Overexpression of normal and mutant Arp1α (centractin) differentially affects microtubule organization during mitosis and interphase

Imran B. Clark and David I. Meyer*
Department of Biological Chemistry, UCLA School of Medicine and the Molecular Biology Institute, University of California, Los Angeles, California 90024-1737, USA
*Author for correspondence: (e-mail: dimeyer@ucla.edu)

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SUMMARY

Dynactin is a large multisubunit complex that regulates cytoplasmic dynein-mediated functions. To gain insight into the role of dynactin’s most abundant component, Arp1α was transiently overexpressed in mammalian cells. Arp1α overexpression resulted in a cell cycle delay at prometaphase. Intracellular dynactin, dynein and nuclear/mitotic apparatus (NuMA) protein were recruited to multiple foci associated with ectopic cytoplasmic aggregates of Arp1α in transfected cells. These ectopic aggregates nucleated supernumerary microtubule asters at prometaphase. Point mutations were generated in Arp1α that identified specific amino acids required for the prometaphase delay and for the formation of supernumerary microtubule asters. The mutant Arp1α proteins formed aggregates in cells that colocalized with dynactin and dynein peptides, but in contrast to wild-type Arp1α, NuMA localization remained unaffected. Although expression of mutant Arp1α proteins had no effect on mitotic cells, in interphase cells expression of the mutants resulted in disruption of the microtubule network. Immunoprecipitation studies demonstrated that overexpressed Arp1α interacts with dynactin and NuMA proteins in cell extracts, and that these interactions are destabilized in the Arp1α mutants. We conclude that the amino acids altered in the Arp1α mutant proteins participate in stabilizing interactions between overexpressed Arp1α and components of the endogenous dynactin complex as well as the NuMA protein.

Key words: Arp1, Mitosis, dynactin, Dynein, Nuclear/mitotic apparatus (NuMA)

INTRODUCTION

The majority of intracellular movements in the minus-direction along microtubules is accomplished by the microtubule-activated ATPase cytoplasmic dynein (reviewed in Walker and Sheetz, 1993). This molecular motor exists as a large protein complex of approx. 1200 kDa (reviewed in Schroer, 1994) and has been implicated in membrane transport and organelle positioning, multiple stages of cell division, axonal transport and protein sorting (reviewed in Vallee, 1993).

The functions of cytoplasmic dynein are highly regulated (Vaisberg et al., 1996; Niclas et al., 1996; Vaughan and Vallee, 1995; reviewed in Asai, 1996). One such regulator of dynein function is the dynactin complex, which was identified by its activation of dynein-mediated membrane vesicle movements along microtubules in vitro (Schroer and Sheetz, 1991). The dynactin complex contains proteins of 160, 150, 62, 50, 45, 37, 32, 27 and 24 kDa (Gill et al., 1991), which together form a highly ordered structure with an approximate stoichiometry of 1:1:1:5:10:1:1:1:1 (Schafer et al., 1994). The 45 kDa peptide was identified as the actin-related protein centractin, or Arp1 (Clark and Meyer, 1992; Lees-Miller et al., 1992), and the 160/150 kDa peptides are homologous to the product of the Glued gene in Drosophila (Holzbaur et al., 1991; Garen and Kankel, 1983).

The dynactin complex, like cytoplasmic dynein, appears capable of association with many cellular constituents in a highly regulated fashion. Components of the dynactin complex are ubiquitous and well conserved, and localize to multiple structures within the cell, including membrane organelles, the centrosome, spindle poles and spindle microtubules during mitosis and prometaphase kinetochores. However, most of the dynactin complex is found in the cytoplasm (reviewed in Schroer, 1996). Genetic evidence from N. crassa (Plamann et al., 1994; Robb et al., 1995; Tinsley et al., 1996), Drosophila (McGrail et al., 1995) and S. cerevisiae (Clark and Meyer, 1994; Muhua et al., 1994) indicates not only that cytoplasmic dynein and the dynactin complex are involved in the same cellular processes of nuclear migration, mitotic spindle dynamics and general cellular trafficking, but that the two complexes directly interact with each other. Evidence for direct interaction of these two complexes in higher organisms is well supported by affinity chromatography (Karki and Holzbaur, 1995), antibody microinjections (Waterman-Storer et al., 1997), immunoprecipitation studies (Vaughan and Vallee, 1995) and...
While several functions for the dynactin complex have been determined and many interacting proteins have been identified, the roles of the individual components of the complex remain largely unexplained. The Arp1 (centractin) component is of particular interest because it is the most highly represented protein in the complex (Schafer et al., 1994), with a 15:1 ratio of Arp1α to Arp1β (Clark et al., 1994). Arp1 shares a significant degree of homology to actin (reviewed in Mullins et al., 1996). Several motifs found in actin are well conserved in Arp1, including residues involved in actin polymerization and the cation and nucleotide binding pockets (reviewed in Mullins et al., 1996). Arp1 forms a short actin-like polymer that associates with the actin-binding proteins CapZ (Schafer et al., 1994) and spectrin (Holleran et al., 1996). Arp1 is capable of binding GTP and ATP (Melki et al., 1993).

Overexpression studies are useful in mammalian systems for addressing the function of a particular protein within a heteromeric complex and for determining the structure/function role of specific peptide sequences. This approach has proved fruitful in the past with individual dynactin components and other interacting proteins (Pierre et al., 1994; Waterman-Storer et al., 1995; Echeverri et al., 1996; Holleran et al., 1996; Burkhardt et al., 1997; Ahmad et al., 1998; Dujardin et al., 1998; Diamantopoulos et al., 1999). Therefore, to elucidate a function for the most abundant component of the dynactin complex, the α isoform of human Arp1 was overexpressed in COS-7 cells. Our results show that Arp1α-overexpressing cells become delayed at prometaphase, presumably due to aberrant configurations of the mitotic spindle. Structure/function relationships within the Arp1α protein were investigated by engineering point mutations into Arp1α at conserved residues. Expression of mutant Arp1α proteins did not lead to mitotic phenotypes, as observed with overexpression of wild-type Arp1α; rather, expression of the mutant proteins resulted in disruption of the interphase microtubule network. Differential interactions of Arp1α and the Arp1α mutants with dynactin components and NuMA protein were also identified.

