

Protease-sensitive Component(s) on the Cell Surface Prevents Self-fusion in a Bisexual Strain of *Dictyostelium discoideum*

Hideko Urushihara and Kazuhiro Aiba

*University of Tsukuba, Institute of Biological Sciences, Tsukuba, Ibaraki 305, Japan

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ABSTRACT. The sexual cycle of the cellular slime mold *Dictyostelium discoideum* offers a suitable system to analyze the mechanism of cell recognition during mating. Sexual cell fusion in *D. discoideum* typically occurs between complementary heterothallic strains. In addition, several bisexual strains are known which undergo sexual cell fusion with heterothallic strains of either mating type, but cannot do so by themselves. In the present study, trypsin digestion of cell surface molecules was found to induce self-fusion in a bisexual strain WS2162, suggesting the presence on the cell surface of a self-recognition molecule whose homophilic interaction interferes with the cell fusion mechanism.

Sexual cell fusion provides opportunities for genetic shuffling, by which rejection of hazardous mutations (14) and accumulation of advantageous characteristics within species (15) are possible. The benefit of sexuality, however, is not fully realized when mating occurs between individuals with the same genetic background. In most higher organisms, mating with non-self is guaranteed via binary mating systems. Even in the organisms whose sexual differentiation at the individual level is not achieved, various self-incompatibility mechanisms prevent self-mating. How this incredible system works is not fully understood, though several possibilities are suggested in higher plants such as participation of a specific RNase to prevent elongation of self pollen tubes (17).

The soil amoebae of cellular slime molds usually propagate by fission but they undergo sexual development called macrocyst formation (3, 16) under certain environmental conditions such as in darkness and at high humidity (8, 18). The initial step in macrocyst formation is the fusion of cells to form zygotic giant cells, which attract surrounding cells and engulf and digest them to develop into macrocysts (11, 19). There are homothallic, heterothallic, bisexual and asexual strains of *Dictyostelium discoideum* (7, 20). Homothallic strains form macrocysts independently, while heterothallic ones require coexistence of complementary strains for macrocyst formation. Two mating types, *mat A* and *mat a*, have been assigned to the strains exhibiting heterothallic mating (30). Bisexual strains can

form macrocysts with heterothallic strains of either mating type but are self-incompatible and cannot do so independently. Asexual strains have never been observed to form macrocysts.

Several cell surface proteins which are involved in sexual cell fusion between NC4 and HM1, complementary heterothallic strains in *D. discoideum*, have been identified. All of them are glycoproteins and present on the surface of sexually mature cells but absent on that of immature cells. One of them, gp70, is specific to HM1 (28), while another one, HH9 antigen, is specific to NC4 (1). All others, gp138 (26), DE1 antigen, and GG6 antigen (1) are commonly found in the two strains. Two genes for gp138 have been isolated and characterized (9, 10).

In terms of sex differentiation, bisexuality is of particular interest, since it apparently represents a form intermediate between homothallism and heterothallism. We previously showed that cells of WS2162, a bisexual strain of *D. discoideum*, are self-incompatible at the step of cell fusion. Results of antibody inhibition of cell fusion and detection of gp138 (2) and its genes (13) suggested that a molecular basis for bisexual and heterothallic mating are common. We presented two models to explain the molecular mechanism of self-incompatibility in bisexual strains (13) (Fig. 1). The first ("imperfect model") assumes that cell fusion molecules are partially defective in WS2162 so that they can interact with functional cell surface receptors of heterothallic strains but cannot do so with their own. The second ("self-recognition model") postulates the presence on the cell surface of a self-recognition molecule whose homophilic interaction interferes with the cell fusion mechanism.

* Address correspondence to H. Urushihara
TEL: 0298-53-4910, FAX: 0298-53-6614, e-mail: d402hu@sakura.
cc.tsukuba.ac.jp

