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Colocalization of cytoplasmic dynein with dynactin and CLIP-170 at microtubule distal ends

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SUMMARY

Cytoplasmic dynein is a minus end-directed microtubule motor responsible for centripetal organelle movement and several aspects of chromosome segregation. Our search for cytoplasmic dynein-interacting proteins has implicated the dynactin complex as the cytoplasmic dynein 'receptor' on organelles and kinetochores. Immunofluorescence microscopy using a total of six antibodies generated against the p150*Glued*, Arp1 and dynamitin subunits of dynactin revealed a novel fraction of dynactin-positive structures aligned in linear arrays along the distal segments of interphase microtubules. Dynactin staining revealed that these structures colocalized extensively with CLIP-170. Cytoplasmic dynein staining was undetectable, but extensive colocalization with dynactin became evident upon transfer to a lower temperature. Overexpression of the dynamitin subunit of dynactin removed Arp1 from microtubules but did not affect microtubule-associated p150*Glued* or CLIP-170 staining. Brief acetate treatment, which has been shown to affect lysosomal and endosomal traffic, also dispersed the Golgi apparatus and eliminated the microtubule-associated staining pattern. The effect on dynactin was rapidly reversible and, following acetate washout, punctate dynactin was detected at microtubule ends within 3 minutes. Together, these findings identify a region along the distal segments of microtubules where dynactin and CLIP-170 colocalize. Because CLIP-170 has been reported to mark growing microtubule ends, our results indicate a similar relationship for dynactin. The functional interaction between dynactin and cytoplasmic dynein further suggests that this these regions represent accumulations of cytoplasmic dynein cargo-loading sites involved in the early stages of minus end-directed organelle transport.

Key words: Cytoplasmic dynein, Dynactin, CLIP-170, Microtubule, Motility

INTRODUCTION

Cytoplasmic dynein is a minus end-directed microtubule motor implicated in a wide variety of intracellular functions, including retrograde axonal transport, endosomal, lysosomal and Golgi transport, and prometaphase chromosome movement (reviewed in Holzbaur and Vallee, 1994; Vallee and Sheetz, 1996). Dynactin is a large multisubunit complex that is required for several, and perhaps all, of the known functions of cytoplasmic dynein (reviewed in Allan, 1994; Schroer et al., 1996). Like dynein, it is composed of multiple subunits: p150*Glued* (Holzbaur et al., 1991; Gill et al., 1991), Arp1 (Lees-Miller et al., 1992; Clark and Meyer, 1992; Paschal et al., 1993), dynamitin (p50) (Paschal et al., 1993; Echeverri et al., 1996), capping proteins α and β (Schafer et al., 1994), and polypeptides of 62 kDa, 27 kDa and 24 kDa of unknown function.

Rotary shadow imaging of dynactin revealed that the complex contains two prominent structural elements: a 37 nm F-actin-like core and a projecting side arm (Schafer et al., 1994). Antibody decoration suggested that the globular 'heads' and rod of the side arm correspond to a portion of p150*Glued*. The remaining dynactin components appear to form the core of the dynactin complex. Arp1 is an actin-related protein thought to assemble into the short microfilament backbone. At opposite ends of the filament are the actin capping proteins and p62. The position of dynamitin (p50) in the dynactin complex is unknown. However, its relatively high stoichiometry and the results of overexpression suggest that it could be involved in linking p150*Glued* and the Arp1 filament (Echeverri et al., 1996).

Dynactin was initially characterized as a factor which stimulated cytoplasmic dynein-mediated vesicle movements in vitro (Schroer and Sheetz, 1991; Gill et al., 1991), although the mechanism of stimulation was unclear. Genetic studies from a variety of organisms have since confirmed that dynactin functions in a common pathway with cytoplasmic dynein (Gill et al., 1991; Eshel et al., 1993; Li et al., 1993; Plamann et al., 1994; Muhua et al., 1994; Clark and Meyer, 1994; Xiang et al., 1995; McGrail et al., 1995; Echeverri et al., 1996; Geiser et al., 1997). As part of our search for cytoplasmic dynein-interacting proteins, we discovered a

direct interaction between the cytoplasmic dynein intermediate chains (ICs) and p150^{Glued} (Vaughan and Vallee, 1995; and see Karki and Holzbaur, 1995). Because the ICs have been localized to the base of the motor (Steffen et al., 1996) and are thought to mediate binding of cytoplasmic dynein to cargo, the binding of the ICs to p150^{Glued} identified dynactin as a potential dynein 'receptor' on membranous organelles and kinetochores. In support of this model, dynactin was found to colocalize with cytoplasmic dynein on prometaphase kinetochores (Echeverri et al., 1996). Furthermore, overexpression of dynamitin (p50) disrupted the dynactin complex, leading to loss of both dynactin and cytoplasmic dynein from prometaphase kinetochores and arrest of mitosis in prometaphase (Echeverri et al., 1996). Subsequent analysis of organelle transport has indicated that dynamitin (p50) expression also perturbs all cytoplasmic dynein-mediated membrane traffic (Burkhardt et al., 1997).

Despite the available evidence for a role for dynactin in membrane transport, defining the relative distributions of dynactin and cytoplasmic dynein during interphase has been problematic. Clear centrosomal staining has been repeatedly observed (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993; Waterman-Storer et al., 1995). However, the preponderance of dynactin staining is associated with relatively weak, fine punctate structures throughout the cytoplasm that can be difficult to resolve. Staining is concentrated in the perinuclear region and is largely detergent-extractable (Paschal et al., 1993; Echeverri et al., 1996). Cytoplasmic dynein and dynactin have been reported to be associated with preparations of purified organelles (Fath et al., 1994; Allan, 1995; Niclas et al., 1996; Blocker et al., 1997), though biochemical analysis has indicated that a significant fraction of dynactin is cytosolic (Gill et al., 1991; Paschal et al., 1993; Fath et al., 1994). The level of dynactin associated with membranes has been reported to be regulated in a cell cycle-dependent manner in *Xenopus* extracts (Niclas et al., 1996).

The large subunit of dynactin, p150^{Glued}, has structural similarities to another protein implicated in membrane trafficking, CLIP-170 (Rickard and Kreis, 1990; Scheel and Kreis, 1991; Pierre et al., 1992). Both proteins contain approx. 80-amino-acid N-terminal microtubule binding motifs followed by an extensive coiled-coil structure of similar length. CLIP-170 has been reported to link endosomes to microtubules in biochemical experiments (Scheel and Kreis, 1991). By immunofluorescence microscopy it is seen to have a unique distribution on punctate, vesicular structures associated with the distal ends of microtubules (Rickard and Kreis, 1990; Pierre et al., 1992). Although similar to p150^{Glued} in overall structure, CLIP-170 does not purify as part of a large multisubunit complex, and interacting proteins have not been detected (Rickard and Kreis, 1990). The relationship between CLIP-170 and cytoplasmic dynein/dynactin in vesicle transport remains unclear.

