Myosin cooperates with microtubule motors during organelle transport in melanophores

Stephen L. Rogers and Vladimir I. Gelfand

Melanophores offer an outstanding system for the study of intracellular motility. These cells aggregate their pigment-filled melanosomes to the cell center or disperse them throughout the cytoplasm in response to hormonal modulation of intracellular cyclic AMP levels in order to effect color changes in lower vertebrates [1]. Previous work from our laboratory demonstrated a role for microtubule-based motors in melanosome transport and we succeeded in reconstituting their regulated motility along microtubules *in vitro* [2,3]. Here we demonstrate that, in addition to microtubule-mediated motility, melanosomes purified from *Xenopus* melanophores exhibit unidirectional movement along actin filaments *in vitro* as well. Immunoblotting analysis shows that these organelles possess the actin-based organelle motor, myosin-V. *In vivo*, melanosomes are able to slowly disperse in the absence of microtubules, and this slow dispersion requires the integrity of the actin cytoskeleton. Furthermore, in cells with dispersed pigment, disruption of filamentous actin induces a rapid, microtubule-dependent aggregation of melanosomes to the cell center. Our results, together with the accompanying paper by Rodionov *et al.* [4], demonstrate that the concerted efforts of both microtubule-based and actin-based motors are required for proper melanosome distribution in melanophores. This is the first example of a biochemically defined organelle in possession of both plus-end and minus-end directed microtubule motors and a myosin; coordinated activity of all three motors is essential for organelle motility *in vivo*.

Address: Department of Cell and Structural Biology, University of Illinois at Urbana-Champaign, B107 Chemical and Life Science Laboratory, 601 S. Goodwin Avenue, Urbana, Illinois 61801, USA.

Correspondence: Vladimir I. Gelfand
E-mail: vgelfand@uiuc.edu

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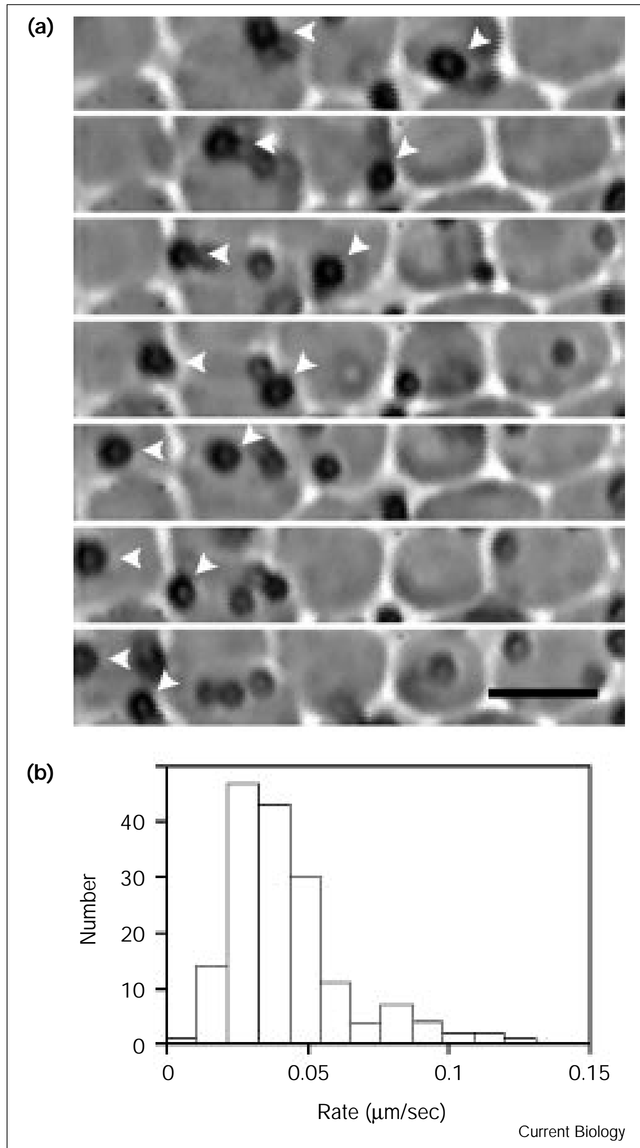
Results and discussion

