Cytoplasmic Dynein Undergoes Intracellular Redistribution Concomitant with Phosphorylation of the Heavy Chain in Response to Serum Starvation and Okadaic Acid

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Abstract. Cytoplasmic dynein is a microtubule-binding protein which is considered to serve as a motor for retrograde organelle movement. In cultured fibroblasts, cytoplasmic dynein localizes primarily to lysosomes, membranous organelles whose movement and distribution in the cytoplasm have been shown to be dependent on the integrity of the microtubule cytoskeleton. We have recently identified conditions which lead to an apparent dissociation of dynein from lysosomes in vivo, indicating that alterations in membrane binding may be involved in the regulation of retrograde organelle movement (Lin, S. X. H., and C. A. Collins. 1993. *J. Cell Sci.* 105:579–588). Both brief serum withdrawal and low extracellular calcium levels induced this alteration, and the effect was reversed upon addition of serum or additional calcium. Here we demonstrate that the phosphorylation state of the dynein molecule is correlated with changes in its intracellular distribution in normal rat kidney fibroblasts. Dynein heavy chain phosphorylation level increased during serum starvation, and decreased back to control levels upon subsequent addition of serum. We found that okadaic acid, a phosphoprotein phosphatase inhibitor, mimicked the effects of serum starvation on both phosphorylation and the intracellular redistribution of dynein from a membrane-associated pool to one that was more soluble, with similar dose dependence for both phenomena. Cell fractionation by differential detergent extraction revealed that a higher proportion of dynein was present in a soluble pool after serum starvation than was found in comparable fractions from control cells. Our data indicate that cytoplasmic dynein is phosphorylated in vivo, and changes in phosphorylation state may be involved in a regulatory mechanism affecting the distribution of this protein among intracellular compartments.

ACCUMULATING evidence indicates that retrograde and anterograde organelle transport along the microtubule network are mediated by the actions of microtubule-dependent motor molecules, cytoplasmic dynein, and kinesin (for review see Vallee and Shpetner, 1990; Walker and Sheetz, 1993). In contrast to the plethora of kinesin and kinesin-like molecules, only some of which may be anterograde organelle motors (for review see Endow, 1991; Goldstein, 1991; Walker and Sheetz, 1993), cytoplasmic dynein has been found in only one form in each cell type and species examined (Koonce et al., 1992; Paschal et al., 1992; Eshel et al., 1993; Li et al., 1993; Mikami et al., 1993; Zhang et al., 1993; Gibbons et al., 1994; Rasmusson et al., 1994). However, this retrograde translocator may carry out multiple cellular functions, as dynein has been localized to both membranous organelles and the mitotic apparatus in a number of cell types (Pfarr et al., 1990; Steuer et al., 1990; Lin and Collins, 1992).

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In our previous work cytoplasmic dynein in cultured fibroblasts was shown to be concentrated on large membrane-bounded organelles, identified as lysosomes (Lin and Collins, 1992). The association of dynein with these organelles was stable under a variety of conditions in which retrograde transport was inhibited, including after disruption of the microtubule network, after cytoplasmic acidification, and during mitosis (Lin and Collins, 1992). These results suggested that specific dynein-organelle binding interactions could be uncoupled from the formation of a functional motile complex. However, regulation of dynein-based organelle transport is likely to occur at several levels, including alteration in the specificity and affinity for membranous organelles. We have chosen to investigate conditions which might lead to changes in the distribution of the cellular pools of dynein, reasoning that this would provide information as to the regulatory pathways and extra- and intracellular signals involved in coordination of organelle transport in a single cell. We have recently shown that brief (4 h) serum withdrawal from cultures of NRK fibroblasts resulted in the apparent loss of lysosome-associated dynein (Lin and Collins, 1993). This alteration in dynein distribution was rap-

idly reversible upon addition of calf serum to the culture medium. These results suggested that the binding of dynein to its organelle targets is regulated by the cell, and can undergo changes in response to extracellular factors. Considering that normal cellular signaling pathways may be involved in this regulatory process, we investigated the effects of protein kinase and phosphatase inhibitors and activators in order to determine whether any of these agents could reproduce the effect of serum starvation on dynein distribution. We found that okadaic acid, a phosphoprotein phosphatase inhibitor, induced a time- and dose-dependent loss of punctate lysosome-associated dynein staining in NRK fibroblasts. Increased phosphorylation of the dynein heavy chain, but not the 74-kD intermediate chain, after serum starvation or okadaic acid treatment correlated with the loss of vesicular staining, suggesting that phosphorylation of the dynein molecule results in decreased affinity for lysosomal binding sites. Biochemical fractionation of the cells by differential detergent extraction provided corroborative evidence for this hypothesis. In conjunction with recent evidence that kinesin is phosphorylated in vivo (Hollenbeck, 1993; Matthies, 1993), and that phosphorylation in vitro using cAMP-dependent protein kinases reduces binding of kinesin to synaptic vesicles (Sato-Yoshitake, 1992), these data suggest that regulation of the microtubule motors kinesin and dynein takes place, in part, by modulation of the binding affinity for their intracellular organelle targets by phosphorylation.

Materials and Methods

Materials

[32]P$_i$ was purchased from Amersham Corp. (Arlington Heights, IL). Okadaic acid was from L. C. Laboratories (Woburn, MA), and was stored at 4°C as a 0.5-mM stock solution in DMSO. Most other drugs and reagents were obtained from Sigma Chem. Co. (St. Louis, MO).

Cell Culture and Immunofluorescence Microscopy

Normal rat kidney (NRK)[1] fibroblasts were grown on coverslips for 2 d after plating in DME (Irvine Scientific, Santa Ana, CA) with 10% calf serum (Hyclone Laboratories, Logan, UT). Control cells or those treated with increasing concentrations of okadaic acid as indicated in the figure legends were fixed in methanol (−20°C) for 5 min. The fixed cells were stained using polyclonal anti-cytoplasmic dynein (Lin and Collins, 1992), monoclonal anti-58 kD protein to label the Golgi apparatus (Bloom and Brashear, 1989), polyclonal anti-cathepsin D to label lysosomes (Sensibar et al., 1990), monoclonal anti-α tubulin (Amersham Corp.), and secondary antibodies (fluorescein- and rhodamine-conjugated goat anti–mouse and anti–rabbit, Jackson Immunoresearch Laboratories, West Grove, PA), viewed and photographed as previously described (Lin and Collins, 1992, 1993).

