Viruses activate a genetically conserved cell death pathway in a unicellular organism

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Given the importance of apoptosis in the pathogenesis of virus infections in mammals, we investigated the possibility that unicellular organisms also respond to viral pathogens by activating programmed cell death. The M1 and M2 killer viruses of *Saccharomyces cerevisiae* encode pore-forming toxins that were assumed to kill uninfected yeast cells by a nonprogrammed assault. However, we found that yeast persistently infected with these killer viruses induce a programmed suicide pathway in uninfected (nonself) yeast. The M1 virus–encoded K1 toxin is primarily but not solely responsible for triggering the death pathway. Cell death is mediated by the mitochondrial fission factor Dnm1/Drp1, the K^+ channel Tok1, and the yeast metacaspase Yca1/Mca1 encoded by the target cell and conserved in mammals. In contrast, cell death is inhibited by yeast Fis1, a pore-forming outer mitochondrial membrane protein. This virus–host relationship in yeast resembles that of pathogenic human viruses that persist in their infected host cells but trigger programmed death of uninfected cells.

Introduction

The evolution of a genetically programmed cell death pathway in animals is strongly supported by the analysis of mice with gene deletions that lead to abnormal retention of specific cell populations. However, the evolutionary selection of a cell death pathway that benefits species of unicellular organisms is less obvious. If programmed death arose before multicellular organisms, it must serve an essential purpose other than sculpting tissues and preventing cancer, such as the ability to respond to environmental stresses. Thus, it is possible that yeast respond to virus infections by undergoing programmed cell death analogous to the model in mammals where virus-induced apoptosis is a host defense response to block virus transmission (for review see Everett and McFadden, 1999). However, there are no viruses known that cause acute de novo infections of *Saccharomyces cerevisiae* to directly test this hypothesis. In an alternative model in mammals, virus-induced apoptosis is responsible for the pathogenesis caused by viruses such as human immunodeficiency virus, mosquito-borne encephalitis viruses, and others (Levine et al., 1993; Hardwick, 1997; Colón-Ramos et al., 2003). These deleterious host cell responses may be the unintended consequences of a program that evolved for another purpose. Alternatively, this process may have been selected through evolution because it benefited the species rather than the cell. Most yeast strains are persistently infected with viruses, such as the double-stranded RNA (dsRNA) L-A virus and associated satellite dsRNA M viruses (Wickner, 1996; see Fig. 1 A). Because these viruses are "cell associated," they are transmitted to a new host by cell–cell fusion during mating rather than by infection with a cell-free virus, consistent with their lack of an envelope gene. Thus, yeast viruses share analogies with some prevalent human viruses (e.g., herpesviruses and retroviruses) that reside in their human hosts throughout life. The satellite M killer viruses of yeast rely on the L-A virus *gag* and *pol* genes to provide virion structural proteins and replication enzymes (Wickner, 2001), analogous in part to the dependence of hepatitis delta virus of man on hepatitis B virus for structural proteins (Taylor, 2003).

The existence of evolutionarily conserved programmed cell death pathways in yeast that resemble mammalian cell death pathways is still regarded with some skepticism, though new evidence continues to support this hypothesis. Three general approaches have been taken to address this question. Survival analyses comparing short- versus long-term yeast cultures supports the idea that yeast require programmed cell death for perpetuation of their species through evolution (Fabrizio et al., 2004). Others have applied traditional mammalian cell death assays for DNA breaks (TUNEL staining) and membrane alterations (Annexin V staining) to define "apoptosis" in yeast (Madeo et al., 1997). Although these DNA and lipid changes

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Abbreviations used in this paper: cfu, colony forming units; dsRNA, double-stranded RNA; YPD, yeast peptone dextrose.

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strongly suggest that yeast undergo programmed cell death, the underlying molecular mechanisms and their causal role in facilitating programmed versus accidental death or survival attempts are not yet delineated. More recently, genetic approaches have mapped death pathways conserved in yeast and mammals (Madeo et al., 2002; Fannjiang et al., 2004; Ahn et al., 2005).

Mitochondria appear to have a central role in programmed cell death. The mammalian mitochondrial fission factor Drp1 is required for normal mitochondrial fission in healthy cells, but also promotes excessive organelle fission and release of cytochrome *c* into the cytosol where it can act as a cofactor for caspase activation (Frank et al., 2001; Breckenridge et al., 2003). The mitochondrial fission and pro-death function of Drp1 is conserved in worms (Jagasia et al., 2005). Furthermore, we showed that the yeast homologue of Drp1, Dnm1, promotes yeast cell death after exposure to conditions that mimic a harsh environment such as heat and acidity (Fannjiang et al., 2004). After treatment, Dnm1 promotes excessive mitochondrial fission and subsequent degradation of these essential organelles (Fannjiang et al., 2004). The possibility that yeast also possess a caspase-like death pathway characteristic of mammalian apoptosis is supported by the observation that the metacaspase Yca1 promotes yeast cell death, though the targets of this presumed protease and the mechanisms by which the resulting cleavage fragments facilitate cell death have not yet been identified in yeast (Madeo et al., 2002). Other molecular corollaries were identified that link superoxide dismutase (Longo et al., 1997), mitochondrial NADH oxidase (Wissing et al., 2004), phosphorylation of histone H2B/chromatin remodeling (Ahn et al., 2005), and hormone osmotin/adiponectin receptor signaling (Narasimhan et al., 2005) to the control of programmed cell death pathways conserved between mammals and yeast.

