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Programmed cell death in the aspergilli and other filamentous fungi

G. D. ROBSON
Faculty of Life Sciences, University of Manchester, Manchester, UK

Programmed cell death (PCD), in which cells actively participate in their own death through the activation of defined pathways, has long been established as an important developmental pathway in metazoan systems but it is only recently that evidence for a primitive form of PCD in the fungi has come to light. While much of the evidence for this comes from studies in the yeast *Saccharomyces cerevisiae*, recent evidence strongly suggests the presence of a metacaspase (primitive orthologues of the mammalian caspases) independent and dependent pathways in the Aspergilli which can be activated by deleterious environmental stimuli such as starvation, oxidative stress and antifungal agents as well as developmentally during sporulation. Bioinformatic evidence for these pathways suggests that the PCD pathway(s) is more complex in the Aspergilli compared to yeasts.

Keywords Programmed cell death, apoptosis

Introduction

Programmed cell death (PCD) is cellular death that involves the active participation of the cell through the activation of a discrete signalling pathway(s) and has been an area of intensive study in multi-cellular organisms for many years (for reviews see [1,2]). Programmed cell death plays an essential role in development, tissue homeostasis and as an important defence mechanism eliminating pathogen-infected cells [3,4]. Moreover, PCD underlies the hypersensitive response of plants by which cells surrounding an invading pathogen die in order to try and limit pathogen spread [5,6]. Until recently, it had been assumed that PCD evolved only with the evolution of multi-cellularity, however, the discovery that apoptosis inducing proteins from mammalian cells such as bax could induce death with an apoptotic phenotype when expressed in the yeast *Saccharomyces cerevisiae* and that this could be prevented by the simultaneous expression of the anti-apoptotic mammalian protein bcl-2, has led to the realization that a primitive PCD pathway evolved earlier than had previously been imagined [7–9].

Correspondence: G. D. Robson, Faculty of Life Sciences, 1.800 Stopford Building, University of Manchester, Manchester M13 9PT, UK. Tel: +44 (0)161 275 5048; E-mail: geoff.robson@manchester.ac.uk

PCD in yeasts

Numerous studies in yeast have since shown that various toxic agents including hydrogen peroxide, acetic acid and aspirin along with cellular stress including hyperosmotic stress, nutrient starvation, DNA damage and defects in *N*-glycosylation, all induce PCD [10–16]. The PCD phenotype includes the flipping of phosphatidylserine from the inner to the outer plasma membrane, an early classical marker of PCD in higher eukaryotes, and the distinctive and specific degradation of DNA, which initially occurs without loss of membrane integrity. Initial genome studies in *S. cerevisiae* revealed few orthologues of the PCD pathway from multi-cellular organisms; however some functional homologues have begun to emerge. Chief amongst these is the metacaspase protein *YCA1*, which is a protease with activity related to the divergent caspase family in higher eukaryotes [17]. Caspases play a critical role in the downstream execution of the PCD pathway in higher eukaryotes and form a family of cysteine proteases with distinctive functions. Disruption of *YCA1* in *S. cerevisiae* has been shown to rescue yeasts from PCD confirming its functional role as an executor of PCD although its target substrates have yet

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to be characterized [18–21]. However, disruption of *YCA1* does not prevent PCD in all cases. For example, PCD induced by ammonia in ageing colonies was able to occur in *YCA1* deleted strains [22]. Moreover PCD induced by overexpression of the yeast homologue of the mammalian apoptosis inducing factor (AIF) was only partially negated by disruption of *YCA1* [23]. Thus it is unclear whether the execution of PCD is mediated through *YCA1* in all cases.

In higher eukaryotes, mitochondria appear to play a central role in the PCD process with PCD inducing agents causing the loss of cytochrome C from the mitochondria which then complexes with the apoptosis activating factor (APAF). The complex then activates caspase 9 which itself activates downstream caspases [1,2] However, no APAF homologue has so far been described in yeast although release of cytochrome C from mitochondria has been demonstrated in conjunction with the development of a PCD phenotype, for example in response to acetic acid and mating pheromone [24,25]. However, recently a homologue of the apoptosis-inducing factor (AIF), Aif1p, a protein which also is normally sequestered in mitochondria but released during PCD, has also been characterized in yeast and shown to mediate PCD [23]. In higher eukaryotes, AIF triggers PCD through a caspase independent pathway suggesting that PCD in yeasts may be regulated through two independent pathways. More recently, another pro-apoptotic mammalian homologue of PCD, AMID, a mitochondrial NADH dehydrogenase, has been identified in yeast and shown to induce PCD when overexpressed [26].

