Regulation of Melanosome Movement in the Cell Cycle by Reversible Association with Myosin V

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Abstract. Previously, we have shown that melanosomes of X. laevis melanophores are transported along both microtubules and actin filaments in a coordinated manner, and that myosin V is bound to purified melanosomes (Rogers, S., and V. I. Gelfand. 1998. Curr. Biol. 8:161–164). In the present study, we have demonstrated that myosin V is the actin-based motor responsible for melanosome transport. To examine whether myosin V was regulated in a cell cycle-dependent manner, purified melanosomes were treated with interphase- or metaphase-arrested X. laevis egg extracts and assayed for in vitro motility along Nitella actin filaments. Motility of organelles treated with mitotic extract was found to decrease dramatically, as compared with untreated or interphase extract-treated melanosomes. This mitotic inhibition of motility correlated with the dissociation of myosin V from melanosomes, but the activity of soluble motor remained unaffected. Furthermore, we find that myosin V heavy chain is highly phosphorylated in metaphase extracts versus interphase extracts. We conclude that organelle transport by myosin V is controlled by a cell cycle-regulated association of this motor to organelles, and that this binding is likely regulated by phosphorylation of myosin V during mitosis.

Key words: myosin • molecular motors • melanophores • melanosomes • regulation

Cytological studies of many different types of organelles in animal cells have revealed that their proper spatial and temporal distribution relies upon transport along elements of the cytoskeleton. The Golgi apparatus, for example, is restricted to its perinuclear position in many differentiated cell types by transport to the minus ends of microtubules by cytoplasmic dynein (Corthesy-Theulaz et al., 1992; Burkhardt et al., 1997). In contrast, the steady-state distribution of the ER in the cell’s periphery is actively mediated by transport to the distal, plus ends of microtubules by kinesin (Feiggin et al., 1994). Other types of organelles are more dynamic and must be transported to specific intracellular destinations to perform their specific tasks. Secretory vesicles travel from the Golgi apparatus, their point of origin, to the plasma membrane for exocytosis (Lafont and Simons, 1996). Endosomes are transported centripetally from the cell’s periphery to its interior for subsequent processing (Bomsel et al., 1990). In neurons, synaptic vesicles move from the cell body to sites of synaptic connections, which may be up to meters away (Amaratunga et al., 1993), while mitochondria are believed to be transported up gradients of ADP to intracellular sites of high ATP consumption (Nangaku et al., 1994). Perhaps the most dramatic example of organelle transport occurs during cell division when, during prophase, chromosomes are transported along microtubules to congress at the metaphase plate, and later, during anaphase, segregate to their respective, opposite poles (Waters and Salmon, 1997).

Many years of study have led to the dogma that the majority of intracellular transport occurs along microtubules, driven by the activities of the dynein and kinesin superfamilies of motor proteins. However, in recent years, this assumption has been challenged by the demonstration that some organelles can move along filamentous actin (Adams and Pollard, 1986; Kuznetzov et al., 1992; Bearer et al., 1993; Mermall et al., 1994; Morris and Hollenbeck, 1995; Krendel et al., 1998). Compelling evidence that a single type of organelle can be transported along both microtubules and actin filaments has resulted from the study of pigment granule transport in melanophores, a model system for the study of intracellular transport. The
role of these cells, present in the dermal layers of fish and amphibians, is to cyclically aggregate pigmented organelles, termed melanosomes, to the center of the cell or disperse them throughout the cytoplasm to effect color changes in the animal's skin (Haimo and Thaler, 1994). In X enopus laevis melanophores, pigment transport is regulated by hormone-induced modulation of intracellular cAMP levels: melanocyte-stimulating hormone (MSH) triggers dispersion by upregulation of cAMP production, while melatonin induces pigment aggregation by downregulating cAMP levels (Daniolos et al., 1990). This hormone-induced organelle transport is regulated by antigenic cycles of kinase and phosphatase activities (Reilein et al., 1996). Until recently, it was believed that melanosomes were exclusively carried along the cell's radially organized microtubule cytoskeleton with a kinesin-related protein, kinesin-II, transporting pigment to the microtubule plus ends during dispersion and dynein moving them to the minus ends during aggregation (Nilson and Wallin, 1997; Tuma et al., 1998). It is now clear, however, that another, actin-based component also contributes to pigment transport in melanophores. Upon disruption of the microtubule cytoskeleton, melanosomes exhibit short, shuttling movements that halt in the presence of actin-depolymerizing drugs (Rodionov et al., 1998). Furthermore, we have demonstrated that purified melanosomes can move along actin filaments in vitro and that the actin-based motor, myosin V, is associated with these organelles (Rogers and Gelfand, 1998). Similar findings of coordinated actin- and microtubule-based transport were also reported for melanosomes in cultured mouse melanocytes (Wu et al., 1998a).

The mitotic cell is confronted with the important task of ensuring that both daughter cells receive their appropriate allotment of each organelle type (Warren, 1993; Warren and Wickner, 1996; Shima et al., 1998). Since the interphase distributions of many organelles rely upon the activities of motor proteins, it stands to reason that their segregation during mitosis must be accompanied by modulation of the activities of associated motors. At present, the A-lan and V-ale laboratories have performed the only studies directly examining this topic. Using X enopus frog egg extracts arrested in metaphase, these groups demonstrated that both plus and minus end directed microtubule-based transport of membranous organelles was inactivated (A-lan and V-ale, 1991). Furthermore, mitotic inhibition of dynein-mediated organelle transport is achieved by dissociation of the motor from its cargo, and this dissociation correlated with phosphorylation of the motor by a mitotic kinase activity (Niclas et al., 1996).

