Dynein, Dynactin, and Kinesin II's Interaction with Microtubules Is Regulated during Bidirectional Organelle Transport

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Abstract. The microtubule motors, cytoplasmic dynein and kinesin II, drive pigmented organelles in opposite directions in *Xenopus* melanophores, but the mechanism by which these or other motors are regulated to control the direction of organelle transport has not been previously elucidated. We find that cytoplasmic dynein, dynactin, and kinesin II remain on pigment granules during aggregation and dispersion in melanophores, indicating that control of direction is not mediated by a cyclic association of motors with these organelles. However, the ability of dynein, dynactin, and kinesin II to bind to microtubules varies as a function of the state of aggregation or dispersion of the pigment in the cells from which these molecules are isolated. Dynein and dynactin bind to microtubules when obtained from cells with aggregated pigment, whereas kinesin II binds to microtubules when obtained from cells with dispersed pigment. Moreover, the microtubule binding activity of these motors/dynactin can be reversed in vitro by the kinases and phosphatase that regulate the direction of pigment granule transport in vivo. These findings suggest that phosphorylation controls the direction of pigment granule transport by altering the ability of dynein, dynactin, and kinesin II to interact with microtubules.

Key words: dynein • kinesin • melanophores • microtubules • phosphorylation

Introduction

The mechanism by which the direction of organelle transport along microtubules is regulated is not understood although the microtubule motor families, kinesin and dynein, responsible for transporting organelles in opposite directions have been extensively studied. Two major models have been proposed to explain how direction of transport could be controlled. Motors might cyclically bind to and dissociate from organelles, thereby dictating, by their presence or absence, the direction of organelle transport. Alternatively, motors might remain on organelles during bidirectional transport, but their activity, i.e, their ability to generate force along a microtubule, may be enhanced or inhibited to regulate the direction of transport (Sheetz et al., 1989; Hirokawa et al., 1990; Hirokawa, 1996). Strong support for one or the other of these models is lacking.

Evidence that motor–organelle interactions may be regulated is provided by various studies. Vesicles that contain dynein but not kinesin move towards the minus ends of microtubules, whereas those that contain both kinesin and dynein move towards the plus ends of microtubules (Hirokawa et al., 1990, 1991; Dahlstrom et al., 1991; Muresan et al., 1996), suggesting that vesicles acquire and lose kinesin to reverse direction. Changes in phosphorylation can affect kinesin's (Sato-Yoshitake et al. 1992; Lee and Hollenbeck, 1995; Okada et al., 1995; Marlowe et al., 1998) or cytoplasmic dynein's (Lin et al., 1994; Niclas et al., 1996) association with membranes. These data suggest that motor–organelle interactions can be controlled, potentially by phosphorylation, but do not reveal if cells utilize control of motor binding to organelles to change the direction in which they transport their organelles.

Other studies indicate that motors remain on organelles during bidirectional transport and that the activity of the motors may be regulated, instead. Immunofluorescent studies suggest that intermediate compartment vesicles possess kinesin regardless of whether they are moving towards the Golgi apparatus or towards the ER (Lippincott-Schwartz et al., 1995), and that cytoplasmic dynein and kinesin remain associated with pigment granules during aggregation and dispersion in fish melanophores (Nilsson et al., 1996). ER-derived vesicles from *Xenopus* eggs possess kinesin, but only move towards the plus ends of microtubules after the vesicles have been exposed to somatic cell extracts, suggesting that the kinesin on the vesicles must be activated to be functional (Lane and Allan, 1999). Changes in phosphorylation can alter kinesin's (Matthies et al., 1993; McIlvain et al., 1994; Lindesmith et al., 1997; De Vos et al., 2000) or dynein's (Hamasaki et al., 1991;

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Dillman and Pfister, 1994; Howard et al., 1994; Allan, 1995; Habermacher and Sale, 1997; Runnegar et al., 1999; Kumar et al., 2000) activity and affect organelle distribution (De Vos et al., 2000). Thus, changes in motor activity may alter the direction of organelle transport.

Here, we sought to determine if the direction of organelle transport is controlled by regulating motor–organelle or motor–microtubule interactions. We used melanophores as a model system as these cells provide several distinct advantages for investigating the control of the direction of organelle transport. A single population of organelles, pigment granules or melanosomes, is coordinately dispersed or aggregated in response to appropriate external stimuli transduced as phosphorylation or dephosphorylation events, respectively (Rozdzial and Haimo, 1986; Sugden and Rowe, 1992; McClintock et al., 1996; Reilein et al., 1998). Kinesin II and cytoplasmic dynein are present on *Xenopus* pigment granules (Rogers et al., 1997), and kinesin II drives their dispersion (Tuma et al., 1998), whereas cytoplasmic dynein is implicated in their aggregation (Nilsson and Wallin, 1997). We report here that cytoplasmic dynein, dynactin, and kinesin II remain on pigment granules during aggregation and dispersion in *Xenopus* melanophores, but exhibit differential microtubule binding activity that can be reversed by the kinases and phosphatase that control the direction of transport in vivo. These findings suggest that the microtubule binding activity of the motors/dynactin is a primary target of regulation in which phosphorylation differentially alters the ability of dynein, dynactin, and kinesin II to interact with microtubules and thereby changes the direction of organelle transport.

Materials and Methods

Cells, Reagents, and Solutions

The immortalized *Xenopus* melanophore cell line (Potenza and Lerner, 1991) used here was a gift from Dr. Michael Lerner (University of Texas, Southwestern Medical Center at Dallas, Dallas, Texas). Cells were washed in 0.7× PBS (100 mM NaCl, 2.69 mM KCl, 3.78 mM Na_2HPO_4, 14 mM NaH_2PO_4, and 1.26 mM KH_2PO_4) before lysis. Lysis buffer consisted of BRB40 (40 mM Pipes, 1 mM EGTA, 1.0 mM $MgCl_2$, pH 6.9) supplemented with 2 mM DTT and a protease inhibitor cocktail containing leupeptin, aprotinin, 4-(2-aminoethyl) benzenesulfonyl flouride (AEBSF), *N*α-p-tosyl-L-arginine methyl ester (TAME), benzamidine, and soybean trypsin inhibitor (SBTI), all at a final concentration of 10 μg/ml. The catalytic subunit of protein kinase (PK)[1] A (bovine heart), the catalytic subunit of PKC (rat brain), protein phosphatase (PP)$2A_1$, and the recombinant catalytic subunit of PP1 (rabbit muscle) were purchased from CalBiochem and diluted into lysis buffer immediately before use. PP2B was purchased from Sigma-Aldrich, rehydrated with lysis buffer, and used immediately. Antibodies used for immunoprecipitation and/or immunoblotting were 70.1, an mAb against the intermediate chain of dynein (Sigma-Aldrich), 74.1, an mAb against the intermediate chain of dynein (a gift from Dr. K. Pfister, University of Virginia, Charlottesville, VA or purchased from Chemicon), p150, an mAb against the 150-kD ($p150^{glued}$) subunit of dynactin (Transduction Laboratories), K2.4, an mAb recognizing the 85-kD subunit of kinesin II (a gift from Dr. V. Gelfand, University of Illinois, Urbana, IL or purchased from BabCo), HD, a pAb recognizing the motor domain of kinesin (a gift from Dr. F. Gyoeva, Institute for Protein Research, Moscow, Russia; Rodionov et al., 1991), and DM1A, an mAb against the α-subunit of tubulin (Sigma-Aldrich). HRP-conjugated secondary antibodies against mouse and rabbit IgGs were purchased from Amersham Pharmacia Biotech or The Binding Site. Proteins were separated on 8.5% polyacrylamide gels and blotted onto NitroBind (Micron Separations Inc.). Enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) were used to visualize the blots.

