and Vallee, 1995; Waterman-Storer, et al., 1995)(gifts from Dr. E. Holzbaur, Univ. of Pennsylvania, Philadelphia, PA), "DART" rabbit polyclonal anti-p150<sub>Glue</sub> antiserum (Vaughan and Vallee, 1995), "L5" affinity-purified rabbit polyclonal anti-cytoplasmic dynein intermediate chain (Vaughan and Vallee, 1995), "74.1" monoclonal anti-cytoplasmic dynein intermediate chain (Dillman III and Pfister, 1994)(gift from Dr. K. Pfister, Univ. of Virginia, Charlottesville, VA), rabbit antiserum against the myc epitope tag (courtesy of Dr. M. A. Gee, Worcester Foundation for Biomedical Research), "SH" human CREST autoimmune antiserum (Simerly, et al., 1990), rabbit polyclonal anti-CENP-E antiserum (Lombillo, et al., 1995)("pAb-1.6", gift from B. Schaar and Dr. T. Yen, Fox Chase Cancer Center, Philadelphia, PA), monoclonal anti-tubulin (DM1A, Amersham), rabbit polyclonal anti-tubulin (gift from Dr. J.C. Bulinski, Columbia University, NY), monoclonal (Boehringer Mannheim Biochemicals) and rabbit polyclonal (5 Prime - 3 Prime Inc., Boulder, CO) anti-β-galactosidase, monoclonal anti-VSV-G epitope ("P5D4", from Sigma); monoclonal anti-rat α-mannosidase II (Babco, Berkeley, CA);
For western blotting, samples were separated by SDS-PAGE, and electrophoretically-transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA). After blocking in 5% nonfat dry milk in TBS + 0.1% Tween-20 (TBST) at 4°C overnight, the blot was incubated with primary antibody diluted in TBST + 1% milk for 1 hr, washed (3x 10 min in TBST+ 1% milk), then incubated for 40 min in HRP-conjugated donkey anti-mouse or anti-rabbit IgG diluted 1:10,000 in TBST + 1% milk. After final washing (3x 10 min in TBST), signal detection was achieved by enhanced chemiluminescence (Amersham Corp.).

For immunofluorescence staining, cells grown on glass coverslips were briefly rinsed with D-PBS, and processed according to one of the following protocols. Some samples were pre-extracted with a 1 min incubation in 0.5% Triton X-100 (Pierce) in PEMG buffer [80 mM PIPES (pH 6.8), 5mM EGTA, 1 mM MgCl₂, 4 M glycerol], followed by either 10 min in 100% methanol at -20°C, or 15 min in 4% formaldehyde (from 16% EM grade, Electron Microscopy Sciences, Ft. Washington, PA) in PEMG. Some samples ("methanol fixation") were simply incubated for 10 min in 100% methanol at -20°C. Other samples were fixed in 4% formaldehyde in D-PBS for 15 min, followed by either incubation in 0.5% Triton X-100 in D-PBS for 2 min, or 10 min in 100% methanol at -20°C. Samples destined for anti-tubulin staining were simultaneously fixed and extracted ("FGE method") in 4% formaldehyde + 0.25% glutaraldehyde (from 8% EM grade, Polysciences Inc., Warrington, PA) + 0.5% Triton X-100 in PEMG for 15 min, rinsed in PBS (3x 5min), and incubated in 0.5 mg/ml sodium borohydride in PBS (3x 5min) to reduce free aldehyde groups.

All samples were then rinsed in PBS (3x 5min), incubated in primary antibody solution for 30-45 min, rinsed again in PBS (3x 5min), and incubated in secondary antibody solution for 30-40 min. All antibodies were diluted in PBS + 1% normal donkey serum (Jackson Immunoresearch Labs., West Grove, PA). All secondary antibodies were made in donkey, conjugated to DTAF, Cy2, Texas Red, Cy3, or Cy5, and made species-specific by cross-adsorption ("ML" series,
Jackson ImmunoResearch Labs. Labeling of chromosomal DNA was achieved with a brief incubation in Hoechst dye #33258 (Pierce). Samples were mounted in 0.1% p-phenylenediamine in PBS + 50% glycerol.

*Microscopy techniques (as described in Chapter 1)*

Conventional immunofluorescence microscopy was carried out on a Zeiss Axiphot photomicroscope (Carl Zeiss Inc., Thornwood, NY) equipped for epifluorescence, and micrographs were taken on Kodak TMAX-400 film. Images were digitized by scanning the negative with a Nikon Coolscan Scanner (Nikon Inc., Electronic Imaging Dept., Melville, NY). Confocal microscopy was carried out on a Bio Rad MRC1000 system (Bio Rad Microscience, Hercules, CA) equipped with Kr/Ar laser, mounted on a Nikon Diaphot 200 microscope. All digitized images (from conventional and confocal microscopy) were cropped using Adobe Photoshop (versions 3.0 and 4.0, Adobe Systems Inc., Mountain View, CA), and imported into CorelDraw (versions 5.0 to 7.0, Corel Corp., Ottawa, Canada) for figure assembly. Figures were printed on a Kodak Colorease PS color printer (Eastman Kodak Comp., Rochester, NY).

*Live cell analysis*

COS-7 cells were seeded at 2x10^5 cells in growth medium (D-MEM + 10% FBS + penicillin + streptomycin + L-glutamine) on glass coverslips affixed to the bottom of 35mm homemade observation chambers, and grown for 24 hrs before transfection. Transfections were carried out for 6 hrs in 2 ml serum-free and antibiotic-free growth medium containing 2 µg total of plasmid DNA (pCMVH50m + pCNG2, or pCNG2 alone for control) and 8 µl of Lipofectamine, mixed according to supplier's instructions. In co-transfected samples, over 90% of overexpressors expressed both constructs. Cells were then returned to normal growth medium, and incubated at 37°C for 30-35 hrs before starting time-lapse observations.
Time-lapse observations were carried out using an inverted Leica DMIRB microscope (Leica, Wetzlar, Germany) equipped with temperature-, humidity- and CO2-controlled stage incubator, motor-driven Z-axis stage control and a 12V/100W halogen lamp (used at 7-9V) for fluorescence illumination. All recordings (made at 3 to 7 second intervals) were obtained using a Leica 40x/1.00 PL Fluotar (Ph3) objective and Photometrics liquid-cooled-CCD camera (CH250, Photometrics, Tucson, AZ). Camera settings, shutter, fine-focusing and epifluorescence filter wheels were all controlled through Metamorph software (version 2.5, Universal Imaging Corp., West Chester, PA).

**Sedimentation analysis**

Cells grown in 100mm dishes were harvested in D-PBS + 10 mM EDTA, counted, pelleted, and resuspended at equal cell density in buffer "A" (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.5 mM AEBSF, 10 µg/ml leupeptin, 1 mM TAME, 1 µg/ml aprotinin, 1 µg/ml pepstatin A), kept at 4°C for 15 min. Alternatively, pelleted cells were resuspended in buffer A without NP-40 present, and homogenized by 2 passes at 2000 rpm in a motor-driven teflon-glass homogenizer kept at 4°C. The cell lysates were airfuged at 30psi for 15 min, and the supernatant (cytosolic extract) was recovered. Sucrose gradients (4.8 ml, 5-20% in buffer A without NP-40) were prepared and 160 µl of each cytosolic extract (corresponding to approximately 5×10^6 cells) was carefully layered on top. These gradients were centrifuged in a SW50.1 rotor (Beckman Instruments, Palo Alto, CA) at 26,500 rpm for 18 hours at 4°C, and collected as 350 µl fractions. Sedimentation standards which included alcohol dehydrogenase (5S), and thyroglobulin (19S), were diluted in buffer A, and run with each experiment. Equal volumes of all fractions were analyzed by SDS-PAGE followed by western blotting, as described above.
Results

Effects of p50 overexpression on Golgi localization

As a first step in investigating p50 and dynactin function in vivo, I transiently transfected cultured mammalian cells with plasmid constructs driving high constitutive expression of the full-length wild type and myc-tagged human p50 coding region, under control of the CMV promoter. Both constructs produced the same striking phenotypic effects throughout the cell cycle.

When analyzed by immunofluorescence microscopy, the overexpressed p50 and p50myc were found diffusely throughout the cytoplasm and were excluded from the nucleus (Fig. 9a). I first examined the effects of p50 overexpression on localization of the Golgi complex in COS-7 and Rat2 cells, using the trans Golgi marker γ-adaptin, and the medial Golgi marker enzyme α-mannosidase I (Fig. 9). In clear contrast to control cells which typically exhibited tightly-clustered perinuclear staining patterns consisting of 1-3 contiguous elements, 80% of interphase p50 overexpressing cells showed numerous small vesicular structures scattered throughout the cytoplasm (Fig. 9b, f), and 15% showed a partial dispersal resulting in 3-5 contiguous structures. The observed range in the severity of Golgi disruption reflected the apparent level of p50 overexpression, as judged by immunofluorescence staining intensity. Similar results were obtained with COS-7 cells transfected to co-express p50myc with either GFP-tagged N-acetylglucosaminyl transferase I (NAGT-I), marking the medial Golgi (Fig. 9h), or VSV-G-tagged sialyl transferase, marking the trans Golgi (Rabouille, et al., 1995)(Fig. 10). Using both of these markers, I observed 80-85% of the co-expressing cells to exhibit p50myc-specific Golgi disruptions similar to those observed previously.

It could be argued that the observed redistribution of Golgi elements is caused either by a direct inhibition of retrograde microtubule-based transport, or more indirectly, by a disruption of the radial organization of the microtubule arrays in these cells. As a first test of the latter hypothesis, I examined the microtubule arrays of p50myc-overexpressing COS-7 cells. I found...
Figure 9: Effects of p50 overexpression on Golgi localization in cultured mammalian cells.

COS-7 (a-d) and Rat2 (e, f) cells were transfected to overexpress either wild type p50myc (a, b, e, f), or β-galactosidase (c, d), and double-labeled to reveal the overexpressed protein (a, c) and either γ-adaptin, a trans-Golgi marker (b, d), or α-mannosidase I, a medial Golgi marker (f). In other experiments, COS-7 cells were transfected to overexpress another medial Golgi marker, NAGT-I, fused to GFP, either alone (g), or with p50myc (h), and the GFP pattern was recorded (g, h). In all cases, p50myc overexpression resulted in extensive fragmentation and dispersal of Golgi elements, in contrast to control cells which typically showed a tightly-clustered, perinuclear Golgi complex.
that indeed, up to half of interphase p50myc-overexpressing COS-7 cells exhibiting a disrupted Golgi apparatus also show a less-focused microtubule organizing center, though the microtubule array often still appears to radiate from the perinuclear region (Fig. 10d). Nevertheless, Golgi fragmentation and dispersal were observed both in p50myc-overexpressing cells with normal, clearly-radial microtubule arrays (Fig. 10a,b), as well as in those with less well-organized arrays (Fig. 10c,d). No changes were observed in the sensitivity of microtubules to nocodazole-induced depolymerization, nor in the rate of centrosome-based microtubule regrowth after nocodazole washout (not shown). Also, no gross changes were detected in the actin cytoskeleton in response to p50myc overexpression (Fig. 10g,h).

In order to distinguish the effects of p50myc overexpression on the microtubule array from any potential effects on transport, it was necessary to observe the retrograde movements of Golgi-destined vesicles in living cells directly. For this purpose, I first treated the transfected cells with brefeldin A, which leads to a dramatic, yet reversible, resorption of the Golgi complex into the endoplasmic reticulum (ER) (Klausner, et al., 1992). Upon removal of the drug, control cells have been shown to reform a normal Golgi complex via microtubule-based retrograde transport of ER-derived vesicles. I therefore transfected COS-7 cells to co-express p50myc with GFP-NAGT-I to permit observations of Golgi elements in living cells using low-light level, time-lapse fluorescence microscopy. Staining of fixed cells showed that over 90% of GFP-positive cells were also overexpressing p50myc throughout these experiments.

As expected, control cells expressing the GFP-NAGT-I alone showed the appearance of GFP-NAGT-I-labeled vesicles throughout the cytoplasm within a few minutes after brefeldin A washout, followed by their rapid centripetal movement along linear tracks consistent with microtubules, leading to the reformation of a perinuclear Golgi complex after 25-30 min (Fig. 11a-e). In cells co-expressing p50myc, similar labeled vesicles also reappeared throughout the cytoplasm after brefeldin A washout, but these exhibited no sustained movements in any direction.
Figure 10: Effects of p50myc overexpression on cytoskeletal structures in COS-7 cells do not correlate with Golgi disruption.

(a-f) COS-7 cells were transiently transfected to co-express VSV-G-tagged ST with either p50myc (a-d), or β-galactosidase (e, f). More than 90% of overexpressing cells in co-transfected cultures were found to express both transfected constructs. Double-labeling with anti-tubulin (b, d, and f) with anti-VSV-G tag (a, c, and e) revealed clear Golgi disruptions in p50myc-transfected cells with normal radial microtubule arrays (a, b), as well as in those showing less well-focused arrays (c, d). Golgi distribution and microtubule organization in β-galactosidase-transfected cells (e, f) were indistinguishable from those of control untransfected cells. (g, h) COS-7 cells overexpressing p50myc alone and double-labeled with anti-myc tag (g) and rhodamine phalloidin (h) showed no apparent perturbations of the F-actin cytoskeleton.