Our interest in dynactin as the putative dynein 'receptor' has led us to prepare new antibodies to p150^{Glued} based on the premise that the N terminus of this polypeptide is likely to be more exposed and accessible than other antigenic sites within dynactin. Using these antibodies, as well as previously characterized antibodies, we have examined the distribution of dynactin in interphase cells. We report a novel population of dynactin-containing structures, which accumulate along microtubule distal ends and colocalize with CLIP-170. After a temperature shift, cytoplasmic dynein colocalized with the microtubule-associated dynactin. The overlapping distributions of dynactin and CLIP-170 suggest that these two proteins may play related roles in dynein-mediated transport. Furthermore, the temperature-sensitive association of cytoplasmic dynein with the microtubule-associated dynactin may indicate a previously undetected initiation stage in minus end-directed vesicle traffic.

MATERIALS AND METHODS

Antibody production

A cDNA sequence encoding the N-terminal 200 amino acids of rat brain p150^{Glued} (Holzbaur et al., 1991) was amplified by PCR and subcloned in the *E. coli* expression vector pET-15b by standard methods. *BL21(DE3)* bacteria were transformed and induced by addition of IPTG to 1 mM. The HIS-tagged fusion protein was purified using nickel-affinity chromatography, cleaved with thrombin to remove the HIS-sequence, and mixed with Freund's adjuvant for immunization. Three New Zealand White rabbits were immunized and sera were characterized by western blot and immunoprecipitation (Vaughan and Vallee, 1995). A monoclonal antibody to p150^{Glued} was obtained from Transduction Laboratories (Lexington, KY). Anti-Arp1 was provided by Sean Clark (Clark et al., 1994). Anti-CLIP-170 was provided by Thomas Kreis (Rickard and Kreis, 1990). Anti-*Glu*-tubulin and anti-*Tyr*-tubulin antibodies were provided by Chloe Bulinski (Gunderson et al., 1984). A monoclonal antibody to dynamitin (p50) has been previously characterized (Paschal et al., 1993; Echeverri et al., 1996). Monoclonal anti-58kD was provided by George Bloom (Bloom and Brashear, 1989). Anti-ERGIC 53 was provided by Hans Hauri (Schweizer et al., 1990), Mitotracker, Lysotracker, Bodipy Ceramide and FITC-WGA were obtained from Molecular Probes (Eugene, Oregon).

Cell culture

COS-7 cells (ATCC, Rockville, MD) were grown in DMEM (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco), penicillin and streptomycin (Sigma, St Louis MO), and glutamine (Sigma). Cells were fixed by immersion in −20°C methanol after culturing for 2-3 days (80-90% confluence) on glass coverslips. Alternatively, cells were fixed for 15 minutes at 37°C in 4% paraformaldehyde or 2.5% glutaraldehyde followed by multiple washes in 50 mM sodium borohydrate. In these cases, cells were permeabilized with 0.5% Triton X-100 prior to incubation with antibodies. When indicated, detergent preextraction was performed by immersing coverslips in PEMG (80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl, 4 M glycerol) containing 0.5% Triton X-100 for 1 minute prior to methanol fixation. Temperature shift was accomplished by incubating coverslips in Dulbecco's phosphate buffered saline (D-PBS) (8 mM $NaPO_4$, 1 mM KPO_4, pH 7.4, containing 140 mM NaCl, and 3 mM KCl) at room temperature for 1 minute prior to methanol fixation. Nocodazole treatment was performed as described previously (Echeverri et al., 1996). DNA transfections were performed using Lipofectamine (Gibco, Grand Island, New York).

Acetate treatment

Coverslips containing subconfluent COS-7 cells were incubated in Ringer's acetate, pH 6.4 (80 mM NaCl, 70 mM sodium acetate, 10 mM Hepes, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $NaPO_4$, 1 mM $MgCl_2$) for 15 minutes in a 37°C incubator. Acetate recovery was achieved by washing the coverslips twice with prewarmed medium and incubating at 37°C for the times indicated. Cells were fixed in methanol as above, or using 4% paraformaldehyde in D-PBS.

Immunofluorescence microscopy

Fixed cells were blocked in D-PBS containing 1% BSA for 1 hour. Antibody incubations were performed for 1 hour at room temperature in D-PBS containing 1% normal donkey serum. Secondary antibodies conjugated to DTAF, Cy3, Cy2 and TRITC were cross-species absorbed (Jackson Immunoresearch, West Grove, PA). Coverslips were mounted in D-PBS containing gelvatol and 0.25% DABCO, or alternatively in Prolong Antifade (Molecular Probes). Microscopy was performed on a Zeiss Axiophot equipped with a 63× planapo lens and a 35 mm camera as well as a Biorad MRC 1024 Laser Scanning Confocal Microscope. Images were photographed using TMAX 400 film and figures were prepared after scanning negatives on a Nikon LS-1000 film scanner. Figures were printed using a Kodak Colorease PS printer.

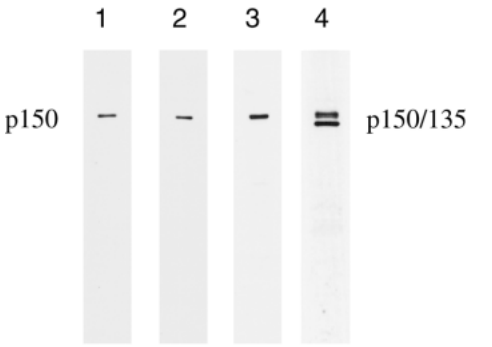

Fig. 1. Characterization of p150^{Glued} antisera by western blotting. COS-7 cell extracts (lanes 1-3) or bovine brain extract (lane 4) were resolved by SDS-PAGE (5-18% acrylamide gel) and transferred to Immobilon-P. Membrane strips were probed with D'Art (lanes 1,4), Portos (lane 2) or Aramis (lane 3) antisera. The antisera detect a single band of 150 kDa in cell extracts and a doublet of 150 kDa and 135 kDa in brain extract.

RESULTS

Distribution of dynactin during interphase

Three polyconal antibodies were prepared against the N-terminal 'head' domain of p150^{Glued} and used to probe extracts of COS-7 cells and bovine brain cytosol by western blot. As observed in Fig. 1, each of the antisera detected a single band at 150 kDa in the cultured cell extracts, though evidence of electrophoretic heterogeneity within the band was observed in some samples. In bovine brain cytosol, a doublet of 150 and 135 kDa was detected.

Each of the p150^{Glued} antisera exhibited fine punctate staining throughout the cytoplasm by immunofluorescence microscopy, as well as centrosomal staining, as has been previously reported for other anti-dynactin antibodies. The punctate staining was most concentrated in the perinuclear region. In addition, prominent punctate linear arrays were also observed throughout the cell (Figs 2A,D, 3-8). The arrays had an overall radial distribution and were most obvious in the cell periphery. When observed at high magnification, the individual spots appeared similar in size to the fine punctate structures observed in the perinuclear region. Within each array, the staining density often appeared graded, diminishing from the periphery cytoplasm by immunofluorescence microscopy, as has been previously reported for other anti-dynactin antibodies.

All three polyclonals and a monoclonal anti-p150^{Glued} antibody (e.g. Fig. 3A,B) revealed the same staining pattern. This pattern was most obvious after methanol fixation, but was also evident after paraformaldehyde or glutaraldehyde fixation. One intriguing difference was the tendency of the monoclonal antibody to stain primarily the p150^{Glued} fraction in the arrays (Fig. 3B), with relatively little of the perinuclear staining observed with the other antibodies.