[1]*Abbreviations used in this paper:* AMP-PNP, adenylyl imidodiphosphate; MSH, melanocyte stimulating hormone; PK, protein kinase; PP, protein phosphatase.

Cell Culture

Xenopus melanophores were cultured at 25°C in L-15 medium (Sigma-Aldrich) containing 15% heat-inactivated FBS (GIBCO BRL), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Subconfluent cultures, grown in 15-cm plates, were transferred to serum-free medium overnight. To induce aggregation and dispersion of pigment granules, cells in serum-free medium were incubated in the dark in 10 μM melatonin or in 100 nM α-melanocyte stimulating hormone (MSH), respectively, for 1 h.

Pigment Granule Isolation from Melanophores

Melanophores with aggregated and dispersed pigment granules were washed twice with 0.7× PBS containing melatonin or MSH, respectively, then scraped with a rubber policeman into 1 ml (per 15-cm plate) cold lysis buffer containing melatonin or MSH and lysed by passage through a 25-gauge needle. Pigment granules were purified as described (Rogers et al., 1998) by centrifugation through 80% Percoll (Sigma-Aldrich) in BRB40. The pellet of purified pigment granules was either prepared for immunoblotting or resuspended in 100 μl cold lysis buffer supplemented with 1% Triton X-100 and extruded 10 times through a 25-gauge needle to shear the membrane and solubilize pigment granule–associated motors. Only a small fraction of the organelle motors could be extracted, possibly because melanin is sticky. Once the pigment granule membrane is solubilized, the motors and other proteins may bind avidly to the pigment. The preparation was then centrifuged at 100,000 *g* for 20 min at 4°C in a 70.1 Ti rotor (Beckman) and the supernatant fraction (the pigment granule extract) was treated with 2.0 U/ml apyrase for 15 min at 4°C before using in microtubule cosedimentation assays.

Cell Lysates

Cells undergoing aggregation or dispersion, or cells with aggregated or dispersed pigment granules, were washed, scraped up with a rubber policeman into lysis buffer, and lysed, as described above. The lysates were then centrifuged at 100,000 *g* for 20 min at 4°C in a 70.1 Ti rotor (Beckman) to obtain clarified lysates. Bradford assays were performed so that the protein concentration of aggregated and dispersed clarified lysates could be equalized, although normally the concentrations were equal and no adjustments were necessary. The clarified lysates were incubated for 15 min on ice in the presence of 2.0 U/ml apyrase (Sigma-Aldrich) to deplete endogenous ATP, and used in either microtubule cosedimentation assays or in microtubule capture assays, which are described below. The protein kinase or phosphatase inhibitors, staurosporine or calyculin A, respectively, were sometimes included during cell lysis, but their presence or absence did not alter the outcome of any of the studies reported here. However, if the clarified lysates were warmed to 30°C, then calyculin A was required to maintain the normal behavior of dynein and kinesin II, but not dynactin, in dispersed lysates.

Purification and Assembly of Bovine Brain Tubulin

Tubulin was isolated from bovine brain by two cycles of microtubule polymerization and depolymerization. Tubulin was then purified from microtubule-associated proteins by ion exchange chromatography on a DEAE-Sephacel column (Sigma-Aldrich; modified from Vallee, 1986). Purified tubulin in 50 mM Pipes, 1 mM EGTA, 0.5 mM $MgSO_4$, 0.1 mM GTP, pH 6.9, was assembled into microtubules by incubation at 37°C in the presence of 20 μM taxol. The resulting microtubules were used in microtubule cosedimentation and microtubule capture studies.

Microtubule Cosedimentation Assays

To examine motor–microtubule binding, the pigment granule extracts or the clarified cell lysates were incubated with microtubules and subjected to sedimentation. A final concentration of 2 mg/ml taxol-stabilized microtubules, 20 μM taxol, and 1 mM adenylyl imidodiphosphate (AMP-PNP) was added to the pigment granule extracts or clarified cell lysates, and the preparations were incubated on ice for 30 min to allow motor–microtubule binding to occur. The mixture was then sedimented through a cushion of 30% glycerol in lysis buffer containing 5 μM taxol and 0.5 mM

AMP-PNP at 100,000 *g* for 45 min at 4°C in an SW 50.1 rotor (Beckman). After centrifugation, the supernatant fraction was collected, and the surface of the glycerol cushion was washed two times with water. The glycerol cushion was then removed, and the microtubule pellet (normally not visible) was resuspended and prepared for gel electrophoresis.

Microtubule Capture Assays

Dynein, dynactin, and kinesin II were immunoprecipitated from aggregated and dispersed clarified lysates with the appropriate antibody immobilized on protein A–Sepharose 4B beads (Sigma-Aldrich). 20 μl of packed protein A–Sepharose 4B beads in 1 ml cold BRB40 was incubated with either 5 μg of 74.1 antibodies, 1.25 μg of p150 antibodies, or 10 μg of K2.4 antibodies for 1 h at 4°C. The beads with bound antibody were washed three times with BRB40 and then incubated in 1 ml of clarified lysate from aggregated or dispersed melanophores. After a 30-min incubation at 4°C, the beads were collected and washed three times with 1 ml cold lysis buffer containing 0.25% gelatin (Bio-Rad Laboratories). In some studies, the immunoprecipitants were then incubated for 30 min at 30°C in either 500 U of the catalytic subunit of PKA and 1 mM ATP; 117 mU of the catalytic subunit of PKC and 1 mM ATP; 50 U of PP2B and 0.2 μM free Ca^{2+} and 0.5 μM calmodulin; 610 mU of PP2A; or 6.66 U of the recombinant catalytic subunit of PP1 in 1 ml BRB40 and again washed. Each immunoprecipitant was resuspended in 1 ml cold BRB40 and incubated with a final concentration of 0.2 mg/ml taxol-stabilized microtubules, 20 μM taxol, and 1 mM AMP-PNP for 30 min at 4°C. The beads were allowed to settle by gravity for 15 min, washed three times with 1 ml cold BRB40, washed once with 1 ml cold BRB40 containing 0.5 M NaCl and 0.05% Tween 20, washed again in 1 ml cold BRB40, and prepared for gel electrophoresis and immunoblotting. In studies to determine if microtubule capture by the motors/dynactin was ATP sensitive, AMP-PNP was omitted during the microtubule capture step and ATP, at a final concentration of 1 mM, was added to each immunoprecipitant–microtubule complex after capture. The beads were incubated for an additional 30 min at 4°C and then washed as described above.

Microscopy and Image Acquisition

Micrographs of melanophores aggregating and dispersing pigment were obtained using bright field optics and acquired by Image 1 (Universal Imaging) using a Dage Newvicon camera. Immunoblots were digitally scanned. All figures were prepared using Adobe Photoshop® software.