Cell Treatments

Cellular ATP was depleted by addition of 5 mM NaN$_3$ to serum-free culture medium (DME lacking glucose) for 1-3 h. No effect on cell viability or changes in cell shape were observed during this period. The effects of other agents on localization of dynein were tested by adding them to culture medium containing serum (to test whether the added agent could itself induce changes in the immunolocalization pattern) or to cells which had been serum starved (to test whether the added agent could induce recovery of lysosomal dynein staining alone, or whether it could prevent recovery upon the addition of serum). In some cases the addition of drugs at their physiologically effective dose led to irreversible changes in cell morphology or decreased viability, and these data were not included. For this reason, the effect of many of the inhibitors could not be tested for their ability to prevent

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1. *Abbreviation used in this paper*: NRK, normal rat kidney.

serum starvation–induced loss of lysosomal dynein, as this redistribution occurred slowly, requiring 2-4 h of incubation.

Metabolic Labeling and Immunoprecipitation

Nearly confluent NRK cells were labeled for 4 h with 0.3 mCi/ml [32]P$_i$ in low phosphate medium (10 μM phosphate in phosphate-free DME with 5% dialyzed serum). Where indicated, okadaic acid was added to the culture medium during the last 45 min of the labeling period. For serum starvation experiments, the 4-h labeling period was initiated 16 h after removal of serum. For serum recovery experiments, labeled, starved cells were allowed to incubate for an additional 45 min in the presence of [32]P$_i$ after addition of dialyzed serum (1%) to the labeling medium. Cells were lysed in 0.5 ml/10-cm plate with 1% Triton X-100 and 50 mM β-glycerophosphate in extraction buffer (Paschal et al., 1987) containing 50 mM Hepes, 50 mM Pipes, pH 7.0, 2 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT and protease inhibitors (1 mM PMSF, 20 μg/ml leupeptin, 2 μg/ml pepstatin A, and 0.03 trypsin inhibitor U/ml aprotinin). A soluble extract was obtained after cell disruption using a Dounce homogenizer by centrifugation at 10,000 g for 10 min at 4°C. Polyclonal antibody to dynein (20 μl antiserum per 0.5 ml cell extract, and protein A-agarose beads (Sigma Chem. Co.), 35-μl beads per 0.5 ml cell extract, were added to the cell extract and incubated overnight at 4°C. The beads were washed four times by sedimentation in extraction buffer and once in 1 M NaCl in extraction buffer. SDS-PAGE sample buffer was added to solubilize protein associated with the protein A beads, and the samples were resolved by SDS-PAGE using 7% acrylamide in the separating gel. The Coomassie blue–stained gels were dried and exposed to x-ray film. Dynein content in the immunoprecipitates was quantitated by laser scanning densitometry of the stained gel bands compared with dynein preparations of known protein concentration, and ^{32}P incorporation was determined by scintillation counting of individual protein bands cut from the dried gel and rehydrated in water. The cpm in each band was normalized according to relative protein content between samples. For some experiments 2 mM unlabeled ATP was added to the homogenization buffer to prevent any postlysis transfer of phosphate (Hollenbeck, 1993). There was no effect of this addition on the level of ^{32}P incorporation into heavy chain under our experimental conditions. The molar incorporation of phosphate into dynein subunits was calculated assuming that the specific activity of the intracellular phosphate pool was identical to that of the medium (Sefton, 1991). An experiment examining a time course of labeling from 2-6 h revealed that the specific activity of several phosphoproteins examined, including dynein, had reached a plateau at 4 h, indicating that this labeling period was sufficient to reach equilibrium in these experiments.

Cell Fractionation

Differential detergent extraction of control and serum-starved cells was performed as described by Hollenbeck (1989). Briefly, confluent dishes of NRK cells were rinsed with warm PBS (50 mM NaPO$_4$, pH 7.4, 150 mM NaCl), and then incubated in 1.5 ml of warm buffer (100 mM Pipes, 5 mM MgSO$_4$, 10 mM EGTA, 2 mM DTT, pH 7.0) containing 4% polyethylene glycol, 10 μM taxol, 0.02% saponin, and the protease inhibitors indicated above. After 10 min at 37°C, the buffer was removed and replaced with the same buffer but with 1% Triton X-100 instead of saponin. After 3 min the buffer was removed and replaced with 0.5 ml of SDS-PAGE sample buffer. The remaining cell remnants were scraped from the plate in this solution and boiled for 5 min. Dynein was recovered from the saponin and Triton X-100 fractions by immunoprecipitation as described above. The dynein content in the two immunoprecipitates and the SDS fraction was quantitated by laser scanning densitometry of the dynein intermediate chain after SDS-PAGE and Western blotting. Previous analyses have shown immunoreactivity to be linear with respect to dynein content in the protein concentration range examined (Lin and Collins, 1993). Dynein was not detected in the SDS fractions. Based on measurements of the sensitivity of this method the dynein content in the SDS fractions was less than 5% of the total. The effect of okadaic acid on dynein fractionation was not evaluated as this drug led to morphological changes in the cells and there was considerable cell loss during extraction procedures.

Phosphopeptide Analysis

Immunoprecipitates of ^{32}P-labeled dynein were fractionated by SDS-PAGE, the region of an unstained gel corresponding to the heavy chain excised, and sample preparation performed as described by O'Connor et al. (1981). Briefly, the gel piece was rinsed three times in 50% methanol for a total of 3 h followed by one wash in 100% methanol for 10 min. The gel

was cut into pieces and incubated in 1 ml of 10 μg/ml trypsin in 50 mM NH$_4$CO$_3$, pH 7.8 for 16 h at 37°C. The gel pieces were sedimented in a microfuge and discarded, and the supernate collected and lyophilized. After resuspension in 50 μl of 10 μg/ml trypsin, the sample was further digested for 3 h at 37°C, lyophilized, and resuspended into electrophoresis buffer. A two-dimensional separation was performed as described by Boyle et al. (1991). Electrophoresis on cellulose thin layer plates was carried out at pH 1.9 (20 ml 88% formic acid, 23 ml glacial acetic acid, 750 ml water) using constant voltage (700 V) for 1 h. After drying, the plates were chromatographed in a solution containing 60 ml n-butyl alcohol, 12 ml acetic acid, 40 ml pyridine, and 48 ml water. After drying, the plates were exposed to x-ray film for autoradiography, and quantitation of ^{32}P content in the phosphopeptide species was performed using a Fuji Bio-Imaging Analyzer.