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Figure 11: Time-lapse analysis of living COS-7 cells during recovery from brefeldin A directly demonstrates dynamitin-induced inhibition of ER-to-Golgi transport.

COS-7 cultures were transiently transfected with a GFP-NAGT-I fusion protein either alone (a-e), or with p50myc (f-j). Over 90% of overexpressing cells in fixed, co-transfected cultures were found to express both transfected constructs. After a 20 min exposure to brefeldin A, the drug was washed out and the GFP-NAGT-I fluorescence was recorded by time-lapse microscopy at 3 to 7 second intervals. Control cells typically exhibited rapid centripetal movements of newly-formed GFP-NAGT-I positive vesicles (arrows in a-e, arrowhead indicates static vesicle for reference), leading to the re-formation of a perinuclear Golgi complex (upper left corner in a-e). Similar labeled vesicles appeared throughout the cytoplasm of p50myc-transfected cells, but no sustained movements were observed in any direction (f-j). Note partial alignments of vesicles in (f-j), forming short linear arrays suggestive of interactions with microtubules. Bars, 1 μm.
(Fig. 11f-j), even after more than one hour of observation. I noted that many of these vesicles did align to form short linear arrays suggestive of static interactions with microtubules (Fig. 11f-j).

These data directly demonstrate that p50myc overexpression inhibits microtubule-based ER-to-Golgi transport, resulting in the accumulation of Golgi elements throughout the cytoplasm, most likely at or near their sites of emergence from the ER.

**Cells overexpressing p50 show prometaphase-like arrest with aberrant spindle morphology**

A higher than normal proportion of p50- and p50myc-overexpressing COS-7 cells appeared to be in mitosis, as judged by rounded morphologies and condensed chromosomes. To determine the magnitude of this effect, I compared the cell cycle index of p50myc-overexpressing cells with those of three separate control categories: untransfected cells, transfected but non-expressing cells, and transfected cells overexpressing an unrelated cytosolic protein, β-galactosidase ("β-gal transfectants") (Fig. 12). From a total of 6838 p50myc-overexpressing cells counted over 4 independent experiments, 9.4 ± 1.5% were in M-phase. Nearly all of these (96.3% of mitotic) showed a prometaphase-like chromosome configuration, having clearly undergone chromosome condensation and NEB but not metaphase chromosome alignment (Fig. 13, 14). Anaphase and telophase configurations were extremely rare in these cells. In striking contrast, the three control categories showed much lower indexes, with 4.2 ± 0.8% of untransfected cells, 4.2 ± 0.6% of transfected non-expressing cells and 3.6 ± 0.4% of β-gal transfectants found to be distributed throughout M-phase (Fig. 12).

The prometaphase-like p50myc-overexpressing cells were found to show significant spindle aberrations, as revealed by anti-tubulin staining (Fig. 13, 14). In nearly all cases, two half-spindles were observed, which typically showed marked asymmetry in overall size, shape and microtubule density, and appeared to be oriented independently. Generally, spindle poles were closer than normal to the cell periphery. In most cases, one half spindle was significantly more developed than the other (e.g. Fig. 13d-i). The microtubules of the larger half spindle were
Figure 12: Effect of p50 overexpression on mitotic progression.

Cell cycle indexes of control untransfected cells (open bars), transfected but non-expressing cells (hatched bars), β-galactosidase transfectants (shaded bars), and p50myc transfectants (black bars). Cell cycle phases were scored based on chromosome configurations. All values are means from 4 independent experiments (507<n<3283 cells/category/experiment) +/- SD. Values for p50myc transfectants were found to be significantly different from the three control categories (p < 0.05).
**Graph Description:**

- **Title:** The graph is labeled, indicating it compares different cell stages.
- **X-axis:** Represents different cell stages: Interphase, Prophase/Prometaphase, Metaphase, Anaphase, Telophase.
- **Y-axis:** Represents the percentage of cells counted.
- **Legend:**
  - Control untransfected cells
  - Transfected, non-expressing cells
  - β-Galactosidase-overexpressing cells
  - p50myc-overexpressing cells

**Observations:**

- **Interphase:** The control untransfected cells show a higher percentage compared to the transfected, non-expressing cells.
- **Prophase/Prometaphase:** Similar to Interphase, control untransfected cells have a higher percentage.
- **Metaphase:** All cell types show a significant drop in percentage, with control untransfected cells remaining the highest.
- **Anaphase:** There is a further decrease in percentage across all cell types, with control untransfected cells still leading.
- **Telophase:** A notable decrease is observed across all cell types, with control untransfected cells remaining significantly higher than the others.

**Conclusion:** The graph illustrates how different cell treatments affect the progression through the cell cycle, with control untransfected cells consistently having a higher percentage throughout the stages compared to the transfected and overexpressing cell types.
**Figure 13:** Effects of p50 overexpression on mitotic spindle morphology.

Immunofluorescence anti-tubulin staining (a-d, f, h) of mitotic β-galactosidase-overexpressing COS-7 cell (a), and p50myc-overexpressing cells (b-i) exhibiting a range of spindle distortions (arrows indicate spindle poles). Panels (b-d, f, h) illustrate pronounced asymmetry in microtubule density and orientation of half-spindles, as compared with the unperturbed spindle of a metaphase β-galactosidase-overexpressing COS-7 cell (a). Staining of overexpressed p50myc (e, g, i) reveals non-random chromosome distribution, seen as unstained regions which co-localize with dense areas of distorted spindles (particularly in e and i). Note non-expressing mitotic cell in panel (f) as further control. All cells were simultaneously fixed and extracted by "FGE" method (see Materials and Methods). All images were acquired by confocal microscopy. Single optical sections are shown for anti-p50myc staining (e, g, i) to facilitate localization of chromosomes. All other panels are through-focus maximal projections of complete x/y optical section stacks (24-40 sections, 0.4 μm step).
noticeably longer than those of control mitotic cells, and often formed a loose bundle which curved along the cell periphery as it splayed apart. These elongated microtubules were invariably found to end within the chromosome mass, which often formed a loose, U-shaped configuration around the microtubule bundle (Fig. 13e,i and Fig. 14a,c). Triple-labeling with anti-p50, anti-tubulin and a human CREST autoimmune serum confirmed that all kinetochores of p50myc transfecteds co-localized with spindle microtubules (Fig. 14c-f).

I also examined the distribution of CENP-E, a kinesin-like protein which accumulates on kinetochores during prometaphase, and has been implicated in chromosome congression (Yen, et al., 1992). Anti-CENP-E kinetochore staining intensity, which normally shows a marked decrease after metaphase (Yen, et al., 1992), remained high in the p50myc-overexpressing cells and clearly revealed that sister chromatids were unseparated (Fig. 14b), both consistent with the prolongation of a prometaphase-like state.

Tetrapolar spindles were occasionally noted in p50myc transfecteds fixed over 35 hours after transfection (as opposed to 27 to 33 hours for most experiments), which were not observed in controls. These cells, which were larger than normal and showed an increased chromosome mass, were probably in their second abnormal mitotic division.

**Overexpression of p50 in cultured cells causes dissociation of the dynactin complex**

Previous work has shown that in brain and testis cytosolic extracts, p50, p150\textsuperscript{Glu96}, and Arp1 all migrate at approximately 20S, indicating that they exist exclusively as a complex (Clark, et al., 1994; Paschal, et al., 1993). To investigate the molecular basis for the cytological defects observed in the present study, cytosolic extracts were prepared from control and transfected cells and analyzed by sucrose density gradient sedimentation (Fig. 15). Extracts of control untransfected and β-gal-transfected COS-7 cultures showed the four dynactin components examined, p150\textsuperscript{Glu96}, p62, p50 and Arp1, co-migrating at 18S. This slight divergence from the 20S value observed
**Figure 14:** Effect of p50 overexpression on kinetochore proteins.

Double immunofluorescence staining of mitotic p50myc-overexpressing cells with anti-p50 (a) and anti-CENP-E antiserum (b) shows bright staining of paired kinetochores. Triple-labelling with anti-p50myc (c), human CREST anti-centromere autoimmune serum (d), and anti-tubulin (e) confirms colocalization of kinetochores with spindle (panel f shows combination of d and e) (arrows indicate spindle poles). All images were acquired by confocal microscopy. Single optical sections are shown for anti-p50myc staining (a, c) to facilitate visualization of chromosomes. All other panels are through-focus maximal projections of complete x/y optical section stacks (21-22 sections, 0.4 μm step). Cell in (a, b) was fixed in formaldehyde followed by methanol, and cell in (c-f) was simultaneously fixed and extracted by “FGE” method (see Materials and Methods).
previously may reflect tissue-specific differences in dynactin subunit composition, as suggested by p150\textsuperscript{Glued} isoform heterogeneity reported in chicken tissue extracts (Gill, et al., 1991). In contrast, all of the dynactin components examined in cultures transfected with either p50myc or untagged p50 (not shown) exhibited the same abnormal behavior. In these samples, p150\textsuperscript{Glued} showed a dramatic shift, exhibiting a major peak at 9S, and additional streaking throughout the lower end of the gradient (Fig. 15). The Arp1 and p62 peaks showed the same small but consistent shift to 16-17S. Overexpressed myc-tagged and endogenous p50 could be distinguished by a slight difference in electrophoretic mobility. Both species migrated as a single peak at 5S. The cytoplasmic dynein ICs migrated at 20S in both control and experimental samples. Identical results were obtained with and without the use of detergent in the cytosol extraction buffer. Thus, these data indicate that dynactin, but not dynein, is specifically disrupted by p50 overexpression.

\textit{Cells overexpressing p50 show decreased dynactin and cytoplasmic dynein staining of prometaphase kinetochores}

To evaluate the effects of p50myc overexpression on dynactin and cytoplasmic dynein within the cells, mitotic transfectants were examined by immunofluorescence microscopy using antibodies to components of both complexes. Because of the large excess of p50myc in transfected cells, an association of recombinant p50 or p50myc with kinetochores could not be evaluated. However, mitotic transfectants expressing high levels of p50myc showed undetectable or lower than normal kinetochore staining with anti-p150\textsuperscript{Glued} and anti-Arp1 antibodies (not shown). The magnitude of this decrease was found to vary in proportion to the level of p50myc overexpression as judged by immunofluorescence staining intensity. In contrast, kinetochore staining with both anti-CENP-E antiserum (Fig.14b), and a human CREST anti-centromere auto-antiserum (Fig.14d) were not affected, arguing that general disruption of kinetochore structure does not occur. Anti-cytoplasmic dynein IC staining of kinetochores, because of its sensitivity to aldehyde fixation (Table 1), was difficult to evaluate in these samples.
It could be argued that the observed loss of p150\textsuperscript{Glued} and Arp1 was not due to p50-induced dissociation of the complex, but rather because the arrest phenotype may have occurred downstream of the normal loss of dynactin from kinetochores (see Chapter 1). Thus, in order to distinguish between these possibilities, I re-examined kinetochore staining in nocodazole-treated cells. The cells are thereby rendered incapable of forming the kinetochore-microtubule interactions which are normally associated with loss of cytoplasmic dynein, dynactin and other antigens, and the kinetochores are permitted to grow larger than usual. This had the added benefits that the immunofluorescence signals were maximized in control kinetochores, and any potential effects of kinetochore microtubules on antibody accessibility were also eliminated.

As expected, transfected non-expressing cells and β-gal-transfected cells showed brighter, enlarged kinetochore patterns under these conditions, often appearing crescent-shaped (Fig. 16, Fig. 17). In cells overexpressing high levels of p50myc, however, both anti-Arp1 (Fig. 16a) and anti-p150\textsuperscript{Glued} (Fig. 16a) showed clearly reduced kinetochore staining. Anti-cytoplasmic dynein IC staining of kinetochores in these cells also showed a clear decrease in intensity (Fig. 17). In general, the morphology of anti-IC-stained kinetochores was more variable than that seen with anti-p150\textsuperscript{Glued} and anti-Arp1, due to the effects of formaldehyde fixation on IC staining. Methanol fixation, which yields optimal kinetochore staining with anti-IC’s (Table 1), caused excessive loss of mitotic transfected cells, and therefore could not be used in these experiments. Nonetheless, careful examination of numerous p50-overexpressing cells over 5 separate experiments indicated that the decrease of IC staining at kinetochores was comparable to that seen for anti-Arp1 and anti-p150\textsuperscript{Glued}. In contrast, anti-CENP-E staining at kinetochores remained unchanged under these conditions (Fig. 17g).
Figure 15: Sedimentation analysis of dynactin and cytoplasmic dynein in p50 overexpressing cultures.

Cytosolic extracts of equal numbers of cells from untransfected, β-galactosidase-transfected, and p50myc-transfected COS-7 cultures were subjected to 5-20% sucrose density gradient sedimentation, followed by SDS-PAGE and Western blotting using antibodies to p50, p150Glued ("UP235"), Arp1 ("A27"), p62, and cytoplasmic dynein IC ("74.1"). Peak positions of sedimentation standards are shown.
Figure 16: Effect of p50 overexpression on anti-Arp1 and anti-p150\textsuperscript{Glued} staining of kinetochores. Nocodazole-treated (10\,\mu m, 3hrs at 37\,^{\circ}\text{C}) COS-7 cells transfected with p50myc (a, b, e, f), or β-galactosidase (c, d, g, h) were stained with “A27" anti-Arp1 (a, c) or anti-p150\textsuperscript{Glued} (e, g). Anti-p50myc (b, f) and anti-β-galactosidase (d, h) staining identifies overexpressing cells, and reveals chromosomal localization as unstained regions. (a, c, e, g) are through-focus maximal projections of complete x/y optical section stacks (14-22 sections, 0.35 \,\mu m step). (b, d, f, h) are single optical sections to facilitate visualization of chromosomes in overexpressing cells. Anti-Arp1 and anti-p150\textsuperscript{Glued} staining are displayed in pseudocolor to show intensity differences (color range shown in panel c). Neighboring non-expressing prometaphase cells offer an internal control in each field of view, to assess the decrease in anti-Arp1 and anti-p150\textsuperscript{Glued} kinetochore staining intensity. All cells were fixed in formaldehyde followed by methanol extraction.
Figure 17: Effect of p50 overexpression on anti-cytoplasmic dynein and anti-CENP-E staining of kinetochores.

