Immunolocalization of cytoplasmic dynein to lysosomes in cultured cells

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Summary

Polyclonal antisera have been raised against cytoplasmic dynein purified from calf brain and rat testis. These antibodies reacted most strongly with the 74 kDa dynein intermediate chain, but also recognized the 410 kDa heavy chain, and the 150 and 45 kDa polypeptides previously observed to copurify with cytoplasmic dynein from rat tissues. Localization studies were performed by indirect immunofluorescence microscopy using a fibroblast cell line. Dynein-specific staining appeared vesicular, distributed throughout the cell, but more concentrated near the nucleus. Double-labeling studies using fluorescent markers for membranous organelles indicated a co-localization of dynein with lysosomes. The distribution of the dynein-positive lysosomes was disrupted by treatment of the cells with microtubule-active drugs, and by acidification of the cytoplasm. Comparison of the distribution of lysosomes with peripheral microtubules indicated a high degree of coincidence. These results are consistent with the hypothesis that cytoplasmic dynein is involved in retrograde-directed movement of membranous organelles. In mitotic cells, dynein staining was also apparent along the microtubules of the mitotic apparatus, though vesicular staining was still conspicuous. The presence of dynein on vesicles as well as on spindle microtubules indicates that dynein distribution between these compartments may be regulated by distinct binding proteins.

Key words: cytoplasmic dynein, lysosomes, microtubule-associated proteins, cell motility.

Introduction

Intracellular movements of many types of membrane-bounded organelles have been proposed to occur along microtubule tracks (reviewed by Schliwa, 1984; Vale, 1987). Studies have shown that disruption of the microtubule network with drugs results in fragmentation of the Golgi apparatus (Wehland et al., 1983; Rogalski and Singer, 1984), collapse of the endoplasmic reticulum (Terasaki et al., 1986), disorganization of endosomes and lysosomes leading to the prevention of endocytic transfer of material (Herman and Albertini, 1984; Oka and Weigel, 1983), and loss of polarity in secretion (Rindler et al., 1987; Eilers et al., 1989). Recovery after drug treatment allows the regrowth of the microtubule network and concomitant recovery of organelle organization and cytoplasmic transport (Terasaki et al., 1986; Matteoni and Kreis, 1987; Swanson et al., 1987; Turner and Tartakoff, 1989).

Recent studies have implicated a role for the microtubule-associated ATPases kinesin and cytoplasmic dynein in organelle transport events. Kinesin, shown in vitro to promote anterograde (plus end)-directed microtubule and organelle motility (Vale et al., 1985a,b), has been localized to membrane vesicles in cultured cells (Pfister et al., 1989; Hollenbeck, 1989). Studies using reconstituted systems indicate a role for kinesin in the maintenance of a membrane network resembling endoplasmic reticulum (Dabora and Sheetz, 1988), and in extension of the tubular lysosome system of macrophages (Hollenbeck and Swanson, 1990). Cytoplasmic dynein has been implicated in retrograde (minus end)-directed movement of organelles in crude lysate motility experiments (Schroer et al., 1989; Schnapp and Reese, 1989), and promotes retrograde-directed movement of microtubules, in vitro (Paschal and Vallee, 1987). In crude homogenates and in vivo, however, bidirectional movement of individual organelles is often observed (Pryer et al., 1986; Matteoni and Kreis, 1987). It is not clear whether the control mechanisms regulating directed movement include alternate binding of motility factors to the same or distinct binding sites, regulation of their activity once associated with the membrane, or a combination of several factors.

Organelles associated with later stages of the endocytic pathway in cells (endosomes, lysosomes and Golgi) generally exhibit retrograde movement and tend to concentrate or localize near the microtubule organizing center of the cell. Their movement, therefore, may in part be regulated by a retrograde (minus-end-directed)
motor, such as cytoplasmic dynein. In order to examine the subcellular localization of dynein and to investigate its role in retrograde transport, we have generated polyclonal antibodies to the native protein and have used them to localize cytoplasmic dynein by immunofluorescence microscopy. We provide evidence that cytoplasmic dynein in cultured cells is associated with organelles in the endocytic pathway, specifically with structures identified as lysosomes.

