Part 2

Role of the cytoplasmic and the intranuclear microtubules on the behavior of pronuclei during conjugation in *Paramecium caudatum*

Abstract

Fertilization process of *Paramecium caudatum* includes a reciprocal exchange of migratory pronuclei across a temporary junction between conjugating cells. To know the role of microtubules on the behavior of pronuclei during nuclear exchange in *P. caudatum*, the localization of microtubules was investigated by indirect immunofluorescence with a monoclonal anti- α -tubulin antibody and transmission electron microscope. The immunofluorescence showed that the migratory pronucleus was surrounded by the cytoplasmic microtubules. The migratory pronucleus seems to be pushed to the cell junction by the cytoplasmic microtubules. Then the migratory pronucleus extended through into the partner cell. An electron-microscopic observation showed that the extranuclear microtubules were assembled at the back of the migratory pronucleus, and the intranuclear microtubules of the pronucleus were aligned along the direction of the extension at the exact moment when the migratory pronucleus was crossing the cell junction. The transfer of migratory pronucleus was inhibited by injection of the monoclonal anti- α -tubulin antibody. These evidences suggest that both the cytoplasmic and the intranuclear microtubules are necessary for the dynamic behavior of gametic pronucleus through the cell junction of conjugating pair. I also suggest that microtubules are involved in the pronuclear migration after entering the partner cell.

Introduction

Migration of migratory pronucleus is the most dynamic nuclear event in conjugation of *Paramecium*. After the selection of meiotic products, one survived meiotic product divides and forms two gametic pronuclei in each cell of mating pair of *P. caudatum*. One is a migratory pronucleus, and the other is a stationary pronucleus corresponding to male and female pronuclei in higher organisms. A reciprocal exchange of migratory pronuclei occurs through a small opening on the membranes of the junction that separates the two conjugating cells. Then, the migratory pronucleus fuses with the stationary pronucleus of the conjugating partner to form fertilized nuclei (Sonneborn, 1947; Hiwatashi, 1969, 1981; Miyake, 1981).

Immunological study about the pronuclear exchange during conjugation of *Paramecium* has not been carried out. In *Paramecium*, microtubules were observed in or out of the migratory pronucleus during nuclear exchange (André and Vivier, 1962; Inaba *et al.*, 1966; Jurand, 1976), but the role of microtubules during nuclear exchange was not be studied in *Paramecium*. What are the roles of microtubules involved in the behavior of pronucleus during conjugation of *P. caudatum*?

It also remains unsettled questions how the migratory pronucleus move to the stationary pronucleus of the partner cell, and it fuses with the stationary pronucleus to form the fertilized nucleus after penetration of the migratory pronuclei.

The main purpose of this part is to investigate the role of microtubules during the nuclear exchange. I will describe the evidences that the migration of the migratory pronucleus result from the cooperative work of the cytoplasmic and the intranuclear microtubules during the nuclear exchange. The other roles of the cytoplasmic and the intranuclear microtubules during the nuclear exchange are discussed.

Materials and Methods

Stocks and culture, Concentration of conjugating pairs, and **Iimmunofluorescence.** These techniques were described in the part 1.

Electron microscopy. The procedures for fixation and block-staining followed those of Orias *et al.* (1983). Cells were fixed with 2% glutaraldehyde in 5mM PB (pH 7.0) containing 1% sucrose, and treated immediately with 1% osmium tetraoxide at room temperature. After 1 h, the cells were washed three times with 50mM PB (pH 7.0) and once with 50% ethanol. Then, the cells were block-stained with 1% uranyl acetate in 50% ethanol, and dehydrate through increasing alcohol concentrations and 100% n-butylglycidyl ether (QY-1, Nisshin EM). After that, the cells were infiltrated in an n-butylglycidyl ether/epoxy rigin (Epon 812, TAAB) mixture and embedded in the epoxy resin at 60℃. Ultrathin sections were cut with a diamond knife (Diatome), mounted on formvar-coated single hole grids. Observations were performed with a JEM-1010 transmission electron microscope (JEOL Ltd.) at 80 kV.

Microinjection. Pairs for the microinjection were deciliated with 5% ethanol (Ogura, 1981) and embedded in mineral oil. Pairs were observed under a Nomarski polarizing microscope (OPTIPHOT, Nikon). Antibodies were injected with a micromanipulator (IM-6, Narishige). About 20-30 pl (protein 160 μg/ml) of an antibody was injected into a cell of the mating pair of *P. caudatum* at the stage of the third prezygotic division by using a glass microinjection needle (inside diameter about 5 μm) (Koizumi, 1974; Mikami, 1979). Then the pairs were placed in K-DS supplemented with BSA (1mg/ml) to heal the injury brought by the operation, and were sequentially observed each 15 min until 2 h after injection.