Double-labeling with anti-tubulin indicated that the linear arrays were aligned along microtubules (Fig. 4, see arrows). The pattern was abolished by nocodazole treatment (see Fig. 7). It was resistant to Triton X-100 pre-extraction (Fig. 6), though the punctate perinuclear staining was diminished by this treatment.

When observed at high magnification in favorable regions

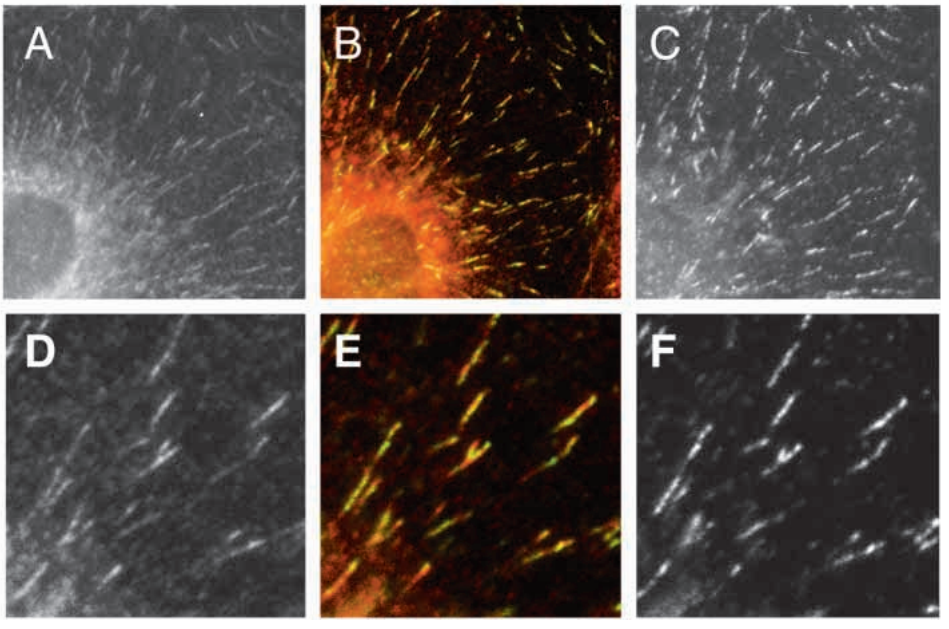

Fig. 2. Immunofluorescence microscopy of p150^{Glued} versus CLIP-170. COS-7 cells were probed with polyclonal anti-p150^{Glued} (A,D) and monoclonal anti-CLIP-170 (C,F); merged images (B,E; red, p150^{Glued}; green, CLIP-170). D-F are higher magnification views of A-C. p150^{Glued} and CLIP-170 colocalized to the same linear punctate arrays, often graded in intensity, diminishing from the cell periphery to the cell center.

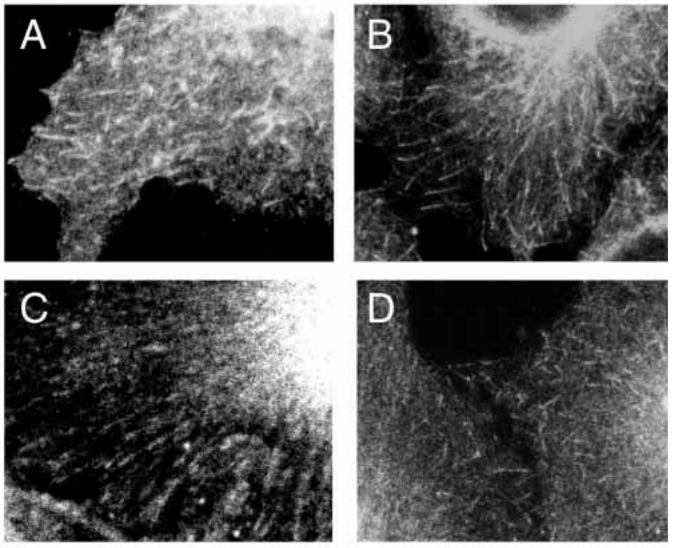

Fig. 3. Immunofluorescence microscopy of dynactin subunits. A second polyclonal antisera to p150^{Glued} (A-Portos), one monoclonal anti-p150^{Glued} antibody (B), a monoclonal antibody against dynamitin (C) and a polyclonal antibody against Arp1 (D) each detected a prominent microtubule-associated dynactin population in cells. This staining was most obvious in the cell periphery where it was not obscured by the dense perinuclear staining. The graded nature of the staining was especially apparent at the cell margin (B, lower left).

of the cytoplasm, the microtubule-associated dynactin fraction was seen to be restricted to microtubule distal ends (Fig. 4, arrows). Furthermore**,** the spots were associated with only a subset of microtubules. To test whether this subset represented stable microtubules, we compared p150^{Glued} labeling to the staining patterns with anti-*Glu*-tubulin and anti-*Tyr*-tubulin antibodies (Fig. 5; Gunderson et al., 1984). In COS-7 cells, the microtubules staining with anti-*Glu*-tubulin antibody represented a small fraction of the total microtubule population. These anti-*Glu*-tubulin-positive microtubules were curved rather than straight with the ends rarely extending to the cell periphery. The overall distribution of anti-*Glu*-tubulin positive microtubules was clearly different from that of p150^{Glued}, which was evident in areas of the cell where no anti-*Glu*-tubulin positive microtubules were detected (Fig. 5C,D). Conversely, the p150^{Glued} staining pattern was often observed along anti-*Tyr*-tubulin-positive microtubules, though on only a subset of these structures (Fig. 5E,F, see arrows).

Colocalization with other dynactin subunits and CLIP-170

To determine whether other subunits of the dynactin complex were associated with the p150^{Glued}-positive arrays, cells were stained with antibodies to Arp1 and dynamitin (p50). Both antibodies revealed the same microtubule-associated dynactin staining pattern (Fig. 3), suggesting that the entire dynactin complex is present in the linear arrays. Comparable results were obtained following detergent extraction (see Fig. 6).

The microtubule-associated dynactin staining pattern was very similar to that reported for CLIP-170 (Rickard and Kreis, 1990; Pierre et al., 1992, 1994). Double-labeling with anti-p150^{Glued} and anti-CLIP-170 antibodies revealed striking colocalization of these two antigens within the linear arrays (Fig. 2B,E). However, in contrast to the results obtained using the anti-p150^{Glued} antibodies, CLIP-170 staining was more clearly restricted to the microtubules, showing relatively little perinuclear staining (Fig. 2C,F). In some examples, CLIP-170 staining appeared more variable in size than p150^{Glued,} which was very uniform. At high magnification (Fig. 2D-F), coincidence of many of the p150^{Glued}- and CLIP-170-positive spots was observed. Virtually all of the microtubule-associated dynactin colocalized with CLIP-170, although examples of punctae that were positive for only dynactin or CLIP-170 were also observed.

p150^{Glued} and CLIP-170 contain homologous 80-amino-acid microtubule-binding motifs near their N termini (Pierre et al., 1992; Holzbaur et al., 1991; Waterman-Storer et al., 1994; Vaughan and Vallee, 1995). Although we did not detect a polypeptide the size of CLIP-170 by immunoblotting (Fig. 1) or immunoprecipitation (Vaughan and Vallee, 1995) using anti-p150^{Glued} antibodies, we further tested for possible cross-reaction by expressing several portions of CLIP-170 in cultured mammalian cells (data not shown). When these cells were stained with anti-p150^{Glued}, reaction with the excess microtubule-bound CLIP-170 was not detected, and the usual limited linear arrays of p150^{Glued} staining along the microtubules were still observed.