Materials and methods

Protein preparative methods

Cytoplasmic dynein was purified from calf brain as previously described (Paschal et al., 1987; Collins and Vallee, 1989). Briefly, tissue was homogenized in 1 volume of extraction buffer (50 mM Pipes-NaOH, 50 mM Hepes, pH 7.0, 2 mM MgCl₂, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 TIU/ml aprotinin, and 0.5 mM diithiothreitol). Following centrifugation to obtain a cytosolic extract, taxol was added to a final concentration of 20 μM to assemble microtubules. Microtubules were collected by centrifugation and washed once by resuspension in extraction buffer containing 10 μM taxol. A soluble fraction enriched in dynein was obtained by incubation of the microtubules in buffer containing 10 mM Mg²⁺-ATP, followed by sedimentation of the microtubules as before. The ATP eluate was further fractionated by sucrose density gradient centrifugation using 11 ml linear gradients of 5% to 20% sucrose in extraction buffer containing 10 μM taxol. A soluble fraction enriched in dynein was obtained by incubation of the microtubules in buffer containing 10 mM Mg²⁺-ATP, followed by sedimentation of the microtubules as before. The ATP eluate was further fractionated by sucrose density gradient centrifugation using 11 ml linear gradients of 5% to 20% sucrose in extraction buffer. Dynein-containing fractions were identified on the basis of ATPase activity and protein profile was determined by SDS-PAGE (Collins and Vallee, 1989). Microtubule and dynein preparations from rat tissues were made similarly.

Production and analysis of antibodies

Gradient-purified cytoplasmic dynein from calf brain was concentrated by ultrafiltration (Amicon, Danvers, MA), and mixed 1:1 with Freund's complete adjuvant. A New Zealand white rabbit was injected with 350 μg dynein intradermally. A boost injection after two weeks consisted of 250 μg dynein in incomplete Freund's adjuvant, also administered intradermally. Venous blood was collected and the serum tested for reactivity and specificity. A second rabbit polyclonal antibody was obtained similarly following inoculation with cytoplasmic dynein purified from rat testis.

The antibodies were characterized by Western immunoblot analysis of dynein preparative fractions from various rat tissues and calf brain. The serum was generally used at a dilution of 1:250, and the secondary antibody was goat anti-rabbit alkaline phosphatase. Color development proceeded using bromochloro indoylphosphate and nitroblue tetrazolium (Blake et al., 1984). Preimmune serum was used at the same dilution as a control for each experiment.

Subtractive adsorption of antibody with MAP 2 or dynein subunits was accomplished by incubation of serum with the appropriate polypeptides transferred to nitrocellulose. Preadsorbed antibodies were tested on immunoblots to ensure that they no longer were reactive against the antigen being tested.

To prepare an affinity column, calf brain dynein pooled from sucrose gradient fractions was further purified by fractionation on DEAE-cellulose in 20 mM Tris-Cl, 1 mM EDTA, 2 mM MgCl₂, pH 7.0, using a linear gradient of 50 mM to 500 mM NaCl. Peak fractions were pooled and the dynein was coupled to Affi-Gel 10 (Bio-rad Laboratories, Richmond, CA) according to the manufacturer's protocol. After an overnight incubation, affinity-purified antibody was eluted from the column with 200 mM glycine, 500 mM NaCl, 0.1% Tween-20, pH 2.5. The eluate was rapidly adjusted to neutral pH by gel filtration over a NAP-10 column (Pharmacia LKB Biotech, Inc, Piscataway, NJ) equilibrated in PBS, or by addition of 1 M Tris-HCl, pH 9. Bovine serum albumin was added to a final concentration of 0.25%. Antibody prepared in this way could be stored for at least a week without loss of reactivity. Affinity-purified antibody was used without further dilution for immunofluorescence microscopy, or following a 1:100 dilution for immunoblot analysis.