M viruses (M1, M2, and M28) of *S. cerevisiae* encode a preprotoxin protein that is cleaved by Kex1 and Kex2 proteases to produce the active secreted toxin (K1, K2, and K28, respectively) composed of a disulfide-linked α and β chain (Wickner, 1996). The organization of these viral preprotoxin domains, their activating proteases, and cleavage site specificities are analogous to mammalian preproinsulin. Each preprotoxin also provides immunity to its own toxin, but not to the other (nonself) toxins. However, the molecular mechanisms of toxin immunity are not understood, and the mechanistic details by which these toxins kill target cells are not fully delineated. K28 toxin is thought to induce nonprogrammed death by inhibiting DNA synthesis and causing cell cycle arrest (Schmitt et al., 1996; Eisfeld et al., 2000). In contrast to K28, K1 toxin forms pores in the target cell plasmalemma that can be measured by patch-clamp analysis (Martinac et al., 1990). This pore-forming function of K1 was presumed to kill cells in a nonprogrammed manner by causing ATP, K^+, and other essential components to leak out of the target cell (Martinac et al., 1990). However, recent evidence suggests that low concentrations of purified K28 and K1 toxins can induce TUNEL staining in yeast spheroplasts that is reduced in the absence of Yca1 (Reiter et al., 2005). Furthermore, pore formation is also a feature of the mammalian Bcl-2 family of apoptosis regulators. Human Bcl-x_L and Bax induce channel openings in membranes measured directly by patch clamping both isolated and intracellular mitochondria (Pavlov et al., 2001; Basañez et al., 2002; Jonas et al., 2004). Bcl-2 proteins also have three-dimensional structures similar to the pore-forming *Diphtheria* toxin (Muchmore et al., 1996). Given the functional and structural commonalities between pore-forming toxins and mammalian Bcl-2 family members, we entertained the idea that yeast harboring killer viruses could trigger programmed cell death in susceptible yeast cells.

We found that killer virus–infected yeast cells induce programmed cell death that is mediated by yeast homologues of mammalian pro-death factors. However, purified viral K1 toxin alone was not sufficient to promote death via the metacaspase Yca1 without the addition of whole cells, implying the requirement for an additional pro-death component to activate Yca1. These findings support the model that mitochondrial factors regulate an evolutionarily conserved cell death pathway that provides single cell organisms with a type of host defense or innate immunity. This model is further supported by our observation that killer viruses confer a survival advantage to their host cells in chronologically aged cultures.

Results

Yeast Fis1 inhibits killer virus–induced death in liquid co-cultures

The M virus genome is preferentially lost from laboratory strains of yeast, rendering these strains susceptible to killing by other M-containing strains of yeast, called killer strains. To determine if the *S. cerevisiae* knockout collection can be used to analyze the mechanisms of yeast virus-induced death, the BY4743 (background strain) was tested in a halo assay and found to be susceptible to the M1 3165 (see Fig. 3 C) and M2 1506 killer strains (not depicted).

It was also necessary to develop an assay capable of distinguishing between programmed cell death and death-by-assault given that high doses of many proapoptotic agents are capable of triggering nonprogrammed death. Fis1 is a small α-helical TPR protein that interacts with Dnm1, a dynamin-like GTPase, and is also required for normal mitochondrial fission in healthy cells (Shaw and Nunnari, 2002; Dohm et al., 2004). Like several mammalian Bcl-2 family proteins, Fis1 is anchored to the cytosolic side of the outer mitochondrial membrane by a COOH-terminal hydrophobic-basic region and causes pH-dependent flux of small molecules from lipid vesicles (Mozdy et al., 2000; Fannjiang et al., 2004). Although Fis1 shares no amino acid sequence similarity with human Bcl-2, strains lacking Fis1 are more susceptible to cell death after exposure to low pH or heat shock (Fannjiang et al., 2004). Therefore, to determine if yeast lacking Fis1 are more susceptible to cell death induced by yeast killer viruses, a co-culture assay was developed in which M1 (MATα) killer cells are co-cultured at a 1:2 ratio with diploid knockout cells lacking *FIS1* (*fis1*Δ) or the wild-type (BY4743) control (diagrammed in Fig. 1 B). Vital dye (FUN-1) staining after 14 h revealed significantly more death in the *fis1*Δ-containing co-cultures, suggesting that Fis1

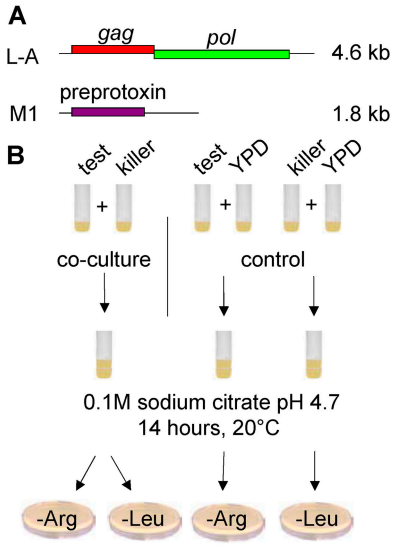

Figure 1. **Co-culture assay for yeast killer virus–induced death.** (A) Map of the L-A and M1 dsRNA genomes. (B) Schematic of the co-culture cell death assay (see Materials and methods).

