Video and Electron Microscopic Studies on Pigment Transport in *Gambusia* Melanophores

Naoshi Nakamura, Yayoi Ikeda and Masataka Obika

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**Abstract** Pigment migration and the role of cortical microtubules in granular transport in melanophores of a teleost, *Gambusia affinis*, were studied by means of video and electron microscopy. In pigment aggregation, the migratory velocity of individual melanosomes was maximal in the initial 20 sec, rapidly decreasing afterward. In dispersion, pigment moved at a uniform, slower rate as compared with aggregation. During aggregation, melanosomes migrated faster in thinner dendrites than in thicker ones, while in dispersion, the rate of migration was rather constant irrespective of the thickness of the path. Withdrawal of melanosomes from the dendritic processes during aggregation was followed by a flattening of the processes and a decrease in the number of cortical microtubules. On pigment dispersion, collapsed dendrites resumed their original morphology prior to the re-entry of melanosomes by gradual refilling of the cytoplasm and re-establishment of cortical structure. Complete disassembly of microtubules by cold treatment made the distance between pigment granules and cell membrane remarkably shorter, indicating a significant correlation between the density of cortical microtubules and distance between cell membrane and pigment granules. These findings suggest that cortical microtubules are essential for the maintenance of the path in which melanosomes migrate. N-ethylmaleimide (NEM) inhibited both aggregation and dispersion, and erythro-9(3-(2-hydroxynonyl))adenine (EHNA) arrested pigment aggregation, while cytochalasin B had no appreciable effect on pigment migration. These pharmacological evidences suggest that the generation of motive force for pigment migration, at least for pigment aggregation, is dynein-dependent. These experiments also clearly show that pigment dispersion in *Gambusia* melanophores can be produced by a mechanism independent of tubulin-dynein or actin-myosin system, though the involvement of other motor molecules in pigment dispersal remains to be studied.

On translocation of pigment granules in fish chromatophores, it has been reported that migratory velocity of pigment granules is different between aggregation and dispersion. Investigations on teleost melanophores show that velocity of melanosome aggregation is generally higher than that of dispersion (Green, 1968; Egner, 1971). The difference of migratory velocity between aggregation and dispersion probably reflects the difference in the mechanism of pigment translocation in these processes, although the motor molecules that provide the motive force for locomotion still remain to be elucidated (see Obika, 1986, for review). Scanning and transmission electron microscopy has revealed that there is a drastic change of cell morphology between the chromatophores with aggregated and dispersed pigment (Obika, 1975, 1976; Byers and Porter, 1977; Schliwa and Euteneuer, 1978).

Teleost chromatophores that respond to stimuli with rapid pigment translocation generally contain well developed microtubule system (Bikle et al., 1966; Schliwa and Bereiter-Hahn, 1973; Murphy and Tilney, 1974; Byers and Porter, 1977). The radial array of cytoplasmic microtubules, which runs from microtubule-organizing center (MTOC) of the centrosphere to the periphery of the cell, provides the cortical, cytoskeletal framework of the dendritic processes (Schliwa et al., 1978; Ochs, 1982). It is widely accepted that melanosome aggregation in teleost melanophores is dependent on the presence of cytoplasmic microtubules (see Schliwa and Euteneuer, 1983; Obika, 1986, for review). Regarding to the generation of motive force, Clark and Rosenbaum (1982) have demonstrated the involvement of dynein-like ATPase in pigment aggregation in their permeabilized melanophore preparations of *Fundulus heteroclitus*.

In the present study, we have investigated the
velocity of pigment migration in aggregation and dispersion in melanophores of *Gambusia affinis* by the use of video microscopy. Electron microscopic studies on the change of cell shape during pigment translocation, and the studies on the effects of N-ethylmaleimide (NEM), erythro-9[3-(2-hydroxynonyl)] adenine (EHNA) and cytochalasin B on the physiological responses were also carried out.

