

MicroCommentary

Another piece of the puzzle of apoptotic cytochrome *c* release

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Summary

Involvement of the mitochondrial permeability transition pore (PTP) in apoptosis and PTP structure are highly controversial. In this issue of *Molecular Microbiology*, experiments based on yeast genetics analyse the roles of the three proteins commonly considered to form the PTP, i.e. porin, ADP/ATP carrier (ACC) and mitochondrial cyclophilin, on apoptosis-like cell death. Whereas knocking out cyclophilin had no effect, the porin-1 knockout yeast showed enhanced apoptosis, suggesting that porin-1 has an antiapoptotic role. Loss of the ACC proteins afforded protection against some causes of death, but enhanced death induced by H₂O₂, suggesting a more complex role for the ACC proteins in regulating apoptosis-like death in yeast.

Apoptosis in biomedical sciences

Apoptosis has become a major research area in biomedical sciences because its deregulation has potential implications for many diseases. There can be too much apoptosis (as appears to be the case in some neurodegenerative diseases) or too little apoptosis (as is the case in autoimmune diseases and cancer) (Lawen, 2003). In addition to apoptosis, several other forms of cell death have now been described and have been more or less well defined (Bredesen *et al.*, 2006). Apoptosis (and other forms of cell death) might have evolved as a protective mechanism against invading pathogens in primitive multicellular organisms (Yuan, 2006). However, nature was able to adopt cell death programmes already developed in unicellular organisms like yeast (Goldfarb and Scheffers,

2004; Gourlay *et al.*, 2006). The cell death nomenclature is anything but clear and the number of death categories and subcategories is continually increasing (Kroemer *et al.*, 2005; Gourlay *et al.*, 2006). As apoptosis was morphologically defined in mammalian cells, however (Kerr *et al.*, 1972), I believe we should refer to 'apoptosis-like cell death' in yeast to avoid even more confusion.

Apoptosis-like yeast cell death has many features in common with mammalian apoptosis – not least being the involvement of mitochondria in a major death pathway (Eisenberg *et al.*, 2007). In the mitochondrial pathway – both in yeast and in mammals – the outer mitochondrial membrane permeabilization (MOMP) and release of pro-apoptotic proteins from the intermembrane space are crucial for death. Many of the pro-apoptotic proteins released are conserved from yeast to human, including cytochrome *c*, apoptosis-inducing factor (AIF) and HtrA/Omi (Gourlay *et al.*, 2006), which makes yeast a good model organism to study the intrinsic pathway. Cytochrome *c* release appears to contribute to but does not appear to be necessary for apoptosis-like yeast cell death (Silva *et al.*, 2005), whereas release of AIF appears to be necessary for yeast apoptosis-like death (Wissing *et al.*, 2004). The nature of the pore that releases these proteins is still unknown (both in yeast and in mammalian cells), however, and the identity of the proteins involved in its formation is highly controversial (Ly *et al.*, 2003; Kinnally and Antonsson, 2007).

Mechanism of cytochrome *c* release

Two channels might be responsible for release of cytochrome *c* and other pro-apoptotic proteins in mammalian cells, the permeability transition pore (PTP) and the mitochondrial apoptosis-induced channel (MAC) (Kinnally and Antonsson, 2007). MAC appears to contain at least Bax or Bak, and Bax/Bak double knockout murine embryonic fibroblasts (MEFs) are resistant to many apoptotic triggers (Wei *et al.*, 2001). Yeast does not contain any Bax or Bak homologues (Manon *et al.*, 1997) and therefore lacks MAC. Thus, the group of Côte-Real decided to analyse the role of the yeast PTP in cytochrome *c* release and apoptosis-like cell death (Pereira *et al.*, 2007).

