Part I

Molecular relationship between odd mating-type substances and even mating-type substances in *Paramecium caudatum*

Introduction

In *Paramecium*, when cells of the odd (O) and even (E) complementary mating types are mixed together under appropriate conditions, they undergo syngen-specific cell-to-cell sexual recognition, called mating reaction (Sonneborn 1937). The substances involved in the mating reaction are called mating-type substances. No one has so far succeeded in identifying them from the cilia, and the biosynthetic processes of both mating-type substances are not known.

To identify the mating-type substances, five monoclonal antibodies (i.e. OmA, OmB, OmC, OmD and OmE) specifically inhibiting the mating reactivity of all strains belonging to O mating type of syngen 3 (O^3) were obtained (Azuma et al. 1996). The localization of the antigens on the ventral surface cilia of the O^3 cells was completely consistent with the cellular localization of the mating-type substance so far postulated (Hiwatashi 1961). Furthermore by indirect immunostaining, the antigens were only detected from the cilia of the mating reactive O^3 cells, but not mating reactive E^3 cilia. These monoclonal antibodies inhibit the mating reactivity not only of O^3, but also of O cells of syngen 1 (O^1), syngen 5 (O^5), and syngen 13 (O^{13}). These results together strongly suggested that these monoclonal antibodies are raised against the O mating-type substances, although the direct identification of the antigen molecules by using these monoclonal

antibodies has not yet been achieved (Azuma 1999).

Kumakura (1997) examined that the strength of antigen signals detected by dot-blot analysis was well correlated with the mating reactivity of the cells in deferent cell-cycle stages. However, when the whole cells were used as samples, the antigen signals were detected both of O^3 and E^3 types. This result is contradictory to the fact that these antibodies do not inhibit the mating reactivity of any E mating type. Are these monoclonal antibodies really raised against O mating type substance? If so, what is the molecular nature of the antigen detected from E^3 mating type cells?

In this part, I had clarified that the antigens recognized from E^3 cells by these antibodies were contained in the cytoplasm, but not on the cilia of E^3 cells. Comparable results have been obtained from syngen 1, syngen 5, and syngen 13 in which these antibodies block the mating reaction of O type cells. Finally, I succeeded to prove that cytoplasmic extracts from both O^3 and E^3 cells contain the materials that induce the mating reaction and the formation of conjugating pairs with living E^3 cells but not O^3 cells. These results proved that mating-reactive O^3 mating-type substances exist in the E^3 mating-type cells.

Materials and Methods

Strains and cultures. The main strains used in the study were Bky13, the O mating type of syngen 3 (O^3), and C103s13 (a selfing progeny of C103, kindly supplied by Dr. T. Watanabe, Tohoku University) belonging to the E mating type of syngen 3 (E^3). Other strains used are listed in Table 1. KU is immature progeny of Ugy144 (O^3) and Kyk 5-2 (E^3). Cells were cultured at 25°C in a 2.5% fresh lettuce juice medium (Hiwatashi 1968) diluted with modified Dryl's solution (Dryl 1959) (called K-DS), prepared by substituting KH_2PO_4 for NaH_2PO_4. They were incubated with *Klebsiella pneumoniae* one day before use. Several hundred cells were inoculated into 2 ml of culture medium and then 4 ml, 8 ml, and 8 ml of fresh medium were added on successive days. One day after the last feeding, cells with high mating reactivity were obtained. To obtain starved cells, about 10^6 mating-reactive cells were washed and transferred into the K-DS. Five days later, the very starved cells without mating reactivity completely were obtained. To obtain well-fed cells, about 5×10^5 mating-reactive cells were transferred into 500 ml of fresh culture medium. Sixteen hours later, the cells in log phase without the mating reactivity were used for the dot-blot analysis.

Sample preparation. Cilia detached from cells were

obtained as described (Azuma et al. 1996). About 10^6 packed cells with high mating reactivity were treated for 15 min with an ice-cold Triton X-100 solution containing 0.005% (v/v) Triton X-100, 20 mM KCl, 10 mM of tripotassium salt of EDTA, and 10 mM MOPS buffer, pH 7.0 (adjusted with KOH). The cells were centrifuged at 600 *g* for 2 min and then transferred into an ice-cold deciliation solution of 20 mM $CaCl_2$ in 10 mM Tris, pH 7.4. Twenty minutes later, a cilium suspension was obtained by slow centrifugation at 600 *g* for 2 min and then filtered through a sheet of TOYO 5A filter paper (Advantec, Tokyo, Japan). The filtrate was centrifuged at 10,000 *g* for 30 min, and the precipitated cilia were washed twice in K-DS using the same centrifugation.