Nocodazole-treated (10 μm, 3 hrs at 37°C) COS-7 cells transfected with p50myc (a-d, g, h), or β-galactosidase (e, f) were stained with anti-cytoplasmic dynein IC (a, c, e), or anti-CENP-E antiserum (g). Anti-p50myc (b, d, f) and anti-β-galactosidase (f) staining identifies overexpressing cells, and reveals chromosomal localization as unstained regions. (a, c, e, g) are through-focus maximal projections of complete x/y optical section stacks (20-26 sections, 0.4 μm step). (b, d, f, h, j, l) are single optical sections to facilitate visualization of chromosomes in overexpressing cells. Anti-IC and anti-CENP-E staining are displayed in pseudocolor to show intensity differences (color range shown in panel g). Neighboring non-expressing prometaphase cells offer an internal control in panels (a, c, e, g) to assess the differences in kinetochore staining intensity. All cells were fixed in formaldehyde followed by methanol extraction.
Discussion

In view of the remarkable disruptive effects of p50 overexpression on the dynactin complex, the Golgi complex, the interphase microtubule organizing center, and mitotic progression, I proposed the name dynamtin for this polypeptide.

Interphase phenotype: disruption of Golgi complex and microtubule organizing center

This study represented the first analysis of the effects of disrupting dynactin function in mammalian cells. I first documented two distinct cytological defects associated with dynamtin overexpression in cultured interphase cells: inhibition of microtubule-based ER-to-Golgi retrograde transport resulting in severe fragmentation and dispersal of the Golgi complex, and disruption of the microtubule-organizing center, resulting in non-radial microtubule arrays. Although it is conceivable that the latter effect may contribute in part to the dispersal of Golgi elements throughout the cytoplasm, my direct observations of living cells demonstrates that the two are distinct consequences of loss of dynactin function. Microtubule-based retrograde transport of Golgi elements is clearly inhibited in these cells, consistent with a loss of cytoplasmic dynein activity. This conclusion was also confirmed recently by similar time-lapse observations showing inhibition of ER-to-Golgi transport of a GFP/VSV G fusion protein in cells overexpressing chicken dynamtin (Presley, et al., 1997).

In a collaborative study with Dr. Janis Burkhardt (Univ. of Chicago, Chicago, IL), this Golgi disruption phenotype was replicated in cells microinjected with a function-blocking monoclonal antibody directed against cytoplasmic dynein ICs (Burkhardt, et al., 1997). This study also revealed that the scattered Golgi elements in dynamtin-overexpressing cells contain several markers of the cis, medial and trans Golgi, all co-localizing with two proteins, ERGIC-53 and ERD-2, which are normally found in the so-called ER-to-Golgi intermediate compartment (Burkhardt, et al., 1997; Schweizer, et al., 1990; Sonnichsen, et al., 1994). Electron microscopy showed that the arrested structures represent short Golgi stacks that closely resemble those
resulting from treatment with microtubule-depolymerizing agents (Burkhardt, et al., 1997; Pavelka and Ellinger, 1983; Thyberg and Moskalewski, 1985). These drug-induced “stacklets” have also been shown to contain the full complement of Golgi and intermediate compartment marker proteins (Cole, et al., 1996). All of these results are therefore consistent with dynactin and cytoplasmic dynein playing an obligate role in the transport of ER-derived vesicles towards the minus ends of microtubules, to form the Golgi complex.

The present results also suggest a role for dynactin and cytoplasmic dynein in maintaining the interphase microtubule organizing center, thus implying the ability of dynactin/dynein to transport nucleating material, and/or microtubules themselves as cargo. A similar function was suggested to underlie the organization of taxol-induced radial arrays assembled in mitotic extracts in vitro (Verde, et al., 1991), and more recently, for assembly of spindle-like bipolar arrays in vitro (Heald, et al., 1996). In both studies, untethered microtubules were observed to move with minus-ends leading along tethered microtubules, indeed consistent with the untethered microtubule being transported by dynein/dynactin. This raises the intriguing, and so far unfuted possibility that this could in fact be the role of the microtubule-binding domain found in p150Glued, i.e. to permit dynactin binding to untethered microtubules as cargo, as opposed to interacting with the microtubule being used for transport. Finally, this also raises the possibility that dynein and dynactin are also directly involved in organizing the mitotic spindle poles. Although, as discussed below, the present mitotic arrest phenotype appeared too complex at this stage to address this possibility unequivocally, further experiments described in Chapter 3 were designed to answer this question more precisely.

Mitotic phenotype

Overexpression of dynamitin also resulted in an increase in mitotic index, which correlated with a clear defect in spindle morphology. Based on the range of mitotic index values observed, it appears that mitosis in the overexpressing cells is prolonged but not completely blocked. This
conclusion is supported by the presence of tetrapolar cells among transfectants fixed at later time points, which, presumably, were in their second abnormal mitosis, having completed the first round without segregating their duplicated pair of centrosomes. Furthermore, the near-absence of anaphase and telophase cells among the dynamitin overexpressors also suggests reentry into G1 without completion of cell division.

Virtually all of the mitotic dynamitin-overexpressing cells had achieved centrosome duplication and separation, chromosome condensation, and NEB, but not metaphase chromosome alignment. Kinetochores remained paired, and showed prominent anti-CENP-E staining (Fig. 14). In all of these regards, the cells may be concluded to be in a state comparable to prometaphase. Thus, these observations represented the first direct in vivo evidence for a role for dynactin and cytoplasmic dynein in prometaphase. Furthermore, in view of the present and previous immunocytochemical localization data (see Chapter 1), these results are also consistent with a direct involvement of both complexes in kinetochore function.

Prometaphase involves a series of events, several of which could conceivably be disrupted by loss of dynactin and cytoplasmic dynein function. Following kinetochore maturation (Rieder, 1982), two types of prometaphase chromosome movements have been described, which are, in general, difficult to discriminate temporally but which can be seen as distinct processes in appropriate cells (reviewed by Rieder and Salmon, 1994). First, in vivo observations of late-attaching chromosomes in newt lung cells revealed the initial capture event to involve a tangential interaction between a kinetochore and the wall of a spindle microtubule (Hayden, et al., 1990; Rieder and Alexander, 1990). The mono-oriented chromosome then exhibited rapid poleward movement along the microtubule at a rate consistent with cytoplasmic dynein-driven organelle motility (Rieder and Alexander, 1990). Interference with dynein motor activity at this stage would be expected to leave chromosomes at their sites of initial capture, distributed at a variety of distances from the spindle poles. The apparently random distribution of chromosomes along the
spindle in dynamitin-overexpressing cells is, therefore, consistent with a role for dynactin and, presumably, dynein in poleward prometaphase movement.

Following the initial kinetochore-microtubule interaction, the kinetochore associates with and stabilizes the plus-ends of additional spindle microtubules (Mitchison, et al., 1986; Nicklas and Kubai, 1985; Spurck, et al., 1990; reviewed by Rieder and Salmon, 1994). Chromosome movement is now considerably slower than before and bidirectional, exhibiting low amplitude oscillations (Skibbens, et al., 1993), and leading to bi-orientation and congression to the metaphase plate. Assembly and disassembly of kinetochore microtubules is also intimately coupled to chromosome movement during this and subsequent mitotic phases (Gorbsky, et al., 1987; Mitchison, et al., 1986; Skibbens, et al., 1993; reviewed by Desai and Mitchison, 1995; Rieder and Salmon, 1994). While it is unclear whether or not this type of chromosome movement involves motor activity at all, its onset occurs while both dynein and dynactin are still prominent at the kinetochore. Thus, our observation of longer than normal and distorted spindle microtubule bundles in dynamitin-overexpressing cells may also reflect a role for dynactin and dynein during congression, in force production, microtubule dynamics, or both.

The basis for the more general distortion of the mitotic spindle and the displacement of the spindle poles toward the cell periphery in many of the dynamitin-overexpressing cells is uncertain. These effects may be secondary consequences resulting from the possible loss of kinetochore function. It should be noted, in this regard, that microinjection of CREST anti-centromere antisera, which were found to disrupt kinetochore assembly (Bernat, et al., 1991), also result in both chromosome misalignment (Bernat, et al., 1990; Simerly, et al., 1990), and spindle deformation (Bernat, et al., 1990). Alternatively, dynactin and cytoplasmic dynein may have additional cellular sites of action beside the kinetochore, which are responsible for the currently observed effects. Consistent with this possibility, mutational analysis of dynein and dynactin in lower eukaryotes has indicated a role in spindle positioning and elongation, suggesting a primary
site of action for the two complexes at the cell cortex (Clark and Meyer, 1994; Eshel, et al., 1993; Li, et al., 1993; Muhua, et al., 1994; Saunders, et al., 1995 Carminati and Stearns, 1997). Conceivably then, dynamitin overexpression may affect a comparable role for dynactin and cytoplasmic dynein in vertebrate spindle positioning. In this regard, microinjection of anticytoplasmic dynein antibodies into cultured vertebrate cells resulted in a blockage of bipolar spindle formation (Vaisberg, et al., 1993), a result which may be consistent with a role for a cortical dynein pool in exerting tension on the spindle poles. It should be noted, however, that the present dynamitin-induced spindle phenotype differs significantly from the phenotype reported by Vaisberg and colleagues. A recent study using microinjection of a different function-blocking antibody, in this case directed against the cytoplasmic dynein ICs, reported phenotypic effects on spindle organization closely similar to those seen with dynamtin overexpression (Gaglio, et al., 1997). An earlier study by the same group also confirmed the dynamtin-induced mitotic arrest phenotype reported here, including the dissociation of the dynactin complex (Gaglio, et al., 1996).

**Molecular Basis for Dynamitin-Induced Cytological Defects**

The molecular basis for the mitotic phenotype following dynamtin overexpression appears to be dissociation of the dynactin complex. The present sedimentation analysis shows that p150Glued and dynamtin detach from the Arp1 filament, which appears to remain intact and maintain its association with p62. This effect may indicate a role for dynamtin in linking p150Glued to the Arp1 filament.

According to one model (see Fig. 18a), the normal assembly of dynactin would involve two stages, the first being the formation of intermediate dynamtin-p150Glued and dynamtin-Arp1 sub-complexes. These sub-complexes would then associate via dynamtin to form the complete dynactin complex in the second stage. The presence of a predicted coiled coil domain within dynamtin indeed supports the possibility of self-association of this protein. Assuming that endogenous control of dynamtin expression levels normally cause this subunit to be the limiting
factor in this process, the second phase of this assembly reaction would be highly favored. However, for the same reason, this second phase of dynactin assembly would also be highly susceptible to inhibition by the presence of excess dynamitin, since this would permit the intermediate sub-complexes to associate with free dynamitin instead of each other (Fig. 18a). The finding that all dynamitin normally present in brain extracts is exclusively within the complete 20S complex (Paschal, et al., 1993) indeed supports the notion of dynamitin as a limiting factor in dynactin assembly. This situation is therefore analogous to that of antigen:antibody excess on immunodiffusion plates, in which suprastoichiometric levels of antigen interfere with precipitin formation. It should be noted, however, that no evidence of the p150$^{Gluad}$-dynamitin and Arp1-dynamitin sub-complexes, predicted by this model to be present in extracts from dynamitin-transfected cultures, were observed in the present sedimentation analysis. Of course, the absence of a multi-protein species, especially an assembly intermediate not normally found in cells, in this type of analysis is of little probative value. Alternatively, a second major model would propose that the excess dynamitin titrates out a limiting structural or regulatory factor critical to the proper assembly of the dynactin complex. Further work will be needed to test these models.

In view of the heterogeneous nature of the transiently-transfected cultures, the near-total fraction of dynactin which was observed to be dissociated in the transfected cell extracts indicates that this effect must have occurred at least in part after cell lysis. Throughout these experiments, approximately 10% of the cells in a typical transfected culture overexpressed dynamitin at high level, though lower levels of expression occurred in a higher fraction of the cells. Nonetheless, the disruptive effects of dynamitin appear to be virtually complete in cytosolic extracts, thus raising the possibility that this disruption occurred only after cell lysis. Preliminary experiments have confirmed that excess bacterially-expressed dynamitin, when added to control COS-7 cell extracts, can induce the same dynactin dissociation (not shown). Nevertheless, the disruption of dynactin within cells is strongly supported by my immunocytochemical results which showed a marked decrease in both p150$^{Gluad}$ and Arp1 immunoreactivity at the kinetochore of cells expressing high

-76-
levels of dynamitin (Fig. 16). These effects were observed in cells accumulated in mitosis by nocodazole treatment, as well as in non-drug-treated mitotic cells. Nocodazole treatment blocks mitosis prior to the stage at which dynactin and cytoplasmic dynein normally dissociate from the kinetochore. Even under these conditions, we observed loss of p150Glued and Arp1 kinetochore staining in dynamitin-overexpressing cells. These results support a direct disruption of dynactin by the excess dynamitin, resulting in inhibited recruitment of the complex to prometaphase kinetochores. Why both p150Glued and Arp1 were displaced is uncertain. This observation could either mean that dynamitin is important in targeting dynactin to kinetochores, or that the complex becomes unstable after dynamitin-induced dissociation.

