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Kinesin is responsible for centrifugal movement of pigment granules in melanophores

(microtubules/organelle movement/antibody inhibition)

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ABSTRACT **Kinesin is a mechanochemical ATPase that induces translocation of latex beads along microtubules and microtubule gliding on a glass surface. This protein is thought to be a motor for the movement of membranous organelles in cells. Recently Hollenbeck and Swanson [Hollenbeck, P. J. & Swanson, J. A. (1990) *Nature (London)* 346, 864–866] showed that kinesin is involved in the positioning of tubular lysosomes in macrophages. However, the role of this protein in the movement of organelles was not yet clear. We used a polyclonal antibody against the kinesin heavy chain that inhibited kinesin-dependent microtubule gliding *in vitro* to study the role of kinesin in the movement of pigment granules in melanophores of the teleost black tetra (*Gymnocorymbus ternetzi*). Microinjection of the antibody into cultured melanophores did not produce any specific effect on the aggregation of pigment granules in melanophores, but it did result in a strong dose-dependent inhibition of the dispersion. Immunoblotting of melanophore extracts showed that the kinesin antibody reacted in these cells with a single protein component with a molecular mass of 135 kDa. Thus, kinesin is responsible for the movement of pigment granules from the center to the periphery of the melanophore.**

The microtubule-dependent ATPase, kinesin, has been purified from and detected in different cells and tissues (for reviews see refs. 1 and 2). Purified kinesin is able to translocate microtubules along a glass surface and plastic beads along a microtubule from the minus to the plus end (3, 4).

The kinesin molecule consists of two heavy and two light chains (5, 6). The heavy chain contains the N-terminal globular domain where ATPase and microtubule-binding sites are localized (7). This N-terminal domain alone is sufficient for inducing microtubule motility *in vitro* (8), and monoclonal antibodies reacting with this portion of the heavy chain inhibit the kinesin-driven microtubule gliding (9).

Immunofluorescent localization of kinesin has shown that this protein is associated with membrane-bound organelles in cultured cells (10). Purified kinesin induces formation of polygonal membrane networks that are closely aligned with microtubules (11). These *in vitro* activities and *in situ* localization studies make kinesin the most likely candidate for the role of microtubule-dependent plus-end-directed (anterograde) organelle translocator.

Recent *in vivo* evidence for the function of kinesin in living cells was obtained by Hollenbeck and Swanson (12). They found that anti-kinesin antibodies introduced into macrophages caused redistribution of tubular lysosomes in a way that suggested the inhibition of centrifugal translocation of these organelles. However, no direct effect of kinesin antibodies on the movement of organelles in the cells was demonstrated in this work.

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We have applied the same tool—an anti-kinesin antibody—to elucidate the role of kinesin in one of the most spectacular forms of intracellular transport, the translocation of pigment granules in fish chromatophores. The main function of these large cells, which have a well-developed radial system of microtubules (13), is a fast and synchronous redistribution of hundreds of pigment granules that is completely dependent on integrity of cytoplasmic microtubules (14, 15) and ATP as an energy source (16–19). As in many other eukaryotic cells, the plus ends of microtubules in melanophores are at the cell periphery, and the minus ends are associated with the centrosome (20).

We have shown that the injection of the kinesin antibody into melanophores does not affect the pigment aggregation but completely suppresses pigment dispersion. Thus, kinesin is the motor responsible for the translocation of pigment granules toward the plus ends of microtubules in melanophores.

MATERIALS AND METHODS

Antibody Against Kinesin. Polyclonal anti-kinesin antibody (antibody HD) was produced in a rabbit and purified by affinity chromatography. As an antigen we used the head portion of the *Drosophila melanogaster* kinesin heavy chain expressed in *Escherichia coli* BL21 (DE3) carrying plasmid pET-HD coding the N-terminal globular part of the heavy chain (this plasmid was kindly provided by L. S. B. Goldstein and M. de Cuevas of Harvard University). The fragment of kinesin was isolated from bacteria in the form of inclusion bodies according to ref. 21, subjected to SDS/10% PAGE, and electroeluted from the gel. For affinity chromatography inclusion bodies were dissolved in 1% SDS and immobilized on CNBr-Sepharose (Pharmacia). Preimmune IgG was precipitated from the serum by 50% saturation with ammonium sulfate and purified on a DEAE-cellulose column (Whatman DE-52).

