Correspondence

Pheromone Induces Programmed Cell Death in *S. cerevisiae*

F.F. Severin and A.A. Hyman

Programmed cell death is a ubiquitous process in multicellular organisms. The pathways by which cells in multicellular organisms trigger cell death have been extensively characterized. A typical scenario for programmed cell death development includes activation of the MAP kinase cascade, accumulation of reactive oxygen species, release of cytochrome *c* from mitochondria into cytoplasm as a consequence of opening the permeability transition pore (PTP) in the mitochondrial membrane, and activation of caspases (reviewed in [1]). It remains unclear whether single cell organisms have evolved such pathways. And it is unclear what benefit would accrue to a single cell organism undergoing programmed cell death. One reason for a unicellular organism to commit altruistic suicide may be to benefit the cell community. Such a mechanism would improve the genetic fund of the community by eliminating the weak individuals.

Yeast show aspects of communal behaviour when they mate. There are two mating types in yeast: α and *a*. Cells of α mating type produce alpha-factor, triggering cells of *a* type to mate and vice versa. High doses or prolonged exposure to sexual pheromone is toxic for yeast (see [2] for review). The altruistic death of yeast cells unable to mate after a long time in contact with cells of the opposite mating type might be beneficial for the cell community. We decided to test whether the toxicity of alpha-factor could be explained by induction of programmed cell death.