Previous studies of mitotic melanophores in vivo documented that these cells do not respond to stimuli which normally induce pigment aggregation and dispersion in interphase, suggesting that melanosomal motors may, indeed, be differentially regulated throughout the cell cycle (Starobudov and Golichenkov, 1998). Melanophores provide a very useful system to study motor protein regulation. The melanosomes present in these cells may be purified rapidly and in large quantities, and have been shown to exhibit both microtubule- and actin-based motility in vitro. Treatment of isolated melanosomes with X enopus egg extracts arrested either in metaphase or interphase allows the study of cell cycle-dependent regulation of the microtubule- and actin-based motors present on these organelles. In this study, we have demonstrated that myosin V is the motor responsible for actin-based transport of melanosomes in X enopus melanophores through the use of a dominant-negative myosin V construct and by immunofluorescent localization of the motor to melanosomes. We then used our system to study the regulation of myosin V during mitosis. Treatment of melanosomes with metaphase, but not interphase, extracts resulted in a dramatic decrease in vitro motility. This decreased motility was due to dissociation of myosin V from pigment granules and not due to inhibition of its motor activity. The myosin V heavy chain exhibited a substantial increase in phosphate incorporation in mitotic extracts, compared with interphase extracts, implicating phosphorylation of myosin V as the regulatory mechanism. To our knowledge, this is the first study documenting a molecular mechanism for the cell cycle-mediated regulation of actin-based organelle transport.

Materials and Methods

Melanophore Cell Culture and Transfection

Immortalized X enopus melanophores were cultured as described previously (Rogers et al., 1997). Immunochemical localization of myosin V was performed using a clonal nonpigmented cell line, clone 47, or gray cells, derived from the original melanophore cell line (Daniolos et al., 1990). Melanophores containing a lower melanin content were selected by freezing the original cell line in 95% FCS and 5% DMSO, according to standard protocols. Approximately 5% of the cells survived thawing and reculturing, many of them possessing large vesicles containing small (∼0.2 μm) particles of melanin. This cycle of freezing and thawing was repeated once again and pigment-deficient cells were cloned twice on 10-cm tissue culture plates using the cloning ring technique. A morphologically stable clone was selected and expanded. Over 50% of the cells in this population contained numerous unmelanized vesicles ∼1-μm diam. These vesicles responded to hormone treatment by aggregating and dispersing as normal melanosomes and were, therefore, considered pigment-free melanosomes. Further melanin production was inhibited by treating the cells for 2 wk with 1 mM phenylthiourea. Cultures isolated in this manner were grown in standard growth medium, supplemented with 1 mM phenylthiourea.

To preserve cellular morphology for microscopy, cells were transfected using the FUGENE 6 transfection reagent (Boehringer Mannheim Corp.) following the vendor’s protocols.

Construction of myc-tagged Headless Myosin V

To prepare the plasmid pcDNA3-Myc-MST, which contains the COOH-terminal 601 amino acids of the mouse myosin V gene fused to the COOH terminus of the myc epitope tag (Evan et al., 1985), the following PCR primers were constructed: 5′-AAA AAG CTT AAA CCA TGG AGC AAA AGC TCA TTT CTG AAG AGG ACC TGG GGA TCC AAG CTG-3′ and 5′-AAA AAG CTT AAA CCA TGG AGC AAA AGC TCA TTT CTG AAG AGG ACC TGG GGA TCC AAG CTG-3′. The product of this PCR was digested with XhoI and HindIII, and cloned into the vector pcDNA 3 (Invitrogen Corp.).

Immunofluorescence Microscopy

Melanophores were plated on acid-washed polylysine-coated glass cover-
slips and cultured for 24 h. Cells were then briefly rinsed in 0.7× PBS and fixed for 20 min in a solution of freshly prepared 3% paraformaldehyde in 0.7× PBS. A solution of 0.3%. Triton X-100 was used to permeabilize cells for 15 min. For immunofluorescent staining, cells were blocked using 3% BSA in the same solution for 10 min. Myc epitope-tagged proteins were stained using the 1-9E 10.2 mAb (E Evans et al., 1985) diluted 1:1,000 into the BSA/Triton buffer for 60 min. Myosin V distribution was visualized with the DIL2 polyclonal antibody (see below) at a dilution of 1:8,000. The cells were then washed with PBS, stained using FITC-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 for 60 min, washed with 0.7× PBS, and mounted in 80% glycerol in 0.1 M sodium borate, pH 8, supplemented with N-propyl gallate. For fluorescent actin staining, rhodamine-conjugated phalloidin (Molecular Probes, Inc.) was diluted to 0.33 nM and included with the secondary antibodies. Images were obtained using a Zeiss Axioskop equipped with a CH250 cooled CCD camera (Princeton Instruments). The gray-scale histograms of the images were stretched to utilize their full dynamic ranges using Adobe Photoshop.

