Heterotrimeric Kinesin II Is the Microtubule Motor Protein Responsible for Pigment Dispersion in *Xenopus* Melanophores

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Abstract. Melanophores move pigment organelles (melanosomes) from the cell center to the periphery and vice-versa. These bidirectional movements require cytoplasmic microtubules and microfilaments and depend on the function of microtubule motors and a myosin. Earlier we found that melanosomes purified from *Xenopus* melanophores contain the plus end microtubule motor kinesin II, indicating that it may be involved in dispersion (Rogers, S.L., I.S. Tint, P.C. Fanapour, and V.I. Gelfand. 1997. *Proc. Natl. Acad. Sci. USA*. 94: 3720–3725). Here, we generated a dominant-negative construct encoding green fluorescent protein fused to the stalk-tail region of *Xenopus* kinesin-like protein 3 (Xklp3), the 95-kD motor subunit of *Xenopus* kinesin II, and introduced it into melanophores. Overexpression of the fusion protein inhibited pigment dispersion but had no effect on aggregation. To control for the specificity of this effect, we studied the kinesin-dependent movement of lysosomes. Neither dispersion of lysosomes in acidic conditions nor their clustering under alkaline conditions was affected by the mutant Xklp3. Furthermore, microinjection of melanophores with SUK4, a function-blocking kinesin antibody, inhibited dispersion of lysosomes but had no effect on melanosome transport. We conclude that melanosome dispersion is powered by kinesin II and not by conventional kinesin. This paper demonstrates that kinesin II moves membrane-bound organelles.

Key words: heterotrimeric kinesin • microtubules • microtubule motors • melanophore • lysosome

A wide variety of intracellular processes depend on directed active transport of organelles along microtubules. These movements are driven by microtubule motors. These proteins have been implicated in many dynamic processes, including fast axonal transport, distribution of organelles such as the Golgi apparatus, lysosomes, and mitochondria, as well as motility of eukaryotic cilia and flagella. Although our knowledge of the genes encoding motor proteins has been increasing rapidly, their role in the cell and their regulation are still not completely understood.

Pigment cells provide a unique model for the study of intracellular transport mechanisms and motor protein function. Their major physiological task is to transport pigment granules within the cytoplasm, which allows certain animals to change their color. Pigment can be distributed in the cells in one of two configurations—either aggregated at the cell center or homogeneously dispersed in the cytoplasm. The bidirectional transport of pigment organelles during aggregation and dispersion is regulated by signaling mechanisms initiated by binding of specific hormones to cell surface receptors, which results in modulation of cAMP concentrations. For *Xenopus* melanophores, dispersion is triggered by melanocyte stimulating hormone (MSH),[1] which increases cAMP levels, whereas aggregation is stimulated by melatonin, which decreases cAMP levels (Daniolos et al., 1990). As with most chromatophores, both pigment dispersion and aggregation in melanophores are microtubule-dependent processes (Schliwa and Bereiter-Hahn, 1974; Obika et al., 1978; Schliwa, 1982; McNiven et al., 1984). As these antagonistic movements are regulated and can be easily induced experimentally,

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; KAP, kinesin-associated protein; KIF, kinesin superfamily protein; MSH, melanocyte stimulating hormone; Xklp3, *Xenopus* kinesin-like protein 3.

pigment cells constitute an attractive model for the study of regulation of microtubule transport. However, to study regulation it is first necessary to determine which motors are responsible for aggregation and dispersion.

The involvement of a member of the kinesin family of motors in pigment dispersion was first established by Rodionov and collaborators by microinjection of a function-blocking antibody against kinesin into fish melanophores (Rodionov et al., 1991). However, as the antibody used in this study was directed against the conserved kinesin motor domain, it cross-reacted not only with conventional kinesin but also with several other kinesin-like proteins (Wright et al., 1993; Johnson et al., 1994; Lombillo et al., 1995). Thus, the inhibition of dispersion observed in fish melanophores indicates only that a kinesin-like protein is involved in this process. Alternative strategies are needed to identify the specific motor proteins involved in dispersion.

Melanosomes purified from *Xenopus* melanophores are able to move along microtubules in vitro in the absence of cytosolic proteins, indicating that the microtubule motors responsible for their aggregation and dispersion are tightly associated with the organelles. Western blot analysis has demonstrated that kinesin II and cytoplasmic dynein are the microtubule motors that copurify with the melanosome fraction (Rogers et al., 1997). Since kinesin II is a plus end–directed motor (Cole et al., 1993) and dispersion of pigment in the cells corresponds to a plus end–directed movement along microtubules, these results suggest that kinesin II is a potential candidate to be the motor responsible for pigment dispersion.

Kinesin II was originally found in sea urchin eggs (Cole et al., 1992, 1993). It is a heterotrimeric protein formed by two distinct motor subunits with molecular masses of 85 and 95 kD, and a nonmotor accessory protein of 115 kD (Rashid et al., 1995; Wedaman et al., 1996). The motor subunits possess an NH_2-terminal motor domain followed by an α-helical coiled-coil region believed to be important for dimerization, and small globular COOH-terminal domains that may play a role in association with the nonmotor subunit KAP115 (kinesin-associated protein; for review see Scholey, 1996). Homologues of kinesin II have been found in mammals (Kondo et al., 1994; Yamazaki et al., 1995; Muresan et al., 1998), *Drosophila* (Stewart et al., 1991), and *Chlamydomonas* (Walther et al., 1994; Vashishtha et al., 1996). The *Xenopus* homologue of the 95-kD subunit of kinesin II, Xklp3 (*Xenopus* kinesin-like protein 3), was found by PCR screening along with other kinesin-like transcripts (Vernos et al., 1993).

To investigate the involvement of kinesin II in *Xenopus* melanophores, we generated a mutant form of Xklp3: a headless mutant in which the motor domain is substituted by the enhanced green fluorescent protein (EGFP). Overexpression of the headless mutant protein leads to a dominant-negative phenotype, dramatically decreasing the rate of pigment dispersion in *Xenopus* melanophores. This effect was direction and organelle specific, as the minus end–directed movement of melanosomes was not affected, nor was the dispersion or aggregation of lysosomes. We conclude that kinesin II is the microtubule-based motor that drives pigment dispersion in melanophores, and that this movement does not require conventional kinesin. Thus, this paper demonstrates that kinesin II is not only bound to membrane organelles, but is also a motor that transports them in vivo.

Materials and Methods

Plasmid Construction

We generated a headless Xklp3 construct in which NH_2-terminal 330 amino acid residues that form the motor domain were replaced by EGFP. A template consisting of the pBluescriptSK+ vector containing the cDNA for Xklp3 was used for PCR amplification. We designed the primers so that unique restriction sites would be added at the ends of the amplified fragments for easy insertion into eukaryotic expression vectors. The headless construct (pEGFP-headless Xklp3) encoding a fusion protein between EGFP and the Xklp3 stalk and tail was created by insertion of the Xklp3 stalk-tail cDNA into the pEGFP-C1 vector (CLONTECH Laboratories, Palo Alto, CA). The primers used were the following: GCGCGGGTACCAAGAGAGGGGTAATACCC (forward primer, including a KpnI site at the 5′ end) and CGCGCGGATCCTTATTTGGGTACGAGGCCTCGTGACTG (reverse primer, including a BamHI site at the 3′ end). The PCR-amplified fragment was digested with appropriate restriction enzymes, gel purified, and cloned into the pEGFP-C1 plasmid that had been previously cut with the same enzymes. Transcription of the construct was driven by the constitutively active cytomegalovirus (CMV) promoter. As a control for transfections and microinjections with pEGFP-headless Xklp3, we used the pEGFP-C1 plasmid encoding EGFP for easy detection of injected cells expressing exogenous protein. The predicted sequence for pEGFP-headless Xklp3 was directly confirmed by sequencing (performed by the Biotechnology Center at the University of Illinois at Urbana-Champaign).