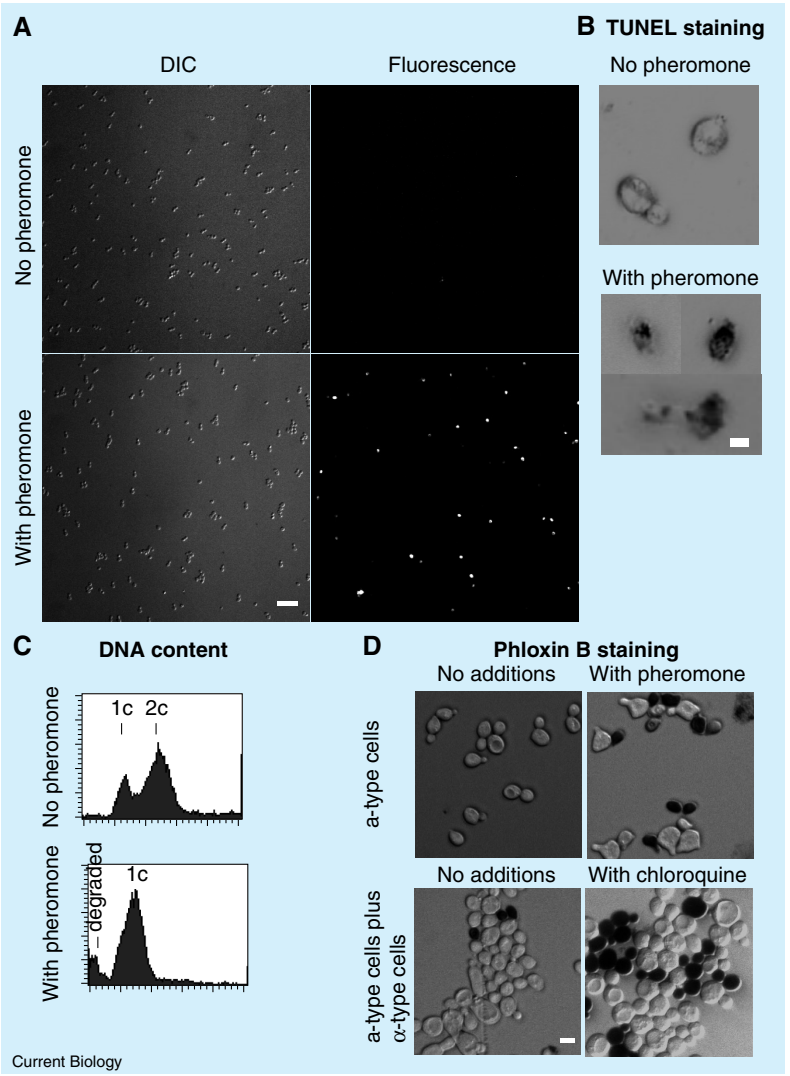

Figure 1.
(A) Reactive oxygen species accumulation in the presence or in the absence of pheromone was visualized by the addition of H_2DCF-DA. Bar 10 μm. (B) TUNEL staining shows DNA breakage in pheromone-treated but not in the control cells. Bar 2 μm. (C) FACS analysis shows accumulation of cells with degraded (less than 1C) DNA in pheromone-treated cells. (D) Cell staining with Phloxin B. Only the dead cells accumulate the dye. Bar 5 μm.

One of the key mechanisms by which cells trigger programmed cell death is by production of reactive oxygen species (see [1] for review). 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) can be used as a probe for reactive oxygen species production; H_2DCF-DA is oxidized by reactive oxygen species to DCF which fluoresces green [3]. We tested whether alpha-factor addition to *a* cells could trigger DCF accumulation. Experiments showed that after 1.5 hr exposure of *a* cells to alpha-factor approximately 30% of *a*-type cells fluoresce, whereas no fluorescing cells are found either in the absence of pheromone (Figure 1A), or when alpha-factor is added to α cells (Table 1). We conclude that addition of alpha-factor to cells of the opposite mating type can induce the formation of reactive oxygen species, a marker of programmed cell death. We titrated the concentration of pheromone and monitored the appearance of shmoos, morphological markers of mating. We found that reactive oxygen species induction requires approximately 10-fold higher

Table 1. Pheromone causes mitochondrion-linked reactive oxygen species production and death of yeast cells.

Additions			Effects	
Cells	**Pheromone (α-factor)**	**Other**	**% ROS-producing cells** (after 1.5 h)	**% Dead cells** (after 3.5 h)
α	+	–	0.8±0.8	0.9±1.0
a	+	–	26.9 ±3.3	26.3±4.0
a	+	cycloheximide	0	2.6±1.3
a, no mitochondria	+	–	0	1.5±0.6
a, cyc1Δ/cyc7Δ	+	–	33.5±12.7	2.6±3.6
a, *ste20*Δ	+	–	0.5±0.6	0.5±0.8
a, *cmd1-6*	+	–	54.9±4.4	51.6±1.0
a	+	CsA	2.6 ±0.6	2.5 ±3.6
a + α	–	-	24.3±4.0	5.8±1.0
a + α	–	chloroquine	nd	33.7±8.7
a	–	chloroquine	nd	1.4±0.2
a	+	chloroquine	nd	32.9±3.4
a + α	–	chloroquine CsA	nd	2.9±1.8

pheromone concentration than schmoo growth (data not shown).

To test whether alpha-factor induces other programmed cell death markers in *a* cells, we scored the accumulation of dead cells, as judged by phloxin B staining, and the appearance of degraded DNA. Figure 1D shows that ~ 30% of the *a* cells are dead after 3.5 hr of treatment with alpha-factor (100 μg/ml), and DNA is being degraded as shown by TUNEL (Figure 1B) and FACS (Figure 1B,C). These data indicate that pheromone-induced cell death shows some features of programmed cell death in higher organisms.

We also examined the pathway for development of pheromone-induced programmed cell death. The mating response activates the MAP kinase pathway, of which a key component is the Ste20 kinase [2]. Deletion of *ste20* prevents alpha-factor-induced death of *a*-type cells, and concomitantly prevents the formation of reactive oxygen species (Table 1). Interestingly, homologues of the MAP kinase pathway have been shown to play a major role in programmed cell death development in higher cells [4], suggesting a parallel between the pathways inducing cell death in yeast and higher cells.