Cell culture and immunofluorescence microscopy

BHK-21 cells were cultured in DME (Irvine Scientific, Santa Ana, CA) with 10% calf serum. Microtubule drugs and sodium acetate were added to the medium as indicated in the figure legends. Cells were fixed for fluorescence microscopy using three different protocols. In method 1, cells were fixed and permeabilized by immersion of coverslips into −20°C methanol for 5 min. Method 2 consisted of a 5 min incubation in 3.75% formaldehyde in PBS (50 mM NaPO₄, pH 7.4, 150 mM NaCl), followed by 2 min in acetone at −20°C. Method 3 involved glutaraldehyde fixation (0.1% glutaraldehyde in PBS) for 7 min in the presence of 0.05% Triton X-100. Comparisons among these fixation conditions revealed no significant differences in staining patterns. Background staining following glutaraldehyde fixation was more obvious, and therefore methods 1 and 2 were used for the studies shown.

Indirect immunofluorescence observation of cytoplasmic dynein was carried out using dynein antiserum at a dilution of 1:20, and goat anti-rabbit secondary antibodies conjugated to either tetramethylrhodamine or fluorescein. Antibody incubations were generally carried out for 3 h (primary antibody) and 30 min (secondary antibody) in a humidified chamber at 37°C. For comparison of dynein and tubulin in double-label experiments, mouse anti-alpha tubulin monoclonal antibody at 1:500 dilution (Amersham Corp., Arlington Heights, IL) and goat anti-mouse secondary antibody were used.

Double-label experiments were also carried out in cells pretreated with fluorescent dextrans. Cells grown on coverslips were incubated in the presence of conjugated dextran in growth medium for 16 hr and then fixed using method 2. Coverslips were incubated with dynein antiserum and fluorescent goat anti-rabbit antibody. Identical results were obtained using either fluorescein- or rhodamine-conjugated dextran. Double-label experiments using fluorescein-wheat germ agglutinin involved incubation of fixed cells with the lectin (1:50 dilution) for 5 min. For visualization of endoplasmic reticulum, 3,3'-dihexylxocarbocyanine iodide (Molecular Probes Inc., Eugene, OR) was included at a concentration of 2.5 μg/ml for 1 min following anti-dynein antibody incubations. In some experiments cells were incubated for 1 min in microtubule stabilizing buffer (extraction buffer as above without protease inhibitors) containing 0.05% saponin, followed by fixation in 3.75% formaldehyde containing 0.05% saponin. Extraction with Triton X-100 (0.1%) was performed for 5 min in the presence of 3.75% formaldehyde in microtubule-stabilizing buffer as above. Following antibody incubations, coverslips were mounted with gelvatol (Monsanto, St. Louis, MO) containing 1 μg/ml 1,4-diazabicyclo-(2.2.2) octane and viewed using a Carl Zeiss Inc. (Thornwood, NY) axiophot microscope with a 6x planachromat-
Localization of cytoplasmic dynein

A

HC-
150-
74-
45-
kDa

B

Fig. 1. Subunit specificities of dynein antibody preparations. (A) Proteins extracted from rat liver microtubules with ATP were fractionated by sucrose density gradient centrifugation. Individual fractions were transferred to nitrocellulose following SDS-PAGE and probed with dynein antiserum. Only the region of the gradient containing the peak of dynein ATPase activity at 20 S (fractions 4-9) is shown. The positions of immunoreactive dynein subunits are indicated. (B) Calf brain cytosol (lane 1) and BHK cell samples (lanes 2-4) were probed with affinity-purified antibody. Whole BHK cell homogenate (lane 4) was further fractionated into cytosol (lane 2) and membrane pellet (lane 3). HC, dynein heavy chain.

tic objective. Photomicrographs were taken using the same microscope using T-MAX 400 film (Eastman Kodak Co., Rochester, NY) processed with T-MAX developer.

Chemicals
Taxol was dissolved in DMSO at a concentration of 10 mM and stored as a stock solution at −80°C. It was kindly provided by Nancita Lomax at the Drug Synthesis and Chemistry Branch, Division of Cancer Research, NCI. Fluorescent secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Reagents for SDS-PAGE were from ICN Biomedicals, Inc. (Costa Mesa, CA) and Bio-Rad Laboratories (Richmond, CA). Enzyme-conjugated secondary antibodies, conjugated dextran and wheat germ agglutinin, reagents for immunoblotting, and most other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Other methods
Protein assays were conducted using BCA reagents (Pierce, Rockford, IL). SDS-PAGE was performed using 7% acrylamide in the separating gel and 3% acrylamide in the stacking gel. Transfer conditions and Western immunoblot analyses were carried out as previously described (Collins and Vallee, 1989).