Role of Dynactin in Cytoplasmic Dynein Function

Dynamitin overexpression caused a clear decrease in the association of cytoplasmic dynein with the kinetochore, implying a role for dynactin in mediating the association of cytoplasmic dynein with kinetochores and other organelles. As noted above, two broad models for dynactin function have been envisaged. First, it may serve to regulate dynein motor activity, and thereby act as an “activator” for transport. Dynactin was, in fact, initially described on the basis of its effect in stimulating the frequency of dynein-mediated organelle movements along microtubules in vitro (Gill, et al., 1991; Schroer and Sheetz, 1991). The present data offer little support for this view. Alternatively, dynactin may serve to mediate the binding of dynein to subcellular structures destined for retrograde transport (Vallee and Sheetz, 1996; Vaughan and Vallee, 1995). According to this model, inactivation of dynactin should serve to dissociate dynein from its subcellular binding sites (Fig. 18b). This is, in fact, what was observed in the present study. This latter model is also strongly supported by the identification of p150Glued as an IC-binding protein (Vaughan and Vallee, 1995), especially in light of its recent localization to the cargo-facing base of the dynein complex (Steffen, et al., 1996), and the role of the axonemal dynein ICs in targeting to the flagellar outer doublet microtubules (King and Witman, 1990).
Figure 18: Models of dynamitin-induced dynactin dissociation and implications for the role of dynactin in cytoplasmic dynein function.

(a) One model of dynamitin-induced dissociation of dynactin, based on disruption of a two-step assembly process involving formation of dynamitin-p150Glued and dynamitin-Arp1 intermediate sub-complexes (step I), followed by dynamitin self-assembly to yield the complete complex (step II). Excess dynamitin would therefore be predicted to inhibit the second step of this process, a situation analogous to that of antibody-antigen interactions in precipitin formation on immunodiffusion plates.

(b) Schematic representation of predicted fate of cytoplasmic dynein and dynactin at the kinetochores of dynamitin-overexpressing cells, according to two major models of dynactin function: model 1, in which dynactin is not involved in dynein binding to its cargo, would predict that dynein remains unaffected at kinetochores in dynactin-disrupted cells. Model 2, corresponding to the "receptor" model, predicts that dissociation of dynactin results in loss of cytoplasmic dynein binding to kinetochores and other cargo organelles destined for retrograde transport. The results of the present study (see Fig. 16, 17) are therefore consistent with the "receptor model" of dynactin function.
Dynactin may, therefore, serve as a “receptor” for cytoplasmic dynein on the surface of membranous organelles and kinetochores. However, it is structurally distinct from known cell surface receptors and its mode of attachment to kinetochores and membranous organelles is unknown. It is also likely to serve as more than a simple anchor for cytoplasmic dynein, especially with regard to kinetochore function. The microtubule-binding domain of the p150Glued subunit of dynactin could conceivably participate in kinetochore capture by mitotic microtubules. However, activity of this domain must be regulated if force production by cytoplasmic dynein is to result in productive poleward chromosome movement. Conceivably, the domain is active transiently, to serve only in loading kinetochores onto the kinetochore-to-pole microtubules. Alternatively, it is possible that dynactin is required throughout the transport process and that its microtubule-binding activity is regulated coordinately with the dynein cross-bridge cycle. Resolution of these issues promises to provide further insight into the transport mechanisms underlying mitosis and organelle movements.
CHAPTER 3

DETAILED ANALYSIS OF
DYNACTIN AND CYTOPLASMIC DYNEIN FUNCTION
IN MAMMALIAN MITOSIS

Introduction

Understanding the full range of cytoplasmic dynein and dynactin functions in vertebrate mitotic events has proven to be a difficult endeavor. First, the strong prometaphase arrest observed in dynamitin-overexpressing COS-7 cells did not allow for testing of possible further roles of dynactin and dynein in later phases of mitosis (and could not refute this possibility). Past efforts at elucidating cytoplasmic dynein function in mitosis have indeed suffered from the same limitations (Vaisberg, et al., 1993). Second, the identification of cellular sites of dynein and dynactin activity during vertebrate mitosis has been complicated by the complexity of the observed disruption phenotypes. Initial characterization of the dynamtin-induced prometaphase arrest phenotype suggested loss of dynactin and dynein function at several possible sites. Lack of chromosome alignment suggested a role in kinetochore function, consistent with the observed inhibition of the normal accumulation of dynein and dynactin at these structures (Chapter 2, Fig. 16, 17).

However, the presence of severe spindle distortions could also account for this lack of chromosome alignment, suggesting a role in spindle organization. Conversely, the spindle distortions could also be a secondary consequence of kinetochore defects, as previously observed with injection of anti-centromere antibodies (Bernat, et al., 1990). Finally, the aberrant positioning of the spindle within dynamitin-overexpressing cells is also consistent with a role in mediating the interaction of astral
microtubules with the cell cortex. Indeed, similar roles have been proposed for dynein and dynactin during mitosis in lower eukaryotes. Phenotypic analyses of mutant strains of *S. cerevisiae* containing cytoplasmic dynein and dynactin disruptions have suggested roles for the two complexes in orienting and positioning the mitotic spindle with respect to the bud neck (Clark and Meyer, 1994; Eshel, *et al.*, 1993; Li, *et al.*, 1993; Muhua, *et al.*, 1994), and in some aspects of anaphase chromosome movements (Saunders, *et al.*, 1995).

The series of experiments described in this chapter was therefore conducted to effectively dissect the dynactin-induced mitotic arrest phenotype, in order to address these issues further. Based on a broader and more detailed immunofluorescence analysis in fixed cells and time-lapse analyses in living cells, the data strongly support three major sites of dynactin/dynein activity in mammalian mitotic cells: prometaphase kinetochores, spindle poles, and the cell cortex. In addition, domain analysis of dynactin was initiated to gain new insights into its own function within the dynactin complex. Dynactin is revealed to be capable of self-association through at least two separate interaction domains and, with data from other sources, it is identified as the kinetochore-targeting subunit of dynactin.
Materials and Methods

Cell culture and transfections

Cell cultures (COS-7 from ATCC, and SA48 from Dr. J. Burkhardt, Univ. of Chicago, Chicago, IL; Rabouille, et al., 1995) were maintained as described in Chapter 1. Briefly, cultures were maintained as subconfluent monolayers in "growth medium": DMEM or F12K (Gibco/BRL) + 10% fetal calf serum (Gibco/BRL) + 100U/ml penicillin + 100 µg/ml streptomycin (Sigma). For SA48 cells, 400 µg/ml G-418 (Sigma) was added to the medium to maintain selection for transfected VSV-G-tagged sialyl transferase (Rabouille, et al., 1995). For immunofluorescence staining experiments, cells were trypsinized, and seeded onto sterile 18x18 mm glass coverslips in 6-well dishes (Corning) to reach 70-80% confluence after 48 hours.

For transient transfections, cells were seeded onto 18x18 mm glass coverslips in 6-well dishes at 1.5-2.5 x 10^5 cells per well, so that they reached ~60-70% confluence at the time of transfection (usually the next day). Once this density was reached, growth medium was rinsed off with Ca++- and Mg++-free PBS (D-PBS), and replaced with transfection mixture (each well contained 1 µg plasmid DNA with 4 µl Lipofectamine reagent (Gibco/BRL) in 1 ml DMEM or F12K, prepared as per supplier's instructions) for 6 hours before replacing again with growth medium. Transfected samples destined for sedimentation analysis were plated at equivalent densities in 100mm or 150mm tissue culture dishes (Corning), and treated as above, except that 3 ml or 8 ml of transfection medium was used per dish, respectively. Transfected cultures were either fixed or harvested 24-45 hours later (typically 24 hours for SA48 and 32-35 hours for COS-7).

cDNA constructs

pCMV-GFP-LpA, encoding the full length green fluorescent protein under the control of the CMV promoter (used in time lapse analyses of living cells) was a gift of Dr. H. Stunnenberg, EMBL, Heidelberg. Mutant dynamitin constructs used for domain analysis were prepared using
standard PCR cloning techniques, and the QuickChange mutagenesis kit (Stratagene). Mutant human dynamitin constructs used for the two-hybrid analysis were obtained from Daniel Starr and Michael Goldberg (Cornell Univ., Ithaca, NY).

**Live cell studies**

COS-7 cells were seeded at 2x10^5 cells in growth medium (F12K + 10% FBS + penicillin + streptomycin + L-glutamine) on glass coverslips affixed to the bottom of 35mm homemade observation chambers, and grown for 24 hrs before transfection. Transfections were carried out for 6 hrs in 2 ml serum-free and antibiotic-free growth medium containing 2 μg total of plasmid DNA (pCMVH50m + pCNG2, or pCNG2 alone for control) and 8 μl of Lipofectamine, mixed according to supplier's instructions. In co-transfected samples, over 90% of overexpressors expressed both constructs. Cells were then returned to normal growth medium, and incubated at 37°C for 30-35 hrs before starting time-lapse observations. Rhodamine-labeled bovine brain tubulin (used at ~2-4 mg/ml) was obtained from Dr. Sally Wheatley (Worcester Foundation).

Time-lapse observations were carried out using an inverted Leica DMIRB microscope (Leica, Wetzlar, Germany) equipped with temperature-, humidity- and CO2-controlled stage incubator, motor-driven Z-axis stage control, a 12V/100W halogen lamp (used at 7-9V) for fluorescence illumination, and a Leica micromanipulator. All recordings (made at 3 to 7 second intervals) were obtained using a Leica 40x/1.00 PL Fluotar (Ph3) objective and Photometrics liquid-cooled-CCD camera (CH250, Photometrics, Tucson, AZ). Camera settings, shutter, fine-focusing and epifluorescence filter wheels were all controlled through Metamorph software (version 2.5, Universal Imaging Corp., West Chester, PA).

**Immunohistochemical methods**

Antibodies used in this chapter were: "50-1A" ascites preparation of monoclonal anti-p50 (Paschal, *et al.*, 1993), "DART" rabbit polyclonal anti-p150GluLd antiserum (Vaughan and Vallee,
1995), rabbit antiserum against the myc epitope tag (courtesy of Dr. M. A. Gee, Worcester Foundation for Biomedical Research), “SH” human CREST autoimmune antiserum (Simerly, et al., 1990), monoclonal anti-tubulin (DM1A, Amersham), rabbit polyclonal anti-tubulin (gift from Dr. J.C. Bulinski, Columbia University, NY), monoclonal (Boehringer Mannheim Biochemicals) and rabbit polyclonal (5 Prime - 3 Prime Inc., Boulder, CO) anti-β-galactosidase, monoclonal ("P5D4", from Sigma) and rabbit polyclonal (from Dr. J. Burkhardt, Univ. Chicago, Chicago, IL) anti-VSV-G epitope, rabbit polyclonal anti-pericentrin “4B” and monoclonal anti-NuMA (“204-41”) were gifts from Dr. Steve Doxsey (Univ. Massachusetts, Worcester, MA), monoclonal anti-γ-tubulin was from Sigma, rabbit polyclonal anti-Mad2 was obtained from J. Waters (Univ. North Carolina, Chapel Hill, NC), with permission from R. Chen and A. Murray (UCSF, San Francisco, CA).

For immunofluorescence staining, cells grown on glass coverslips were briefly rinsed with D-PBS, and processed according to one of the following protocols. Some samples were pre-extracted with a 1 min incubation in 0.5% Triton X-100 (Pierce) in PEMG buffer [80 mM PIPES (pH 6.8), 5mM EGTA, 1 mM MgCl₂, 4 M glycerol], followed by either 10 min in 100% methanol at -20°C, or 15 min in 4% formaldehyde (from 16% EM grade, Electron Microscopy Sciences, Ft. Washington, PA) in PEMG. Some samples ("methanol fixation") were simply incubated for 10 min in 100% methanol at -20°C. Other samples were fixed in 4% formaldehyde in D-PBS for 15 min, followed by either incubation in 0.5% Triton X-100 in D-PBS for 2 min, or 10 min in 100% methanol at -20°C. Samples destined for anti-tubulin staining were simultaneously fixed and extracted ("FGE method") in 4% formaldehyde + 0.25% glutaraldehyde (from 8% EM grade, Polysciences Inc., Warrington, PA) + 0.5% Triton X-100 in PEMG for 15 min, rinsed in PBS (3x 5min), and incubated in 0.5 mg/ml sodium borohydride in PBS (3x 5min) to reduce free aldehyde groups.
All samples were then rinsed in PBS (3x 5min), incubated in primary antibody solution for 30-45 min, rinsed again in PBS (3x 5min), and incubated in secondary antibody solution for 30-40 min. All antibodies were diluted in PBS + 1% normal donkey serum (Jackson Immunoresearch Labs., West Grove, PA). All secondary antibodies were made in donkey, conjugated to DTAF, Cy2, Texas Red, Cy3, or Cy5, and made species-specific by cross-adsorption ("ML" series, Jackson Immunoresearch Labs). Labeling of chromosomal DNA was achieved with a brief incubation in Hoechst dye #33258 (Pierce). Samples were mounted in 0.1% p-phenylenediamine in PBS + 50% glycerol.