Preparation of Xenopus Egg Extracts and Endogenous Egg Organelles

Interphase and metaphase-arrested X enopus egg extracts were prepared as described (Murray, 1991; A Ilan, 1993), with the following modifications. CSF-arrested frog eggs were activated by treatment with 10 μM A23187 for 5 min and allowed to progress to interphase in the presence of 100 μg/ml cycloheximide before extract preparation. M etaphase-arrested extracts were prepared by treating interphase extracts with 0.13 mg/ml bacterially expressed Myosin V. Initial extracts were supplemented with 5 μM latrunculin A (BIOMOL) to depolymerize actin (Spector et al., 1989). Histone HI kinase assays were used to monitor activity of cdc2/p34 kinase activity during every experiment (Allan, 1993). Histone H1 kinase assays were used to monitor activity of cdc2/p34 kinase activity during every experiment (Allan, 1993).

Treatment of Melanosomes with Xenopus Egg Extracts

Melanosomes were isolated from cultured melanophores, essentially as described in Rogers et al. (1998). In brief, melanophores were grown in 10-cm tissue culture plates to confluency. Cells were rinsed with IMB50 supplemented with 1 mM ATP then scraped into 2 ml of the same buffer supplemented with 2 mM N-ethyl maleimide for 5 min to poison the plant’s endogenous cytoplasmic streaming and ensure that all motility observed was due to the activity of melanosome-associated myosin. NItella cells were then dissected into buffer containing 2 mM dithiothreitol, 2 mM ATP, and 10 μM phallolidin to stabilize filamentous actin. Melanosomes or protein A beads were then pipetted onto dissected NItella filaments and observed using time-lapse video-enhanced bright-field microscopy with a 40× long-working distance objective mounted on a Diaphot 300 inverted microscope (Nikon, Inc.). Images were captured using a Newcon camera and an Ar gus-10 video processor (Hamamatsu Photonics) and recorded onto s-VHS tapes with a time-lapse video recorder (Panasonic).

To examine the activity of myosin V in egg extracts, protein A–agarose beads (Sigma Chemical Co.) were incubated with affinity-purified DIL2 polyclonal antibody at a protein concentration of 25 μg/ml, or with preimmune serum for 1 h at 4°C. The beads were then washed in IMB50 by re-suspension and centrifugation five times to remove unbound antibody. The preimmune-bound bead pellets (∼50 μl vol) were then resuspended in 200 μl of interphase or metaphase egg extracts clarified of membranes by centrifugation at 150,000 g in a TLA 100.3 rotor (Beckman Instruments, Inc.) to preclear for 1 h at 4°C. The beads were pelleted, and each aliquot of extract was then incubated either with DIL2- or preimmune-conjugated beads for 1 h at 4°C. A filter washing five times in IMB50 supplemented with 2 mM ATP, these beads were used in the NItella assay, as described above.

32P-Labeling and Immunoprecipitation of Myosin V from Egg Extracts

X enopus egg extracts arrested in interphase or metaphase were labeled with 200 μCi 32P in 20 μl reactions and incubated for 30 min at room temperature. Reactions were stopped by the addition of 1 ml ice-cold IP buffer (10 mM Tris, 80 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, pH 7.5) supplemented with 1% Triton X-100, and a protease inhibitor cocktail (10 μg/ml each leupeptin, pepstatin, and chymostatin, and 1 mM PM SF) and incubated on ice for 10 min. The samples were clarified by centrifugation at 16,000 g for 15 min and precleared for 90 min by incubation with normal rabbit serum, prebound to 25 μl of 50% protein A–agarose bead suspension. A fer centrifugation, 25 μl of 0.2 mg/ml affinity-purified DIL2 antibody was added and incubated for 4 h at 4°C. The samples were then incubated with 30 μl of protein A beads for 30 min, and the beads were collected by centrifugation at 10,000 g for 10 min. The following regimen of washes was then performed: four times with ice-cold IP buffer, one time with IP buffer supplemented with 500 mM sodium chloride, and once with 30 mM Tris, pH 8. Pellets were resuspended in 20 μl sample buffer and analyzed by SDS-PAGE and autoradiography.

Results

Myosin V Is Responsible for Melanosome Transport Along Actin Filaments

U sing an in vitro assay, we previously demonstrated the presence of an actin-based motor on the surface of melanosome purified from Xenopus melanophores (Rogers and Gefland, 1998). Tentative identification of this motor as myosin V was based on the observation that this motor is enriched in purified melanosome fractions relative to number of organelles. Immunoblotting was performed using the protocol of Towbin et al. (Towbin et al., 1979) and bound antibody was detected by chemiluminescence using SuperSignal (Pierce Chemical Co.). Myosin V was detected using a polyclonal antibody. DIL2, raised against bacterially expressed neck domain of the dilute isoform of mouse myosin V, and affinity-purified as described (Wu et al., 1997). Kinase-II was detected using an α mAb cross-reactive with the motor’s 85-kD subunit, K2.4 (Cole et al., 1993). The intermediate chain of cytoplasmic dynein was probed with an α mAb, m74-1 (Steffen et al., 1997).