Results
Characterization of anti-dynein antibody
Rabbit polyclonal serum was obtained which specifically recognized polypeptides characterized as subunits of cytoplasmic dynein. As shown in Fig. 1A, the immunoreactive polypeptides in a sucrose gradient fractionation of rat liver dynein include the 410 kDa dynein heavy chain and the 74 kDa subunit. The 150 and 45 kDa species, shown previously to copurify with the other dynein polypeptides from this tissue (Collins and Vallee, 1989), are also recognized by the antibody. A similar analysis of rat brain dynein revealed reactivity against the same polypeptides as well as with MAP 2 present in fractions higher in the sucrose gradient (not shown). To avoid potential problems of non-specific reactivities in localization experiments, crude antiserum was purified by affinity chromatography. Purified antibody reacted with the heavy chain and 74 kDa subunits of brain dynein on immunoblots (Fig. 1B), and did not react with purified MAP 2 (not shown). A second polyclonal antibody was raised against cytoplasmic dynein purified from rat testis, which does not contain detectable MAP 2 (Collins and Vallee, 1989; Neely and Boekelheide, 1988). This antibody recognized the same dynein subunits as the anti-calf brain dynein antiserum, but showed no reactivity against MAP 2. Both antibodies recognized native protein as demonstrated by reaction with dynein fractions adsorbed to nitrocellulose without denaturation by SDS, and by immunoprecipitation of dynein polypeptides from crude cytosolic extracts (data not shown).

Western immunoblots containing microtubule protein from a variety of rat and calf tissues were probed with the polyclonal antiserum (data not shown). In all cases the dynein heavy chain and 74 kDa bands were detected, while the 150 kDa species was most obvious in preparations from testis and liver.

Localization of dynein in cultured cells
Several cultured cell lines, including BHK-21, NIH-3T3, Hela and R4909 rat bladder epithelial cells, were tested for their content of cytoplasmic dynein by Western blot analysis and immunocytochemistry. The abundance and distribution of dynein was found to be
Fig. 2. Immunofluorescence localization of cytoplasmic dynein in fibroblasts. BHK cells were fixed with methanol for indirect immunofluorescence using whole dynein antisera (A), preimmune serum (B), rhodamine-conjugated secondary antibody alone (C), or affinity-purified antibody (D). All micrographs were prepared using the same exposures for photography and printing. Bar, 10 μm.

similar in all cases, and BHK cells were chosen for further study. The crude antisera recognized dynein heavy chain and 74 kDa subunits on Western blots containing BHK fractions (not shown), while the affinity-purified antibody recognized primarily the 74 kDa species (Fig. 1B). As shown in Fig. 2, the specific staining pattern determined with whole antisera was comparable to that obtained using affinity-purified antibody. Punctate staining was observed throughout the cytoplasm, with the highest concentration of fluorescently labeled structures in the perinuclear region of the cell. Serum preabsorbed against MAP 2 revealed a comparable staining pattern, and the results of immunofluorescent labeling studies using the antitestis dynein antibody yielded similar results. Preimmune serum (Fig. 2B) and antiserum preabsorbed with dynein polypeptides revealed no punctate staining pattern. All studies shown were performed using anticalf brain antibody, either as whole serum or following affinity purification.

As one test of the nature of the structures stained by the dynein antisera, cells were extracted in detergent solutions in the presence of a microtubule-stabilizing buffer. In the presence of a low concentration of saponin, bright punctate patterns could still be observed (not shown). However, low-intensity diffuse staining was eliminated by this procedure, indicating that a soluble pool of dynein may have been extracted. Extraction of the cells with Triton X-100 eliminated positive staining of vesicles with the dynein antibody but preserved the microtubule network (not shown). These results are consistent with the identification of the punctate spots as membrane-bounded organelles.