Results

Dynamic organization of microtubules during nuclear exchange in P. caudatum.

In each cell of the conjugating pairs of *P. caudatum*, a surviving haploid nucleus undergoes the third prezygotic division to form two gametic pronuclei, a migratory and a stationary pronuclei. Then the migratory pronuclei exchange reciprocally through the junction of the conjugating pair in the paroral region.

To investigate the interaction between microtubules and pronuclear behavior during the migration of the gametic pronucleus into the partner cell, I observed the localization of α-tubulin in detail using confocal microscopy. When the third prezygotic division is complete, the cytoplasmic microtubules assemble radially around the migratory pronucleus in paroral region (Fig. 5A, arrowheads). Each migratory pronucleus then changes to biconvex lens shape and is piled up in the junction of the conjugating cell (Fig. 5B). The assemblies of the cytoplasmic microtubules around the migratory pronucleus become to flatten against the migratory pronucleus at that time (Fig. 5B, arrowhead). The space between the nuclear envelop and the cell junction is hardly distinguishable. Immediately after this stage, the migratory pronucleus extended into the partner cell (Fig. 5C). The microtubules were also present in the vicinity of the stationary pronuclei at that time (Fig. 5C, arrowheads), although in fewer numbers than the microtubules around the migratory pronucleus. These results strongly suggest that the cytoplasmic microtubules work on the migration of the pronucleus into the partner cell.

Ultrastructual observation of microtubules during conjugation.

To reveal the organization of the microtubules, which are involved

in the pronuclear exchange, I observed the localization of microtubules by transmission electron microscopy.

Figure 6 shows that the migratory pronucleus is just crossing the junction of conjugating pair. Numerous numbers of cytoplasmic microtubules are assembled at the back of the migratory pronucleus (Fig. 7, arrowheads). In contrast to the cytoplasmic microtubules, the intranuclear microtubules are formed parallel to the envelope (arrows). In the lateral side of the migratory pronucleus, the intranuclear microtubules are aligned along the direction of extension (Fig. 8, arrows). In the tip of the pronucleus a number of intranuclear microtubules are aligned along the direction of extension (Fig. 9, arrows). The cytoplasmic microtubules are also observed in the vicinity of the stationary pronucleus (Fig. 10, arrowheads). The smooth edges of the boundary membrane invading the partner cell are seen in Figs. 8 and 10 (circles).

Dynamic organization of microtubules during formation of the fertilized nucleus.

After entering the partner cell, the migratory pronucleus migrates to the cytoplasm of the partner cell and fuses with the stationary pronucleus to form the fertilized nucleus. To know the distribution of microtubules after penetration of the migratory pronuclei I examined the conjugating cells using the confocal microscopy. The migratory pronucleus extends toward the stationary pronucleus of the partner cell and keeps connection with the junction (Fig. 11A). The intranuclear microtubules of the migratory pronucleus seem to extend along the direction of the movement. The migratory pronucleus then fuses with the stationary pronucleus of the partner cell to form the fertilized nuclei connecting to the cell junction (Fig. 11B, arrows), but their chromatin is not yet homogenous in appearance. The intranuclear microtubules of the migratory pronucleus

might be responsible for this connection between the nucleus and cell junction. These microtubules disappear before post-zygotic division (Fig. 11C).

Effect of injection of anti-α-tubulin antibody on the nuclear exchange during conjugation.

To investigate whether microtubules participate in pronuclear exchange, anti-α-tubulin antibody was injected into the cytoplasm of conjugating cells at the stage of the third prezygotic division. The injected cells were sequentially observed under the Nomarski polarizing microscope every 15 min after injection.

When the anti-mouse IgG was injected into the cytoplasm of conjugating cells as a control, all pairs underwent the normal conjugation process and performed the pronuclear exchange and the formation of the fertilized nucleus (Table 2). On the contrary, the conjugating cells injected with the anti-α-tubulin antibody failed to conduct the nuclear exchange. All the injected cells completed the third prezygotic division, but the nuclear exchanges were blocked (Table 2). Two pronuclei of nearly all pairs existed apart from the cell junction in the paroral region. Fertilized nuclei did not form in 46 out of 47 pairs injected with the anti-α-tubulin antibody (Table 2). In a few of the cells injected with the anti-α-tubulin antibody, the unfused pronucleus underwent mitotic division 120 min after the injection, which is the time of the first postzygotic division in the control cells.

Discussion

Cytoplasmic and intranuclear microtubules are essential for nuclear exchange during conjugation.