NItella In Vitro Motility Assays

The NItella-based motility assay was performed essentially as described (Sheetz et al., 1998), with the following modifications. Before dissection, each NItella filet was incubated with 2 mM N-ethyl maleimide for 5 min to poison the plant’s endogenous cytoplasmic streaming and ensure that all motility observed was due to the activity of melanosome-associated myosin. NItella cells were then dissected into buffer containing 2 mM dithiothreitol, 2 mM ATP, and 10 μM phallolidin to stabilize filamentous actin. Melanosomes or protein A beads were then pipetted onto dissected NItella filaments and observed using time-lapse video-enhanced bright-field microscopy with a 40× long-working distance objective mounted on a Diaphot 300 inverted microscope (Nikon, Inc.). Images were captured using a Newcon camera and an Ar gus-10 video processor (Hamamatsu Photonics) and recorded onto s-VHS tapes with a time-lapse video recorder (Panasonic).

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Myosin V Is Responsible for Melanosome Transport Along Actin Filaments
whole cell extracts, while members of several other myosin classes were absent. To determine conclusively whether myosin V was actually involved in melanosome transport in this system, we sought to disrupt its function in melanophores and to observe the effects on pigment granule transport. Since latrunculin A-induced disruption of the actin cytoskeleton in Xenopus melanophores results in microtubule-dependent aggregation of the pigment to the cell center (Rogers and Gelfand, 1998), we hypothesized that inhibition of the melanosome-associated myosin would produce the same effect. Melanophores were, therefore, transfected with a construct encoding an epitope-tagged fragment of mouse myosin Va (Wu et al., 1998a). This fragment, lacking the NH₂-terminal motor domain, consists of the COOH-terminal 601 amino acids of myosin V, and includes part of the central stalk domain believed to be important in homodimerization of myosin V heavy chains by coiled-coil interaction, and the globular tail domain, thought to be the cargo-binding site. This construct, termed myosin V short tail (MST), has been shown to act as a dominant-negative inhibitor of myosin V in mouse melanocytes; its expression mimicking the naturally occurring genetic-null phenotype in this cell type (Wu et al., 1998a).

Melanophores were transfected with the MST construct; 48 h later cultures were fixed and transfected cells identified by immunofluorescent staining for the myc epitope-tag present at the NH₂ terminus of the mutant construct. Xenopus melanophores usually grow in culture with their melanosomes dispersed throughout their cytoplasm unless induced to aggregate to a tight, central mass by treatment with melatonin. Without exception, every cell expressing MST aggregated its pigment to the cell center (n = 250; Fig. 1, A and B). Immunolocalization of expressed myc-tagged MST revealed that the protein was present throughout the entire volume of the cell. Melanosome aggregation was not due to a disruption of the actin cytoskeleton, as staining with fluorescent phalloidin revealed a filamentous actin distribution in these cells, which was similar to nontransfected cells (data not shown). Control cells transfected with green fluorescent protein (GFP) underwent cycles of pigment aggregation and dispersion, indicating that pigment aggregation was an effect specifically caused by MST (Fig. 1, C and D).

To verify the involvement of myosin V in melanosome transport, we sought to determine whether or not the motor was present on melanosomes in situ by immunofluorescent staining. The chemical properties of melanin make this approach problematic, however, for two reasons. First, melanin is a polymer composed of modified tyrosine residues and is rife with charged carboxyl and nonpolar aromatic side chains (Sarna, 1992). Previous studies have documented the high affinity of melanin for various compounds, and it has been our experience that melanin avidly binds many proteins present in cell extracts (Ogers et al., 1998). Second, as melanin has been evolved to act as a light-absorbing pigment, it interferes with fluorescence microscopy, especially in aggregated cells where the pigment mass often obscures staining. To circumvent these problems, we developed a melanin-free clonal melanophore subline. The original Xenopus melanophore cell line was subjected to two freeze-thaw cycles to select for melanophores possessing a low melanin-content. After two rounds of cloning, a morphologically stable cell line was isolated. Any residual melanin was eliminated by culturing the cells in 1 mM phenylthiourea, a potent tyrosinase inhibitor, for two weeks. This cell line, designated clone 47 or gray cells, possesses numerous vesicles which are ~1 μm in diameter, and respond to treatment with MSH and melatonin by dispersing to the cell periphery or aggregating to the cell center (Fig. 2, A and B). Therefore, we believe that these vesicles are melanosomes devoid of pigment.

Gray cells were fixed and immunofluorescently stained with an antibody specific for myosin V. The antibody was highly enriched on punctate vesicular structures that aggregated to the cell center upon treatment with melatonin (Fig. 2 C) and dispersed throughout the cells after exposure to MSH (Fig. 2 D). We conclude that myosin V is present on melanosomes in vivo in Xenopus melanophores and remains bound to these organelles, at least to some degree, during aggregation and dispersion.

**Cell Cycle-dependent Regulation of Myosin V**

Previous studies examining Xenopus melanophores in situ have shown that these cells fail to transport their pigment in response to MSH or melatonin during mitosis (Starobudov and Gribbonkov, 1988). Work from our lab has established that melanosome transport occurs along both the
microtubule and actin cytoskeletons in a coordinated manner (Rogers and Gelfand, 1998). We considered the possibility that the melanosome-associated motors are differentially regulated throughout the cell cycle. Since nothing is known about whether actin-mediated organelle transport is regulated during cell division, we chose to focus upon this question by treating melanosomes with *Xenopus* egg extracts arrested in metaphase or interphase and examining their motility in vitro.