The cilia, cell bodies or whole cells from about 10^6 cells were treated for 30 min on ice with a solution containing 0.5% sucrose monolaurate (SM 1200) (Dojindo, Kumamoto, Japan), 10 mM Tris, pH 7.4, 10 mM EDTA, pH 7.0 (adjusted with KOH), and 10μg/ml leupeptin hemisulfate monohydrate (Wako Chemicals, Osaka, Japan). Then, they were centrifuged at 12,000 *g* for 30 min. The supernatants were centrifuged again at 100,000 *g* for 1 h at 4 °C. Supernatants were used as the samples in the dot-blot experiments. In the experiments of testing mating reactivity and mating-type specificity of cell body extracts, the samples after centrifugation at 100,000 *g* were ultrafiltrated with Microcon100 (Amicon Millipore, Bedford, USA), and the filtrates were used as the samples in dot-blot analysis and bioassay. The filtrate

passing through the Microcon100 contains proteins smaller than 100 kDa. Since detergent SM1200 in the cytoplasmic extracts kills the tester cells, it must be removed from the cytoplasmic extracts in advance. This was done using Microcon10 (Amicon Millipore) by changing buffer with 10mM Tris, pH 7.4 containing 10μg/ml leupeptin hemisulfate monohydrate. Microcon10 can retain proteins with molecular weight larger than 10 kDa.

Dot-blot Method. Fifty μl of samples as described in **Sample preparation** and their 1: 2 or 1 : 4 dilution series in 10 mM Tris, pH 7.2 were applied to Immobilon™ Transfer membranes (Millipore) with a Millipore dot-blot system. The membranes were blocked with 3% skim milk (Wako chemicals, Osaka, Japan) in a Tris buffer sodium solution (TBS; 150 mM NaCl in 10 mM Tris, pH 7.2) for 2 h at room temperature. The membranes were incubated with the supernatant of the hybridoma culture containing monoclonal antibodies (1 : 4 dilution in TBS) for 2 h at room temperature and then washed with TBS-T solution (0.5% (v/v) Tween-80 in TBS) 3 times and with TBS twice. The membranes were incubated with HRP (horse-radish-peroxidase)-conjugated goat anti-mouse IgG (Biosource international, Camarillo, USA) for 2 h at room temperature and then washed as indicated in the previous steps. The membranes were reacted with substrates using Konica immunostain HRP-1000 (Konica, Tokyo, Japan).

Indirect immunofluorescence. To determine the

localization of antigens by indirect immunofluorescence, cells were incubated with the antibody OmA before or after fixation. The cells were fixed on ice for 20 min with 2% formalin solution containing 0.005% Triton X-100, 25 mM KCl and 2 mM phosphate buffer, and washed 3 times with 25 K·PB (25 mM KCl in a 2 mM phosphate buffer, pH 7.2) at 4 °C. Then, the cells were incubated for 4 h on ice with the supernatant of the hybridoma culture containing antibody OmA (1 : 4 dilution in PB pH 7.2). In another experiment, living cells of E^3 and O^3 were incubated with the antibody OmA at room temperature for 20 min and then fixed with 2% formalin solution for 20 min at 4 °C. In both cases, after three washes with 25 K·PB at 4 °C, the cells were incubated on ice for 4 h with fluorescein-conjugated goat anti-mouse IgG (Cappl, USA, 1 : 1000 dilution). The treated cells were mounted in 90% glycerol with 1% *p*-phenylene diamine and observed with a confocal laser-scanning microscope (CLSM) (Zeiss, Germany).

Bioassay. To determine mating reactivity and the mating-type specificity, a geometric series of 1 : 4 dilution of the extracts of O^3 and E^3 cell bodies (i.e. the original protein concentrations of the O^3 and E^3 cytoplasmic extracts were 0.23 mg/ml, 0.28 mg/ml, respectively) was prepared after removal of SM 1200 as described in **Sample preparation.** Samples (5 μl) from each dilution were added to 45 μl of mating-reactive tester cells of O^3 and E^3 at a cell density of about 2,000 cells/ml.

