Viral killer toxins induce caspase-mediated apoptosis in yeast

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In yeast, apoptotic cell death can be triggered by various factors such as H_2O_2, cell aging, or acetic acid. Yeast caspase (Yca1p) and cellular reactive oxygen species (ROS) are key regulators of this process. Here, we show that moderate doses of three virally encoded killer toxins (K1, K28, and zygocin) induce an apoptotic yeast cell response, although all three toxins differ significantly in their primary killing mechanisms. In contrast, high toxin concentrations prevent the occurrence of an apoptotic cell response and rather cause necrotic, toxin-specific cell killing. Studies with Δ*yca1* and Δ*gsh1* deletion mutants indicate that ROS accumulation as well as the presence of yeast caspase 1 is needed for apoptosis in toxin-treated yeast cells. We conclude that in the natural environment of toxin-secreting killer yeasts, where toxin concentration is usually low, induction of apoptosis might play an important role in efficient toxin-mediated cell killing.

Introduction

The production of cytotoxic proteins (killer toxins) is a widespread phenomenon among a great variety of yeast genera and is typically associated with the secretion of a protein or glycoprotein toxin that kills susceptible yeast cells in a two-step receptor-mediated manner (Bussey et al., 1990; Magliani et al., 1997; Schmitt and Breinig, 2002). In *Saccharomyces cerevisiae*, three different killer toxins (K1, K2, and K28) have been identified so far, which are all encoded by cytoplasmic persisting double-stranded RNA viruses encoding the unprocessed precursor proteins of the secreted α/β toxins (Tipper and Schmitt, 1991; Wickner, 1996). Although most viral killer toxins, like the *S. cerevisiae* K1 toxin and the *Zygosaccharomyces bailii* toxin zygocin, act as ionophores and disrupt cytoplasmic membrane function by forming cation-specific plasma membrane pores (Martinac et al., 1990; Weiler et al., 2002; Breinig et al., 2002; Weiler and Schmitt, 2003), the *S. cerevisiae* K28 toxin enters susceptible cells by receptor-mediated endocytosis, travels the secretion pathway in reverse, and induces a cell cycle arrest at the G1/S boundary (Schmitt et al., 1996; Eisfeld et al., 2000).

In higher multicellular organisms, it is well known that pore-forming toxins like *Staphylococcus aureus* α toxin and/or inhibitors of protein synthesis like diphtheria toxin produced and secreted by *Corynebacterium diphtheriae* are able to induce apoptosis (Weinrauch and Zychlinsky, 1999).

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Abbreviations used in this paper: DHR, dihydrorhodamine; MBA, methylene blue agar plates; PS, phosphatidylserine; ROS, reactive oxygen species.

The finding of cell death with apoptosis-like features in yeast (Madeo et al., 1997) was unexpected, as a unicellular organism seems to have no advantages in committing suicide. Further research in this field demonstrated that in yeast apoptotic cell death can be induced by different exogenous and intrinsic stresses like H_2O_2, UV irradiation, acetic acid, cell aging, and high pheromone concentration (Madeo et al., 1999; Laun et al., 2001; Ludovico et al., 2001; Severin and Hyman, 2002; Del Carratore et al., 2002; Herker et al., 2004). Similar to mammalian apoptosis, reactive oxygen species (ROS) play a central role in most of these apoptotic scenarios. The similarity between yeast and mammalian apoptosis was further underlined by the finding of yeast orthologues of a caspase, a proapoptotic serine protease, AIF, and the transkingdom Bax-inhibitor BI-1 (Madeo et al., 2002; Chae et al., 2003; Fahrenkrog et al., 2004; Wissing et al., 2004). It was shown that debilitated cells die for the benefit of the whole cell population saving limited nutrients for healthy cells to enable survival of the whole population (Fabrizio et al., 2004; Herker et al., 2004). Another natural cell death situation for yeast is the exposure to killer toxins produced and secreted by concurring killer strains. Therefore, we investigated if killer toxins are able to induce the apoptotic process and if apoptosis is responsible for cell death under natural environmental conditions in the presence of moderate or low toxin concentrations closely reflecting the situation in the natural yeast habitat.