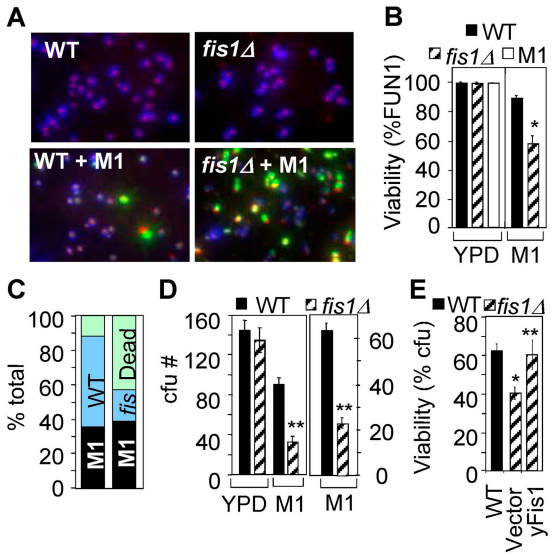

Figure 2. **Fis1 inhibits killer virus–induced yeast cell death.** (A) FUN-1 staining of 14-h co-cultures of M1 killer plus wild-type (WT) or *fis1Δ* yeast. Blue and red, viable; green, dead/dying cells. (B) FUN-1 viability (percentage of total cells in each sample) for three independent experiments (mean ± SEM; *, $P < 0.002$ for wild type vs. *fis1Δ*) as described for A. (C) Stack graph showing the relative proportions of cells in co-cultures (see text) calculated separately for viable test and killer cells as: [cfu selection plates/(cfu test + cfu killer selection plates)] × %FUN1 viable cells for the same three experiments shown in B. (D) Numbers of cfu for test strains in co-culture with YPD only or M1 killer cells (left) were used to calculate the percentage of viability (right) for the same three experiments shown in B and C. **, $P < 4 \times 10^{-5}$ for wild type vs. *fis1Δ*. (E) Viability of wild type and *fis1Δ* transformed with yeast Fis1 or empty vector in five independent M1 co-culture assays (killer/susceptible = 2:1). *, $P < 0.0001$ for wild type vs. *fis1Δ*; **, $P < 0.008$ for vector vs. yFis1 in *fis1Δ*.

inhibits cell death induced by the M1 killer strain (Fig. 2, A and B). To distinguish the diploid test strains from the M1 killer strain in the same co-cultures, cells were simultaneously plated on selective medium for enumeration. By integrating the results from both assays, we determined that M1 killer cells comprised approximately one-third of the total cells (consistent with the initial 1:2 ratio) in both the wild type and *fis1*Δ co-cultures (Fig. 2 C, black bars). However, there were significantly fewer viable *fis1*Δ cells compared with wild type (Fig. 2 C, blue bars). The increased death of *fis1*Δ cells required the M1 killer strain because the viabilities of wild type and *fis1*Δ were indistinguishable in companion (yeast peptone dextrose [YPD] only) co-culture controls lacking M1 killer cells as determined by vital dye (Fig. 2 B, left) and by colony formation (Fig. 2 D, left). The vital dye analysis also confirms that differences in colony formation are due to cell death rather than differences in cell growth rates. When viability of test strains was recalculated for the same dataset as the percentage of colony forming units (cfu) of the test strain in co-culture with M1 compared with the same test strain co-cultured with YPD only, the same results were obtained (Fig. 2 D, right). Therefore, this strategy was used for further analyses. To confirm that *fis1* deficiency was responsible for the enhanced death, FIS1 was restored in the *fis1*Δ strain using a plasmid. Expression of exogenous Fis1, but not the empty vector, fully restored survival of *fis1*Δ to wild-type levels (Fig. 2 E). Fis1 overexpression was confirmed by immunoblot analysis (unpublished data). Together, these results suggest that the yeast M1 killer strain triggers a genetically programmed death pathway in susceptible yeast.

M virus is required for killer strain–induced programmed cell death

To pursue the importance of M viruses for induction of cell death by killer strains, a second killer strain, M2 (1506), was tested and also found to induce more cell death in *fis1*Δ (Fig. 3 A). Cell death triggered by both M1 and M2 killer strains was dose dependent and further enhanced by deletion of *FIS1* (Fig. 3 B). Sodium citrate, which reduces the pH of the medium to 4.7 for optimal K1 toxin activation (Woods and Bevan, 1968; Wickner, 1996), is required for the observed death (Fig. 3 A). Furthermore, co-culturing the test strains with a strain lacking M1 (wild-type MATα BY4742) failed to induce death even in the presence of sodium citrate (Fig. 3 A).

To confirm that the M1 virus is required for induction of cell death, the M1 killer strain was cured of its M1 dsRNA. The replication of M1 dsRNA is known to be more sensitive than the L-A virus to inhibitors of translation and to host cell mutations affecting the 60S ribosomal subunit (Wickner, 1996; Peltz et al., 1999). Consistent with the loss of M1, two cycloheximide-treated strains, CUR1 and CUR2, were defective for killing/halo formation when grown on methylene blue plates embedded with the wild-type susceptible strain (Fig. 3 C). Because M1 also encodes immunity to K1, these strains should gain sensitivity to purified K1 toxin. As expected, spotted K1 toxin killed the CUR1 and CUR2 strains that were embedded in agar (Fig. 3 D). RT-PCR analyses of the CUR1 and CUR2 strains confirmed the absence of M1 dsRNA, but not the L-A dsRNA (Fig. 3 E). This analysis also established that the wild-type (BY4743) strain contains an L-A virus, as do many yeast strains. Importantly, the cured strains were completely defective for inducing cell death when co-cultured