Purification of Proteins. Bovine brain kinesin was purified as described by Kuznetsov and Gelfand (22). Microtubule proteins were isolated from bovine brain by the cycles of polymerization–depolymerization (23) in 50 mM imidazole·hydrochloride, pH 6.7/50 mM KCl/0.5 mM $MgCl_2$/0.1 mM EDTA/1 mM EGTA/1 mM 2-mercaptoethanol (buffer A). Tubulin was separated from microtubule-associated proteins by chromatography on phosphocellulose (Whatman P-11) in buffer A (24). Protein concentration was determined by Bradford assay (25).

Analytical Methods. Electrophoresis was performed in the presence of SDS by the method of Laemmli (26), in a 10% polyacrylamide gel at a 100:1 (wt/wt) ratio of acrylamide to *N,N'*-methylenebisacrylamide. Gels were stained with Coomassie blue R-250.

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Immunoblotting was performed according to Towbin *et al.* (27).

Motility was assayed at room temperature in buffer A supplemented with 20 μM taxol and 5 mM MgATP. Microtubules were used at 20 μg/ml. Kinesin in buffer A (8 μl, 0.36 mg/ml) was adsorbed onto a glass coverslip for 10 min in a humidified chamber, the glass was washed with buffer A, and one of the following variants of assay was performed: (*i*) 10 μl of antibody solution in buffer A was added, and after a 10-min incubation the coverslip was washed with buffer A and 10 μl of microtubule suspension was applied; or (*ii*) 10 μl of microtubule suspension was applied onto a kinesin-coated coverslip, and after 1 min the glass was washed with buffer A and 10 μl of antibody solution was added.

Kinesin-driven gliding of microtubules was analyzed by video-enhanced differential interference contrast microscopy. We used a Zeiss Axiophot microscope fitted with a 100× 1.3 NA Plan-Neofluar objective. The microscope was equipped with a Hamamatsu 2400-01 Chalnicon video camera and an Argus 100 image processor. Images were recorded on a Sony VO-7630 videocassette recorder in real time and analyzed directly on a TV screen. Magnification was calibrated with a stage micrometer.

Melanophore Cultures and Microinjection. Primary cultures of melanophores were obtained from black tetras (*Gymnocorymbus ternetzi*) as described earlier (28). Cells were grown on carbon-coated glass coverslips for 24 hr before microinjection experiments. For immunoblotting, melanophores were collected by the following procedure: The fish scales were incubated for 60 min at 30°C in Ringer solution supplemented with collagenase (Fluka) at 1 mg/ml and bovine serum albumin at 5 mg/ml. Melanophores were removed from scales by pipetting and washed by mild centrifugation with three portions of Ringer solution with bovine serum albumin at 5 mg/ml and protease inhibitors. The final pellet was suspended in Laemmli SDS sample buffer and immediately boiled. Melanophores from 800 scales were loaded on a lane.

Microinjection was performed essentially as described by Graessmann and Graessmann (29). Antibody in a buffer containing 114 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, and 3 mM NaH_2PO_4, pH 7.0, was clarified by centrifugation at 100,000 × *g* for 30 min and kept at −70°C. Injected cells were incubated for 1–2 hr at 30°C; epinephrine was then added to the culture medium to the final concentration of 10 μM to induce aggregation. After the aggregation was complete, the medium was replaced with Ringer solution containing 5 mM caffeine to initiate dispersion.

Movement of pigment granules was observed by phase-contrast microscopy on a Zeiss Photomicroscope III equipped with a Hamamatsu 2400-08 silicon-intensified target camera. Observation was carried out at a low level of illumination to avoid heating of the cells. Analysis of the rates of the aggregation and dispersion was performed on a Hamamatsu Argus 100 image processor using images recorded in real time.

To calculate the rate of pigment movement the images of the cells were taken every 15 sec and the intensity profile of transmitted light was measured in a lane with a width of 85 μm as a function of a distance from the cell center. The point where intensity was equal to 50% of maximal was considered as a position of the pigment front (Fig. 1). The rate of movement of the pigment front was determined from linear interpolation of the front position in 8–12 sequential images versus time.