Using three viral killer toxins that either disrupt cytoplasmic membrane function or arrest cells at the G1/S boundary of

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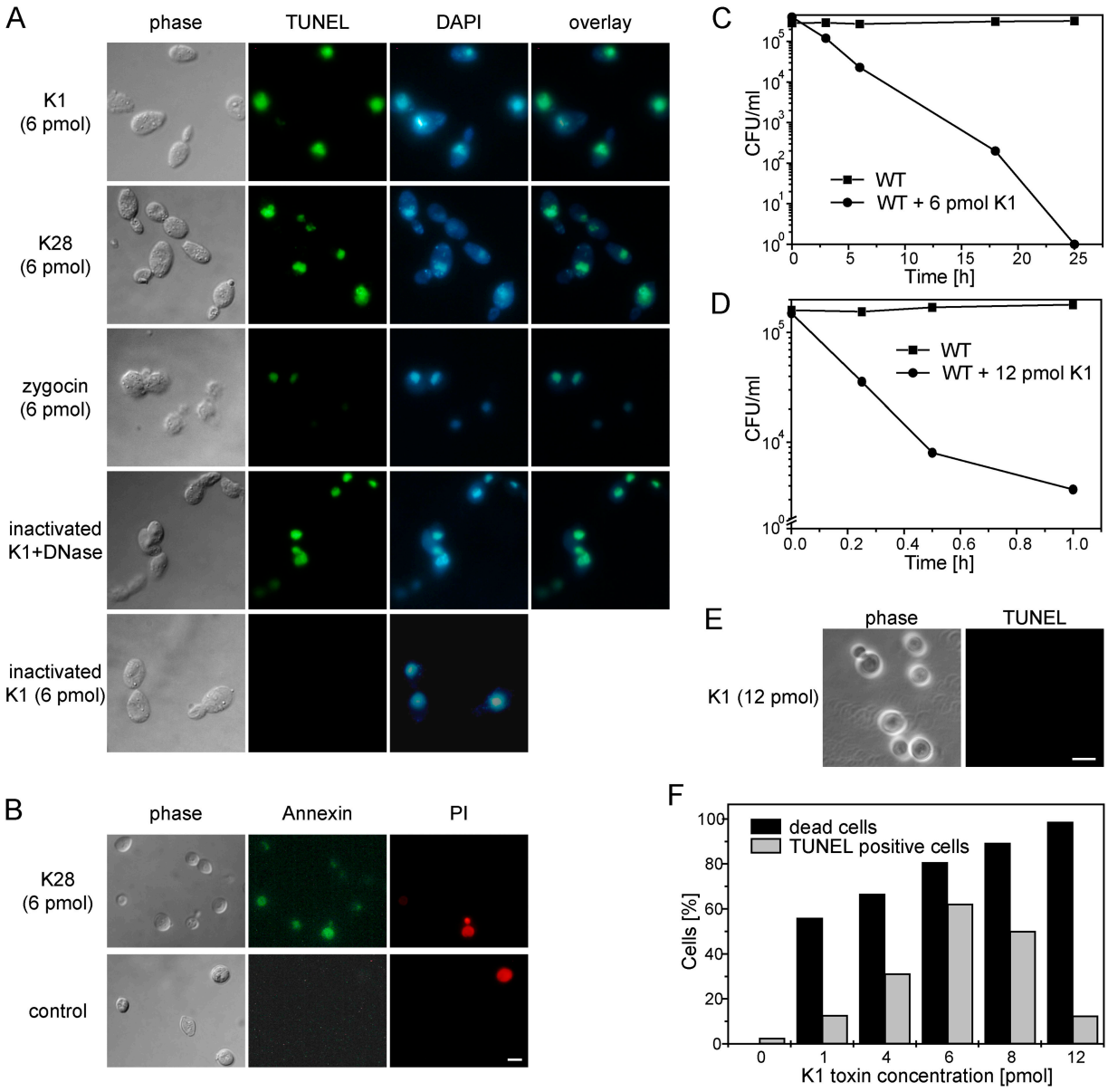

