Molecular Characterization of the 50-kD Subunit of Dynactin Reveals Function for the Complex in Chromosome Alignment and Spindle Organization during Mitosis

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Abstract. Dynactin is a multi-subunit complex which has been implicated in cytoplasmic dynein function, though its mechanism of action is unknown. In this study, we have characterized the 50-kD subunit of dynactin, and analyzed the effects of its overexpression on mitosis in living cells. Rat and human cDNA clones revealed p50 to be novel and highly conserved, containing three predicted coiled-coil domains. Immunofluorescence staining of 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. Overexpression of p50 in COS-7 cells disrupted mitosis, causing cells to accumulate in a prometaphase-like state. Chromosomes were condensed but unaligned, and spindles, while still 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 represent clear evidence for dynactin and cytoplasmic dynein codistribution within cells, and for the presence of dynactin at kinetochores. The data also provide direct in vivo evidence for a role for vertebrate dynactin in modulating cytoplasmic dynein binding to an organelle, and implicate both dynactin and dynein in chromosome alignment and spindle organization.

Cyttoplasmic dynein is a ubiquitous, multi-subunit ATPase responsible for minus end-directed microtubule-based organelle transport (Paschal and Vallee, 1987; Holzbaur and Vallee, 1994). It is thought to be responsible for retrograde axonal transport (Paschal and Vallee, 1987; Schnapp and Reese, 1989; Schroer et al., 1989; Lacey and Haimo, 1992) and the perinuclear distribution of a number of organelles, including the Golgi apparatus (Corhész-Theulaz et al., 1992; Fath et al., 1994), late endosomes, and lysosomes (Lin and Collins, 1992; Aniento et al., 1993). Several lines of evidence have also implicated cytoplasmic dynein in mitosis, though its precise role has remained uncertain. The motor has been localized in some studies to the prometaphase kinetochore of vertebrate chromosomes (Pfarr et al., 1990; Steuer et al., 1990; but see Lin and Collins, 1992). Consistent with this result, rapid minus end-directed chromosome movements have been observed in cells during early prometaphase (Rieder and Alexander, 1990; for review see Rieder and Salmon, 1994). Kinetochores of isolated chromosomes have also been reported to exhibit retrograde motor activity (Hyman and Mitchison, 1991). Although these findings suggest a role in chromosome movement, microinjection of anti-cytoplasmic dynein antibodies (Vaisberg et al., 1993) has been found to result instead in spindle collapse, apparently implicating the motor in some aspect of spindle organization. Phenotypic analysis of yeast cytoplasmic dynein disruption strains has indicated a role in spindle positioning (Eshel et al., 1993; Li et al., 1993) and some participation in anaphase chromosome segregation (Saunders et al., 1995). These functions seem to be manifestations of a more general role in nuclear positioning and migration in yeast (Eshel et al., 1993; Li et al., 1993) and other fungi (Plamann et al., 1994; Xiang et al., 1994).

In addition to uncertainties over the full range of cytoplasmic dynein functions, little is understood about the regulation of its activity and the mechanism by which it is targeted to a specific subset of intracellular organelles. One reported regulatory factor is dynactin, which was identified as a fraction that could be separated out of sucrose gradient-purified cytoplasmic dynein preparations, and which stimulated the frequency of minus-end directed
particle movements in an in vitro assay (Gill et al., 1991; Schroer and Sheetz, 1991). Components of this fraction could also be communoprecipitated out of brain cytosol (Paschal et al., 1993), and appear as a discrete particle by electron microscopy (Schafer et al., 1994). Purified dynactin contains at least nine polypeptides ranging from 24 to 150 kD (Gill et al., 1991; Paschal et al., 1993; Schafer et al., 1994). It consists of an F-actin–like core filament composed of the actin-related protein Arp1 (Clark and Meyer, 1992; Lees-Miller et al., 1992; Paschal et al., 1993), with a capping protein heterodimer bound at one extremity, and a 62-kD component at the other (Schafer et al., 1994). Extending laterally from the filament is the largest subunit of the complex, p50Glued (Schafer et al., 1994). This polypeptide has been found to be homologous throughout its length to the product of the Glued gene in Drosophila (Holzbaur et al., 1991; Gill et al., 1991), which is involved in neuronal development and possibly other more general cellular functions (Harte and Kankel, 1982).