Results

Microtubule Motors and Dynactin Remain Associated with Pigment Granules during Bidirectional Transport

To determine if the direction of pigment granule transport in *Xenopus* melanophores is regulated by the cyclic association of microtubule motors with pigment granules, we isolated these organelles from cells that had either aggregated or dispersed pigment. Previous studies have demonstrated that cytoplasmic dynein and kinesin II are present on *Xenopus* pigment granules (Rogers et al., 1997). Here, we find these motors on the organelles, regardless of whether they have undergone aggregation or dispersion (Fig. 1). In addition, dynactin is also present and at equivalent amounts on granules isolated from melanophores with aggregated or dispersed pigment (Fig. 1). These findings suggest that net attachment or detachment of motors/dynactin to these organelles does not occur as a function of aggregation and dispersion and is not the mechanism regulating the direction of pigment granule transport.

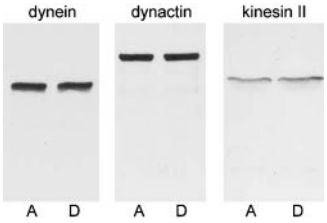

Figure 1. Dynein, dynactin, and kinesin II remain on pigment granules during bidirectional transport. Immunoblot analysis of purified pigment granules isolated from equal numbers of aggregated (A) or dispersed (D) melanophores and probed with antibodies to dynein (74.1 antibody against dynein intermediate chain), dynactin (p150 antibody against the $p150^{glued}$ subunit), and kinesin II (K2.4 antibody against the 85-kD subunit).

Dynein, Dynactin, and Kinesin II on Pigment Granules Only Bind Microtubules Following One Direction of Transport

To determine if the direction of pigment granule transport in melanophores is regulated by controlling the activity of the microtubule motors, we examined the microtubule binding behavior of motors and dynactin extracted from pigment granules that had been transported in opposite directions. Pigment granules were isolated from melanophores that had either aggregated or dispersed pigment, and the motors and dynactin were extracted from these organelles by shearing them in 1% Triton X-100. The extracts were incubated with microtubules under conditions that promote motor–microtubule binding, and the microtubules were collected by centrifugation through a glycerol cushion. Similar amounts of dynein were extracted from pigment granules obtained from melanophores with aggregated or dispersed pigment (Fig. 2 A, a, lanes 1 and 2), but only that extracted from the aggregated but not from the dispersed pigment granules is capable of binding to microtubules (Fig. 2 A, a, lanes 3 and 4). Dynactin, which contains a microtubule binding site (Waterman-Storer et al., 1995), also only binds to microtubules when it is extracted from aggregated, but not from dispersed, pigment granules (Fig. 2 A, b, lanes 3 and 4). Kinesin II is at too low a concentration to be detected in the extracts of the pigment granules. Nevertheless, if present in equal amounts, then kinesin II preferentially cosediments with microtubules when extracted from dispersed, but not aggregated, pigment granules (Fig. 2 A, c, lanes 3 and 4). Simultaneous blotting of microtubule pellets for both dynactin and kinesin II, to ensure that gel loading anomalies do not account for the observed differences, further demonstrates that dynactin cosediments with microtubules when extracted from aggregated pigment granules whereas kinesin II exhibits the opposite behavior (Fig. 2 A, d). These findings suggest that pigment granule–associated dynein, dynactin, and kinesin II preferentially interact with microtubules when obtained from cells that had transported pigment in the direction in which each is used in vivo.

The Soluble Pool of Dynein, Dynactin, and Kinesin II Is Also Regulated during Bidirectional Transport

To determine if dynein, dynactin, and kinesin II on the pigment granules are specifically targeted for regulation, we assessed the ability of the soluble pool of these proteins to bind to microtubules as a function of the direction of pigment granule transport. Melanophores with aggregated or dispersed pigment were lysed, and a high speed super-

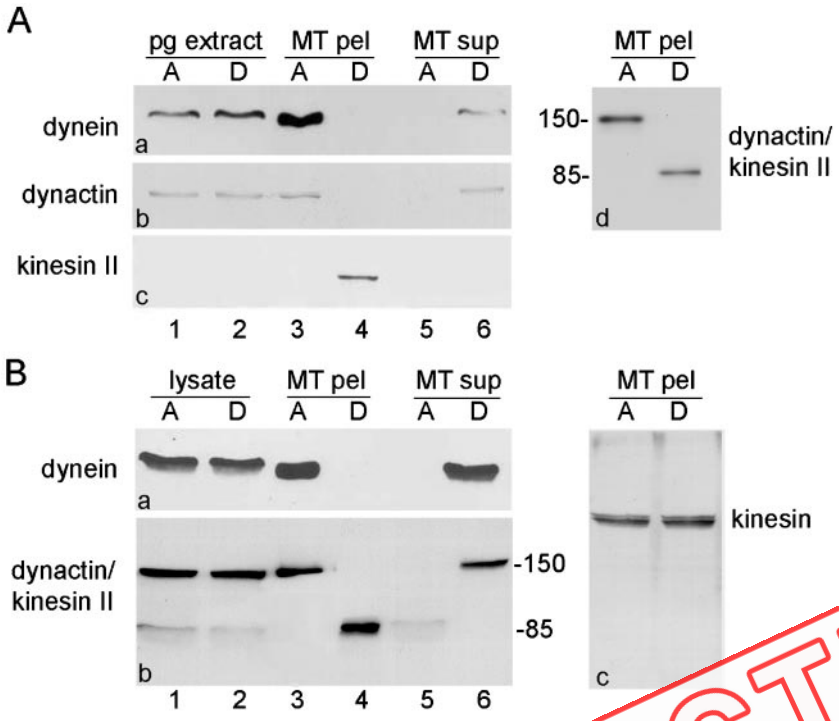

Figure 2. Dynein, dynactin and kinesin II display differential microtubule cosedimentation behavior that varies with the direction of pigment transport. (A) Pigment granule–associated motors/dynactin differentially bind microtubules. Triton X-100 extracts of pigment granules (pg extract, lanes 1 and 2) isolated from aggregated (A) and dispersed (D) melanophores, and the microtubule pellets (MT pel, lanes 3 and 4) and supernatants (MT sup, lanes 5 and 6) obtained after cosedimentation of microtubules with the extracts were immunoblotted for (a) dynein, (b) dynactin, and (c) kinesin II. Microtubule pellets were simultaneously blotted for dynactin and kinesin II (d). (B) The soluble pool of dynein, dynactin, and kinesin II differentially binds microtubules. Clarified lysates (lanes 1 and 2) from aggregated and dispersed melanophores, and the microtubule pellets (MT pel, lanes 3 and 4) and supernatants (MT sup, lanes 5 and 6) obtained after cosedimentation of microtubules with the lysates were probed for dynein (a) or simultaneously for dynactin and kinesin II (b). Microtubule pellets were also probed for conventional kinesin (c). 150, $p150^{glued}$ subunit of dynactin; 85, 85-kD subunit of kinesin II. Dynein, dynactin, and kinesin II exhibit differential microtubule binding as a function of the direction of pigment granule transport whereas conventional kinesin does not.

