Caspase-like activity in programmed nuclear death during conjugation of *Tetrahymena thermophila*

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Abstract

Apoptosis, or programmed cell death, is common in a variety of eucaryotes, from unicellular protozoa to vertebrates. The ciliated protozoan *Tetrahymena thermophila* has a unique apoptosis-like nuclear death during conjugation, called programmed nuclear death. This death program involves nuclear condensation (pyknosis) and oligonucleosomal DNA fragmentation in the parental macronucleus. Subsequently, the condensed nucleus is entirely resorbed in the autophagosome. Here we demonstrate that caspase-8- and -9-like activity was detected, but no caspase-3-like activity, by *in vitro* assay during the nuclear resorption process, suggesting that caspase-like activity is associated with both programmed cell death and apoptosis-like nuclear death in *Tetrahymena*. The use of indicator dye to detect the loss of mitochondrial membrane potential suggested the uptake of mitochondria and the degenerating macronucleus by the autophagosome. An involvement of mitochondria in the programmed nuclear death is discussed.

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Abbreviations: PND, programmed nuclear death; Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline; Ac-IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline; Ac-LEHD-pNA, *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroaniline; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-IETD-CHO, *N*-acetyl-Ile-Glu-Thr-Asp-aldehyde; Ac-LEHD-CHO, *N*-acetyl-Leu-Glu-His-Asp-aldehyde.

Introduction

Programmed cell death (PCD) is an essential mechanism for the development and homeostasis of multicellular organisms.^{1,2} In the last decade, many molecules involved in apoptosis have been identified. Of these molecules, caspases, a family of cysteine protease, are unequivocally

important.^{3,4} Caspases are a key factor in DNase activation, cytoskeleton breakage, and nuclear and chromatin condensation, and result in final cell death. In *Caenorhabditis elegans*, CED-3 is homologous to caspase-1 (ICE: interleukin-1 β -converting enzyme) and plays a central role in PCD.⁵ Caspase family proteins are conserved in apoptosis or PCD among many animal species from hydra to humans.⁶ Apoptosis-like cell death has also been reported in plants, unicellular protists, such as the cellular slime mold, kinetoplastids, dinoflagellates, ciliates and heterokonts.^{7–15} In the ciliated protozoan *Tetrahymena*, apoptosis- or PCD-like death has been observed as well as the above unicellular protists. When *Tetrahymena thermophila* was cultured in a condition of low cell density, they died within a few hours.^{15,16} This cell death is thought to be a suicide because the cell death can be inhibited by actinomycin D.¹⁵ Apoptosis-like cell death in *T. thermophila* or *T. pyriformis* is also known to be inducible with a treatment of staurosporine, C₂ ceramide or Fas-ligand.^{17–19}

In addition to the PCD, *T. thermophila* has a unique apoptosis-like process.²⁰ *T. thermophila* have two functionally and morphologically distinct nuclei in a single cell; one nucleus is a reproductive somatic macronucleus and the other is a germinal micronucleus. Both nuclei are derived from the micronucleus during conjugation. Conjugation in *Tetrahymena* is initiated by cell-to-cell interaction between different mating types.^{21,22} After pair formation, conjugating cells undergo meiosis, resulting in the formation of four haploid meiotic products, one of which selectively survives while the remaining three products degenerate. The selected nucleus divides mitotically once, leading to the production of two pronuclei. Conjugating partners in a pair reciprocally exchange either of the pronuclei, and a synkaryon (fertilized nucleus) is formed in each conjugating cell. After two successive postzygotic divisions, a new macronucleus and micronucleus differentiate and the old parental macronucleus is replaced by the newly formed macronucleus. The parental macronucleus destined to degenerate behaves in a similar manner to the nucleus in apoptotic cells.^{20,23,24} The DNA is fragmented in a chromatin-sized ladder and the parental macronucleus is then completely resorbed.^{20,24} This process is controlled by specific gene expression and is referred to as programmed nuclear death (PND).²⁰ PND is thought to be a primitive form of PCD, since only the nucleus dies and not the exconjugant cell. At present, it is unclear which molecules are involved in *Tetrahymena* nuclear death. So far, there is only one report suggesting that a caspase inhibitor blocks nuclear death *in vivo*.²⁵