Melanophore Cultures

Immortalized *Xenopus* melanophores (a gift of Dr. Michael Lerner, University of Texas Southwestern Medical Center, Dallas, TX) were cultured at 27°C in 0.7× Leibowitz L-15 medium (GIBCO BRL; Life Technologies, Gaithersburg, MD) supplemented with 5% fetal calf serum (HyClone Labs, Logan, UT), 5 mg/ml insulin, and 100 μg/ml each of penicillin and streptomycin (see details in Daniolos et al. [1990] and Rogers et al. [1997]). For microscopic analysis, cells were plated on acid-washed poly-L-lysine–coated coverslips in serum-containing medium. 24 h before the experiment, cells were shifted to serum-free medium. Aggregation or dispersion of pigment was induced by treating melanophores with either 10 nM melatonin or 100 nM MSH, respectively, in serum-free medium for 1 h. Cells were fixed with 4% formaldehyde, and the state of pigment distribution was scored under an upright microscope (model Microphot-SA; Nikon, Inc., Melville, NY) as follows: dispersed, aggregated, and partially dispersed/aggregated (Reilein et al., 1998). Examples are shown in Fig. 3 *A* for dispersed and 3 *B* for partially dispersed; in cells considered aggregated, the pigment forms a tight mass in the cell center (not shown). A minimum of 100 cells was counted per experiment, and each experiment was repeated at least three times.

Electroporation

Melanophores were transfected by electroporation (Graminski et al., 1993) as modified by Reilein and collaborators (1998). In brief, cells were detached with trypsin/EDTA, resuspended in medium (0.7× L-15) containing 5% fetal calf serum, pelleted, resuspended in cold 0.7× PBS, pH 7.3, pelleted again, and then resuspended in 0.2 ml of cold 0.7× PBS, pH 7.0. The cell suspension ($\sim 2 \times 10^6$ cells) was mixed with 10 μg of DNA, incubated on ice for 20 min, transferred into 2-mm electroporation cuvettes (Invitrogen, Carlsbad, CA), and electroporated at 450 V, 200 Ω, 250 μF, using a GenePulser II (Bio-Rad Laboratories, Hercules, CA). Immediately after electroporation, cells were resuspended in growth medium and plated onto 100-mm tissue culture plates. Transfected cells were replated onto polylysine-coated coverslips after 24 h. The following day, the growth medium was replaced with serum-free medium, and cells were used for the experiments 24 h later. Thus, cells were assayed for pigment dispersion and aggregation 72 h after transfection. We scored cells using a 10× lens on an upright fluorescent microscope to detect cells expressing EGFP or an EGFP fusion protein (see below). By doing this, we assured

that only cells expressing high levels of exogenous proteins (very bright) were scored.

Microinjection

Microinjection was performed essentially as described by Graessmann and Graessmann (1976). Melanophores were plated on coverslips in 35-mm dishes and were observed with an inverted microscope (model Diaphot 200; Nikon, Inc.) using a long-distance 40× phase-contrast objective lens and a long-distance condenser. Needles were pulled from borosilicate capillary tubes containing a filament with a micropipette puller (model P-97; Sutter Instrument Co., Novato, CA). DNA for nuclear injections and antibodies for cytoplasmic injections were prepared in microinjection buffer (90 mM KCl and 10 mM NaH_2PO_4, pH 7.2) and clarified by centrifugation at 265,000 *g* for 10 min immediately before use. Injections were performed with a micromanipulator (model PLI-188; Narishige, Greenvale, NY) and a Nikon microinjector. After DNA injections, cells were cultured in serum-free medium for 48–72 h before assays. After antibody injections, cells were allowed to recover for 2–4 h.

Immunofluorescent Staining and Fluorescence Microscopy

For detection of melanophores expressing EGFP or visualization of injected SUK4 antibody, cells were briefly rinsed with 0.7× PBS, fixed in 3.7% formaldehyde in 0.7× PBS for 15–30 min, and then rinsed with 0.7× PBS again. For tubulin staining of transfected cells, cultures were fixed in methanol at −20°C for 6 min before staining with the monoclonal antibody DM-1α (Blose et al., 1984) at a dilution of 1:2,000, followed by a Texas red–conjugated goat anti–mouse secondary antibody at a dilution of 1:100. For detection of cells injected with the SUK4 antikinesin antibody (Ingold et al., 1988), cells were permeabilized with staining buffer (0.1% BSA, 0.1% Triton X-100 in PBS) before staining with a FITC-conjugated anti–mouse antibody at 1:100. All antibodies were diluted in staining buffer. After staining, coverslips were mounted in Elvanol. Epifluorescence microscopy was performed with an upright microscope (model Microphot-SA; Nikon, Inc.). By doing this, we assured that only very bright cells, expressing high levels of exogenous protein, were scored. This procedure was used to minimize differences due to different levels of expression. Pictures were acquired with a cooled CCD camera (model CH250, Photometrics, Tucson, AZ) controlled by Oncor Image (Gaithersburg, MD) software. To show the pigment distribution in transfected cells, bright field and fluorescence images were overlaid using Adobe Photoshop software.

Antibodies

For immunofluorescent staining of microtubules, we used the antitubulin monoclonal antibody DM-1α (Blose et al., 1984) (ascites diluted at 1:2,000). For Western blots, we used the following antibodies: a polyclonal anti-Xklp3 prepared against the Xklp3 tail (residues 489–744) at a dilution of 1:2,000 (Le Bot et al., 1998); K2.4, a monoclonal antibody to the sea urchin 85-kD subunit of kinesin II, at 1:200 (Cole et al., 1993); a polyclonal antibody raised against mouse KIF3C at 1:1,000 (Muresan et al., 1998); and a polyclonal antibody against the sea urchin KAP115 (Wedaman et al., 1996). A function-blocking antibody against conventional kinesin (SUK4) was used for microinjections (Ingold et al., 1988). SUK4 was partially purified from mouse ascites fluid by ammonium sulfate precipitation and dialyzed against microinjection buffer (90 mM KCl and 10 mM NaH_2PO_4, pH 7.2). SUK4 was used at 10 mg/ml for microinjection experiments. All secondary antibodies were purchased from Jackson ImmunoResearch Labs (West Grove, PA). Fluorescent antibodies were used at 1:100 and HRP-conjugated antibodies at 1:10,000.

Immunoprecipitation and Western Blots

Cells transfected with pEGFP-C1 or pEGFP-headless Xklp3 were rinsed briefly with PBS to remove the medium, detached with 1 mM EDTA in 0.7× PBS, and then pelleted and resuspended in 150 μl of lysis buffer per 10-cm plate. The lysis buffer contained 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EGTA and 1 mM EDTA, and 1 mM β-mercaptoethanol, supplemented with protease inhibitors. We lysed the cells in suspension by passing them through a syringe with a 27-gauge needle as previously described (Rogers et al., 1998). The extract was spun at 16,000 *g*, and Triton X-100 was added to the supernatant to a final concentration of 1%. The extract was then spun once more at 16,000 *g* to remove nonsolubilized membranes. Extracts were precleared for 1.5 h with 10 μl of normal rabbit serum prebound to 25 μl of a 50% suspension of protein A beads (Sigma Chemical Co., St. Louis, MO). The precleared extracts were incubated for 12 h at 4°C with 5 μg of polyclonal antibody against GFP (gift of Dr. Kevin Sawin, Cell Cycle Laboratory, Imperial Cancer Research Fund, London, UK). Pelleted beads were washed twice with lysis buffer, once with 0.3 M NaCl in lysis buffer, and once more with lysis buffer. Beads were resuspended in 15 μl of Laemmli sample buffer (Laemmli, 1970).