**MATERIALS AND METHODS**

**Transfection vectors**

All plasmids used in transient transfections were derived from the pCMVβ vector (Clontech), from which a β-galactosidase cDNA flanked by NotI restriction sites was replaced with the cDNAs indicated below. The pCMVHS0A plasmid encoding full-length p50 was a gift from Richard B. Vallee. The pCMVαCA plasmid encodes full-length Arp1α protein; pCMVHSVαCA encodes full-length Arp1α with a VSV G tag at the N terminus (see below); the pCMVmt1, pCMVmt2, pCMVmt1/2 plasmids encode Arp1α-m1, Arp1α-m2 and Arp1α-m1/2, respectively (see below); and pCMVHSVmt1, pCMVHSVmt2 and pCMVHSVmt1/2 encode the VSV G-tagged Arp1α mutants (see below). All the Arp1 cDNAs used for transfections had the 3' untranslated regions removed flush using PCR techniques.

**Construction of VSV G-tagged Arp1α**

The C-terminal eleven amino acids of the VSV G (Vesicular Stomatitis Virus glycoprotein) were fused to the N terminus of Arp1α using standard PCR techniques. A 72mer primer was synthesized that contained the following sequences: a vertebrate Kozak consensus (Kozak, 1987), an ATG, the 11 C-terminal codons of VSV G, five glycine codons as a linker, and the first 15 5’-nucleotides of the Arp1α coding region starting with the first ATG. This primer was used in conjunction with an anti-sense primer that spans the downstream Arp1α coding sequence, and standard PCR methods were employed to generate an Arp1α cDNA fragment that contained the VSV G sequence at the 5’ end.

**Construction of Arp1α mutants**

The Arp1α point mutations, Arp1α-m1, Arp1α-m2 and Arp1α-m1/2, were generated by Olivier Staub in our laboratory using standard PCR methods. Briefly, primers were synthesized that were complimentary to Arp1α cDNA sequence except for specific nucleotides, which were changed in order to alter specific codons. One primer, (5'TCCTCTCTGAGTTTGAGATT) was used to generate Arp1α-m1, which contains the amino acid replacements E210A and E212A. Another primer, (5'AAAGATGTGAGATCAGATTCACCG3'), was used to generate Arp1α-m2, which contains the amino acid replacements K327A and R329A. Arp1α-m1 and Arp1α-m2 were combined to create Arp1α-m1/2 using standard restriction endonuclease and ligation procedures.

**Immunochemistry**

Affinity-purified A27 rabbit anti-Arp1α (see below) was generated in our laboratory. Affinity-purified rabbit anti-dynein heavy chain was a gift from Eugeni Vaisberg; mAb50-1A mouse anti-p50 and p150DART affinity-purified rabbit anti-p150Glued were gifts from Richard B. Vallee; mAb61F1 mouse anti-NuMA were gifts from Don W. Cleveland and Duane A. Compton. Monoclonal mouse anti-β-galactosidase was purchased from GibcoBRL; monoclonal mouse anti-VSV G, monoclonal mouse anti-α-tubulin, monoclonal anti-γ-tubulin and monoclonal mouse anti-cytoplasmic dynein intermediate chain (clone 70.1) were purchased from Sigma, and rabbit anti-tubulin was purchased from ICN Biomedicals Inc.

For indirect immunofluorescence, cells that had been transfected and seeded into 24-well plates containing 12 mm glass coverslips were processed by fixing the cells in 3% p-formaldehyde, pH 7.2, for 15 minutes at room temperature, and then permeabilizing with 20°C methanol for 5 minutes. In some cases (for detection with the p150DART, NuMA 1F1 and 70.1-anti-dynein antibodies), 20°C methanol for 20 minutes only was used for fixation. Cells were incubated at room temperature for 30 minutes in blocking buffer (0.2% fish skin gelatin, 0.02% sodium azide, 1x PBS), for 30 minutes with primary antibodies, then for 20 minutes with secondary antibodies. To avoid fluorescence bleed-through, the overexpressed proteins were detected using Texas Red-conjugated secondary antibodies and the endogenous proteins were detected using fluorescein-conjugated secondary antibodies, except in the case of tubulin, which was always visualized with Texas Red. For staining of DNA, 0.2 µg/ml bisbenzamide (Hoechst; Sigma) was included with the secondary antibody. Coverslips were mounted on glass microscope slides with glycerol containing 10 mM Tris-HCl, pH 8.6 and 2% Dabco (1,4-diazabicyclo(2.2.2.)octane; Kodak), and then sealed around the edge with clear nail polish. Immunofluorescence was viewed using a Nikon Microphot-FXA epifluorescence microscope equipped with a Photometrics CCD camera. Digitized images were captured on a Silicon Graphics Indy5000 computer using ISee 4.0 (c) software (Inovision).

For western blotting, immunoprecipitated fractions from transfected cell lysates were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Micron Separations, Inc.). Membrane strips were incubated in blocking buffer (3% non-fat milk, 1x PBS, 0.05% Triton X-100, 0.02% sodium azide) for 30 minutes at room temperature. Primary antibody incubations were carried out for 1-2 hours at room temperature or overnight at 4°C, then incubated for 1-2 hours at room temperature with alkaline
phosphatase-conjugated secondary antibody and detected by development in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Promega).