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The idea that PTP might be involved in mammalian cytochrome *c* release stems from observations that it can be inhibited by PTP inhibitors like bongkreikic acid and cyclosporin A (CsA). However, there are also many reports showing that PTP inhibition has no effect on cytochrome *c* release (e.g. Grubb *et al.*, 2001; Ly *et al.*, 2003). The notion that PTP opening can lead to MOMP is also controversial. The most commonly suggested mechanisms propose either the PTP-dependent formation of a specific releasing pore in the outer mitochondrial membrane (OMM) or OMM rupture and cytochrome *c* release as result of PTP-dependent mitochondrial swelling (Garrido *et al.*, 2006). The latter hypothesis, however, does not account for the fact that not all intermembrane space proteins are released into the cytosol.

The PTP

The mammalian PTP is commonly considered to be composed of the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane (IMM), the voltage-dependent anion-selective channel (VDAC) in the OMM and cyclophilin D (CyPD) in the mitochondrial matrix (Fig. 1A) with a range of associated proteins, including hexokinase I (HKI), creatine kinase (CK) and the benzodiazepine receptor (BR) (Desagher and Martinou, 2000; Ralph *et al.*, 2006; Javadov and Karmazyn, 2007). Yeast possess homologues of the core PTP proteins [yeast VDACs 1 and 2 (POR1 and POR2), a yeast mitochondrial cyclophilin (CPR3) and three ADP/ATP carrier proteins (AAC1, AAC2 and AAC3)] that are believed to function in a similar manner, forming a yeast PTP (Fig. 1B) that is also known as the yeast mitochondrial unselective channel or MUC (Manon *et al.*, 1998).

Mitochondrial cyclophilin

Cyclophilins are peptidyl-prolyl *cis/trans* isomerases that catalyse the *cis/trans* isomerization of prolyl peptide bonds (Fischer and Aumüller, 2003). They can be inhibited by the immunosuppressive drug CsA (Lawen, 1996). The mammalian mitochondrial cyclophilin is CyPD, while

the yeast homologue is CPR3 (Wang and Heitman, 2005). The PTP is reportedly CsA-sensitive and CyPD (CPR3) is believed to associate with the ANT (ACC) on the matrix side (see Fig. 1). CsA-sensitive and -insensitive cytochrome *c* release have both been reported in mammals (reviewed in Ly *et al.*, 2003) but the strongest evidence comes from the use of CyPD knockout mice (Baines *et al.*, 2005; Nakagawa *et al.*, 2005). As expected, PTP is no longer sensitive to CsA in these mice but the CyPD knockout was without effect on apoptosis, although some forms of necrotic cell death were inhibited. The paper from the **Côte-Real group** analyses apoptosis-like cell death in a $\Delta cpr3$ yeast strain (Pereira *et al.*, 2007). As in CyPD knockout mice, $\Delta cpr3$ yeast did not show any resistance to either H_2O_2 - or diamide- [a drug that in mammalian systems triggers PTP opening (Zamzami *et al.*, 1998)] induced apoptosis-like cell death. Thus, it appears that in yeast, as in mammals, mitochondrial cyclophilin is not involved in regulating of cytochrome *c* release and apoptosis-like cell death.

The ADP/ATP carrier

The mammalian PTP can be inhibited by bongkreikic acid and atractyloside [both ligands of ANT (Grubb *et al.*, 2001)], supporting a role of ANT in PTP structure. However, mice lacking both endogenous ANT isoforms still show CsA-sensitive permeability transition and cytochrome *c* release (Kokoszka *et al.*, 2004). These data suggest that ANT is a non-essential structural component of mammalian PTP and is not involved in cytochrome *c* release. The situation in yeast appears to be quite different. Yeast has three isoforms of the ANT homologue, AAC1–3. When Pereira *et al.* (2007) used a $\Delta aac1/2/3$ yeast strain – lacking all three yeast AAC isoforms, they observed strong protection against acetic acid and diamide-induced apoptosis-like cell death. This effect is not likely to be due to an impaired mitochondrial electron transport, as complex III and V inhibitors sensitized wild-type yeast to acetic acid, rather than protecting them, a result reproduced with the $\Delta atp2$ strain that lacks the β -subunit of complex V. Importantly, the authors observed