We initially prepared *Xenopus* egg extracts arrested in interphase or metaphase essentially according to the protocols of Murray (1991), as modified by Allan (1993). Frog eggs were activated using calcium ionophore in the presence of cytochalasin D to prevent actin polymerization. Cycloheximide was included to arrest them in interphase, and extracts were obtained by centrifugal crushing. Extracts at this stage were found to remain stably in interphase. Metaphase arrested-extracts were prepared by further supplementing these extracts with the nondegradable cyclin derivative, Δ90 (Glotzer et al., 1991). Mitotic extracts were found to stably maintain high MPF kinase activity (data not shown). Both interphase and metaphase extracts prepared this way completely inhibited actin-based motility of melanosomes in vitro using the *Nitella* assay, as compared with untreated control organelles. Biochemical studies of purified myosin V demonstrated that this motor binds to actin with high affinity, even in the presence of ATP (Nascimento et al., 1996). Therefore, we speculated that cytochalasin-capped fragments of actin filaments bound to myosin V present on the melanosomes, effectively blocking the motor from interacting with exogenous *Nitella* actin filaments. To circumvent this possibility, latrunculin A, a drug which binds to monomeric actin and, unlike cytochalasin, induces complete depolymerization of filamentous actin, was included during extract preparation (Spector et al., 1989).

To establish a basal level of melanosome motility, organelles were purified from melanospheres and scored for their ability to move in vitro using the *Nitella* assay. In agreement with our previous results (Rogers and Gelfand, 1998), we found that ~90% of the total number of pigment granules exhibited unidirectional motility (Fig. 3). When isolated, melanosomes were preincubated in interphase frog egg extracts supplemented with latrunculin and purified by density gradient centrifugation. The fraction of organelles transported along actin filaments was ~85%, virtually indistinguishable from the untreated control. However, treatment with metaphase-arrested extracts dramatically inhibited actin-based motility; only ~10% of the melanosomes exhibited motility in vitro. Incubation of the melanosomes with metaphase extracts, therefore, decreased motility nearly eightfold, compared with interphase extracts or untreated organelles.

**Myosin V Dissociates from Melanosomes in Metaphase-arrested Frog Egg Extracts**

We hypothesized that the inhibition of motility observed following metaphase extract treatment could be due to one of two possible mechanisms: myosin V may dissociate from melanosomes or the motor could be rendered inactive during mitosis. To test this first possibility, melanosomes were isolated, treated with either interphase- or metaphase-arrested extracts, and examined by electrophoresis and immunoblotting for myosin V (Fig. 4 A). The number of melanosomes in each sample was normalized by optical density to ensure that an equal number of or-
metaphase organelles (Mo) from frog extracts. Equal amounts of metaphase extracts (M ex), interphase organelles (I o), and equal. C, Immunoblot for myosin V in interphase extracts (Iex), demonstrating that the protein loaded in each lane is approximately 10 kD, compared to solubility of melanin at 550 nm. Myosin V is designated by MV.

Figure 4. A, Immunoblot for myosin V of melanosome fractions treated with mitotic extracts (M), interphase extracts (I), or untreated organelles (MS). Myosin V is absent from metaphase treated organelles. The samples for each lane were normalized to load equivalent numbers of melanosomes by measuring the absorbance of melanin at 550 nm. Myosin V is designated by MV. B, Coomassie blue stained gel of the samples shown for A, demonstrating that the protein loaded in each lane is approximately equal. C, Immunoblot for myosin V in interphase extracts (I ex), metaphase extracts (M ex), interphase organelles (I o), and metaphase organelles (M o) from frog extracts. Equal amounts of protein were loaded for each sample. D, Immunoblot for myosin V on melanosomes treated with interphase (I) and metaphase (M) high-speed supernatants prepared from egg extracts.

Endogenous egg organelles from both types of extracts were purified by flotation through a sucrose gradient to exclude soluble proteins and analyzed for the presence of myosin V (Fig. 4 C). Interestingly, the motor was found to remain associated to these organelles in both interphase and metaphase extracts. This result suggests that the dissociation of myosin V may be an organelle-specific phenomenon. Furthermore, it conclusively rules out the possibility that melanosome fractions became contaminated with endogenous organelles during treatment with the egg extracts. We noted that the mobility of myosin V bound to organelles did not exhibit a shift in molecular weight in either population of organelles or as compared with soluble myosin V from either type of extract.

If melanosome-bound myosin V was able to exchange with the soluble pool of egg-derived motor, then our observations might also reflect a cell cycle-dependent association of egg myosin V with melanosomes. To test whether this was the case, we immunodepleted myosin V from both interphase- and metaphase-arrested extracts before melanosome addition. We reasoned that if there were an exchange between the organelles and the extracts, then we would detect a net release from interphase-treated melanosomes in myosin V-depleted extracts. Immunoblots of organelles treated with mitotic extract depleted of myosin V lacked the motor, whereas immunodepleted interphase extracts retained myosin V (data not shown). However, when we compared the relative amount of myosin V bound to melanosomes after treatment with motor-depleted extracts with organelles treated with extracts immunodepleted using preimmune serum, we noted a quantitative difference. In myosin V-depleted extracts, melanosomes retained less motor, indicating that egg-derived myosin V was able to exchange with the melanosome-bound protein in interphase-arrested extracts (data not shown). These results indicate that melanosomal myosin V is able to exchange with myosin V derived from interphase extracts, but neither the egg nor melanosomal myosin can bind to organelles in metaphase-arrested cytosol.

