

Part 3

Role of γ -tubulin on the behavior of germinal nuclei during conjugation in *Paramecium caudatum*

Abstract

In the part 1 and 2, I suggested that microtubules are involved in the nuclear behaviors, which are essential for the gametogenesis and the formation of fertilized nucleus during conjugation of *Paramecium caudatum*. In order to characterize the MTOCs responsible for nuclear behavior during conjugation, I produced an antibody (Pcg 3) raised against *P. caudatum* γ -tubulin and analyzed localization of γ -tubulin during conjugation, especially at the stage of the selection of meiotic products and the nuclear exchange in *P. caudatum*. The immunofluorescence showed that γ -tubulin exhibited in the micronucleus, basal bodies of cilia and oral apparatus throughout the cell cycle. While the macronucleus only labeled with Pcg 3 during cell division. During mitosis and meiosis, fluorescence of Pcg 3 was accumulated in the spindle pole and the separation spindle of dividing micronucleus. When the cytoplasmic microtubules were assembled around the survived meiotic product and the migratory pronucleus, dots stained by Pcg 3 accumulated around these nuclei during conjugation. After the nuclear exchange, the migratory pronucleus extends to the stationary pronucleus without separating from the cell junction. The dots stained by Pcg 3 accumulated at end of or out the migratory pronucleus at the cell junction sides. In the amiconucleate partner cell of conjugating partner, however, these dots did not accumulate, and the cytoplasmic microtubules were not assembled around the paroral region. The movements of the meiotic products and the pronuclei were inhibited by injection of Pcg 3. These results suggest that γ -tubulin are essential for the dynamic behaviors of germinal nuclei during selection of meiotic products and nuclear exchange. I also suggest that the appearance of these cytoplasmic microtubule assemblies and accumulations of γ -tubulin around the paroral region are dependent on the existence of the germinal nuclei.

Introduction

In the parts 1 and 2, I showed that the microtubules are involved in the nuclear migrations during conjugation of *P. caudatum*. The consistent labeling by the anti- γ -tubulin antibodies has been reported on basal bodies of cirri, on oral membrane consisting of three parallel rows of basal bodies and on the micronuclei in interphase cell of *E. octocarinatus* (Liang *et al.*, 1996). In *T. thermophila* and *P. tetraurelia*, labels of the basal bodies and oral apparatus with the anti- γ -tubulin antibody were also observed (Liang *et al.*, 1996; Ruiz *et al.*, 1998). However, none reported where and how localize the MTOCs during conjugation in ciliates, especially at two stages of the nuclear movement.

In this part, I focused on the dynamics and the role of γ -tubulin at the stage of the selection of meiotic product and the nuclear exchange during conjugation in *P. caudatum*. The results suggest that the γ -tubulin is involved in the nuclear behavior during conjugation.

Materials and Methods

Stocks and culture. The stocks used were 27aG3 9417, mating type V (a selfing progeny derivative of 27aG3), C103s8, mating type VI (selfing progeny of C103, kindly supplied by Dr. T. Watanabe, Tohoku University) and KNZ2, mating type VI (kindly supplied by Dr. H. Endoh, Kanazawa University) belonging to syngen 3 of *P. caudatum*. The amiconucleate clone was prepared artificially by removing the micronucleus of C103sII (a segregant from two rounds of selfing of C103). The other techniques were the same as those described in the part 1.