Figure 1. **Yeast virus toxins kill susceptible cells in a dose-dependent manner by inducing either apoptotic or necrotic pathways.** (A) TUNEL reaction, fluorescence and the corresponding phase-contrast display, and DAPI staining after 10-h treatment of *S. cerevisiae* 192.2d at 20°C at moderate concentrations in the range of 6 pmol each of K1, K28, zygocin, heat-inactivated K1 toxin (negative control), and heat-inactivated K1 toxin plus 10-min DNase I treatment (positive control). (B) Detection of PS exposure via Annexin V staining in *S. cerevisiae* wild-type cells (strain 192.2d) treated for 10 h at 20°C with or without killer toxin K28 (6 pmol) and propidium-iodide staining. Bar, 5 μm. (C) Kinetics of cell survival of *S. cerevisiae* 192.2d (wild type) in the presence of moderate K1 toxin concentrations (6 pmol). (D) Kinetics of cell survival of *S. cerevisiae* 192.2d (wild type) in the presence of high K1 toxin concentrations (12 pmol). (E) TUNEL staining of wild-type cells of *S. cerevisiae* 192.2d after treatment with high concentrations of K1 toxin (12 pmol). Bar, 5 μm. (F) Dose–response relationship between yeast cell survival and apoptotic cell response (determined by TUNEL staining) of *S. cerevisiae* 192.2d in dependence of increasing K1 toxin concentrations (each experiment was initiated with 1.1×10^6 colony forming units/ml). At the indicated K1 toxin concentration, the frequency of TUNEL-positive cells was determined for at least 400 cells in three independent experiments.

the eukaryotic cell cycle, we found that all toxins induce cell death in *S. cerevisiae* when added in moderate or low concentrations, always accompanied by the production of ROS, DNA fragmentation, typical phenotypic changes, and translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the cytoplasmic membrane—the defining phenotype of apoptotic cell death. A yeast Δ*yca1* disruptant showed significantly decreased toxin sensitivity, whereas yeast mutants blocked in endogenous glutathione biosynthesis were hypersensitive. In contrast, high concentrations of all three toxins led to nonapoptotic cell death independent of yeast caspase 1 and ROS.

Results and discussion

Killer toxins can induce both apoptotic and necrotic cell death in yeast

Treatment of yeast cells with low concentrations of three different viral killer toxins resulted in a moderate rate of cell death

Table I. **Major properties of the viral killer toxins K1, K28, and zygocin**

Toxin	Size	Structure	Receptor on target cell	Lethal effect
	D			
K1	19,088	α/β heterodimer	β-1,6-D-glucan	ionophore
K28	21,496	α/β heterodimer	α-1,6-mannoprotein	G1/S cell cycle arrest
Zygocin	10,421	monodimer	α-1,6-mannoprotein	ionophore

Data for K1, K28, and zygocin were taken from Bostian et al. (1984), Schmitt and Tipper (1995), and Weiler et al. (2002), respectively (adapted from Breinig et al., 2004).

(Fig. 1 C). We tested wild-type yeast cells (strain 192.2d) treated under these conditions for typical apoptotic markers by TUNEL assays and DAPI staining. After 10 h of treatment with either K1, K28, or zygocin, a strong green fluorescence indicating DNA fragmentation could be detected, whereas negative control cells treated with heat-inactivated killer toxin did not show any DNA fragmentation (Fig. 1 A). The fluorescence was located in the nucleus, as could be proven by DAPI counter staining (Fig. 1 A). Furthermore, DAPI staining revealed an atypical nuclear phenotype with condensed chromatin in the toxin-treated samples (Fig. 1 A). Thus, all three killer toxins tested likewise induce genomic DNA fragmentation and chromatin condensation, although their primary mode of action and mechanism of cell killing differs significantly (Table I).