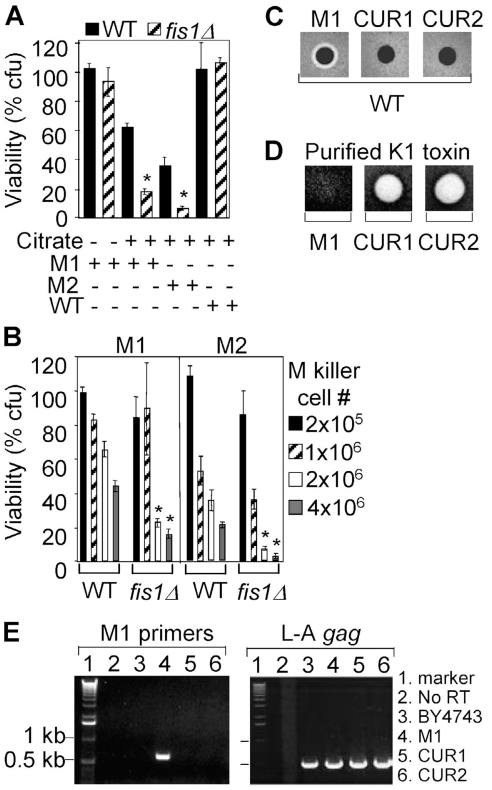

Figure 3. **M1 killer virus is required to induce programmed cell death.** (A) Viability of indicated test strains for the indicated co-culture conditions was determined as described for the right panel in Fig. 2 D. Data are the mean ± SEM for three or more independent experiments; *, $P < 0.0005$ for wild type versus *fis1Δ*. (B) Viability of test strains in two or more independent co-culture assays done in triplicate (Fig. 2 D, right) with the indicated number of M1 or M2 killer cells. Data are the mean ± SEM; *, $P < 0.0005$ for wild type versus *fis1Δ*. (C) Halo assay showing that the M1 strain, but not M1-derived strains CUR1 and CUR2 (cured of M1 virus), forms a halo (clear dead cell zone confirmed by microscopy) when grown on methylene blue plates embedded with the wild-type (BY4743) test strain. (D) Toxin-induced dead cell zone (clear area) in methylene blue plates embedded with the indicated test strains indicate sensitivity to purified K1 toxin (10 μl undiluted). (E) Ethidium bromide–stained agarose gel of RT-PCR products (using RNA isolated from the indicated strains as starting template) shows the expected 600-bp M1 and 500-bp products of L-A *gag*.

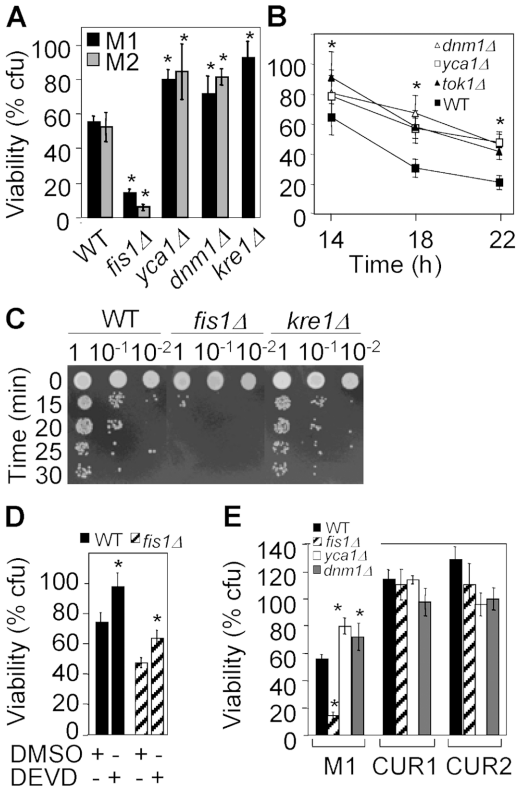

Figure 4. **Mapping the genetic pathway of killer strain–induced death.** (A and B) Viability of the indicated test strains in M1 or M2 co-culture assays for 14 h or in a separate set of experiments at the indicated times with M1 (Fig. 2 D, right). Data presented are the mean ± SEM for three or more independent experiments; *, $P < 0.039$ for wild type vs. knockouts. (C) Growth of the indicated strains (and serial 10× dilutions) after 55°C heat shock treatment for the indicated times. One of two similar experiments is shown. (D) Viability of test strains in co-culture assays with M1 for 10 h showing partial rescue of wild type and *fis1Δ* by treatment with DEVD-CHO (see Materials and methods). Data are the mean ± SEM for four or more independent experiments; *, $P < 0.01$ comparing ± DEVD for both wild type and *fis1Δ*. (E) Viability of the indicated test strains in co-culture with M1, CUR1, or CUR2 (Fig. 2 D, right). Data presented are the mean ± SEM for four or more independent experiments; *, $P < 0.04$ for wild type versus mutants.

with wild type or *fis1Δ* (see Fig. 4 E). Thus, the M virus is essential for the programmed cell death phenotype of the killer yeast strain.