Cloning and mutation. Two pairs of primers were designed from 3' end of the *P. caudatum* γ -tubulin sequence (Mikami *et al.*, personal communication), which exhibits minimal homology to the α - and β -tubulin sequences for PCR amplification of cDNA. To amplify and change TAA and TAG to CAA and CAG, several PCR steps were performed using plasmid pT7 blue (Takara). Primer pair combinations as follows: For the first primer pair, 5'-oligonucleotide 1 (corresponding nucleotides 673-697) 5' **CGGGATCCGTTTCT**CAGACAAACTCTCTT-GTTGC3', which contains nucleotide exchange (underline) and has an additional *Bam*HI restriction site (bold), and 3'-oligonucleotide 2 (corresponding nucleotides 1027-1052) 5' *TTGGACCCCACTCAATAAA-GCTAGC*3', which has an internal *Eco*47I restriction site (italic). For the second primer pair, 5'-oligonucleotide 3' (corresponding nucleotides 1043-1067) 5' *GGGGTCCAACATCAATT*CAAGTAGC3', which has an internal *Eco*47I restriction site (italic) and contains nucleotide exchange (underline), and 3'-oligonucleotide 4 (corresponding nucleotides 1392-1353) 5' **CGGGATCCATTCGCATATTTGTCATCATT**CCCC3', which has an additional *Bam*HI restriction site (bold). PCRs were performed using KOD dash polymerase (TOYOBO). Cycling started with an initial

denaturalization step for 1 min at 94°C, and followed by 30 cycles with 30s 94°C, 10s 45°C, 30s 74°C and a final extension of 5 min at 74°C. Amplification products were purified and cloned into pT7 blue vector, and cut restriction endonuclease and ligated two PCR fragments. The construct was then confirmed by sequencing the complete *P. caudatum* γ -tubulin ORF after cloning into pT7 bleu vector.

Expression and purification of recombinant γ -tubulin. The γ -tubulin cDNA with TAA and TAG changed to CAA and CAG was amplified by PCR using 5'-oligonucleotide 1 and 3'-oligonucleotide 2. The resulting product was cloned in-frame into the expression vector pGEX-6P-2 (Amersham Pharmacia Biotech) using the *Bam*HI site. Expression and purification of recombinant γ -tubulin was performed essentially as described by Smith and Johnson (1988). Solubilization and purification of recombinant were performed according to John and Benjamin (1993). *Escherichia coli* strain BL21 (Amersham Pharmacia Biotech) was transformed with a pGEX-6P-2- γ -tubulin. Protein expression was induced with 0.1mM IPTG. Pellet of bacteria were suspended by repeated pipetting in ice-cold buffer A (20mM PBS, pH 7.3, 200mM NaCl, 1mM EDTA, 2mM DTT) containing 1mg/ml of lysozyme, and incubated on ice for 30 min. Cells were sonicated on ice for 1 min (power level 8, 80% duty cycle) in Ultrasonic disruptor UD-201 (TOMY). After sonication, Triton X-100 was then added to final concentration of 4%. The lysate was incubated at 4°C for 30 min. After incubation, the lysate was clarified by centrifuging for 10 min in a microfuge, 7000 rpm, 4°C. And the pellet was washed three times with buffer A containing 0.5% Triton X-100 and 1mM EDTA. Then the pellet were incubated in 0.4% N-laurylsarcosine (sarkosyl) in buffer A at 4°C for 30 min. After dissolution, the lysate was clarified by centrifuging for 30 min in a microfuge, 14500 rpm, 4°C. The supernatant was transferred to a new

tube, Triton X-100 was added to the desired final concentration 1% from a 20% stock in buffer A, and the lysate was vortexed for 5 s. Swollen glutathion-Sepharose 4B (Amersham Pharmacia Biotech) suspension (50% v/v in buffer A) was added. The lysate was incubated for 45 min at 4°C. The glutathion beads were washed 5 times with ice-cold buffer A containing 1% Triton X-100 by repeated centrifugation and resuspended in cleavage buffer (500mM Tris-HCl, pH 7.0, 150mM NaCl, 1mM EDTA, 1mM DTT). To purify γ -tubulin, GST- γ -tubulin-bound beads were incubated in Cleavage buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM DTT) containing PreScission Protease (Amersham Pharmacia Biotech, 100 cleavage units/ml beads) for 16 h at 4°C. The resulting pellet including γ -tubulin, GST and GST- γ -tubulin were recovered by centrifugation.

