EVIDENCE FOR RAPID STRUCTURAL AND FUNCTIONAL
CHANGES OF THE MELANOPHORE MICROTUBULE-
ORGANIZING CENTER UPON PIGMENT MOVEMENTS

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ABSTRACT
Melanophores of the angelfish, Pterophyllum scalare, have previously been shown
to display ~2,400 microtubules in cells with pigment dispersed; these microtubules
radiate from a presumptive organizing center, the central apparatus (CA), and
their number is reduced to ~1,000 in the state with aggregated pigment (M.
Schliwa and U. Euteneuer, 1978, J. Supramol. Struct. 8:177–190). In an attempt
to elucidate the factors controlling this rapid reorganization of the microtubule
apparatus, structure and function of the CA have been investigated under different
physiological conditions. As a function of the state of pigment distribution,
melanophores differ markedly with respect to CA organization. A complex of
dense amorphous aggregates and associated fuzzy material, several micrometers
in diameter, surrounds the centrioles in cells with pigment dispersed, and numerous
microtubules emanate from this complex in a radial fashion. In the aggregated
state, on the other hand, few microtubules are observed in the pericentriolar
region, and the amount of fibrous material is greatly reduced. These changes in
CA morphology as a function of the state of pigment distribution are associated
with a marked difference in its capacity to initiate the assembly of microtubules
from exogenous pure porcine brain tubulin in lysed cell preparations. After
complete removal of preexisting microtubules, cells lysed in the dispersed state
into a solution of 1–2 mg/ml pure tubulin have numerous microtubules associated
with the CA in a radial fashion, while cells lysed in the aggregated state nucleate
the assembly of only a few microtubules. We conclude that it is the activity of the
CA that basically regulates the expression of microtubules. This regulation is
achieved through a variation in the capacity to initiate microtubule assembly.
Increase or decrease in the amount of dense material, as readily observed in the
cell system studied here, seems to be a morphologic expression of such a physio-
logic function.
The development of conditions for the polymerization of microtubules in vitro (29) allows detailed studies of cellular structures involved in the control of microtubule distribution in the living cell. Such studies have already been performed on a variety of mitotic centers, and other sites involved in microtubule organization (e.g., references 1, 4, 7, 10, 12, 25, 26, 27, and 30). Slightly modified procedures have been used here. We show that the material comprising the CA may serve as an initiation center for the in vitro assembly of microtubules in an aster-like fashion, and that this material undergoes a profound structural and functional reorganization in association with different physiological activities of the cell. The results indicate that it is the constantly changing activity of the CA which determines the expression of microtubules.

**MATERIALS AND METHODS**

**Preparation of Cells**

Melanophores of the angelfish, *Pterophyllum scalare*, were isolated from excised scales as described (22). Briefly, scales were treated with collagenase (5 ml/ml) in calcium-free Ringer's solution for 20-40 min. Melanophores were then collected with a micropipette and allowed to settle on a cover slip in the presence of calcium-Ringer's. To induce spreading of the cells on the substrate, isolated cells were kept in a 1:3 mixture of amphibian culture medium (Biocult: Grand Island Biological Co., Grand Island, N. Y.) and Ringer's solution for up to 4 h at room temperature.

**Preparation of Brain Tubulin**

Microtubule protein from porcine cerebrum was purified by three cycles of temperature-dependent assembly and disassembly in polymerization buffer (0.1 M piperazine-\(\cdot\)H\(\cdot\)2SO, \(\cdot\)2-ethane sulfonic acid, 2 mM EGTA, 1 mM MgSO\(_4\), 1 mM GTP, pH 6.4), as described (24). Tubulin was separated from microtubule protein by phosphocellulose chromatography in the cold (26), as described (8, 9): phosphocellulose was equilibrated and loaded with microtubule protein in polymerization buffer. Homogeneous tubulin was eluted with this buffer and the microtubule-associated proteins were obtained from the same column by elution with polymerization buffer, 0.8 M in NaCl. Pure tubulin (7 mg/ml) was stored in aliquots in polymerization buffer at \(-70^\circ\)C. This tubulin preparation was free of polypeptides with molecular weights higher than that of tubulin as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and only a few bands of lower molecular weight polypeptides could barely be detected on very heavily overloaded slab gels (for documentation, see references 8 and 9). These preparations of phosphocellulose-purified tubulin did not show in light-scattering analysis any significant self assembly in polymerization buffer after a 25-min incubation at 37°C at a protein concentration of 3 mg/ml. Addition of microtubule-associated proteins immediately restored a fast and extensive polymerization (8, 9).