One of the consequences of MAP kinase cascade activation is the induction of a number of proteins [4]. To test whether this downstream effect of the pheromone action is important for programmed cell death development, we checked whether inhibition of protein synthesis prevents pheromone-induced cell death. As shown in Table 1, cycloheximide inhibits reactive oxygen species production and the accumulation of dead cells.

The downstream events of programmed cell death in higher cells usually require mitochondria (see [1] for review). We tested whether yeast without functional mitochondria undergo pheromone-induced programmed cell death. Table 1 shows that the destruction of mitochondrial DNA by ethidium bromide completely abolishes the effect of alpha-factor on reactive oxygen species formation and accumulation of dead cells. On the other hand the disruption of mitochondrial functions does not prevent cell shmooing in response to alpha-factor addition (data not shown) suggesting that the mating response is still intact.

Mitochondria-related programmed cell death events generally include generation of reactive oxygen species, opening of the mitochondrial permeability transition pore and release of cytochrome *c* into the cytoplasm (see [1] for review). Is a similar pathway involved in pheromone-induced programmed cell death in yeast? As shown in Table 1, yeast cells without cytochrome *c* still generate reactive oxygen species but fail to complete programmed cell death. On the other hand, inhibition of permeability transition pore formation by cyclosporin A prevents both reactive oxygen species accumulation and cell death (Table 1).

Cyclosporin A inhibits not only permeability transition pore formation but also the calcineurin/calmodulin system [5], which regulates programmed cell death development in animal cells [6]. To test how cyclosporin A interferes with cell death development we checked whether yeast cells with a compromised calcineurin/calmodulin system — a *cmd1-6* mutant strain — undergo pheromone-induced cell death. As shown in Table 1, reactive oxygen species accumulation and cell death are approximately doubled in the *cmd1-6* mutant. As yeast with mutations in calmodulin and calcineurin are supersensitive to pheromone treatment [7,8] it seems likely that the calcineurin/calmodulin system

inhibits programmed cell death. Therefore we propose that cyclosporin A inhibits programmed cell death through its effect on the permeability transition pore. Our data on pheromone-induced programmed cell death cascade are schematically presented in Supplementary Figure 1. The cascade is assumed to include calmodulin/calcineurin-controlled MAP kinase pathway, reactive oxygen species generation and the formation of permeability transition pore triggering the release of cytochrome *c* and cell death.

We were interested in whether pheromone-induced programmed cell death occurs in native conditions. We mixed cells of α and *a* type and monitored the appearance of reactive oxygen species-positive and dead cells. As shown in Table 1 and Figure 1D, ~ 30% of the cells were reactive oxygen species-positive under these conditions, similar to the experiments with added pheromone. The percentage of dead cells was higher than control cells (cells without any pheromone additions) but lower than *a*-type cells with alpha-factor added. Apparently local concentrations of pheromones in the mating-induced agglutinates of α and *a* cells are potentially high enough to induce cell death, but successful mating reverses or prevents programmed cell death. Indeed, it is logical to expect that the altruistic suicide of a yeast cell is a relatively rare event under normal physiological conditions. In line with such an explanation once two cells fuse and form a diploid they lose their sensitivity to pheromones [2]. To check the possibility that cell fusion during mating prevents programmed cell death, we incubated a mixture of α and *a* cells with 5 mM chloroquine. (Chloroquine at 5 mM does not significantly affect vegetative growth but does prevent zygote formation by inhibiting cell wall degradation necessary for fusion [9].) As shown in Table 1, row 10 and Figure 1D, ~ 30% of mating cells die in the presence of chloroquine. Chloroquine is not toxic to non-mating cells (Table 1, row 11), nor does it affect the percentage of *a* cells killed by α-factor (compare rows 12 and 2, Table 1). As expected, the anti-apoptotic agent cyclosporin A rescues the chloroquine-induced death in the mixture of *a* and α cells (Table 1, row 13 and Figure 1D). Thus in yeast, mating-induced cell agglutination without fusion leads to programmed cell death. These data on mixtures of α and *a* cells suggest that programmed cell death is a natural part of the yeast mating process. Interestingly, the plant antibiotic osmotin activates one of the components of the yeast mating kinase cascade [10] which leads to apopotosis-like cell death [11], suggesting that plants can use this natural process of mating-linked cell death to defend themselves against pathogenic fungi.