Cells were also transfected with a C-terminal deletion mutant of p150^{Glued} designed to correspond to the Gl^{1} mutation in *Drosophila* (Swaroop et al., 1987). The Gl^{1} polypeptide, like

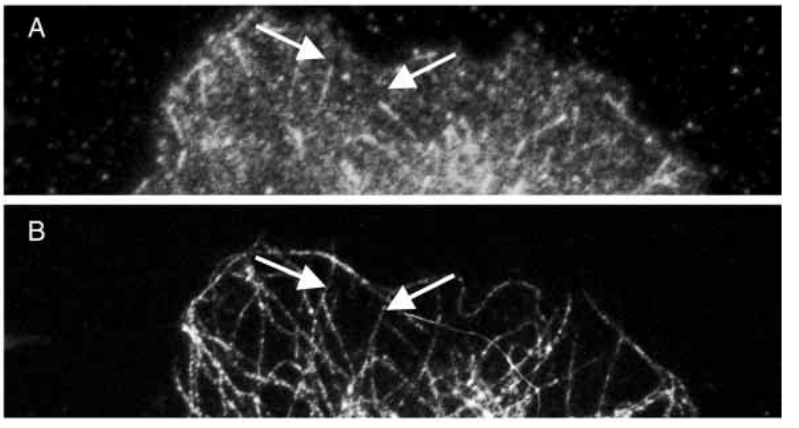

Fig. 4. Immunofluorescence microscopy of p150^{Glued} and tubulin. COS-7 cells were probed with the D'art polyclonal anti-p150^{Glued} (A) and monoclonal anti-tubulin (B). In addition to more generalized punctate staining, especially around the nucleus, polyclonal anti-p150^{Glued} antibodies detected linear arrays of p150^{Glued}. These linear arrays of p150^{Glued} staining coincided with microtubules (see arrows), and were observed at microtubule ends in regions at the cell periphery where microtubule density was low.

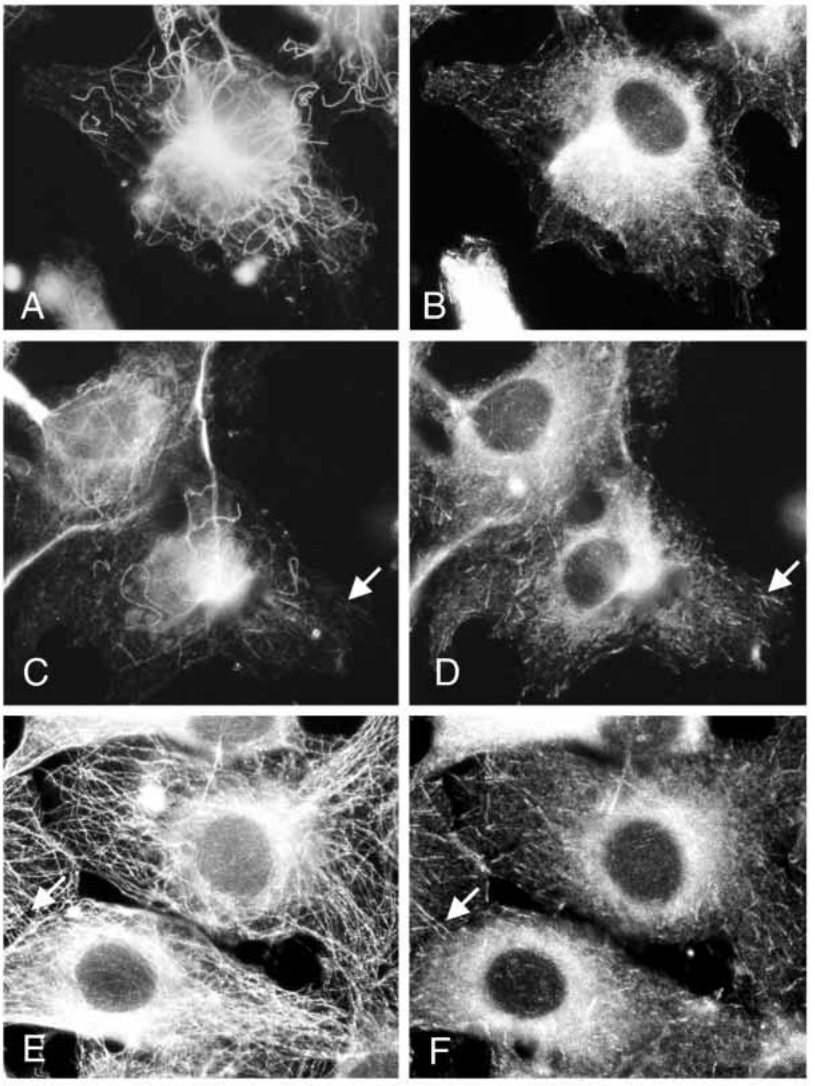

Fig. 5. Immunofluorescence microscopy of p150^{Glued} versus *Glu*- and *Tyr*- tubulin. p150^{Glued} (B,D,F) was compared with tubulin modification forms using polyclonal anti-*Glu* (A,C) or *Tyr*-tubulin (E) antibodies. Dynactin staining often coincided with anti-*Tyr*-tubulin positive microtubules (E,F, see arrows) but only a subset of them.

other C-terminal truncation mutants and the full-length p150^{Glued} polypeptide (Waterman-Storer et al., 1992) decorated microtubules and stabilized the microtubule array (data not shown). We observed no binding of CLIP-170 antibody to the p150^{Glued} polypeptides, nor was there any noticeable effect of the Gl^{1} fragment on CLIP-170 distribution.

The co-distribution of dynactin with CLIP-170 suggested that the dynactin-positive structures represent membranous vesicles (Scheel and Kreis, 1991; Pierre et al., 1992), though only limited data exist for codistribution of the latter protein with membrane markers in cells. To compare the distribution of dynactin with known membrane compartments, we examined the distribution of the Golgi marker p58 (Bloom and Brashear, 1989), ERGIC 53 (Schweizer et al., 1990), wheat germ agglutinin (WGA), Bodipy ceramide, Mitotracker, Lysotracker, GFP-tagged NAGT (N-acetyl-glucosamine transferase) during BFA treatment and recovery, and a temperature-sensitive mutant of VSV-G at restrictive and permissive temperatures. Although we did observe occasional colocalization with individual dynactin-positive spots, we did not observe convincing colocalization with any of these markers along the entire length of the dynactin arrays.

Temperature-dependent colocalization of cytoplasmic dynein with dynactin

To test for the presence of cytoplasmic dynein on the dynactin-positive structure, cells were stained using two polyclonal anti-intermediate chain antibodies (L5 and M5), three monoclonal anti-intermediate chain antibodies (74.1, 70.1, 70.2) and an IC-2-specific anti-peptide antibody (Vaughan and Vallee, 1995). Convincing colocalization with the microtubule-associated dynactin pattern was not observed. Although the cytoplasmic dynein intermediate chains and p150^{Glued} are thought to interact in the cell (Vaughan and Vallee, 1995; Karki and Holzbaur, 1995; Echeverri et al., 1996), the lack of detectable colocalization was not attributed to steric inhibition because colocalization of cytoplasmic dynein with dynactin was observed on prometaphase kinetochores on the same coverslips using the same antibodies (data not shown).