Results

Okadaic Acid Induces Redistribution of Cytoplasmic Dynein

We found previously that the immunofluorescent staining pattern of cytoplasmic dynein in fibroblasts under standard culture conditions is distinctly punctate, with the highest concentration of staining colocalizing with markers for lysosomes (Lin and Collins, 1992, 1993). However, we found that dynein became diffusely distributed throughout the cytoplasm of NRK cells after brief (4 h) withdrawal of serum from the cell culture medium (Lin and Collins, 1993). After addition of serum, a punctate lysosomal pattern of dynein staining comparable to that of control cells was restored within an hour. To further examine this phenomenon, we searched for conditions which would lead to a similar intracellular redistribution of cytoplasmic dynein, as these might provide clues as to the regulatory processes involved in the distribution and activity of this motor protein.

We examined many drugs and treatment conditions for their ability to affect the immunofluorescent staining pattern of cytoplasmic dynein in control, serum-starved and serum-recovered NRK cells. As previously described (Lin and Collins, 1992), disruption of the microtubule network and alteration in vesicle trafficking due to cellular acidification (Heuser, 1989) did not affect the association of dynein with lysosomes in control cells, though the lysosomes themselves became more peripherally distributed than in untreated controls. Chloroquine, which increases lysosomal pH and also disrupts the endocytic pathway (Merion and Sly, 1983), similarly had no effect on the colocalization of dynein with lysosomal markers. ATP depletion induced by NaN$_3$ treatment during serum withdrawal was found to prevent changes in the dynein distribution, but ATP depletion alone did not lead to recovery of lysosomal staining in serum-starved cells (not shown).

Calcium depletion in the presence of serum was previously found to lead to loss of punctate dynein staining, and chelation of calcium in the culture medium prevented the recovery of vesicular staining upon addition of serum to serum-starved cells (Lin and Collins, 1993). This result suggested that intracellular calcium stores, depleted in the presence of chelators or low calcium medium, were required for maintenance of a lysosomal staining pattern. Calcium ionophores could not replace serum in restoring vesicular staining, indicating that an increase in intracellular calcium was not sufficient to recover a lysosomal dynein distribution. Calmodulin antagonists were also without effect. A variety of additional agents which stimulate or inhibit signal transduction pathways were tested for their effects on dynein localization (Table I). Of all the drugs tested which affect protein kinase and phosphatase activities, only okadaic acid led to reproducible changes in the intracellular distribution of cytoplasmic dynein, inducing the loss of vesicular dynein staining similar to that observed during serum starvation.

Okadaic acid is a specific inhibitor of phosphoprotein phosphatases types 1 and 2A (Bialojan and Takai, 1988; Haystead et al., 1989). Since it is cell membrane permeable, it has been used to advantage in the investigation of the role of phosphorylation in cellular events (Cohen et al., 1990). We observed that treatment of NRK fibroblasts with okadaic acid resulted in the dose-dependent loss of punctate cytoplasmic dynein immunofluorescence patterns (Fig. 1 A). Studies have shown that okadaic acid has effects on morphology and integrity of the Golgi apparatus (Lucocq et al., 1991; Thyberg and Moskalewski, 1992) and on dynamics of cytoskeletal elements (Erikkson et al., 1992; Gliksman et al., 1992; Sacher et al., 1992; Thyberg and Moskalewski,

Table I. Agents Tested for Effects on Loss and Recovery of Vesicular Dynein Staining

Treatment		Induces loss of vesicle staining	Induces recovery in absence of serum	Prevents recovery with serum present
Kinase/phosphatase reagents				
PMA	(10 ng/ml)	no	no	no
Forskolin	(100 μM)	no	no	no
dibutyryl-cAMP	(5 mM)	no	no	no
8-bromo cAMP	(1 mM)	no	—	—
Kt5720	(200 nM)	—	no	no
Staurosporine	(50 nM)	—	—	no
Okadaic acid	(1.5 μM)	YES	—	—
Calcium/calmodulin reagents				
Calmidazolium	(5 μM)	no	—	no
W-7	(10 μM)	no	—	no
Tonomycin	(300 nM)	no	no	—
A23187	(10 μM)	—	no	no
EGTA	(3 mM)	YES	no	YES
Ca-free DME		YES	no	YES

Control, serum-starved, and serum-refed NRK cells were treated under the conditions listed.
PMA, phorbol myristic acid; (−), not determined.