**Materials and methods**

Scale melanophores of the mosquitofish, *Gambusia affinis*, were used throughout the experiments. Scales were taken from the antero-dorsal region of the trunk with fine forceps and rinsed in a physiological saline (128 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, adjusted to pH 7.3 with 0.1 N NaHCO₃). Melanosome aggregation and dispersion were induced by perfusing the scales with the saline containing 10 µM epinephrine (Sigma) or 10 mM theophylline (Tokyo Kasei), respectively.

**Video microscopy.** The behavior of melanosomes during pigment aggregation and dispersion was recorded on video tapes with a TOSHIBA IK-1550 video camera attached to a Nikon AFX light microscope. Recording was made on the individual melanosomes and the migratory velocity and distance traveled were calculated by frame analysis.

**Electron microscopy.** Melanophores in the physiological saline (with fully dispersed pigment), samples transferred into 10 µM epinephrine for 1 to 5 min (melanophores with partially to fully aggregated pigment) and those preincubated in epinephrine and then transferred into 10 mM theophylline for approximately 2 min (melanophores with partially dispersed pigment) were used. Some scales kept at −3 to −5°C for 10, 20 and 30 min, and those treated in the cold for 60 min and then placed at room temperature with or without the presence of colchicine (1 mM) for the period of 10 to 30 min were also utilized. Scales were fixed in 3% glutaraldehyde for 60 min at room temperature, postfixed in 1% OsO₄ for 60 min at room temperature, dehydrated through a graded series of alcohol and embedded in Epon 812. Thin sections were cut on an LKB 4802A ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and observed in a JEOL 100S electron microscope at 80 kV.

For scanning electron microscopy, glutaraldehyde and OsO₄ fixed, ethanol dehydrated samples were dried in a critical point drier (Hitachi HCP-1) in liquid CO₂. In order to observe the inner structure, a part of plasma membrane was manually broken by tungsten needles and spatter-coated with gold in an ion-spatter (Hitachi E101). Samples were observed in a Hitachi S510 SEM at an accelerating voltage of 25 kV.

**Inhibitors.** Prior to the application of the inhibitors, scales were mechanically shaken for 30 to 60 min in the presence of 0.25% collagenase (Worshington, type II), 0.15% trypsin (Merck) and 2% bovine albumin (Sigma), and then epidermis was removed manually with fine forceps. N-ethylmaleimide (Kokusan Kagaku, 0.2–2 mM) and erythro-9[3-(2-hydroxynonyl)]adenine (Burroughs Wellcome, 1 mM) were dissolved directly in the physiological saline immediately before use. Cytochalasin B (Aldrich) was dissolved in dimethylsulfoxide (DMSO) to make up a stock solution. The stock solution was diluted 400 times by adding physiological saline. Final concentration of cytochalasin B was 10 µg/ml in 0.25% DMSO. DMSO at this final concentration had no appreciable effect on melanophores. Responses of the melanophores to these drugs were examined under a Nikon inverted microscope at room temperature (20–25°C).

**Results**

**A. Migratory velocity of melanosomes in pigment aggregation and dispersion.** The migratory pattern of individual melanosomes was obviously different in two phases of pigment migration (Fig. 1). In aggregation, the migratory velocity of individual melanosomes showed the maximum rate in the initial 20 sec of the response (0.88 ± 0.35 µm/sec), rapidly decreasing afterwards. The velocity was higher in distal parts of dendrites, but became lower as melanosomes approached near the proximal end of the dendrites. In dispersion, where melanosomes migrated at much reduced rate (0.25 ± 0.14 µm/sec) as compared with aggregation, the velocity was more or less uniform irrespective of the region of dendrites. Relationship between the migratory velocity and the width of the dendrite was also examined. In aggregation, there is an apparent correlation between the velocity (V) and the thickness of the
Fig. 1. Velocity of pigment migration in aggregation and dispersion.