We noted a hint of positive staining with antibodies to cytoplasmic dynein when cells were washed extensively in D-PBS prior to fixation. Because membrane traffic is differentially affected by temperature, we examined the relative effects of this variable on dynein and dynactin distribution. A brief shift of temperature from 37°C to room temperature prior

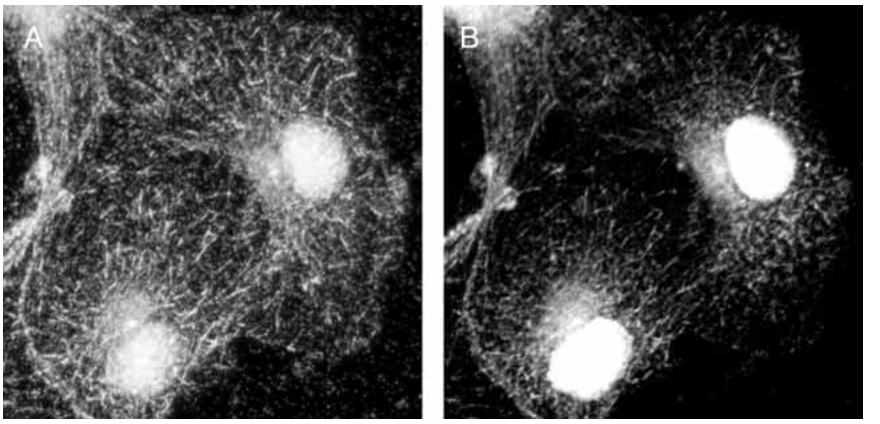

Fig. 6. Effect of Triton X-100 preextraction on dynactin staining patterns. We performed brief detergent preextraction and assessed the dynactin staining pattern using antibodies to p150^{Glued} (A) and Arp1 (B). The microtubule-associated dynactin resisted detergent extraction whereas the dense perinuclear staining was abolished. The overall size and pattern of microtubule-associated dynactin was not affected. Bright nuclear staining was not observed under normal conditons and is thought to be an artifact of detergent preextraction. Prominent staining along the length of the microtubule was not observed.

to fixation resulted in positive staining for cytoplasmic dynein along the microtubule-associated arrays (Fig. 7). This staining was coincident with dynactin staining, consistent with an association between the two complexes. Whereas only a subset of the microtubules contained the dynactin arrays, cytoplasmic dynein was strictly associated with the same microtubules.

The temperature shift also led to an increase in the dynactin staining intensity along the microtubule-associated arrays as well as an increase in the length of the dynactin-positive arrays. Whereas the length of the arrays in control cells was 2.22 μm (±1.23 μm), the length of the arrays increased to 3.45 μm (±1.02) after the shift. We observed a tendency for the increased microtubule-associated staining to correlate with a decrease in the more diffuse perinuclear staining, suggesting a shift in equilibrium toward the microtubule-associated state. These changes peaked at approximately 1 minute of room temperature incubation and staining intensity decreased at longer times of incubation.

Manipulation of microtubule-associated dynactin

To learn more about the relationship of the microtubule-associated dynactin fraction to microtubule structure and motility, the effect of a number of potential perturbants was examined. In general, the radial microtubule distal-end staining pattern was abolished by nocodazole. During depolymerization the number of microtubules exhibiting distal end staining appeared to be reduced, and an increase in staining along the length of intact microtubules was observed (Fig. 8). Those few microtubules that remained were stained very densely along their entire length by anti-p150^{Glued} (Fig. 8), obscuring the punctate nature of the pattern. The coated microtubules also tended to give a weaker than normal immunofluorescence signal when stained with anti-tubulin, suggesting steric interference by components of the dynactin complex.

Brief acetate treatment**,** which leads to a change in pH to 6.4, has been reported to cause a redistribution of lysosomes (Heuser, 1989) and endosomes (Parton et al., 1991) to the cell periphery. While the detailed mechanism underlying this behavior is unclear, it is strikingly reminiscent of the results of dynamitin overexpression (Burkardt et al., 1997), and may involve an effect on cytoplasmic dynein.

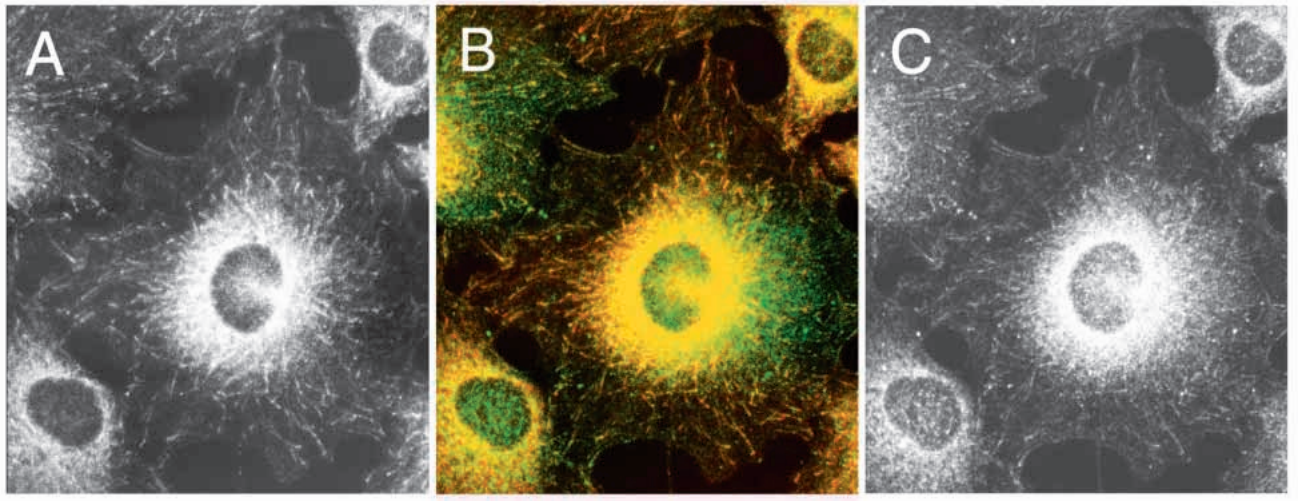

Fig. 7. Colocalization of cytoplasmic dynein with dynactin after temperature shift. Subconfluent COS-7 cells were incubated at room temperature for 1 minute prior to fixation and then stained with antibodies to p150^{Glued} (A) or the cytoplasmic dynein intermediate chains (C). The digital images in A and C were pseudocolored red and green, respectively, and superimposed to compare staining patterns (B). Unlike control cells in which cytoplasmic dynein does not colocalize with dynactin, cells subjected to temperature shift show positive staining for cytoplasmic dynein coincident with the dynactin and an increase in staining intensity and length with dynactin antibodies.