Our data show that *S. cerevisiae* can induce what seems in outline to be similar to programmed cell death development in animal cells. However, yeast do not have caspases, components of the major apoptotic machinery in animal cells [12]. Programmed cell death has also been described for plant cells, although genes encoding typical caspases have not been found in plants [13]. Similarly, bacteria lack caspases and still undergo programmed cell death under certain conditions [14,15]. On the other hand, programmed cell death pathways exist that do not involve caspases. The extent to which caspase-independent cell death is used in animal cells is unclear, but the data in more primitive organisms suggest that caspase-independent programmed cell death might represent a more ancient form of active cell death.

References

1. Skulachev, V.P. (1996). Why are mitochondria involved in apoptosis? Permeability transition pores and apoptosis as selective mechanisms to eliminate superoxide-producing mitochondria and cell. FEBS Lett. *397*, 7–10.
2. Kurjan, J. (1993). The pheromone response pathway in *Saccharomyces cerevisiae*. Annu. Rev. Genet. *27*, 147–179.
3. Possel, H., Noack, H., Augustin, W., Keilhoff, G. and Wolf, G. (1997). 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. FEBS Lett. *416*, 175–178.
4. Dan, I., Watanabe, N.M. and Kusumi, A. (2001). The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol. *11*, 220–230.
5. Liu, J., Farmer, J.D. Jr., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807–815.
6. Lotem, J., Kama, R. and Sachs, L. (1999). Suppression or induction of apoptosis by opposing pathways downstream from calcium-activated calcineurin. Proc. Natl. Acad. Sci. U.S.A. *96*, 12016–12020.
7. Cyert, M.S. and Thorner, J. (1992). Regulatory subunit (CNB1 gene product) of yeast Ca^{2+}/calmodulin- dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell Biol. *12*, 3460–3469.
8. Moser, M.J., Geiser, J.R. and Davis, T.N. (1996). Ca^{2+}-calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca^{2+}-calmodulin-dependent protein kinase. Mol. Cell Biol. *16*, 4824–4831.
9. Doi, S., Tanabe, K., Watanabe, M. and Yoshimura, M. (1989). Chloroquine, a lysosomotropic agent, inhibits zygote formation in yeast. Arch. Microbiol. *151*, 20–25.
10. Yun, D.J., Ibeas, J.I., Lee, H., Coca, M.A., Narasimhan, M.L., Uesono, Y., Hasegawa, P.M., Pardo, J.M. and Bressan, R.A. (1998). Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. Mol. Cell *1*, 807–817.
11. Narasimhan, M.L., Damsz, B., Coca, M.A., Ibeas, J.I., Yun, D.J., Pardo, J.M., Hasegawa, P.M. and Bressan, R.A. (2001). A plant defense response effector induces microbial apoptosis. Mol. Cell *8*, 921–930.
12. Aravind, L., Dixit, V.M. and Koonin, E.V. (2001). Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. Science *291*, 1279–1284.
13. Lam, E., Pontier, D. and del Pozo, O. (1999). Die and let live - programmed cell death in plants. Curr. Opin. Plant Biol. *2*, 502–507.
14. Raff, M. (1998). Cell suicide for beginners. Nature *396*, 119–122.
15. Lewis, K. (2000). Programmed death in bacteria. Microbiol. Mol. Biol. Rev. *64*, 503–514.

Supplementary material

Supplementary material for this article is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank F. Sherman, T. Davis and K. Nasmyth for providing the mutants and Arshad Desai, Heidi Woods, Christiane Walch-Solimena and Kai Simons for the critical reading of the manuscript. We thank V.P. Skulachev for intellectual support and encouragement.

Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany. severin@mpi-cbg.de