Killer virus–infected cells induce Tok1-, Dnm1-, and metacaspase-dependent death

Yeast Fis1 was previously shown to inhibit a cell death pathway that is mediated by the mitochondrial fission factor Dnm1 and by Yca1, which shares sequence similarity to the cysteine proteases that mediate mammalian apoptosis (Shaw and Nunnari, 2002; Madeo et al., 2002; Cerveny and Jensen, 2003; Fannjiang et al., 2004). To pursue the potential involvement of these pro-death factors in killer virus–induced death, these knockout strains were tested for susceptibility to M1 and M2 killer strains in co-culture assays. Survival of both *yca1Δ* and *dnm1Δ* was significantly improved compared with wild-type cells in a dose-dependent manner (Fig. 4, A and B). To provide further evidence for the involvement of cysteine proteases, yeast were treated with DEVD-CHO, a cell-permeable peptide substrate/inhibitor of mammalian caspases-3 and -7. Treatment with this inhibitor provided a significant but partial (~20%) protection against M1 strain–induced death of both wild type and *fis1Δ* compared with DMSO carrier control (Fig. 4 D). Because the peptide substrate specificity of Yca1 does not include DEVD (Madeo et al., 2002), it is likely that the action of yet unidentified proteases explains the modest protective effect of DEVD-CHO. These findings indicate the existence of core cell death machinery in yeast composed of mitochondrial fission factors and proteases that regulate survival/death decisions induced by environmental stresses and viruses. However, the existence of alternative death pathways in yeast is also suggested by our observation that deletion of Dnm1 or Yca1 did not completely block cell death (Fig. 4 E, compare co-cultures with M1 vs. CUR1/2 cells).

To further delineate the killer virus–induced death pathway, additional knockout strains were tested for sensitivity to

killer yeast strains. Like many animal viruses, K1 toxin is thought to have a dual receptor mechanism, first binding to the cell wall component β-1,6-glucan and subsequently to its high affinity receptor Kre1 (Breinig et al., 2002). Yeast lacking Kre1 are potently resistant to the toxicity of relatively high concentrations of purified K1 toxin. As expected, *kre1Δ* was also resistant to the M1 killer strain in our co-culture assays (Fig. 4 A). To verify that Kre1 is specific for M1 virus–induced death and is not required for other nonviral death stimuli, *kre1Δ* was subjected to heat shock at 55°C for increasing times. Cells lacking Kre1 were indistinguishable from wild type, whereas cells lacking Fis1 retained sensitivity as previously reported (Fig. 4 C; Fannjiang et al., 2004). Hog1 MAP kinase phosphorylates nuclear transcription factors as well as the plasma membrane (outwardly rectifying) potassium channel Tok1 in response to hyperosmotic stress (Proft and Struhl, 2004). Tok1 was previously identified as a target of K1 toxin, and addition of K1 toxin to susceptible cells activates potassium flux (Ahmed et al., 1999). Conversely, inhibition of Tok1 channels in killer strains was suggested to mediate toxin immunity (Sesti et al., 2001). However, Tok1 is apparently not the binding receptor for K1 as deletion of Tok1, in contrast to Kre1, does not protect from higher concentrations of toxin (Breinig et al., 2002). Consistent with earlier work with purified toxin, deletion of Tok1 rendered yeast resistant to the killer strain in co-culture assays, suggesting that Tok1 facilitates killer virus–induced programmed cell death (Fig. 4 B).

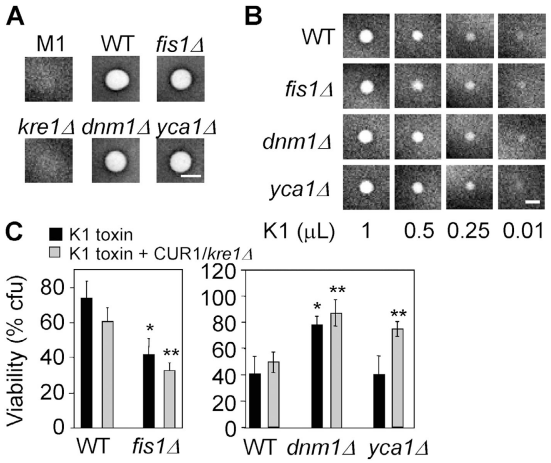

Figure 5. **Distinguishing Yca1-dependent and -independent death pathways.** (A) Toxin-sensitivity assay. The indicated agar-embedded strains were spotted with purified K1 toxin. One of three similar independent experiments is shown. Bar, 10 mm. (B) Dose–response of indicated amounts of K1 (spotted in 1-μl volumes) as described for A. Bar, 4 mm. (C) Viability of the indicated test strains in reconstituted co-culture assays (see Materials and methods) containing purified K1 toxin or K1 toxin + CUR1/*kre1Δ* cells (see Materials and methods). Data represented are the mean ± SEM of four or more independent experiments. *, $P < 0.02$ for wild type and mutants treated with toxin; **, $P < 0.003$ for wild type and mutants treated with toxin + cells.

High K1 toxin concentrations induce nonprogrammed death

To pursue the possibility that K1 toxin is solely responsible for the killing effects of killer virus–infected strains, K1 toxin was partially purified from the supernatants of M1 (3165) cultures. In contrast to the co-culture assay, only the parental M1 killer strain and the Kre1 receptor knockout strain were resistant to killing by purified K1 toxin, as deletion of either the anti- or pro-death genes had no effect on the size of the dead cell zone after spotting purified K1 toxin on plates embedded with these knockout strains (Fig. 5 A). Further dilutions of purified K1 toxin still failed to reveal programmed cell death defined by these genetic markers (Fig. 5 B). By calculating toxin concentrations in this plate assay compared with the co-culture assays (Fig. 1 B), we concluded that the amount of toxin required to induce programmed death in co-cultures is not sufficient to produce a detectable response in the plate assay. Therefore, the plate assay appears to measure only nonprogrammed death, and we could not determine from this approach if K1 toxin alone triggers programmed death.