The specific role of dynactin in modulating cytoplasmic dynein function is unknown. Although the two complexes partially copurify through the microtubule sedimentation and ATP extraction steps of the cytoplasmic dynein preparative procedure (Collins and Vallee, 1989; Gill et al., 1991, Paschal et al., 1993), they show limited evidence of an interaction after extraction from microtubules, or when either is immunoprecipitated directly from cytosolic extracts (Paschal et al., 1993). Nonetheless, recent evidence has revealed direct binding between recombinant cytoplasmic dynein intermediate chains (IC) and p150Glued (Vaughan and Vallee, 1995; Karki and Holzbaur, 1995). These data indicate that the two complexes can interact directly and suggest that the interaction between the complete complexes must be regulated. An interaction between dynein and dynactin in cells has been difficult to test by immunocytochemical means. This is in part due to the high density of fine punctate staining observed for dynactin in interphase cells (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993). Furthermore, during mitosis, dynactin was not observed to associate with kinetochores (Gill et al., 1991). Nevertheless, homologues of Arp1 and p150Glued have been deduced to function in a common pathway with cytoplasmic dynein based on phenotypic similarities and suppressor analysis in S. cerevisiae (Muhua et al., 1994; Clark and Meyer, 1994), Neurospora (Plamann et al., 1994), and Drosophila (McGraIl et al., 1995).

The present study was initiated to gain further insight into the function of dynactin and its relationship to cytoplasmic dynein. We have cloned p50, the second most abundant component of dynactin (4–5 moles per mole of complex; Paschal et al., 1993; Schafer et al., 1994) and analyzed its distribution and the effects of its overexpression in cultured mammalian cells. We report that dynactin, like cytoplasmic dynein, is recruited to prometaphase kinetochores, and present direct in vivo evidence for a specific role for vertebrate dynactin in prometaphase chromosome alignment and spindle organization during early eukaryotic mitosis.

**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, BioRad Laboratories, Richmond, CA), and electroelastically 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 C8 reverse-phase HPLC as previously described (Aebesold, 1989). NH2-terminal amino acid sequence from six peptides was determined on a 477A Sequenator (Applied Biosystems, Foster City, CA), with a 120A phenylisothiocyanate analyzer by Dr. J. Leszyk of the Worcester Foundation for Biomedical Research Protein Chemistry Facility (Shrewsbury, MA).

**Cloning of p50**

The peptide No. 21 sequence was used to design an inosine-containing, partially degenerate oligonucleotide, composed of equal amounts of two oligonucleotides: 5′-AAT-GA-GA-GAT-TAT-GA-GA-A-CI-A-G-C-T-GA-CT-T-TG-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-3′ and 5′-AAT-GA-GA-GAT-TAT-GA-GA-A-CI-A-G-C-T-GA-CI-GC-A-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-GA-3′. This probe was [32P] end-labeled, and used for hybridization screening of a bovine brain λgt10 cDNA library (No. 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), 5× Denhardt’s solution, 0.6% SDS, 100 μg/ml denatured salmon sperm DNA, at 53°C for 18 h. 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 h. The TMAC was removed from the filters by three 10-min washes in 2× 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, labeled with [32P]dCTP by random priming (Boehringer Mannheim Biochemicals, Indianapolis, IN), and used for hybridization screening of a rat brain λZAPII cDNA library (No. 936515, Stratagene, La Jolla, CA). Hybridization was performed overnight at 65°C, in Rapid-Hyb solution (Amersham Corp., Arlington Heights, IL), and washed 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 rescreen 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 (EST05385) (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 (re-named 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 screened 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 e-mail 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 (Novagen, Madison, WI) after PCR mutagenesis and standard cloning techniques. After transformation into the E. coli expression strain BL21(DE3) and induction with 0.4 mM IPTG, bacteria were lysed in standard SDS-PAGE sample buffer, and analyzed 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, labeled with [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

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1. Abbreviations used in this paper: CREST, calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia; EST, expressed sequence tag; HTTH, helix-turn-helix; IC, intermediate chain; NEB, nuclear envelope breakdown.
65°C, in Rapid Hyb solution, followed by washes according to the sup-
plier’s instructions, except that SSPE was used instead of SSC. Results
were detected on a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA).