A double-labeling experiment using affinity-purified anti-dynein antibody and a monoclonal antibody to alpha tubulin is shown in Fig. 3. In most cases labeled
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Fig. 3. Comparison of dynein and microtubule distribution in BHK cells by double immunofluorescence. Cells were fixed with methanol and double-stained with monoclonal antibody to alpha tubulin (A) and affinity-purified anti-dynein antibody (B). Bar, 10 μm.

vesicles were heavily concentrated around the nucleus, especially toward the region of highest microtubule density. Vesicles located in the periphery of the cell appeared to colocalize with microtubules (see below).

Identification of the dynein-associated organelles

Because the dynein-associated vesicles appeared to be concentrated near the microtubule-organizing center, we investigated the possibility that they were derived from or associated with the Golgi apparatus. Wheat germ agglutinin has been used as a marker for the identification of the trans-Golgi network (Tartakoff and Vassalli, 1983). A double-labeling experiment using fluorescein-conjugated wheat germ agglutinin and the anti-dynein antibody is shown in Fig. 4. The region of the Golgi network exhibiting a tubular morphology overlying the microtubule organizing center identified using the lectin (Fig. 4A, arrowhead) did not stain especially strongly with the dynein antibody. However, wheat germ agglutinin also appeared to label a population of vesicles that co-localized with punctate structures labeled with the dynein antiserum. This lectin is known to label plasma membrane and lysosomal membranes as well as the Golgi apparatus in certain cell types (Virtanen et al., 1980; Hiller and Weber, 1982), and the general distribution of the vesicular structures was consistent with the possibility that they were lysosomes.

To investigate further the nature of the dynein-positive organelles, a double-label experiment using rhodamine-dextran was performed. This ligand is taken up by the cell in pinocytic vacuoles and is selectively concentrated in lysosomes (Decker et al., 1985; Heuser, 1989). As illustrated in Fig. 5, the staining pattern obtained with the dextran marker is largely coincident with that using the dynein antibody, suggesting that this population of organelles is lysosomal in origin.

To examine the association of dynein with lysosomes and endosomes, we incubated the cells with sodium acetate, a treatment found to reduce the pH of the cytoplasm to ~6.5. This results in the redistribution of lysosomes and late endosomes to the periphery of the cell (Heuser, 1989; Parton et al., 1991). As shown in Fig. 6, cells treated in this manner contain increased numbers of fluorescein-dextran-labeled lysosomes in the cell processes and throughout the cytoplasm. This is in contrast to cells that have been allowed to recover by a return to normal culture medium, which exhibit the usual perinuclear concentration of these organelles (Fig. 6C,D). The staining pattern in these cells again demonstrates the co-localization of cytoplasmic dynein with fluorescein-positive lysosomes. However, smaller dynein-positive vesicles that are not labeled by the dextran marker are occasionally observed in the periphery of the cell. These may represent newly formed pinocytic vesicles (early endosomes). The inclusion of a 3 h wash step in the protocol for dextran loading in this experiment reduces the amount of fluorescein label associated with these earlier compartments (cf. Fig. 5).

There has been some indication that kinesin is responsible for formation of tubular processes from endoplasmic reticulum in vitro (Dabora and Sheetz, 1988; Allan and Vale, 1991). To determine whether dynein was associated with the ER network in these cells, a double-labeling experiment using the lipid marker diOC₆(Terasaki et al., 1986) was performed. As shown in Fig. 7, there was no obvious coincidence in the two staining patterns.

Dynein association with microtubules

We have not observed anti-dynein staining of the interphase array of microtubules in any cell type examined. To determine whether microtubule-associated dynein could be detected by the antibody, rat brain
and testis microtubule preparations containing cytoplasmic dynein were adsorbed to a coverslip and fixed and stained for immunofluorescence. Dynein staining could be observed along the length of the microtubules (data not shown).