In this paper, we demonstrate caspase-like activity *in vitro*, and report a correlation between the appearance of the activity and PND during conjugation in *T. thermophila*. The possible involvement of mitochondria in the apoptosis-like phenomenon is suggested by the localization of a fluorescent dye used as an apoptosis indicator. The evolutionary

significance of PND is discussed with reference to apoptosis or PCD.

Results

Macronuclear degeneration process

PND was observed during *T. thermophila* conjugation.^{20,24} Although Davis *et al.*²⁰ and Mpoke and Wolfe²⁴ previously gave the details of the PND, here we show the PND process to easily comprehend the timing in our experimental condition. The first morphological changes in the degenerative parental macronucleus occurred at macronuclear development stage I (Mac I), after the nuclear differentiation stage. After two postzygotic divisions of the fertilized zygotic nucleus, two of the four resultant nuclei were located in the anterior region and the other two in the posterior region (Figure 1b). At the nuclear differentiation stage, the anterior nuclei differentiated into new macronuclei (macronuclear anlagen; MA, USA), while the posterior nuclei remained as new presumptive micronuclei

without any change. Subsequently, the old parental macronucleus began to condense at 8 h, and continued until 10 h (Figure 1a). The size of the nucleus decreased by 65%. No further remarkable size reduction of the condensed macronucleus was observed until 15 h when it began to shrink again (Figure 1d). By 18 h, the condensed macronucleus was rapidly resorbed (Figure 1d), and at the same time one of the two presumptive micronuclei was eliminated (Figure 1e). In this experiment, exconjugants were not fed again, so that there was no subsequent development, and they maintained one micronucleus and two macronuclear anlagen (Figure 1e). If fed again, the micronucleus divides mitotically, while the anlagen become mature macronuclei after DNA amplification.

DNA degradation in the parental macronucleus during conjugation is shown in Figure 1f. DNA degradation began at 8 h after the induction of conjugation (Figure 1f, lane 4). At this time, the majority of cells were in the Mac I or Mac IIp stage (Figure 1c). As conjugation proceeded, chromatin-sized DNA fragments were discernible at 10 h (Figure 1f lane 5). The DNA ladder fragments consisted of multiple units of an approximately 180-bp monomer unit, as previously demonstrated (Figure 1f, lane 5, arrowhead).^{20,24} These chromatin-sized DNA fragments with a ladder pattern are generally observed in apoptotic cells.²⁶ The DNA degradation was complete by 18 h, coinciding with the completion of old macronucleus resorption (Figure 1e, f, lanes 9–10). The degraded DNA remnant seen after 18 h is presumably because of the mixture of delayed conjugants from the asynchronous progress of conjugation. These results suggest that parental macronucleus condensation and DNA degradation occur almost simultaneously. The initial DNA degradation into the ladder is suspended once between 10 and 12 h, and final DNA loss rapidly occurs between 16 and 18 h together with nuclear degradation.