**Generation of affinity-purified A27 antibody**

A27 rabbit anti-Arp1α was raised against the Arp1α peptide segment (CLSINPQKDETLETKAQQ), corresponding to amino acids 223-240. The peptide was conjugated to KLH-maleimide, which was already coupled to liposomes, according to the manufacturer’s instructions (Pierce) for injection into rabbits. Sera were affinity-purified using GST-Arp1α (see Clark et al., 1994, for construction and purification of GST-Arp1α). The GST-Arp1α was immobilized on an Actigel ADL column (Sterogene Bioseparations) and affinity purification of A27 antibodies was carried out using a Quick Pure kit according to the manufacturer (Sterogene).

**Transient transfections**

COS-7 cells were grown at 37°C, 5% CO₂, in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 200 mg/ml L-glutamine and a penicillin/streptomycin cocktail (Gemini Bio-Products Inc.). At 12-16 hours before transfection a 90% confluent 10 cm polystyrene plate (Becton Dickson Co.) of cells was split 1:6 into 10 ml of medium and seeded onto a 10 cm plate. Cells were then transfected with Qiagen purified plasmids using FuGene™ reagent (Boehringer Mannheim), which typically resulted in 30-60% transfection efficiency.

FuGene™ transfections were carried out according to the manufacturer’s instructions. Transfected cells were incubated at 37°C, 5% CO₂, overnight, then processed for use in immunoprecipitation or immunofluorescence experiments. Cells transfected for use in immunofluorescence experiments were split into 24-well polystyrene plates containing 12 mm glass coverslips coated with 0.02% poly-L-lysine, incubated overnight, and then processed approximately 40 hours post-transfection for immunofluorescence.

**Cell cycle synchronization**

COS-7 cells were grown and transfected as described. For cell cycle synchronization, cells transfected the previous day were incubated in 5 mM thymidine for 18 hours. The thymidine was removed by three rinses with 37°C medium and a fourth rinse for 10 minutes at 37°C, 5% CO₂. The medium was replaced in each plate with 5 ml of medium containing 2 μg/ml nocodazole and incubated at 37°C, 5% CO₂, for 6-8 hours. Cells were collected by mitotic shake-off and either processed for immunoprecipitation or transferred to coverslips in 24-well plates and processed for immunofluorescence at various times after removal of the nocodazole block.

**Immunoprecipitation**

For each immunoprecipitation six 10 cm plates of COS-7 cells were transfected with plasmids encoding VSV G-tagged Arp1α or the VSV G-tagged Arp1α mutants. Cells were synchronized in prometaphase as described above. Cells were collected by mitotic shake off and, and the six plates of cells from a single transfection (approximately 2×10⁸ cells in each collection) were pooled into 5 ml medium containing 2 μg/ml nocodazole and 20 μg/ml cytochalasin B, then incubated at 37°C for 30 minutes. The cells were rinsed twice with ice cold 1×PBS containing 2 μg/ml nocodazole and 20 μg/ml cytochalasin B. The cells were pelleted and resuspended in 0.5 ml of ice-cold Lysis Buffer A (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 440 μM PMSF and Complete™ protease inhibitor (Boehringer Mannheim)) containing 2 μg/ml nocodazole and 20 μg/ml cytochalasin B and transferred to a 1.7 ml tube on ice. The cells were incubated on ice for 10 minutes to permit lysis, then centrifuged in a microfuge at 10,000 rpm, 4°C, for 10 minutes, and the cytosol removed to a fresh 1.7 ml tube on ice. The remaining pellet was rinsed once with Lysis Buffer A, resuspended in 0.5 ml Lysis Buffer A containing 200 mM NaCl, and transferred to a glass Dounce homogenizer. The pellet was homogenized with 50-60 strokes of a tight-fitting glass pestle, incubated on ice for 5 minutes, and microfuged at 10,000 rpm, 4°C, for 10 minutes. The salt extract was removed to a fresh 1.7 ml tube on ice. To the cytosol and the salt extract 25 mg/ml IgG of anti-VSV G was added and incubated with rotating at 4°C for 4 hours to overnight. To this was added 50 μl of a 1:1 slurry of proteinG-agarose in Lysis Buffer A for 4 hours at 4°C. The immunoprecipitate was pelleted for 30 seconds in a microfuge and the depleted cytosol or salt extract removed to a fresh 1.7 ml tube on ice. The immunoprecipitate was rinsed four times with ice-cold Dilution Buffer (one tenth Lysis Buffer A, with Tris-Cl brought to 50 mM and NaCl brought to 150 mM), pelleted for 30 seconds in a microfuge, and as much of the final wash as possible removed using a 25-gauge syringe needle. The immunoprecipitate was boiled for 5 minutes in 65 μl of 1× gel loading buffer (without reducing agent), microfuged for 15 seconds, the gel loading buffer removed to a fresh 1.7 ml tube, and dithiothreitol added to 50 mM. The depleted cytosol and depleted salt extract were precipitated with TCA and resuspended in 65 μl of 2× gel loading buffer. 10 μl of each fraction was run on 10% polyacrylamide minigels and transferred to NitroBind nitrocellulose membranes (Micron Separations Inc.) for immunodetection.

**RESULTS**

**Overexpression of Arp1α results in an increase in the percentage of cells in prometaphase**

Arp1α was overexpressed in COS-7 cells. Initial immunofluorescence studies revealed that a greater percentage of Arp1α-transfected cells appeared to be in mitosis compared to non-transfected cells. This observation, and the report by Echeverri et al. (1996) that COS-7 cells overexpressing the p50 component of the dynactin complex had an increased mitotic index, prompted closer examination of the mitotic figures in our experiments.