**Polymerization of Microtubules onto Melanophore Nucleating Sites**

To test the initiating capacity of the CA, cells were prepared according to one of the following procedures: (a) isolated melanophores were kept at 0°C for 30-40 min either in Ringer's solution alone or in Ringer's supplemented with colchicine (0.05 mM) to depolymerize preexisting microtubules. To free the cells from the calcium-containing culture medium, they were transferred to polymerization buffer without added tubulin. After this brief wash, cells were lysed with polymerization buffer containing 0.1% Triton X-100 (30-60 s), followed...
by application of tubulin in polymerization buffer. All steps described were carried out at 0°C. Cells were then brought to 37°C for the desired time period using an incubator.

(b) The second procedure involved a brief wash of the cells in polymerization buffer, followed by lysis in polymerization buffer plus 0.1% Triton X-100. With this detergent concentration, the cell membrane is ruptured within 5-20 s. Upon lysis, all movements cease immediately and some of the pigment granules are washed out into the medium. The outline of the cell as seen in the light microscope, however, remained unchanged. The cells were kept in this medium for at least 10 min before the tubulin solution was applied. All these steps were performed at room temperature.

Electron microscopy revealed that both procedures (a) and (b) lead to essentially microtubule-free open cell preparations before application of exogenous tubulin.

**Electron Microscopy**

For electron microscopy, cell preparations were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) for 20-30 min, followed by osmium tetroxide in cacodylate buffer for 30-40 min. After rapid dehydration in ethanol, specimens were embedded in an Epon/Araldite mixture. The cover slip was removed by a cold shock in liquid nitrogen. Serial thin sections embedded in a Hitachi H500 electron microscope.

**Microtubule Counts**

Microtubules were counted on prints with a final magnification of 40,000. Counts were made in an area 5 x 5 μm wide of sections that included the centriole pair in the center of the square studied.

**Reagents and Solutions**

Ringer's solution was of the same composition as described (21). To induce pigment aggregation and dispersion of isolated cells, solutions of 0.1 mM adrenalin and atropin, respectively, were freshly prepared in Ringer's solution before each experiment. Colchicine (SERVA, Heidelberg, Federal Republic of Germany) was used in a concentration of 0.05 mM in Ringer's solution.

**RESULTS**

Microtubules of angelfish melanophores show a precise radial arrangement relative to the central cell area (22, 23). The latter is characterized by the CA, an accumulation of osmiophilic material (dense aggregates embedded in a finely fibrillar or granular matrix) associated with the centrioles. A comparison of melanophores in different states of pigment distribution reveals distinct alterations of the structure of the CA. In the dispersed state (Fig. 1) dense material is scattered in the vicinity of the centrioles. The diameter of the zone occupied by this material may reach several micrometers, and dense aggregates may be found at a distance of up to 5 μm from the centrioles (20). Numerous microtubules are associated with this complex in a radial fashion, as demonstrated by immunofluorescence microscopy (23) and electron microscopy (20, 22).

In the aggregated state (Fig. 2), the CA has a different appearance. Fibrous material comparable to that found in cells with pigment dispersed is identified only in the immediate vicinity of centrioles. As judged from serial thin sections, there are only a few distinct dense aggregates, and the number of microtubules in the pericentriolar region is greatly reduced. An investigation of the time-course of the transition from the aggregated to the dispersed state reveals a gradual increase in the amount of fibrous material, dense aggregates, and associated microtubules (Fig. 3). The whole process resembles a "condensation" of amorphous material that leads to a re-establishment of the system of dense aggregates and microtubules (see Fig. 1) within <1 min.