Figure 1 A process of PND. (a) Change in size of parental macronucleus during conjugation. The initial size reduction by nuclear condensation (pyknosis) started at 8 h and extended over 2–3 h. Resorption started at 15 h and was complete by 18 h. A total of 17–28 nuclei were measured at each point. B, C, D and E (italic) on the top correspond to conjugation stage shown in (b)–(e). (b, c, d, e); Fluorescence microscopy of DAPI-stained nuclei during the late stages of conjugation. (b) Typical configuration of postzygotic division II (PZD II). Division products localized in the anterior and posterior regions. A degenerating meiotic product remained visible. (c) Macronuclear development stage II (MAC IIp). The conjugant is still in a pair and has a condensed parental macronucleus. (d) Early macronuclear development stage III (Mac III). One micronucleus has been eliminated, and the parental macronucleus has gradually degenerated. (e) Late macronuclear development stage III (MAC III). The parental macronucleus has been resorbed. Abbreviations: M, macronucleus, mi, micronucleus, MA, macronuclear anlage; dM, degenerating macronucleus. The scale bar in E indicates 10 μ m. (f) Apoptosis-like DNA degradation during conjugation. Total DNA was isolated from conjugating cells every 2 h after the induction of conjugation, and loaded on a 2.0% agarose gel. DNA degradation started at 8 h. Thereafter, a chromatin-sized ladder appeared and lasted until 16 h. Arrowheads indicate the positions of the nucleosome-sized DNA ladder. M denotes a 100-bp ladder size marker

Caspase-like protease activity during conjugation

Caspases are part of the cysteine protease family and have significant roles in apoptosis in various organisms. Caspase colorimetric assay was used to elucidate whether caspase family proteases are associated with macronuclear degeneration during conjugation in *T. thermophila*. Cell extracts were prepared at 16 h after mating and were incubated with different caspase substrates. Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, which are known to be specifically cleaved by caspase-3, -8 and -9, respectively, were used as substrates.²⁷ Figure 2 shows a caspase-like protease assay performed with extracts prepared from cells in the macronuclear degeneration stage (Mac IIe to Mac III). Although all samples showed a time-dependent increase in absorbance (Figure 2), the increase was higher in the Ac-IETD-pNA and Ac-LEHD-pNA samples than in the Ac-DEVD-pNA samples. To verify that this increase was because of a substrate-dependent activity, a caspase assay with a specific competitive inhibitor was carried out. The absorbance of the Ac-DEVD-pNA samples did not change, irrespective of the presence or absence of the inhibitor Ac-DEVD-CHO (Figure 3b). Therefore, extract activity in the Ac-DEVD-pNA assay appeared to be small or absent. On the other hand, the absorbance of Ac-IETD-pNA samples was inhibited by the addition of the inhibitor Ac-IETD-CHO (Figure 3d). Similarly,

Figure 2 Caspase-like activity in cells during macronuclear degradation. Extracts from cells at 16 h after the initiation of conjugation were incubated with the caspase substrates Ac-DEVD-pNA (squares), Ac-IETD-pNA (circles) and Ac-LEHD-pNA (triangles) ($100 \mu\text{M}$ each). The assay samples included each substrate and $300 \mu\text{g}$ protein, and were performed at 37°C

Figure 3 Caspase-like activity assay with competitive inhibitors. Extracts were prepared from cells at 12 (a, c, e) and 18 h (b, d, f) after the initiation of conjugation. Extracts were incubated with substrates (black square) Ac-DEVD-pNA (a, b), Ac-IETD-pNA (c, d) and Ac-LEHD-pNA (e, f) ($100 \mu\text{M}$ each), or the competitive inhibitors (open circle) Ac-DEVD-CHO (a, b), Ac-IETD-CHO (c, d) and Ac-LEHD-CHO (e, f) ($100 \mu\text{M}$ each). Samples included $200 \mu\text{g}$ of protein in (a)–(d) and $450 \mu\text{g}$ in (e) and (f)

extract activity in the Ac-LEHD-pNA assay was inhibited by Ac-LEHD-CHO (Figure 3f). These results indicate that caspase-like activity towards Ac-IETD-pNA and Ac-LEHD-pNA is present in cell extracts derived from conjugating *T. thermophila*.