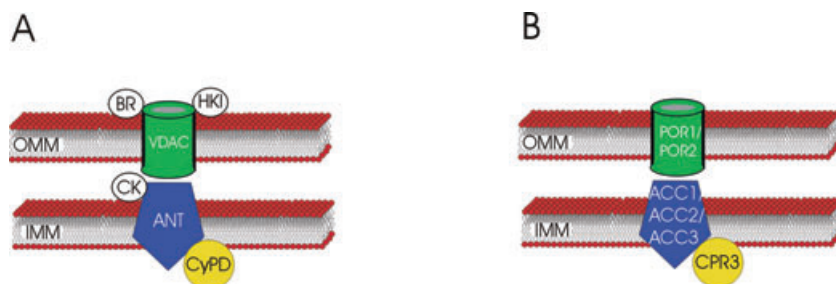


Fig. 1. Suggested structures of the mammalian (A) and yeast (B) PTP. For abbreviations used, see text.

almost complete inhibition of cytochrome *c* release in the $\Delta aac1/2/3$ yeast strain after acetic acid treatment (the cytosolic cytochrome *c* observed in Fig. 3 of Pereira *et al.* (2007) appears to be mainly attributable to unspecific membrane rupture, as some other mitochondrial proteins were also released). The resistance of the $\Delta cpr3$ yeast strain to acetic acid appears not to be the result of the loss of ADP/ATP translocation activity, as the expression of a loss of function mutant of *aac2* in the $\Delta aac1/2/3$ yeast strain reversed the resistance phenotype observed in $\Delta aac1/2/3$. In further contrast with the mammalian system, the authors do not observe any differences in metacaspase YCA1 activation between wild type and $\Delta aac1/2/3$, despite clear differences in cytochrome *c* release. A further indication that cytochrome *c* release might not be directly linked to metacaspase activation and apoptosis-like death was provided by the results of an experiment in which H_2O_2 was used to induce death. As with acetic acid treatment, cytochrome *c* release was inhibited in the $\Delta aac1/2/3$ yeast strain, but cell death was dramatically enhanced.

Porin (VDAC)

Finally, the authors analysed a VDAC1 knockout strain for its capacity to undergo apoptosis-like cell death. VDAC is believed to be the IMM component of the PTP (Fig. 1). Mammals have three VDAC isoforms, whereas yeast has only two (De Pinto *et al.*, 2003). Many reports suggest a role for VDAC in cytochrome *c* release (e.g. Zheng *et al.*, 2004) and apoptosis (e.g. Zaid *et al.*, 2005). However, recent data suggest a more peripheral role for VDAC in apoptosis, as mouse cells lacking all three VDAC isoforms were still capable of inducing apoptosis through the intrinsic pathway, releasing cytochrome *c* in response to Bax and Bid, pro-apoptotic members of the Bcl-2 family of proteins. Moreover, their mitochondria still showed permeability transition (Baines *et al.*, 2007). As with CyPD, VDAC now appears to be more important for non-apoptotic forms of cell death (Yagoda *et al.*, 2007). When Côte-Real's group tested a $\Delta por1$ strain, lacking the yeast VDAC1 gene, they observed enhanced apoptosis-like cell death induced by all three agents tested. Unfortunately, they did not study a porin 2 knockout or a double knockout, so we do not know whether the effects observed are specific for porin 1. Although it is commonly believed that yeast VDAC2 is not able to form pores, the literature does not contain any data directly demonstrating this. The $\Delta por2$ strain does not present any obvious phenotype, but *por2* can partially reverse the phenotype of the $\Delta por1$ strain, although POR2 was unable to form pores in a liposomal system (Blachly Dyson *et al.*, 1997). Together, these data suggest that there is at least some redundancy between POR1 and POR2.

Conclusion – what have we learned?