Mitotic indices were determined for populations of COS-7 cells transiently transfected with either Arp1α, p50 (positive control), or with β-galactosidase (negative control). In three separate experiments, populations of cells that overexpressed Arp1α or p50 showed a significant increase in mitotic index when compared to untransfected and β-galactosidase-expressing control cells (Fig. 1A). This increase in mitotic index was due solely to an increase in the percentage of cells in a prometaphase-like state (Fig. 1A); the other stages of mitosis appeared unaffected. Overexpression of Arp1α also resulted in an increased mitotic index in Rat-2 and HeLa cells (data not shown). These results suggest a possible function for Arp1α in progression through the prometaphase of mitosis, presumably via its role in the dynactin complex.

**Point mutations engineered into Arp1α reveal essential residues**

To address the structure/function relationship of Arp1α to the prometaphase phenotype, two pairs of point mutations were engineered into Arp1α. The first pair, referred to as Arp1α-m1, consisted of an E210A and an E212A replacement. The second pair, referred to as Arp1α-m2, is a K327A and an R329A replacement. A protein with the combined sets of mutations, referred to as Arp1α-m1/2, was also constructed. The rationale for making these mutations was based upon the study by Wertman et al. (1992), in which several mutations in yeast actin were generated in an alanine scanning mutational analysis. Two of these mutations, referred to as act1-14 and act1-26
cells overexpressing Arp1. Cells were counted through an epifluorescence microscope and proteins and for microtubules. DNA was viewed using Hoechst stain. For double immunofluorescence visualization of the overexpressed Arp1 in four separate experiments, a total of 4,195 cells overexpressing Arp1 non-transfected controls (data not shown). (B) Expression of the transfected, but did not overexpress protein, appeared identical to vector alone or salmon sperm DNA, and cells that had been mitosis, other than prometaphase, in Arp1 cells were near control levels. Cells that were mock-transfected with cDNAs encoding Arp1, p50 or β-galactosidase, and processed for double immunofluorescence visualization of the overexpressed proteins and for microtubules. DNA was viewed using Hoechst stain. Cells were counted through an epifluorescence microscope and scored according to their different stages of mitosis. In three separate experiments, a total of 11,411 cells overexpressing Arp1α, 7,131 cells overexpressing p50, 6,617 cells expressing β-galactosidase, and 15,982 wild-type non-transfected cells were counted. All phases of mitosis, other than prometaphase, in Arp1α- and p50-transfected cells were near control levels. Cells that were mock-transfected with vector alone or salmon sperm DNA, and cells that had been transfected, but did not overexpress protein, appeared identical to non-transfected controls (data not shown). (B) Expression of the Arp1α mutant proteins failed to result in an increased mitotic index. In four separate experiments, a total of 4,195 cells overexpressing Arp1α, 4,730 cells expressing Arp1α-m1, 7,625 cells expressing Arp1α-m2, 5,436 cells expressing Arp1α-m1/2, 3,520 cells expressing β-galactosidase, and 61,407 wild-type non-transfected cells were counted. All phases of mitosis in transfected cells were near control levels.

Overexpression of Arp1α results in the formation of supernumerary mitotic spindles in prometaphase cells

Since a multimeric complex comprising the dynactin complex, cytoplasmic dynein and NuMA protein is required for mitotic spindle pole formation (Gaglio et al., 1996; Merdes et al., 1996), one could predict that overexpression of Arp1α perturbs mitosis through either a disruption of spindle formation or the assembly of additional spindles. To distinguish between these two possibilities, COS-7 cells transiently transfected with β-galactosidase (negative control) or Arp1α were processed for double immunofluorescence analysis of the overexpressed proteins and endogenous microtubules. Untransfected (Fig. 2a-c) and β-galactosidase-expressing (data not shown) mitotic cells possessed normal bipolar microtubule spindles. Prometaphase cells that overexpressed Arp1α displayed supernumerary spindle asters and microtubule foci that co-localized with ectopic aggregates of Arp1α (Fig. 2d-l). Interestingly, these ectopic microtubule asters reappeared in prometaphase cells upon recovery from nocodazole treatment (Fig. 2m-r), suggesting that aggregates of ectopic Arp1α actively nucleate microtubule asters, instead of localizing to preformed asters. Normal bipolar spindles were not observed in prometaphase cells that overexpressed Arp1α. However, the spindles of Arp1α-transfected cells appeared completely normal at later stages of mitosis (see below).

Since a complex containing dynactin, cytoplasmic dynein and NuMA is required for formation of the bipolar microtubule spindle during mitosis (reviewed in Merdes and Cleveland, 1997), we interpreted the formation of multiple microtubule asters to represent the recruitment of dynactin, dynein and NuMA to ectopic aggregates of overexpressed Arp1α. Double immunofluorescence techniques were initially employed to observe potential interactions between overexpressed Arp1α and the dynactin complex, cytoplasmic dynein and/or NuMA protein in transfected cells, as many of these interactions may be transient and of low affinity. Dynactin, dynein and NuMA
were normally localized in prometaphase cells to two crescents on each side of the forming metaphase plate, representing the location of the two spindle poles (Arp1α, p50 and NuMA are shown in Fig. 3a-f). Expression of β-galactosidase had no effect on localization of dynactin and dynein components (data not shown). Upon transfection with Arp1α, prometaphase cells accumulated numerous ectopic cytoplasmic aggregates of Arp1α protein, as described above. Endogenous p50 and p150, dynein intermediate chain and NuMA proteins were seen to co-localize with many of these ectopic Arp1α aggregates (Fig. 3g-r). However, γ-tubulin continued to localize as two spots, indicating the presence of only two centrosomes (Fig. 3s-u). No obvious effect of Arp1α overexpression on kinetochore localization of dynein/dynactin was observed, differing from normal localization.