Figure 4 Changes in caspase-like activity during conjugation. Extracts were prepared from conjugants or exconjugants every 2 h during conjugation and incubated with the substrates Ac-DEVD-pNA (a), Ac-IETD-pNA (b) and Ac-LEHD-pNA (c) at 37°C for 3 h

To trace possible changes in caspase-like activity, cell extracts were prepared every 2 h during conjugation (Figure 4). The Ac-DEVD-pNA assay showed a low level of extract activity that changed little throughout conjugation as expected for the above result (Figure 4a). By contrast, Ac-IETD-pNA and Ac-LEHD-pNA assays showed a remarkable increase in extract activity during the late stage of conjugation (Figure 4b, c). When compared with premating (0 h) or early stage of conjugation (2 h) by Dunnett's multiple comparison test ($P < 0.05$), the increase of the activities was not evident until 14 h, but it was significant after 16 h. Macronuclear condensation, the first step in macronuclear degeneration, occurred at 8 h, while resorption began at 16 h (Figure 1). Caspase-like activity began to increase after 16 h. This correlation between morphological changes in the macronucleus and the increase

Table 1 Selectivity of caspase inhibitors on caspase-like activity

Cell extract (h)	Ac-IETD-pNA with Ac-LEHD-CHO	Ac-LEHD-pNA with Ac-IETD-CHO
12	89.2 (± 6.0)	98.8 (± 4.8)
14	75.0 (± 2.4)*	91.3 (± 3.4)
16	77.5 (± 2.8)*	94.2 (± 1.5)

The results shown are the comparative values ($\pm$ S.E., $N=15$) to control activity in each extract. Cell extracts were prepared at 12, 14 and 16 h after mating. Samples included 100 μ M substrate and 100 μ M inhibitor or 1% DMSO in control * $P<0.05$ in Student's t -test

in caspase-like activity strongly suggests that the two are closely associated. In these experiments, activity tended to increase transiently at around 6 h during the early stage of conjugation, when most conjugating cells were in the nuclear exchange stage. This may be related to the degradation of extra meiotic products.

As mentioned above, the caspase-like activity in Ac-IETD-pNA and Ac-LEHD-pNA assays increased in the process of nuclear death during conjugation. However, basal activity was comparatively higher in these assays than in Ac-DEVD-pNA assays at all stages. In order to demonstrate that the activity in the assays was indeed caspase-like, we performed caspase assays using a specific competitive inhibitor and extracts from the cells before (12 h) and after (18 h) the increase in activity. Activity in the Ac-DEVD-pNA assay was similar with and without the inhibitor (Ac-DEVD-CHO) at the two different stages (Figure 3a,b). In contrast, activity in the Ac-IETD-pNA assay was inhibited by Ac-IETD-CHO at 18 h, whereas no inhibition was observed at 12 h (Figure 3c, d). The response to the inhibitor Ac-LEHD-CHO was different, however, with activity inhibited at both 12 and 18 h. This result suggests the constant presence of caspase-9-like activity, which further increases later. The net activity in the Ac-IETD-pNA and Ac-LEHD-pNA assays significantly increased during the nuclear degeneration stage, suggesting that caspase-8- and -9-like activity may be associated with nuclear death. In the present study, caspase-3-like activity was not clearly detected during conjugation.

To explore the selectivity of the inhibitors used above, we investigated the effect of Ac-IETD-CHO on Ac-LEHD-pNA cleavage, and of Ac-LEHD-CHO on Ac-IETD-pNA cleavage (Table 1). It is generally known that in this assay system, substrate specificities are not necessarily strict.²⁷ Inhibitory assay was done using the extracts obtained 12, 14 and 16 h after conjugation. When caspase-8 inhibitor, Ac-IETD-CHO, was incubated with Ac-LEHD-pNA, no significant effect ($P<0.05$) was detected although a slight decrease of the activity was observed. In contrast, when caspase-9 inhibitor, Ac-LEHD-CHO, was incubated with Ac-IETD-pNA, a significant inhibition was observed in the extract derived from conjugation of 14 and 16 h. This inhibition could be interpreted by the attribute of the imperfect specificity unless we assume a cascade from caspase-8 to caspase-9 in *Tetrahymena*.