Proteins from melanophore extracts, pure melanosomes (Rogers et al., 1998), or immunoprecipitates were run on 6–10% gradient SDS-PAGE gels and transferred to nitrocellulose (Towbin et al., 1979). Blots were blocked overnight in 0.1% Tween in TBS (TBST) supplemented with 5% nonfat dry milk. The blots were then incubated for 30 min with primary antibodies, rinsed three times in TBST, incubated for 30 min with HRP-conjugated secondary antibodies, and rinsed three more times. All antibodies were diluted into TBST with 5% dry milk. SuperSignal (Pierce Chemical Co., Rockford, IL) was used for secondary antibody detection.

Video Microscopy

For real-time analysis of pigment movement, cells were plated onto poly-L-lysine–coated coverslips that were sealed to holes at the bottom of 35-mm plates. Cells were observed by bright-field microscopy using a microscope (model Diaphot 300; Nikon, Inc.) equipped with a 40×, 1.0 NA oil immersion lens. To minimize the effect of light on pigment movement, the light source was additionally equipped with a red filter with a cut-off of 695 nm (Chroma Technology, Brattleboro, VT), as *Xenopus* melanophores react to visible light of wavelength less than 550 nm by dispersing pigment (Daniolos et al., 1990). Images were captured with a Newvicon camera (model C2400-07; Hamamatsu City, Japan) and processed for background subtraction and contrast enhancement using an Argus-10 video processor (Hamamatsu Photonics, Hamamatsu City, Japan). Electronic contrast enhancement allowed visualization of cells without the use of phase-contrast or any other optical method of contrast generation, thus minimizing the amount of light required to visualize melanophores. Pigment migration in individual cells was recorded onto super-VHS videotapes with a time-lapse S-VHS recorder (Panasonic, Secaucus, NJ). Video sequences were digitized and subsequently analyzed using the NIH Image software version 1.61 (National Institutes of Health, Bethesda, MD).

To evaluate total pigment distribution, the ratio of the area of cytoplasm occupied by the pigment mass to the total cell area was calculated. As the pigment area increases, it approaches the total cell area, and in fully dispersed cells these areas are the same (ratio = 1). The areas were obtained by manual tracing of the boundaries of a cell and of its pigment in 30 frames (1 frame every 2 min) of each video sequence, using NIH Image version 1.61. These ratios were plotted versus time, and from these curves, the time points corresponding to 50% dispersion (midpoint between the initial ratio and 1) or aggregation were calculated. The half-time values from each individual cell are shown in Table I. A minimum of five cells was analyzed for each experimental condition (n = 5–10).

Table I. Overexpression of Headless Xklp3 Decreases the Rate of Pigment Dispersion

Cell group	Average half-times for dispersion and aggregation ± SD	
	Dispersion	Aggregation
	min	
Nontransfected	7.0 ± 2.9‡	19.0 ± 9.8*
pEGFP-C1	7.5 ± 1.4‡	20.6 ± 10.8*
pEGFP-headless Xklp3	16.9 ± 7.1§	22.8 ± 9.5*
Nocodazole-treated	14.0 ± 4.7§	ND

*There is no significant difference between the values of half-times for aggregation for any two groups among them all. (Aggregation does not occur in the absence of microtubules, and therefore aggregation rates were not determined in nocodazole-treated cells.)

‡The half-times for dispersion for nontransfected cells and cells transfected with pEGFP-C1 are not significantly different.

§The half-time for dispersion for cells expressing headless Xklp3 or treated with nocodazole are significantly different from those obtained in nontransfected or EGFP-expressing cells ($P < 0.01$).

The movement of individual melanosomes was tracked and classified into two categories: slow movements in random directions and fast directed saltations. A movement was classified as a rapid saltation when it occurred on a straight line at a rate higher than 0.1 μm/s. The saltations were further classified as directed toward the cell periphery (plus saltations) or toward the cell center (minus saltations). In each of 15 cells analyzed by this protocol for each experimental treatment, 20 melanosomes were randomly selected and the number of plus and minus saltations was counted over a period of 5 min.

Lysosome Labeling and Motility Assay

A pH-based lysosome motility assay (Heuser, 1989) was modified for melanophores. Lysosomes were labeled with 0.05 mg/ml Texas red dextran (Molecular Probes, Eugene, OR). Because pinocytosis in melanophores occurs at a very low rate, consistent lysosome labeling required incubation of cells with dextran for 48 h. To induce lysosome movement, cells were incubated for 45 min in one of the experimental solutions described below and then fixed in 3.7% formaldehyde. Three experimental solutions were used: normal frog Ringer's solution (115 mM NaCl, 2.6 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.6), acidification solution (45 mM NaCl, 70 mM Na acetate, 2.6 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes, pH 6.9), and alkalinization solution (20 mM NH_4Cl in normal Ringer's solution, pH 7.6).

For quantitation of lysosomal distribution, we measured the distance from each lysosome in the cell to the cell center using the NIH Image software. Average distances were calculated for each cell and then divided by the square root of the cell area to normalize for differences in cell size. We analyzed a minimum of five randomly selected cells per treatment (alkaline or acidic) for each experimental group, using cells from at least two independent experiments.

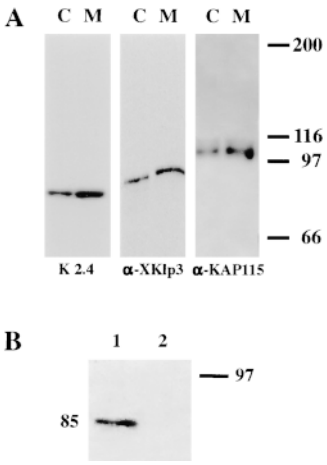

Figure 1. (*A*) All subunits of the heterotrimeric motor protein, kinesin II, are present on melanosomes. Western blots of cell extracts and purified melanosomes probed with antibodies against the motor subunits of kinesin II. Lanes *C* are cell extracts, and lanes *M* are purified melanosomes. The first two lanes were probed with K2.4 to detect the 85-kD motor subunit, the following two lanes were probed with a polyclonal anti-Xklp3 to detect the 95-kD motor subunit, and the last two lanes were probed with a polyclonal anti–sea urchin KAP 115 to detect the 115-kD accessory subunit. Notice that all the subunits are enriched in the melanosome fraction. (*B*) The overexpressed EGFP-headless Xklp3 protein forms a complex with the endogenous 85-kD motor subunit. Melanophore extracts were prepared from cells transfected with pEGFP-C1 or pEGFP-headless Xklp3, immunoprecipitated with an anti-GFP antibody, and probed with K2.4 to detect the 85-kD motor subunit. Shown are Western blots from both immunoprecipitates. The 85-kD subunit coimmunoprecipitates with EGFP-headless Xklp3 (lane *1*), but not with EGFP alone (lane *2*).