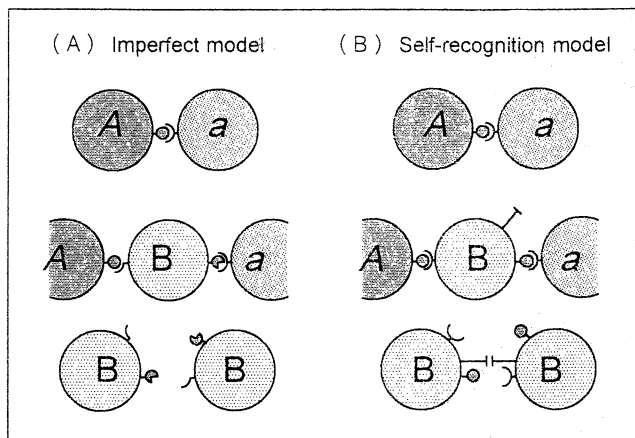


Fig. 1. Two models for mechanisms of self-incompatibility in bisexual strains of *D. discoideum* (9). Cell fusion occurs by interaction of *mat A* (—●) and *mat a* (—○) cell fusion molecules. In the imperfect model (A), bisexual strains possess partially defective cell fusion molecules (—●, —○), while in the self-recognition model (B), a self-recognition molecule (—→) interferes with function of cell fusion molecules in bisexual strains.

Here we present data indicating that a cell surface molecule(s) susceptible to trypsin digestion is responsible for prevention of self-mating in WS2162 as predicted by the self-recognition model.

MATERIALS AND METHODS

Strains and cell culture. One bisexual strain, WS2162 (Clone 4) (13), and two heterothallic ones, NC4 (*mat A*) and HM1 (*mat a*), of *D. discoideum* were used. All the strains were maintained as stock fruiting bodies on nutrient A-medium agar in association with *Klebsiella aerogenes* as a food. To induce sexual maturation, growth-phase cells were recovered from A-medium agar plates, suspended in Bonner's salt solution (BSS: 10 mM NaCl, 10 mM KCl, 2.7 mM CaCl₂) (4) containing *K. aerogenes* and cultured in the dark with shaking for 16 hr at 22°C. The cells thus cultured were called mature cells, while those obtained from agar plates were called immature cells. For experiments, cells were freed of bacteria by repeated centrifugation in cold BSS and were kept on ice until use.

Assay for cell fusion. The bacteria-free cells were suspended in BSS at 5×10^6 cells/ml. Equal volumes of two cell suspensions were mixed and incubated on a reciprocal shaker (120 strokes/min) for 30 min at 22°C (22). To assay competence of self-fusion, cells of only one type were incubated in the same manner. Extent of cell fusion was expressed in terms of the fusion index (FI) which was calculated as follows:

$$FI = \frac{N_0 - N_{30}}{N_0} \times 100 (\%)$$

where N_0 and N_{30} corresponded to the numbers of unfused cells at 0 and 30 min of incubation, respectively.

Trypsin treatment of cells. Trypsin treatment of cells was performed as follows unless otherwise noted. Bacteria-free cells were suspended in a 20 mM Tris buffer at pH 8.0 containing 1 mM EDTA (TE) at 1×10^7 cells/ml, and incubated with 0.1 mg/ml of trypsin (Sigma, type III) without shaking for 30 min at 22°C in the dark. At the end of incubation, a twofold excess of soybean trypsin inhibitor (Sigma, Type I-S) was added to completely inactivate the trypsin. The cells were then washed and resuspended in cold BSS.

Nuclear staining of cells. Cells were fixed with 100% methanol at 4°C for 1 hr or longer. After fixation, the cells were washed with TE, and stained with 10 µg/ml of Hoechst 33258. One drop of stained cell suspension was mounted on a slide glass and observed with a fluorescence microscope (Nikon: Optiphot). Micrographs were taken with Tri-X pan film (Kodak).

RESULTS

Trypsin induces syncytium formation in WS2162. If the self-recognition model of bisexuality is true, removal of the postulated self-recognition molecule may allow self-fusion in WS2162, assuming that the mechanism of cell fusion is kept intact. To investigate this possibility, trypsin treatment of the cells was employed, because cell fusion between NC4 and HM1 had been found not to be interfered with by preceding treatment of cells with trypsin (unpublished observation). Mature WS2162 cells undergo extensive cell fusion when incubated with either HM1 or NC4, while the incubation of WS2162 cells alone did not cause such cell fusion. However, if mature WS2162 cells were subjected to treatment with 0.1 mg/ml trypsin in TE and then incubated alone, many large cells appeared in the suspension (Fig. 2). Those cells were indistinguishable from the giant cells formed by synchronous fusion of WS2162 and HM1 or NC4. Nuclear staining of the large cells indicated that they were multinucleated (Figs. 2c–2e), confirming that the large cells were cell fusion products. The fusion was so extensive that the fusion index was comparable to those between WS2162 and heterothallic strains (Table I). Thus, a cell surface component(s) susceptible to trypsin digestion seems to be responsible for self-incompatibility of WS2162 as predicted by the self-recognition model. Trypsin treatment of WS2162 cells did not affect their ability of giant cell formation with NC4 or HM1 cells (data not shown).