The data presented by Pereira *et al.* (2007) do not solve the puzzle of cytochrome *c* release, but they do add an important piece to the puzzle. In mammals, the data suggest that PTP is mainly involved in non-apoptotic forms of cell death, like necrosis (Tsujiimoto and Shimizu, 2007). Either CyPD, ANT or VDAC appears to be dispensable elements of PTP. In this recent addition to the puzzle, the data from the Côte-Real group support a role for both POR1 and AAC in apoptosis-like yeast cell death. However AAC appears to be protective in some regimens while enhancing cell death in others. POR1, on the other hand, at least for the treatments tested, appears to protect from apoptosis-like cell death. The data on CPR3, deletion of which had no effect, support the claim that at least the CsA-sensitive yeast PTP is not necessary for apoptosis-like cell death. Whether the effects observed with yeast strains lacking porin 1 and the three AAC isoforms are due to their association with the yeast PTP or due to some other functions and whether they are indispensable components of yeast PTP or not will be revealed by further experiments. Mammalian VDAC is also localized to the plasma membrane, where it can also regulate apoptosis (Elinder *et al.*, 2004; Lawen *et al.*, 2005); thus, at least porin 1 might have functions in addition to those in the OMM. Côte-Real's group is certainly well placed and the tools established in this communication will be useful in answering these questions.

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References

- Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H., Hambleton, M.A., *et al.* (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* **434**: 658–662.
- Baines, C.P., Kaiser, R.A., Sheiko, T., Craigen, W.J., and Molkentin, J.D. (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* **9**: 550–555.
- Blachly-Dyson, E., Song, J., Wolfgang, W.J., Colombini, M., and Forte, M. (1997) Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein. *Mol Cell Biol* **17**: 5727–5738.
- Bredesen, D.E., Rao, R.V., and Mehlen, P. (2006) Cell death in the nervous system. *Nature* **443**: 796–801.
- De Pinto, V., Messina, A., Accardi, R., Aiello, R., Guarino, F., Tommasello, M., *et al.* (2003) New functions of an old protein: the eukaryotic porin or voltage dependent anion selective channel (VDAC). *Ital J Biochem* **52**: 17–24.
- Desagher, S., and Martinou, J.-C. (2000) Mitochondria as the