Results

All Three Subunits of Kinesin II Are Present on Melanosomes

To investigate the role of kinesin II in dispersion of melanosomes, the first step was to show that this protein is actually present on isolated organelles. Previous Western blotting analysis of isolated *Xenopus* melanosomes with the monoclonal antibody K2.4, which specifically recognizes the 85-kD motor subunit of kinesin II, showed the presence of this motor in the organelle fraction (Rogers et al., 1997). The presence of the 95-kD motor subunit was only verified with a pan-kinesin antibody, HIPYR, which recognizes motor domains of many kinesin-like proteins (Rogers et al., 1997). However, the 85-kD subunit of mouse kinesin II (KIF3A) can form complexes with two different motor subunits of ~95 kD: KIF3B and KIF3C (Muresan et al., 1998; Yang and Goldstein, 1998). Therefore, we needed to know which of these two 95-kD subunits associates with the 85-kD subunit in the complex copurified with melanosomes. To answer this question, we probed Western blots of purified melanosomes with antibodies directed against the two motor subunits known to form a complex with the 85-kD subunit: an antibody against Xklp3, the *Xenopus* homologue of KIF3B, and an antibody against mouse KIF3C. Western blots showed that Xklp3 is present not only in the cell extract, but also on purified melanosomes (Fig. 1). The homologue of KIF3C in *Xenopus* is present in the extract, but unlike Xklp3, it does not copurify with melanosomes (data not shown). Thus, Xklp3, and not the *Xenopus* form of KIF3C, is the 95-kD motor subunit that, together with the 85-kD subunit, is present on melanosomes. Xklp3 has been fully characterized and shown to share a high degree of homology with sea urchin KRP95 and with mouse KIF3B. The motor domain has 90% amino acid similarity with KIF3B, suggesting that Xklp3 is the *Xenopus* counterpart of mouse KIF3B (Le Bot et al., 1998). Also, like the mouse and the sea urchin proteins, Xklp3 forms a complex with another motor subunit of 80 kD, and with a 100-kD accessory protein that cross-reacts with an antibody against the sea urchin KAP115 (Le Bot et al., 1998). To test whether the accessory subunit was present on melanosomes, we used a polyclonal antibody against the sea urchin KAP115 and verified that the third subunit of the kinesin II complex also copurifies with the pigment organelles. Thus, the presence of all the three subunits of kinesin II on melanosomes represents a strong indication that kinesin II might play a role in transporting these organelles.

Overexpression of Headless Xklp3 Inhibits Pigment Dispersion

To target the role of kinesin II in pigment transport, we generated a dominant-negative construct that encodes an EGFP-tagged headless form of Xklp3 (pEGFP-headless Xklp3). This headless form of the protein lacks the motor domain but contains the domain responsible for dimerization with the 85-kD subunit (Rashid et al., 1995). Consequently, it should complex with the 85-kD subunit but be unable to hydrolyze ATP and generate movement. The functional significance of dimerization of motor subunits, as proposed for conventional kinesin (Hackney, 1994; Gilbert et al., 1995; Vale et al., 1996), may be to allow the complex to move processively along microtubules in a "hand-over-hand" fashion, transporting its vesicular cargo

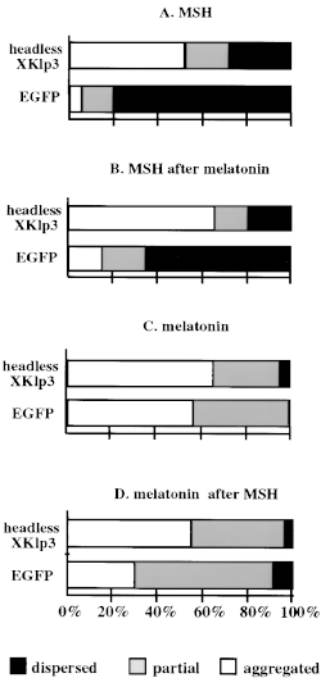

Figure 2. Quantitative analysis of pigment distribution in cells transfected with either control DNA (pEGFP-C1) or headless Xklp3 (pEGFP-headless Xklp3). Melanophores were transfected by electroporation and plated on coverslips, and after 72 h of expression, cells were incubated in serum-free medium containing MSH (*A*) or melatonin (*C*) for 1 h. In sequential treatments (*B* and *D*), cells were incubated for 1 h in melatonin followed by 1 h in MSH (*B*) or vice versa (*D*). The horizontal axis shows the percentage of cells that were scored as aggregated (*white*), partially dispersed (*gray*), or dispersed (*black*). For each treatment, 100 cells were scored. Data shown here are representative from one of four independent experiments.

(Cole and Scholey, 1995). As this mutant protein is overexpressed, it should compete with the endogenous Xklp3 pool for dimerization with the 85-kD subunit, thus behaving as a dominant-negative. We transfected melanophores with pEGFP-headless Xklp3 or the control plasmid pEGFP and used an anti-GFP antibody to immunoprecipitate the expressed proteins. The efficiency of transfection was typically ~10% for the headless Xklp3 construct and ~50% for pEGFP-C1. Immunoprecipitates were probed by Western blotting with K2.4, a mouse monoclonal antibody that recognizes the 85-kD subunit of kinesin II. Fig. 1 *B* shows that the anti-GFP antibody immunoprecipitated the 85-kD protein from headless transfected cells, but not from EGFP-transfected cells. Thus, the headless 95-kD subunit of kinesin II forms a complex with the 85-kD subunit, and therefore it can compete with the endogenous pool of 95-kD subunit for binding to the other components of the heterotrimeric kinesin II. Identical results were obtained in the accompanying paper with a similar headless Xklp3 construct (Le Bot et al., 1998). 72 h after transfection, cells were treated with MSH to induce pigment dispersion, or with melatonin to induce aggregation. Upon treatment with MSH, a smaller percentage of cells expressing headless Xklp3 had their pigment dispersed, compared with control cells expressing EGFP (Fig. 2 *A*). When transfected cells were induced to aggregate pigment by melatonin and then stimulated to redisperse in response to MSH, the number of headless-expressing cells with fully dispersed pigment was further reduced compared with EGFP-expressing cells (Fig. 2 *B*). Overexpression of wild-type Xklp3 did not have any effect on the ability of the cells to disperse their pigments (data not shown).

Comparison between control cells transfected with pEGFP-C1 and nontransfected cells showed that transfected cells had slightly reduced ability to disperse pigment in response to MSH, suggesting that electroporation has a subtle effect on the ability of cells to disperse pigment. To verify that our results did not reflect the side effects of electroporation, we used nuclear microinjection as an alternative way to introduce exogenous DNA into cells. Injected cells were kept in serum-free medium for 72 h and then induced to disperse or aggregate pigment with MSH or melatonin, respectively. Analysis of pigment distribution in microinjected cells confirmed the results obtained with electroporation. MSH treatment of cells overexpressing headless Xklp3 induced no more than 30% of the cells to completely disperse their pigment, as opposed to 70–80% of the cells expressing EGFP-C1 (data not shown).

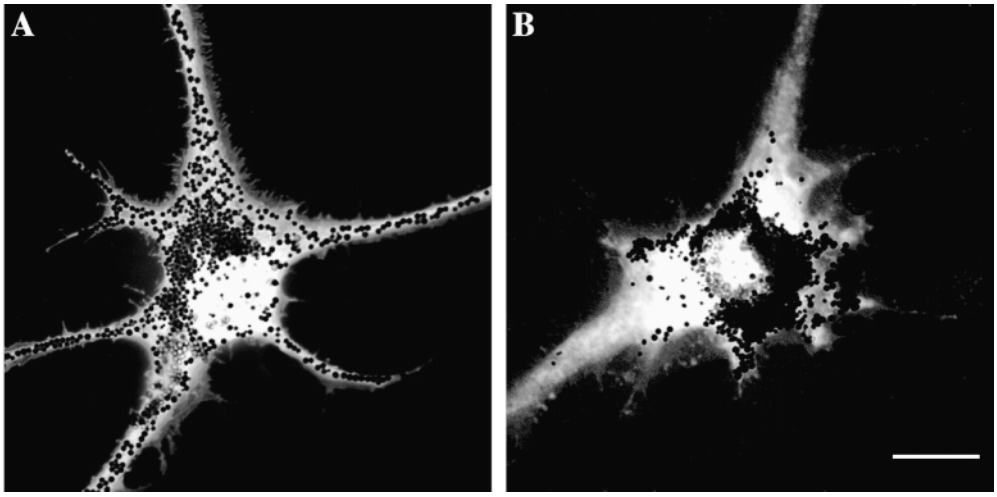

Figure 3. Overexpression of headless Xklp3 inhibits pigment dispersion. Cell nuclei were injected with plasmids pEGFP-C1 (*A*) or pEGFP-headless Xklp3 (*B*). Cells were allowed to recover and express exogenous protein for 48 h and then treated with melatonin for 1 h to induce pigment aggregation, followed by 1 h in MSH, to disperse pigment. A cell expressing EGFP (*A*) dispersed its pigment normally, while a cell expressing the EGFP-headless Xklp3 fusion protein (*B*) dispersed melanosomes only partially. Bright field and fluorescence images of cells were overlaid to show pigment distribution in cells expressing EGFP-tagged proteins. Bar, 20 μm.