Aggregation and dispersion of pigment in melanophores is mediated, at least in part, by microtubule-based motors [2,3]. However, in addition to microtubule-dependent movement, several studies have pointed to the activity of a non-microtubule-based component as well [5–7]. These observations, together with the fact that pigment granules in mammalian melanocytes are transported centrifugally by a myosin [8,9], suggest that an actin-based transport mechanism may be a ubiquitous feature of melanosome transport. This concept is also lent credence by the results of Rodionov *et al.*, who demonstrate an association of melanosomes with actin filaments in fish melanophores [4]. To test directly the hypothesis that actomyosin-driven motility contributes to melanosome transport, we examined the ability of these organelles to move along actin filaments *in vitro*. Using the *Nitella* myosin motility assay [10], we found that melanosomes purified from *Xenopus* melanophores attached to actin bundles, individually and in clusters, and moved unidirectionally in the presence of 1 mM ATP with an average velocity of 41 ± 20 nm/sec ($n = 169$) (Figure 1a,b). The polarity of this movement was identical to that exhibited by endogenous *Nitella* organelles, demonstrating that the melanosomes were transported by a motor directed towards the barbed end [11].

Several observations indicated that melanosome motility was due to a motor carried on the surface of the organelles and was not due to adsorbed *Nitella* myosin. First, the velocity of melanosome transport was three orders of magnitude slower than that of *Nitella* organelles (> 60 μm/sec) [12]. Second, pretreatment of the algae with the sulfhydryl alkylating reagent *N*-ethylmaleimide (NEM), an irreversible general inhibitor of myosins [12], abolished the faster *Nitella* organelle movements without affecting melanosome motility. Third, actin-based motility of melanosomes was completely inhibited by pretreatment with 2 mM NEM or by heating the organelles to 95°C for 5 minutes. Fourth, melanin granules that had been stripped of membrane by detergent were unable to move along actin. Melanosome movement along actin filaments *in vitro* was, therefore, driven by a motor bound to the organelle membrane.

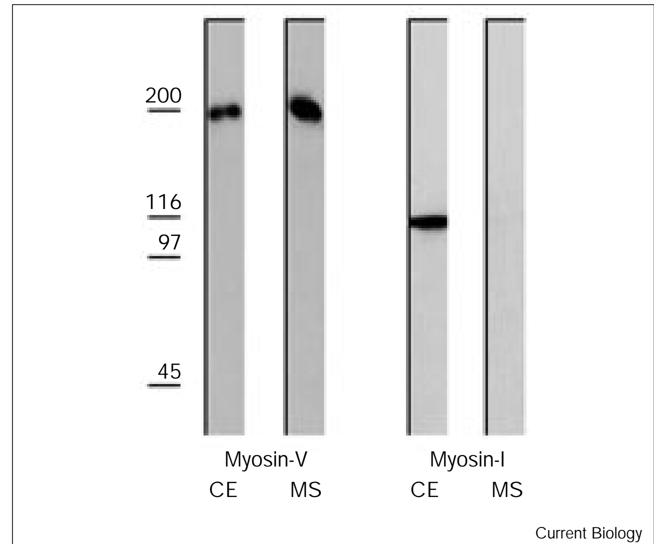
Given the abundant evidence for the involvement of myosin-V in organelle motility [13,14] and its role in pigment transport in mammalian melanocytes [8,9], this motor seemed a likely candidate for the melanosome-associated myosin. Purified melanosomes were analyzed by western blotting, using a polyclonal antibody raised against the rod domain of the mouse *dilute* gene product, myosin-V [9]. This antibody cross-reacted with a 200 kDa protein in melanophore cell extract which showed an

Figure 1



(a) Melanosomes moving on a substrate of *Nitella* actin filaments *in vitro*. Frames were taken at 100 sec intervals. Scale bar = 5 μm. **(b)** Distribution of melanosome velocities *in vitro*. Movements of 109 organelles are shown.

Figure 2



Myosin-V is present on purified melanosomes. Immunoblots probed with an antibody specific for myosin-V in cell extract (CE) and purified melanosomes (MS) show an enrichment for this motor in melanosome fractions. In contrast, myosin-I is detected only in cell extract and is excluded from melanosomes.

enrichment in purified melanosome fractions (Figure 2, lanes 1,2). To eliminate the possibility that myosin-V co-purified with melanosomes through a nonspecific association, pigment granules were also probed with an antibody raised against the tail of myosin-I [15], another membrane-associated actin-based organelle motor [16]. This antibody recognized a 115 kDa protein in cell extracts that was completely absent from melanosomes (Figure 2, lanes 3,4). Likewise, myosin-II was also found to be absent from pigment granules (data not shown). Myosin-V, therefore, selectively co-purifies with melanosomes.