Polyclonal antisera. Recombinant γ -tubulin described above was further subjected to SDS-PAGE (17% running gel including 4M Urea with a 3% stacking gel) and cut out from gel. After dialysis against PBS, the gel slices containing γ -tubulin alone were emulsified with Freund's complete adjuvant and injected into two guinea pigs and two rabbits. Booster injections using Freund' incomplete adjuvant were carried out at 7-day intervals. Following booster injections, anti- γ -tubulin antisera were produced in guinea pigs and two rabbits. The anti- γ -tubulin antisera were affinity purified on expressed *P. caudatum* γ -tubulin (225 a.a.-451 a.a.) isolated by one dimensional gel electrophoresis.

Gel electrophoresis and Immunoblot analysis. These techniques were described in the part 1.

Indirect immunofluorescence. Cells were fixed with 1.5% formaldehyde in a PHEM buffer (60mM PIPES, 25mM HEPES, 10mM

EGTA, 2mM MgCl₂) as described by Schliwa and Van Blerkom (1981) at room temperature for 45 min and treated with acetone at -20°C for 20 min. The cells were washed five times with the PBS (pH 7.0) containing 2mM MgCl₂ and 10mM EGTA (PBSm) as described by Keryer *et al.* (1989) and then transferred into the PBSm buffer (pH7.0) containing 2% BSA. The cells were incubated with a monoclonal anti- α -tubulin antibody (1:100 dilution with PBSm containing 1% BSA, Amersham Pharmacia Biotech) and Pcg 3 (1:8 dilution with PBSm containing 1% BSA) for 120 min. The cells were washed four times with the PBSm containing 0.03% Triton-X 100. The secondary antibodies consisted of FITC-conjugated goat anti-mouse IgG (1:100 dilution with PBSm containing 1% BSA, Jackson Immuno Reserch) and Rhodamine-conjugated goat anti guinea pig IgG (1:100 dilution with PBSm containing 1% BSA, Kirkegaard & Petty Laboratories) were incubated for 120 min at room temperature. Finally, the cells were mounted in VECTA SHIELD Mounting Medium (Vector Laboratories) and observed under a confocal microscope Lsm 510 (Carl Zeiss). Each image was constructed about 10 sections. Each Z axis of sections was 0.8 μ m.

Results

Expression of P. caudatum γ -tubulin in E. coli.

I examined whether monoclonal and polyclonal antibodies raised to human γ -tubulin (38 a.a. - 53 a.a.) can be used for *Paramecium*, because γ -tubulin is known to be highly conserved among species. No antibody was able to recognize the *P. caudatum* γ -tubulin. Therefore, I decided to raise polyclonal antisera to *P. caudatum* γ -tubulin. To prepare an anti- γ -tubulin antiserum, I made C-terminal region of *P. caudatum* γ -tubulin (231 a.a.) containing the C-terminal sequence (220 a.a. - 451 a.a.) in *E. coli* transformed with the expression vector, because this sequence exhibits minimal homology to α - and β - tubulin sequences and interacts with the other centrosomal (or spindle pole body) proteins (Roy, 1995). The same as in other ciliates, *Paramecium* employs the abnormal genetic code using the universal stop codons TAA and TAG for glutamine (Preer *et al.*, 1985). To express *P. caudatum* C-terminal γ -tubulin in *E. coli*, I changed a TAA and a TAG codons in this gene to glutamine codons by PCR. The 3' end of *P. caudatum* γ -tubulin gene (693 bp) with all TAA and TAG changed to CAA and CAG was subcloned into the expression vector pGEX-6P-2.

The *P. caudatum* C-terminal γ -tubulin was expressed in *E. coli* and was purified by glutathione-Sepharose 4B (Fig. 13 A). Whole-cell extracts of the transformed bacteria with pGEX-6p-2 recombinant before and after IPTG induction were resolved by SDS-PAGE and stained by Coomassie brilliant blue (Fig. 13 A, lanes 1 and 2). Since the GST- γ -tubulin fusion protein was found in the completely insoluble fraction of total bacterial sonicates (Fig. 13 A, lanes 3 and 4), I solubilized the insoluble fraction using 0.4% N-lauroylsarcosine as modified method of John and Benjamin (1993). The GST- γ -tubulin fusion protein then subsequently was bound to glutathione -Sepharose 4B (Fig. 13 A, lane 5).