natant fraction containing the soluble proteins was prepared and mixed with microtubules, which were then collected by centrifugation, as described above, and blotted for dynein (Fig. 2 B, a), or simultaneously for dynactin and kinesin II (Fig. 2 B, b). More dynein and dynactin in an aggregated cell lysate cosediment with microtubules than in a dispersed cell lysate (Fig. 2 B, a and b, lanes 3 and 4). Conversely, more kinesin II cosediments with microtubules when this motor is derived from a dispersed cell lysate than from an aggregated cell lysate (Fig. 2 B, b, lanes 3 and 4). Conventional kinesin is present in *Xenopus* melanophores but is not on pigment granules and does not drive their transport (Rogers et al., 1997; Tuma et al., 1998), and this kinesin exhibits no differential binding to microtubules as a function of the direction of organelle transport (Fig. 2 B, c). These findings indicate that those motors involved in driving pigment transport are specifically targeted by the regulatory system in melanophores. Furthermore, the entire pool of these motors, not just those on the pigment granules, is regulated. Accordingly,

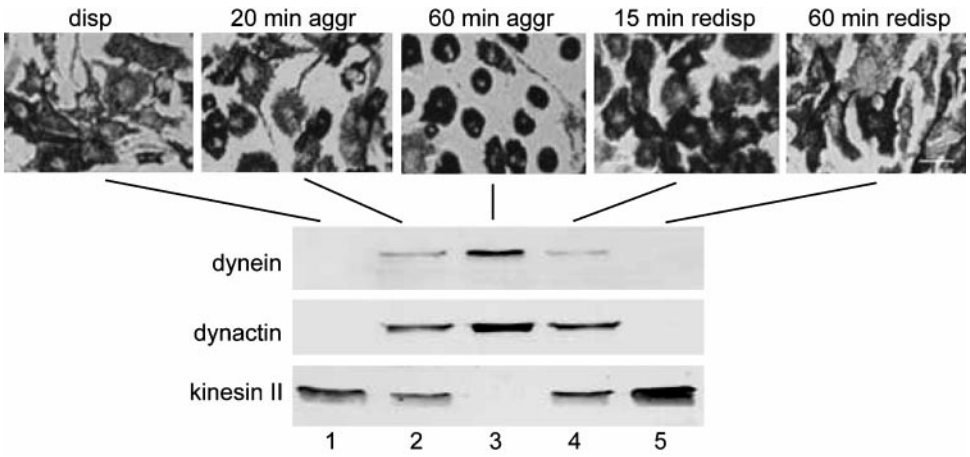

Figure 3. Dynein, dynactin, and kinesin II's microtubule binding activity rises and falls during aggregation and dispersion. Light micrographs of dispersed (disp) melanophores undergoing aggregation (aggr) and then redispersion (redisp) of pigment for the times indicated. The corresponding immunoblots, resulting from cosedimentation of microtubules with soluble motors/dynactin isolated from melanophores at the time points indicated, have been probed for dynein, dynactin, and kinesin II.The pool of active dynein and dynactin increases during aggregation and decreases during dispersion, whereas kinesin II displays the opposite behavior. Bar, 50 μm.

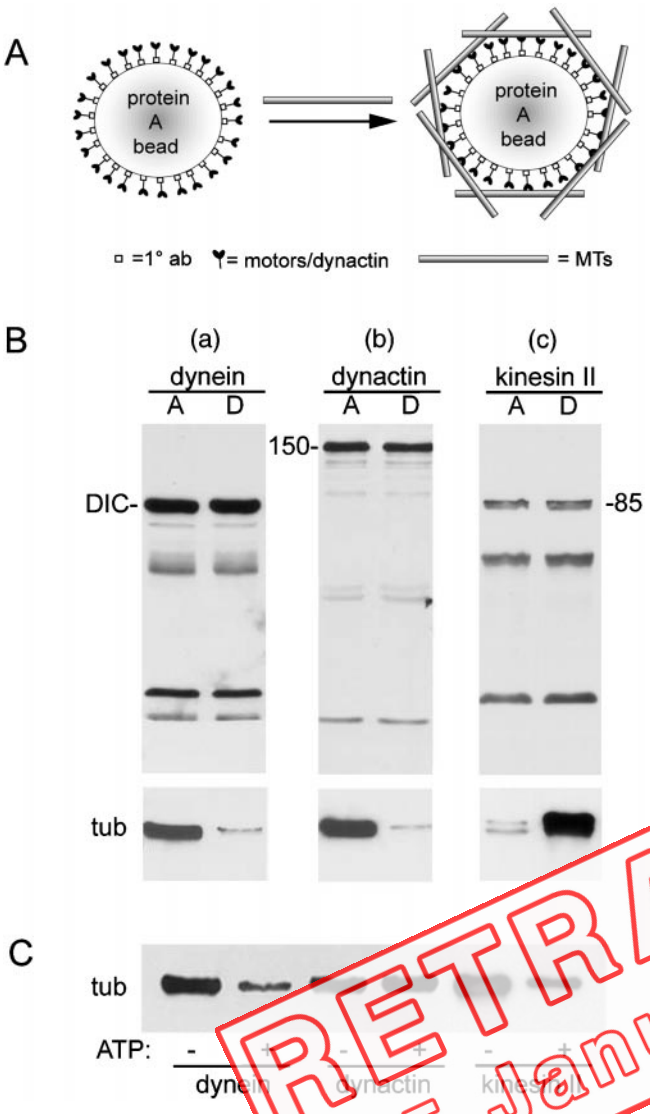

Figure 4. Microtubule capture by immobilized dynein, dynactin, and kinesin II varies as a function of the direction of pigment transport. (A) Model for microtubule capture assay. Protein A beads with bound antibodies (primary antibodies [1° ab] 74.1, p150, or K2.4) are incubated with aggregated or dispersed *Xenopus* melanophore lysates to immunoprecipitate the appropriate motor/dynactin. Motor/dynactin antibody–bead complexes (left) are then challenged to bind microtubules (MTs). Microtubules are captured by active dynein, dynactin, or kinesin II immobilized on the beads (right). (B) Dynein (a), dynactin (b), and kinesin II (c) from aggregated (A) and dispersed (D) melanophore lysates were immunoprecipitated, incubated with taxol-stabilized microtubules, and probed either for motors/dynactin (top) or for tubulin (bottom). Heavy and light chains of the immunoprecipitating antibodies are also detected in the top panels. DIC, intermediate chain of cytoplasmic dynein; 150, $p150^{glued}$ subunit of dynactin; 85, 85-kD motor subunit of kinesin II; tub, tubulin. Dynein and dynactin from aggregated cells and kinesin II from dispersed cells capture microtubules. (C) Microtubule capture by motors is ATP sensitive. Dynein and dynactin immunoprecipitated from aggregated melanophore extracts and kinesin II immunoprecipitated from dispersed extracts were incubated with taxol-stabilized microtubules, washed, and incubated in the presence (+) or absence (−) of 1 mM ATP. Immunoblotting for tubulin reveals that microtubules dissociate from dynein and kinesin II, but not from dynactin, in the presence of ATP.

because it is regulated in concert with the pigment granule–associated motors/dynactin and is much more abundant, we use the soluble pool as the source of dynein, dynactin, and kinesin II in all subsequent studies. We refer to dynein, dynactin, or kinesin II that is competent to bind microtubules as active and that which is incompetent to bind microtubules as inactive.