A depolymerization of microtubules induced by either cold treatment (0°C for 30 min) alone or in combination with 0.05 mM colchicine does not affect the material of the dense aggregates. In cells with pigment dispersed, patches of dense material occupy the space near the centrioles (Fig. 4), while cells in the aggregated state show only a few dense aggregates in the pericentriolar region (Fig. 5). Microtubules and microtubule fragments are apparently completely absent from these cells, even in the vicinity of the centrioles. This conclusion is based on the examination of numerous serial thin sections.

Hence a change in morphological appearance of the CA is associated with changing patterns of microtubule organization in different states of pigment distribution, and these differences are preserved after removal of microtubules. An important role of the CA in the establishment of the microtubule system may therefore be suggested. To test for the capacity of the CA to initiate the assembly of microtubules from exogeneous tubulin provided, isolated cells were Triton-extracted according to the procedure outlined in Materials and Methods. To allow a direct comparison between the initiating capacity of CA's of cells with either pigment dispersed or aggregated, all polymerization experiments were performed on cells attached to cover slips to introduce as little perturbation of the cellular structures (including the CA) as possible. Observation of microtubule polymerization in negatively stained preparations would clearly circumvent the tedious embedding and thin-sectioning work and thus would be less time consuming.

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FIGURES 4 and 5  Cold-plus-colchicine treatment, intact cells.

FIGURE 4  Pigment dispersed. Numerous dense aggregates (arrows), but no microtubules.

FIGURE 5  Pigment aggregated. Few dense aggregates (arrows). Figs. 4 and 5: × 33,000.

FIGURES 1-3  Central apparatus of melanophores fixed in different states of pigment distribution under normal conditions.

FIGURE 1  Pigment dispersed. Note numerous electron-dense aggregates near centriole.

FIGURE 2  Pigment aggregated. Some fibrous material near centriole.

FIGURE 3  Cell fixed during pigment dispersion. Some electron-dense aggregates and microtubules are present near the centriole. Figs. 1-3: × 33,000.

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consuming. This approach was not taken since unwanted alterations of the CA cannot be excluded. Therefore, all subsequent descriptions are based on an analysis of thin sections.

Melanophores lysed into polymerization buffer alone without exogeneous tubulin are essentially free of microtubules, even in the immediate vicinity of the CA, although the persistence of very short (<0.1 μm) microtubule fragments undetectable with the electron microscope methods applied here cannot be excluded with absolute certainty. The CA of lysed cells consists of centrioles, dense aggregates, interspersed fibrous material, and some associated intermediate-sized filaments and ribosomes. Figs. 6 and 7 compare sections taken at the level of the centrioles of melanophores with pigment dispersed and aggregated, respectively. A difference in the amount of dense material is demonstrated also in these lysed cell preparations. When isolated melanophores are lysed in the cold into a solution of pure tubulin (1 mg/ml), followed by a rewarming period of only 2-5 min at 37°C, short microtubules are observed only in the vicinity of the CA, demonstrating that microtubule formation is initiated at this complex (not shown).

In the presence of 2 mg/ml tubulin and after an incubation time of 20-25 min at 37°C, the CA of cells with pigment aggregated nucleates the growth of only a small number of microtubules, while in cells with pigment dispersed microtubules are associated with the CA in greater number and in an aster-like arrangement comparable to that found in normal cells (Figs. 8 and 9). To document quantitative differences in microtubule number, microtubules have been counted in horizontal sections taken at the level of the centrioles. Counts in areas of identical size show that the number of microtubules initiated at the CA is in both the aggregated and dispersed state roughly comparable to that observed in the corresponding normal, untreated cells (Table 1). Their number is apparently only slightly increased by raising the tubulin concentration (data not shown).