Possible involvement of mitochondria in nuclear death

It is well known that procaspase-9 is activated by cytochrome *c* released from mitochondria in mammalian apoptosis.²⁸ In addition to cytochrome *c*, other molecules are also released

Figure 5 DePsipher-stained conjugants and exconjugant. (a, b) Conjugants in PZD II. Green fluorescence is sporadically visible around degenerating meiotic products (arrow), but not on any nuclei. Most mitochondria show red fluorescence, indicating their living state. (c, d): Conjugants in MAC IIp. Green fluorescence is visible only on degenerating macronucleus. (e, f): Exconjugant in MAC III. Similar to (d), the degenerating macronucleus appears green. (a, c, e) were stained with DAPI and (b, d, f) were stained with DePsipher. Arrow, degenerating meiotic product; dM, degenerating macronucleus. The scale bar in F indicates 10 μ m.

from mitochondria in apoptotic cells: apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase, direct IAP binding protein with low pH (Smac/DIABLO).^{29–31} Release of these molecules is accompanied by a change in mitochondrial membrane potential. To clarify the involvement of mitochondria in nuclear death during conjugation, conjugating cells were stained with a unique cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (DePsipher), which is a useful indicator for the loss of mitochondrial membrane potential. This dye fluoresces in a bright red multimeric form in normal mitochondria. When mitochondrial membrane potential is lost, the dye cannot accumulate and remains in a green fluorescent monomeric form. In vegetative phase cells, or before the degeneration of the parental macronucleus, the great majority of mitochondria showed red fluorescence, and only some demonstrated green fluorescence around the degenerating meiotic products (Figure 5). Surprisingly, green fluorescence was specifically

located not in mitochondria, but in the degenerating macronucleus (Figure 5c–f). Before condensation, the parental macronucleus, the presumptive micronucleus and two developing macronuclear anlagen never fluoresced (Figure 5a–f). This localization of the dye only in the parental macronucleus reinforced the belief that mitochondria containing the dye were taken into the phagolysosome and broken down, resulting in the release of factors such as cytochrome *c*. In this context, DNA degradation should be restricted to the degenerating macronucleus.

Discussion

Caspase-like activity during conjugation

Apoptosis is induced by various signals or stimulation (e.g. Fas-ligand, TNF, irradiation and drugs).³² These signals are transduced to initiate the suicide process. Caspase family proteases have an essential role in apoptosis, and can be divided into three distinct groups by their biological role.⁴ Group I (caspase-1, -4, -5) has a role in cytokine maturation, group II (caspase-3, -7) activates the apoptosis executioner and group III (caspase-8, -9) activates the group II caspases. In this study, we tested caspase-like activity using substrates for caspase-3, -8 and -9. Ac-IETD-pNA and Ac-LEHD-pNA were cleaved by extracts prepared from cells during macronuclear degeneration.

Caspase-3-like activity was not detected during PND in *Tetrahymena*. Caspase-3-like activity is thought to be essential for the final step in apoptosis. Given the importance and omnipresence of this enzyme in a wide variety of organisms, it is possible that caspase-3-like activity existed, but it was too low to detect. It is also possible that the enzyme may be active in PCD, but not in the nuclear death, suggesting that different cascades are responsible for the two processes. Alternatively, *Tetrahymena* may not have simply acquired caspase-3-like activity, or may have lost caspase-3-like activity in the course of evolution, and caspase-8- and -9-like activity or other factors may be responsible for caspase-3-like activity in nuclear death. In *Dictyostelium*, caspase-like activity does not seem to be required for PCD, although caspase inhibitors prevent their development.³³ In any unicellular organisms, so far no caspase gene homolog has been identified in their genome, although metacaspase genes have been identified in a few protistans.³⁴ These facts suggest that the role of caspase or the caspase cascade in certain protistans is different from that in higher eucaryotes. If the lack of caspase-3-like activity in *Tetrahymena* is true, PND might be achieved by other effectors such as Omi/HtrA2 or endonuclease G seen in mammalian cell death, or by caspase-8- and -9-like activity itself.^{35,36} Now, *Tetrahymena* genome project is in progress (<http://www.tigr.org/tdb/tgi/ttgi/>). Although no caspase gene has been identified at this time when 10% of the genome (ca. 3500 genes) was analyzed, a future study will elucidate whether there exists a caspase-3-like gene or not in *Tetrahymena*.