Previous work from our lab has demonstrated that the bidirectional transport of pigment granules along microtubules in Xenopus melanophores is due to the activities of the plus-end directed motor, kinesin-II, and the minus end directed motor, cytoplasmic dynein (Tuma et al., 1998; Karcher, R., and V. Gelfand, unpublished data). Niclas et al. (1996) demonstrated that dynein-driven motility of Golgi membranes and ER membranes exhibits cell cycle-dependent regulation in X. enopus mitotic egg extracts, and this inhibition is due to dissociation of the motor. Our system allowed us to test if this is a general phenomenon for dynein-driven motility. Consistent with the results of Niclas et al. (1996), quantitative immunoblotting for dynein using an antibody raised against the intermediate chain demonstrated that this motor is released from melanosomes in metaphase extracts (Fig. 5 A). Interestingly, dynein intermediate chain in extract-treated melanosome fractions exhibits a mobility shift of ~10 kD, compared with its apparent molecular weight of 83 kD on untreated melanosomes. This mobility shift has been observed by other groups who have attributed it to represent posttranslational modification (Huang et al., 1999) or the recruitment of an egg-specific isoform of the motor (Lane and...
A Ilan, 1999). In an effort to distinguish between these two possibilities, we treated egg extracts with alkaline phosphatase to determine whether this mobility shift was due to phosphorylation. The migration of dynein intermediate chain remained unaffected by this treatment (data not shown), indicating that melanosomes likely recruited an egg-specific isoform. Kinesin-II was found to remain associated with metaphase-extract-treated pigment granules by immunoblotting with an antibody that recognizes the 85-kD subunit of the motor (Fig. 5 B).

Our experiments with the dominant-negative myosin V construct demonstrated that the activity of this motor is necessary for proper dispersion of melanosomes in melanophores. It is, therefore, possible that modulation of myosin V’s activity is a key regulatory event during the cycles of aggregation and dispersion within these cells. Since elevation of intracellular cAMP and the subsequent activation of protein kinase A (PKA) plays a key role in triggering pigment dispersion, we considered the possibility that the activity of this kinase may play a role in the association of myosin V with melanosomes throughout progression of the cell cycle. The activity of PKA in cycling Xenopus egg extracts has been extensively studied, and has been found to play an important role during the transition of mitosis to interphase (Greico et al., 1996). The basal level of cAMP production and PKA activity was found to decrease during the transition from interphase to mitosis, and to exhibit a peak in activity just before the transition to interphase (Greico et al., 1994). If PKA activity were essential for myosin V attachment to melanosomes, then the diminished activity of the kinase in metaphase-arrested extracts might account for the motor’s dissociation. To test this hypothesis, we incubated purified melanosomes in metaphase egg extracts in the presence of 50 μM cAMP and 1 mM 1-isobutyl 3-methyl xanthine to inhibit phosphodiesterases. Comparison of immunoblots of treated versus untreated metaphase extracts showed no difference in the amount of myosin V present on melanosomes; the motor dissociated from the organelles in both cases (data not shown). A ddition of the PKA catalytic subunit to metaphase extracts, likewise, did not prevent this dissociation (data not shown). Mitotic release of myosin V from melanosomes was, therefore, not due to the inactivity of PKA.

The Motor Activity of Myosin V Is Not Differentially Regulated Throughout the Cell Cycle

In addition to dissociation from its cargo, another potential regulatory mechanism for myosin V could be inhibition of its motor activity. To test this possibility, affinity-purified myosin V antibody was bound to protein A-conjugated agarose beads. The beads were then used to isolate myosin V from interphase- and metaphase-arrested egg extracts that had been clarified of membranes by ultra-centrifugation. Beads with attached myosin V were analyzed in the Nitella motility assay.

Myosin V immunosolated on beads from interphase (Fig. 6 A) and mitotic (Fig. 6 B) extracts exhibited vigorous motility in the Nitella assay. Virtually every bead from both samples exhibited motility, with average velocities of 84.2 ± 19 nm/s for interphase beads and 52.8 ± 17 nm/s for metaphase beads. These velocities are somewhat faster than those observed for untreated melanosomes in vitro (41 ± 20 nm/s). Melanosomes treated with interphase extracts exhibited average velocities of 32.8 ± 7.8 nm/s, while metaphase treated melanosomes traveled at 27.8 ± 10.5 nm/s. Beads conjugated with preimmune serum from the same rabbit in which the DIL2 antibodies were generated exhibited no motility, indicating that the movements we observed were due to immunoadsorbed myosin V. We conclude that the motor activity of myosin V is not inhibited in mitotic extracts.