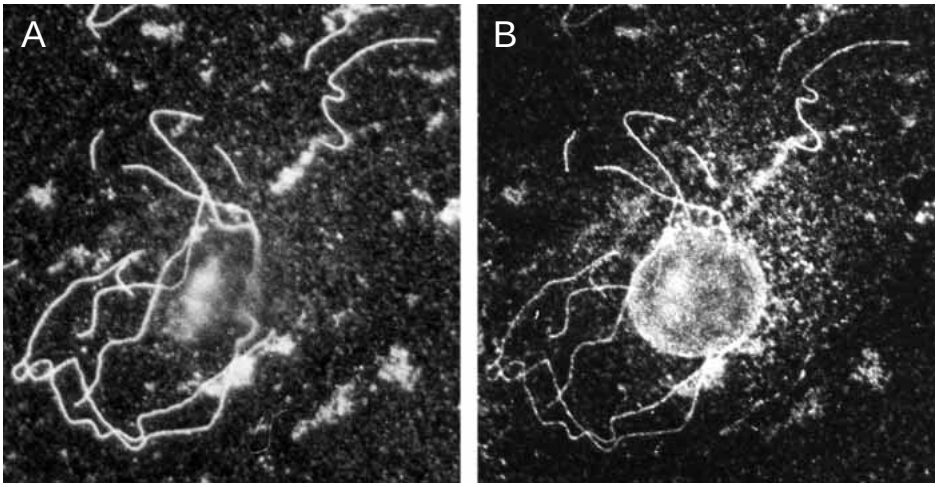

Fig. 8. Redistribution of dynactin following nocodazole treatment. Nocodazole-treated cells were preextracted briefly with Triton X-100 and probed with antibodies to p150^{Glued} (A) or anti-tubulin (B). The few microtubules that resisted nocodazole treatment (B) were uniformly decorated with p150^{Glued} (A). The density of staining in these cases obscure the punctate nature of staining, and diminishes the intensity of tubulin staining.

We monitored the distribution of the Golgi apparatus as an indicator of cytoplasmic dynein activity in these cells (Fig. 9). Rapidly reversible dispersion and reclustering was observed upon acidification and return to neutrality. Concurrent with this effect was a dramatic and rapid redistribution of dynactin (Fig. 10). After 15 minutes exposure to acetate, punctate staining with anti- p150^{Glued} was abolished, and a high level of diffuse staining could be seen throughout the cytoplasm (Fig. 10A).

We performed a time course of acetate treatment to follow the apparent dynactin dissociation. Dynactin staining was observed to shift from punctate to soluble as early as 3 minutes after exposure to Ringer's acetate (not shown). Complete dissociation was apparent by 10 minutes. No effects on the microtubule array were detected in these cells, and no effect on the ability of p150^{Glued} to bind microtubules was observed in cells overexpressing the *GI*^{1} fragment (data not shown). Furthermore, no effect on kinetochore-associated dynactin was observed.

Following return to normal medium, dynactin rapidly returned to a punctate distribution. Within 3 minutes of recovery, punctate dynactin was observed and microtubule end-labeling was detected (see Fig. 10B). The regions of microtubule labeling at these early time points were quite restricted in length relative to those in control cells. Recovery to a normal dynactin pattern was gradual, reaching a normal distribution by 1 hour (Fig. 10C), at which point the Golgi apparatus had reclustered in the perinuclear region of the cell (Fig. 9F). The ability to recover from acetate persisted even after 2 hours of pretreatment.

We also used dynamitin expression as a tool to disrupt membrane traffic. Surprisingly, there was no apparent effect on the microtubule distal end distribution of p150^{Glued} (Fig. 11C) or CLIP-170 (not shown). However, Arp1 was displaced from the linear arrays (Fig. 11A), suggesting that the dynactin complex had become dissociated, as we had previously reported based on biochemical analysis (Echeverri et al., 1996). The microtubule-associated p150^{Glued} retained the same punctate appearance as in control cells, and maintained the ability to bind cytoplasmic dynein after temperature shift (not shown).

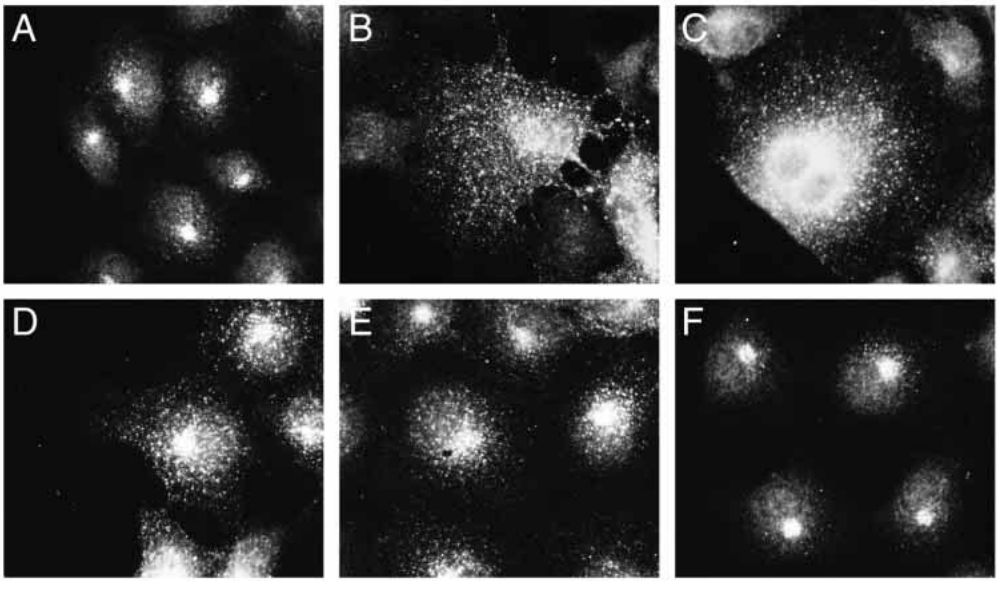

Fig. 9. Redistribution of the Golgi apparatus during cytoplasmic acidification. COS-7 cells were treated in Ringer's acetate buffer as above and stained using a monoclonal antibody against the 58 kDa Golgi component. Normally tightly focussed in control cells (A), the Golgi apparatus dispersed after brief acetate treatment (B). During recovery in growth medium (C-F), vesicles detected by the anti-58 kDa antibody were observed in short arrays (C,D). These vesicles reclustered resembling a normal Golgi apparatus (E,F).