PND in *Tetrahymena*

As demonstrated in Figure 1, it is known that macronuclear death is distinctly separated into two processes, condensation

(pyknosis) and degradation (resorption).³⁷ In pyknosis, macronuclear DNA is partially digested into large fragments and then chromatin condensation is thought to occur.²⁴ Thereafter, macronuclear DNA degrades into oligonucleosomal fragments (Figure 1). During conjugation of Nulli 3 mutants that lack chromosome 3 in the micronucleus, conjugants undergo successful pyknosis but the subsequent process is abolished, resulting in the failure of parental macronucleus resorption.²⁰ This suggests that both processes are controlled by different sets of genes. After pyknosis, the condensed macronucleus is surrounded by the autophagosomal membrane, followed by the fusion of lysosomes, resulting in auto(phago)lysosome formation and resorption of the parental macronucleus.^{38,39} During apoptosis in animals, an apoptotic body is formed after a series of changes. The apoptotic body is taken into the macrophage by endocytosis and is entirely resorbed. The condensed parental macronucleus in *Tetrahymena* may correspond to the apoptotic body, while resorption of the macronucleus in the autophagosome is equivalent to digestion in the macrophage.

The appearance of caspase-like activity correlated with the beginning of parental macronucleus resorption in the present experiment (Figures 1, 4). This corresponds to the timing of autolysosome formation. Nuclear resorption is generally executed by various enzymes from the lysosome, and caspase-like activity may activate such enzymes in the lysosome or autolysosome. To induce mating reactivity, cells must be under conditions of mild starvation. Indeed *Tetrahymena* cells have numerous autophagosomes in the cytoplasm under these conditions.⁴⁰ The slightly high background level found in premating cells may reflect on this kind of activity, although statistical analysis gave no significant difference from the early stage of conjugation. Considering these observations, caspase-like activity in an ancestral ciliate may have activated lysosomal enzymes to digest materials in food vacuoles and degrade organelles in the autophagosome for its turnover. In the course of evolution, spatial differentiation of germ line/soma in the same cytoplasm may have facilitated the elaboration of intracellularly localized nuclear degradation by diversion of the original caspase cascade. Therefore, PND may be different from PCD. The Fas ligand–Fas receptor system participates in PCD in *Tetrahymena* as it does in apoptosis in general.¹⁹ However, the Fas system may not be involved in PND.

A role for mitochondria in PND

Generally, caspase-9 is involved in mitochondrial pathways in mammalian apoptosis. Pro-caspase-9 is activated when Apaf-1 and cytochrome *c* are released from mitochondria.²⁸ In protistan apoptosis, a mitochondrial pathway for apoptosis is observed in *Dictyostelium discoideum* and *Leishmania major*.^{41,42} The use of a dye to detect mitochondrial membrane potential change in this study brought an unexpected result; the dye was localized in the degenerating macronucleus, but not in the cytosol (Figure 5). This may be explained as follows. (1) Mitochondria are simultaneously taken into the autophagosome when the phagosomal membrane surrounds the degenerative nucleus and, as suggested by the change of fluorescent color from red to

green, key molecules such as cytochrome *c* are released by mitochondrial breakdown. (2) Mitochondria may selectively fuse with the autophagosome, and then lose membrane potential. In either case, the dye is obligatorily localized in mitochondria and the autophagosome can acquire the key molecules from the restricted mitochondria. Unfortunately, there is no direct evidence that such molecules derived from mitochondria are localized in the autophagosome. Finally, we cannot deny the possibility that the dye may bind directly the degraded macronucleus. Further investigation into this aspect, an involvement of mitochondria, might contribute to the elucidation of a mechanism of *Tetrahymena* nuclear death.