RESULTS

Antibody Characterization. In our study we used an affinity-purified polyclonal antibody against the head portion of the *Drosophila* kinesin heavy chain (antibody HD).

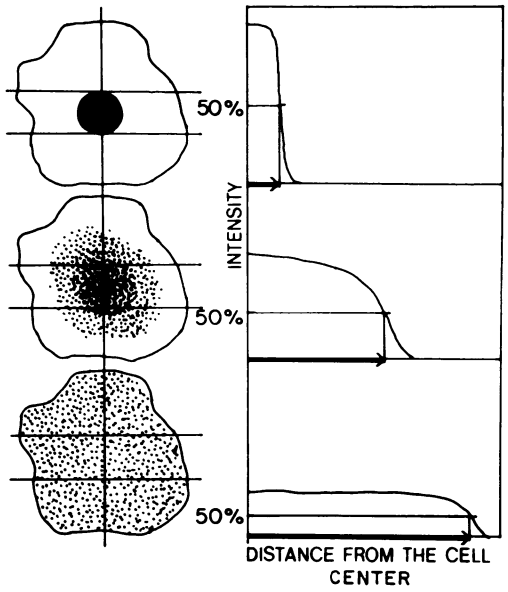

FIG. 1. Principle of measurement of pigment aggregation and dispersion in cultured melanophores. The position where intensity of the transmitted light was equal to 50% of the plateau value was taken as the position of the pigment front.

The results of immunoblotting (Fig. 2 *A* and *B*) showed that antibody HD was highly specific to the kinesin heavy chain—it recognized a single band with a molecular mass of 120 kDa in bovine brain homogenate and reacted also with the heavy chain of purified bovine brain kinesin. This result shows also that the antibody is not species specific, as it was raised against *Drosophila* kinesin but reacted equally well with bovine kinesin.

We tested the effect of antibody HD on the gliding of microtubules, promoted by bovine brain kinesin. The total IgG fraction isolated from preimmune serum was used as a control. An *in vitro* motility assay was carried out in two variations. For the first variation, the antibody was applied onto the coverslip after kinesin but before the microtubules and the gliding was observed immediately after the addition of microtubules. For the second variation, the antibody was added to the complete reaction mixture (after microtubules),

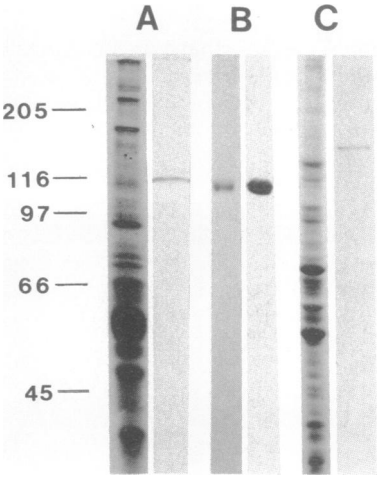

FIG. 2. Immunoblotting with antibody HD. (*A*) Homogenate of bovine brain. (*B*) Bovine brain kinesin. (*C*) Melanophore lysate. In each panel the left lane is the acrylamide gel and the right lane is the corresponding immunoblot. Positions and molecular masses (in kDa) of markers are shown at the left.

Table 1. Effect of antibody HD on the rate of the kinesin-based gliding of microtubules

Additions	Gliding rate, μm/sec, at antibody concentration			
	0 mg/ml	0.3 mg/ml	0.4 mg/ml	0.5 mg/ml
HD + MTs*	0.86 ± 0.07	ND	0.89 ± 0.08	No MTs attached
MTs + HD†	0.85 ± 0.08	0.91 ± 0.10	0	ND
Preimmune IgG + MTs‡	ND	ND	ND	0.97 ± 0.05

The gliding rates are given as mean ± SD (n = 50). MTs, microtubules; ND, not determined.
*Antibody HD was added after kinesin but before microtubules.
†Antibody HD was added after kinesin and microtubules.
‡Preimmune IgG was added after kinesin but before microtubules.

and the gliding was analyzed 10 min after the addition of the antibody.