![Fig. 2. Cells that overexpress Arp1α possess aberrant spindle morphologies. COS-7 cells transiently transfected with a cDNA encoding Arp1α were processed for double immunofluorescence. Each row illustrates a single field visualizing Arp1α (a,d,g,j,m,p), microtubules (b,e,h,k,n,q), and the chromosomes (c,f,i,l,o,r). Endogenous Arp1α localized to the spindle poles and polar microtubules (a). Non-transfected cells (a-c) contained a well-organized bipolar microtubule spindle by prometaphase. Overexpression of Arp1α resulted in prometaphase cells that contained supernumerary microtubule spindle asters and foci (d-l). When microtubules were completely depolymerized in 2 µg/ml nocodazole (m-o) multiple new asters organized around Arp1α aggregates 5 minutes after washout of the nocodazole (p-r).

![Fig. 3. Colocalization of dynactin, dynein and NuMA proteins with ectopic aggregates of overexpressed Arp1α in prometaphase cells. COS-7 cells transiently transfected with a cDNA encoding Arp1α (or VSVG-Arp1α) were processed for double immunofluorescence of overexpressed Arp1α (or VSVG-Arp1α) and either p50, p150 (in the case of VSVG-Arp1α), dynein intermediate chain, NuMA protein or γ-tubulin. Chromosomes were viewed using Hoechst stain (c,f,i,l,o,r,u). In non-transfected prometaphase cells dynein/dynactin components (Arp1α (a,d) and p50 (b) are shown) and NuMA (e) colocalized to crescents on both sides of the forming metaphase plate. Prometaphase cells that overexpressed Arp1α (or VSVG-Arp1α) contained multiple Arp1α aggregates (g,j,m,p,s). Endogenous p50 (h), p150 (k), dynein intermediate chain (n) and NuMA (q) colocalized with several of the Arp1α aggregates (arrows). Localization of γ-tubulin (t) is also shown. Bar, 10 µm.]

![Arp1α](image1.png)

![Tubulin](image2.png)

![DNA](image3.png)
substantially from the phenotype produced by p50 overexpression (data not shown; Echeverri et al., 1996). Based on these data, overexpressed Arp1α appears to recruit endogenous dynactin complex, cytoplasmic dynein and NuMA to aggregates that retain the microtubule nucleating function (of the multimeric dynactin/dynein/NuMA mitotic complex) during prometaphase.

Late stages of mitosis appear unaffected by overexpression of Arp1α

The statistical data in Fig. 1 suggest that stages of mitosis beyond prometaphase remain unaffected by overexpression of Arp1α. Two explanations are possible: (1) only a sub-population of the overexpressing cells becomes blocked at prometaphase or (2) all the transfected cells are delayed at prometaphase and then all these cells recover and continue normally through mitosis. Our observations support the latter alternative. All prometaphase cells overexpressing Arp1α were seen to possess an aberrant spindle morphology, all cells in late mitosis had a bipolar spindle (see below), and no multipolar divisions were observed. Cell cycle synchronization experiments verified that all Arp1α-overexpressing cells cycle into and out of mitosis and (2) all the transfected cells were delayed at prometaphase or (2) all the transfected cells are blocked at prometaphase (data not shown; Echeverri et al., 1996). Correspondingly, microtubule spindle morphology was unaffected in cells that expressed the mutant Arp1α proteins (Fig. 6a-i), differing substantially from the behavior of cells overexpressing wild type Arp1α (Fig. 2).

Biochemical analysis supports immunofluorescence observations

Echeverri et al. (1996) reported that overexpression of the p50 component resulted in fragmentation of the dynactin complex. Our observations suggest that excess Arp1α recruits the dynactin complex to ectopic aggregates in a manner that does not disrupt the complex. This assumption was substantiated by sucrose sedimentation analysis, which demonstrated that expression of the mutants would not form the same kind of aggregates in mitotic cells as seen with overexpression of wild-type Arp1α. Accordingly, COS-7 cells transiently transfected with Arp1α-m1, Arp1α-m2 or Arp1α-m1/2 were examined by double immunofluorescence for localization of the mutant proteins and for the localization of p50, p150, cytoplasmic dynein intermediate chain, NuMA and microtubules. The Arp1α mutants formed aggregates in the cytoplasm of mitotic cells and endogenous dynactin and dynein peptides colocalized to these aggregates (Fig. 5a-i), identical to overexpression of wild-type Arp1α (Fig. 3). However, in Arp1α mutant expressing cells, most of the NuMA protein retained its normal distribution as two crescent shapes on either side of the forming metaphase plate (Fig. 5j-l), indicative of localization to two, well-organized spindle poles. Correspondingly, microtubule spindle morphology was unaffected in cells that expressed the mutant Arp1α proteins (Fig. 6a-i), differing substantially from the behavior of cells overexpressing wild type Arp1α (Fig. 2).

Table 1. Distribution of synchronized mitotic cells

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<td>18.2±0.25</td>
<td>27.3±0.10</td>
</tr>
<tr>
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<td>100.0</td>
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<tr>
<td>8</td>
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COS-7 cells were transiently transfected with Arp1α, synchronized to prometaphase, then processed at various times after release from cell cycle arrest for immunofluorescence viewing of Arp1α, microtubules and chromosomes, and scored according to specific stages of mitosis.

From two separate experiments a total of 16,212 cells were scored, of which 6,254 were overexpressing Arp1α.
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To better address the existence of these potential stable protein-protein interactions, cells expressing VSV G-tagged Arp1α, or VSV G-tagged Arp1α mutant proteins, and synchronized to prometaphase of the cell cycle, were subjected to immunoprecipitation analysis using an anti-VSV G antibody. Dynactin peptides were observed to co-immunoprecipitate with VSV G-Arp1α, and to a much lesser extent with the VSV G-Arp1α mutant proteins (Fig. 7).