An accumulation of reactive oxygen species (ROS) as a result of mitochondrial dysfunction during PCD appears to be a central phenomenon in most instances of PCD. Reactive oxygen species accumulation during PCD development has also been reported in many cases of induced PCD in *S. cerevisiae* [10,27–29]. In addition, PCD was shown to be delayed by reducing ROS accumulation with free radical scavengers, further implicating ROS as a core component of PCD signalling [27,30–32]. Initially it was hypothesized that ROS played a central role in the execution of PCD although it is now unclear as to whether ROS accumulation plays a secondary role or may act as both signalling molecules and as PCD regulators [9].

While the downstream targets and machinery of yeast apoptosis is still to be discovered, it is becoming more apparent that many mutations in yeast can lead to PCD and that PCD may be induced when normal cellular functions are impaired. For example, whilst it is known that aberrant protein glycosylation, DNA replication and mRNA stability can lead to PCD [16,29,33,34], it has recently been discovered that PCD may also be linked to actin and the cytoskeleton. Agents and mutations that decrease actin turnover led to an accumulation of ROS and induced PCD, whereas increasing actin turnover led to an increase in long-term viability [35], thus implicating the actin cytoskeleton in chronological ageing.

Cell death in filamentous fungi

In contrast to the increasing number of studies that are beginning to unravel the PCD pathway in yeasts, relatively little work has, as yet, focused on the filamentous fungi (Table 1). Due to the larger genome size and developmental complexity, it is possible that the PCD pathway(s) in filamentous fungi may be more complex than that present in yeast. Preliminary studies of the genomes of filamentous fungi have already identified homologues of mammalian PCD components that appear to be absent in yeasts though experimental studies have yet to confirm their function [36]. For example, the Aspergilli possess a number of proteins which include the STAND domain, a group including NACHT NTPases and NB-ARC ATPases which regulate PCD in animals and plants, subunits of mitochondrial NADH ubiquinone oxidoreductase GRIM-19 and NDUFS1, poly(ADP-ribose) polymerase (PARP), a key regulator of mammalian caspase independent PCD, TRAF3 and a number of APAF homologues that in mammalian cells complex with caspase 9 to activate PCD [36–38].

Autolysis

Autolysis is a highly regulated and natural process that occurs after filamentous fungi in liquid culture become nutrient-limited and enter the stationary phase [39,40]. Autolysis is the process of enzymatic self-degradation that occurs during the stationary phase of growth following nutrient depletion and involves the coordinated activation of several key enzyme classes including glycosyl hydrolase and proteases resulting in the catabolism of macromolecules within the hyphae cell [40,43]. The liberated sugars and amino acids can be utilized by the remaining mycelium through a process known as cryptic growth [39,40]. The sequence of events in fungal autolysis therefore bears some similarities to PCD of higher eukaryotes; for example, both phenomena are energy-dependent in contrast to necrosis, which has no energy requirement. In *Aspergillus fumigatus*, entry into the stationary phase has been shown to be associated with a rapid decrease in viability which was associated with the development of an

Table 1 Summary of programmed cell death phenotypes observed in filamentous fungi

Treatment/ condition	Programmed cell death marker							Reference
	DNA cleavage	PS flipping	Membrane integrity maintained	Mitochondria involvement	Dependence on protein synthesis	Metacaspase activity	Inhibited by caspase inhibitor	
Stationary phase*	Yes	Yes	Yes	?	Yes	Yes	Yes	Mousavi & Robson 2003 [44]
Hydrogen peroxide*	Yes	Yes	Yes	?	Yes	No	No	Mousavi & Robson 2004 [46]
Amphotericin B*	Yes	Yes	Yes	?	Yes	No	No	Mousavi & Robson 2004 [46]
Sphingoid long chain bases†	Yes	Yes	Yes	Yes	Yes	No	?	Cheng *et al.* 2003 [47]
Antifungal protein†	Yes	Yes	Yes	Yes	Yes	No	?	Leiter *et al.* 2005 [48]
Asexual sporulaton	?	?	?	?	?	Yes	?	Thrane *et al.* 2004 [56]
Farnesol‡	Yes	Yes	Yes	Yes	?	?	?	Semeghini *et al.* 2006 [49]
Vegetative incompatibility‡	Yes	?	?	?	?	?	?	Marek *et al.* 2003 [59]
RAS overexpression§	Yes	Yes	Yes	Yes	?	?	?	Chen & Dickman 2005 [57]
Meiosis defect¶	Yes	?	?	?	?	?	?	Lu *et al.* 2003 [55]