The trypsin sensitivity profile of the self-incompatibility system in WS2162 was further characterized. As shown in Fig. 3A, the 30-min treatment with trypsin at as low as 1 µg/ml induced detectable self-fusion, and the fusion was most extensively induced in the case of 0.1 mg/ml of trypsin. Unexpectedly, at higher concentrations than this, the extent of self-fusion decreased. This fact indicates that treatment with high trypsin con-

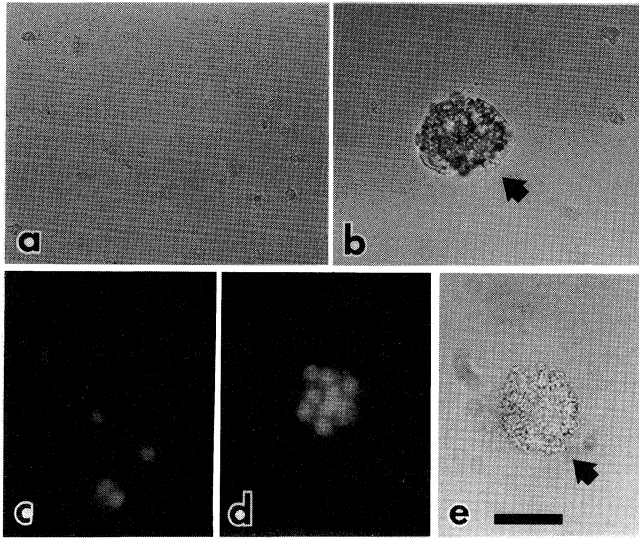


Fig. 2. Syncytium formation by trypsin-treated WS2162 cells. Mature WS2162 cells were treated with trypsin and incubated for 0 (a) or 30 min (b). To examine nuclearity after the incubation, cells with (d, e) or without (c) trypsin treatment were stained with Hoechst 33258. Fluorescence micrographs (c, d) and a phase-contrast one (e; the same field as d) are shown. Arrow indicates a Syncytium. Bar represents 50 μ m.

centrations impaired the fusion mechanism itself in WS2162. Abolition of self-incompatibility by trypsin occurred quickly, and near the maximum level of self-fusion was achieved after 10-min treatment with trypsin

(Fig. 3B).

Addition of EDTA to the buffer used for trypsin treatment was done for two reasons: first, to inhibit cell fusion, which was known to be Ca^{2+} -dependent, during the treatment; second, to prevent possible Ca^{2+} -protection of relevant proteins against trypsin attack. However, trypsin treatment in the presence of 1 mM Ca^{2+} gave similar results as those in the presence of 1 mM EDTA; that is, cells did not fuse, at least extensively, during the treatment even in the presence of Ca^{2+} , if incubated without shaking. Rapid and extensive cell fusion detected in our assay system seems to require enhanced collision of cells. In addition, self-fusion did occur after trypsin treatment in the Tris buffer containing 1 mM Ca^{2+} (TC). The slight but significant difference in the extent of self-fusion between the cells after trypsin treatment in TE and in TC (Table I) may have some bearing, but what this difference is is currently unclear.

Induction of self-fusion is specific. To investigate the possibility that the induction of self-fusion in WS2162 observed above was nonspecific and independent of sexuality, immature WS2162 cells obtained from agar plates and mature NC4 and HM1 cells were each treated with trypsin, and then assayed for competence of self-fusion. As is evident from Table 2, none of the treated cells underwent self-fusion except for mature WS2162. Since coincubation of trypsin-treated mature NC4 and HM1 cells resulted in normal giant cell formation, lack of self-fusion was not due to disruption of a fusion mechanism in those cells. Thus, induction of