- central control point of apoptosis. *Trends Cell Biol* **10**: 369–377.
- Eisenberg, T., Büttner, S., Kroemer, G., and Madeo, F. (2007) The mitochondrial pathway in yeast apoptosis. *Apoptosis* **12**: 1011–1023.
- Elinder, F., Akanda, N., Tofighi, R., Shimizu, S., Tsujimoto, Y., Orrenius, S., and Ceccatelli, S. (2004) Opening of plasma membrane voltage-dependent anion channels (VDAC) precedes caspase activation in neuronal apoptosis induced by toxic stimuli. *Cell Death Differ* **12**: 1134–1140.
- Fischer, G., and Aumüller, T. (2003) Regulation of peptide bond *cis/trans* isomerization by enzyme catalysis and its implication in physiological processes. *Rev Physiol Biochem Pharmacol* **148**: 105–150.
- Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C., and Kroemer, G. (2006) Mechanisms of cytochrome *c* release from mitochondria. *Cell Death Differ* **13**: 1423–1433.
- Goldfarb, D., and Scheffers, L. (2004) Editorial: 'Thematic issue: apoptosis-like death programs in yeasts'. *FEMS Yeast Res* **5**: 99–100.
- Gourlay, C.W., Du, W., and Ayscough, K.R. (2006) Apoptosis in yeast – mechanisms and benefits to a unicellular organism. *Mol Microbiol* **62**: 1515–1521.
- Grubb, D.R., Ly, J.D., Vaillant, F., Johnson, K.L., and Lawen, A. (2001) Mitochondrial cytochrome *c* release is caspase-dependent and does not involve mitochondrial permeability transition in didemnin B-induced apoptosis. *Oncogene* **20**: 4085–4094.
- Javadov, S., and Karmazyn, M. (2007) Mitochondrial permeability transition pore opening as an endpoint to initiate cell death and as a putative target for cardioprotection. *Cell Physiol Biochem* **20**: 1–22.
- Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**: 239–257.
- Kinnally, K.W., and Antonsson, B. (2007) A tale of two mitochondrial channels, MAC and PTP, in apoptosis. *Apoptosis* **12**: 857–868.
- Kokoszka, J.E., Waymire, K.G., Levy, S.E., Sligh, J.E., Cai, J., Jones, D.P., *et al.* (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* **427**: 461–465.
- Kroemer, G., El-Deiry, W.S., Golstein, P., Peter, M.E., Vaux, D., Vandenabeele, P., *et al.* (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* **12**: 1463–1467.
- Lawen, A. (1996) Biosynthesis and mechanism of action of cyclosporins. *Prog Med Chem* **33**: 53–97.
- Lawen, A. (2003) Apoptosis – an introduction. *Bioessays* **25**: 888–896.
- Lawen, A., Ly, J.D., Lane, D.J.R., Zarschler, K., Messina, A., and De Pinto, V. (2005) Voltage-dependent anion-selective channel 1 (VDAC1) – a mitochondrial protein, rediscovered as a novel enzyme in the plasma membrane. *Int J Biochem Cell Biol* **37**: 277–282.
- Ly, J.D., Grubb, D.R., and Lawen, A. (2003) The mitochondrial membrane potential ($\Delta\Psi_m$) in apoptosis; an update. *Apoptosis* **8**: 115–128.
- Manon, S., Chaudhuri, B., and Guérin, M. (1997) Release of cytochrome *c* and decrease of cytochrome *c* oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-x_L. *FEBS Lett* **415**: 29–32.
- Manon, S., Roucou, X., Guérin, M., Rigoulet, M., and Guérin, B. (1998) Characterization of the yeast mitochondria unselective channel: a counterpart to the mammalian permeability transition pore? *J Bioenerg Biomembr* **30**: 419–429.
- Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., *et al.* (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* **434**: 652–658.
- Pereira, C., Camougrand, N., Manon, S., Sousa, M.J., and Côté-Real, M. (2007) ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome *c* release in yeast apoptosis. *Mol Microbiol* (in press). doi:10.1111/j.1365-2958.2007.05926.x
- Ralph, S.J., Low, P., Dong, L., Lawen, A., and Neuzil, J. (2006) Mitocans: mitochondria targeted anti-cancer drugs as improved therapies and related patents. *Recent Patents Anti-Canc Drug Disc* **1**: 327–346.
- Silva, R.D., Sotoca, R., Johansson, B., Ludovico, P., Sansonetty, F., Silva, M.T., *et al.* (2005) Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. *Mol Microbiol* **58**: 824–834.
- Tsujimoto, Y., and Shimizu, S. (2007) Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis* **12**: 835–840.
- Wang, P., and Heitman, J. (2005) The cyclophilins. *Genome Biol* **6**: 226.
- Wei, M.C., Zong, W.-X., Cheng, E.H.-Y., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., *et al.* (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**: 727–730.
- Wissing, S., Ludovico, P., Herker, E., Büttner, S., Engelhardt, S.M., Decker, T., *et al.* (2004) An AIF orthologue regulates apoptosis in yeast. *J Cell Biol* **166**: 969–974.
- Yagoda, N., von Rechenberg, M., Zaganjor, E., Bauer, A.J., Yang, W.S., Fridman, D.J., *et al.* (2007) RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature* **447**: 864–868.
- Yuan, J. (2006) Divergence from a dedicated cellular suicide mechanism. *Mol Cell* **23**: 1–12.
- Zaid, H., Abu-Hamad, S., Israelson, A., Nathan, I., and Shoshan-Barmatz, V. (2005) The voltage-dependent anion channel-1 modulates apoptotic cell death. *Cell Death Differ* **12**: 751–760.
- Zamzami, N., Marzo, I., Susin, S.A., Brenner, C., Larochette, N., Marchetti, P., *et al.* (1998) The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. *Oncogene* **16**: 1055–1063.
- Zheng, Y., Shi, Y., Tian, C., Jiang, C., Jin, H., Chen, J., *et al.* (2004) Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome *c* release induced by arsenic trioxide. *Oncogene* **23**: 1239–1247.