A
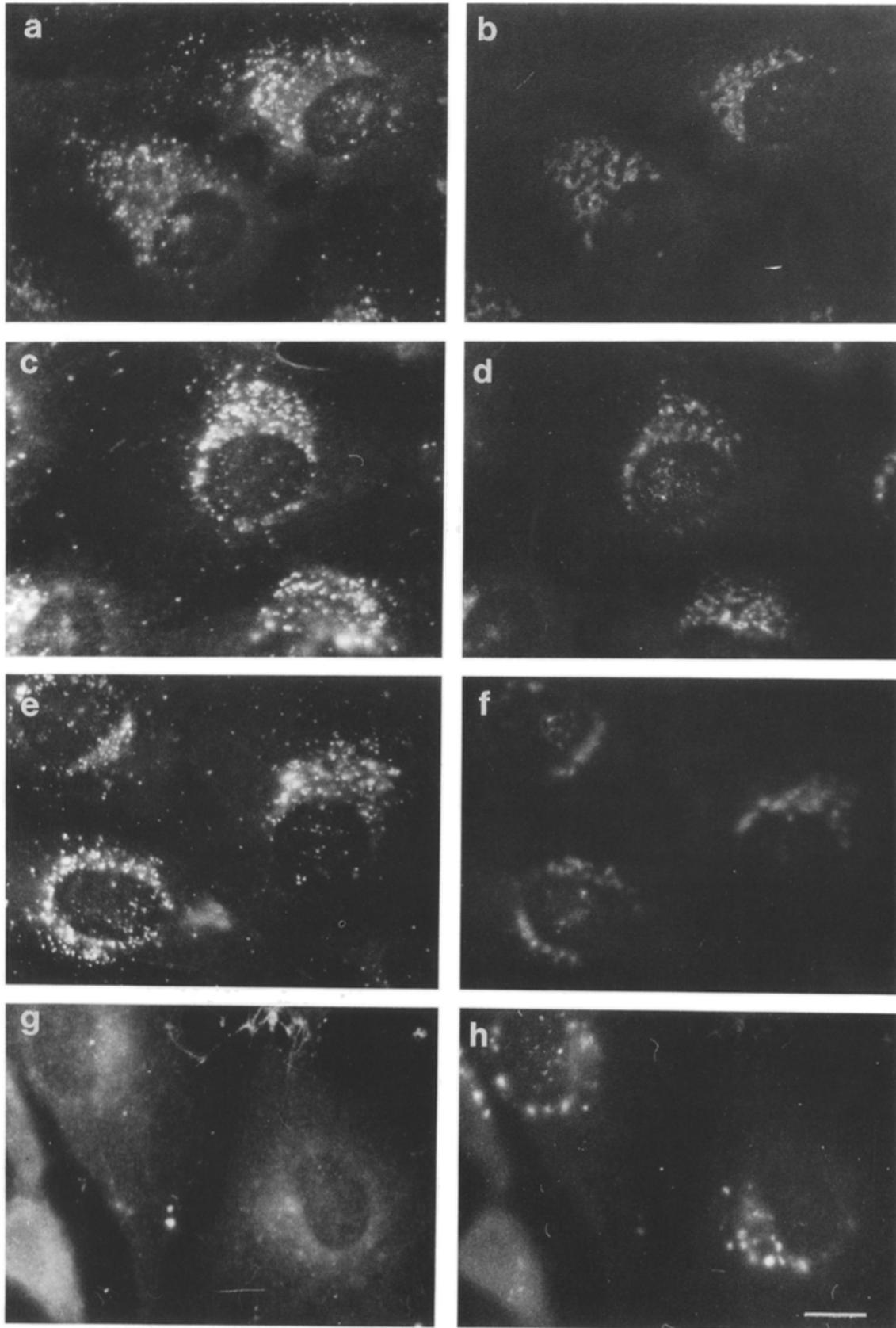
Figure 1. Dose dependent effects of okadaic acid on the intracellular localization of cytoplasmic dynein and morphology of cellular organelles. (*A*) NRK cells were treated with okadaic acid for 45 min, and then fixed and stained for cytoplasmic dynein (*a, c, e,* and *g*) and the Golgi apparatus (*b, d, f,* and *h*). (*B*) NRK cells similarly treated with okadaic acid were fixed and labeled with antibodies to cathepsin D (*a, c, e,* and *g*) and tubulin (*b, d, f,* and *h*). Cells were incubated without drug (*a* and *b*), or with 10 nm (*c* and *d*); 100 nM (*e* and *f*); or 1.5 μM (*g* and *h*) okadaic acid. Bar, 10 μm.
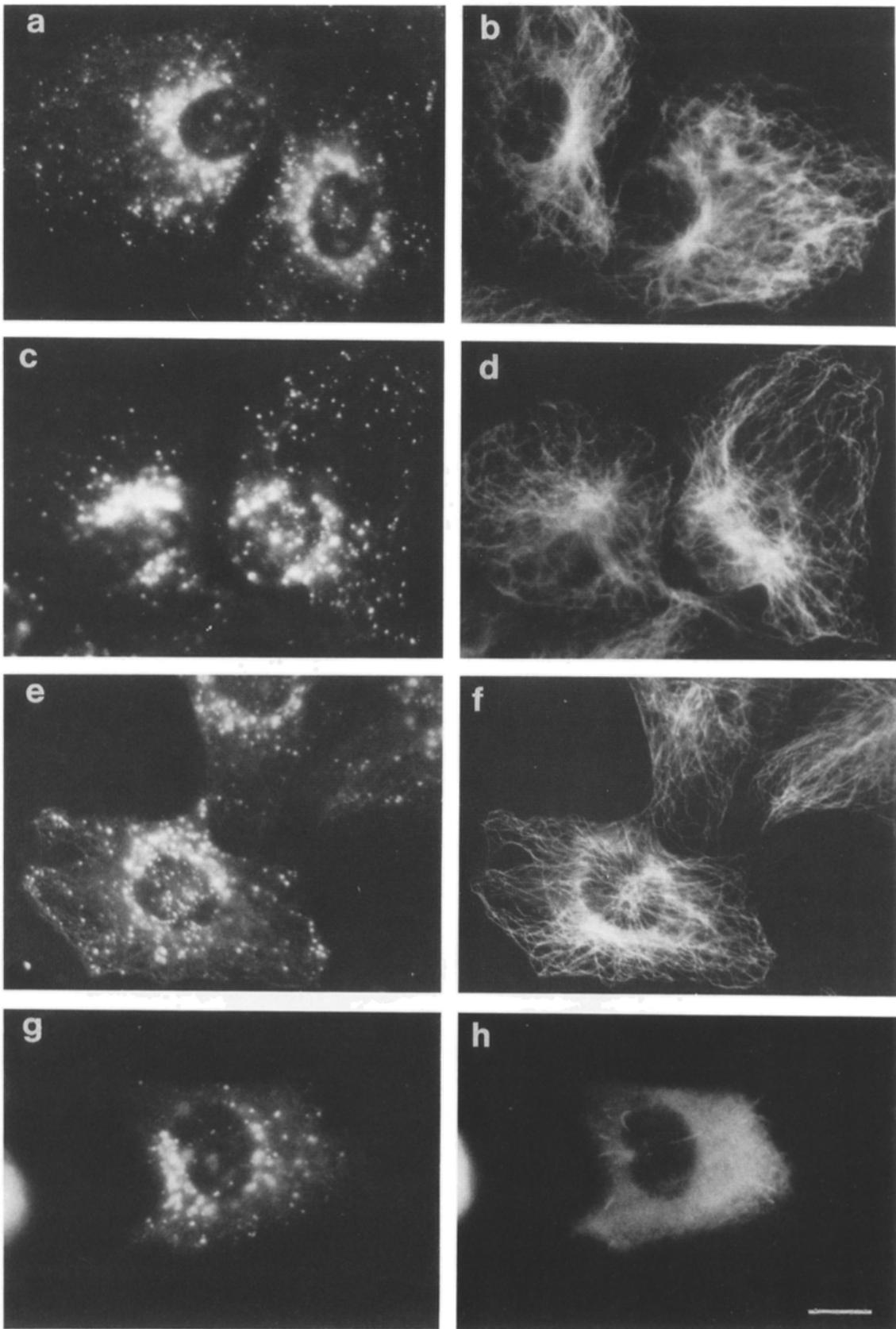
B

Figure 1.