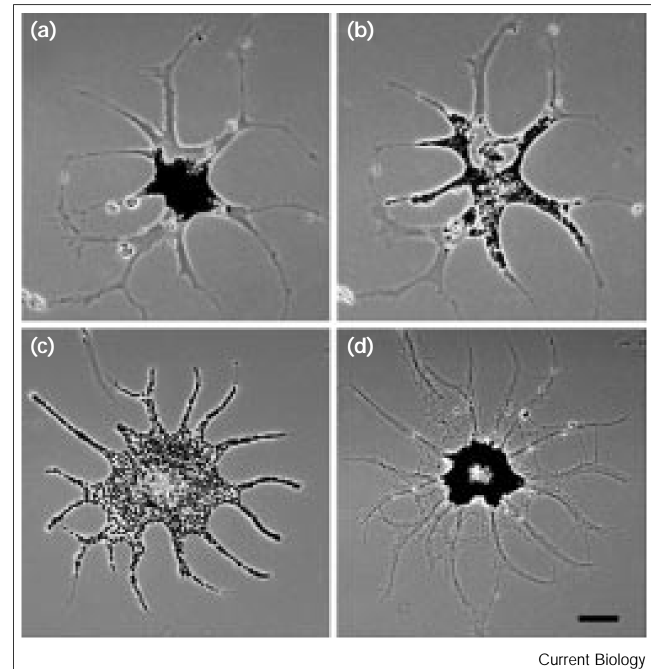
In order to determine the relative contributions of microtubules and actin to melanosome transport, we examined the intracellular motility of melanosomes in the absence of either cytoskeletal system. Melanophores were incubated at 0°C in the presence of 10 μg/ml nocodozole to induce microtubule depolymerization or perfused with 5 μM latrunculin A or 20 μg/ml cytochalasin B to depolymerize filamentous actin, and individual cells were imaged using video-enhanced time-lapse microscopy. In the absence of microtubules, melanosomes in dispersed cells exhibited undirected, shuttling movements. When treated with melatonin to induce aggregation, pigment granules in these cells formed clusters and exhibited a slow, shuttling movement towards the cell periphery, leaving the central cytoplasmic region surrounding the nucleus bare. In the converse experiment, melanophores were aggregated with melatonin prior to microtubule depolymerization. In these cells, melanosomes left the central pigment mass and made a slow migration to the cell periphery with an average velocity of 36 ± 19 nm/sec (n = 64) (Figure 3a,b). This slow dispersion was unidirectional, as opposed to untreated cells in which melanosome motility is punctuated by frequent pauses and reversal of direction. Treatment with 100 nM melanocyte-stimulating hormone (MSH), a stimulus that normally induces pigment dispersion, did not appreciably alter this movement. Melanosome motility in the absence of microtubules was not due to passive diffusion, but was actin dependent, as treatment with either 5 μM latrunculin A or 20 μg/ml cytochalasin B reversibly inhibited this movement.

The previous results implicated a role for the actin cytoskeleton in directing melanosome transport towards the cell periphery. To test this possibility, dispersed melanophores were perfused with 5 μM latrunculin A or 20 μg/ml cytochalasin B. This treatment resulted in aggregation of pigment to the cell center with a velocity of 0.29 ± 0.28 μm/sec (n = 53), even without addition of melatonin (Figure 3c,d). The rate distributions for this motility were wide, but the average corresponds well with what we have observed for normal pigment aggregation *in vivo*, and the maximal velocities induced by latrunculin treatment approached those exhibited by microtubule minus-end directed transport *in vitro* (around 1 μm/sec) [3]. Addition of 100 nM MSH to the medium did not reverse this effect, but melanosomes could be induced to redisperse upon washout and recovery from latrunculin. When these actin-perturbing drugs were applied to cells pretreated with nocodozole, however, pigment aggregation was inhibited, indicating that centripetal movement in the absence of actin is due to microtubule-based transport and not merely to cytoplasmic contraction. This result also demonstrates that the integrity of the actin cytoskeleton is necessary to maintain the dispersed state.

Our data demonstrate that the pigment granules of *Xenopus* melanophores can move along actin filaments, *in vitro* and *in vivo*. This actin-based transport appears to be necessary for pigment dispersion and maintenance of the dispersed state, as depolymerization of filamentous actin induces aggregation. Several observations lead us to believe that myosin-V is the motor involved in melanosome transport. First, this motor is selectively enriched in purified melanosome fractions; second, the rates of melanosome motility *in vitro* are consistent with velocities of myosin-V motility measured in the *Nitella* assay [17]; and third, myosin-V has been implicated in melanosome transport in mammalian melanocytes [8,9]. We postulate a model for melanosome transport in which motility along microtubules, driven by cytoplasmic dynein, is required for pigment aggregation. Melanosome dispersion and maintenance of the dispersed state, however, are mediated by the coordinated actions of a microtubule-dependent motor, kinesin-II, and an actin-dependent motor, myosin-V.