Identical results were obtained with immunoprecipitation from asynchronous populations of transfected cells (data not shown). Co-immunoprecipitation of the NuMA protein from mitotic cytosol could not be addressed, as the NuMA in COS-7 cells pelleted with insoluble material (data not shown). However, a minimum concentration of 200 mM NaCl was found to solubilize NuMA. A significant amount of dynactin complexes, in contrast to the overexpression of p50 (data not shown).

Dynactin and dynein proteins, but not NuMA protein, colocalize with aggregates of Arp1α mutant proteins in prometaphase cells. COS-7 cells transiently transfected with cDNAs encoding Arp1α-m1 (or VSVG-Arp1α-m1), Arp1α-m2 (or VSVG-Arp1α-m2) or Arp1α-m1/2 (or VSVG-Arp1α-m1/2) were processed for double immunofluorescence viewing of the Arp1α mutants and either p50, p150 (in the case of VSVG-Arp1α mutants), dynein intermediate chain or NuMA protein. Chromosomes were viewed using Hoechst stain (c,f,i,l). Since the subcellular distributions of the three mutants and their effects on the localization of dynactin, dynein and NuMA proteins were identical, only the data for Arp1α-m2 is shown here, since Arp1α-m2 expression was consistently equivalent to wild-type Arp1α overexpression. Prometaphase cells expressing the Arp1α mutant proteins contained multiple aggregates of mutant protein (a,d,g,j) and endogenous p50 (b), p150 (e) and dynein intermediate chain (h) were seen to colocalize with several of these ectopic aggregates of Arp1α mutant protein (arrows). But most of the NuMA protein (k) continued to localize to only two regions on either side of the forming metaphase plate. Bar, 10 μm.
overexpressed Arp1α and dynactin peptides could also be extracted from this insoluble pellet (Fig. 7), consistent with the previously observed insolubility of dynactin at mitotic spindle poles (Dionne et al., 1999). Much of the VSV G-Arp1α proteins could be immunoprecipitated from the salt extracts, and dynactin components, as well as NuMA protein, were seen to co-precipitate with VSV G-Arp1α and not with the VSV G-Arp1α mutants (Fig. 7). Dynein intermediate and heavy chains were not observed to co-precipitate with the VSV G-Arp1α proteins (data not shown), suggesting that interactions between these molecules are unstable or transient.

Sucrose sedimentation analysis of cytosol from cells that express the VSV G-tagged proteins demonstrated unequivocally that the mutant Arp1α proteins did not assemble into 20S complexes, while much of the overexpressed wild-type Arp1α did (data not shown). Expression of VSV G-Arp1α resulted in an increase in the proportion of cells in a prometaphase-like state and in the formation of supernumerary microtubule spindle asters, comparable to overexpression of untagged Arp1α (data not shown). Also, low level expression of VSV G-Arp1α, as well as the VSV G-tagged mutant proteins, demonstrated an immunofluorescence pattern identical to that of endogenous Arp1α, suggesting the overexpressed proteins are properly folded and targeted within the cell (data not shown).

These data lend further support to the hypothesis that the amino acids mutated in Arp1α for this study are required to maintain the stable association of overexpressed Arp1α with the dynactin complex and NuMA protein.

### Expression of Arp1α-m1, Arp1α-m2 or Arp1α-m1/2 disrupts the organization of interphase microtubules

Overexpression studies have revealed a function for cytoplasmic dynein in the organization of interphase microtubules at the microtubule organizing center (MTOC; Koonce and Samso, 1996). Overexpression of the p150 subunit of the dynactin complex resulted in cells that possess a microtubule array no longer focused at the MTOC (Waterman-
Storer et al., 1995). Based on these reports, and the pronounced effect in this study of overexpression of Arp1α on the organization of microtubules in prometaphase cells, we examined the effect of overexpression of Arp1α on the organization of microtubules in interphase cells.

COS-7 cells were transiently transfected with β-galactosidase (negative control), Arp1α, Arp1α-m1, Arp1α-m2 or Arp1α-m1/2 and processed for immunofluorescence. Most interphase cells overexpressing Arp1α contained aggregates in the cytoplasm that accumulated at the MTOC (Fig. 8a-c). On occasion, transfected interphase cells contained filament-like structures in the cytoplasm, sometimes trailing out of larger aggregates, composed of overexpressed Arp1α (data not shown), similar to the observations of Holleran et al. (1996). Arp1α-m1, Arp1α-m2 and Arp1α-m1/2 proteins also accumulated in interphase cells as cytoplasmic perinuclear aggregates (Fig. 8d-I). In cells overexpressing Arp1α, microtubules remained focused at the MTOC and well-organized in the cytoplasm, identical to microtubule organization in non-transfected (Fig. 8a-c) and β-galactosidase-expressing control cells (data not shown). In contrast, cells that expressed Arp1α-m1, Arp1α-m2 or Arp1α-m1/2, even at very low levels, displayed an interphase microtubule array that was no longer well-focused at the MTOC (Fig. 8d-l). The number of cells expressing Arp1α mutants that displayed these disrupted interphase microtubule arrays was extensive: 44% of the Arp1α-m1 transfected cells, 61% of the Arp1α-m2 transfected cells and 56% of the Arp1α-m1/2 transfected cells. In contrast, only about 8% of the cells overexpressing wild-type Arp1α and less than 4% of non-transfected cells, exhibited microtubule arrays that were only slightly less focused at the MTOC.