To remove GST carried from the fusion protein, GST- γ -tubulin-bound beads were incubated with PreScission Protease. After centrifugation, however, all the C-terminal γ -tubulin proteins were found in the pellet with GST and the GST- γ -tubulin proteins (Fig. 13 A, lane 6). To isolate the C-terminal γ -tubulin protein from GST protein, proteins in the pellet were separated by SDS-PAGE using 17% polyacrylamide gel containing 4M urea (Fig.13 B, lane 2), and the band of the C-terminal γ -tubulin was cut out from the gel. The protein samples obtained were used as antigens to immunize guinea pigs and rabbits.

*Antibody Pcg3 specifically recognized γ -tubulin in *P. caudatum* cell extract.*

Guinea pigs and rabbits were immunized with the expressed *P. caudatum* C-terminal γ -tubulin protein. The specificity of a polyclonal antibody for the whole cell extract of *P. caudatum* (Fig. 14, lane 1) was analyzed. This analysis was made by immunoblotting using an affinity selected fraction of the antiserum of a guinea pig against *P. caudatum* C-terminal γ -tubulin (Pcg 3) derived from the fusion protein in bacterial extract. Pcg3 (relative molecular mass 50 kDa) specifically recognize a single band, corresponding to *P. caudatum* γ -tubulin from whole cell extracts of *P. caudatum* (Fig. 14, lane 3). No such a band is present in the preimmune serum of guinea pig (Fig. 14, lane 2). Moreover, no cross reactivities with other α - and β -tubulin are apparent, because the patterns of α - and β -tubulin are different from Pcg3 (Fig. 14, lanes 4 and 5). Thus, Pcg3 recognize only *P. caudatum* γ -tubulin polypeptide. All further experiments were performed with the affinity-purified Pcg 3.

*Localization of γ -tubulin in interphase cell of *P. caudatum*.*

To determine the localization of γ -tubulin in interphase cells of *P. caudatum*, I performed immunofluorescence experiments with Pcg3. Pcg

3 stains micronucleus, basal bodies of cilia and those of oral apparatus, but not macronucleus and cilia (Fig. 15 b). No staining is observed with the pre-immune serum (Fig. 15 a). These localization agree with the localization of γ -tubulin reported in other ciliates.

*Localization of γ -tubulin in dividing cell of *P. caudatum*.*

To study the localization of γ -tubulin in dividing cell of *P. caudatum* and compare with the localization of microtubules, I performed immunofluorescence experiments with Pcg 3 and the anti- α -tubulin antibody. In dividing cells, the localization patterns between microtubules and γ -tubulin are very similar in micronucleus. However, γ -tubulin is localized at both spindle poles, but α -tubulin is not localized (Fig. 16 a - c, arrowheads). Furthermore, localization of γ -tubulin is quite different from that of α -tubulin in the parental and the new oral apparatuses (Fig. 16 a - c). γ -tubulin seems to be localized in basal bodies of somatic ciliary rows, quadrulus and two peniculi (Fig. 16 d and e). The fluorescence of γ -tubulin is localized along with microtubule arrays in amitotic macronucleus (data not shown). The fluorescence is generally the dots, not the continuous filaments as observed with the anti- α -tubulin antibody in macronucleus (Fig. 16 a - e). The fluorescence observed in food vacuoles in the control cells may be self-fluorescence of the chloroplast of the food (Fig. 16 f and g).

Localization of γ -tubulin during gametogenesis.

In *P. caudatum*, the diploid micronucleus undergoes meiosis and forms the four haploid nuclei. The localization of γ -tubulin is similar to that of α -tubulin except for the spindle poles in telophase of meiotic division (Fig 17 a - d, arrowheads). Moreover, the distribution of γ -tubulin is dotted and scattered throughout the cross section of the separation spindle, while α -tubulin is localized at the edge of the spindle

(Fig. 17 b – d, ss). γ -tubulin can be detected in a micronucleus at all stages of the cell cycle (Figs. 15, 16 and 17).