Preparation of Tissue Samples
Calbin brain and tissue samples from adult male Sprague-Dawley rats
(Charles River Laboratories, Wilmington, MA) were homogenized as previ-
ously 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
pepsin A. Cytosolic extracts were prepared by centrifugation at 100,000
g for 1 h 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 and Transfections
Cell cultures (Rat2, HeLa, COS-7, and PtK1, all from Amer. Type Cul-
ture Collection, Rockville, MD) were maintained as subconfluent mono-
layers in “growth medium”: DMEM (GIBCO/BRL, Life Technologies,
Gaithersburg, MD) + 10% FCS (GIBCO/BRL) + 100 U/ml penicillin +
100 μg/ml streptomycin (Sigma Chem. Co., St. Louis, MO). For immuno-
fluorescence staining experiments, cells were trypsinized, and seeded onto
sterile 18-mm² coverslips in six-well dishes (Corning Glass Works, Corning,
NY) to reach 70–90% confluency after 48 h.

For transient transfections, cells were seeded onto 18-mm² coverslips in
six-well dishes at 2–5 x 10⁴ cells per well. After 24 h, 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 3.5 lx
lipofectamine reagent (GIBCO/BRL) in 1 ml DMEM, prepared as per
transfection protocol). Some samples were preextracted with 1 min incubation
in 0.5% Triton X-100 (Pierce, Rockford, IL) in PEGM buffer [80 mM Pipes
(pH 6.8), 5 mM EGTA, 1 mM MgCl2, 4 μM glycerol], followed by either 10
min in 100% methanol at ~20°C, or 15 min in 4% formaldehyde from (16% EM grade.

Sedimentation Analysis
Cells grown in 100-mm dishes were harvested in D-PBS + 10 mM EDTA,
counted, pelleted, and resuspended at equal cell density in buffer “A” (50
mM Tris-HCl [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
pepsin A), kept at 4°C for 15 min. Alternatively, pelleted cells were re-
suspended in buffer A without NP-40 present, and homogenized by two
passes at 2,000 rpm in a motor-driven teflon glass homogenizer kept at
4°C. The cell lysates were centrifuged at 30 psi for 15 min, and the superna-
tant (cytosolic extract) was recovered. Sucrose gradients (4.5 ml, 5–20% in
buffer A without NP-40) were prepared and 160 μl of each cytosolic ex-
tact (corresponding to ~5 x 10⁶ cells) was carefully layered on top.

The plasmid used for p50 transfections was made by subcloning the full
H50A coding region into the Not I site of pCMVβ (Clontech), which it-
self was used as is for β-galactosidase overexpression. The myc epitope
tag (MEQKLISEED-stop)(Evan et al., 1985) was inserted after the last
5’ codon by PCR mutagenesis and subcloning through a shuttle vector
(pARK2mycSTOP, courtesy of Dr. M. A. Gee, Worcester Foundation for
Biomedical Research).

Sedimentation Analysis
Cells grown in 100-mm dishes were harvested in D-PBS + 10 mM EDTA,
counted, pelleted, and resuspended at equal cell density in buffer “A” (50
mM Tris-HCl [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
pepsin A), kept at 4°C for 15 min. Alternatively, pelleted cells were re-
suspended in buffer A without NP-40 present, and homogenized by two
passes at 2,000 rpm in a motor-driven teflon glass homogenizer kept at
4°C. The cell lysates were centrifuged at 30 psi for 15 min, and the superna-
tant (cytosolic extract) was recovered. Sucrose gradients (4.5 ml, 5–20% in
buffer A without NP-40) were prepared and 160 μl of each cytosolic ex-
tact (corresponding to ~5 x 10⁶ cells) was carefully layered on top.

These gradients were centrifuged in a SW50.1 rotor (Beckman Instru-
ments, Palo Alto, CA) at 26,500 rpm for 18 h at 4°C, and collected as 350-
μl fractions. Sedimentation standards which included alcohol dehydroge-

nease (5 S), apoferritin (13 S), and thyroglobulin (19 S), were diluted in
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% formal-
dehyde 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 16% EM grade,
Polysciences Inc., Warrington, PA) +0.5% Triton X-100 in PEMG for 15 min, rinsed in PBS (3 x 5 min), and incubated in 0.5 mg/ml
sodium borohydride in PBS (3 x 5 min) to reduce free aldehyde groups.