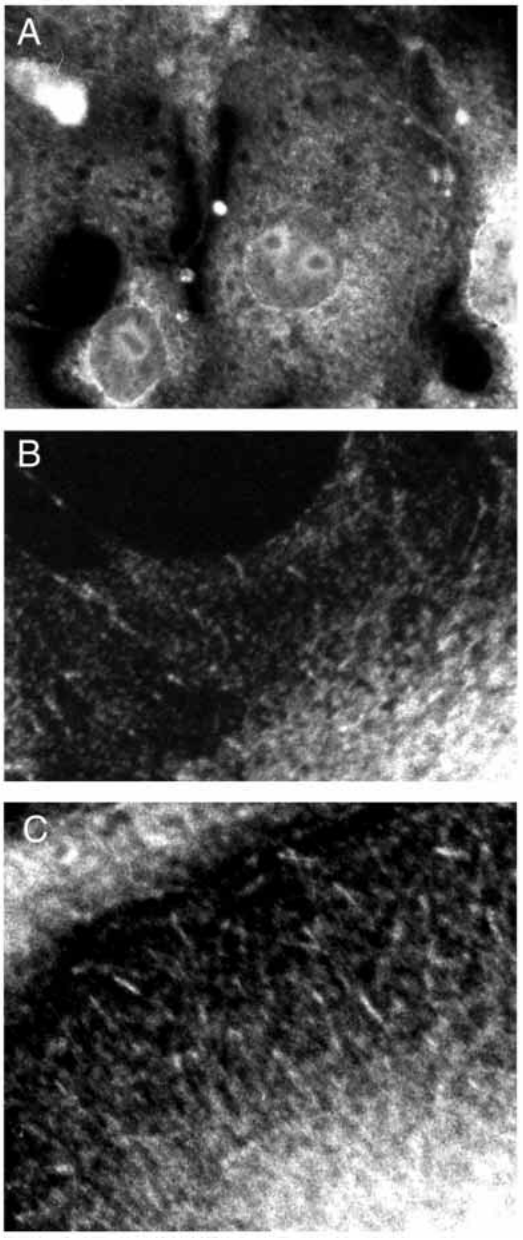

Fig. 10. Reversible effects of brief acetate treatment on microtubule-associated dynactin. COS-7 cells were incubated in Ringer's acetate for 15 minutes (A) and then returned to normal growth medium for either 3 (B) or 60 minutes (C). p150^{Glued} staining (A-C) became diffuse in treated cells (A). Upon return to growth medium, the p150^{Glued} staining pattern rapidly became punctate and aligned with microtubules (B) and was essentially normal by 60 minutes (C).

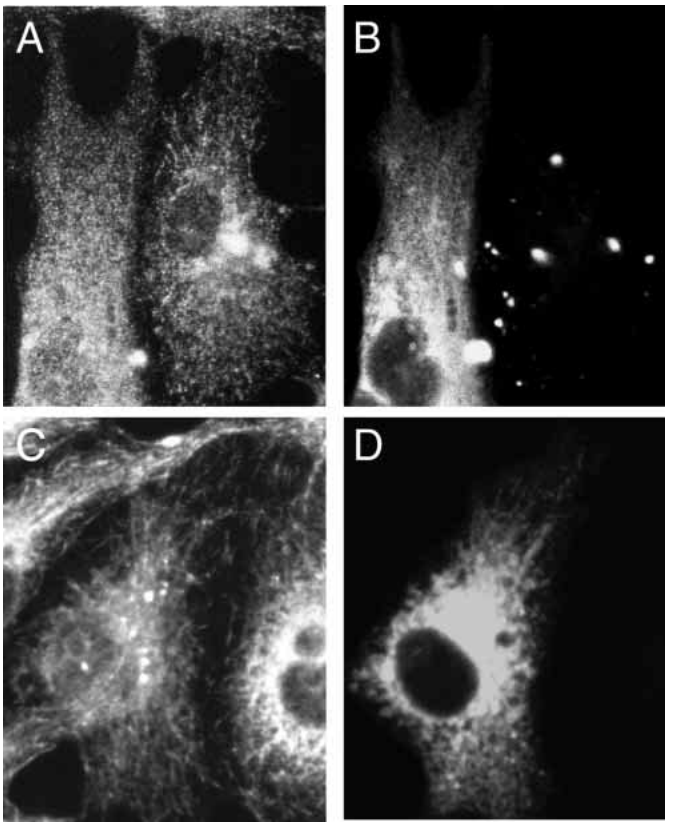

Fig. 11. Effect of dynamitin overexpression on microtubule-associated dynactin. COS-7 cells were transfected with dynamitin and stained for Arp1 (A), p150^{Glued} (C) or dynamitin (B,D). The punctate microtubule-associated p150^{Glued} staining pattern was unaffected in dynamitin-expressing cells, whereas Arp1 was dissociated, consistent with biochemical characterization of dynamitin-expressing cells (Echeverri et al., 1996).

DISCUSSION

Previous attempts to localize dynactin during interphase have shown weak staining of fine punctate structures throughout the cytoplasm, as well as prominent labeling of the centrosome (Gill et al., 1991; Paschal et al., 1993; Clark and Meyer, 1992; Waterman-Storer et al., 1994; Echeverri et al., 1996). Using a panel of antibodies to p150^{Glued}, dynamitin (p50) and Arp1, we now identify a clear microtubule-associated dynactin fraction which, surprisingly, colocalizes with CLIP-170. After slowing membrane transport by temperature-shift, we observed colocalization of cytoplasmic dynein with this dynactin fraction as well. The colocalization of CLIP-170 with dynactin suggests a potential role for CLIP-170 in the cytoplasmic dynein-dynactin pathway. Furthermore, in view of evidence for a direct interaction between dynein and dynactin, our results provide new insight into the steps involved in cytoplasmic dynein-mediated transport during interphase.

Composition of dynactin-containing structures

The dynactin distribution pattern we observe differs in two ways from that reported in previous studies: the resolution of the dispersed punctate staining into individual structures, and the colocalization with microtubules. The latter staining pattern, although appearing to be continuous in some images, generally appears to consist of linear arrays of punctae of equivalent size to the cytoplasmic structures. The nature of these spots remains uncertain. Dynactin has been found by immunoelectron microscopy to be associated with Golgi (Fath et al., 1994) and phagosome membranes (Blocker et al., 1997), suggesting that the punctae observed in the current study are membranous. This conclusion is supported by the detergent

extractability of the free dynactin-positive structures, together with the colocalization of the microtubule-bound arrays with dynein and with CLIP-170, which has been reported to mediate the interaction of endosomes with microtubules using a biochemical assay (Scheel and Kreis, 1991) and to show limited colocalization (Pierre et al., 1992) with endosomal vesicles using immunofluorescence microscopy. The size uniformity of the structures we observe suggests that dynactin may associate preferentially with smaller vesicles, though we cannot rule out the possibility that the dynactin punctae represent non-membranous aggregates, or pre-membrane-binding intermediates.

The microtubule-associated structures, unlike the dispersed structures, were sufficiently distinctive in distribution to allow us to gain insight into their composition and organization, as we have been able to do with kinetochore-associated dynactin and dynein (Echeverri et al., 1996). We detected the presence of the three major dynactin subunits in the microtubule-associated particles, consistent with the presence of the entire dynactin complex. Excess dynamitin, which dissociates p150^{Glued} from the Arp1 filament (Echeverri et al., 1996), surprisingly failed to affect the microtubule association of p150^{Glued}, but ablated Arp1 from the microtubules. This result provides important in situ confirmation of the dissociation of the dynactin complex in the cell. However, it contrasts with the effect of dynamitin on kinetochores, from which both Arp1 and p150^{Glued} became dissociated. This disparity can be readily explained if different subunits of dynactin are assumed to be responsible for anchoring the complex to kinetochores versus microtubules. In the case of the kinetochore, dynamitin itself has been implicated as the anchoring polypeptide (Starr et al., 1998), whereas p150^{Glued} is suggested by our current results to be involved in microtubule binding. The latter conclusion is entirely consistent with the presence of a microtubule binding site within this polypeptide (Waterman-Storer et al., 1994; Vaughan and Vallee, 1995). Furthermore, it is a likely explanation for why the microtubule-associated dynactin-positive structures are detergent-resistant: Triton X-100 treatment is expected to disperse the protein components of vesicle membranes, whereas the distribution of a microtubule-bound component should be unaffected.