The results suggest that microtubule formation in these preparations results from an initiation step of the CA. The question remains, however, which of the components of the CA, notably centrioles or dense aggregates, are responsible for this effect. Efforts were therefore made to separate these two components from one another. Several more vigorous washes with polymerization buffer before addition of tubulin seem to separate the centrioles from the dense aggregates at least in some of the cells. While centrioles free of dense material give rise to microtubules in an axoneme-like pattern but not in a radial array (Fig. 10), the complex of dense aggregates, even when lacking centrioles, is capable of initiating the growth of microtubules in a characteristic aster-like arrangement (not shown). We did not observe growth of microtubules from centrioles still associated with dense material, which suggests that the association of the two components somehow prevents this mode of microtubule formation.

DISCUSSION

Observations from a variety of cell types suggest that the distribution of microtubules may be controlled by distinct structures or foci for which Pickett-Heaps (14) coined the term microtubule-organizing centers (MTOCs). MTOCs may have mainly two functions: they precisely define the locus where a microtubule should begin to grow (initiation) and then help to maintain the position of the microtubule initiated there (anchorage) (7). Regulation of the initiating/anchoring site itself would therefore be an effective means for the cell to control the gross distribution of microtubules. The melanophore apparently provides an excellent example for MTOC regulation in terms of a rapid "disassembly/reassembly of the assembly site." The observations presented here demonstrate profound morphological and functional changes of the CA in the course of pigment movements. In

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**Figures 6-9** Lysed cell preparations.

**Figure 6** Pigment dispersed, lysis procedure 1 (see Materials and Methods), no exogenous tubulin added. (a) Overview of the pericentriolar region. (b) Higher magnification of the dense material near the centrioles.

**Figure 7** Pigment aggregated, lysis procedure 1, no exogenous tubulin added. (a) Overview of the pericentriolar region. (b) Higher magnification of the material near the centrioles. Figs. 6a and 7a: x 26,000; Figs. 6b and 7b: x 79,000.

**Figure 8** Pigment dispersed, lysis procedure 1, 2 mg/ml exogenous tubulin.

**Figure 9** Pigment aggregated, lysis procedure 1, 2 mg/ml exogenous tubulin. Figs. 8 and 9: x 26,000.
the dispersed state, the CA consists of numerous
electron-dense aggregates and associated fibrous
material. An analysis of serial thin sections has
shown that the vast majority of microtubules are
situated with their proximal ends embedded in this
complex in such a way that they originate in the
dense aggregates or at least start in their vicinity
(20). In the aggregated state, this complex is re-
placed by a narrow zone of fibrous material asso-
ciated with the centrioles, and a much lower num-
ber of microtubules is observed in its vicinity. The
process of pigment dispersion is characterized by
a gradual increase in the number of both dense
aggregates and microtubules, demonstrating the
dynamics of the changes which this site can un-
dergo.

The in vitro experiments with lysed cell prepa-
trations of melanophores show the competence of
the CA to initiate the assembly of microtubules
from tubulin free of microtubule-associated pro-
teins that otherwise is incompetent of self-initia-
tion. More importantly, however, these experi-
ments show that the remarkable difference in
structure of the CA between the two extreme states
of pigment distribution is retained under these

| Pigment state | Normal cells | Lysed cells, 2 mg/ml tubulin |
|---------------|--------------|-----------------------------|
| Pigment dispersed | 388 ± 70 (n = 5) | 348 ± 65 (n = 8) |
| Pigment aggregated | 60 ± 7 (n = 6) | 25 ± 8 (n = 5) |

Microtubules were counted in horizontal sections taken
at the level of the centrioles. Number of cells in which
counts were made in parentheses.