All samples were then rinsed in PBS (3 x 5 min), incubated in primary
antibody solution for 30–45 min, rinsed again in PBS (3 x 5 min), 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, 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 No. 33258 (Pierce). Samples were mounted in 0.1% p-phenylenedi-
amine in PBS + 50% glycerol.

Microscopy Techniques
Conventional immunofluorescence microscopy was carried out on a Zeiss
Axiopt 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 an MRC1000 system (BioRad Mic-
roscience, 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 (version
3.0, Adobe Systems Inc., Mountain View, CA), and imported into
CorelDraw (version 5.0, Corel Corp., Ottawa, Canada) for figure assem-
blage. Figures were printed on a Kodak Colorese PS color printer (East-
man Kodak Comp., Rochester, NY).

Results
Molecular Characterization of Mammalian p50
To isolate cDNA clones encoding mammalian p50, amino
acid sequences of six tryptic peptides from immunoprecip-

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panied calf brain p50 were obtained. One of these (peptide
21, Fig. 1, a and b) was used to design a mixed oligonucle-
otide 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. 1, a and b). The
B14A insert was then used as a hybridization probe to iso-
late 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 1,221 bp open reading frame (ORF) encoding
407 residues (Fig. 1 a). A homology search of nucleic acid
databases also identified a partially characterized human
cDNA clone (EST06385, Adams et al., 1993, renamed
RllC, RllD) were found to share the same,
complete ORF found in the rat clones (Fig. 1, a and b). The de-
duced 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 re-
sulting whole cell lysates analyzed by SDS-PAGE and
Western blotting using a p50-specific monoclonal antibody
(Paschal et al., 1993) (Fig. 2 a). 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 constit-
tute the full-length coding regions for the 50-kD com-
ponent of human and rat dynactin complex. We also note the
identification of a single p50 chromosomal locus in mouse,
thus strongly suggesting the existence of a single p50 gene
in mammals (Vaughan, K., A. Mikami, B. Paschal, E.
Holzbaur, S. Hughes, C. Echeverri, N. Jenkins, D. Gilbert,
K. Moore, N. Copeland, and R. Valle, manuscript in
preparation).

Northern blot analysis of adult rat poly(A)RNA re-

![Figure 1. Identification and sequence analysis of mammalian p50-encoding cDNA clones. (a) Alignment

diagram of bovine (B14A), and full-length hu-
mman (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 hexa-
nucleotide polyadenylation signal sequence (AT-
TAAC 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
cDNA sequence H50A. Note the Kozak consensus box
(Kozak, 1991) at the predicted translational initia-
tion site (AUG in bold, at 79), which was preceded
by no other inframe methionine codons in this or
any other clones characterized in this study. A hexa-
nucleotide polyadenylation signal (ATTAAA in
H50A, and ATTAAA 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). (c) Summary
diagram of p50 showing probability of predicted
colai-coil domains (in parentheses) and location of
prokaryotic HTH motif are shown. Coiled-coil
structure was predicted using NEWCOILS (Lupas
et al., 1991) with a 28-residue window.}
Dyactin Function in Mitosis

 Dynactin Components

 Immunofluorescence microscopy was used to examine the subcellular distribution of p50 in several cultured mammalian cell lines, including HeLa, PK1, COS-7, and Rat2, all of which yielded equivalent patterns. Fine punctate staining densely filled the entire cytoplasm throughout the cell cycle, and was excluded from the nucleus during interphase (Fig. 3 a). Prominent centrosomal staining (Fig. 3, a and b) was also observed, appearing as a closely spaced group of 2-4 brighter spots (inset in Fig. 3 a). 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. 3 c, g-k). Detergent extraction of the cells before fixation abolished most of the fine punctate staining, while the centrosomal spots remained prominent (Fig. 3 b). These patterns are consistent with previous reports of anti-Arpl (Clark and Meyer, 1992), and anti-p150\textsubscript{Glued} (Gill et al., 1991; Paschal et al., 1993) staining in cultured cells.

Staining of mitotic cells with anti-p50 also showed a strong apparent kinetochore pattern (Fig. 3, c and g, 4 a) beginning after nuclear envelope breakdown (NEB), and disappearing from each chromosome upon its alignment at the metaphase plate (Fig. 3 c and i). 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 nonaligned metaphase chromosomes (Fig. 3 c).