1992; Vandre and Wills, 1992). We examined the staining pattern of the Golgi apparatus (Fig. 1 *A*) and microtubules (Fig. 1 *B*) after treatment with increasing concentrations of okadaic acid and found disruption of these compartments as previously reported. The morphology and intracellular localization of the lysosomes themselves were not significantly affected by okadaic acid treatment (Fig. 1 *B*).

Doses lower than 1.5 μM okadaic acid also led to changes in dynein distribution, but required longer incubation periods in order to have an effect. In the presence of 250 nM okadaic acid, punctate dynein staining was not observable after 3.5 h of incubation. At this time point, cells incubated in 10 nM or 100 nM okadaic acid were indistinguishable from controls (data not shown). The effect of okadaic acid on dynein distribution was reversible. Vesicular dynein staining patterns were recovered in cells incubated with either 250 nM (for 3.5 h) or 1.5 μM (for 45 min) okadaic acid within 2 h after washing the cells and incubation in drug-free medium (not shown).
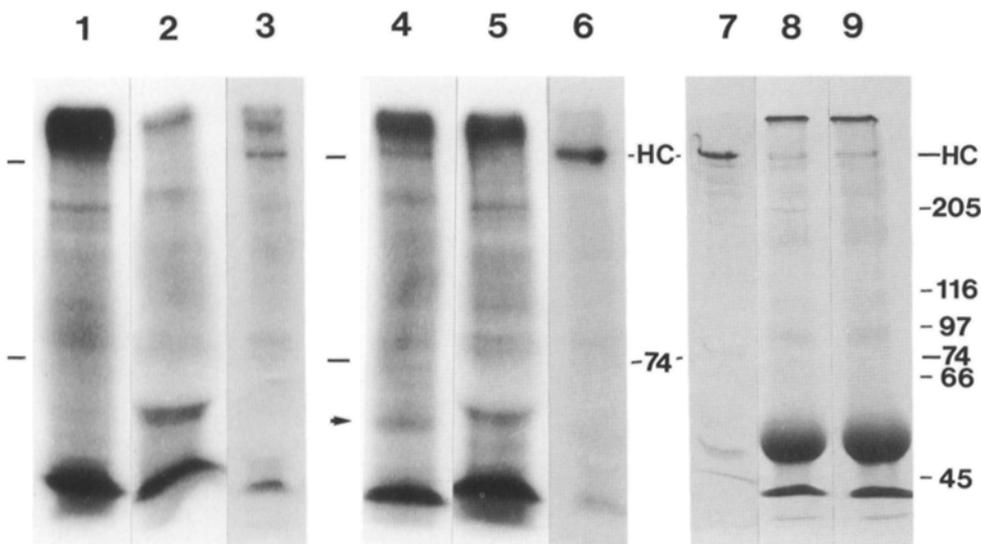
Cytoplasmic Dynein Is Phosphorylated In Vivo

The ATP requirement for the loss of lysosomal dynein staining and the promotion of this loss by okadaic acid suggested that protein phosphorylation may be involved in the changes in localization observed. To determine whether cytoplasmic dynein was phosphorylated in vivo, and whether okadaic acid exerted its effect via increased phosphorylation of dynein subunits, cytoplasmic dynein was isolated from ^{32}P-labeled NRK cells incubated in the presence and absence of okadaic acid by immunoprecipitation. As shown in Fig. 2, cytoplasmic dynein heavy chain and 74 kD intermediate chain are both phosphorylated in immunoprecipitates prepared from control cultures (lane *3*). After a 45-min incubation with 1.5 μM okadaic acid, there was a fivefold increase in ^{32}P incorporation into the dynein heavy chain (lane *6*) compared with the control. The amount of dynein in the immunoprecipitates was comparable, as shown in the Coomassie blue–stained samples (lanes *8* and *9*). There was some variability between experiments in the extent of the okadaic

acid effect, ranging from 2–5-fold increased labeling of the heavy chain relative to untreated cells, but in all cases treatment with 1.5 μM okadaic acid resulted in changes in dynein distribution examined in corresponding coverslips processed for immunofluorescence.

Immunoprecipitation of dynein appeared to be quantitative, as the high molecular mass ^{32}P-labeled band was absent from the unbound fraction (compare lanes *4* and *5*), and Western blot analysis of the unbound supernates did not reveal dynein immunoreactivity (data not shown). The average increase in phosphate incorporation in immunoprecipitated dynein after okadaic acid treatment was 4.0 ± 1.6-fold (Fig. 4). The molar stoichiometry of phosphate incorporation into dynein in control cells was estimated to be 0.2 ± 0.1 mol/mol of heavy chain, and after treatment with 1.5 μM okadaic acid was 0.7 ± 0.3 mol of phosphate/mol of heavy chain (average of five determinations). Phosphorylation of the 74-kD subunit appeared to be relatively unaffected by okadaic acid, and the extent of modification was estimated to be 0.05 ± 0.02 mol phosphate/mol 74 kD species under control and treatment conditions. As shown in the autoradiograms of cell extracts (Fig. 2, lanes *1* and *4*) cytoplasmic dynein heavy chain appears to be one of the major phosphorylated species to increase in labeling after okadaic acid treatment. The other major ^{32}P-labeled species to be affected by drug treatment is ∼55 kD (lane *4*, *arrowhead*) and is likely to be vimentin (Eriksson et al., 1992).

Increases in Dynein Phosphorylation Correlate with Changes in Immunofluorescence Staining Patterns

To establish a tighter correlation between phosphorylation of the heavy chain and changes in dynein localization observed, we examined the dose dependence of ^{32}P incorporation into dynein after treatment of NRK cells with increasing concentrations of okadaic acid (Fig. 3). Only the highest concentration tested resulted in significantly increased labeling of the heavy chain, correlating with the dose-dependent effect on dynein localization shown in Fig. 1 *A*. As indicated above,