Results

Correlation between the detection of the antigen and the mating reactivity of the cells. At first, I confirmed the correlation between the antigen detection and mating reactivity of O^3 cells by dot-blot analysis. Very starved cells and the cells in log phase (called well-fed cells in the figures) without any mating reactivity and mating-reactive cells were used as samples. Strong dot-blot signals were only detected from the mating-reactive cells (Fig. 1, lane 1). No antigen signals were detected from the well-fed or very starved cells, which had no mating reactivity (Fig. 1, lanes 2 and 3). On the other hand, no signals were detected in the control where the supernatant of a myeloma culture was used instead of the antibodies (Fig. 1, Lanes 4, 5, and 6). Moreover, dot-blot signals were never detected from the cells during the immature period of the life cycle (Table 1). These results show that the antigens detected as dot-blot signals were tightly correlated with the mating reactivity of O^3 cells, and are consistent with previous study.

Antigens recognized by the O mating-type-specific antibody exist in the E^3 cell bodies but not in the E^3 cilia. Next to clarify the molecular nature of the antigen detected from E cells, the prerequisite is to know where the antigen exists. In order to clarify the cellular localization of the antigen detected from E^3

cells, I analyzed the cilia and deciliated cell bodies of both O^3 and E^3 mating types separately (Fig. 2). The antigen was detected from both the cilia and deciliated cell bodies of O^3 (Fig. 2A, lanes 2 and 3). However, the signals appeared only from the cell bodies of E^3 and never from its cilia (Fig. 2B, lanes 2 and 3). Thus, some substances recognized by the monoclonal antibody OmA exist in the E^3 cytoplasm, although the antibody never inhibits the mating reactivity of E^3 living cells. The same results were also obtained with antibodies OmB, OmD, and OmE, and only the results of dot-blot analysis with OmD were shown on Fig. 2C. To avoid the problem of the amount of protein loading, I increased the ciliary protein concentration of both mating types up to 0.3 mg/ml, and used them did the dot blot analysis. Clear signals of antigen were detectable from O^3 cilia at the dilution of 1 to 64, but no antigen was detected from the E^3 cilia (Fig. 2C, lanes 3 and 6).

The samples of O^3 cilia and O^3 cell bodies in Fig. 2A were made from the same 10^6 cells. Consequently, comparing the signals in the minimum detectable dilutions, the total amount of antigens contained in the cell bodies of O^3 and E^3 was estimated to be roughly 8 times that of O^3 cilia per cell (Fig. 2A, B). Therefore, I tried to detect the antigen from cytoplasm of E^3 cells by the improved indirect immunostaining. Photographs of 4 typical cells are shown in Fig. 3. When living cells with mating reactivity were treated with the antibodies and then fixed, only the cilia of O^3 cells were labeled with FITC (Fig. 3B), and not the

cilia of E^3 cells (Fig. 3D). However, when cells were incubated with OmA after being fixed with 2% formalin solution containing Triton X-100 and all steps were carried out on ice, the cytoplasm of both O^3 and E^3 cells showed the fluorescence (Fig. 3F, H), although the fluorescence detected from the E^3 cytoplasm seemed to be comparatively weaker than that from the O^3 cytoplasm.

Detection of antigens in the other syngens cross-reacting with these antibodies. Antibodies OmA, OmD, and OmE not only inhibit the mating reactivity of O^3 but also show inhibiting effect on the mating reactivity of O mating types of syngen 1 (O^1), syngen 5 (O^5), and syngen 13 (O^{13}). However, the cross reaction was not seen among syngens 4, 6, and 12 (Azuma et al. 1996). To determine whether there is a correlation between the inhibiting effects on the mating reactivity of O cells and the detection of the antigen from E type cells, I examined the samples of the mating-reactive O and E cells among 7 syngens. Comparable results showing cross-reaction were obtained from some syngens (Table 1). The antigens were detected not only from O^1, O^5, and O^{13} cells, but also from E cells of syngen 1 (E^1), syngen 5 (E^5), and syngen 13 (E^{13}), although they were weaker than those detected from O^3. No antigen was detected from both mating types of syngen 4, syngen 6, and syngen 13. These results showed that the detection of the antigen in the E mating type of one syngen was associated with the inhibiting effect on the mating reactivity

of O mating types of this syngen (Table 2).