Because kinetochore staining was not observed previously in cells stained with anti-p150\textsubscript{Glued} antibodies (Gill et al., 1991), we examined the effects of multiple fixation conditions on mitotic staining obtained with a battery of monoclonal and polyclonal antibodies to dynactin components (Table I, Fig. 4). Three independent affinity-purified monoclonal antisera directed against p150\textsubscript{Glued} (UP236), and Arpl (RA1/10 and A27) gave kinetochore staining patterns comparable to that seen with the anti-p50 monoclonal antibody (Table I, Fig. 4). Both a monoclonal ("74.1", Table I) and an affinity-purified polyclonal anti-cytoplasmic dynein IC antibody (Vaughan and Vallee, 1995) produced the same kinetochore pattern (Table I, Fig. 4 e). Detergent preextraction increased the clarity of kinetochore staining (Table I) apparently by decreasing the fine punctate cytoplasmic staining generally seen with these antibodies (Fig. 3 a and b). Importantly, all six antibodies also showed clear kinetochore staining in cells fixed without detergent preextraction, thus minimizing the risk of antifactual redistribution of cytosolic antigens (Table I, Fig. 4).

The kinetochore localization of anti-dynactin staining was confirmed by double labeling with a CREST human autoimmune antiserum (Fig. 4, g-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. 4 j, Figs. 9 and 10) clearly reminiscent of previous electron microscopic observations made under similar microtubule-depolymerizing conditions (Rieder, 1982). 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 (Paschal, B., L. Wordeman, and R. Vallee, unpublished observations).

**Cells Overexpressing p50 Show Prometaphase-like Arrest with Aberrant Spindle Morphology**

As a first step in investigating p50 and dynactin function in vivo, we transfected COS-7 cells with full-length wild-type and myc-tagged p50. Both constructs produced the same striking phenotypic effects observed throughout the cell cycle. While the present work focuses on the mitotic abnormalities, disruption of the Golgi complex (Echeverri, C.J., and R. Vallee, unpublished observation) and redistribution of endocytic organelles were also noted, and will be described separately (Burkhardt, J., C.J. Echeverri, K.T. Vaughan and R. Vallee, manuscript in preparation).

When analyzed by immunofluorescence microscopy, the overexpressed p50 and p50myc were found diffusely throughout the cytoplasm (Figs. 6 and 7), and were excluded from the nucleus (not shown). A higher than normal proportion of p50- and p50myc-overexpressing cells appeared to be in mitosis, as judged by rounded morphologies and condensed chromosomes. To determine the magnitude of this effect, we compared the cell cycle index of p50myc-overexpressing cells with those of three separate control categories: untransfected cells, transfected but nonexpressing cells, and transfected cells overexpressing an unrelated cytosolic protein, β-galactosidase ("β-gal transfectants") (Fig. 5). From a total of 6,838 p50myc-overexpressing cells counted over four 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 (Figs. 6 and 7). 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 nonexpressing cells and 3.6 ± 0.4% of β-gal transfectants found to be distributed throughout M-phase (Fig. 5).

The prometaphase-like p50myc-overexpressing cells were found to show significant spindle aberrations, as revealed by anti-tubulin staining (Figs. 6 and 7). 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, few or no astral microtubules were visible, and spindle poles were closer than normal to the cell periphery. In most cases, one half-spindle was significantly more developed than the other, as seen in Fig. 6, d-i, which shows three clear examples of this phenotype. The microtubules of the larger half-spindle were 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. 6, e and i and Fig. 7, a and c).

Triple labeling with anti-p50, anti-tubulin and a human CREST autoimmune anti-centromere antiserum confirmed that all kinetochores of p50myc transfectants colocalized with spindle microtubules (Fig. 7, c-f). We also examined the distribution of CENP-E, a kinesin-like protein which accumulates on kinetochores during prometaphase (Yen et al., 1992). Anti-CENP-E staining intensity, which normally shows a marked decrease after metaphase (Yen et al., 1991; Echeverri, C., and R. Vallee, unpublished observations), remained high and revealed kinetochores to be paired in the p50myc-overexpressing cells (Fig. 7 b), consistent with the prolongation of a prometaphase-like state.