There is growing evidence that actin-based transport may be a mechanism common to many types of organelles. Squid axoplasmic vesicles are able to move along actin filaments *in vitro,* although the identities of these vesicles remain unknown [18,19]. Mitochondria use both filamentous actin and microtubules for transport in neurons, but the motor proteins responsible have yet to be determined [20]. Recently, an actin-based mechanism was implicated in pigment granule transport in retinal pigment epithelium [21]. In some instances, the

Figure 3



Melanosome transport in the absence of microtubules or filamentous actin. **(a)** Phase-contrast image of a melanophore that was induced to aggregate pigment with melatonin followed by treatment with cold and nocodozole to depolymerize microtubules. **(b)** The same cell as in (a) 60 min later. Melanosomes migrated from the central pigment mass to the cell periphery. **(c)** Melanophore with pigment dispersed. **(d)** Pigment aggregation in the same cell as in (c) following a 60 min treatment with latrunculin A to depolymerize actin. Note that the melanosomes in these images appear phase-bright despite their black pigmentation. Scale bar = 10 μm.

myosin carrying a particular organelle has been identified. For example, myosin-I has been implicated in *Dictyostelium* organelle motility [16]. This motor, along with cytoplasmic dynein, is also present on Golgi-derived membranes, where it may assist in exocytosis from the apical surface of epithelial cells [22]. As mentioned above, genetic analysis has implicated myosin-V as a motor essential for proper melanosome transport and positioning in mouse melanocytes and for vesicle motility in yeast [8,9,23].

Our results complement the study of Rodionov *et al.* [4], which shows that pigment transport along actin filaments is involved in maintenance of uniform organelle distribution in fish melanophores. The apparent difference in the polarity of transport between fish and frog cells may simply represent a difference in the polarity of actin filaments in these two systems. It is also interesting to note that in frog melanophores, the melanosome-associated myosin is essential for long-range pigment dispersion and maintenance of the dispersed state, but in fish cells, actin-mediated motility appears to have an accessory role

in short-range pigment distribution. As pigment transport in *Xenopus* melanophores requires both microtubules and filamentous actin, this process may represent an evolutionary midpoint between a microtubule-dominated mechanism in fish cells and a solely actin-based system in mammalian melanocytes.

In summary, these results represent the first direct demonstration of organelle motility requiring the activity of both plus-end and minus-end directed microtubule motors and a myosin. Further study of pigment granule transport promises to illuminate how both cytoskeletal systems are regulated and act in concert to mediate directed organelle transport in melanophores as well as in other cellular systems.

Materials and methods

Cell culture and drug treatments

Immortalized *Xenopus* melanophores were cultured as described previously [3,24] and transferred to serum-free medium 24 h prior to experiments. Pigment aggregation was induced by treatment with 10 nM melatonin, while pigment dispersion was triggered using 100 nM MSH. Microtubule depolymerization was induced by chilling the culture dishes to 0°C for 60 min in culture medium containing 10 μg/ml nocodozole. This treatment caused a complete loss of microtubules as determined by immunofluorescent staining with the monoclonal antibody DM1α (data not shown). Similarly, actin depolymerization was induced with 5 μM latrunculin A or with 20 μg/ml cytochalasin B, where described.

In vitro *motility assay*

The *Nitella* actin-based motility assay was performed as described [25]. In some experiments, *Nitella* cells were pretreated with 2 mM NEM for 30 min before microdissection into buffer containing 1 mM dithiothreitol (DTT). Melanosome purification and demembranated pigment isolation were carried out as described previously [3]. In some experiments, melanosomes were treated with 2 mM NEM for 20 min, then diluted 10-fold to an OD_{550} of 0.1 with buffer containing 2 mM DTT.

Microscopy

Live cells and in vitro assays were imaged by video-enhanced phase-contrast and bright-field microscopy using the system previously described [3].

Immunoblotting

SDS–PAGE and western blotting were performed as described [3]. The DIL2 polyclonal antibody raised against the rod domain of myosin-V (gift of J. Hammer) [9] was used at a dilution of 1:40,000. A monoclonal antibody generated against the tail of mammalian myosin-I (gift of J. Albanesi) was used at a dilution of 1:1000 [15]. The MHC-B polyclonal antibody against the carboxyl terminus of non-muscle myosin-II (gift of R. Adelstein) [26] was used at a dilution of 1:500. Blots were visualized using the SuperSignal CL-HRP chemiluminescent detection system (Pierce).

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