In contrast, treatment with high concentrations of killer toxins resulted in fast cell killing (Fig. 1 D) resembling necrosis as DNA fragmentation could not be detected (Fig. 1 E). To determine if an in vivo breakpoint exists at which efficient cell killing cuts in while apoptotic cell responses simultaneously disappear, a dose–response curve was created that correlates yeast cell survival with the appearance of TUNEL-positive apoptotic cells in dependence of increasing K1 toxin concentrations. As illustrated in Fig. 1 F, the K1 virus toxin caused a rapid cell killing as well as a TUNEL-positive yeast cell response, both continuously increasing until K1 reached molar concentrations of up to 6 pmol. At higher concentrations (8 and 12 pmol K1), necrotic cell killing dramatically increased, whereas the percentage of TUNEL-positive, apoptotic cells steadily declined down to 12% (Fig. 1 F). Thus, in the case of the K1 virus toxin, a concentration close to 6 pmol resembles the breakpoint at which efficient necrotic cell killing occurs, whereas apoptotic cell responses are of minor importance for in vivo toxicity.

Translocation of PS from the inner leaflet to the extracellular side of the plasma membrane is an early event in apoptosis and can be detected by Annexin V, a protein with strong affinity to PS (Martin et al., 1995). To demonstrate that yeast cell spheroplasts are still intact, a propidium iodide staining was performed. Because both zygocin and K1 kill their corresponding target cell by disruption of plasma membrane integrity, almost all cells were stained with propidium iodide (unpublished data), and, therefore, Annexin V staining was not performed on cells treated with these toxins. In contrast, K28 has no effect on membrane integrity as it irreversibly inhibits nuclear DNA synthesis and arrests cells at the G1/S boundary of the cell cycle (Schmitt et al., 1996). Indeed, only 10% of K28-treated cells had taken up propidium iodide and ~20% of the cells were PS positive, showing PS translocation to the outer leaflet of the plasma membrane (Fig. 1 B).

ROS mediate cell death in killer toxin–treated cells

ROS play a central role in inducing apoptotic markers and mediating cell death in yeast (Madeo et al., 1999; Laun et al., 2001; Ludovico et al., 2001; Mazzoni et al., 2003; Weinberger et al., 2003). We incubated cells with dihydrorhodamine (DHR) 123, which is a cell permeable leukodye that converts to a red light emitting fluorochrome in the presence of ROS. After 10 h of killer toxin treatment, an intensive red fluorescence could be detected for all three killer toxins (Fig. 2 A), whereas no ROS were produced in negative control cells that had been treated with heat-inactivated toxin (Fig. 2 A). To determine the initial time point when ROS first appeared, samples of K1-treated cells were taken at 1-h intervals. A weak signal appeared after 2 h of toxin treatment, which became significantly more intense thereafter. The majority of the cells (99%) were stained within two cell generations (~8 h), whereas in the control (heat-inactivated toxin) only 1% of the cells were fluorescent (unpublished data).

Interestingly, in phase contrast of K28-treated cells we could observe an apoptosis-typical shrinking and condensation of only those cells that showed a positive staining for ROS (Fig. 2 B). After incubation with higher doses of the toxins, which resulted in faster killing kinetics (0.5–1 h), cells died without accumulation of ROS (Fig. 2 C).

To further analyze the role of intracellular ROS in toxin-induced cell death, we tested yeast Δ*gsh1* mutant cells that are genetically blocked in glutathione biosynthesis. Glutathione acts as a redox buffer and protects cells from damage by reducing the amount of ROS (Carmody and Cotter, 2001). Toxin sensitivity assays determined in the well-test on methylene blue agar plates (MBA assay) indicated that Δ*gsh1* mutant cells were significantly more sensitive against killer toxin treatment than the isogenic wild type and displayed a considerably larger zone of growth inhibition (Fig. 2 D). Considering the linear relationship between the diameter of the inhibition zone and the logarithm of the killer toxin concentration, the hypersensitive phenotype of Δ*gsh1* mutant cells portrays the evident involvement of ROS in toxin-induced cell death.