Figure 2. Dynein is phosphorylated in vivo, and the heavy chain subunit exhibits increased labeling upon treatment of cells with okadaic acid. Dynein was immunoprecipitated from control cells or those treated for 45 min with 1.5 μM okadaic acid. Lanes *1*, *2*, and *3* are autoradiograms of cell extract, unbound supernate, and immunoprecipitate pellet from control ^{32}P-labeled cells. Lanes *4*, *5*, and *6* are equivalent samples from ^{32}P-labeled okadaic acid–treated cells. Lanes *8* and *9* are Coomassie blue–stained gels of the immunoprecipitates shown in lanes *3* and *6*, respectively, and lane *7* is a purified cytoplasmic dynein standard, also stained with Coomassie blue. The positions of dynein heavy chain (*HC*) and intermediate chain (*74*), vimentin (*arrow*), and molecular mass standards are indicated.
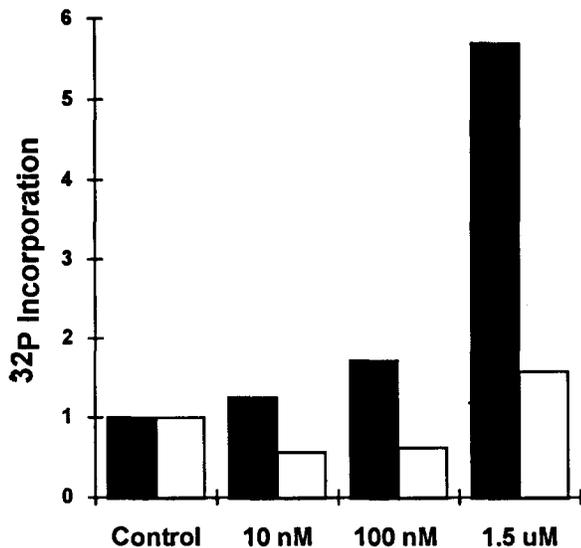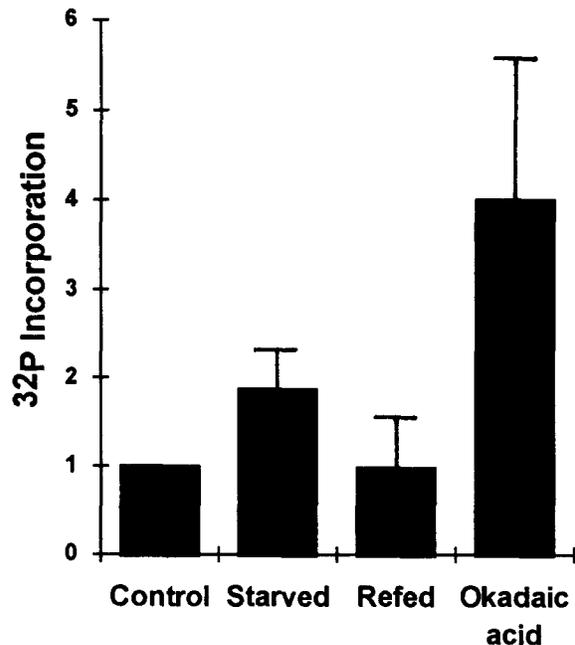
Figure 3. Incorporation of ^{32}P into dynein subunits in okadaic acid–treated cells. Quantitation of ^{32}P in dynein subunits after treatment of cells with the indicated concentrations of okadaic acid was performed as described in Materials and Methods. The values shown are the ^{32}P incorporation in each subunit relative to controls after normalizing to the cytoplasmic dynein content in each of the immunoprecipitates. (*Closed bars*) Dynein heavy chain; (*open bars*) 74-kD subunit. The data shown are from a single study, and are representative of data from three separate experiments.

no concentration of okadaic acid tested led to significant increases in the labeling of the 74-kD subunit.

Since our initial observations concerning changes in dynein immunofluorescence patterns involved brief serum starvation, we investigated the link between phosphorylation and dynein distribution under these conditions (Fig. 4). There was a reproducible 1.9-fold increase in ^{32}P incorporation into the dynein heavy chain after serum starvation. Upon return to serum-containing medium, the lysosomal dynein distribution in cells was significantly recovered by 45 min (not shown, see Lin and Collins, 1993), and the heavy chain phosphorylation level determined at this time point had decreased to control levels. As found for okadaic acid treatment, there was no effect of serum starvation on phosphorylation levels of the 74-kD intermediate chain (data not shown).

To examine the complexity of the dynein heavy chain phosphorylation pattern, and to investigate the relationship between okadaic acid and serum starvation–induced phosphorylation of dynein heavy chain, we performed phosphopeptide mapping of dynein labeled under these treatment conditions. One major phosphopeptide and several minor species were detected in the control sample (Fig. 5 *a*). The same phosphopeptides were present in the okadaic acid–treated sample (Fig. 5 *b*), but labeled to a greater extent. The proportion of ^{32}P in each of the radioactive species was determined for each treatment group. There was no obvious increase in any individual phosphopeptide relative to total incorporation after okadaic acid treatment compared with the control. The phosphopeptide maps of both serum-starved and serum-recovered samples were also comparable to those of the control and okadaic acid–treated samples (data not shown). These results suggest that okadaic acid treatment


Figure 4. Incorporation of ^{32}P into dynein heavy chain after serum starvation and recovery. Dynein immunoprecipitates were obtained from ^{32}P-labeled control and serum-starved cells, and serum-starved cells which were allowed to recover for 45 min in 1% dialyzed serum. Data are expressed as ^{32}P incorporation relative to control values, and error bars indicate SD of four experiments. For comparison, the extent and variability of labeling after treatment with 1.5 μM okadaic acid is shown.

and serum starvation lead to similar alterations in phosphorylation patterns on dynein heavy chain. These changes appear to represent proportional increases in the phosphorylation of all sites on the molecule. It is possible that the phosphopeptides in these samples do not represent unique proteolytic cleavage products. However, more extensive proteolysis of the dynein samples did not result in changes in the phosphopeptide pattern (data not shown). Upon acid hydrolysis and electrophoresis of amino acid products obtained from the major phosphopeptide (Boyle et al., 1991), the labeled species was identified as phosphoserine (data not shown).