Tetrapolar spindles were occasionally noted in p50myc...
Figure 3. Subcellular distribution of p50 throughout the cell cycle in cultured mammalian cells. (a, b, c, e, i, k, and m) Anti-p50 staining (d, f, h, j, l, and n); HOECHST DNA staining. (a and b) Interphase HeLa cells (c and d); late prometaphase COS-7; (e and f) prophase Rat2; (g and h) early prometaphase Rat2; (i and j) metaphase Rat2; (k and l) anaphase Rat2; and (m and n) telophase Rat2. (a) Interphase cells fixed in formaldehyde without detergent preextraction 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 preextraction. Centrosomal staining persists prominently, but most of the fine punctate staining is lost. Centrosomal spots are visible during prophase (e and f), but become obscured by an accumulation of fine punctate staining along spindle microtubules, particularly near the poles, from prometaphase (c, d, g, and h) to anaphase (k and l). Spindle pole staining is often notably dimmer in telophase cells (m and n). Kinetochores were observed in prometaphase cells (c, d, g, and h) found most prominent in late-attaching chromosomes before their alignment at the metaphase plate (arrows in c and 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 I. Bars: a and b shown in b; c and d shown in d; for e–n shown in n, 5 μm.
transfectants fixed over 35 h after transfection (as opposed to 27–33 h 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, having completed the first round without segregating their duplicated pair of centrosomes.

**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{Glued}, and Arpl all sediment at ~20 S, indicating that they exist exclusively as a complex (Paschal et al., 1993; Clark et al., 1994). 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. 8). Extracts of control untransfected and β-gal–transfected COS-7 cultures showed the four dynactin components examined, p150\textsuperscript{Glued}, p62, p50, and Arpl, comigrating at 18 S. This slight divergence from the 20 S value observed 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 9 S, and additional streaking throughout the lower end of the gradient. The Arpl and p62 peaks showed the same small but consistent shift to 16–17 S. Overexpressed myc-tagged and endogenous p50 could be distinguished by a slight difference in electrophoretic mobility. Both species migrated as a single peak at 5 S. The cytoplasmic dynein ICs migrated at 20 S 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.

**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 transfecteds 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 transfecteds expressing high levels of p50myc showed undetectable or lower than normal kinetochore staining with anti-p150\textsuperscript{Glued} and anti–Arpl 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 antisemur (Fig. 7 b), and a human CREST anti-centromere auto-antisemur (Fig. 7 d) 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 I), was difficult to evaluate in these samples.

To maximize the immunofluorescent signal at the kinetochores, and to eliminate potential effects of kinetochore microtubules on antibody accessibility, we examined kinetochore staining in nocodazole-treated cells. As expected, transfected nonexpressing cells and β-gal–transfected cells showed brighter, enlarged kinetochore patterns under these conditions, often appearing crescent shaped (Figs. 9 and 10). In cells overexpressing high levels of p50myc, both anti–Arpl (Fig. 9 a) and anti-p150\textsuperscript{Glued} (Fig. 9 e) showed clearly reduced kinetochore staining. Anti-cytoplasmic dynein IC staining of kinetochores in these cells also showed a decrease in intensity (Fig. 10). In general, the morphology of anti–IC–stained kinetochores was more variable than that seen with anti-p150\textsuperscript{Glued} and anti–Arpl, due to the effects of formaldehyde fixation on IC staining. Methanol fixation, which yields optimal kinetochore staining with anti–ICs (Table I), caused excessive

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

| Method | Anti-p50 (mAb “50-1”) | Anti-p150\textsuperscript{Glued} (pAb “UP236”) | Anti-Arpl (pAb “RA1/10”) | Anti-Arpl (pAb “A27”) | Anti-IC (pAb “L5”) | Anti-IC (mAb “74.1”) |
|--------|----------------------|---------------------------------|-------------------|-------------------|-------------------|-------------------|
| MeOH   | ++                   | ++                              | ++                | ++                | ++                | ++                |
| Tx-100, then MeOH | +                  | +                               | +                 | +                 | +                 | +                 |
| Form., then Tx-100 | +                  | +                               | +                 | +                 | +                 | +                 |
| Form., then MeOH | +                  | +                               | +                 | +                 | +                 | +                 |
| Tx-100, then Form. | +                  | +                               | +                 | +                 | +                 | +                 |

Abbreviations: form., formaldehyde; glut., glutaraldehyde; simult., simultaneous.