AGGREGATION (n = 40)

DISPERSION (n = 37)

Fig. 2. Relation between the migratory velocity and the width of the dendrite.
path (T) \( V = 2.34 e^{-0.34T}, \quad r = 0.705; \quad \text{Fig. 2a}. \)

While in dispersion, the rate of migration was rather constant regardless of the thickness of the path \( V = 0.25, \quad r = 0.086; \quad \text{Fig. 2b}. \). In addition, melanosomes migrated unidirectionally in aggregation, while in dispersion, they migrated bidirectionally in a short distance, showing back and forth movements in cycles of several seconds.

**B. Change of cell shape during pigment migration.** In order to examine the relationship between pigment migration and morphological changes of dendrites, scale melanophores in various stages of pigment migration were examined by electron microscopy. In dispersed melanophores, melanosomes distributed uniformly in dendrites. The proximal half of the dendrite was

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Fig. 3.  
(a): Longitudinal section of an aggregating dendrite. Arrow indicates collapsed dendritic process.  
(b): Longitudinal section of a dispersing dendrite. Note that the proximal half of the dendrite has regained its thickness before the re-entry of melanosomes. Arrow indicates thin distal portion of the dendrite. Scale bar = 1 \( \mu m \).
Fig. 4. Schematic drawings of cell shape change during aggregation and dispersion.

Fig. 5. a: Cross sectional profile of a dendrite with aggregating pigment. Note decreased number of cortical microtubules. b: A dendrite with dispersing pigment. Microtubules in cross section are present. Scale bar=1 μm.
Fig. 6.  

a: Cross section of a dendrite (control). Note numerous cortical microtubules. Inset: Scanning electron micrograph showing the arrangement of pigment granules in untreated melanophores. Melanosomes appear to be associated with cytoskeletal elements. ×7500. 

b: Cross section of a dendrite treated in the cold for 60 min. Cortical microtubules are absent. Inset: Scanning electron micrograph showing internal surface of cell membrane with depressions. Cytoskeletal structure is missing. ×7500. Scale bar=1 μm.
1.5 to 2.0 μm in diameter. The cell morphology changed drastically when melanosomes started to aggregate. The diameter of the proximal portion of the dendrites gradually became thicker, owing to the centripetal migration of melanosomes and other cytoplasmic constituents. At the same time, distal, melanosome-free portions became extremely thin (Fig. 3a). In pigment redispersion, gradual refilling of the cytoplasm into the dendrites took place in advance to the re-entry of melanosomes (Fig. 3b). Based on the electron microscopic observation in transitional stages, schematic drawings of cell shapes during pigment aggregation and dispersion are depicted in Fig. 4. The observation on the cross sections of dendrites during pigment aggregation and dispersion indicated that disassembly and reassembly of cortical microtubules took place concomitantly with the pigment translocation. In dendrites where melanosomes were moving inward, the density of cortical microtubules (N) remarkably decreased (N=0.32 microtubules/μm length of plasma membrane) and the distance between cell membrane and pigment granules (D) became significantly shorter (D=54.35±24.91 nm) as compared to those in dispersing phases (N=2.21/μm, D=115.46±58.79 nm; Fig. 5, Table 1).

C. The effect of low temperature. Complete disassembly of microtubules by cold treatment not only significantly blocked the responses to epinephrine and theophylline but also made the space between cell membrane and pigment granules considerably narrower. This alteration in intracellular organelle distribution is clearly shown in scanning electron micrographs shown in Fig. 6. Three-dimensional schematic representations of dendrites with and without microtubules are given in Fig. 7. Microtubule reassembly, which is totally blocked by the presence of colchicine, took place when the materials were transferred to room temperature. Based on the ultrastructural studies of disassembly and reassembly of cortical microtubules produced by the cold treatment and the following recovery, the relation between the density of cortical microtubules (N) and the distance between cell membrane and pigment granules (D) was examined by the method shown in Fig. 8. There was a significant correlation between them (D=29.5N+46.3, r=0.980; Fig. 9).