Phosphorylation of Myosin V in Metaphase Extracts

Since many cellular processes, including dynein-mediated transport, are modulated in a cell cycle-dependent manner by phosphorylation, we tested the possibility that myosin V might be regulated similarly. Interphase- and metaphase-arrested egg extracts were treated with [32P]orthophosphate to produce an endogenous pool of labeled ATP to act as a substrate for kinases present in the extracts. Myosin V was then immunoprecipitated and equal amounts of protein were analyzed by electrophoresis and autoradiography. In both interphase and metaphase extracts, a 200-kD myosin V heavy chain was immunoprecipitated and found to be phosphorylated (Fig. 7). Quantitation of the amount of radioactive phosphate incorporated into the protein in each treatment revealed that in mitotic cytosol it was labeled approximately fivefold greater than in interphase extract. Myosin V is, therefore, more highly phosphorylated in metaphase, compared with interphase. Interestingly, several additional phosphoproteins coimmunoprecipitated with myosin V. In interphase extracts, pro-
teins with molecular weights of 70 and 20 kD exhibited labeling, while in metaphase extracts, species of 85, 100, 120, and 140 kD remained associated (Fig. 7). The molecular weights of these proteins do not correlate with any known subunits of this motor, and the significance of these possible associations is unknown.

The metaphase-arrested extracts that we used were prepared by treatment of interphase extracts with D\textsubscript{90}, a sea urchin cyclin B construct modified to delete its ubiquitination sequence (Glotzer et al., 1991).

D\textsubscript{90} associates with and activates cdc2/p34 kinase, driving the extracts into metaphase, but since the cyclin's degradation sequence has been removed, the kinase activity of the cyclin B/p34 complex remains constitutively active and the extracts cannot progress further. We speculated that myosin V might be phosphorylated directly by cdc2/p34 mitotic kinase. To test this hypothesis, we purified melanosomes and treated them in vitro with commercially available recombinant cyclin B/cdc2 kinase. After the melanosomes were repurified by density gradient centrifugation, immuno- 

Discussion

It has become evident over the past several years that cells partition membrane-bound organelles to their daughters by precisely regulated, yet unique, mechanisms. Intracellular membranes undergo a specific choreography within the spindles of living PtK2 cells, first collecting along the microtubules to gather at the poles through prometaphase, followed by an abrupt exclusion from the spindle at metaphase (Waterman-Storer et al., 1993). Using a GFP-labeled resident Golgi apparatus protein, Shima et al. documented the fragmentation of the Golgi apparatus in living HeLa cells during mitosis and observed that the resultant vesicles segregate to each daughter cell via a static association with each pole of the spindle in a microtubule-dependent process (Shima et al., 1997, 1998). GFP-labeled peroxisomes, which are transported vectorally along microtubules during interphase, lose their association with the cytoskeleton during cell division and appear to be segregated randomly (Weimer et al., 1997). In frog melanophores, melanosomes are excluded from the mitotic spindle and fail to respond to the hormonal stimuli that induce them to aggregate or disperse during interphase (Starobudov and Golichenkov, 1988). One common feature between these examples is the apparent downregulation of microtubule-based transport during metaphase. This hypothesis is borne out by the work of the Allan and Vale

Figure 6. Immunoadsorbed myosin V from interphase (A) or metaphase (B) extracts is able to transport Sepharose beads in the Nitella motility assay. The beads appear as large, refractile spheres, whereas the smaller oval objects are the underlying Nitella chloroplasts. The centers of three beads in each panel were tracked over 30 min; each white dot overlaid on the video frames represents the position of the beads in 1 min increments. Bar, 20 μm.

Figure 7. Phosphorylation of myosin V in cell cycle-arrested frog egg extracts. A, Extracts were \textsuperscript{32}P-labeled, and myosin V was immunoprecipitated from interphase- and metaphase-arrested extracts using affinity-purified DIL2 antibody (I and M, respectively), separated by SDS-PAGE, and analyzed by autoradiography. Myosin V (MV) is more highly phosphorylated in metaphase extracts. B, Coomassie blue stained gel as in A, to demonstrate approximately equal protein load for both treatments.
laboratories, which demonstrated that dynein-mediated microtubule minus end-directed transport, along with plus end transport, of the ER and Golgi membranes are inhibited in metaphase-arrested Xenopus egg extracts (A.Ilan and V ale, 1991; Niclas et al., 1996).

In the present study, we examined whether myosin V was subjected to cell cycle-dependent regulation. Our previous work demonstrated that melanosomes purified from Xenopus melanophores exhibit actin-based motility in vitro (Rogers and Gelfand, 1998). Furthermore, the unconventional myosin, myosin V, was found to be enriched in melanosomes in fractions, compared with whole melanophore extracts, suggesting that this motor is responsible for movement along actin filaments. In this study we have confirmed this hypothesis both in vivo and in vitro by using a dominant-negative approach to block myosin V function and by immunolocalizing myosin V to melanosomes in unfragmented melanophores. To test cell cycle governed regulation of myosin V bound to melanosomes, we prepared Xenopus egg extracts arrested either in interphase or metaphase. Melanosomes incubated in interphase extracts and untreated organelles exhibited vigorous motility in the Nitella assay, but mitotic-treated melanosomes showed an eightfold decrease in their in vitro movement. Furthermore, this mitotic inhibition was caused by dissociation of myosin V from melanosomes without an accompanying inhibition of its motor activity. We used a polyclonal antibody raised against a 27-kD fragment of myosin V for detection of this motor and do not believe that our inability to detect it on blots of metaphase-treated melanosomes is due to masking of the epitope by posttranslational modification. Membranous organelles purified from egg extracts did not exhibit cell cycle induced dissociation, however, suggesting that mitotic release of myosin V may be specific for certain organelles. Interestingly, we did observe a slight difference in the velocities of soluble myosin V between interphase and mitotic extracts, which may indicate a second level of regulation of this motor. Dissociation of myosin V was accompanied by increased phosphorylation of its heavy chain in mitotic extracts, relative to interphase extracts. Therefore, we propose a mechanism whereby myosin V-driven organelle transport is activated during mitosis by phosphorylation-induced dissociation from melanosomes.