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Figure 4. Dynactin and cytoplasmic dynein components localize to kinetochores. Prometaphase COS-7 cells show clear kinetochore staining with anti-p50 (a), anti-p150\textsuperscript{Glued} (c), anti–Arpl ("A27") (g), and anti-cytoplasmic dynein IC (e). b, d, and f are DNA staining patterns corresponding to a, c, and e, respectively. Double labeling using anti–Arpl (g and j) and human CREST autoimmune serum (i and l) confirms kinetochore localization of dynactin. The dynactin pattern is restricted to nonaligned kinetochores in control cells (g–i), but appears on all kinetochores in nocodazole-treated (10 μm, 3 h at 37°C) cells (j–l). h and k show superimposition of g and i and j and l, re-
respectively, showing signal colocalization 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 and f which was fixed directly in methanol. All kinetochores in this figure would be scored as "++" in Table I, except for panel j which would be scored "+++". Bars: a–d shown in d; e–l shown in l, 10 μm.
That dynactin, like cytoplasmic dynein, associates with four different antibodies to three dynactin polypeptides (Lin and Collins, 1992). Furthermore, a comparable pattern was not observed in cells stained with an antibody against p150

seen as distinct processes in appropriate cells (for review see 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 ac-
Figure 6. Effects of p50 overexpression on mitotic spindle morphology. Immunofluorescence anti-tubulin staining (a-d, f and h) of mitotic β-gal-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 and h illustrate pronounced asymmetry in microtubule density and orientation of half-spindles, as compared with the unperturbed spindle of a metaphase β-gal-overexpressing COS-7 cell (a). Staining of overexpressed p50myc (e, g, and i) reveals nonrandom chromosome distribution, seen as unstained regions which colocalize with dense areas of distorted spindles (particularly in e and i). Note nonexpressing 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, and 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). Bar, 5 μm.
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 p50-overexpressing cells is, therefore, consistent with a role for dynactin and, presumably, dynein in poleward prometaphase movement.

After 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; for a review see 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 biorientation 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 (Mitchison et al., 1986; Gorbsky et al., 1987; Skibbens et al., 1993; for reviews see Rieder and Salmon, 1994; Desai and Mitchison, 1995). 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 abundant at the kinetochore. Thus, our observation of longer than normal and distorted spindle microtubule bundles in p50-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 p50-overexpressing cells is uncertain. These effects may be secondary consequences resulting from the 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; Simmerly 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 (Eshel et al., 1993; Li et al., 1993; Clark and Meyer, 1993).
Conceivably then, p50 overexpression may effect a comparable role for dynactin and cytoplasmic dynein in vertebrate spindle positioning. In this regard, microinjection of anti-cytoplasmic 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. We note, however, that the present p50-induced spindle phenotype differs significantly from the phenotype reported in the earlier study, and further work will be needed to reconcile these results.

**Molecular Basis for Mitotic Phenotype**

The molecular basis for the mitotic phenotype resulting from p50 overexpression appears to be disruption of the dynactin complex. Our sedimentation analysis indicates that p150\textsuperscript{Glued} and p50 detach from the Arpl filament, which appears to remain intact and maintain its association with p62. This partial dissociation of the dynactin complex may indicate a role for p50 in linking p150\textsuperscript{Glued} to the Arpl filament. In this view, overexpressed p50 would compete for both p150\textsuperscript{Glued} and Arpl binding sites. This situation is analogous to that of antigen:antibody excess, in which suprastochiometric levels of antigen interfere with precipitin formation. Alternatively, the excess p50 may titrate out some limiting structural or regulatory factor critical to the proper assembly of the dynactin complex. Further work will be aimed at discriminating between these models.