Altered Immunofluorescent Staining Patterns Reflect a Change in the Intracellular Distribution of Cytoplasmic Dynein

To examine whether the altered staining patterns for dynein in control vs serum-starved cells reflected a redistribution of the protein from a membrane bound to a cytoplasmic pool, we employed differential detergent extraction as described for the analysis of kinesin distribution (Hollenbeck, 1989). As shown in Table II, 53% of the dynein in control cells was present in a saponin extract, with the remainder in a Triton-extractable fraction, implying a roughly equal proportion of protein in membrane bound and soluble compartments. No dynein reactivity was found in the SDS extractable fraction (the residual cytoskeleton). Upon serum starvation, the bulk of the dynein (75%) was present in the saponin extract, implying that a greater amount of the protein was soluble, or in a more easily extractable form. As in the control cells, no dynein was detected in the SDS fraction.
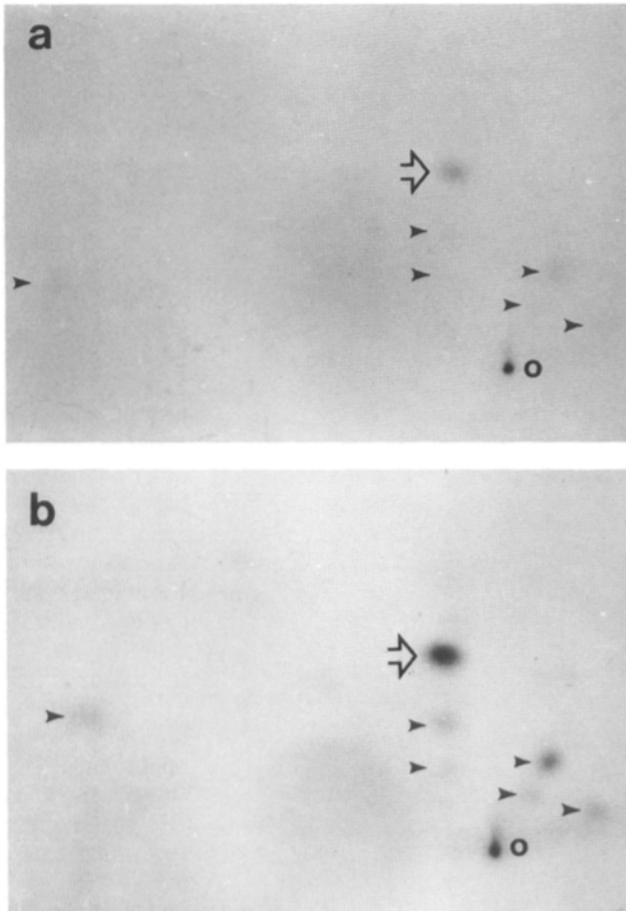
Figure 5. Phosphopeptide mapping reveals labeled peptides present in control and okadaic acid–treated samples. Phosphopeptide mapping was performed as described in Materials and Methods. Autoradiograms of samples from control (*a*) and okadaic acid–treated (*b*) cells are shown. The origin (*o*) and the positions of the major (*open arrow*) and minor (*arrowheads*) phosphopeptide species are indicated.

Table II. Quantitation of Dynein Pools after Differential Detergent Extraction of NRK Cells

Treatment	Cell fraction	Cytoplasmic dynein content
		%
Control	Saponin extract (soluble)	53 (51–56)
	Triton X-100 extract (membrane)	47 (44–49)
	SDS extract (cytoskeleton)	ND
Serum-starved	Saponin extract (soluble)	75 (72–80)
	Triton X-100 extract (membrane)	25 (20–28)
	SDS extract (cytoskeleton)	ND

Detergent fractions of NRK cells were prepared and quantitative immunoblotting performed as described in Materials and Methods. The percent of dynein in each fraction was calculated, and the means plus the range for three (serum starved) or four (control) experiments are shown rounded off to the nearest whole number.
ND, not detected.

Discussion

Our previous results and those presented here suggest that the association of cytoplasmic dynein with specific organelle binding sites is under cellular regulation, and that alterations in intracellular distribution may in part be mediated by the phosphorylation state of the dynein heavy chain. Phosphorylation changes have been shown to be involved in the regulation of several motility systems. Dispersion and aggregation of pigment granules in fish scale melanophores have been shown to be correlated with phosphorylation and dephosphorylation events (Lynch et al., 1986a,b; Rozdial and Haimo, 1986; Palazzo et al., 1989; Thaler and Haimo, 1990; Sammak et al., 1992). Kinesin is thought to be involved in the dispersion of pigment granules on the basis of antibody inhibition studies (Rodionov et al., 1991), but phosphorylation of kinesin in these cells has not been examined, and the role, if any, that cytoplasmic dynein may play in pigment aggregation remains to be determined. Kinesin has, however, been shown to be phosphorylated both in vitro (Matthies et al., 1993; Sato-Yoshitake et al., 1993) and in neuronal cells (Hollenbeck, 1993; Matthies et al., 1993). Calmodulin binding and cAMP-dependent phosphorylation in vitro were shown to affect kinesin's ATPase activity (Matthies et al., 1993). Axonemal dynein has been shown to be phosphorylated (Chilcote and Johnson, 1990; Dey and Brokaw, 1991; Hamasaki et al., 1991), and this modification has been shown to correlate with effects on ATPase activity and motility in in vitro assays. Phosphorylation of myosin has been shown to alter actomyosin mechanochemical coupling efficiency, and affects other structural and enzymatic properties of the enzyme as well (Tan et al., 1992). Phosphorylation by cdc2/H1 kinase has been implicated in many of the events that occur at the onset of mitosis, including inhibition of vesicle trafficking (Nurse, 1990). Both endocytosis and secretion are blocked, perhaps at the level of vesicle fusion, by increased phosphorylation as a result of okadaic acid treatment (Davidson et al., 1992; Woodman et al., 1992), and it has been suggested that okadaic acid can induce many of the changes in organelle trafficking and cytoskeletal organization that normally occur during mitosis (Yamashita et al., 1990; Lucocq et al., 1991; Thyberg and Moskalewski, 1992). As we showed previously, cytoplasmic dynein remains associated with vesicles during mitosis, and localizes to the mitotic apparatus in the same cells (Lin and Collins, 1992, 1993). The association of dynein with the mitotic apparatus, but not with interphase microtubules, suggests that there may be additional means of modulating the affinity and specificity toward intracellular binding sites.

Based on in vitro experiments, it has been concluded that phosphorylation of kinesin decreases binding to synaptic vesicles (Sato-Yoshitake et al., 1992). Our results provide in vivo evidence for the modulation of the organelle binding activity of cytoplasmic dynein by phosphorylation. Changes in the equilibrium between bound and free pools of motor molecules might be expected under conditions of increased or decreased organelle transport activity. Phosphorylation of cytoplasmic dynein has also been suggested as a means of regulating its activity and membrane association in the axon, where dynein associated with anterograde vesicles has relatively less phosphate in the heavy chain than dynein isolated from the whole nerve (Dillman and Pfister, 1994).