Purified K1 toxin induces metacaspase-independent programmed death

To circumvent the sensitivity problems of the plate assays, purified K1 toxin was tested for killing activity in a liquid assay mimicking the co-culture conditions outlined in Fig. 1 B. Consistent with the co-culture assays, results with *fis1Δ* and *dnm1Δ* deletion strains indicate that purified K1 toxin, in the absence of killer cells, is capable of inducing a programmed death (Fig. 5 C, black bars). In contrast, deletion of the metacaspase (*yca1Δ*) failed to enhance survival in the same assays. This finding suggests that purified K1 toxin fails to activate a Yca1-mediated death pathway, and that killer yeast cells contribute an additional unidentified component to the death pathway. To confirm this possibility, the M1 killer strain was replaced with a combination of purified K1 toxin plus CUR1 cells (M1 strain cured of M1 dsRNA) in the co-culture assay. However, to prevent CUR1 cells from being killed by the purified K1 toxin in these reconstituted co-cultures, *KRE1* was deleted in the CUR1 strain to generate CUR1/*kreΔ*. Under these conditions, the protective effects of Yca1 deletion were restored (Fig. 5 C, gray bars). However, the extra contribution to death from the CUR1/*kreΔ* cells was without consequence to wild-type, *fis1Δ*, and *dnm1Δ* strains. Thus, K1 toxin alone induces a death pathway that depends on Dnm1 but not on Yca1, and in this manner differs from the death pathway induced by killer virus–infected cells.

Killer yeast have a survival advantage in aged cultures

To further address the physiological significance of killer virus–induced programmed cell death, competition experiments were performed in aged cultures. M1 killer cells and wild-type susceptible cells (BY4743) were co-cultured for over 20 d without addition of fresh medium. Aliquots were removed periodically for enumeration of the two cell types on selection plates. Consistent with the importance of active K1 toxin for cell death, the killer strain rapidly dominated the co-cultures if sodium citrate was present (Fig. 6 A). Even in the absence of sodium citrate, the pH of the co-culture medium decreased to ~4–5 by day 17, coincident with the more rapid gain of killer over wild-type cells at later times in co-cultures lacking sodium

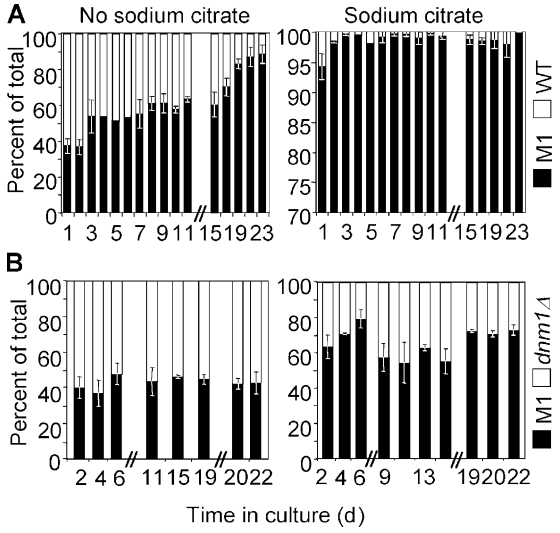

Figure 6. **Survival advantage with M1 in aged cultures.** (A and B) Ratio of M1 to wild-type or dnm*1Δ* strains at the indicated times in co-cultures ± sodium citrate. Data presented are the mean ± SEM for three independent experiments.

citrate. However, the *dnm1Δ* strain that is defective for programmed cell death was resistant to takeover by M1 killer cells in this time frame, even in the presence of sodium citrate (Fig. 6 B). This simulation of environmental competition between yeast strains suggests that harboring M1 or other toxin-producing viruses is advantageous in nature, consistent with the relative prevalence of these viruses in wild strains compared with laboratory strains that do not need to fend off invaders.

Discussion

Using a co-culture assay to mimic the competition of yeast strains in the environment, we found that yeast harboring a killer virus are capable of inducing genetically programmed cell death in yeast cells lacking the same killer virus. These cell-associated killer viruses provide their hosts with a survival advantage that is based on the ability to distinguish "self" from "non-self." That is, highly related species of yeast that carry the same virus passed on to them by their parents are immune to their (and their family member's) killer virus, but are capable of triggering nonimmune (i.e., nonself) yeast to orchestrate suicidal death. By analyzing yeast knockouts, it is clear that the pro-death genes encoded by the cell destined to die are responsible for the observed death. These pro-death genes include the mitochondrial fission factor Dnm1, the plasma membrane K^+ channel Tok1, and the protease Yca1. This same death machinery can be activated in the immune killer strains, but only after they are cured of their killer virus, which also encoded the immunity. The K1 toxin encoded by the M1 virus constitutes most but not all of the pro-death signal from killer strains of yeast. That is, partially purified K1 toxin is sufficient to induce death that is mediated by Dnm1 but not Yca1, suggesting that yeast have more than one death pathway. This finding with Yca1 seems contrary to a recent report that Yca1 does promote purified K1 toxin–induced DNA degradation (TUNEL staining) and reactive oxygen species production (Reiter et al., 2005). However, the more dramatic effects observed in their "apoptosis" assays contrasted with the lesser effects of Yca1 on cell viability (Reiter et al., 2005). This raises the possibility that detectable levels of DNA damage and reactive oxygen species production do not mark the commitment point in the yeast cell death pathway, beyond which the cell cannot recover. This possibility is consistent with our earlier finding that yeast can recover from the excessive mitochondrial fission induced by Dnm1, but not the subsequent autophagic destruction of mitochondria (Fannjiang et al., 2004).