For western blotting, samples were separated by SDS-PAGE, and electrophoretically-transferred to a PVDF membrane (Immobilon P, Millipore, Bedford, MA). After blocking in 5% nonfat dry milk in TBS + 0.1% Tween-20 (TBST) at 4°C overnight, the blot was incubated with primary antibody diluted in TBST + 1% milk for 1 hr, washed (3x 10 min in TBST+ 1% milk), then incubated for 40 min in HRP-conjugated donkey anti-mouse or anti-rabbit IgG diluted 1:10,000 in TBST + 1% milk. After final washing (3x 10 min in TBST), signal detection was achieved by enhanced chemiluminescence (Amersham Corp.).

**Microscopy techniques (as described in Chapters 1, 2)**

Conventional fluorescence microscopy was carried out on a Zeiss Axiophot photomicroscope (Carl Zeiss Inc., Thornwood, NY) equipped for epifluorescence, and micrographs were taken on Kodak TMAX-400 film. Images were digitized by scanning the negative with a Nikon Coolscan Scanner (Nikon Inc., Electronic Imaging Dept., Melville, NY). Confocal microscopy was carried out on a Bio Rad MRC1000 (Bio Rad Microscience, Hercules, CA) equipped with Kr/Ar laser, mounted on a Nikon Diaphot 200 microscope. All digitized images were cropped using Adobe Photoshop (versions 3.0 and 4.0, Adobe Systems Inc., Mountain View, CA), and imported into CorelDraw (v. 7.0, Corel Corp., Ottawa, Canada) for figure assembly. Figures were printed on a Kodak Colorease PS printer (Eastman Kodak Comp., Rochester, NY).
**Sedimentation analysis (as described in Chapter 2)**

Cells grown in 100mm dishes were harvested in D-PBS + 10 mM EDTA, counted, pelleted, and resuspended at equal cell density in buffer "A" (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.5 mM AEBSF, 10 μg/ml leupeptin, 1 mM TAME, 1 μg/ml aprotinin, 1 μg/ml pepstatin A), kept at 4°C for 15 min. Alternatively, pelleted cells were resuspended in buffer A without NP-40 present, and homogenized by 2 passes at 2000 rpm in a motor-driven teflon-glass homogenizer kept at 4°C. The cell lysates were airfuged at 30 psi for 15 min, and the supernatant (cytosolic extract) was recovered. Sucrose gradients (4.8 ml, 5-20% in buffer A without NP-40) were prepared and 160 μl of each cytosolic extract (corresponding to approximately 5x10^6 cells) was carefully layered on top. These gradients were centrifuged in a SW50.1 rotor (Beckman Instruments, Palo Alto, CA) at 26,500 rpm for 18 hours at 4°C, and collected as 350 μl fractions. Sedimentation standards which included alcohol dehydrogenase (5S), apoferritin (13S), and thyroglobulin (19S), were diluted in buffer A, and run with each experiment. Equal volumes of all fractions were analyzed by SDS-PAGE followed by western blotting, as described above.

**Two-hybrid assay**

Two-hybrid assays based on the GAL4 system (Bai and Elledge, 1996) were conducted using full-length, wild-type human dynamin as "bait" (fused to the GAL4 DNA-binding domain in the pAS2 vector), and a panel of dynamin truncation mutants as potential "prey" (fused to the activation domain, in pACT2). Appropriate bait and prey combinations were co-transformed into the yeast host strain, Y190, plated onto selective media, and grown for 3 days. Colony growth was then recorded, and colonies were picked and transferred to a fresh selective plate in a grid pattern, and grown overnight. Colonies were transferred onto a #1 3M filter disk, and an X-gal colony filter assay was conducted to detect β-galactosidase activity as outlined in (Bai and Elledge, 1996).
Results

Time-lapse analysis of mitotic arrest phenotype in living dynamitin-overexpressing cells

In order to better understand the specific defects which gave rise to the spindle distortions observed in fixed dynamitin overexpressing cells, a study of living cells was initiated using time-lapse microscopy. Cultures of COS-7 cells were transiently transfected to co-express dynamitin and GFP, or alternately, GFP alone for control observations. More than 90% of overexpressing cells in fixed, co-transfected cultures were found to express both transfected constructs, and the intensity of GFP fluorescence was found to vary in proportion with the level of overexpressed dynamitin (data not shown). The GFP fluorescence therefore permitted the identification of living cells overexpressing dynamitin, and could be used as an indicator of the level of dynamitin overexpression. Cells were either observed by time-lapse phase contrast microscopy or, in some cases, microinjected with rhodamine-labeled tubulin to permit time-lapse fluorescence microscopy observations of their microtubule arrays. Injections typically occurred at prometaphase or metaphase, and images were recorded at 3-7 second intervals over the following hour or more.

Control cells (untransfected or transfected with GFP alone, with or without tubulin injections) (Fig. 19) were found to progress from nuclear envelope breakdown to the end of telophase within ~20 min, spending most of that time (13-18 min) in prometaphase and metaphase. Although early prometaphase spindles sometimes showed slight asymmetry or axial curvature, all became highly-symmetrical within a few minutes, showing little or no changes in axis orientation throughout the rest of mitosis. The events following anaphase onset (as judged by sister chromatid separation) were always completed quickly with little or no variation, within 4-6 min.

Co-transfected cells which showed very intense GFP fluorescence, indicating high levels of exogenous dynamitin expression, were invariably found to remain arrested in a prometaphase-like state for over one hour, and in some cases, more than 4 hours. These cells exhibited severely-distorted microtubule arrays (Fig. 20), similar to those observed previously in fixed cells.
Figure 19: Time-lapse analysis of mitosis in control cells.

(a-h) Single frame images from time-lapse series recorded by phase-contrast microscopy of an untransfected, uninjected control cell reveals typical kinetics and characteristics of major mitotic events, starting just after NEB (time 0, a), reaching metaphase after ~13 min (d), followed by rapid anaphase A (f) and B (g), and telophase (h). Note the late disjunction of one chromatid pair which becomes stretched during anaphase B, and is finally separated at telophase (h). This was occasionally seen in control cells, and in all cases observed, the forces pulling the two half spindles apart during anaphase B clearly outweighed those keeping the chromatids together, resulting in separation, as seen here. (Elapsed time shown in minutes:seconds.milliseconds)

(i-o) Single frame images from time-lapse series recorded by phase contrast (i, o) and fluorescence microscopy of a control, untransfected cell starting approx. 2 minutes after microinjection of rhodamine-tubulin to visualize microtubules.

Bars, 5 μm.
Figure 20: Time-lapse analysis of microtubule array in COS-7 cell overexpressing high levels of dynamitin.

Single frame images from time-lapse series recorded over 50 min, using fluorescence microscopy to visualize microinjected rhodamine tubulin (b-h) in a cell co-expressing high levels of GFP (a) and dynamitin. Cell was microinjected approx. 3 min before start of time lapse recording. The bulk of the microtubule mass appears disorganized, coursing throughout the cytoplasm with no apparent spindle shape. Two putative poles are found at one extremity of cell, as focus plane is brought near top of cell (g, h). Multiple small microtubule foci were noted around the periphery of the main microtubule mass (arrows in c-f): these showed no sustained movements in any direction. This cell remained in this prometaphase-like state for over 3 hours. Bar, 5 μm.
Two major microtubule foci, presumably the poles, were identified in most cases, but the bulk of the microtubule mass was disorganized, coursing throughout the cell, and leaving chromosomes scattered around it (not shown). Smaller supernumerary microtubule foci were observed often in such cells (arrows in Fig. 20c-f), and their connections to the rest of the microtubule array were usually tenuous.

Cells overexpressing moderate levels of dynamitin were also examined, with the expectation that a partial inhibition of dynactin/dynein function would yield more interpretable and informative phenotypes. Co-transfected cells exhibiting lower levels of GFP fluorescence, indicating moderate levels of dynamitin overexpression, were indeed found to exhibit more subtle spindle defects, although these cells, too, remained arrested prior to anaphase onset for more than one hour. Such cells were found to assemble normal-looking bipolar spindles with congressed chromosomes, usually forming a clear metaphase plate (Fig. 21, 22, 23). These features proved to be useful landmarks which helped reveal several distinct anomalies.

First, astral microtubules directed towards the cell periphery were found to be over-elongated and much more sinuous than observed in control cells (Fig. 21, arrows). Such microtubules are normally relatively short, highly dynamic and under tensional forces which render them straight, connecting the spindle poles to the cell cortex, and helping to orient the spindle within the cell (reviewed by Hyman and Karsenti, 1996; Rieder and Salmon, 1994). The increased curvature and length of these microtubules in dynamitin-overexpressing cells is therefore consistent with a loss of tension and changes in growth/shrinkage dynamics permitting excessive growth. As seen in high-level overexpressors, multiple aster-like microtubule foci were also observed in moderate overexpressors (Fig. 21, arrowheads). However, in this case, the foci exhibited sustained movements along linear trajectories ending at spindle poles (Fig. 21). Since no chromosomes were observed in association with these foci, and in view of the nature and destination of the transport events, it appears most likely that these observations reveal the normal process of spindle
Figure 21: Time lapse analysis of COS-7 cell overexpressing moderate levels of dynamitin: effects on astral microtubules and spindle pole assembly.

(a-e) Single frame images from time-lapse series recorded using fluorescence microscopy to visualize microinjected rhodamine tubulin (b-n) in a COS-7 cell co-expressing moderate levels of GFP (a), and dynamitin. Cell was microinjected approx. 3 min before start of time-lapse recording. Cell maintained a symmetrical, control-like bipolar spindle with fully-congressed chromosomes for over 1 hour. Astral microtubules were found to be much longer and more sinuous (arrows) than those of control cells, and several ectopic microtubule foci (arrowheads) were observed to undergo sustained movements along linear trajectories towards the two main poles, eventually fusing with them. Bar, 5 μm.
pole assembly, rendered significantly less efficient due to the partial inhibition of dynactin/dynein function by the moderate excess of dynamitin.

Consistent with this conclusion, other cells expressing moderate levels of dynamitin, which remained arrested for over 20 minutes with well-formed bipolar spindles and fully-congressed chromosomes were seen to undergo dramatic spindle pole fragmentation events. Figure 22 shows an example of such an event, giving rise to a third pole and associated half-spindle which both migrate within the cell to the opposite side of the facing pole, resulting in the presence of two bipolar spindles joined at the intact pole, with two metaphase plates. The very distinctive spindle and chromosome configurations which result from these fragmentation events were observed very rarely in control cultures, but in contrast, very commonly in fixed populations of dynamitin-overexpressing cells (Fig. 22i-n). Since these cells exhibited fully-congressed chromosomes, it appears unlikely that the observed defects resulted from loss of dynactin/dynein function at kinetochores, and, therefore, these data offer strong support for dynactin/dynein activity at spindle poles, both for initial assembly and subsequent maintenance. These data also strongly suggest that the severe spindle distortions observed in dynamitin-overexpressing cells result in part from repeated spindle pole fragmentation events.

Finally, two examples of dynamitin-overexpressing cells were noted wherein metaphase configurations were successfully formed, and anaphase onset was achieved, with the same aberrant results. In both cases, anaphase A movement of separated chromatids appeared normal (Fig. 23b-d), but anaphase B movement of poles with associated half-spindles and chromatids failed, apparently due to the presence of a single unseparated chromatid pair (Fig. 23f, arrow). This caused one half-spindle to rotate off-axis as one side of it continued to move outwards, while the other, containing the unseparated chromosome, was apparently dragged back in the opposite direction by the other half spindle moving toward its extremity of the cell. The displaced half-spindle was then observed in both cases to become “trapped” within the cleavage furrow.
Figure 22: Time lapse and fixed cell analyses of COS-7 cells overexpressing moderate levels of dynamitin: spindle pole fragmentation events reveal dynactin/dynein role in pole maintenance.

(a-h) Single frame images from time-lapse series recorded by phase-contrast (a, h), and fluorescence microscopy to image microinjected rhodamine tubulin (c-g) in COS-7 cell co-expressing moderate levels of GFP (b), and dynamitin. Cell was microinjected in late prometaphase, approx. 3 min before start of time-lapse recording. Cell maintained a symmetrical, control-like spindle with fully-congressed chromosomes and two normal-looking poles (arrows in c) for approx. 25 min. One spindle pole then fragmented approximately in half, and the new pole (arrow with asterisk in d-g) migrated to the opposite end of the cell (d-g), on the other side of the intact pole. A second, smaller bipolar spindle was thereby formed, apparently still joined to the first spindle, and complete with a second metaphase plate (h). This cell remained arrested in this state, with 3 poles, for at least 2 hours.

(i-n) Fixed populations of COS-7 cells overexpressing moderate levels of dynamitin reveal characteristic spindle configurations consistent with pole fragmentation events, including apparently split poles (i, j), tripolar spindles (k), ejected poles (l, m), and fully-fragmented poles (n).

Bars, 5 μm.
Figure 23: Time-lapse analysis of dynamitin-overexpressing COS-7 cell after anaphase onset reveals role for dynactin/dynein in anaphase B movements.