E^3 cells contain O^3 mating-type substance. The results of dot-blot analysis and indirect immunofluorescence showed that E^3 cells contained the substances recognized by monoclonal antibodies exclusively inhibiting specifically on the mating reactivity of O cells. If the substances in the E cytoplasm are the real O mating-type substances, it may be possible to induce mating reaction of E cells with the extracts from E cell bodies. The preliminary result revealed that the Microcon100 filtrates still contained some antigens when deciliated cell bodies of mating-reactive O^3 and E^3 cells were solubilized by the SM 1200 solution (Fig. 4A). To examine the activities inducing the mating reaction, the Microcon100 filtrates were used as starting samples. Since SM 1200 in the filtrates kills the living tester cells, it was removed by changing the buffer 6 times using a Microcon10 filter apparatus. Both samples from E^3 and O^3 cell bodies were obtained. Then, their mating reactivity and mating-type specificity were tested by mixing with both mating-type living tester cells (Fig. 4B). Although the mating reaction is mating-type specific, pair formation can be formed without mating-type specificity (Hiwatashi 1951). Therefore, in the mixture of the two mating types, both of homotypic pairs (selfing pairs) and heterotypic pairs (cross pairs) can be formed. Using mating-reactive cilia or membrane vesicles constituted from cilia

with high mating reactivity, it has been so far succeeded to induce mating reaction and the subsequent pair formation because they specifically stick to living cells of the complementary mating types and induce them to show mating reaction and conjugate (Kitamura and Hiwatashi 1976; Takahashi et al. 1974). If there are mating-type substances in the cytoplasm, the extract from the cell bodies might induce mating reaction with complementary mating-type cells and the homotypic conjugating pair of tester cells would form subsequently. As expected samples from O^3 cell bodies induced the E^3 testers to show mating reaction and to form conjugation pairs (Fig. 4 B). The samples from E^3 cell bodies did not induce a mating reaction with O^3 cells. On the contrary, the samples from E^3 cell bodies induced a mating reaction in 3--5 min only with the same mating-type E^3 tester cells. Furthermore, conjugating pairs of E^3 testers were observed about 90 min later. This result shows that the mating-reactive O^3 mating-type substances exist in the E^3 cytoplasm.

Discussion

Mating-type substances are thus far characterized as intrinsic membrane proteins of ventral surface cilia that induce a mating reaction and the formation of conjugating pairs in *P. caudatum* (Kitamura 1988). The cytoplasm of both mating types contained the O mating-type substances because extracts from cell bodies of both mating types induced a mating reaction with E^3 cells and the subsequent formation of conjugating pairs (Fig. 4B). Time schedule of the mating reaction and conjugating pair formation was consistent with that observed in a previous report (Hiwatashi 1951; Kitamura and Hiwatashi 1976). I have not yet succeeded in finding direct evidence to show that these cytoplasmic molecules, the molecules inducing mating reaction with living E cells and the molecules recognized by these antibodies, are the same molecules. However, the results strongly suggest that they are the same. First, the antibodies used in the present study were raised against the O mating-type substance of *P. caudatum* (Azuma et al. 1996). In the present study, the antigen as dot-blot signals was only detected with the mating-reactive cells (Fig. 1, Table 1). Second, the activities inducing the mating reaction were always achieved with the samples containing the antigens recognized by the antibodies (Fig. 4). When the antigen was not detected in the extracts of the cell bodies by some undesirable conditions, these samples failed to

induce the mating reaction with E^3 testers.

The simplest system exhibiting activities of the mating-type substances so far obtained was that of ciliary membrane vesicles isolated by treatment of reactive cilia of *P. caudatum* using the urea-EDTA method (Hiwatashi and Kitamura 1977; Kitamura 1988; Kitamura and Hiwatashi 1976). When the supernatant was treated with high-salt solution, it did not show any signals recognized by the monoclonal antibodies (Kaku 1997). The signals from the supernatant following ultracentrifugation were obtained only after treatment with the detergent SM1200. Therefore, it is reasonable to suppose that the materials inducing the mating reaction were solubilized by the SM 1200 from cell bodies, and contained in the membrane vesicles that were formed after removal of the detergent.