D. The effects of inhibitors. Preincubation of scale melanophores in the saline containing 0.2 mM NEM for 5 min produced a strong but incomplete inhibition of epinephrine- and theophylline-induced pigment responses. NEM at 2 mM, however, perfectly inhibited pigment migration. When melanophores were transferred from saline solution into NEM-saline containing epinephrine, they responded with a transitory full pigment aggregation immediately followed by a

<p>| Table 1. Comparison of number of cortical microtubules (CMT) and pigment granule (PG)-cell membrane (CM) distance in aggregating and dispersing melanophores. |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Number of CMT/μm CM length (N)</th>
<th>PG-CM distance (D)</th>
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<tr>
<td>Aggregating 0.32/μm (23/71.41 μm)</td>
<td>54.35±24.91 nm (n=51)</td>
</tr>
<tr>
<td>Dispersing 2.21/μm (209/94.48 μm)</td>
<td>115.46±58.79 nm (n=50)</td>
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Fig. 8. Method of measurement of distance between cell membrane and pigment granules. CM, cell membrane; D, distance between cell membrane and pigment granules; MT, microtubule; PG, pigment granule.

Fig. 9. Correlation between the number of cortical microtubules and pigment granule-cell membrane distance. C, control; L&C, cold and colchicine treatments; L10, 10 min in the cold; L20, 20 min; L30, 30 min; R10, recovery period (room temp.) 10 min; R20, 20 min; R30, 30 min.

partial redispersion. Preincubation of scale melanophores in the saline containing 1 mM EHNA for 5 min totally inhibited pigment aggregation, but not pigment dispersion. Preincubation of the melanophores in the saline containing 10 μg/ml cytochalasin B for 60 min did inhibit neither pigment aggregation nor dispersion.

Discussion

The present experiments clearly indicate that 1) melanosome aggregation and dispersion are accompanied by drastic changes of cell shape, 3) cortical microtubules somehow keep the distance between cell membrane and pigment granules, and 4) rigid cortical structure facilitates smooth movements of the granules.

Regarding to the velocity of granular movements, it is generally accepted that centripetal translocation of pigment granules is faster than centrifugal movement. For example, in angelfish Pterophyllum scalare melanophores, the velocity measured as mass movement in aggregation is about 1.3 μm/sec while that in dispersion is about 0.56 μm/sec (Egner, 1971). In Fundulus melanophores, pigment aggregation proceeds at a rate of 2 to 5 μm/sec, while in dispersion, melanosomes travel 1 to 2 μm/sec (Green, 1968). In swordtail erythrophores, pigment particles aggregate at an average velocity of 1 μm/sec and disperse at 0.03 to 0.1 μm/sec (Matsumoto et al., 1984). Our current results of Gambusia melanophores are also in accordance with these previous reports, although the movements in this species are much slower than those reported previously.

In the present investigation, it is also noted that melanosomes migrate faster in thinner dendrites than in thicker ones in pigment aggregation, while in dispersion, the rate of migration is rather constant irrespective of the thickness of the path. This might be an indication that cellular cortex is involved in the generation of motive force for aggregation. The observed differences in the velocity and pattern of pigment migration between aggregation and dispersion show that these processes are driven by different mechanisms, presumably by different motor molecules. Melanosome aggregation in melanophores of Fundulus and Oryzias is energy driven and probably supported by tubulin-dynein system (Clark and Rosenbaum, 1982, 1984; Negishi et al., 1985). Recently, Vale et al. (1985a, b) described a novel translocator (kinesin) that moves cytoplasmic particles unidirectionally along a microtubule, thus producing the anterograde axoplasmic flow in the squid giant axon. Although the polarity of microtubules in Gambusia melanophores has not yet been examined, those of angelfish melanophores are arranged with the plus end toward the periphery (Euteneuer and McIntosh, 1981) and kinesin translocate particles along microtubules from their minus end toward the fast growing, plus end (Vale et al., 1985c). Since the outward movement (dis-
persion) of melanosomes was not affected by a dynein ATPase inhibitor, EHNA, it is not likely that this depends on tubulin-dynein system. However, the process was potently inhibited by NEM. In contrast, the kinesin-based movement is found to be insensitive to NEM and vanadate (Vale et al., 1985c). Our results of inhibitor experiments on pigment aggregation (corresponds to retrograde transport in the axon) suggest that the movement is driven by dynein ATPase-like molecule, while in axoplasmic transport, the retrograde flow is induced by a forcegenerating protein that is distinct from kinesin (Vale et al., 1985c).