(a-j) Single frame images from time-lapse series recorded by phase-contrast microscopy of COS-7 cell co-expressing moderate levels of GFP and dynamitin. Recording started at anaphase onset (a). Anaphase A movement of chromatids toward the poles (white arrows) appeared normal (b-d). Anaphase B movement of the right-hand half-spindle was disrupted, apparently by one non-disjoined chromatid pair (black arrow in f) which caused it to turn off-axis, becoming parallel to the growth substrate (clearest in f, g), and get “dragged” back into the furrow area as the other half-spindle underwent apparently normal anaphase B.
(Fig. 23i, j), which formed and constricted with the same kinetics seen in control cells. These data are consistent with a loss of outward pole-to-cortex pulling forces during anaphase B, with pole-to-pole outward pushing forces being apparently unaffected. These data are therefore consistent with a loss of dynactin/dynein function both at kinetochores, resulting in the defect in sister chromatid disjunction, and at the cell cortex, resulting in a loss of outward pulling forces on astral microtubules during anaphase B.

Analysis of spindle pole components in fixed dynamitin-overexpressing cells

The observations of multiple microtubule foci and pole fragmentation events in living dynamitin-overexpressing cells clearly warranted an investigation of the localization of centrosomal components in these cells. Three spindle pole markers were examined in fixed dynamitin-overexpressing cells. The first two, γ-tubulin and pericentrin, have been localized to the pericentriolar material of centrosomes, and have been directly implicated in microtubule nucleation (Doxsey, et al., 1994; Zheng, et al., 1995). The third marker, NuMA, has been localized inside the nucleus during interphase, and along spindle microtubules during mitosis, accumulating near their minus ends and forming “collars” just outside the pericentriolar region of spindle poles (Lyderson and Pettijohn, 1980). Several lines of evidence have implicated NuMA in spindle function (reviewed in Cleveland, 1995), and it was recently shown to co-immunoprecipitate with dynactin and cytoplasmic dynein from Xenopus egg extracts (Merdes, et al., 1996).

Of the three, NuMA localization was found to be most disrupted in dynamitin-overexpressing cells, showing numerous scattered accumulations of staining in almost all mitotically-arrested cells examined (Fig. 24). Pericentrin and γ-tubulin, both of which normally show a more discrete localization strictly restricted to the poles themselves (Fig. 24), also revealed smaller accumulations of staining in the ectopic microtubule foci of dynamitin-overexpressing cells (Fig. 24). The level of pericentrin and γ-tubulin staining always appeared high and relatively constant within two poles of all prometaphase dynamitin-overexpressing and control cells,
**Figure 24**: Immunofluorescence analysis of spindle pole components in fixed dynamitin-overexpressing COS-7 cells.

Confocal immunofluorescence microscopy of fixed control (a, b) or dynamitin-overexpressing (c-f) COS-7 cells double-labeled with anti-tubulin (a, c) and anti-NuMA (b, d), or triple-labeled (e, f) with anti-tubulin (red in e, f), anti-dynamitin (blue in e, f), and either anti-γ-tubulin (green/yellow in e) or anti-pericentrin (green/yellow in f). In contrast to the “collar-like” distribution near poles in control cells, anti-NuMA staining reveals multiple small ectopic foci in all mitotic dynamitin-overexpressing cells. Both anti-γ-tubulin and anti-pericentrin show normal-looking staining at both poles in dynamitin-overexpressing cells, but also show significant accumulations at major ectopic poles, as seen in these two examples (e, f).
presumably corresponding to centriole-containing poles, but showed considerable variability in the smaller ectopic foci. Cells exhibiting numerous smaller foci showed proportionally less detectable pericentrin and γ-tubulin staining in all except two foci (presumably again corresponding to the centriole pair-containing poles). These data are consistent with a decreased cohesion of pericentriolar material within the spindle poles of dynamitin-overexpressing cells, permitting repeated fragmentation events which result in the formation of ectopic poles and severe spindle distortions.

Analysis of kinetochore-bound checkpoint protein and spindle orientation in fixed SA48 cells

To gain further insight into dynactin/dynein function during mammalian mitosis, the dynamitin-induced arrest phenotype was examined in a second cell line, SA48, a derivative of the human epithelial HeLa line which expresses a stably-transfected epitope-tagged Golgi enzyme (Rabouille, et al., 1995). As noted in COS-7 cells overexpressing moderate levels of dynamitin, many SA48 cells overexpressing wild type dynamitin were found to exhibit more subtle mitotic defects. Cell cycle index graphs indicate a less robust arrest in prometaphase, more cells progressing past anaphase onset, and interestingly, a second arrest point at metaphase (Fig. 25). Fewer spindle distortions were observed among prometaphase-arrested cells, although the higher overexpressors did exhibit severe distortions similar to those observed in COS-7 cells. Overexpression of a mutant form of dynamitin, called ΔHTH (see domain analysis, below), yielded a stronger phenotype, with severe spindle distortions observed in the wide majority of prometaphase-arrested cells, similar to those reported for COS-7 cells. These data suggest that overexpression of wild type dynamitin blocks dynactin function less efficiently in SA48 than in COS-7, perhaps because of differences in the natural turnover rates of the complex in the two cell lines (see dissociation model, Chapter 2, Fig. 18). Nevertheless, a second arrest point at metaphase was still noted with the more potent ΔHTH construct, suggesting an additional function for dynactin/dynein.
**Figure 25:** Effect of dynamitin overexpression on mitotic progression in SA48 cells.

Cell cycle indexes of control untransfected cells (grey bars), dynamitin-overexpressing cells (red bars), and ΔHTH-overexpressing cells (green bars). Results from COS-7 cells (a, from Fig. 12) are included for comparison with results from SA48 cells (b). Cell cycle phases were scored based on chromosome configurations. All values are means from 3 (for SA48) or 4 (for COS-7) independent experiments (+/- SD).
Untransfected control COS cells
Dynamitin-overexpressing COS cells

Control Untransfected SA48 cells
Dynamitin-overexpressing SA48 cells
ΔHTH-overexpressing SA48 cells
The finding of a second arrest point at metaphase was of interest for several reasons. Although this suggested that the role of dynactin and dynein in prometaphase kinetochore function may not be absolutely essential for congression in these cells, it also suggested a new role in the anaphase onset checkpoint pathway, downstream of congression, and apparently independent of spindle organization. In order to better understand the nature of this arrest state with respect to the known checkpoint machinery, I examined the fate of Mad2, a kinetochore-bound component of this machinery (Chen, et al., 1996; Li and Murray, 1991; Li and Benezra, 1996). Mad2 normally accumulates transiently on kinetochores after NEB, and its release, during congression, has been correlated with establishment of the kinetochore-microtubule interactions which are necessary for passage of the anaphase onset checkpoint (Waters, et al., 1997). As expected, 100% of control and dynamitin-overexpressing prometaphase cells were found to contain numerous Mad2-positive kinetochores (not shown). In control cultures, 47% of cells which exhibited a metaphase chromosome configuration still contained at least one Mad2-positive kinetochore, while the other 53% showed a total absence of anti-Mad2 staining from all kinetochores (Fig. 26). In contrast, approximately 71% of dynamitin-overexpressing cells in metaphase were found to exhibit Mad2-positive kinetochores, indicating that these cells retain Mad2 significantly longer than control cells. These data therefore suggest a new role for dynactin/dynein in kinetochore function, i.e. establishing the functional kinetochore-microtubule interactions which are necessary for release of Mad2 and for passage of the anaphase onset checkpoint.

The finding of metaphase-arrested dynamitin-overexpressing cells was also of value because these exhibited a clear, single spindle axis which could be compared to that of control cells to clearly assay for effects on spindle orientation. In control SA48 cultures, the wide majority of metaphase cells (86%) exhibited a spindle axis oriented parallel to the growth substrate (Fig. 27). In the metaphase dynamitin-overexpressing cells, however, this number was reduced to only 57%, and a concomitant increase was noted in the proportion of cells showing a clearly tilted spindle axis (Fig. 27). Since all of these spindles appeared otherwise well-formed, and since no correlation was
Figure 26: Effect of dynamitin overexpression on kinetochore localization of Mad2 in SA48 cells.

Metaphase cells from control SA48 cultures were scored for the presence or absence of Mad2-positive kinetochores, and compared with SA48 cultures overexpressing either β-galactosidase or wild type dynamitin. Examples of representative cells are shown, triple-labeled for dynamitin (blue), Mad2 (green), and CREST anti-centromere serum (red). Dynamitin-overexpressing SA48 cells retain Mad2-positive kinetochores longer than control cells.
Analysis of MAD2p Staining in Metaphase Cells

- Control cells
- beta-Gal-overexpressing cells
- Dynamin-overexpressing cells

% of cells analyzed

MAD2p-positive
MAD2p-negative
Figure 27: Analysis of spindle axis orientation in metaphase dynamitin-overexpressing SA48 cells.

Spindle axis orientation was examined in metaphase cells from control untransfected SA48 cultures (grey bars), and in dynamitin-overexpressing SA48 cell populations (red bars). Spindle axis orientation was scored as either parallel (first category), tilted (second category), or perpendicular (third category) with respect to the growth substrate, as determined on the basis of DAPI-stained chromosome position and anti-tubulin staining to reveal spindle microtubules. All results are from 3 independent experiments (+/- SD). Differences between control and dynamitin-overexpressing cells in the first two categories were found to be statistically significant (p<0.05).
Control untransfected SA48 cells
Dynamitin-overexpressing SA48 cells

% of cells analyzed
found between spindle axis orientation and retention of Mad2 staining, these data are most consistent with a defect in interactions of astral microtubules with the cell cortex, resulting in aberrant spindle orientation. These observations may therefore represent a second manifestation of the same defect which gave rise to the defective outward pulling forces observed during anaphase B in living dynamitin overexpressing cells (see above).

**Domain analysis of dynamitin**

While the above series of experiments yielded new insights into the function of dynactin during mitosis, the following experiments were designed primarily to gain further insights into the function of dynamitin itself. A battery of mutant human dynamitin constructs was prepared, including C- and N-terminal truncations, internal deletions, and point mutations (Table 2). Locations and types of mutations were chosen primarily based on structural predictions (Chapter 1, Fig. 3). Each construct was transfected into cultured SA48 cells, to be assayed for its ability to induce the same mitotic and Golgi defects observed using wild type dynamitin (Chapter 2). The results of this analysis are summarized in Table 2. First, overexpression of either N-terminal or C-terminal truncation constructs (N105myc, N136myc, C311myc, C218myc) was found to cause little or no detectable effects on either mitotic progression or Golgi organization, indicating that these constructs apparently did not affect the dynactin/dynein pathway (Fig. 28). Both N-terminally-truncated proteins, N105myc and N136myc, exhibited clear aggregation within the cytoplasm of overexpressing cells (Fig. 28b), suggesting inappropriate folding of these polypeptides and reduced solubility. This conclusion was supported by bacterial expression of the N105myc construct, which yielded less than 10% soluble protein, in contrast to wild type dynamitin which typically yields more than 60% soluble protein (not shown).
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</table>
Constructs containing smaller internal modifications were therefore examined. Single proline insertions meant to disrupt each of the three highly-charged putative α-helices (see Chapter 1, Fig. 3, Table 2) revealed a striking effect when the putative coiled coil region between residues 105 and 135, was targeted. Overexpression of this construct, named V122Pmyc, caused the same mitotic arrest and Golgi disruption phenotypes observed with wild type dynamitin, but with significantly higher potency (Fig. 29). Although the range of cytological defects remained the same, the apparent minimal threshold of V122Pmyc overexpression required to cause these defects was clearly lower than that of wild-type dynamitin. Proline insertion constructs targeting the other two major helices, named L236Pmyc and V295Pmyc, showed no changes in potency compared to wild type dynamitin (Fig. 29).

An increase in dominant negative potency similar to that of V122Pmyc was also observed with ΔTHmym, another mutant construct in which the 18 residues immediately following the putative coiled coil domain were deleted (Fig. 29). These residues correspond to the part of the putative HTH-like motif (see Chapter 1) which does not overlap with the predicted coiled coil region. While this domain is not expected to confer a DNA-binding ability, structural predictions do suggest that these residues, predicted as a highly-charged random coil followed by a short helix, should appear on the polypeptide surface, making them good candidates for mediating protein-protein interactions.

These results warranted a close comparison with another construct, ΔHTHmym, wherein the entire 25-residue HTH-like motif is deleted, affecting both the coiled coil structure and the subsequent region targeted in ΔTH. Overexpressed ΔHTHmym was found to cause the same mitotic arrest and Golgi disruption phenotypes, but with even higher apparent potency than V122Pmyc and ΔTHmym (Fig. 29). In this case, disruption phenotypes appeared more uniformly severe, exhibiting an even lower expression threshold for causing dominant negative inhibition of dynactin function. Conversely, however, sedimentation analysis revealed that ΔHTHmym is
Figure 28: Domain analysis of human dynamitin using N- and C-terminal truncation mutants

(a) Cell cycle index graph showing effect of overexpression of dynamitin truncation mutants on mitotic progression in SA48 cells. All results are means of 3 experiments (+/- SD).