The effects of cytoplasmic acidification address the nature of a potential dynactin-vesicle interaction. Previous studies have indicated an effect of acidification on the distribution of endosomes and lysosomes (Heuser, 1989; Parton et al., 1991; Burkhardt et al., 1997). Lin and Collins (1992), indeed, reported a dispersal of cytoplasmic dynein immunoreactivity under these conditions, consistent with such an hypothesis. We also note that the Golgi apparatus is dispersed by this treatment in these cells (see Fig. 9B), suggesting the possibility of a generalized effect on dynein-mediated motility. Acetate treatent of MDCK cells did not result in dispersal of the Golgi apparatus (Parton et al., 1991). However, many aspects of organelle transport are thought to differ between polarized epithelial cells and more fibroblastic cultured cells such as COS-7 (Fath et al., 1994; Lafont et al., 1994).

Our current finding that dynactin becomes diffuse during acidification is strongly consistent with our model that dynactin serves an important role in mediating the attachment of cytoplasmic dynein to vesicles, perhaps as a ‘receptor’ or anchor. We observed that dissociation of dynactin occurred within as little as 3 minutes after transfer to Ringer’s-acetate buffer; considering the likelihood of a lag in the change in cytoplasmic pH, the effect on dynactin may be virtually immediate. In view of our finding that the effect of acidification on minus end-directed organelles is more general than had previously been reported, the immediacy of the effect on dynactin and the previously reported effect of dynactin dissociation on dynein (Echeverri et al., 1996; Burkhardt et al., 1997), we propose that the primary effect of acetate treatment may be on the dynactin-vesicle interaction.

Relationship between CLIP-170 and dynactin

Recent evidence has revealed that CLIP-170 is colocalized with dynein and dynactin at the prometaphase kinetochore, and can be displaced by dynamitin overexpression (Dujardin et al., 1998). Our finding that many, if not most, of the microtubule-associated dynactin-positive particles contain CLIP-170 (see Fig. 2) provides further evidence that dynactin and CLIP-170 may be functionally related. This possibility is supported by the limited, although significant, homology and overall secondary structure similarity between CLIP-170 and the p150^{Glued} subunit of dynactin.

Whether the two proteins interact directly is uncertain. We found no evidence for an interaction of CLIP-170 with either the cytoplasmic dynein intermediate chains or with p150^{Glued} using blot overlay and affinity chromatographic assays (Vaughan and Vallee, 1995), though such an interaction cannot be excluded. Also significant is the relative behavior of the two proteins after acidification of the cytosol. We found both to be dissociated from microtubules under acidic conditions. However, dynactin clearly reassociated with microtubules more rapidly than CLIP-170 (not shown), indicating that the two proteins can act independently of each other. It was also of interest that CLIP-170 was less clearly dispersed into an apparent soluble state than was p150^{Glued}. This observation raises the possibility that under acidic conditions CLIP-170 may remain attached to membranes despite their dissociation from microtubules, in contrast to dynactin (see below).

Relevance to dynein and dynactin function

Why the dynactin-containing structures are concentrated toward the peripheral ends of microtubules rather than being uniformly distributed along the full length remains a question of considerable interest. The same issue has arisen in the investigation of CLIP-170, but the current study offers the promise of new insight because of the demonstrated role of dynactin in dynein-mediated vesicle motility.

We reason that the microtubule-associated dynactin-containing structures are immotile. This conclusion is based in part on the absence of dynein staining under normal temperature conditions. In addition, it is difficult to reconcile the distal end staining pattern with the expected distribution patterns of moving vesicles. Vesicle movement along microtubules in vivo and in vitro tends to be relatively uniform in rate (e.g. Schroer and Sheetz, 1991; Gill et al., 1991; Hamm-Alvarez et al., 1993), and vesicles appear to reach their terminal velocity instantaneously. This behavior is consistent with direct coupling of ATP hydrolysis to the translocation via a simple enzymatic process. Given these features of motor protein-driven transport, vesicles in motion should maintain a relatively constant spacing along microtubules. The distribution of a

particular class of vesicles, such as those under the control of cytoplasmic dynein, should be affected only by the site along the microtubule where they initially bind and their inherent rate of movement.

Given these assumptions, a number of mechanisms may be envisaged which could yield a non-uniform distribution of dynactin-containing structures. One possibility is a preferential association with the distal portion of the microtubule. This possibility is supported by recent studies with CLIP-170, which suggest a biochemical preference for microtubule plus ends (Diamantopoulos et al., 1999). One well-characterized biochemical distinction between distal and proximal microtubule ends is the state of the tubulin-bound guanine nucleotide along the microtubule length. However, the 'GTP cap' at the microtubule plus end is calculated to consist of no more than 20-40 tubulin subunits (Drechsel and Kirschner, 1994; Caplow, 1992), comprising a much shorter length of microtubule (<<2 μm) than we observe to be decorated by dynactin. We also find that the dynactin-containing structures are capable of coating the entire length of stable microtubules following nocodazole treatment (Fig. 8). Thus, a simple model involving a differential affinity of dynactin for GTP- versus GDP-tubulin alone cannot explain our observations. However, it is still possible that guanine nucleotide state could control dynactin distribution by a more complicated mechanism. Such a model has recently been proposed for CLIP-170 based on its preferential association with growing microtubules (Perez et al., 1999).

It is also possible that those vesicles destined for minus end-directed transport may originate from non-uniformly distributed sites within the cell. This prospect may be supported by the results of cytoplasmic acidification. Organelles whose distribution is thought to be partially or fully under dynein control, including endosomes, lysosomes and the Golgi apparatus, rapidly move to the cell periphery under these conditions, which also appear to release dynactin (Fig. 9) and cytoplasmic dynein (Lin and Collins, 1992). Upon return to control medium and restoration of minus end-directed motility dynactin reappears in more sharply focused arrays at the very tips of microtubules (Fig. 10), consistent with an outward migration of the source for the dynactin-positive structures.

What is the relationship of the dynactin-positive structures to microtubule-based motility? One possibility is that these structures are premotile. This hypothesis is, in part, based on the acquisition of dynein staining at reduced temperature. We suggest that these conditions serve to isolate a normally undetectable kinetic intermediate in the initiation of dynein-based motility. In this scenario, vesicles first associate with microtubules through dynactin, followed subsequently by dynein binding. This state may normally be difficult to detect because of the rapid onset of movement, a transition which we envisage to be temperature-sensitive. In this view, the concentrations of dynactin staining along microtubules may identify 'entry sites' into the dynein transport pathway. Once dynein binding has occurred, the low-temperature state we have identified would represent an additional and novel intermediate step preceding activation of dynein-mediated movement. A puzzling consequence of evidence for a direct dynein-dynactin interaction (Vaughan and Vallee, 1995; Karki and Holzbaur, 1995) has been the existence of domains within the supercomplex for both dynamic (dynein) and a static (p150^{Glued}) microtubule interactions. As a minimum we propose that activation of minus end motility must involve the inactivation of microtubule binding by p150^{Glued}, though activation of dynein motor activity may also occur.

Finally, assuming that dynactin, like CLIP-170 (Perez et al., 1999), associates with growing microtubules, it is worth considering that the fraction of dynactin which we observe associated with distal ends may be undergoing transport to the cell periphery. In this view, dynein may interact with the distal-end dynactin as a means for its own transport to the cell membrane. How these views may be reconciled must await further studies.

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