Normally, interphase microtubules are nucleated by the centrosome and subsequently released from the centrosome, yet are maintained as a radial array focused at the MTOC (Ahmad et al., 1998; Keating et al., 1997; Yu et al., 1993; reviewed in Stearns and Wines, 1997). Merdes et al. (1996) demonstrated that in mitotic cells most microtubules maintain focused spindle poles in a dynein/dynactin/NuMA-dependent manner but are not directly attached to the centrosomes. Therefore, it was predicted that expression of the Arp1α-m1, Arp1α-m2 or Arp1α-m1/2 proteins may disrupt interphase microtubule organization by hindering the ability of new microtubules to nucleate from the centrosome or by diminishing the ability of microtubules to maintain a radial array once they have been released from the centrosome. Each of these possibilities was examined.

When interphase microtubules were completely depolymerized with medium containing 2 μg/ml nocodazole and then allowed to repolymerize in fresh medium, microtubule regrowth from centrosomes occurred in 100% of all cell types examined (Fig. 9a-f,j-o). However, if the interphase microtubule network was allowed to repolymerize for 30 minutes or more, cells that expressed the Arp1α mutant proteins lost their MTOC-focused microtubule array (Fig. 9p-r), whereas repolymerized interphase microtubule arrays in untransfected and Arp1α-transfected cells remained densely focused at the MTOC indefinitely (Fig. 9g-i, and non-overexpressing cell in Fig. 9p-r). Quantitation of disorganized microtubule arrays in transfected cells 60 minutes after nocodazole washout demonstrated ratios comparable to those previously observed for transfected cells not treated with nocodazole.

Aggregates of overexpressed wild-type Arp1α recruited key components implicated in microtubule aster formation in mitotic cells. The mutants showed differences in this regard. A simple explanation for the interphase phenotype would be differential sequestration of essential components by the Arp1α mutant proteins. In transfected interphase cells immunofluorescence showed that p50, p150 and dynein intermediate chain all colocalized to aggregates of Arp1α, as well as to aggregates of Arp1α-m1, Arp1α-m2 and Arp1α-m1/2 (data not shown). These data suggest that the mutant proteins are disrupting interphase microtubules by a mechanism other than differential recruitment of dynein and dynactin. The following observations were also made: (1) the Golgi apparatus and lysosomes were dispersed in these mutant-expressing cells, similar to observations made with p50 overexpression (Burkhardt et al., 1997); (2) expression of the Arp1α mutants specifically resulted in the disruption of microtubules and not in a generalized perturbation of the cytoplasm in the vicinity of the MTOC, as organization of vimentin intermediate

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**Fig. 8.** Interphase cells that express the Arp1α mutant proteins possess an aberrant microtubule array. COS-7 cells were transiently transfected with cDNAs encoding Arp1α, Arp1α-m1, Arp1α-m2, or Arp1α-m1/2, and processed for double immunofluorescence visualization of the overexpressed proteins and for tubulin. DNA was viewed using Hoechst stain (c,f,i,l). Interphase cells that overexpressed Arp1α had an increased cytoplasmic fluorescence, as well as several aggregates that accumulated as a large mass at the MTOC (a). Microtubule organization was unaffected in these cells (b). The Arp1α mutant proteins were also observed in the cytosol, and they formed several aggregates that accumulated in the perinuclear region (d,g,j). The interphase microtubule network of cells expressing mutant proteins was no longer organized as a radial array focused at the MTOC (e,h,k). Non-transfected cells served as an internal control. Bar, 20 μm.
Arp1α transiently transfected with cDNAs encoding Arp1α and Arp1α was identical for all three of the mutants, only the data for Arp1α of expression of mutant Arp1α-m2 is shown here. COS-7 cells which had been transiently transfected with cDNAs encoding Arp1α, Arp1α-m1, Arp1α-m2 or Arp1α-m1/2 were incubated for 2 hours in medium containing 2 μg/ml nocodazole to depolymerize the microtubule network. Cells were then incubated in fresh medium to allow microtubules to repolymerize for varying amounts of time (0, 1, 3, 7, 15, 30, 45 and 60 minutes), before being processed for double immunofluorescence. Cells are shown at 0 minutes (a-c and j-l), 7 minutes (d-f and m-o) and 60 minutes (g-i and p-r) post-nocodazole treatment. Cells were viewed for overexpressed Arp1α (a,d,g) or Arp1α-m2 (j,m,p) and for microtubules (b,e,h and k,n,q). DNA was viewed using Hoechst stain (c,f,i and l,o,r). Bars, 20 μm.

Fig. 9. Interphase cells that express the Arp1α mutant proteins are not able to maintain microtubule organization at the MTOC once microtubules have been nucleated by the centrosome. Since the affect of expression of mutant Arp1α proteins on microtubule organization was identical for all three of the mutants, only the data for Arp1α and Arp1α-m2 is shown here. COS-7 cells which had been transiently transfected with cDNAs encoding Arp1α, Arp1α-m1, Arp1α-m2 or Arp1α-m1/2 were incubated for 2 hours in medium containing 2 μg/ml nocodazole to depolymerize the microtubule network. Cells were then incubated in fresh medium to allow microtubules to repolymerize for varying amounts of time (0, 1, 3, 7, 15, 30, 45 and 60 minutes), before being processed for double immunofluorescence. Cells are shown at 0 minutes (a-c and j-l), 7 minutes (d-f and m-o) and 60 minutes (g-i and p-r) post-nocodazole treatment. Cells were viewed for overexpressed Arp1α (a,d,g) or Arp1α-m2 (j,m,p) and for microtubules (b,e,h and k,n,q). DNA was viewed using Hoechst stain (c,f,i and l,o,r). Bars, 20 μm.