Representative images of cells injected with pEGFP-C1 and pEGFP-headless Xklp3 are shown in Fig. 3.

Since a minor fraction of cells expressing headless Xklp3 for 72 h were still able to disperse the pigment, we tested whether longer expression of the mutant protein would result in stronger inhibition because of a more complete displacement of wild-type Xklp3 protein from the motor complex. We compared dispersion of pigment in melanophores expressing headless Xklp3 for different periods of time and found that even after 10 d of expression of the mutant protein, ~20–30% of the cells were still able to disperse the pigment (not shown). These results indicate that either the endogenous motor was not completely displaced by the headless protein or that melanophores can use an alternative, although significantly less effective, mechanism of pigment dispersion (see below).

If suppression of dispersion, which corresponds to the movement of melanosomes to the plus ends of microtubules, is indeed explained by inhibition of kinesin II, then movement of melanosomes toward the minus ends of microtubules should not be affected. We therefore tested whether or not the headless Xklp3 had any effect on pigment aggregation. We found that the majority of the headless-expressing cells and EGFP-expressing cells aggregated pigment in response to melatonin alone (Fig. 2 *C*) or after dispersion with MSH (Fig. 2 *D*). Therefore, the effect of overexpression of headless Xklp3 is highly specific for plus end motility along microtubules.

Since pigment aggregation in melanophores depends on microtubules and transfected cells aggregate their pigment normally, microtubules are most likely not affected by overexpression of headless Xklp3. To directly confirm this, microtubules were stained with an antitubulin antibody. Cells overexpressing headless Xklp3 showed normal density and pattern of microtubule organization, with individual microtubules radiating from the cell center and forming a highly organized polar array (Fig. 4). Hence, overexpression of headless Xklp3 specifically interfered with the plus end motility involved in dispersion, but affected neither microtubule organization nor pigment aggregation. Taken together, these results show a direct role of kinesin II in melanosome dispersion.

Pigment Dispersion Rates Are Much Slower in Cells Expressing Headless Xklp3

Since we observed that a small fraction of cells overexpressing the headless form of Xklp3 were still able to disperse their pigment in response to MSH in 60 min (Fig. 2, *A* and *B*), we examined whether the duration of MSH treatment would change this behavior. Longer incubations in MSH only slightly increased the proportion of cells expressing mutant Xklp3 with fully dispersed pigment. Even after a 24-h incubation in MSH, no more than 20–30% of the cells were fully dispersed. The presence of partially or completely dispersed cells after prolonged incubations with MSH suggests that mutant cells can possibly disperse melanosomes, although much less efficiently. To confirm this suggestion, we used video microscopy.

We characterized pigment movement in individual transfected cells by means of time-lapse video microscopy. This analysis demonstrated that the character of melanosome movement is dramatically altered in cells overexpressing headless Xklp3. Spreading of the pigment mass to the periphery of the cells in the presence of MSH was much slower in cells overexpressing headless Xklp3 than in cells expressing EGFP. To quantitate this difference, we measured the ratio of the area occupied by the pigment mass to the total cell area and plotted this ratio over time. Fig. 5 *A* shows typical plots for nontransfected cells and cells transfected with pEGFP-C1 and pEGFP-headless Xklp3. These curves demonstrate that cells expressing headless Xklp3 are indeed able to disperse their pigment, but at a much slower rate. From these curves, we calculated the time required for individual cells to reach 50% of maximal dispersion (midpoint between the initial ratio at the fully aggregated state and the final ratio of 1, equivalent to complete dispersion). The values obtained for cells transfected with pEGFP-headless Xklp3 (8–20 min) were substantially higher than the values for control cells (4–6 min) (Table I). The rates of pigment aggregation in headless-expressing cells were similar to those of control EGFP-expressing cells (Fig. 5 *B*, Table I). This confirms the indication obtained with the fixed time point assay that the effects of overexpression of headless Xklp3 are specific for dispersion.

Kinetic studies of pigment mass motion showed that dispersion is specifically delayed in cells expressing headless Xklp3. To understand the role of Xklp3 in movement of pigment organelles, we used the same video sequences to analyze the motility of individual melanosomes. In control nontransfected cells and in cells expressing EGFP, dispersion consists of slow and random movements of individual melanosomes interspersed with fast saltations in radial directions (toward or away from the cell center). For quantitation of movement, we considered fast saltations all those with rates higher than 0.1 μm/s, although most of them were faster than 0.2 μm/s. The frequency of fast saltations

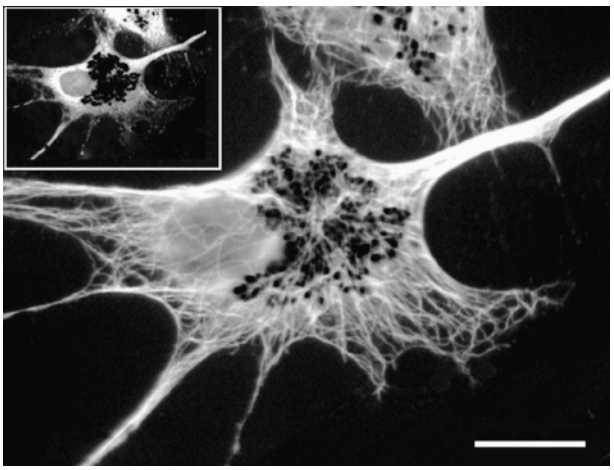

Figure 4. Distribution of microtubules is not affected by expression of headless Xklp3. Immunofluorescence staining with an antitubulin primary antibody followed by a Texas red secondary in a melanophore overexpressing the headless Xklp3. Microtubules radiate from the cell center, forming a highly organized polar array. The inset is a fluorescence image taken at the green channel, showing that the cell in the large picture expresses the EGFP-headless Xklp3 fusion protein. Bar, 20 μm.

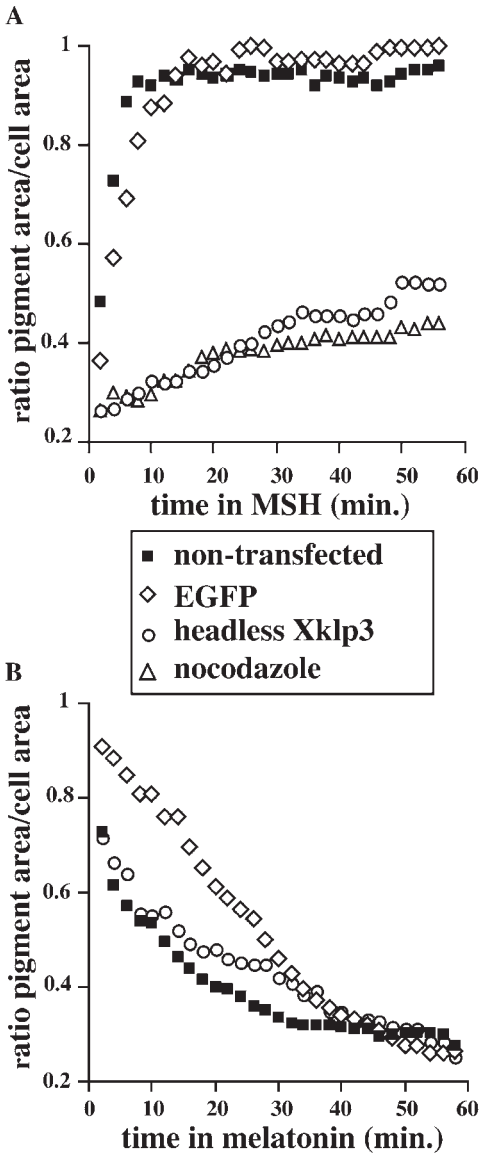

Figure 5. Headless Xklp3 affects the kinetics of pigment dispersion but not aggregation. Video-microscopic analysis of pigment movement was conducted on nontransfected melanophores, on melanophores 72 h after transfection, or immediately after treatment with nocodazole (see detailed description in Materials and Methods). Representative plots of changes in pigment distribution, determined by the ratio of pigment area to total cell area over time: (*A*) dispersion; (*B*) aggregation.

directed to the cell periphery (plus saltations) was reduced by ~50% in cells overexpressing EGFP-headless Xklp3, compared with the control cells overexpressing EGFP (Table II), while the frequency of saltations toward the microtubule minus ends was not reduced, but rather slightly increased (Table II).