The Pool of Active Motor/Dynactin Rises and Falls during Bidirectional Transport

Pigment granules saltate during both directions of transport in *Xenopus* melanophores, and isolated pigment granules show biased rather than unidirectional movements on microtubules (Rogers et al., 1997). These observations suggest that dynein and kinesin II might be active during both directions of movement. To determine if there is opposing motor activity during aggregation or dispersion, we examined the microtubule binding behavior of dynein, dynactin, and kinesin II obtained from melanophores in the process of aggregating and dispersing pigment. Cells with dispersed pigment were induced to undergo pigment aggregation and redispersion (Fig. 3). At various times during transport, cells were lysed, and the motors/dynactin were examined for their microtubule binding activity. During aggregation, the pool of active dynein and dynactin increases, whereas that of kinesin II decreases (Fig. 3, compare lane 2 with lane 1). In cells with fully aggregated pigment, the size of the active pool of dynein and dynactin is at a maximum whereas no active kinesin II is detected (Fig. 3, lane 3). During redispersion of pigment, the pool of active dynein and dynactin falls as that of kinesin II rises (Fig. 3, lane 4) until, in fully redispersed cells, the size of the active pool of kinesin II is at a maximum whereas no active dynein or dynactin is detected (Fig. 3, lane 5). These observations suggest that some opposing motor is active during each direction of transport, a finding that may explain why pigment granules saltate during aggregation and dispersion. Therefore, change in the net direction of pigment granule transport likely occurs when the ratio of active dynein to active kinesin II reaches some critical threshold.

Immunoprecipitated Motors/Dynactin Differentially Capture Microtubules

The differential binding to microtubules is either an inherent property of the motors/dynactin or is due to some other factor in the pigment granule extract or the cell lysate. Therefore, we sought to determine if purified motors/dynactin retain their differential microtubule binding behavior. To conduct these studies, we developed a "microtubule capture" assay. Dynein, dynactin, or kinesin II was immunoprecipitated to purify it from melanophore lysates, and the immobilized motors/dynactin on protein A–antibody beads were used to capture microtubules (Fig. 4 A). Equal amounts of dynein, dynactin, or kinesin II were immunoprecipitated from aggregated and dispersed cells (Fig. 4 B, top), but the immunoprecipitated dynein or dynactin from aggregated melanophores captures considerably more microtubules than does dynein or dynactin from dispersed cells (Fig. 4 B, bottom, a and b). Conversely, the immunoprecipitated kinesin II from dispersed

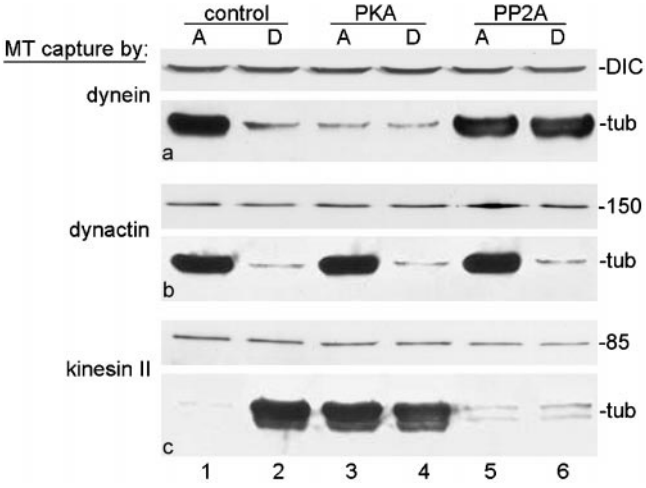

Figure 5. Microtubule capture behavior by dynein and kinesin II is reversed by PKA and PP2A. Dynein (a), dynactin (b), and kinesin II (c) were immunoprecipitated from aggregated (A) and dispersed (D) melanophore lysates and then incubated with either ATP alone (control, lanes 1 and 2), with ATP and the catalytic subunit of PKA (lanes 3 and 4), or with PP2A (lanes 5 and 6). Immunoprecipitants were subsequently washed free of ATP and kinase or phosphatase, incubated with taxol-stabilized microtubules, and then assessed for the presence of bound microtubules by immunoblotting for tubulin. The preparations were also blotted for dynein, dynactin, or kinesin II. DIC, dynein intermediate chain; 150, $p150^{glued}$ subunit of dynactin; 85, 85-kD subunit of kinesin II; tub, tubulin. PKA inhibits microtubule (MT) capture by dynein and enhances microtubule capture by kinesin II from aggregated cells, whereas PP2A enhances microtubule capture by dynein and inhibits microtubule capture by kinesin II from dispersed cells. The ability of dynactin to capture microtubules is unaffected by PKA or PP2A treatment. Note that tubulin captured by kinesin II appears as a doublet (c; and see also in Figs. 4 and 6), suggesting that tubulin may be modified by the kinesin II immunoprecipitate.

cells captures considerably more microtubules than that from aggregated cells (Fig. 4 B, bottom, c). Upon ATP addition, captured microtubules are released from dynein and kinesin II, but not from dynactin, which is not an ATPase (Fig. 4 C). These findings indicate that the microtubule capture assay measures a functional interaction between motor and microtubule. Thus, because immunoprecipitated motors/dynactin exhibit the same differential microtubule binding behavior as they do in a pigment granule extract or cell lysate, the motors/dynactin, themselves, or associated coimmunoprecipitating proteins, are likely modified during aggregation and dispersion to regulate their interaction with microtubules.

PKA Inhibits Dynein–Microtubule Interactions and Activates Kinesin II–Microtubule Interactions

PKA induces complete dispersion in *Xenopus* melanophores (Reilein et al., 1998). To determine if this kinase can alter motor/dynactin–microtubule interactions, we treated the immunoprecipitated motors or dynactin with PKA before addition of microtubules. PKA-treated dynein from aggregated cells exhibits a significantly reduced ability to capture microtubules, compared with untreated dynein from aggregated cells (Fig. 5 a, compare tubulin in lane 3 with lane 1). In contrast, PKA-treated kinesin II from aggregated cells exhibits significantly enhanced ability to capture microtubules compared with untreated kinesin II from aggregated cells (Fig. 5 c, compare tubulin in lane 3 with lane 1). In both cases, the amount of microtubules captured by the PKA-treated motors from aggregated cells is equivalent to that captured by untreated motors from dispersed cells (Fig. 5, a and c, compare tubulin in lane 3 with lane 2). The behavior of dynein and kinesin II from dispersed cells is unaltered by PKA treatment (Fig. 5, a and c, compare tubulin in lane 4 with lane 2), as expected if these motors have already been affected by PKA during dispersion in vivo. Unlike dynein and kinesin II, dynactin's ability to capture microtubules is unchanged by treatment with PKA (Fig. 5 b, compare tubulin in lanes 3 and 4 to lane 1 with lane 2). These findings suggest that phosphorylation mediated by PKA induces dispersion in vivo by inactivating dynein– and activating kinesin II–microtubule interactions so that plus end–directed microtubule transport is favored.