Determination and inheritance of mating types in *P. caudatum* have been extensively analyzed by inter-syngenic crosses, revealing that three genes (i.e. *Mt, MA,* and *MB*) are involved in these events (Tsukii and Hiwatashi 1983). *Mt* is epistatic to *MA* and *MB* and determines the syngen-specific E mating type. *MA* and *MB* are expressed only in the recessive homozygote (*mt/mt*) at the *mt* locus and determine the O mating-type specificity of each syngen. However, the relationship between the three genes and the O and E mating-type substances is not known. In the present study, I directly verified that the mating-reactive O^3 mating-type

substances exist in the cytoplasm of E^3 cells producing E^3 mating-type substances on its cilia. On the basis of the three-gene hypothesis, the present study provides a possible explanation for the biogenesis of E mating-type substances (Fig. 5). In E cells, O mating-type substances are produced by *MA* and *MB* as precursor molecules of/or one part of E mating-type substances, and mating reactive domain of E mating-type substances were exposed under the modification of the *Mt* gene on the E cilia before being transferred to the E cilia (Fig. 5). Such a precursor hypothesis was first suggested by Butzel (1955): the O mating-type substances are the basic substances, and the activity of the *Mt* locus changes the O mating-type substances into the E mating-type substances. This hypothesis explained the fact that the unidirectional mating type change of selfing stocks occurs naturally only in E mating types (Myohara and Hiwatashi 1975; Taub 1966). In selfing stock, some E cells change into O type by some cytoplasmic control or environmental factors, which switch *Mt* locus from an active to a repressed state, and mating reaction occurs during the sexually reactive phase of one culture. My results strongly support Butzel's hypothesis.

The function of the *Mt* gene is still unknown but it is suggested that the *Mt* gene enzymatically controls the change from the O to the E mating-type substances (Kimball 1939). In *P. aurelia*, a protein of 31kDa, pI 6.8 in the ciliary membrane is absent in the log phase, increases in the early stationary phage

and decreases in the starved cells (Adoutte et al. 1980). However, at the resolution allowed by gel separation and staining, no difference in protein pattern has been observed between mating reactive O and E cilia in *P. aurelia* (Adoutte et al. 1980) and in *P. caudatum* (Kitamura 1988; Hori and Fujishima 1992). Moreover, I found that the mating-reactive O^3 mating-type substances exist in the E^3 cytoplasm. The achievement of mating reactivity of E mating-type substance might occur by exposure of the necessary domain of the protein, changing its conformation in the ciliary membrane. Hori et al. (1994) did a very interesting research in the phenotypic conversion of mating type specificity induced by micro-transplantation of macronucleoplasm in *P. caudatum*. When macronucleoplasm of E^3 was injected into the O^{12} macronucleus (odd mating type of syngen 12), some recipients changed to E mating type of syngen specificity of donor macronucleoplasm (E^3) as expected. Notable result is that some others changed to E mating type of recipient syngen (E^{12}). The fact that intersyngenic mating reaction occurs very commonly in *Paramecium* shows that there is a low difference in the molecule level of mating type substance (Butzel 1974b; Tsukii 1988; Kanzawa 2001). The monoclonal antibodies used in this study also inhibited the mating reactivity of O^1, O^5, and O^{13}, besides O^3, indicated that O mating-type substances among syngens have high homology. The appearance of E^{12} among the recipients might be the case that the product of Mt^3 gene modifies the O^{12}

mating-type substance into E^{12} mating-type substance. Although the modification changing O mating-type substance into E mating-type substance is not known, the facts that neither glycosidases nor phospholipases has any effect on mating reactivity of isolated cilia indicated that at least the glycosylation may be not involved in the modifying the mating-reactive domain of O and E mating-type substances (Kitamura 1988). Once the O mating-type substances have been changed into the E mating-type substances by *Mt* gene and expressed on the cilia, the activity of the O mating-type substances cannot be recovered by treatment of the E mating-type cilia with detergent (Fig. 1).