As discussed above, electroporation with any DNA slightly affects movement of pigment granules in both directions, but the comparison between melanosomes from cells expressing EGFP-headless Xklp3 and EGFP shows that in addition to this nonspecific effect, the headless protein induced strong inhibition of plus end–directed motility without affecting minus end–directed movement.

Table II. Overexpression of Headless Xklp3 Decreases the Frequency of Melanosome Saltations

	Saltations	
Cell group	Plus	Minus
Nontransfected	91	63
pEGFP-C1	62	47
pEGFP-headless Xklp3	34	68
Colcemid-treated	13	12

Individual melanosomes were analyzed by video microscopy. 20 melanosomes were analyzed per cell, from three cells per treatment. Each melanosome was observed for a period for 5 min, during which the number of saltations was counted. The numbers per treatment are the total number of saltations in all three cells (i.e., 80 melanosomes).

There are two possibilities to explain the fact that cells overexpressing headless Xklp3 can slowly disperse pigment. The slow dispersion may reflect residual activity of microtubule motors (incomplete displacement of wild-type kinesin II, activity of single-headed kinesin II, or activity of another motor that could be involved in dispersion). Otherwise, it can be explained by the presence of a microtubule-independent mechanism. To investigate this second possibility, we used colcemid and nocodazole treatment to depolymerize microtubules in melanophores and thus, by definition, completely stop all microtubule-dependent transport. Staining with an antibody against tubulin showed that incubation of melanophores with 10 μM colcemid or 10 μM nocodazole for 1 h at 4°C depolymerizes all microtubules—only short fragments of microtubules were randomly scattered in the cytoplasm of less than 5% of all cells (data not shown). Moreover, transfer of cells from 4 to 25°C in the presence of colcemid or nocodazole did not result in repolymerization of microtubules. To analyze pigment dispersion in the absence of microtubules, melanosomes were aggregated by melatonin treatment before microtubule depolymerization, and cells were transferred to 25°C and treated with MSH, in the presence of colcemid or nocodazole. Real-time analysis showed that unlike control cells, dispersion in colcemid-treated cells occurs by random slow movements of melanosomes. In these cells, rapid radial saltations of organelles either toward the periphery or to the cell center were virtually absent (Table II). Comparison of the kinetics of pigment dispersion in nocodazole-treated cells and cells overexpressing headless Xklp3 (Fig. 5 *A*, Table I) showed that both occur with similar rates. These data show that the slow dispersion observed in cells expressing headless Xklp3 can not be explained by the residual activity of a plus end–directed microtubule motor, but rather by the existence of a microtubule-independent mechanism. The presence of such a mechanism is further supported by recent data showing the involvement of actin filaments and myosin V in the movement of melanosomes in *Xenopus* and teleost melanophores (Rodionov et al., 1998; Rogers and Gelfand, 1998).

Headless-Xklp3 Does Not Affect Microtubule-dependent Movement of Lysosomes

Overexpression of headless Xklp3 inhibited pigment dis-

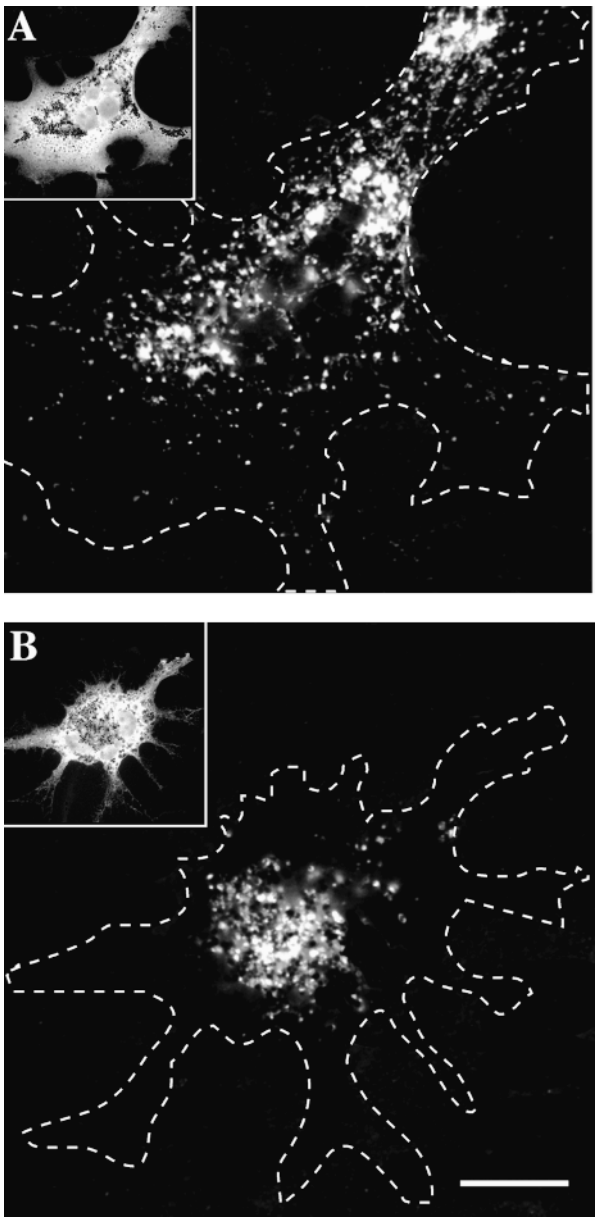

Figure 6. Distribution of lysosomes is not affected in melanophores overexpressing headless Xklp3. Cells exhibit normal lysosomal movement in response to changes in pH (dispersed in acidic conditions, clustered in alkaline conditions). Upon treatment with low pH, lysosomes redistribute through the entire cytoplasm (*A*). Normal perinuclear distribution is reestablished upon treatment with high pH (*B*). Transfected cells expressing the EGFP-headless Xklp3 fusion protein are shown in the insets, while in the large pictures the cell outlines are traced, and lysosomes stained with Texas red dextran are shown. Bar, 20 μm.

Table III. Effect of Headless Xklp3 and SUK4 on Lysomal Movement

	Treatment	
Cell group	Acidic	Alkaline
Nontransfected*	0.35 ± 0.039	0.21 ± 0.044
pEGFP-C1*	0.39 ± 0.095	0.18 ± 0.038
pEGFP-headless Xklp3*	0.36 ± 0.054	0.18 ± 0.012
SUK4-injected‡	0.24 ± 0.050	0.22 ± 0.072

Average distances between lysosomes and the cell center were measured in nontransfected/noninjected cells, cells transfected with either pEGFP-C1 or pEGFP-headless Xklp3, and cells injected with SUK4 (10 mg/ml). To compensate for cell size differences, the averages were normalized by being divided by the square root of the cell area.

*For each group, acidic and alkaline are significantly different ($P < 0.01$); there is no significant difference between any two groups (exception of SUK4) under the same treatment.