*, *Aspergillus fumigatus*; †, *Aspergillus nidulans*; ‡, *Neurospora crassa*; §, *Colletotrichum trifolli*; ¶, *Coprinus cinereus*; ?, Unknown.

apoptotic phenotype [44]. Such a rapid decline in cell viability (>90% within 6 h of reaching the stationary phase) is not confined to *Aspergillus fumigatus* as similar rapid declines in viability have also been observed in *Aspergillus niger* and *Aspergillus nidulans* [GD Robson, unpublished observations]. The development of an apoptotic phenotype was prevented by the addition of the protein synthesis inhibitor cycloheximide immediately prior to the onset of stationary phase, indicating a dependence on *de novo* protein synthesis. Moreover, the apoptotic phenotype was prevented by the addition of a broad-spectrum caspase inhibitor and entry into the stationary phase was associated with a rise in intracellular activity against substrates specific for caspase 1 and 8. By contrast, yeasts on entering stationary phase lose viability much more slowly, taking several days to reach viabilities of less than 10% and have been used as a model of chronological ageing [18,27]. Nonetheless, loss of viability is associated with a PCD phenotype and can be reduced in the metacaspase-deleted strain or by addition of the broad-spectrum caspase inhibitor. By contrast, despite blocking the development of a PCD phenotype in *A. fumigatus* by addition of the broad-spectrum caspase inhibitor, no effect was seen on the loss of viability. The development of a PCD phenotype early during carbon starvation has also been reported in *A. nidulans* [45] although the authors suggest that this pathway is separate from subsequent autolysis as the PCD phenotype was also seen in mutants in which autolysis of the mycelium did not occur. Entry into the stationary phase and loss of viability is also associated with an accumulation of ROS (Fig. 1) which initially was found to be compartmentalized before later accumulating in the majority of hyphae. Reactive oxygen species accumulation appeared to precede cell death [GD Robson, unpublished observation].

Extracellular agents

Compared to yeasts, there have been comparatively few studies investigating whether exposure to toxic treatments triggers the development of an apoptotic phenotype. Treatment of *A. fumigatus* with low but toxic levels of hydrogen peroxide or amphotericin B both induced a strongly apoptotic phenotype and cell death [46]. However, unlike death induced during the stationary phase, this could not be blocked by the broad-spectrum caspase inhibitor Z-VAD-fmk, nor was a rise against any caspase specific substrate observed, suggesting a caspase-independent pathway may operate in filamentous fungi or that additional metacaspase(s) not blocked by the broad-spectrum caspase inhibitor or

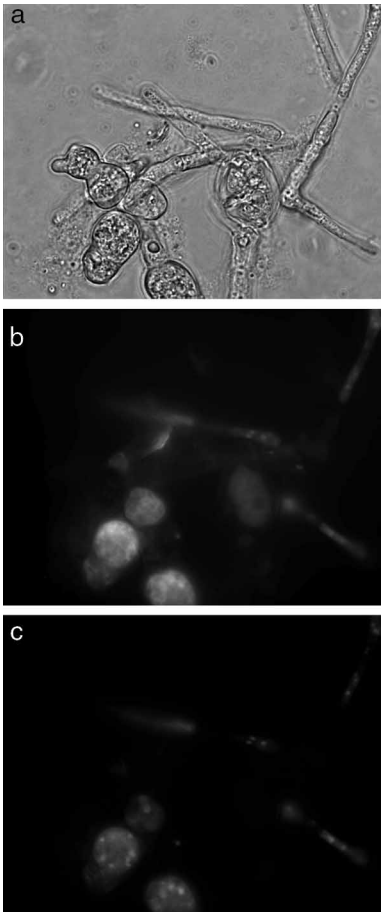

Fig. 1 Accumulation of reactive oxygen species (ROS) and loss of membrane integrity in stationary phase of *Aspergillus niger*. Mycelium from cultures 4 h into the stationary phase were counterstained with 40 μM 2′,7′-dichlorodihydrofluorescein diacetate (40 ROS) and 30 μg ml^{-1} propidium iodide (PI) (membrane integrity) and examined under (a) bright-field and (b) fluorescence microscopy excited at 490 nm (ROS) and then (c) at 575 nm (PI). Note that ROS accumulation appears before loss of membrane integrity.