In the part 1, I revealed that the cytoplasmic microtubules are essential for the selection of the meiotic products. To analyze the localization of γ -tubulin related to the selection of meiotic products, I examined the distribution of γ -tubulin after meiosis in conjugating cells. In addition, to analyze whether the localization of γ -tubulin and the cytoplasmic microtubules are related to the presence of the germinal nucleus or not, conjugation was induced between a micronucleate cell and an amiconucleate cell. When the cytoplasmic microtubules assemble around the survived meiotic product (Fig. 18 b and c, arrowhead, right cell), dots stained by Pcg 3 accumulate around the nucleus (Fig. 18 a and c, arrow, right cell). In amiconucleate cell of the mating pairs, however, the dots of γ -tubulin do not accumulate around the paroral region, and the assembly of the cytoplasmic microtubules do not appear around it (Fig. 18 a - c, left cell). Then the survived nucleus undergoes the third prezygotic division. The weak fluorescence by Pcg 3 appears at both ends of the nucleus and along the microtubule arrays in anaphase of the third prezygotic division (Fig. 18 d - f, right cell). The fluorescence of γ -tubulin then is remarkably accumulated at the spindle poles and remains in the separation spindle at telophase (Fig. 18 d - f, left cell).

Effect of microinjection of anti- γ -tubulin antibody on the survival of the meiotic products during conjugation.

To prove the function of γ -tubulin on the migration of a meiotic product to the paroral region, Pcg 3 was injected into the cytoplasm of conjugating cells at the stage of the second meiotic division. The injected cells were sequentially observed using the Nomarski polarizing microscope every 15 min after injection.

When the preimmune serum was injected as a control into the

cytoplasm of conjugating cells during the second meiotic division, all control pairs underwent the normal meiosis and formed the four meiotic products. Then only one of the four meiotic products moved to the paroral region and survived (Table 3). On the contrary, the conjugating cells injected with Pcg 3 failed in the selection of the meiotic products (Table 3). All pairs injected with Pcg 3 completed the meiotic division and formed the four meiotic products. However, no meiotic products moved to the paroral region in 20 out of 24 pairs injected with Pcg 3 (Table 3). Furthermore, all of the four meiotic products out side of the paroral region degenerated in 20 out of 24 pairs injected with the antibody (Table 3).

Localization of γ -tubulin during nuclear exchange and formation of fertilized nucleus.

The results in the part 2 suggested that the cytoplasmic and the intranuclear microtubules were working on the nuclear exchange. To investigate the localization of γ -tubulin during the nuclear exchange and the formation of the fertilized nucleus, I observed the distribution of γ -tubulin in conjugating cells. Furthermore, to analyze the relationship between localization of γ -tubulin and the presence of the micronucleus during the nuclear exchange, conjugation between a micronucleate and an amiconucleate cells at the stage of the nuclear exchange was observed.

After the third prezygotic division, the cytoplasmic microtubules are assembled around the migratory pronucleus in the paroral region (Fig. 19 b and c, arrowhead, left cell). Dots stained by the fluorescence of Pcg 3 accumulate around the migratory pronucleus (Fig. 19 a and c, arrow, left cell), while these dots do not accumulate around the stationary pronucleus and the paroral region of the amiconucleate cell (Fig. 19 a and c). The cytoplasmic microtubules are also not assembled around the paroral region in amiconucleate cell (Fig. 19 b and c, right cell). When the migratory

pronucleus extends through the cell junction and into the partner cell (Fig. 19 d - f), γ -tubulin is localized at the end of or back side of the migratory pronucleus (Fig. 19 d and f). When the intranuclear microtubules of the migratory pronucleus are aligned along the direction of extension of the pronucleus during the formation of the fertilized nucleus, the dots stained by Pcg 3 remarkably accumulate at the end of or out the migratory pronucleus (Fig. 19 g and i, arrowheads). When the connection between the fertilized nucleus and the cell junction disappeared just after the formation of the fertilized nucleus, γ -tubulin and microtubules remain at the cell junction (Fig. 19 j - l, arrowheads). As soon as the fertilized nucleus go away from the cell junction, these localizations disappear before the post-zygotic division (data not shown).