(b, c) Immunofluorescence staining of SA48 cell overexpressing N105myc showing aggregation of overexpressed protein (detected with anti-myc in b) and lack of disruptive effects on Golgi organization, as revealed by sialyl transferase localization (c).
Control untransfected cells
Dynamitin-overexpressing cells
N105myc-overexpressing cells
N136myc-overexpressing cells
C311myc-overexpressing cells
C218myc-overexpressing cells

Interphase
Prophase/Prometaphase
Metaphase
Anaphase
Telophase

% cells counted

b

c
Figure 29: Domain analysis of human dynamitin using internal deletions and point mutations
(a) Cell cycle index graph showing effect of overexpression of dynamitin constructs containing internal deletions and point mutations on mitotic progression in SA48 cells. All results are means of 3 experiments (+/- SD).
(b-e) Immunofluorescence staining of SA48 cells overexpressing ΔHTHmyc (detected with anti-myc in b, d) showing prometaphase arrest with severe spindle distortions (c), and complete disruption of Golgi organization revealed by sialyl transferase localization (e).
Control untransfected cells
Dynamitin-overexpressing cells
V122Pmyc-overexpressing cells
L236Pmyc-overexpressing cells
V295Pmyc-overexpressing cells
THmyc-overexpressing cells
ΔTHmyc-overexpressing cells
ΔHTHmyc-overexpressing cells

% cells counted

Interphase
Prophase/Prometaphase
Metaphase
Anaphase
Telophase

b
c
d
e
actually less potent than wild type dynamitin at causing dynactin dissociation (Fig. 30, Table 2). Together, these data suggested that overexpression of V122Pmyc, ΔTHmyc, and ΔHTHmyc all result in dynactin loss-of-function through a mechanism which is distinct from that of wild-type dynamitin overexpression. It therefore appears that the residues targeted in these mutants, encompassing the putative coiled coil and downstream HTH-like region, define a functional domain which is important for dynamitin function within the dynactin complex. Likely functions for this region would include mediating self-association of dynamitin, as suggested by this subunit’s stoichiometry within the dynactin complex, or interaction with other proteins.

The first of these hypotheses, that this domain mediates dynamitin self-association, was tested using a two-hybrid assay (Bai and Elledge, 1996). Full-length, wild type human dynamitin was used as a Gal4 fusion “bait” construct, and challenged with a panel of mutant dynamitin “prey” constructs (all constructs courtesy of D. Starr and M. Goldberg, Cornell Univ., Ithaca, NY) (Table 3; unpublished results, CJE, J. Garces, R. Vallee). Interestingly, this analysis revealed that dynamitin can indeed self-associate, but that the putative coiled coil region and HTH-like domain do not significantly contribute to the interaction. Instead, it appeared that at least two separate regions of dynamitin, within residues 1-91 and 156-406, can mediate self-association.
**Figure 30:** Analysis of dynactin dissociation induced by wild type dynamitin vs. ΔHTH.

Sucrose gradient sedimentation analysis was performed on extracts from control COS-7 cultures, and compared to COS-7 cultures transfected to overexpress either wild type dynamitin, or ΔHTH. Western blotting reveals sedimentation behavior of p150\textsuperscript{Glied} and dynamitin (endogenous and overexpressed, including ΔHTH). Contrary to control samples where both components co-peak at ~18S, samples from both transfected cultures show a new p150\textsuperscript{Glied} peak at ~9S, and a ~5S peak for the overexpressed dynamitin (same for wild type and ΔHTH). Note, however, the persistence of a significant proportion of p150\textsuperscript{Glied} at high S values in the ΔHTH sample, as well as the residual dynamitin at high S, indicating that some dynactin remained intact in this sample.
Table 3: Results of dynamitin two-hybrid assays: grey symbols represent results from (Starr et al., 1997), included here for comparison.

<table>
<thead>
<tr>
<th>Name</th>
<th>“Prey“ constructs</th>
<th>Dynamitin Bait</th>
<th>ZW10 Bait</th>
</tr>
</thead>
<tbody>
<tr>
<td>p50 (1-406)</td>
<td></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>p50 (1-163)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p50 (1-143)</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>p50 (1-116)</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>p50 (91-406)</td>
<td></td>
<td>+/--</td>
<td></td>
</tr>
<tr>
<td>p50 (91-163)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p50 (91-143)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p50 (121-163)</td>
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<td>-</td>
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<tr>
<td>p50 (121-406)</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td>p50 (160-406)</td>
<td></td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

New insights into dynamitin structure and function

The domain analysis of dynamitin offers several new insights regarding this polypeptide’s functional organization. The data suggest that dynamitin can self-associate through at least two separate interaction domains, consistent with the possibility of oligomerization. This is consistent with the possibility that the four moles of dynamitin found in each mole of dynactin (Gill, et al., 1991; Paschal, et al., 1993; Schafer, et al., 1994) may actually represent a tetramer. This is also supported by the sedimentation properties of overexpressed dynamitin from COS-7 extracts (see Chapter 2, Fig. 15), appearing as a broad peak at ~5S, a value which would be very high for a ~43 kDa monomer.

This analysis also shows dynamitin to contain at least one other important functional domain, encompassing the putative coiled coil and HTH-like regions, which may mediate heterotypic protein-protein interactions. It is proposed (Fig. 31) that constructs containing small deletions or point mutations within this domain can integrate successfully within endogenous dynactin through natural turnover of the complex, and when overexpressed at moderate levels, do not cause its dissociation. Instead, contrary to wild type dynamitin, the mutant dynamitin can disable the “infiltrated complexes” even at relatively low levels of overexpression, accounting for the higher potency observed with such constructs. At high expression levels however, some or all of these constructs may also cause dynactin dissociation, as observed with both wild type dynamitin and ΔHTH (Fig. 30). This model, however, does not address one important issue: what is the exact function of this domain which apparently confers upon dynamitin an essential role in dynactin function?

One possible answer has recently emerged from an independent study conducted by Daniel Starr and Michael Goldberg (Cornell Univ., Ithaca, NY). Primarily using two-hybrid assays, these researchers found that the human ZW10 protein, a kinetochore component originally identified in
Figure 31: Model for dynamitin-mediated inhibition of dynactin function

At high expression levels, both wild-type and mutant dynamitin constructs have been shown to cause dynactin loss-of-function by dissociating the complex (see Fig. 18a). At low and moderate expression levels, however, wild-type dynamitin (shown in yellow) is thought to integrate into endogenous dynactin without causing dissociation, and therefore, having no inhibitory effects on dynactin and dynein function. Similarly, some mutant constructs of dynamitin (shown in blue), such as ΔHTH, are thought to be capable of integrating into endogenous complex, but not capable of normal dynamitin function. Thus, when expressed at low or moderate levels, such constructs can also cause dynactin loss-of-function simply by infiltrate the dynactin pool. This difference may underlie the higher apparent potency of such constructs in causing the cytological defects associated with dynactin loss of function in the present study.
Legend:

- Arp1 filament
- p150GLUED "arm"
- Wild-type dynamitin (functional)
- Mutant dynamitin (non-functional)
Drosophila (Smith, et al., 1985), can interact with dynamitin (Starr, et al., 1997). Most interestingly, the region mediating this interaction was mapped within dynamitin to residues 91-143, corresponding to the functional domain identified in the present study. This important finding implies that dynamitin directly mediates the interaction of dynactin, and therefore also cytoplasmic dynein, with at least one type of cargo structure, i.e. the kinetochore. Such a function is indeed completely consistent with the differences in mutagenic potency observed amongst the constructs in the present study. It is also consistent with my previous observation that both p150^Glued and Arp1 are lost from prometaphase kinetochores in dynamitin-overexpressing cells (Chapter 2, Fig. 16). However, the observed increase in potency of V122Pmyc, ΔTHmyc, and ΔHTHmyc was not only noted for mitotic defects, but also for Golgi disruption. This strongly suggests that dynamitin also mediates similar interactions through the same functional domain with other types of cargo structures destined for dynein/dynactin-mediated transport. Taken together, therefore, these data are consistent with dynamitin being the universal cargo-targeting subunit of dynactin. It will be of great interest to determine whether or not all types of retrograde cargo structure contain the same dynamitin-binding protein, i.e. ZW10, a possibility which remains untested today.

Roles of dynactin and cytoplasmic dynein in kinetochore function

Despite the clear observations of dynactin and cytoplasmic dynein localization to eukaryotic kinetochores (Echeverri, et al., 1996; Pfarr, et al., 1990; Steuer, et al., 1990; Wordeman, et al., 1991), the exact role of these components in kinetochore function has remained unclear. Recent evidence has supported microtubule depolymerization itself as providing the motive force underlying both congression and poleward anaphase A movements (Lombillo, et al., 1995, reviewed in Desai and Mitchison, 1995). Although the timing of dynactin and dynein accumulation at kinetochores would suggest a role in congression, in vitro assays mimicking this process have failed to implicate dynein, but rather, did point towards another kinetochore-bound motor protein, CENP-E, as a strong candidate for mediating these movements (Lombillo, et al., 1995). In fact, this conclusion has also been supported by my own observation that kinetochores
of prometaphase-arrested dynamitin-overexpressing cells, which are defective for dynactin and dynein but not CENP-E, still appear to interact with spindle microtubules (Echeverri, et al., 1996). Also consistent with this view is the present finding that some cell lines (such as SA48) are able to achieve full congression of all chromosomes despite partial dynamitin-mediated inhibition of dynactin/dynein activity (Fig. 25). Thus, together, these results suggest that the functional contributions of kinetochore-bound dynactin and dynein towards initial microtubule capture and chromosome congression may vary significantly in importance between cell types.

However, the present results also reveal a new and important role for kinetochore-bound dynactin and dynein. Dynamitin-overexpressing SA48 cells exhibit a second clear arrest point in metaphase, and such cells retain Mad2-positive kinetochores significantly longer than control metaphase cells. Since these cells show otherwise normal bipolar spindles and no correlation with aberrant spindle axis orientation, it appears unlikely that the defect underlying Mad2 retention occurred at sites other than kinetochores, such as spindle poles or the cell cortex. These findings therefore strongly implicate dynactin and cytoplasmic dynein activity upstream from Mad2 in the kinetochore-based machinery which controls the anaphase onset checkpoint signaling pathway.

The normal loss of Mad2 from kinetochores in control metaphase cells has recently been found to correlate with the establishment of the functional kinetochore-microtubule interaction, occurring independent of congression but before tension generation (Waters, et al., 1997). It can therefore be concluded that kinetochore-bound dynactin and cytoplasmic dynein are involved in establishing this functional or mature kinetochore-microtubule interaction which is necessary for passage of the anaphase onset checkpoint.

Based on all of the available data, one might consider the following model of kinetochore structure and function (Fig. 32). According to this view, the accumulation of dynactin and dynein in the kinetochore’s fibrous corona serves to facilitate the initial microtubule capture event by effectively increasing the available area of contact. The fibrous corona can then be viewed as a
Figure 32: Proposed model of kinetochore structure and function

(a) Schematic diagram illustrating a proposed model for composition and structure of the mammalian kinetochore corona region, based on the results of this and other recent studies (see text).

(b) Schematic diagram illustrating a proposed model for the roles of dynein and dynactin in prometaphase kinetochore function (see text).
"gripping hook" assembly which serves primarily to grab onto nearby microtubules. This would most likely occur along the wall of the microtubule, resulting in a lateral interaction (Fig. 32b, step 1), as has been observed in vivo in favorable cell types (Hayden, et al., 1990; Rieder and Alexander, 1990). Dynein activity can then account for the rapid poleward chromosome movements observed in vivo (Rieder and Alexander, 1990) and in vitro (Hyman and Mitchison, 1991), and which have actually been proposed to accelerate the formation of kinetochore microtubule bundles by bringing the kinetochores into areas of higher microtubule density (Rieder and Salmon, 1994).

Thus, in this model, the second major role of dynein/dynactin is to facilitate the interaction of the plus end of the spindle microtubule with the appropriate components of the kinetochore needed to permit passage of the anaphase onset checkpoint and subsequent chromosome movements. The shift from the initial tangential interaction to the "mature" head-on interaction is an important process which must occur as an early step to establish functional kinetochore-microtubule bundles. Depolymerization of the initial kinetochore microtubule's free plus end, a process which occurs at increased frequency during mitosis (Hyman and Karsenti, 1996; Rieder and Salmon, 1994), will eventually bring the end in close proximity to its kinetochore interaction site (Fig. 32b, step 2). At this point, the multiple dynein molecules which can then interact with the microtubule end around its entire circumference will pull it towards the kinetochore outer plate (Fig. 32b, step 3). The continuing dynein activity can effectively insert the microtubule into the appropriate complex of components in the outer plate (possibly including CENP-E and MCAK), and should maintain it there until the end abuts the inner surface of the complex (Fig. 32b, step 4). Electron microscopy studies have indeed shown kinetochore microtubules to end head-on at the kinetochore outer plate in late prometaphase and metaphase cells (Rieder, 1982). With further dynein-based movement being physically blocked this way, tension generation should result, which could conceivably cause the release of dynein/dynactin - as well as tightly-associated components of the fibrous corona - from the kinetochore outer plate (Fig. 32b, step 5). The released
components can then undergo dynein-mediated poleward transport along the spindle microtubules, accumulating at the poles, as seen for dynein and dynactin in late prometaphase and metaphase cells (Chapter 2, Fig. 6). This is also consistent with the reported loss of fibrous corona material observed in electron microscopy studies of metaphase cells (Rieder, 1982). Dynein and dynactin functions at the kinetochore are now terminated, and subsequent kinetochore functions are mediated by other components which maintain their kinetochore association beyond metaphase, such as CENP-E and MCAK (Wordeman and Mitchison, 1995; Yen, et al., 1991).