The biochemical function of K1 that triggers programmed cell death may be the same or distinct from its ability to induce nonprogrammed death at higher concentrations. The same pore-forming function that results in a sudden dramatic increase in plasma membrane permeability at high K1 levels could formulate an intracellular signal to the death pathway at low K1 levels (Bortner and Cidlowski, 2004). However, extensive analyses of the pore-forming domain of *Diphtheria* toxin, which shares structural similarity to mammalian Bcl-2 family proteins, revealed mutants that retain function but have lost detectable pore-forming activity (Muchmore et al., 1996; Lanzrein et al., 1996; Falnes and Sandvig, 2000; for review see Lazebnik, 2001). Thus, an alternative possibility is that K1 triggers programmed death by a second uncharacterized activity. In contrast to K28, K1 is not thought to enter target cells by endocytosis after receptor binding (Eisfeld et al., 2000). Therefore, K1 could potentially initiate a signaling pathway or other event by activating the Tok1 K^+ channel or by binding to its GPI-anchored Kre1 receptor on yeast cell surfaces, perhaps analogous to the binding of *Diphtheria* toxin to its GPI-anchored receptor (Lanzrein et al., 1996). Although Kre1 functions in cell wall biogenesis (Breinig et al., 2004), no role for Kre1 in signaling has been described.

Irrespective of its biochemical function, the consequence of K1 activity feeds into a common downstream death pathway involving the mitochondrial fission factor Dnm1 that is conserved in yeast, worms, and mammals. This dynamin-like GTPase is required for yeast cell death induced by a variety of stimuli including killer viruses. A similar pathway was identified in mammals and more recently in *Caenorhabditis elegans*, where *C. elegans* Drp1 (homologue of yeast Dnm1) is required for mitochondrial fragmentation and caspase (CED3)-mediated death (Jagasia et al., 2005). Interestingly, genetic evidence indicates that the Bcl-2 homologue of *C. elegans*, CED-9, activates *C. elegans* Drp1 to kill cells. Thus, even though CED-9 is widely known as an inhibitor of cell death, it apparently undergoes a conversion process leading to activation of Drp1 and cell death. This idea is consistent with our observation that antiapoptotic Bcl-2 and Bcl-xL can be converted into pro-death factors (Cheng et al., 1997; Clem et al., 1998). We suggested a related model to explain the surprising anti-death activity of yeast Fis1, a Dnm1-interacting factor that is required for normal Dnm1-mediated mitochondrial fission in healthy cells. Because of its role in promoting mitochondrial fission in healthy cells, Fis1 was initially expected to promote death, as was reported for its mammalian homologue (James et al., 2003; Lee et al., 2004). In contrast, yeast Fis1 appears to limit the action of Dnm1 and to promote

cell survival (Fannjiang et al., 2004). In a similar manner, Bcl-2 proteins and yeast K1 toxin may have modulatory functions.

A genetic screen for yeast mutants resistant to K1 toxin led to the identification of genes involved in expression and synthesis of glucan (especially β-1,6-glucan), N-glycosylation, cell wall biogenesis, maintenance of cell integrity, and signaling pathways suggested to be involved in intracellular trafficking (Page et al., 2003). This screen was based on cell killing by purified K1 toxin in a plate assay. Consistent with our observation in plate assays, their screen did not identify Dnm1, Fis1, or Yca1. However, Yca1 may not be detected in this assay without the required pro-death component contributed by whole cells. This component may be a cell-associated factor based on our observation that addition of cells, but not crude supernatants or flow-through fractions from the toxin preparation, reconstitutes the cell death phenotype (unpublished data).

This study provides a different perspective on the role of programmed cell death in yeast. After a virus enters a new host organism, the process of adaptation begins. Many if not all viruses develop mechanisms to regulate, or at least adapt to, the host cell programmed death pathway. Herpesviruses encode several mechanisms to inhibit cell death, including Bcl-2 homologues (Hardwick and Bellows, 2003; Hammerschmidt and Sugden, 2004). Similarly, yeast killer viruses encode immunity to their own death stimulus. Yeast killer viruses can also be compared with infections with human immunodeficiency virus and Sindbis virus that result in the death of large numbers of uninfected lymphocytes and neurons, respectively, altering disease pathogenesis (Darman et al., 2004; Holm et al., 2004). Any benefit provided by programmed cell death to the individual dying cell is arguable, for both yeast and man. However, it is possible that yeast killer strains benefit from the nutrients liberated by cells that are willing to die, as suggested for nutrient-deprived yeast (Fabrizio et al., 2004) and toxin-producing bacteria (Gonzalez-Pastor et al., 2003), thereby contributing to the selection of such a process during evolution.

Materials and methods

Strains, plasmids, and media

Genotypes are listed in Table S1 and PCR primer sequences are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200503069/DC1). The killer strains of *S. cerevisiae*, 3165 (M1) and 1506 (M2), were obtained from R.B. Wickner (National Institutes of Health, Bethesda, MD). Test strains are from the diploid knockout consortium (Giaever et al., 2002). For overexpression of yeast *FIS1* under the constitutive ADH promoter, plasmid (pII115) was constructed by inserting PCR-amplified (see Table S2 for primer sequences) YIL065C into the XbaI and SalI sites of the vector BB1641 (J. Boeke, Johns Hopkins University, Baltimore, MD), transformed into the wild type (BY4743) and *fis1Δ* diploids, and selected on SC-Leu. To generate CUR1/*kre1Δ* in the M1-cured CUR1 strain, *kre1Δ* (MATa) was mated with CUR1 (MATα). Diploid strains (provided by V. Culotta, Johns Hopkins University, Baltimore, MD) were selected on SC-Arg-Met and spores from dissected tetrads were selected for MATα haploids that lacked *KRE1* by replica plating on G418 and by PCR. All yeast strains were grown in standard YPD medium (20% peptone, 20% dextrose, and 10% yeast extract) unless otherwise stated.