A though myosin V is one of the best characterized unconventional myosins, its regulation is poorly understood. To date, only one other study has addressed this issue directly. Prekeris and Terrian (1997) demonstrated a calcium-induced release of myosin V from synaptic vesicles in vitro, as well as in isolated synaptosomes (Prekeris and Terrian, 1997). They showed that this dissociation occurred in the absence of ATP and was, therefore, not due to phosphorylation of the motor. We believe that the metaphase-induced release of myosin V from melanosomes occurs through a different regulatory mechanism for two reasons. First, in our study, melanosomes and Xenopus egg extracts were prepared in the presence of the calcium chelator, EGTA. Second, treatment of purified melanosomes with exogenous calcium failed to affect the amount of myosin V bound to the organelles (data not shown). Tissue-specific isoforms of myosin V are produced as the result of differential RNA splicing; brain and epidermal myosin V each possess different protein domains as a result (Seperack et al., 1995). It is possible that this difference in calcium sensitivity may be specific to the neuronal isoform. Alternatively, it may be that organelle receptor proteins for the motor respond to different signals; those on synaptic vesicles, synaptophysin and synaptotagmin II, release myosin V upon exposure to calcium, whereas the unidentified receptor on melanosomes does not (Prekeris and Terrian, 1997).

It is interesting to compare the regulatory mechanisms that govern cytoplasmic dynein with those of myosin V. Although both transport membrane-bound organelles along different cytoskeletal filaments during interphase, they dissociate from their cargo during mitosis. Cytoplasmic dynein has been implicated in other processes during cell division, such as spindle formation, chromosome transport, and spindle orientation (Waters and Salmon, 1997; Palazzo et al., 1999). It is possible that dynein dissociates from its membrane-bound organelle cargo so that it may be recruited to perform these other tasks during mitosis. Alternatively, it may also be that the motor is subjected to identical regulatory mechanisms during interphase and mitosis, but specificity of the cargo transported by dynein changes during the cell cycle, and this differential targeting is modulated during mitosis. A recent study of the distribution of myosin V during mitosis has shown that this motor is present in the spindle and midbody of dividing cells, suggesting that it too may play a role during mitosis (Espreafico et al., 1998; Wu et al., 1998b). However, the fact that dilute myosin V null mice do not exhibit gross mitotic defects suggests that if it plays a role in cell division, it is either nonessential or it is compensated by other factors (Searle, 1952).

The best understood example of unconventional myosin regulation has resulted from the study of ameboid myosin I, M yosins IA, IB, and IC from Acanthamoeba all exhibit increased actin-based motility and ATPase activity upon phosphorylation of a conserved serine or threonine present in an actin binding loop in the motors’ heads (Brzeska and Korn, 1996; Carragher et al., 1998). Bement and M ooseker, after subjecting all known myosin sequences to a comprehensive sequence comparison, noted that in all other known myosins, except for myosin VI, this residue is replaced with either glutamate or aspartate (Bement and M ooseker, 1995). This observation led them to postulate the TEDS rule, in which they hypothesized that the requirement for phosphorylation on this residue in the majority of myosins may have been evolutionarily relieved by the substitution of an acidic amino acid residue (Bement and M ooseker, 1995). If this is true, then it is logical to assume that differential regulation of other unconventional myosins, such as myosin V, could be achieved by differential attachment to the motors to their cargoes. This argument is borne out in the case of myosin V by the results of our study, as well as those of Prekeris and Terrian (1997). Other mechanisms should not be excluded, however. M yosin V is a phosphoprotein in nervous tissue and is a substrate for calcium-calmodulin activated kinase II in vitro (Larson et al., 1988, 1990). We have shown here that myosin V is phosphorylated in interphase egg extracts, albeit at lower levels than in metaphase-arrested extracts, without affecting the amount of motor bound to melanosomes.
This observation suggests the possibility that the motor possesses multiple phosphorylation sites, which may be modified differentially throughout the cell cycle. The significance of this phosphorylated state is unknown, however, and the physiological relevance of these observations has yet to be established. In addition to the dissociation of myosin V from synaptic vesicles, in vitro studies of the motor have demonstrated that its motility is inhibited by calcium and this inhibition is likely due to loss of myosin V-associated calmodulin light chains (Cheney et al., 1993). Paradoxically, calcium treatment also increases the motor’s actin-stimulated A T Pase activity (Nascimento et al., 1996). Intracellular modulation of calcium levels also may be a potential mechanism of myosin V function.

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