Roles of dynactin and cytoplasmic dynein at the cell cortex

Several important aspects of the dynamitin-induced mitotic arrest phenotype reported here cannot be attributed to defects in kinetochore function. Clear changes in spindle axis orientation were observed in moderate-level dynamitin-overexpressing metaphase cells which show otherwise normal bipolar spindles, full chromosome congression, and no detectable tendency towards Mad2 retention. This strongly suggested defects in the interactions between astral microtubules and the cell cortex, which are normally expected to underlie spindle positioning within cells. Consistent with this view, time-lapse analysis of living metaphase dynamitin-overexpressing cells revealed over-elongated and sinuous astral microtubules, indicating a clear lack of tension and altered astral microtubule dynamics. Time-lapse data also revealed apparent defects in anaphase B movements of half-spindles away from each other. Together, these results strongly suggest that dynactin and cytoplasmic dynein are present at the cell cortex, and normally mediate these interactions with astral microtubules, thereby generating the outward pulling forces which position the entire spindle within the cell, and which contribute to anaphase B movements. Thus, if it is confirmed that astral microtubule-cortex interactions are normally end-on (as they appear to be from fixed cell studies), this would be notably similar to the end-on spindle microtubule-kinetochore interactions, including both the geometry and the apparent co-regulation of microtubule dynamics. It is therefore appealing to consider that parallels may also exist in the molecular machinery and mechanisms which underlie the two situations. However, localization of dynactin and cytoplasmic dynein to the
cell cortex has been notoriously difficult to confirm in cultured cells, mainly due to the high concentration of both complexes found throughout the cytoplasm (e.g., Chapter 1, Fig. 6).

Nonetheless, dynactin and dynein staining has recently been noted in discrete accumulations at the cell periphery in fixed, pre-extracted polarized epithelial cells undergoing mitosis (Busson, et al., 1997). Overexpression of dynamtin in these cells was also found to result in aberrant spindle axis orientation (Busson, et al., 1997).

These defects may actually represent manifestations of a more universal function for dynactin and cytoplasmic dynein, possibly conserved throughout eukaryotic evolution.

Dynein/dynactin activity has now been implicated at the cell cortex in multiple intracellular transport processes throughout the cell cycle. Perhaps the closest parallel to the result of this study is the proposed role of dynactin and cytoplasmic dynein in positioning and orienting the spindle with respect to the bud neck and mother-bud axis in dividing S. cerevisiae (Clark and Meyer, 1994; Eshel, et al., 1993; Li, et al., 1993; Muhua, et al., 1994; Saunders, et al., 1995). Recent time lapse analyses of this process in dynein disruption strains (Carminati and Stearns, 1997) have revealed sinuous, over-elongated cytoplasmic microtubules analogous to those observed here (Fig. 21). Anti-dynactin staining has also been detected at the residual body in two-cell stage C. elegans embryos, a cortical site which has been implicated in the capture of microtubules required for the rotation of the centrosome-nucleus complex in the P1 blastomere. This process has recently been shown to be disrupted by RNA-mediated inhibition of p150Glued and dynamtin homologues (pers. comm., A. Skop and J. White, Univ. Wisconsin, Madison, WI). Dynactin and dynein have also been implicated at the cell cortex in several forms of nuclear migration in filamentous fungi (Plamann, et al., 1994; Xiang, et al., 1994), and using in vitro assays in Xenopus egg extracts (Reinsch and Karsenti, 1997). Most recently, dynein/dynactin at the cell cortex have also been proposed to mediate microtubule transport into growing axons in cultured neurons (Ahmad, et al., 1998).
Roles of dynactin and cytoplasmic dynein in spindle pole organization

While the severe spindle distortions observed in dynamitin overexpressing cells are not easily attributable to cortical defects, they could conceivably result from kinetochore defects. Mitotic arrest phenotypes resulting from microinjection of certain anti-kinetochore antibodies have included slight spindle deformations (Bernat, et al., 1990). Those studies however did not report spindle pole fragmentation events, nor the presence of ectopic microtubule foci, as seen here. Thus, in view of the normal localization of dynactin and dynein in and around centrosomes and spindle poles (Chapter 1, Fig. 6; Clark and Meyer, 1992; Gill, et al., 1991; Paschal, et al., 1993), the present phenotypic observations are also consistent with a loss of dynactin and dynein activity at spindle poles.

Recent in vitro assays using Xenopus oocyte extracts have implicated cytoplasmic dynein in the assembly of bipolar spindles induced either with sperm nuclei, which contain a centrosome, or with DNA-coated beads, which do not (Heald, et al., 1996). Cell-free aster formation assays using HeLa cell extracts, and in vivo studies of cells injected with anti-dynein antibodies have also implicated dynactin and dynein activity in mitotic pole assembly (Gaglio, et al., 1997). From these studies, models of spindle pole self-assembly have been proposed whereby cytoplasmic dynein can bind free microtubules as cargo, presumably through dynactin, and transport them towards the minus ends of other microtubules used for transport (Heald, et al., 1996). This process is also thought to require NuMA, which was recently found to co-immunoprecipitate with dynactin and cytoplasmic dynein from Xenopus egg extracts (Merdes, et al., 1996). NuMA also has the capacity to bind to and bundle microtubules, and could thereby serve as a cross-linking agent to stabilize spindles near the poles (Merdes, et al., 1996). The results of the present study are completely consistent with such a model, adding to it the notion that such a process is apparently needed constantly throughout mitosis, to maintain the structural integrity and cohesion of the poles.
GENERAL CONCLUSIONS

AND

FUTURE DIRECTIONS

This thesis work has yielded several new insights in the study of vertebrate dynactin and cytoplasmic dynein. Dynamin has emerged as a powerful tool for studying dynactin and cytoplasmic dynein function in vertebrate cell systems. Since the first two chapters of this thesis work were published (Burkhardt, et al., 1997; Echeverri, et al., 1996), several studies, collaborative and independent, have been initiated based the use of dynamin overexpression to specifically inhibit dynactin function. In the process, several independent laboratories have now confirmed most aspects of the dynamin-induced mitotic arrest phenotype (Dujardin, et al., 1996; Gaglio, et al., 1996; pers. comm., I. Clark and D. Meyer, UCLA, Los Angeles, CA), and the Golgi dispersal phenotype (Ahmad, et al., 1998; Presley, et al., 1997). A collaborative study with Dr. Peter Baas (Univ. Wisconsin, Madison, WI) has recently revealed that injection of bacterially-expressed dynamin into cultured neurons inhibits the outward transport of microtubules from the centrosome to the cell periphery and into growing axons (Ahmad, et al., 1998). This result is particularly interesting because, contrary to conventional expectations, movement of microtubules with plus-ends leading is also attributed to dynactin and cytoplasmic dynein function. This is thought to occur as a result of dynein-based transport processes taking place along microtubules which have been released from the centrosome, and as a result meet with less resistance to movement than do the associated cargo structures. Similar recent observations of microtubule release from the centrosome and their subsequent movements outward in non-neuronal cells (Keating, et al., 1997) suggests that this type of dynactin/dynein-based “reverse transport” of microtubules may be a universal process which will require further investigation.
With this in mind, one important caveat which still permeates in the dynactin field to date will have to be addressed: the specificity of dynactin function only to dynein-based forms of transport has never been thoroughly tested. While all data from this and other studies have supported the involvement of dynactin in all dynein pathways, no findings to date have refuted its possible contribution to other motors. The only advance on this issue to date has been the finding that lysosomes and endosomes in dynamtin-overexpressing cells tend to accumulate at the extreme cell periphery in a microtubule-dependent manner, suggesting an anterograde form of transport which is unaffected by dynactin loss of function (Burkhardt, et al., 1997). It nevertheless remains formally possible that dynactin may play a role, direct or indirect, in other as-yet untested forms of anterograde transport. This will be an important gap to fill in the years to come.

In the meantime, another important challenge will be to continue characterizing the full range of dynein/dynactin-based forms of organelle transport. This would greatly benefit from the identification of dynactin-binding proteins on the various cargo organelles. Indeed, the first of these, ZW10, has recently been found to mediate the interaction of dynactin, through dynamtin, with kinetochores (Starr, et al., 1997). It remains unclear whether all retrogradely-transported organelles contain the same dynactin-binding protein, i.e. ZW10, or whether each is different. If the hypothesis is correct, the identification of these proteins will be particularly important, as these represent a useful point of divergence in dynein/dynactin pathways which could be targeted to inhibit the transport of specific subsets of organelles within cells. This capability should then offer powerful new methods for further dissecting the highly complex events of intracellular organelle motility, morphogenesis and cell division.
APPENDIX

I include below selected sections from the documentation pages for the current (Nov. '97) version of the COILS program which is accessible from the ISREC web server. These include very useful information on the different variations and scoring options available with this program, to better understand the predictions it produces. More detailed information can be obtained at: http://ulrec3.unil.ch/coins/COILS_doc.html.

COILS version 2.2
by A. N. Lupas
programmed by J. M. Lupas

1. Introduction
COILS is a program that compares a sequence to a database of known parallel two-stranded coiled-coils and derives a similarity score. By comparing this score to the distribution of scores in globular and coiled-coil proteins, the program then calculates the probability that the sequence will adopt a coiled-coil conformation. COILS is described in:

and is based on a prediction protocol proposed by David Parry:
2. Scoring options

After asking for input and output filenames, the program will offer the choice of two scoring matrices that it can compare a sequence to:

MTK - is a matrix derived from the sequences of myosins, tropomyosins and keratins (intermediate filaments type I and II). It is the one described in Science, 252:1162 (1991).

MTIDK - is a new matrix derived from myosins, paramyosins, tropomyosins, intermediate filaments type I-V, desmosomal proteins and kinesins.

While the MTIDK matrix provides for a somewhat better resolution between the scores of globular and coiled-coil proteins as well as for a more consistent evaluation of the different families of coiled-coil proteins, the MTK matrix yields fewer high scoring segments in a database of globular sequences. Current data are consistent with the assumption that the MTK matrix is more specific for two-stranded structures and that the MTIDK matrix gives a more realistic assessment for other types of coiled coils.

3. Weighting options

Because coiled coils are generally fibrous, solvent-exposed structures, all but the internal a and d positions have a high likelihood of being occupied by hydrophilic residues. A program that gives equal weight to all positions is therefore going to be biased towards hydrophilic, charge-rich sequences. While this does not pose a problem for the vast majority of natural sequences, some highly charged sequences obtain high coiled-coil probabilities in the obvious absence of heptad periodicity and coiled-coil-forming potential. An extreme case is that of polyglutamate which obtains a coiled-coil-forming probability > 99%. To counter this problem, COILS2 contains a weighting option, which allows the user to assign the same weight to the two hydrophobic positions a and d as to the five hydrophilic positions b, c, e, f and g. This leads to an only slightly worse
performance of the program (see Section 7: PERFORMANCE) and permits the identification of
the class of false positives described above. It is recommended to run a weighted and unweighted
scan in parallel and to compare the outputs. A drop of more than 20-30% in the probability is a
clear indication of a highly-charged false positive.

4. Recommendations for using the program

COILS is specific for solvent-exposed, left-handed coiled coils. Other types of coiled-coil
structure, such as buried coiled coils (e.g. the central coiled coil in catabolite repressor protein, or
some transmembrane domains) and right-handed coiled coils, are not detected by the program.
COILS does not reach yes-or-no decisions based on a threshold value. Rather, it yields a set of
probabilities that presumably reflect the coiled-coil forming potential of a sequence. This means
that even at high probabilities (e.g. >90%), there will be (and should be) sequences that in fact do
not form a coiled coil, though they may have the potential to do so in a different context.

COILS is biased towards hydrophilic, highly charged sequences. For this reason, all scans
should be performed with a weighted and an unweighted matrix, and the results compared.
Differences of more than 20-30 percentage points in the probabilities should be taken to indicate
that a coiled-coil structure is unlikely, the elevated scores being mainly due to the high incidence of
charged residues (note, though, that this would have marked human mannose-binding protein as a
false positive).

The MTK and MTIDK matrices both assign high probabilities to known coiled coils
segments, but identify different helices at high probability in a database of globular proteins. This
is a surprising feature whose reason is as yet unclear, but which can be exploited for predictive
purposes. It is therefore useful to compare the results of scans made with the two matrices. Again,
differences of more than 20-30 percentage points in the probabilities should be taken to indicate
that a coiled-coil structure is unlikely (note, though, that this threshold would make the replication
terminator protein a border-line case).

The resolution between globular and coiled-coil score distributions decreases strongly with a decreasing size of the scanning window. The prediction of new coiled-coil segments should therefore be made using a 28 residue window, or in special cases a 21 residue window. 14 residue windows should normally be reserved for the analysis of local parameters (such as the frame) in known or predicted coiled coils.

The ends of coiled-coil segments appear to be most accurately identified in a 21 residue window. In general, I assume that residues with probabilities >50% are part of a coiled-coil segment. In addition, a search for the most likely helix ends using CAPS is generally useful (see also the CAPS documentation).

Sequences with high coiled-coil probability from globular proteins rarely exceed a length of 30 residues. None is longer than 35 residues. Sequences with probabilities >80-90% that extend for more than 35 residues are therefore more likely to assume a coiled-coil structure than is indicated by the obtained probabilities.

Where possible, sequences related to the protein of interest should also be analyzed for predicted coiled-coil segments (see the section on the ALIGNED programs). It should be kept in mind, though, that the sequences must be related in the region of high scores in order for the comparison to be significant. Comparison of the coiled-coil prediction with predictions of the secondary structure are generally useful, particularly if multiple related sequences are available.


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