‡Acidic and alkaline are not significantly different ($P < 0.05$); SUK4 acidic significantly differs from nontransfected acidic ($P = 0.01$).

persion but had no effect on pigment aggregation and therefore interfered specifically with the plus end-directed microtubule transport of these organelles. If the pEGFP-headless XKlp3 acts as a dominant-negative mutant, it should only inhibit movement mediated by kinesin II but not by other plus-end directed microtubule motors. To test this assumption, we analyzed the pH-induced movement of lysosomes along microtubules, which is driven by conventional kinesin (Hollenbeck and Swanson, 1990; Nakata and Hirokawa, 1995; Tanaka et al., 1998). Movement of lysosomes in living cells can be induced by pH shifts: an increase in pH leads to clustering of lysosomes in the perinuclear area of the cytoplasm, whereas a decrease in pH leads to their dispersion throughout the cytoplasm (Heuser, 1989). This assay was adapted to melanophores (see Materials and Methods) and used to evaluate the specificity of the effect of headless Xklp3.

In nontransfected melanophores, lysosomes are normally clustered near the nucleus. Incubation in acidic conditions leads to their dispersion throughout the cytoplasm, and this movement is reversed by alkaline conditions (data not shown). These responses to pH were not changed in melanophores expressing either EGFP or headless Xklp3 (Fig. 6). We quantitated the distribution of lysosomes by measuring the average distance between them and the cell center (see Materials and Methods). For all groups (nontransfected, transfected with pEGFP-C1, or transfected with pEGFP-headless Xklp3), the normalized average distance between the lysosomes and the cell center was substantially higher in acidic than in alkaline conditions (Table III). Moreover, there was no difference in the average normalized distance between the groups either in alkaline or in acidic conditions (Table III). Thus, neither dispersion nor aggregation of lysosomes was affected by overexpression of headless Xklp3, indicating that the effect observed on melanosomes is specific and that movements powered by conventional kinesin are not affected by the dominant-negative kinesin II.

Movement of Melanosomes Does Not Depend on Conventional Kinesin

The experiments described above implicate the involvement of kinesin II in pigment dispersion. However, conventional kinesin, in addition to kinesin II, could play a role in this process. Although conventional kinesin is absent from purified melanosomes, it is possible that it is lost from the organelle fraction during isolation or that it is only present at levels undetectable by immunoblotting. The role of conventional kinesin in melanosome transport must be addressed in light of the findings that function-blocking antibodies raised against the kinesin heavy chain prevent pigment dispersion (Rodionov et al., 1991). Since

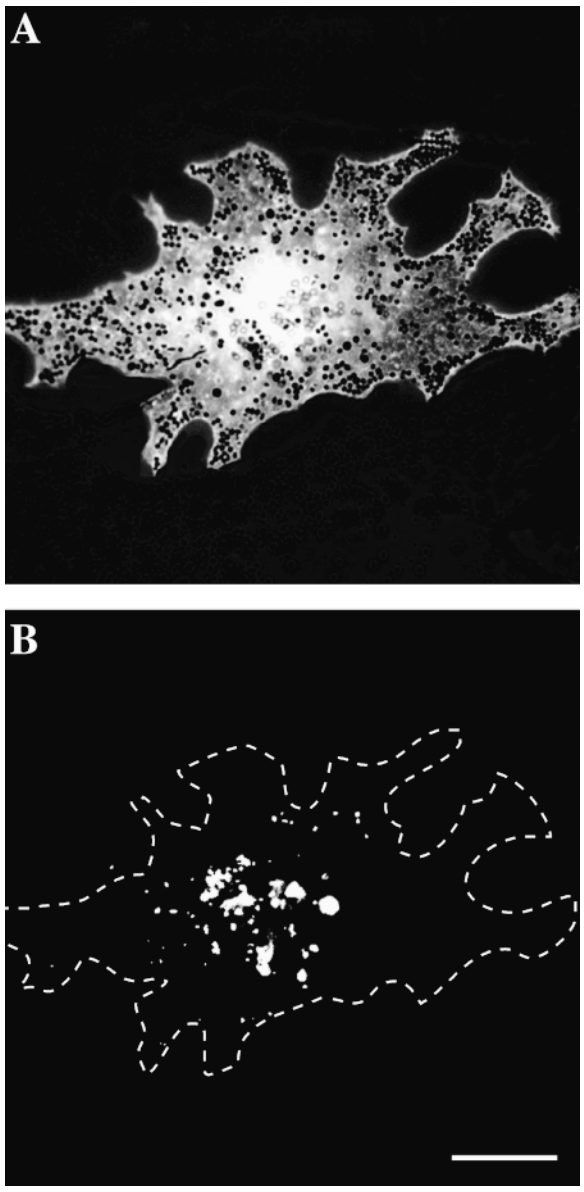

Figure 7. SUK4 inhibits dispersion of lysosomes but does not affect melanosomes. Melanophores with labeled lysosomes were injected with SUK4 and then exposed to acidic conditions in presence of MSH. Injected cells were identified by staining with a rhodamine anti–mouse antibody. (*A*) Overlay of bright field and fluorescence images, showing that melanosomes are normally dispersed in an injected cell. (*B*) Clustered distribution of lysosomes, labeled with FITC-dextran, in acidic conditions. Instead of normally dispersed through the cytoplasm, they remain clustered in the cell center. Bar, 20 μm.

then, however, it has become clear that the polyclonal antibody used in those experiments (HD) cross-reacts with other members of the kinesin superfamily, possibly including kinesin II (Wright et al., 1993; Johnson et al., 1994; Lombillo et al., 1995). To determine the role, if any, of conventional kinesin during melanosome dispersion, we examined the behavior of pigment granules in cells microinjected with SUK4, a monoclonal antibody that specifically blocks the function of conventional kinesin (Ingold et al., 1988).

Melanophores were injected with SUK4, allowed to recover for 4 h, and then were incubated in melatonin to induce pigment aggregation, followed by MSH, to induce dispersion. Dispersion of melanosomes was not affected by the antibody injections (Fig. 7 *A*). Although SUK4 recognizes conventional kinesin from *Xenopus* melanophores on Western blots (Rogers et al., 1997), we did not know if this antibody would inhibit its motor activity, as all the inhibition data were obtained with either sea urchin or mammalian kinesins (Hollenbeck and Swanson, 1990; Wright et al., 1993; Bi et al., 1997). We therefore wanted to determine if SUK4 inhibits organelle movement powered by *Xenopus* kinesin. To do so, we analyzed lysosomal movement in cells injected with SUK4. Lysosomes were labeled with Texas red dextran, and then cells were injected with SUK4. The pH shift assay performed 4 h after injection. Lysosome dispersion in response to low pH was strongly inhibited by SUK4 (Fig. 7 *B*), but aggregation of these organelles to the cell center in response to high pH was not affected, as reflected by the quantitative measurements of lysosomal distribution (Table III). We conclude that conventional kinesin does not play a role in the dispersion of melanosomes.

Discussion

The main goals of this study were to establish the role of kinesin II in pigment dispersion and to determine whether conventional kinesin was involved in this process. We replaced the motor domain of Xklp3 with EGFP to generate a dominant-negative mutant of kinesin II. This strategy has been previously used for the study of motor protein functions (Burns et al., 1995; Bi et al., 1997; Arhets et al., 1998) and has proven a useful tool in our system. Transfected melanophores could be readily identified, and overexpression of this headless protein had a clear effect: it dramatically decreased the rate of pigment dispersion. This effect was direction specific, as no alteration was observed on pigment aggregation in transfected cells. We conclude that kinesin II is indeed required for dispersion of melanosomes. Thus, not only is this motor bound to membrane organelles, but it also moves them along microtubules.