Effect of microinjection of anti- γ -tubulin antibody on the nuclear exchange during conjugation.

In order to know whether γ -tubulin is involved in the migration of the pronucleus during nuclear exchange, Pcg 3 and the preimmune serum as a control were injected into the cytoplasm of conjugating cells undergoing the third prezygotic division. All pairs injected with preimmune serum as control underwent the normal conjugation process, performing the pronuclear exchange and the formation of the fertilized nucleus (Table 4). All conjugating cells injected with Pcg 3 completed the third prezygotic division, but were blocked the migration of the pronucleus (Table 4). The fertilized nucleus was not formed in all pairs injected with Pcg 3 (Table 4).

Discussion

Localization of the MTOCs responsible for nuclear behaviors during conjugation are important for our understanding how microtubules are assembled. Analyses of the localization of MTOCs has been hampered by difficulty of expression and purification of *P. caudatum* C-terminal γ -tubulin. I have overcome this difficulty and succeeded to make an anti- γ -tubulin antibody (Pcg 3).

Pcg 3 specifically recognize P. caudatum γ -tubulin.

Pcg 3 recognized only about 50 kDa protein in the cell extract of *P. caudatum* by immunoblotting, and did not recognized α - and β -tubulin (Fig. 14). This molecular mass, 50 kDa, agrees with expected molecular mass of *P. caudatum* γ -tubulin. Furthermore, Pcg 3 recognized basal bodies of the cilia and oral apparatus, micronucleus, spindle pole and separation spindle of the micronucleus in *P. caudatum* (Figs. 15, 16 and 17). These localization of γ -tubulin were different from the localization of α -tubulin (Figs. 16 and 17). The macronucleus was only labeled with Pcg 3 during cell division (Fig. 16 a - e). These suggest that Pcg 3 really recognizes the γ -tubulin of *P. caudatum*.

The γ -tubulin localized in separation spindles during mitosis and meiosis.

Microtubules constitute the major skeleton of the midbody in animal and plant cells (McIntosh and Landis, 1971; Smirnova and Bajer, 1992) and separation spindle in *P. aurelia* (Stevenson, 1972). It has been thought that the staining of the midbody by anti- γ -tubulin antibodies might be due to cross-reaction of the antibodies with similar sequences in unrelated proteins. Julian *et al.* (1993) proved that γ -tubulin localized to not only the centrosome and the spindle poles in mammalian cells, but

also transiently present at the microtubule bundles of the midbody during cytokinesis by four polyclonal antibodies against specific epitopes of γ -tubulin. I showed that γ -tubulin localized in the separation spindles during mitosis and meiosis in *P. caudatum* (Figs. 16 a - e and 17 a - d). The intensity of these fluorescence of γ -tubulin on the separation spindles were similar to that observed on the micronucleus in the same cell. In the cross section of the separation spindle, localization of γ -tubulin was different from that of α -tubulin (Fig. 17 b - d). This result suggests that the staining of separation spindles by Pcg 3 does not due to cross-reaction of the other tubulin family proteins; α - and β -tubulins, and the separation spindles are specifically labeled by Pcg 3 in *P. caudatum*. Julian *et al.* (1993) suggested that the γ -tubulin in the midbody has the transient nucleating activity of the special microtubule organizing centers distinct from the centrosomes, because microinjection of anti- γ -tubulin antibody perturbed the formation of the midbody in mammalian cell. The γ -tubulin in the separation spindle of *P. caudatum* may also have the nucleating activity .

The intranuclear microtubules related to nuclear migration extend from the end to the top of the pronucleus after nuclear exchange.