The large fraction of dynactin dissociated in the transfected cell extracts indicates that this effect must have occurred at least in part after cell lysis, in view of the heterogeneous nature of the transfected cultures. We estimate that ~10% of the cells in a typical transfected culture overexpress p50 at high level, though lower levels of expression occur in a higher fraction of the cells. Nonetheless, the disruptive effects of p50 appear to be virtually complete in cytosolic extracts. That p50 overexpression disrupts dynactin in the cell as well is strongly supported by our immunocytochemical results, which showed a marked decrease in both p150\textsuperscript{Glued} and Arpl immunoreactivity at the kinetochore of cells expressing high levels of p50 (Fig. 9). 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 before the stage at which dynactin and cytoplasmic dynein normally dissociate from kinetochores (Figs. 4, 6-1 and 9). Even under these conditions, we observed loss of p150\textsuperscript{Glued} and Arpl kinetochore staining in p50-overexpressing cells. These results support a direct disruption of dynactin by the excess p50, resulting in inhibited recruitment of the complex to prometaphase kinetochores. Why both p150\textsuperscript{Glued} and Arpl were displaced is uncertain. This observation could either mean that p50 is important in tar-
Figure 9. Effect of p50 overexpression on anti-Arp1 and anti-p150\textsubscript{Glu}ed staining of kinetochores. Nocodazole-treated (10 μm, 3 h at 37°C) COS-7 cells transfected with p50myc (a, b, e, and f), or β-galactosidase (c, d, g, and h) were stained with “A27” anti-Arp1 (a and c) or anti-p150\textsubscript{Glu}ed (e and g). Anti-p50myc (b and f) and anti-β-galactosidase (d and h) staining identifies overexpressing cells, and reveals chromosomal localization as unstained regions. Neighboring nonexpressing prometaphase cells offer an internal control in each field of view, to assess the decrease in anti-Arp1 and anti-p150\textsubscript{Glu}ed kinetochore staining intensity. a, c, e, and g are through-focus maximal projections of complete x/y optical section stacks (14–22 sections, 0.35 μm step). b, d, f, and h are single optical sections to facilitate visualization of chromosomes in overexpressing cells. Anti-Arp1 and anti-p150\textsubscript{Glu}ed staining are displayed in pseudocolor to show intensity differences (color range shown in panel c). All cells were fixed in formaldehyde followed by methanol extraction. Bar, 5 μm.

geting dynactin to the kinetochore, or that the complex becomes relatively unstable after p50-induced dissociation.

In view of the distinctive disruptive effects of p50 overexpression on both the dynactin complex and mitotic progression, we propose the name dynamitin for this polypeptide.

Role of Dynactin in Cytoplasmic Dynein Function

In addition to its effect on other components of the dynactin complex, dynamitin/p50 overexpression caused a clear decrease in the association of cytoplasmic dynein with the kinetochore. This result implies a role for dynamitin in mediating the association of cytoplasmic dynein with kinetochores and other organelles. Two general models for dynactin function may be envisaged. First, it may serve to regulate dynein activity. 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). Alternatively, dynactin may serve to mediate the binding of dynein to subcellular structures destined for retrograde transport. According to this model, inactivation of dynactin should serve to dissociate dynein from its subcellular binding sites, and this is, in fact, what we observe. This latter model is also strongly supported by our recent identification of p150\textsubscript{Glu}ed as an IC-binding protein, especially in light of the role of the axonemal dynein ICs in...
Figure 10. Effect of p50 overexpression on anti-cytoplasmic dynein and anti-CENP-E staining of kinetochores. Nocodazole-treated (10 μm, 3 h at 37°C) COS-7 cells transfected with p50myc (a-d, g and h), or β-galactosidase (e and f) were stained with anti-cytoplasmic dynein IC (a, c, and e), or anti-CENP-E antisera (g). Anti-p50myc (b, d, and h) and anti-β-galactosidase (f) staining identifies overexpressing cells, and reveals chromosomal localization as unstained regions. Neighboring nonexpressing prometaphase cells offer an internal control in panels a, c, e, and g to assess the differences in kinetochore staining intensity. a, c, e, and g are through-focus maximal projections of complete x/y optical section stacks (20–26 sections, 0.4 μm step). b, d, f, and h 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). All cells were fixed in formaldehyde followed by methanol extraction. Bar, 5 μm.

targeting to the flagellar outer doublet microtubules (King and Witman, 1990; Paschal et al., 1992; Vaughan and Vallee, 1995; Karki and Holzbaur, 1995).

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 an anchor for cytoplasmic dynein, especially with regard to kinetochore function. The p150Glued subunit of dynactin has been found to contain a microtubule-binding domain (Pierre et al., 1992; Waterman-Storer et al., 1995). This domain 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 in loading kinetochores onto the kinetochore-to-pole microtubules. This process itself could be complex, involving a sequential tangential and end-on attachment of kinetochores to 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 mechanism of mitosis and of organelle movement.

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