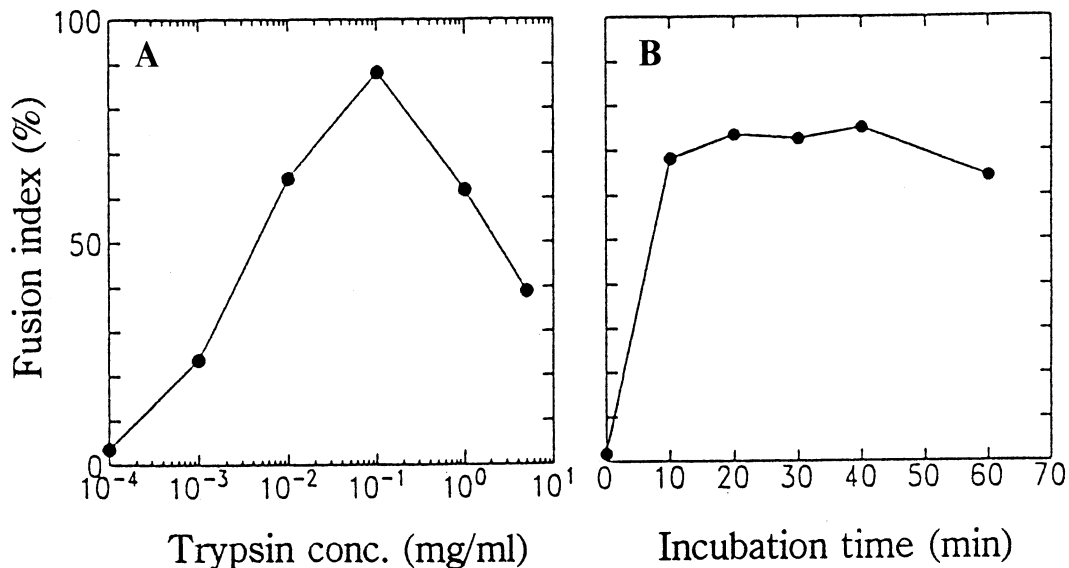


Fig. 3. Factors affecting trypsin-induced self-fusion of WS2162. A: Dependence on trypsin concentration. Mature WS2162 cells were incubated with various concentrations of trypsin for 30 min at 22°C. At the end of incubation, trypsin inhibitor was added to a final concentration of 10 mg/ml, and then the cells were washed with cold BSS and assayed for self-fusion. B: Dependence on duration of trypsin treatment. Mature WS2162 cells were incubated with 0.1 mg/ml of trypsin for indicated periods, and then assayed for self-competence.

Table I. INDUCTION OF SELF-FUSION IN WS2162 BY TRYPSIN TREATMENT.

Treatment	Partner for fusion	Fusion index (%)
Without trypsin	NC4	73.0 \pm 2.5
	HM1	78.2 \pm 3.8
	Self	0.1 \pm 6.2
With trypsin in TE	Self	72.6 \pm 3.0
With trypsin in TC	Self	54.8 \pm 5.7

Mature WS2162 cells were incubated in either TE or TC, with or without 0.1 mg/ml of trypsin, for 30 min at 22°C. At the end of incubation, trypsin inhibitor was added to each incubation to twofold excess, the buffer was changed to BSS, and then treated cells were incubated for fusion assay with partner cells. Mean values \pm standard deviations of three independent experiments are shown.

self-fusion was specific to mature WS2162 cells; these cells appeared to be potentially self-compatible, while the self-compatibility is normally masked by the presence of a trypsin susceptible component(s) on the cell surface.

Macrocyst development from trypsin-induced syncytia does not occur. The ability of trypsin-induced syncytia to develop into macrocysts was examined as follows. Two hundred microliters of cell suspensions obtained after the assay of cell fusion was inoculated into wells of 24-well titer plates with the addition of 300 μ l of BSS containing 1×10^6 cells used for fusion assay. The plates were sealed and incubated at 22°C without agitation. After 3 days of incubation, macrocyst formation was scored under a light microscope.

WS2162 cells formed abundant macrocysts by coinubation with HM1 cells (Fig. 4a), while single cells and amorphous cell clumps were observed if incubated alone (Fig. 4d). Although the syncytia formed by self-fusion of trypsin-treated WS2162 cells were very similar in appearance to giant cells formed with heterothallic strains, their further incubation did not result in macro-