PP2A Activates Dynein–Microtubule Interactions and Inhibits Kinesin II–Microtubule Interactions

Aggregation of pigment granules in *Xenopus* melanophores requires the participation of PP2A (Reilein et al., 1998). To determine if this phosphatase alters the microtubule binding behavior of motors/dynactin, we incubated the immunoprecipitated proteins with PP2A before microtubule addition. PP2A treatment increases the amount of microtubules captured by dynein from dispersed cells (Fig. 5 a, compare tubulin in lane 6 with lane 2) and diminishes the amount of microtubules captured by kinesin II from dispersed cells (Fig. 5 c, compare tubulin in lane 6 with lane 2). In both cases, the amount of microtubules captured by the PP2A-treated motors from dispersed cells is equivalent to that captured by untreated motors from aggregated cells (Fig. 5, a and c, compare tubulin in lane 6 with lane 1). The microtubule binding behavior of dynein and kinesin II immunoprecipitated from aggregated cells is unaffected by PP2A treatment (Fig. 5, a and c, compare tubulin in lane 5 with lane 1), as expected if the behavior of these motors has already been altered by PP2A during aggregation in vivo. In contrast to dynein and kinesin II, dynactin's microtubule binding behavior is unchanged by treatment with PP2A (Fig. 5 b). PP2A has the opposite effect on the motors' microtubule binding properties as PKA, and dephosphorylation by PP2A may induce aggregation in vivo by activating dynein- and inactivating kinesin II–microtubule interactions so that minus end–directed transport is favored.

Dynactin–Microtubule Binding Is Inhibited by PKC

Dynactin's microtubule binding behavior is unaffected by treatment with either PKA or PP2A (Fig. 5 b). Nevertheless, because we observe that dynactin binds to microtubules differentially as a function of the direction of pigment granule transport in the melanophores from which the dynactin is isolated, dynactin's behavior might be modified by other kinases or phosphatases in vivo. PKC induces partial dispersion in *Xenopus* melanophores (Reilein et al., 1998). Treatment of immunoprecipitated dynactin from aggregated cells with this kinase diminishes

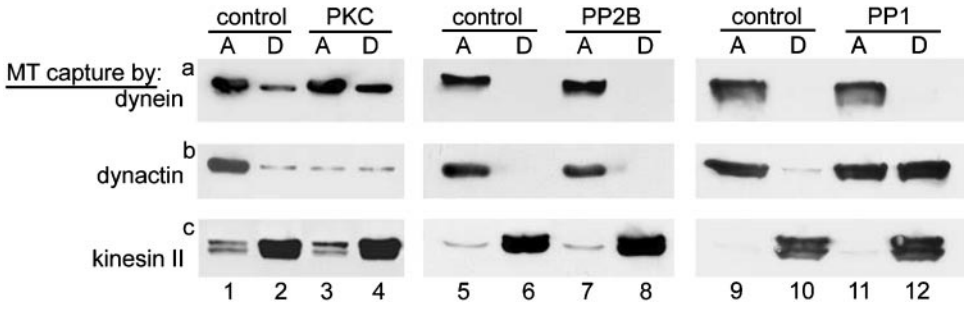

Figure 6. Microtubule capture by dynactin is inhibited by PKC and enhanced by PP1. Dynein (a), dynactin (b), and kinesin II (c) were immunoprecipitated from aggregated (A) and dispersed (D) melanophore lysates and then incubated with either ATP alone (control, lanes 1 and 2) or with ATP and the catalytic subunit of PKC (lanes 3 and 4). Other immunoprecipitants were either incubated in Ca^{2+}/calmodulin (control, lanes 5 and 6) or incubated with Ca^{2+}/calmodulin and PP2B (PP2B, lanes 7 and 8) or were untreated (control, lanes 9 and 10) or incubated with the catalytic subunit of PP1 (lanes 11 and 12). Immunoprecipitants were subsequently washed, incubated with taxol-stabilized microtubules, and assessed for the presence of bound microtubules by immunoblotting and probing for tubulin. PKC inhibits microtubule (MT) capture by dynactin immunoprecipitated from melanophores with aggregated pigment, but has marginal effect on the ability of dynein or kinesin II to capture microtubules. The ability of dynein, dynactin, and kinesin II to capture microtubules is unaffected by PP2B treatment. PP1 treatment enhances microtubule capture by dynactin immunoprecipitated from dispersed melanophore lysates, whereas dynein and kinesin II are unaffected.

dynactin's ability to capture microtubules (Fig. 6 b, compare lane 3 with lane 1), converting its microtubule binding behavior to one mimicking dynactin in dispersed cells (Fig. 6 b, compare lane 3 with lane 2). Unlike dynactin, dynein from aggregated cells remains active after treatment with PKC (Fig. 6 a, compare lane 3 with lane 1). Dynactin isolated from dispersed cells is unaffected by PKC treatment (Fig. 6 b, compare lane 4 with lane 2), as expected if dynactin's behavior has already been altered by PKC during dispersion in vivo. PKC may partially enhance dispersed dynein's activity (Fig. 6 a, compare lane 4 with lane 2), but we have not observed this effect routinely. PKC also has little effect on the behavior of kinesin II's microtubule binding properties (Fig. 6 c, compare lanes 3 and 4 with lanes 1 and 2). These findings suggest that dynactin's ability to bind microtubules varies as a function of phosphorylation, as does dynein's and kinesin II's, and reveal that dynactin is affected by a distinct kinase.

Dynactin–Microtubule Binding Is Enhanced by PP1

Because dynactin–microtubule interactions can be inhibited by a kinase, dynactin–microtubule interactions should be enhanced by a phosphatase. PP2B (calcineurin) is required for aggregation in fish melanophores (Thaler and Haimo, 1990), but this phosphatase has no effect on *Xenopus* dynactin–microtubule binding or on dynein– or kinesin II–microtubule binding (Fig. 6, lanes 5–8). The phosphatase PP1 has not previously been implicated in regulating organelle transport in melanophores (Reilein et al., 1998), but has been implicated in other microtubule-dependent movements (Allan, 1995; Habermacher and Sale, 1996; Lindesmith et al., 1997). Unlike PP2A or PP2B, PP1 enhances the amount of microtubules captured by immobilized dynactin from dispersed melanophore extracts (Fig. 6 b, compare lane 12 with lane 10), converting the microtubule binding behavior of this dynactin to one mimicking that of dynactin isolated from aggregated cells (Fig. 6 b, compare lane 12 with lane 9). PP1 has no effect on the microtubule binding behavior of dynactin obtained from melanophores with aggregated pigment (Fig. 6 b, compare lane 11 with lane 9), as expected if a dephosphorylation event during aggregation in vivo has already altered dynactin's behavior. Neither dynein– nor kinesin II–microtubule binding is affected by PP1 treatment (Fig. 6, a and c, compare lanes 11 and 12 with lanes 9 and 10). Dynactin's microtubule binding ability, like that of dynein's, is enhanced by a kinase and inhibited by a phosphatase, yet dynactin and dynein may be regulated independently as they are affected by different kinases and phosphatases.