The results of the motility test (Table 1) demonstrate that in the first variation of the assay antibody HD at 0.4–0.5 mg/ml completely prevented the attachment of microtubules to the kinesin-covered surface. In the second variation (addition of the antibody after microtubules) the antibody did not interfere with the attachment, but it inhibited the gliding of all microtubules, probably inducing the formation of a rigor complex between kinesin and microtubules. Control preimmune IgG had no effect on the gliding in either of the variations of the assay (Table 1; second variation not shown). The concentration of antibody HD inhibiting the microtubule gliding was much higher than that found for the monoclonal antibodies to kinesin by Ingold *et al.* (9), probably because only a small fraction of the antibodies in the polyclonal antiserum HD were directed against the epitope(s) of the kinesin molecule essential for the motile activity.

Effect of Antibody HD on Pigment Granule Movement in Melanophores. To test the possibility of using antibody HD in microinjection experiments, we performed an immunoblotting of antibody HD with the extract of black tetra melanophores. The results of immunoblotting (Fig. 2*C*) showed that antibody HD recognizes in melanophores a single component with a molecular mass of 135 kDa. Thus, teleost melanophores contain kinesin and antibody HD reacts with it.

Antibody HD was then injected into melanophores with dispersed pigment, and aggregation was induced by the addition of epinephrine. After completion of aggregation the cells were washed with Ringer solution and dispersion was