Materials and Methods

Stocks

The *T. thermophila* strains used in this study were CU813 and CU428.2, and were kindly supplied by P Bruns (Cornell University, Ithaca, NY, USA).

Culture methods and induction of conjugation

The conditions for cell culture, starvation and conjugation were described previously.⁴³ Cells were grown in 0.25% proteose peptone (DIFCO), 0.25% yeast extract (DIFCO) and 3.5% glucose at 26°C. To induce mating reactivity, the cell density was adjusted to 1.0×10^5 cells/ml in 10 mM Tris-HCl (pH 7.5) and the cells were incubated at 26°C overnight. To induce conjugation, equal numbers of both strains were mixed and kept at 26°C.

DNA isolation and agarose gel electrophoresis

Total DNA was extracted by standard methods from cells at respective times during conjugation.⁴⁴ DNA concentration was quantified by UV spectrophotometry at 260 nm; 10 µg of total *Tetrahymena* DNA was loaded and electrophoresed on 2% agarose gels in TBE and stained with ethidium bromide.

Preparation of cell extracts

During conjugation, 10^6 cells were kept on ice for 5 min and collected by centrifugation. The packed cells were resuspended in ice-cold cell lysis solution (50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100) and incubated for 10 min on ice. Then, resuspended cells were homogenized by vortex for 10 s three times. Cell lysates were centrifuged at $10\,000 \times g$ for 10 min at 4°C. The supernatant (cell extract) was transferred to new microtubes and kept on ice until use. Protein Assay CBB Solution (Nacalai) was used to quantify protein concentrations in cell extracts, and standard curves were prepared with BSA (Sigma).

Caspase colorimetric assay

To perform caspase colorimetric assay, Ac-DEVD-pNA (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline), Ac-IETD (*N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline)-pNA and Ac-LEHD (*N*-acetyl Leu-Glu-His-Asp-*p*-nitroaniline)-pNA (BIOMOL) were used as substrates.^{27,45} Assays were performed in $1 \times$ reaction buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 100 mM NaCl, 10% glycerol), and 200–400 µg of protein per 70 µl of cell extract was used for each assay. The cell extract was preincubated

at 37°C for 15 min before adding 25 µl of modified $4 \times$ reaction buffer and 5 µl of 2 mM substrate. Assays were performed at 37°C and read every 15 min for 5 h at 405 nm with a Benchmark microplate reader (BIORAD). For caspase assays with inhibitor, 60 µl of cell extract was preincubated at 37°C for 30 min with 25 µl of modified $4 \times$ reaction buffer and 1 µl of 10 mM inhibitor, Ac-DEVD-CHO (BIOMOL), Ac-IETD-pNA (BIOMOL) or Ac-LEHD-pNA (Peptide Ins.). After incubation, 15 µl of 0.67 mM substrate was added to each sample.

Cytological analysis

To examine the stages of conjugation, cells were fixed with formalin (final 3%), stained with 1 µg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and observed by fluorescence microscopy.

We used a DePsipher Kit (TREVIGEN) to detect changes in the mitochondrial membrane potential. Conjugated cells were transferred to 5 µg/ml Depsipher solution in $1 \times$ reaction buffer with stabilizer solution, and incubated for 1.5–2 h at 26°C. Then, the cells were transferred to $1 \times$ reaction buffer with stabilizer solution. Cells were observed immediately under a fluorescence microscope with FITC and green filters. For photography, the cells were fixed with formalin (final 0.5%) and stained with DAPI to visualize the nucleus. Photographs were adjusted with Adobe PhotoShop 5.0.

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