To investigate the association of dynein-associated vesicles with microtubules, we examined conditions that lead to the reorganization or depolymerization of the microtubule network. Fig. 8 shows the results from an experiment using vinblastine and taxol. Vinblastine treatment leads to the accumulation of tubulin para-crystals within the cytoplasm (Bensch and Malawista, 1969), while taxol addition results in the formation of microtubule bundles that are no longer nucleated from the microtubule organizing center (Schiff and Horwitz, 1980; De Brabander et al., 1981). Consistent subtle changes in the dynein/lysosome pattern were observed following these treatments. Vinblastine treatment generally led to a peripheral redistribution of the dynein-containing organelles (Fig. 8A, B). No obvious co-

Fig. 4. Dynein localization compared with that of the Golgi apparatus. Fluorescein-conjugated wheat germ agglutinin was used to label BHK cells following methanol fixation and incubation with dynein antibody and rhodamine anti-rabbit secondary antibody as described in Materials and methods. The tubular region of the trans-Golgi labeled by wheat germ agglutinin is indicated by the arrow (A). (B) Anti-dynein staining. Bar, 10 μm.

Fig. 5. Co-localization of dynein and lysosomes in BHK cells. Lysosomes were labeled by incubation with 1.33 mg/ml rhodamine-conjugated dextran for 16 h. Cells on coverslips were fixed with formaldehyde containing 0.02% saponin and incubated with dynein antiserum and fluorescein anti-rabbit secondary antibody. (A) Rhodamine-dextran; (B) dynein. Bar, 10 μm.
Fluorescein-dextran

Localization of cytoplasmic dynein

Fig. 6. Redistribution of lysosomes in response to acetate treatment. Lysosomes in BHK cells were labeled with 2 mg/ml fluorescein-dextran for 16 h followed by a 3 h wash in fresh medium without dextran. After treatment with 70 mM sodium acetate for 15 min at 37°C, cells were either fixed and stained immediately using the methanol procedure (A,B), or washed in fresh medium for 15 min before fixation (C,D). (A,C) Fluorescein-conjugated dextran; (B,D) dynein. Bar, 10 μm.

localization of the vesicles with tubulin paracrystals was found. In fact, the vesicles were generally excluded from these areas. Disruption of the normal distribution of dynein-labeled organelles was also found following taxol treatment. In the example shown in Fig. 8C and D, the lysosomes appear to be clustered more toward one end of each microtubule bundle, similar to the results obtained examining taxol effects on Golgi distribution (Wehland et al., 1983).

Disruption of the microtubule network with depolymerizing drugs has been shown to cause reorganization of several membrane systems in the cell, including ER, Golgi and lysosomes (Terasaki et al., 1986; Rogalski and Singer, 1984; Matteoni and Kreis, 1987). The peripheral redistribution of lysosomes following nocodazole treatment is demonstrated in Fig. 9A,B. Following a return to normal culture medium, the lysosomes move back to their normal perinuclear location (Fig. 9C-F). In most untreated cells, it is difficult to examine the association of the lysosomes along microtubules, since both are present in high concentration in the center of the cell. However, the treatment with nocodazole and subsequent recovery allowed us to examine the colocalization of peripheral organelles with individual microtubules. Transparent overlays were made from micrographs of the dynein staining pattern, and used to align with the microtubules in a comparable micrograph. This analysis has shown that where individual vesicles and microtubules can be visualized, more than 80% of the lysosomes are found in close association with a microtubule. This colocalization has also been examined using laser scanning confocal microscopy to examine non-drug-treated cells (not shown). At the limit of resolution of this technique, over 80% of total dynein-staining vesicles co-align with microtubules.

To examine further the association of dynein with microtubules, cells at various stages in the cell cycle were examined. Cells at mitosis also exhibited a punctate vesicular pattern when stained using dynein.
Fig. 7. Comparison of endoplasmic reticulum and dynein distribution. BHK cells were double-labeled with the ER marker 3,3′-dihexyloxacarbocyanine iodide (A) and dynein antibody (B) as described in Materials and methods. Bar, 10 μm.