From these studies, two conclusions can be drawn. The first is that in mitotic cells, Arp1α appears to play an integral role in the sequestering of components needed for spindle pole formation. The second is that in interphase cells, the dynactin complex may also function in the maintenance of a focused microtubule array at the MTOC. Key amino acids in Arp1α have been identified that are essential for these functions. It remains possible that aggregates of overexpressed Arp1α protein exert a non-physiological effect during prometaphase. However, the fact that the mutant Arp1α aggregates do not affect mitosis strongly suggest that overexpression of wild-type Arp1α results in a hyperphysiological activity. Also, the observed phenotypes are consistent with functions previously ascribed to or predicted for the dynactin complex.

Arp1α-transfected cells became delayed in a prometaphase-like state, presumably due to the presence of supernumerary spindle poles. One might expect the Arp1α-transfected cells to divide along several axes in the presence of multiple spindles, yet these cells re-establish a bipolar spindle by metaphase, and then continue through an otherwise normal mitosis. We propose that this is a necessary event for these cells to complete mitosis. The metaphase-to-anaphase transition is an important cell cycle checkpoint (reviewed in Murray, 1994) that is sensitive to the overall tension on the mitotic spindle (Nicklas et al., 1995, 1998). In this context, the supernumerary spindles resulting from overexpression of Arp1α may upset the balance of tension forces acting on the spindle, thereby preventing the onset of anaphase. This raises the question of how Arp1α-transfected cells recover a bipolar microtubule array. Brunet et al. (1998) showed that when multiple spindle poles were generated in mouse oocytes they tended to reform the more stable bipolar configuration. Studies have shown that, despite the presence of other factors capable of bundling microtubule minus-ends, centrosomes are the dominant sites of microtubule spindle pole formation (Heald et al., 1997; Zhang and Nicklas, 1995; reviewed by Hyman and Karsenti, 1998). Immunofluorescence detection of γ-tubulin indicated the presence of only two centrosomes throughout mitosis in Arp1α-transfected cells. In light of these facts, it is conceivable that in Arp1α-transfected cells the two centrosomes eventually out-compete the ectopic Arp1α aggregates for the tethering of dynamic microtubule minus-ends, and a stable bipolar spindle is eventually established.

Since expression of the Arp1α mutants failed to mis-localize NuMA, or to result in a prometaphase phenotype, recruitment of NuMA to ectopic Arp1α aggregates appears necessary for production of supernumerary spindles. The mutated amino acids can be inferred to play an important role. A recent study by Mattagajasingh et al. (1999) has identified an isoform of red blood cell protein 4.1 as a specific binding partner for NuMA and shown that these two proteins colocalize to mitotic spindle poles. At cell membranes protein 4.1 binds to spectrin/adducin (Correas et al., 1986), which in turn directly binds the Arp1 component of the dynactin complex (Holleran et al., 1996, 1998). If a similar structural hierarchy is preserved at the spindle poles, then Arp1 may be a direct binding partner for NuMA protein.

Differences in the interaction between Arp1α, or the Arp1α mutants, with NuMA protein observed in vivo were
reproducible in vitro. Although the mutant proteins appeared to associate with dynactin components in vivo, the mutants were not observed to co-immunoprecipitate p150 and p50 to the same extent as overexpressed Arp1α. Arp1 has previously been shown to directly bind the p150 component of the dynactin complex (Waterman-Storer et al., 1995). Therefore, it may be possible that a stable association of the dynactin complex with overexpressed Arp1α occurs through p150, and is dependent upon the presence of the specific amino acids changed in the Arp1α mutants. The differential protein interactions observed in this study could be a reflection of the ability of overexpressed wild-type Arp1α, and the inability of mutant Arp1α, to incorporate into the Arp1 filament of the endogenous dynactin complex. This idea would be consistent with a hypothesis where the mutated residues affect the polymerization characteristics of the Arp1α monomer, as is believed to be the case with the actin molecule (Lorenz et al., 1993; reviewed in Milligan, 1993; Wertman et al., 1992). This is supported by the fact that the mutant Arp1α proteins fail to sediment with 20S complexes or to immunoprecipitate other dynactin components, whereas overexpressed wild-type Arp1α does. Unfortunately, until in vitro polymerization of recombinant Arp1α can be achieved, polymerization studies with mutant Arp1α proteins will not be possible.

Evidence presented in this study indicates that dynactin may function during interphase in the maintenance, but not the nucleation, of radial arrays of microtubules. This is no surprise considering previous studies have shown that formation of microtubule asters, even in the presence of centrosomes, requires dynein and dynactin (reviewed in Merdes and Cleveland, 1997). Overexpression of p50 resulted in microtubule bundling (Waterman-Storer et al., 1995), and COS-7 cells transfected with a cDNA encoding p50 possessed a microtubule array that was slightly less focused at the MTORC (Burkhardt et al., 1997). In accord with these studies, we found that expression of specific Arp1α mutant proteins disrupted the radial array of interphase microtubules. We further demonstrated that this is a disruption in the maintenance, and not the initial establishment, of MTORC-focused microtubules. Thus, an interaction of Arp1α with a factor essential to the organization of interphase microtubules may be sensitive to the presence of the Arp1α mutant proteins. Prime candidates for this factor would include a structural component directly involved in the maintenance of the interphase MTORC, analogous to the role of NuMA at mitotic spindle poles. Such a role might be fulfilled by the γ-tubulin/pericentrin complex (Dictenberg et al., 1998), by one of the centrin proteins (Paoletti et al., 1996), or by a PCM-1 related protein (Balczon et al., 1994). Protein 4.1 has recently been localized to the centrosome (Krauss et al., 1997), raising the possibility that a spectrin-like network at the interphase centrosome may be dependent upon the presence of the specific amino acids in Arp1α.

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