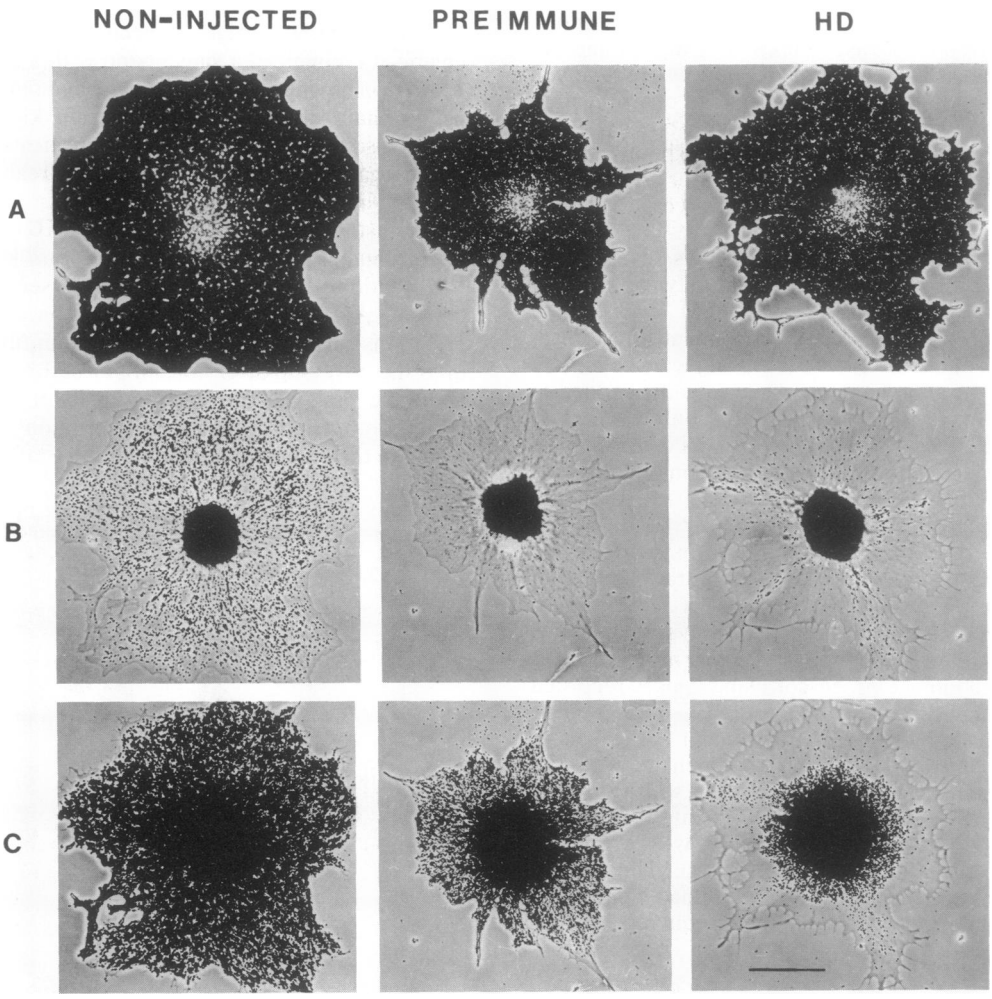

FIG. 3. Phase-contrast micrographs of a noninjected cell (*Left*), a cell injected with preimmune IgG at 12 mg/ml (*Center*), and a cell injected with antibody HD at 12 mg/ml (*Right*). (*A*) Before addition of epinephrine. (*B*) Five minutes after induction of aggregation by the addition of 10 μM epinephrine. (*C*) Fifteen minutes after induction of dispersion by 5 mM caffeine. Aggregation was started 60 min after microinjection. (Bar, 100 μm.)

induced by addition of Ringer solution supplemented with caffeine. The movement of granules was observed by phase-contrast optics at a low light level by using a silicon-intensified target camera, as we found that a high level of illumination reduces the rate of the pigment granule movement in the course of aggregation as well as during dispersion (not shown).

Fig. 3 shows a cell after antibody HD injection, a cell injected with preimmune IgG, and a control noninjected cell at different stages of the aggregation–dispersion cycle. Within 5 min after the addition of epinephrine, aggregation was complete both in intact and in injected cells and the pigment formed a compact spherical aggregate at the center of the cell (Fig. 3*B*). However, in the injected cells the granules moved more slowly. Sometimes at the intermediate stages of the aggregation they formed an unusual ring of pigment around the cell center that was never seen in noninjected cells, but later aggregation went to completion. This effect was observed both in the cells injected with antibody HD and in the cells injected with preimmune IgG. A similar pattern of aggregation was found after the injection with the microinjection buffer (data not shown). Thus, we consider this partial disturbance of aggregation to be the consequence of microinjection itself. Immunofluorescent staining with a monoclonal anti-tubulin antibody showed that the microinjection of antibody HD, preimmune IgG, or buffer had no effect on the density or distribution of cytoplasmic microtubules (not shown).

In contrast with aggregation, the dispersion process was dramatically and specifically affected by antibody HD. In intact cells, the initial event in the process of dispersion was the loosening of the tight globular pigment aggregate. Then, melanosomes started to move centrifugally, and in 15 min they always reached the cell margins (Fig. 3*C* and Fig. 4, lower left cell). In melanophores injected with antibody HD, the addition of caffeine also induced the loosening of the central mass, but the directed migration of melanosomes was much slower, and the granules never reached the cell margins during the observation period (Fig. 3*C* and Fig. 4, upper right cell). The dispersion process in the cells injected with the control IgG was indistinguishable from that in the noninjected cells.

We measured the velocities of the aggregation and dispersion of the pigment in injected and noninjected cells. The results of these measurements are shown in Table 2. In good agreement with the results of the morphological observations, the data of the quantitative analysis demonstrate that injection slightly slowed down the aggregation rate, and this effect was identical for antibody HD and the control IgG solution. The dispersion, however, was dramatically inhibited by antibody HD but not by the control IgG. The effect of antibody HD on the dispersion was dose dependent: the inhibition was more pronounced after the injection of the antibody at 12 mg/ml than at 6 mg/ml (Table 2). This result, together with the absence of the effect of the control IgG, shows the specificity of the dispersion inhibition.

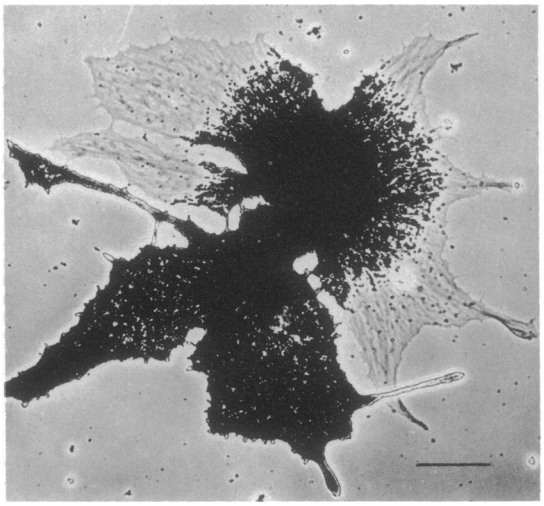

FIG. 4. A noninjected cell (lower left) and a cell injected with antibody HD at 6 mg/ml (upper right) 15 min after the beginning of dispersion. (Bar, 50 μm.)