Co-culture cell death assay

Overnight cultures (rotated at 30°C) were diluted into fresh YPD and grown for approximately two doubling times, and co-cultures were prepared from 1 ml of the test strains (OD_{600} = 0.2, 4×10^6 cells/ml) plus 1 ml of the killer strains (OD_{600} = 0.1, 2×10^6 cells/ml) unless otherwise indicated. Sodium citrate (0.1 ml of 2 M, pH 3.9, for final 0.1 M, pH 4.7) was added and co-cultures were rotated for 14 h at 20°C. Control co-cultures were prepared in parallel by replacing test or killer strain with 1 ml YPD (Figs. 1 B and 2 D). After incubation, control co-cultures (e.g., test strain without killer cells) were counted in a hemocytometer and the calculated volume corresponding to 200 cells in the control was plated for both the control and corresponding co-culture on SC-Arg (to detect test strains in M1 co-cultures), SC-Lys (to detect test strains in M2 co-cultures), or SC-Leu (to detect killer strains), and colonies were counted after 2 d at 30°C. The percentage of survival was calculated as cfu/cfu (of test strain in YPD control co-culture) × 100 except where indicated. For yeast strains overexpressing Fis1, YPD was substituted with SC-Leu and cells were plated on SC-Leu-Arg at 300/plate.

FUN-1 staining, heat shock, and peptide inhibitors

FUN-1 viability staining with FUN 1 and Calcofluor White (F-7030; Molecular Probes) and heat shock assays were performed on diploid strains as previously described (Fannjiang et al., 2004). Yeast cell images were captured on a fluorescence microscope (Eclipse E800; Nikon; oil immersion Nikon 100× Plan Fluor DLL, NA = 1.30) using a Spot RT camera (Diagnostic Instruments) with Spot Advanced v4.0.9 software at ambient temperature. The figure was assembled in Adobe Photoshop and converted to a tiff file. For treatment with the peptide inhibitor, M1 co-cultures and parallel YPD controls were prepared as described in the Co-culture cell death assay section, except that test strains were preincubated with either 20 μM DEVD-CHO (Sigma-Aldrich) or the equivalent volume of DMSO carrier control (1% final) for 1 h at 20°C before addition of killer culture/YPD. Cultures were incubated for an additional 10 h with readdition of DEVD-CHO or DMSO after 5 h.

Curing yeast of the M1 killer virus

To cure the killer strain 3165 of its M1 dsRNA, a fresh colony was streaked onto YPD + cycloheximide (0.4 μg/ml; Fink and Styles, 1972). After 2 d at 30°C, five colonies were streaked onto fresh YPD plates (2 d at 30°C), and two of the five colonies selected (one from each plate) were negative for M1 toxin (see text) and designated CUR1 and CUR2 (Table S1). The absence of M1 dsRNA was confirmed by RT-PCR (Superscript RT/Platinum Taq; Invitrogen; Table S2).

K1 toxin purification

Mature secreted K1 toxin was purified from the medium of late log phase M1 killer strain 3165 as reported previously (Brown et al., 1994). In brief, yeast were grown in minimal medium plus 350 mg/liter of arginine and threonine, and the supernatant from 1.6 liters (OD_{600} = 0.7) was concentrated to 15 ml using the Centricon plus-80 centrifugal filter devices (molecular weight cut-off 10 kD; Millipore). Toxin was further purified and concentrated to 1.6 ml in an Amicon ultrafiltration unit (molecular weight cut-off 10 kD; Millipore) under 20 psi of N_2 gas. The partially purified toxin (retantate) was stored (in 100 μl aliquots) at −20°C.

Halo and toxin spot assays

The methylene blue plate assay was modified from Brown et al. (1994). The bottom layer (9 ml of 2% agar in 60-mm dishes) was overlaid with 1 ml of 1% agar containing 10^6 cells of the test strain. Plates were spotted with purified toxin or cells (15 μl, OD_{600} = 7).

Reconstituted co-culture assay

The reconstituted assay (replacing killer cells with killer-free cells + purified K1 toxin) was the same as the co-culture assay (Co-culture cell death assay section) except scaled down 10-fold. In brief, K1 toxin and CUR1/*kre1Δ* (10^5 cells) were combined and added to test strains (2×10^5 cells) in 200 μl. Controls included toxin storage buffer (Brown et al., 1994) and YPD to replace K1 toxin and CUR1/*kre1Δ* cells, respectively. Viability was calculated for test strains as cfu (+toxin)/cfu (−toxin) × 100 and cfu (+toxin, +cells)/cfu (+cells) × 100 (because the presence of CUR1/*kre1Δ* without toxin increases survival of the test strain ∼10%; Fig. 4 E).

Competition experiments in aged cultures

Co-culture assays were performed as described in the Co-culture cell death assay section except approximately equal ratios of susceptible and killer cells (1 ml each, OD_{600} = 0.1) were co-cultured without addition of fresh medium and enumerated by plating equal volumes (300/plate) on YPD (to detect killer + susceptible cells) and SC-Arg (susceptible cells only).

Online supplemental material
Yeast strains and complete genotypes are listed in Table S1 and the nucleotide sequence of all PCR primers are listed in Table S2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200503069/DC1.

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