TABLE I

*Microtubule Counts in Normal and Lysed Cells*

*FIGURE 10* Pigment dispersed, lysis procedure II, followed by several washes with polymerization buffer.
2 mg/ml exogenous tubulin. Isolated centriole with microtubules polymerized in an axoneme-like pattern.
Section thickness ~0.3 μm. × 26,000.
pigment dispersed: high number of microtubules in vivo
prominent CA
high initiation capacity in vitro

pigment aggregated: lower number of microtubules in vivo
reduced CA
low initiation capacity in vitro

From these observations we conclude:
(a) The capacity to initiate the assembly of microtubules in an aster-like fashion comparable to that observed in vivo resides in the dense material comprising the CA. Centrioles alone give rise to a few microtubules in an axoneme-like pattern but not in a radial orientation. This conclusion supports the results of Gould and Borisy (7) obtained in studies of mitotic centers of Chinese hamster ovary cells in culture. It seems that the dense aggregates of the CA are functionally comparable to the fuzzy pericentriolar material observed in a variety of cell types (e.g., references 15, 17, and 18). Thus, the CA may in fact represent a hypertrophied centrosome.

(b) It is the activity of the CA that basically determines the expression of microtubules in the melanophore. Such a regulation seems to be achieved through a variation in the capacity to initiate microtubule assembly. Increase or decrease in the amount of dense material, as readily observed in the cell system studied here, may be a morphologic expression of such a physiologic property. Taking into account the results of Margolis and Wilson (11) who described opposite-end assembly and disassembly at steady state in vitro, then MTOCs could not only serve as a nucleation center for microtubule growth, if primary assembly and disassembly ends were also present on microtubules in vivo, and if the primary disassembly site were the proximal microtubule end embedded in the centrosomal material, then MTOCs were able, through their rapid disintegration, to provide also an effective trigger for microtubule disassembly in that they expose the proximal microtubule ends to depolymerizing stimuli. However, this hypothesis has to be tested in the future. That the changes described here can take place within a few seconds makes the melanophore an excellent model system for the study of MTOC physiology. Unfortunately, however, the system is not yet susceptible to extensive biochemical investigation because of limitations imposed by sample size.

Although the melanophore with its dramatic rearrangement of the centrosome material so far appears somewhat unusual, similar but possibly more subtle changes may also take place in other cells. That the capacity of the centrosome to initiate microtubule assembly may change at least at the onset of cell division is indicated by studies of mitotic centers. Weisenberg and Rosenfeld (30) observed morphological changes of mitotic centers after egg activation in the surf clam, and Snyder and McIntosh (25) describe a change in the capacity of mitotic centers of mammalian cells to initiate microtubule assembly in lysed cell preparations at the time of nuclear envelope breakdown. Spindle pole bodies of yeast cells apparently undergo a similar change during division (10). The results of the present study suggest that MTOCs may change their activity not only at the time of mitosis but also at any time of the cell cycle in association with different cellular activities.

Currently, we cannot exclude the possibility that initiating sites are being washed out into the medium during or after cell lysis. Particularly in cells with pigment aggregated, such (inactivated?) sites may be present in the cytoplasm without any structural association with the CA. It is not clear, however, whether such free sites, if present, function in initiation. If the number of initiation sites were the same in both the aggregated and dispersed state, and if all sites, free or CA-associated, would function in microtubule nucleation, the number of microtubules should be the same in both states. This, however, is not the case.

In vivo, pigment distribution in chromatophores is under neural and/or humoral control. Signals reaching the pigment cell membrane are transmitted to the cell interior, possibly via a system involving cAMP (5, 6; see reference 2 for review). Regulation of the microtubule apparatus might thereby be a key event in a series of structural processes involved in pigment movements. The present study suggests that, with regard to microtubule regulation, the centrosomal MTOC might be an important target for signals transmitted from the cell surface to its interior.

This study was supported in part by the Deutsche Forschungsgemeinschaft. M. Schliwa is a recipient of a Heisenberg Stipend from the Deutsche Forschungsgemeinschaft.

Received for publication 11 April 1979, and in revised form 26 July 1979.
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