Discussion

The Microtubule Binding Activity of Dynein, Dynactin, and Kinesin II Is Regulated by Specific Kinases and Phosphatases during Bidirectional Organelle Transport

Our results support the hypothesis that motor–microtubule, rather than motor–cargo, interactions are regulated to control the direction of pigment granule transport in melanophores. Dynein and dynactin bind microtubules when these proteins are isolated from melanophores with aggregated, but not dispersed, pigment. Kinesin II binds microtubules when it is isolated from melanophores with dispersed, but not aggregated, pigment. Given that dynein drives aggregation (Nilsson and Wallin, 1997), whereas kinesin II drives dispersion (Tuma et al., 1998), the in vitro microtubule binding properties of each of these proteins correlates with its expected in vivo activity. Moreover, conventional kinesin, which does not drive pigment granule transport (Tuma et al., 1998), is not regulated in concert with aggregation and dispersion. Therefore, control of the direction of pigment granule transport appears to be accomplished by specifically regulating the microtubule binding activity of the molecules that drive this transport, dynein, and dynactin, and kinesin II.

The microtubule binding behavior of the soluble pool of dynein, dynactin, and kinesin II mimics that of the pigment granule–associated proteins (see Fig. 2), suggesting that the regulatory system does not specifically target the motors/dynactin on these organelles, but, instead, modifies the entire pool of these proteins. Because pigment granule–associated motors cannot be extracted in quantity, we have used the soluble pool in several of our studies. We infer a mechanism underlying regulation of the direction of pigment granule transport, the accuracy of which depends on whether the behavior of dynein, dynactin, and kinesin

II in the soluble pool reflects that of the cognate pigment granule–associated proteins. Nevertheless, these findings reveal an important feature of dynein, dynactin, and kinesin II that has not been recognized previously: their ability to interact with microtubules can be regulated.

Protein phosphorylation and dephosphorylation control the direction of pigment granule transport in melanophores (Rozdzial and Haimo, 1986). We find that the microtubule binding behavior of the motors can be reversed in vitro by treatment with PKA and PP2A, the kinase and phosphatase involved in regulating opposing directions of pigment granule transport in *Xenopus* melanophores in vivo (Reilein et al., 1998). Dynein's microtubule binding activity is activated by PP2A and inhibited by PKA, whereas kinesin II's is inhibited by PP2A and activated by PKA. Our findings suggest that PKA induces dispersion by activating kinesin II's ability to interact with microtubules, whereas simultaneously inhibiting dynein's. Conversely, PP2A induces aggregation by inhibiting kinesin II's ability to interact with microtubules, while simultaneously activating dynein's. Dynactin, like dynein, is activated by phosphatase treatment and inhibited by kinase treatment, but a distinct kinase and phosphatase, PKC and PP1, respectively, are responsible for its modulation. PKC induces partial pigment granule dispersion in vivo, but a role for PP1 in regulating pigment granule transport in melanophores has not been recognized previously (Reilein et al., 1998).

We believe that the change in motor/dynactin–microtubule binding behavior that we observe upon treatment of these molecules with specific kinases or phosphatases reveals the underlying mechanism controlling direction of transport in vivo for the following reasons. (a) The in vitro activities of dynein, dynactin, and kinesin II were each altered by a specific kinase and phosphatase, and not by the other kinase and phosphatases examined, suggesting that specific phosphorylation alters the microtubule binding properties of each protein. (b) The kinases and phosphatases that do alter motor/dynactin behavior do so in a way that correlates with the expected in vivo activity of these proteins. For example, dynein drives aggregation and PP2A is required to induce aggregation; we find that dynein is active when isolated from aggregated cells and that PP2A converts inactive dynein from dispersed cells to active dynein. (c) PKA, PKC, and PP2A have been demonstrated to regulate pigment granule transport, and PP1 has been implicated in other microtubule-dependent movements, suggesting that the alterations in behavior conferred on the motors/dynactin by these enzymes are not an in vitro artifact. (d) These enzymes only alter the behavior of the motors or dynactin from melanophores that have transported pigment in one direction and not the other. For example, PKA inhibits the microtubule binding activity of dynein that has been isolated from aggregated cells. Dynein that is isolated from dispersed cells is already in its inactive state and is not further inhibited by PKA. (e) Finally, when motors are isolated from cells with aggregated or dispersed pigment, one motor is active, whereas the other is not. PKA and PP2A reciprocally reverse the behavior of the motors in vitro and retain this feature. One motor is active, whereas the other is inactive, after treatment with either of these enzymes. These findings suggest that cyclic phosphorylation and dephosphorylation induce dispersion and aggregation in melanophores (Rozdzial and Haimo, 1986) by cyclically activating and inhibiting motor/dynactin–microtubule interactions, thereby controlling the direction of transport; this system is modeled in Fig. 7.

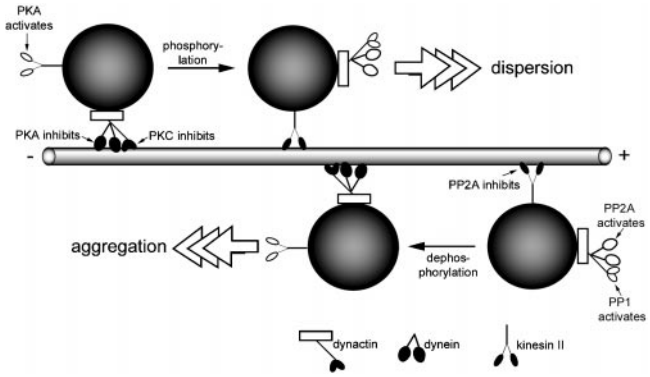

Figure 7. Model for the bidirectional transport of pigment granules along microtubules. Protein phosphorylation and dephosphorylation control the direction of transport in melanophores. A pigment granule at the minus end of a microtubule (left) is bound to the microtubule by active dynein and dynactin (filled heads), whereas inactive kinesin II (clear heads) is unable to bind to the microtubule. PKA and PKC, activated upon stimulation of melanophores with MSH, convert dynein and dynactin to their inactive forms (clear heads), whereas PKA converts kinesin II to its active form (filled heads), allowing it to bind to microtubules. Active kinesin II transports the pigment granule towards the plus end of the microtubule, the direction of pigment granule transport corresponding to dispersion in vivo. Active kinesin II (filled heads) on a pigment granule at the plus end of the microtubule (right) is converted to its inactive form (clear heads) by PP2A when PKA activity is depressed upon stimulation of melanophores with melatonin. Simultaneous modification by PP2A of dynein activates it (filled heads) to its microtubule binding form. Active dynein transports the pigment granule towards the minus end of the microtubule. Activation of dynactin to its microtubule binding form (filled heads) by PP1 may enhance dynein-mediated transport or may anchor pigment granules at the minus ends of microtubules after their transport.