We tested the involvement of conventional kinesin in pigment dispersion by microinjecting melanophores with a function-blocking antibody against kinesin, SUK4 (Ingold et al., 1988; Hollenbeck and Swanson, 1990; Bi et al., 1997). SUK4 had no effect on movement of melanosomes, even though dispersion of lysosomes was blocked in injected cells, confirming that the antibody is effective in blocking kinesin-dependent motility in *Xenopus* melanophores. Hence, we conclude that conventional kinesin is not involved in pigment dispersion. Earlier, the role of a kinesin-like protein in pigment dispersion was established by injection of teleost melanophores with HD, a function-blocking antibody raised against the kinesin motor domain (Rodionov et al., 1991). We see two possibilities to explain the differences between the HD and SUK4 results. First, it is possible that whereas teleosts use conventional kinesin, *Xenopus* uses kinesin II. Second, and more likely, the results obtained with HD could reflect inhibition of more than one kinesin-like motor. As HD targets the motor do-

main, which is highly conserved among the different members of kinesin superfamily, it is possible that it cross-reacts with one or more kinesin-related motors other than conventional kinesin. Support for this explanation comes from other studies in which HD blocked a mitotic kinesin-like protein (Wright et al., 1993) and CENP-E (Lombillo et al., 1995) and also recognized a flagellar kinesin-like protein (Johnson et al., 1994). Considering the effects of HD in many systems and our results with SUK4, we conclude that kinesin II, not conventional kinesin, is involved in pigment dispersion.

The dominant-negative effect of headless Xklp3 on pigment dispersion implies that the motility of organelles powered by other plus end–directed microtubule motors should not be affected by overexpression of the mutant protein. To test this assumption, we used the kinesin-dependent movement of lysosomes along microtubules (Hollenbeck and Swanson, 1990; Nakata and Hirokawa, 1995). Transport of these organelles in response to pH was not affected by overexpression of the headless Xklp3, implying that the dominant-negative effects are specific for kinesin II–based transport. Hence, this construct should prove to be a very reliable tool to investigate putative functions of Xklp3 in other cell types. In fact, a similar headless construct has been used to study the involvement of Xklp3 on the transport of Golgi vesicles (Le Bot et al., 1998).

Structural, genetic, and biochemical data support the idea that melanosomes are specialized lysosomes (for reviews see Orlow, 1995; Schraermeyer, 1995). Both organelles express many proteins in common, such as the lysosomal protein LAMP-1 and several hydrolases (Luo et al., 1994; Diment et al., 1995). Despite the same origin, melanosomes do not respond to changes in pH, and lysosomes do not move in response to melatonin or MSH. Moreover, the two organelles use different plus end–directed microtubule motors for dispersion. This implies that at some point during the process of organelle differentiation, different receptors are expressed on the surface of lysosomes and melanosomes, allowing the specific docking of kinesin or kinesin II, respectively. The development of two independent transport mechanisms may directly reflect the different functional roles of lysosomes and melanosomes. The major function of melanophores is to transport pigment to the cell center or to the periphery in response to physiological stimuli. Thus, one way to guarantee proper pigmentary responses without disturbing the housekeeping cellular functions is to make use of a motor molecule different from those used by the other organelles, as well as to develop separate regulatory systems.

We demonstrated that kinesin II has a role in dispersion of melanosomes. Previously, there have been only two reports that provide direct evidence for kinesin II function. Both of them demonstrated its role in the assembly of cilia and flagella: one using antibody microinjection into sea urchin eggs (Morris and Scholey, 1997), and another using a genetic approach in *Chlamydomonas reinhardtii* (Cole et al., 1998). This motor has also been shown to colocalize with membrane-bound organelles in the sea urchin mitotic apparatus (Henson et al., 1995) and in neurons (Pesavento et al., 1994; Yamazaki et al., 1995). Based on these associations, kinesin II has been implicated in membrane vesicle transport. Nevertheless, localization data have not been paired with functional studies. Thus, these colocalizations could reflect passive redistribution or recycling of the motor, which could be carried as a cargo. We directly demonstrated that kinesin II is required for microtubule-dependent movement of membrane-bound organelles. This paper together with the accompanying paper showing a role for kinesin II on the transport of Golgi vesicles (Le Bot et al., 1998) are the first demonstrations that kinesin II is a motor able to transport membrane organelles.

Our observations that cells expressing headless Xklp3 displayed slow and often incomplete dispersion of pigment point to the existence of a nonmicrotubular component involved in pigment movement. Two recent studies have documented that pigment organelles can also move along actin filaments, in addition to microtubules (Rodionov et al., 1998; Rogers and Gelfand, 1998). In *Xenopus* melanophores, we showed that integrity of the actin cytoskeleton is required for pigment dispersion, because in the absence of actin filaments melanosomes cannot disperse properly, remaining aggregated at the cell center even in the presence of MSH (Rogers and Gelfand, 1998). Moreover, isolated melanosomes are able to move along actin filaments in vitro (Rogers and Gelfand, 1998). Rodionov and collaborators, using fish melanophores, showed that actin depolymerization results in pigment hyperdispersion, with the melanosomes accumulated at the cell periphery (Rodionov et al., 1998). Both of these studies implicate the actomyosin system in promoting homogeneous distribution of pigment throughout the cytoplasm. In agreement with these studies, we observed that inhibition of plus end–directed movement on microtubules by overexpression of headless Xklp3 resulted in melanosome movement by random short walks. We believe that this slow movement is powered by myosin V, which in *Xenopus* is bound to melanosomes (Rogers and Gelfand, 1998). Although the polarity of actin has yet to be determined in *Xenopus* melanophores, we hypothesize that it is random, as it is in fish melanophores (Rodionov et al., 1998). Such an actin organization would be consistent with its role in facilitating homogeneous distribution of pigment. Nondirected movement of melanosomes away from the central mass would ultimately lead to an overall migration toward the cell periphery. If the role of the actin-based transport system is to promote uniform distribution of melanosomes, why does depolymerization of actin lead to pigment hyperdispersion in fish but to aggregation in *Xenopus* melanophores? We believe that this difference can be explained by properties of the microtubule-dependent transport in these systems. Pigment dispersion in fish melanophores is much faster than in frog cells, suggesting that plus end microtubule–based transport is more efficient in fish. This higher efficiency of the microtubule plus end motor system could be accomplished by different strategies, such as a higher ratio of motors per organelle. Assuming that the microtubule motor is the same in the two systems, faster movement results from more motor molecules bound to the surface of melanosomes. Consequently, in the absence of actin, fish melanosomes end up hyperdispersed because the plus end motor overrides the minus end motor. On the other hand, in *Xenopus* the plus end microtubule motor may not be as abundant or as efficient, and as a consequence, it cannot

override cytoplasmic dynein even in the presence of MSH, resulting in incomplete dispersion.

The characteristic saltatory nature of pigment movement in *Xenopus* melanophores both in vivo and in vitro has been carefully described (Rogers et al., 1997). Melanosomes saltate in both directions during aggregation and dispersion, suggesting a constant competition among the motors, the end result reflecting the winning force. Our analysis of the kinetics of individual melanosomes indicated that the fast saltations toward the microtubule plus ends are driven by kinesin II, as their frequency was greatly reduced in cells overexpressing headless Xklp3. This observation suggests that the net direction of motility might be regulated by modulating the frequency of saltations. Similar results have been reported for movement of individual lipid droplets from *Drosophila* embryos (Welte et al., 1998). In this system, regulation of transport seems to occur through changes in persistence time, which could be accomplished by altering either the number of motors present on the organelles or the activity of individual motors. It will be interesting to determine which of these mechanisms, if any, are common to regulation of melanosome movement.

In conclusion, by using a dominant-negative approach we were able to show that kinesin II is required for dispersion of pigment granules on melanophores. The strategy described here has proven useful to address the specific function of a motor, and it could be easily applied to other candidate motor proteins in many experimental systems. Furthermore, in this paper we present one of the first demonstrations that kinesin II is able to transport a distinct set of membrane-bound organelles in vivo.

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