The alterations in dynein distribution and phosphorylation levels observed in the present study were induced both by serum starvation and by treatment with the phosphatase inhibitor, okadaic acid. Effects of okadaic acid on cells are considerable, ranging from alterations in the activity of many metabolic enzymes and ion transporters to rearrangement or disassembly of cytoskeletal elements (Eriksson et al., 1992; Sacher et al., 1992; Thyberg and Moskalewski, 1992). In most cases the effects of the drug have been shown to be mediated by inhibition of phosphoprotein phosphatases types 1 and 2A. The high dose of okadaic acid required in this study suggests that phosphatase type 1 may be involved in the phenomena described here (Cohen et al., 1989). Calyculin A is also a potent inhibitor of phosphatase type 1 (Cohen et al., 1990), and a similar effect of this drug would strengthen the conclusion that phosphorylation changes were important for the dynein redistribution effects seen. In our hands, calyculin A led to drastic changes in cell shape and we were unable to assess the distribution of dynein under these conditions by immunofluorescence. However, a study investigating the effects of calyculin A on phosphorylation of cytoskeletal proteins in 3T3 cells found that ^{32}P incorporation into a ~440-kD polypeptide was increased after drug treatment (Chartier et al., 1991). This polypeptide was not identified, but was found to comigrate with the α-heavy chain of axonemal dynein. It is likely that the labeled species in 3T3 cells was cytoplasmic dynein heavy chain, providing further evidence that inhibition of phosphatase activity is involved in the response found in the present study. However, decreased phosphatase activity alone will not lead to an increase in phosphorylation levels, and it is likely that the continued activity of a protein kinase is required to see this effect. We have examined the effects of inhibiting or activating cAMP- and Ca^{2+}/calmodulin-dependent kinases, as well as protein kinase C. However, we have so far been unable to identify which protein kinase might be involved in the dynein phosphorylation observed in NRK cells.

The dynein heavy chain was determined to be the major phosphorylated subunit under control and treatment conditions. Though the 74-kD dynein subunit was found to be phosphorylated in control cells, the level of labeling was quite low. We do not know whether there are phosphates with much longer turnover times on this polypeptide. However, since the extent of labeling did not change during the okadaic acid treatment nor during serum starvation, we can rule out phosphorylation changes in the 74-kD species as contributing to changes in dynein distribution. Though rough calculations of the stoichiometry of heavy chain phosphorylation indicated somewhat less than 1 mol/mol after okadaic acid treatment, the phosphopeptide maps indicate that most of the labeling occurs at one site, suggesting that phosphorylation changes at this amino acid may function as an off-on switch in terms of binding affinity. Mapping of the phosphorylation site(s) on the heavy chain should provide clues as to how modification may affect the conformation or activity of the entire dynein molecule. Data from both serum-starvation and okadaic acid experiments indicate that as little as a twofold increase in heavy chain phosphorylation correlates with observable effects on dynein localization. The increased phosphorylation levels observed with okadaic acid in some cases (up to fivefold over controls) did not seem to be as sociated with either more drastic loss of punctate dynein staining or changes in the time course of these effects.

Cell fractionation by differential detergent extraction revealed that a greater amount of dynein was present in an easily extracted (soluble) pool after serum starvation. In detergent extracted control cells, approximately half of the dynein was soluble, with the remainder in the Triton extractable membrane-bound pool. Using this method Hollenbeck (1989) found that 68% of kinesin in chick epidermal fibroblasts was in soluble form, with the remainder associated with membranous organelles. In both studies, the quantitation of dynein (or kinesin) in these cell fractions is consistent with the apparent localization of the motor molecule as detected by immunofluorescence microscopy. It is possible that the antibody used in our studies does not recognize the total population of cytoplasmic dynein, or that epitope masking prevents the immunolocalization of modified dynein in serum-starved or okadaic acid–treated cells. However, the alterations in distribution of immunoreactive dynein found by immunoprecipitation after differential extraction, and by immunofluorescence microscopy, suggest that at least a subset of cellular dynein is subject to regulation of its intracellular localization under these treatment conditions.

Though there is a correlation between dynein distribution and phosphorylation changes, this does not prove that there is a direct effect of heavy chain phosphorylation on organelle binding affinity. The effects of other phosphorylated proteins, such as potential membrane receptor molecules, must also be considered. For example, the putative endoplasmic reticulum receptor for kinesin, kinectin (Toyoshima et al., 1992), has been shown to be phosphorylated in vivo (Hollenbeck, 1993), and changes in affinity for binding sites may result from a combination of effects on the motor molecule, its organelle receptor, and changes in the availability of other intracellular binding sites. Increased phosphorylation of dynein could be secondary to its redistribution induced by other mechanisms. Regardless of mechanism, alterations in the size and location of intracellular pools of cytoplasmic dynein could provide one means of regulation of retrograde motility. We don't as yet have direct evidence for alterations in retrograde transport activity due to changes in dynein distribution, but we can speculate on a model suggested by several experimental results. Our previous results (Lin and Collins, 1992, 1993) indicated that the dynein-lysosome association was stable when lysosomes were dispersed peripherally in the cytoplasm, under conditions where retrograde traffic was inhibited. We also found that dynein remained associated with lysosomes in mitotic cells, when organelle traffic is blocked. It is possible that the lysosomal dynein presents a stably bound, inactive pool of cytoplasmic dynein, which remains associated under most circumstances with these organelles at the end of the retrograde pathway, that is, accumulated in the perinuclear region of the cell. On the basis of results from the present study, we suggest that dephosphorylation leads to stable binding, the situation for much of the dynein in the cells under our control conditions. This form of regulation may hold true for dynein associated with anterograde vesicles in the axon (see above) and for mitotic organelles as well. Organelle motility in mitotic extracts has been shown to be decreased compared with that of interphase extracts, though microtubule gliding activity

was comparable (Allan and Vale, 1991). As was suggested in this report, the motor molecules associated with mitotic organelles may be inactive. In another study, addition of okadaic acid was found to increase the frequency and velocity of vesicle movements in CV-1 cells (Hamm-Alvarez et al., 1993). If phosphorylation were required in order to release bound dynein (or kinesin) from the organelle membrane into an active "soluble" pool, changes in binding affinity alone could allow for regulation of motility. However, it is also likely that phosphorylation may play a role in the regulation of other aspects of cytoplasmic dynein function, such as ATPase activity and mechanochemical coupling efficiency, and appropriate modification of dynein using in vitro phosphorylation and activity assays will be required to test these hypotheses. We have been able to use our in vivo assay to examine effects of dynein modification in terms of organelle binding, and it will be interesting to examine further the extent to which changes in dynein phosphorylation level and intracellular distribution are involved in the regulation of cellular activities suspected to rely on this retrograde microtubule motor.

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