Dynactin Regulation May Not Be Required for Bidirectional Transport

The biological function of dynactin is not well understood. Dynactin is required by dynein to transport organelles (Gill et al., 1991). It may do so by enhancing dynein's processivity and thereby prevent organelles from diffusing away from the microtubule (King and Schroer, 2000). Dynactin may also control dynein's phosphorylation state, and thereby its activity (Kumar et al., 2000). We show here not only that the interaction of motors with microtubules can be regulated, but also that the interaction of dynactin with microtubules can be similarly regulated. Accordingly, studies seeking to elucidate the role of dynactin will need to consider that this protein has two functional states. Dynactin is in its active state when dynein drives pigment granule aggregation. How, then, does net displacement of the granules occur if active dynactin tethers them to the microtubules in a non–ATP-dependent manner? We observe that relative to dynactin both dynein and kinesin II become concentrated on microtubules in cosedimentation assays

(see Fig. 2), suggesting that dynactin's affinity for microtubules may be significantly weaker than the motors'. Thus, dynactin might contribute to dynein's processivity by stochastically tethering the granules to the microtubules, yet not interfere significantly with force generation if it also dissociates frequently from the microtubules. Activated dynactin's affinity for microtubules needs be measured to determine if this proposal is viable. Because dynactin's behavior is modified by a different kinase and phosphatase than is dynein's, these two proteins may, in any case, behave independently during aggregation and dispersion in vivo. Dynactin is converted into its active microtubule binding form by treatment with PP1, yet aggregation can be induced in *Xenopus* melanophores under conditions in which PP1 is inhibited (Reilein et al., 1998). Given that aggregation can occur in the absence of PP1 activity, then dynein apparently is capable of driving aggregation without dynactin being in its active microtubule-binding form. Active dynactin might serve only a modulatory role in pigment aggregation, facilitating longer excursions by the pigment granules towards the minus ends of microtubules. Alternatively, active dynactin might anchor pigment granules at the minus ends of microtubules only after aggregation, mediated by active dynein, has been completed.

Partial dispersion can be induced in *Xenopus* melanophores by PKC without simultaneous activation of PKA (Sugden and Rowe, 1992; Reilein et al., 1998). We find here that PKC alters the behavior of dynactin, but not dynein or kinesin II, converting dynactin to its inactive form. The extent of dispersion induced by PKC (Reilein et al., 1998) appears similar to the reported "relaxation" of the aggregated pigment mass after microinjection into fish melanophores of an antibody that disrupts dynein–dynactin interactions (Nilsson and Wallin, 1997). If activated dynactin keeps organelles tethered at the minus ends of microtubules, then inactivation of dynactin by PKC would result in release of that tether and subsequent dispersion of pigment granules away from the cell center. Alternatively, basal amounts of active kinesin II may be sufficient to drive partial dispersion once dynactin is inactivated. Inactivation of dynactin, however, is apparently not a prerequisite for pigment to disperse. Complete dispersion can be induced by PKA even when PKC activity is inhibited (Reilein et al., 1998). Thus, although dynactin regulation clearly occurs during aggregation and dispersion, this regulation may enhance, but not be an essential component of, bidirectional transport.

Opposing Active Motors May Generate Saltatory Movements during Net Directional Transport

In *Xenopus* melanophores, pigment granules saltate during each direction of transport (Rogers et al., 1997), a feature shared by many other organelles in other cell types (Rebhun, 1964; Pryer et al., 1986; Weiss et al., 1986). Saltations are characterized by short excursions of organelles in both directions along microtubules. Our findings that some active dynein and dynactin can be detected during dispersion and that some active kinesin II can be detected during aggregation suggest that a small pool of active motor may be able to drive these transient movements stochastically against the net direction of transport. Supporting this hypothesis is the observation that microtubules undergo reversals of direction in gliding assays performed in the presence of both active kinesin and cytoplasmic dynein (Vale et al., 1992). Thus, even if one motor overrides the other (Muresan et al., 1996), detachment of the overriding motor from the microtubule would provide the other motor an opportunity, if only briefly, to generate transport in the opposite direction. We propose that the size of each motor's active pool determines the net direction of pigment granule transport along microtubules. As phosphorylation causes the pool of active dynein and dynactin to decrease and that of kinesin II to increase, net dispersion occurs. As dephosphorylation causes the pool of active dynein and dynactin to rise and that of kinesin II to fall, net aggregation occurs.

Regulating Transport of Different Organelles

Different organelles possess unique members of the kinesin superfamily (for reviews see Hirokawa, 1996; Goldstein and Philp, 1999) and may also possess unique isoforms of cytoplasmic dynein (Vaisberg et al., 1996). These motors likely vary in their sensitivity to regulation by particular kinases and phosphatases. For example, in the crayfish giant axon, activation of PKA inhibits vesicle, but not mitochondrial, anterograde transport (Okada et al., 1995). Changes in the distribution of pigment granules during aggregation and dispersion are not mimicked by the other organelles in melanophores. Lysosomes do not aggregate and disperse along with pigment granules, but such displacements of the former, but not the latter, organelles can be induced when the pH of the cells is altered (Tuma et al., 1998). Lysosomes, unlike pigment granules, are transported by conventional kinesin (Tuma et al., 1998), and we find that microtubule binding by this kinesin is not regulated during aggregation and dispersion of pigment. An increase in cytoplasmic pH results in lysosomal clustering at the cell center (Heuser, 1989), and an increase in pH has also been reported to inhibit conventional kinesin–microtubule binding (Verhey et al., 1998). This pH change may mimic a dephosphorylation event on conventional kinesin, but not on kinesin II, thereby altering the activity of kinesin. Differential regulation of various motors residing on different organelles would provide the cell the ability to control with precision the distribution of its organelles.

The Role of Myosin V in Regulation of the Direction of Pigment Granule Transport

Dispersion in *Xenopus* melanophores involves force generation mediated not only by kinesin II on microtubules, but also by myosin V on actin filaments (Rogers and Gelfand, 1998; Tuma et al., 1998; Rogers et al., 1999). Transport of pigment granules from microtubules onto actin filaments by myosin V may provide a mechanism for achieving a uniform distribution of pigment throughout the cytoplasm of dispersed cells (Tuma and Gelfand, 1999). Therefore, pigment granules might be expected to accumulate at the cell periphery during dispersion when myosin V cannot function. In fact, the reverse occurs in *Xenopus* melanophores, and pigment granules spontaneously aggregate in the absence of a functional actin–myosin V system (Rogers and Gelfand, 1998; Rogers et al.,

1999). Accordingly, it has been proposed that dynein activity may predominate over kinesin II activity in *Xenopus* melanophores (Tuma et al., 1998). This proposal is difficult to resolve with the current finding that dynein and dynactin are inactive, and kinesin II is active in dispersed cells. It is possible that signal transduction and, thus, the normal state of dynein, dynactin, and/or kinesin II activation is altered in the absence of a functional actin–myosin V system; therefore, pigment granules aggregate when they would be predicted to disperse. A complete understanding of the mechanism regulating the direction of transport will require that the activity of and relationship between these two motor systems be more fully elucidated.

In summary, we provide evidence that cytoplasmic dynein, dynactin, and kinesin II are cyclically activated and inactivated, either directly or via coimmunoprecipitating proteins, by phosphorylation and dephosphorylation to control their interaction with microtubules, and as a result the direction of organelle transport. It will be necessary to identify the targeted phosphorylation sites on these motors/dynactin or associated proteins and determine how their modification affects the ability of these proteins to interact with microtubules. In addition, it will be of great interest to determine if the transport of other organelles is also regulated by changes in the microtubule binding activity of their microtubule motors.

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