I focused on the role of microtubules during the pronuclear exchange. The results obtained strongly suggest that the cooperative work of the cytoplasmic and intranuclear microtubules is essential for the migration of the pronucleus during the nuclear exchange. The cytoplasmic microtubules surround the migratory pronucleus after the third prezygotic division (Figs. 5 A and 12 A) and then may push it to the cell junction (Figs. 5 B and 12 B). This idea is supported by electron microscopic observations that when the migratory pronucleus entered the partner cell the cytoplasmic microtubules assembled densely the posterior hemisphere of the migratory pronucleus (Figs. 7 and 12 C). Furthermore, the transfer of the pronucleus was inhibited by microinjection of the anti-α-tubulin antibody (Table 2). The other is the intranuclear microtubules of the migratory pronucleus. At the moment of nuclear exchange, the migratory pronucleus changed from a biconvex lens shape to an extended shape (Fig. 5 C). The numerous intranuclear microtubules were aligned along the direction of extension (Figs. 8, 9 and 12 C).

In *H. erhadi* and *Tetrahymena*, it is observed that cytoplasmic microtubules appear around the migratory pronucleus during pronuclear exchange (Lanners, 1980; Orias *et al.*, 1983). Although the observation of nuclear exchange was preformed in *Paramecium* (André and Vivier, 1962; Inaba *et al.*, 1966), the presence of cytoplasmic microtubules in the vicinity of the migratory pronucleus has been only observed by Jurand (1976) in *P. aurelia*. However, it has been thought that a main motive force for migration of the pronucleus is the amoeboid movement of migratory pronucleus itself in *Paramecium*. I observed the pronucleus at the moment passing through the cell junction. The active migration of a

nucleus may result from an extension by migratory pronucleus itself but never from amoeboid movement as previously reported, indicating that participation of both the cytoplasmic and the intranuclear microtubules is necessary in the nuclear exchange. A 49-kDa filament-forming protein, fenestrin and TCBP-25 (the *Tetrahymena* Ca^{2+}-binding protein of 25 kDa) have been detected in the vicinity of the pronuclei during nuclear exchange (Numata *et al.*, 1985; Nelsen *et al.*, 1994; Hanyu *et al.*, 1995). Takagi *et al.* (1991) observed that the immunofluorescence patterns for tubulin were the same as those for the 49-kDa protein during nuclear exchange in *T. termophila*. Such proteins localized around the pronuclei might act together microtubules during nuclear exchange even in *Paramecium*.

Cytoplasmic microtubules are necessary for positioning of stationary pronucleus.

It has been observed that the microtubules were assembled in the vicinity of the migratory and the stationary pronuclei in *H. earhardi* (Lanners, 1980), and surrounded the both pronuclei in *T. thermophila* (Gaertig and Fleury, 1992). Not only around the migratory pronucleus but also in the vicinity of the stationary pronucleus, the cytoplasmic microtubules appeared during the nuclear exchange (Figs. 5 C and 10). What is the role of these microtubules? When the anti-α-tubulin antibody was injected into the cytoplasm of the conjugating cells, the positioning in the cells of both the stationary and the migratory pronuclei changed. The pronuclei usually stay near the cell junction. However, in the cells injected with the anti-α-tubulin antibody the pronucleus was apart from the junction. The antibody into the cytoplasm may inhibit polymerization cytoplasmic microtubules related to the proper positioning of the pronuclei in the cell.

Microtubules are involved in pronuclear migration for formation of fertilized nucleus.

The question how the migratory pronucleus moves toward the stationary pronucleus after entering the partner cell has not been solved. In *P. caudatum*, the migratory pronucleus moved to the stationary pronucleus in a stretching manner, and the intranuclear microtubules of the migratory pronucleus seemed to be aligned in the direction of movement at that time (Fig. 11 A). This observation suggests that the extension of the intranuclear microtubules may be involved in directing the movement of the migratory pronucleus after the nuclear exchange. In *Tetrahymena*, the migratory and the stationary pronuclei were connected by microtubules to the cell junction just after nuclear exchange (Gaertig and Fleury, 1992). Although such an assembly of microtubules was not observed in *P. caudatum* using confocal microscopy, the cytoplasmic microtubules must be working. Because in the cell injected with the anti-α-tubulin antibody, the migratory pronucleus exists apart from the junction the same as the stationary pronucleus, resulting in the failure of the formation of a fertilized nucleus. This suggests that the cytoplasmic microtubules are also involved in the movement of the migratory pronucleus after a pronuclear exchange. I think that both the intranuclear and the cytoplasmic microtubules are also involved in the pronuclear migration after entering the partner cell.