active against the specific substrates tested may be present. At least two metacaspase genes are present in *A. fumigatus* genome (and in all other filamentous fungal genomes examined to date) whereas only one has been identified in the yeasts [36]. Treatment of *A. nidulans* with the phytoshingolipids or the antifungal protein PAF have also both been reported to lead to the development of PCD including DNA degradation, translocation of phosphatidylserine and ROS accumulation and appeared to be independent of metacaspase activity at least in the case of the AFP [47,48].

More recently, farnesol, a quorum sensing isoprenoid secreted by *Candida albicans* was shown to inhibit growth, induce ROS accumulation and cause the development of a PCD phenotype in *A. nidulans*, with the authors suggesting that this might be an antagonistic mechanism for suppressing fungal growth in the environment [49].

Developmental cell death

Heterokaryon or vegetative incompatibility is a form of PCD that is triggered when the hyphae of two individuals carrying incompatible *het* loci fuse, leading to growth inhibition, hyphal compartmentalization, repression of sporulation and ultimately death of the fused cells (for reviews see [50–52]). Although widely studied, particularly in Podospora and Neurospora, the downstream PCD pathway remains largely unresolved.

In the development of macrofungi, highly organized and specific death of hyphae appears to occur early during mushroom development which creates for example, separate gills in the mature fruitbody. Such developmental cell death echoes developmental PCD in multi-cellular organisms although to date, there is no firm biochemical evidence that death in these hyphae is occurring through PCD [53,54]. More recently, mutants of *Coprinus cinereus* in which defects in meiosis occur have been shown to induce apoptosis in the basidia but not the neighbouring somatic cells [55]. Programmed cell death is also initiated in yeast with mutations in DNA replication and is thought to be activated in response to DNA damage [32]. Metacaspase activity has also been associated with asexual sporulation in *Aspergillus nidulans* and the same authors also detected an orthologue of the mammalian DNA repair protein poly (ADP-ribose) polymerase (PARP), a known caspase substrate which is degraded during PCD [56]. In filamentous fungi, vacuolization and protein breakdown in the mycelium occurs at the same time as asexual sporulation and metacaspase-mediated degradation of the *A. nidulans*. PARP protein was found to occur early in the sporulation process indicating that PCD is also important in asexual development. Recently, evidence that signalling pathways interact with PCD has come to light. In the plant pathogen *Colletotrichum trifolli*, expression of the small GTP-binding protein Ras (Ct-ras) with a dominant activating mutation was found to cause aberrant hyphal growth and defects in polarized growth which was associated with ROS accumulation and PCD phenotype under nutrient-poor but not nutrient-rich conditions. These effects were inhibited by the inclusion of the antioxidant praline [57]. Recently, the Ras-cAMP pathway has also been implicated as a modulator of PCD induced by acetic acid and hydrogen peroxide in *C. albicans* [58]; thus PCD may play a number of roles in the developmental biology of filamentous fungi and may be modulated by signalling pathways.

Conclusion

Experimental evidence from fungi, principally yeasts, demonstrates that an ancestral PCD evolved early in the eukaryotes and that a number of components are remarkably functionally conserved across both animals and plants. Compared to yeasts, experimental evidence for PCD in the filamentous fungi is sparse, nonetheless, like yeasts; the development of classical markers of PCD in response to numerous stimuli demonstrates the conservation of PCD in the filamentous fungi. Genomic analysis of recently published *Aspergillus* genomes have revealed that the PCD in these organisms is likely to be more complex than the yeasts as they contain homologues of the mammalian PCD machinery not apparently present in the yeast genome. The Aspergilli may therefore prove valuable as alternative model eukaryotes in which to unravel the components of PCD pathway(s). Such studies will not only throw light on the evolution of PCD in eukaryotes, but may also reveal the role of PCD in response to environmental stresses, mycelial development, and may highlight novel targets for antifungal drug development. To date, evidence for PCD in filamentous fungi has been largely investigated in laboratory culture. Future studies should also focus on whether PCD occurs naturally in the environment, for example during the oxidative burst of engulfed spores/hyphae as part of the host defence mechanism, or in the environment in older regions of the mycelium where nutrients have been exhausted.

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