Fig. 8. Effects of microtubule reorganization on dynein distribution. BHK cells were treated for 16 h with either 10 μM vinblastine (VBL) (A,B), or 10 μM taxol (C,D), fixed with methanol, and double-stained for tubulin and dynein. In this experiment sufficient fluorescent signal from the anti-dynein label (B,D) was present in the microtubule channel to allow comparison of the distribution of the dynein-positive organelles with the tubulin (A,C).
Fig. 9. Disruption of the microtubule (MT) network and lysosome distribution with nocodazole. BHK cells were treated with 5 μM nocodazole for 16 h, and fixed and stained immediately (A,B), or washed in fresh medium lacking the drug for 1 h (C,D) or 6 h (E,F) prior to fixation. (A,C,E) Anti-tubulin; (B,D,F) anti-dynein. Bar, 10 μm.

antibody (Fig. 10). Weak fibrous staining was also occasionally observed in the region of the mitotic apparatus. This staining was more obvious in cells that had been extracted with detergent prior to fixation (Fig. 10C,D). The dynein staining pattern in extracted cells appeared identical to that obtained with the tubulin antibody. Experiments using affinity-purified dynein antibody gave similar results.

Discussion

We have raised polyclonal antibodies that recognize the
Fig. 10. Localization of dynein and microtubules in mitotic BHK cells. Cells on coverslips were fixed with methanol and double-labeled for dynein (A,C) and tubulin (B,D). For C and D, the cells were extracted with 0.2% saponin for 1 min in microtubule stabilizing buffer prior to fixation. Bar, 20 μm.

There is evidence that cytoplasmic dynein is present in other cellular compartments as well. From detergent extraction experiments, we and others (Steuer et al., 1990) have obtained evidence for a soluble pool of cytoplasmic dynein. In fact, previous biochemical fractionation data have indicated that most of the cytoplasmic dynein is recovered in cytosol (Paschal et al., 1987; Lye et al., 1987; Collins and Vallee, 1989; Koonce and McIntosh, 1990). However, we find that a fraction of cytoplasmic dynein from rat liver and brain tissue remains associated with membranous pellet fractions, particularly those that are enriched in lysosomes (P. Tuma and C. Collins, unpublished data). The data obtained in this study using BHK cells (Fig. 1B) indicate that the majority of cytoplasmic dynein in this cell type is present in a crude membrane fraction.

Heavy-chain and 74 kDa subunits of cytoplasmic dynein, as well as species of 150 and 45 kDa. These latter polypeptides copurify with dynein and probably represent additional subunits or dynein-associated proteins (Collins and Vallee, 1989; Holzbaur et al., 1991). The antibodies recognize dynein polypeptides in all tissues and cell types examined, indicating the importance of this protein for the maintenance of general cellular function. The immunolocalization results shown here demonstrate that cytoplasmic dynein is associated with cytoplasmic vesicles in cultured cells. We have used double-labeling techniques to co-localize organelle markers with the dynein-positive vesicles. The results of wheat germ agglutinin- and rhodamine-dextran-labeling studies are consistent with the identification of these dynein-containing structures as lysosomes.
These results provide additional evidence in support of the immunolocalization of some fraction of dynein to lysosomes.

Recently, cytoplasmic dynein has been localized to the mitotic spindle of PtK2 cells (Yoshida et al., 1990), to spindle microtubules and kinetochores of dividing PtK2 cells (PFarr et al., 1990) and of chick embryo fibroblasts (Steuer et al., 1990), and to kinetochores of Indian muntjac chromosomes (Zinkowski et al., 1991). These results indicate that dynein may play a role in mitotic microtubule and chromosome movements. We also observe spindle staining, but it is faint compared to the labeling of organelles in the same cell, and thus far has only been observed in detergent-extracted preparations. It remains to be determined whether cytoplasmic dynein is a functional component of the mitotic apparatus. It is possible that cytoplasmic dynein exists in equilibrium between a soluble pool, membrane-bound forms and microtubules of the mitotic spindle, and that soluble factors (Schroer et al., 1989) are involved in the process of membrane or microtubule (kinetochore) recognition and binding.