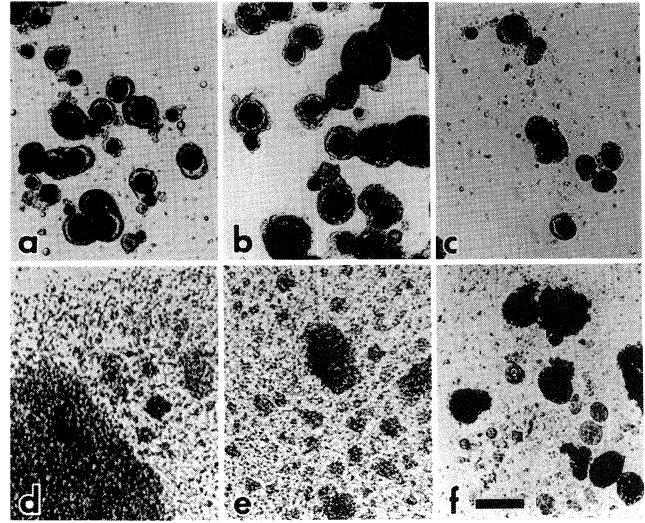


Fig. 4. Abortive macrocyst development from syncytia formed by self-fusion of trypsin-treated mature WS2162 cells. Trypsin-treated (b, c, e, f) or untreated (a, d) mature WS2162 cells with either untreated (a, b) or trypsin-treated mature HM1 cells (c), or without HM1 cells (d, e, f) were incubated for 30 min to allow cell fusion, and then incubated 3 further days for macrocyst development. Bar represents 100 μ m.

cyst formation (Fig. 4e). In a rare case, tiny pseudo-macrocysts were observed (Fig. 4f). Thus, the trypsin-induced syncytia were unable to develop into macrocysts.

There are at least two possibilities to explain this: One is that trypsin treatment may have caused damage to cellular components necessary for macrocyst development. The other is that WS2162 is self-incompatible also at stages of sexual development later than cell fusion. Since the giant cells formed by trypsin-treated NC4 and HM1 cells developed into normal macrocysts, the former possibility appeared to be less likely than the latter. Furthermore, trypsin-treated WS2162 cells did develop into macrocysts when incubated with mature NC4 (data not shown) or HM1 cells, either trypsin treated (Fig. 4b) or untreated (Fig. 4c). Therefore, we believe that a non-self genetic background is necessary also for post-fusion development of WS2162.

DISCUSSION

In the present study, self- and non-self-discrimination in a bisexual strain of *D. discoideum* was attributed to the presence of an inhibitory self-recognition molecule(s) on the cell surface. We assume that the homophilic interaction of this self-recognition molecule interferes with the function of cell fusion mechanisms.

Cell recognition during fertilization is ordinarily achieved by specific binding of surface molecules on gametes, several relevant proteins having been identified

Table II. CELL-TYPE SPECIFIC INDUCTION OF SELF-FUSION BY TRYPSIN TREATMENT.

Cell type	Partner for fusion	Fusion index (%)
Mature WS2162	Self	72.6 \pm 3.0
Immature WS2162	Self	0.0 \pm 6.0
Mature NC4	Self	0.0 \pm 5.5
	Mature HM1*	67.7 \pm 5.3
Mature HM1	Self	0.0 \pm 6.8
	Mature NC4*	70.7 \pm 4.3

Cells were incubated with 0.1 mg/ml of trypsin in TE for 30 min at 22°C. At the end of incubation, trypsin inhibitor was added to twofold excess, the buffer was changed to BSS, and then the treated cells were incubated for fusion assay. Mean values \pm standard deviations of two independent experiments are shown.

* Without trypsin treatment.

and characterized. Those include sperm surface binding (29) and its egg receptor (12) in sea urchin, PH30 in guinea pig (23), *Pex 1* in *Zea mays* (21) and so on. In unicellular organisms, plus- (5) and minus-agglutinins (6) in *Chlamydomonas reinhardtii* and gp70 in *D. discoideum* (28) are also argued to be involved in mating type recognition. While these proteins are all indispensable for sexual cell fusion to proceed, the postulated cell recognition molecule in the present study is opposite in function; its presence on the cell surface inhibits cell fusion. The appearance of such inhibitory self-recognition molecules may have contributed to the evolution of sexual system from homothallism to heterothallism which can appreciate the benefit of sex.

Mechanisms of self- and non-self-discrimination is also indispensable in various other biological systems. For example, it is the central requirement in the immune responses, where various antigenic molecules on lymphocyte surfaces function as recognition molecules via ligand-receptor type interactions (25). Homophilic interactions of surface molecules are often relevant to construction of multicellular systems as the case of cadherins in vertebrates (27) and gp80 contact site A protein in *D. discoideum* (24). Structural and functional similarities of the present self-recognition molecule to those molecules are an interesting subject for future analysis.

The fact that the self-fusion products of WS2162 cells did not develop into macrocysts despite apparent resemblance to zygotic giant cells indicates that WS2162 was self-incompatible also at stages of sexual development later than cell fusion. Self-incompatibility seems to be guaranteed by a double checkpoint system in WS2162. Since we found that trypsin treatment allows cells to evade the first checkpoint, the later checkpoint(s) is now open for analysis.

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