Whether microtubules undergo cycles of disassembly and reassembly during melanosome movement has been studied only in a few species. In Fundulus, Murphy and Tilney (1974) have shown that the number of cortical microtubules remains unchanged during melanophore responses, while Schliwa and Euteneuer (1978) have described a dramatic decrease in the number of microtubules during aggregative phases in angelfish melanophores. Our present observation on Gambusia melanophores indicates that cycles of microtubule disassembly and reassembly are actually taking place concomitantly with or prior to pigment movements. In our preliminary experiment, pigment aggregation and dispersion did occur in the presence of colchicine at 1 mM, the dose sufficient to block reassembly of microtubules (Obika et al., 1978b). This indicates that the reformation of cortical microtubules during pigment dispersion has nothing to do with the force generation for pigment dispersal, but cortical microtubules are probably required for the maintenance of rigid cellular cortex and the pathway in which melanosomes and other cytoplasmic constituents travel toward the cell center. Disassembly of microtubules itself does not induce pigment aggregation, as has been shown by cold treatment.

In goldfish xanthophores (Obika et al., 1978a; Lo et al., 1980) and in swordtail erythrophores (Akiyama and Matsumoto, 1983; Akiyama, 1985), involvement of actin filament in pigment translocation is strongly suggested although in melanophores, including the present system, there is little evidence supporting that actin or actin-myosin system plays a major role in pigment movement.

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**Literature cited**


(OB and YI: Department of Fish Physiology, Tokyo University of Fisheries, Konan 4–5–7, Minato-ku, Tokyo 108, Japan; MO: Department of Biology, Keio University, Hiyoshi, Yokohama 223, Japan; NN, present address: Dainippon Pharmaceutical Co. Ltd., Fukuoka Branch, Nakagofuku-cho 6–10, Hakata-ku, Fukuoka 812, Japan)

カタツシム黑色素胞における色素顆粒移動のビデオおよび電子顕微鏡による解析
中村直志・池田弥生・小比良正敬
カタツシム黑色素胞の色素顆粒移動および微小管の役割について考察した。色素顆粒凝集速度は反応初期の10–20秒間で最大で、その後は急激に減少した。一方拡散はほぼ等速移動であり、速度は色素顆粒凝集に比較して小さかった。また凝集において満られる細い部分で速く、太い部分で遅かったが、拡散においては縮まりの太さにかかわらずほぼ一定であった。色素顆粒凝集に伴って凝集の帯が起こることがあるが、これに付随して細胞膜を裏打ちする微小管（CMT）も減少した。一方拡散においては、色素顆粒移動に先駆して凝集中への細胞質基質の乱流が起こり全元の形態を回復することが認められた。さらに低温処理による微小管の消失は、細胞膜と色素顆粒間の距離を有意に短縮し、このようにCMTは色素顆粒移動を円滑にするための構造的役割を担っていると考えられる。また数値の代謝阻害剤の実験結果から、色素顆粒凝集はダイミン依存性であることが示唆された。色素顆粒拡散はシェルピングダイミン系あるいはアクチノミコン系の何れにも非依存性であることが示唆された。

(中村・池田：108 東京都港区港南4–5–7 東京水産大学水産生理学講座；小比良：223 横浜市港北区日吉 慶應義塾大学生物学教室；中村，現住所：812 福岡市博多区中州服町6–10 大日本製菓株式会社福岡支店)