Our data, which show that dynein is associated with organelles concentrated in the perinuclear region of the cell, support the hypothesis that cytoplasmic dynein functions as a retrograde motility factor (Vallee et al., 1989; Schroer et al., 1989; Schnapp and Reese, 1989). Lysosomes generally occupy a juxtanuclear position in cells (Pastan and Willingham, 1981; Collot et al., 1984; Herman and Albertini, 1984). Following endocytosis, material enclosed in endosomes is transported in a vectorial fashion toward the nucleus (Herman and Albertini, 1984; Matteoni and Kreis, 1987; De Brabander et al., 1988; Gruenberg and Howell, 1989). Material destined to be degraded or modified is transferred to the lysosomes, and material to be modified prior to recycling back to the plasma membrane is transferred to the Golgi apparatus (Fishman and Cook, 1986; Gruenberg and Howell, 1989; Kornfeld and Mellman, 1989). This retrograde flow of material taken in from the plasma membrane is dependent upon an intact microtubule network, and has been proposed to be powered by means of a retrograde-directed microtubule motor (Gruenberg et al., 1989; Heuser, 1989; Bomsel et al., 1990). Our data that indicate a close correlation between the location of the dynein-positive organelles and the intact or reorganized microtubule network provide additional evidence for this proposal.

However, lysosomes have been reported to undergo saltations bidirectionally with regard to the microtubule organizing center (Matteoni and Kreis, 1987; De Brabander et al., 1988). Under certain conditions, net lysosomal translocation in the anterograde direction is also known to occur. Lysosomes of macrophages, which ordinarily exhibit an extended tubulovesicular pattern, become vesicular and juxtanuclear following depolymerization of the microtubules with nocodazole (Swanson et al., 1987). Extension of the tubular network of lysosomes in the outward direction (anterograde) occurs after removal of the drug. Antibody to kinesin has been found to inhibit this outward extension of the lysosomal network (Hollenbeck and Swanson, 1990). Lysosomes have also been found to accumulate at the cell periphery in cultures acidified through the addition of sodium acetate to culture medium (Heuser, 1989), and return to their perinuclear location once the pH returns to normal. Similar treatment of MDCK cells and cultured rat hippocampal neurons resulted in the microtubule-dependent shift of late endosomes toward the basal region of the MDCK cell, and out into the processes of the neuronal cells, consistent with the involvement of a (+)end-directed microtubule motor (kinesin) in this reorganization (Parton et al., 1991). We have also used the acidification protocol and find that peripherally located lysosomes and endosomes in fibroblasts treated with sodium acetate are labeled using anti-dynein antibody, indicating that dynein remains associated with these anterogradely moving organelles.

The results described above suggest that kinesin and dynein are both present on the lysosomal membrane and are differentially regulated. The reversible activation of both retrograde and anterograde motors on lysosomes and endosomes could explain the bidirectional saltatory movements of these organelles observed in vivo. Further evidence to support this model comes from a study by Hirokawa et al. (1990), in which cytoplasmic dynein was found in association with both anterogradely and retrogradely transported organelles in peripheral nerves. These data suggest that dynein is differentially activated in order to allow retrograde transport of specific organelles. The final question raised by the current study concerns the time course of appearance of dynein in association with endocytic or lysosomal organelles. We have not observed anti-dynein labeling of the plasma membrane, indicating that association of dynein with endosomes or lysosomes must take place following internalization of the endocytic vesicle. Studies of the movement of endosomes and lysosomes in intact cells indicate that immediately following internalization, primary endosomes at the periphery of the cell move in a random, salutatory fashion over short distances (Herman and Albertini, 1984; De Brabander et al., 1988). The initial events of endocytosis, receptor internalization and recycling, have, in fact, been found not to rely on an intact microtubule network (Gruenberg et al., 1989). Several minutes following internalization, the vesicles appear to bind to a cytoplasmic track, thought to be microtubules, and move centripetally in either a continuous motion (Herman and Albertini, 1984) or by discontinuous, bidirectional movement, with net translocation toward the cell center (Matteoni and Kreis, 1987; De Brabander et al. 1988). This biphasic pattern of movement may reflect the necessity of binding appropriate cytoplasmic motility factors and finding a nearby microtubule on which to translocate. Thus, regulation of directed movements of membranous organelles is likely to involve reversible binding of the motility factors kinesin and dynein to unidentified membrane binding sites (receptors) as well as the
modulation of motor activity once they become associated with the organelles.

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