Table 2. Effect of antibody HD injection on the rate of melanosome movement

Antibody injected	Velocity, μm/min	
	Aggregation	Dispersion
None	60.2 ± 13.7 (n = 16)	18.5 ± 3.3 (n = 11)
Preimmune IgG (12 mg/ml)	35.5 ± 8.5 (n = 24)	20.1 ± 6.5 (n = 31)
HD (6 mg/ml)	36.4 ± 9.8 (n = 24)	4.0 ± 0.8 (n = 31)
HD (12 mg/ml)	31.7 ± 17.9 (n = 15)	1.3 ± 0.6 (n = 16)

The velocities are given as mean ± SD; n is the number of cells measured.

DISCUSSION

The main conclusion of this study is that kinesin is the motor protein responsible for the centrifugal movement of the pigment granules in melanophores. Inhibition of the kinesin activity in the cells with antibody HD had no effect on the centripetal movement of the organelles. These findings are in good agreement with the results of the *in vitro* studies by Vale *et al.* (4), who showed that kinesin is responsible for the plus-end-directed transport of latex beads along microtubules and does not induce movement in the opposite direction. Our results are also in good agreement with those of Hollenbeck and Swanson (12), who demonstrated that polyclonal and monoclonal antibodies to kinesin block the extension of tubovesicular lysosomes in macrophages.

The results of this study seem to be in contrast with the data of Brady *et al.* (30), who showed that a monoclonal antibody against kinesin inhibited both anterograde and retrograde transport of vesicles in isolated squid axoplasm. They suggested that, unlike the situation *in vitro* (4), kinesin in axoplasm is involved in both plus- and minus-end-directed movement. However, binding of kinesin to the antibody may result in sequestration of a soluble kinesin-binding factor essential for both anterograde and retrograde transport (31) or in formation of cross-linked immunoprecipitate in axoplasm that would be expected to impede motion in both directions.

Rozdzial and Haimo (32) showed that dispersion of pigment in *Tilapia mossambica* lysed cell models is correlated with phosphorylation of a 57-kDa polypeptide and could be promoted by adenosine 5′-[γ-thio]triphosphate (ATP[γS]) in the absence of exogenous ATP. This result seems to be inconsistent with the role of kinesin as a motor for the centrifugal pigment movement because sea urchin kinesin does not use ATP[γS] for microtubule gliding (33). At present we do not know the real reason for this apparent discrepancy, but we suggest at least two explanations. First, melanophore kinesin may differ in substrate specificity from the sea urchin kinesin and can use ATP[γS] as a substrate. Second, in a crude system such as the whole scale treated with detergent, some sequence of metabolic reactions may generate ATP from ATP[γS] and this ATP is used by kinesin for pigment dispersion.

On the basis of our results as well as the data on the action of microtubule inhibitors on pigment dispersion one can

distinguish two phases in this process. The first phase, loosening of the tight central pigment aggregate, is independent of microtubules and can be observed after disruption of microtubules with nocodazole (V.I.R. *et al.*, unpublished observations). It is possible that this initial stage can be induced just by switching off the centripetal motor without activation of the centrifugal motor. The second stage, dispersion of the granules released from the aggregate, is completely dependent on microtubules. This stage is energy dependent (16–19) and, as shown here, is inhibited by anti-kinesin antibody. Therefore, one can postulate that after the addition of caffeine the centripetal motor is inhibited, granules diffuse out of the aggregate, and the diffused granules are moved to the cell periphery along microtubules by the plus-end-directed motor, kinesin.

Thus, antibody inhibition experiments show that kinesin is involved in microtubule-dependent organelle movement. Antibody HD can effectively and specifically inactivate kinesin in living cells, and thus it can be used to study the role of kinesin in the motility and cytoplasmic organization of cultured mammalian cells.

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