I showed in the part 2, that the migratory pronuclei were moved by the extension of the intranuclear microtubules after the penetration of the migratory pronuclei. However there is no information about the direction of the polymerization of the intranuclear microtubules in that part. I found that γ -tubulin localized strongly at the end of or the back side of the extending migratory pronuclei (Fig. 19 d - i). The intensity of the fluorescence of γ -tubulin localized at this portion of the pronucleus was the highest in the cell. The result suggests that γ -tubulin is involved in the extension of the intranuclear microtubules and that the intranuclear microtubules extend from the end to the top of the migratory pronucleus

during extension after nuclear exchange.

The γ -tubulin located in the cytoplasm is necessary for polymerization of the cytoplasmic microtubules.

In the parts 1 and 2, I reported that the cytoplasmic microtubules work on the nuclear movement during conjugation in *P. caudatum*. Where are the MTOCs of the cytoplasmic microtubules responsible for the nuclear behavior during conjugation? The dots stained by the anti- γ -tubulin antibody accumulated around the survived meiotic product and the migratory pronucleus (Figs. 18 a - c; 19 a - c). Moreover, the anti- γ -tubulin antibody blocked the migration of meiotic products and the pronucleus without affecting the meiotic and the third prezygotic divisions (Tables 3 and 4). These experiments suggest that the polymerization of the cytoplasmic microtubules is inhibited by the injection of the anti- γ -tubulin antibody into the cytoplasm, but not of the intranuclear microtubules. The anti- γ -tubulin antibody may not enter into the nuclear envelope. These results suggest that the γ -tubulin localized in the cytoplasm is necessary for the polymerization of the cytoplasmic microtubules on the nuclear migration during conjugation of *P. caudatum*.

Germinal nucleus responsible for accumulation of γ -tubulin and assembly of cytoplasmic microtubules around the paroral region.

I revealed that the cytoplasmic microtubules are essential for the selection of meiotic products and the nuclear exchange of *P. caudatum* described in the parts 1 and 2. In other ciliates, the cytoplasmic microtubules were observed around the survived meiotic product and the migratory pronucleus (Lanners, 1980; Orias *et al.*, 1983; Gaerting and Fleury, 1992). Takagi *et al.* (1991) observed that the network structure of the 49-kDa protein involved in nuclear behavior during conjugation of *T. thermophila* when conjugation was induced between the star-strain with

defective micronuclei and wild-type cells. The network structure appeared in both cells of conjugating pair at the stage of the selection of meiotic products. Do the assembly of the cytoplasmic microtubules and the accumulation of γ -tubulin depend on the presence of the germinal nucleus? Mating pairs between a micronucleate and an amiconucleate cells clearly showed that the accumulation of γ -tubulin was dependent on the presence of meiotic products or gametic pronuclei (Figs. 18 a and c; 19 a and c). Because none of the accumulations of γ -tubulin appeared around the paroral region of the amiconucleate cells, although the dots of γ -tubulin presented in the cytoplasm. Furthermore, the cytoplasmic microtubules were not assembled around the paroral region in amiconucleate cells at that time (Figs. 18 b and c; 19 b and c). These results strongly suggest that the presence of the germinal micronucleus is indispensable for the accumulation of γ -tubulin and the formation of the cytoplasmic microtubules around the paroral region in *P. caudatum*. In *Drosophila*, the study on the γ -tubulin distribution suggested that centrioles play a role in the spatial organization of the nucleating material containing γ -tubulin (Debec *et al.*, 1995). Microtubule nucleation on the centrosome and the SPB is activated at the onset of mitosis (Oakley, 1992; Zheng *et al.*, 1995; Erickson and Stoffler, 1996). In *P. caudatum*, the nuclear migration to the paroral region is specific phenomenon of germinal nucleus. Because I have revealed that when the macronuclear fragments, almost the same size as meiotic products, were transplanted into the cytoplasm of conjugating cell after meiosis, the macronuclear fragments never moved to the paroral region (Nakajima *et al.*, 2002). Only germinal nucleus may have the roles of the spatial organization and the activation of the MTOCs containing γ -tubulin in *Paramecium*.