In addition, experiments in liquid medium confirmed the central role of ROS in toxin-mediated cell killing as toxin-treated Δ*gsh1* cells showed a more intense DHR 123 staining (not depicted) and were killed more effectively than the isogenic wild type (Fig. 2 E). The time course of cell survival was reproduced in three independent experiments. Supplementation of the growth medium with 20 μl/ml of reduced glutathione (50 mM) protected the cells to some extent, and after 24 h killer toxin treatment cell survival rate in glutathione-

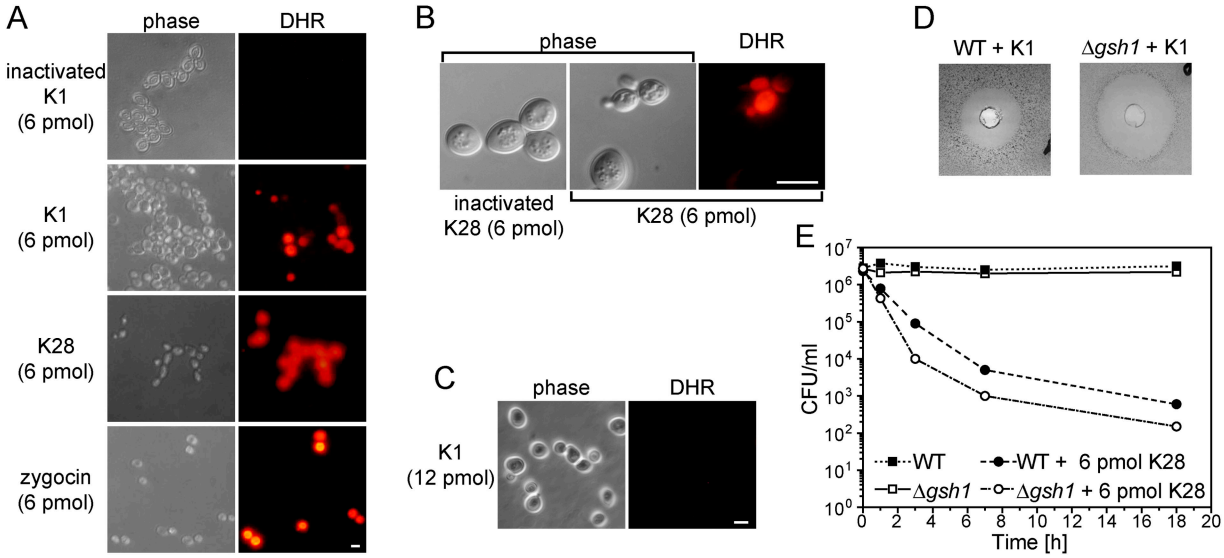

Figure 2. **ROS mediate apoptotic cell death induced by killer toxins.** (A) DHR staining and corresponding phase contrast of *S. cerevisiae* 192.2d (wild type) treated for 10 h at 20°C with moderate concentrations of either K1, K28, or zygocin (6 pmol each). Samples of heat-inactivated toxin were used as negative control. Bar, 5 μm. (B) Phase contrast and DHR staining of K28-treated cells and phase contrast of negative control cells treated with heat-inactivated K28 toxin. Bar, 5 μm. (C) DHR staining of *S. cerevisiae* 192.2d (wild type) after treatment with high concentrations of K1 toxin (12 pmol). Bar, 5 μm. (D) Toxin sensitivity assay on MBA illustrating K1 hypersensitivity of yeast Δ*gsh1* mutant cells compared with its isogenic Gsh1$^+$ wild type. (E) Kinetics of cell survival of a yeast Δ*gsh1* mutant and its isogenic Gsh1$^+$ wild type under moderate K1 toxin concentrations (6 pmol).

supplemented yeast cultures was increased by a factor of two (unpublished data). These results suggest that ROS act as effectors of apoptosis in toxin-treated cells and trigger the subsequent mechanisms.

Toxin-mediated apoptotic cell killing is dependent on *YCA1*

Recently, a caspase homologue was identified in yeast that was shown to mediate apoptosis in this unicellular microorganism (Uren et al., 2000; Madeo et al., 2002). We analyzed the involvement of Yca1p in killer toxin action and cell death. Deletion of *YCA1* had only little effect on toxin sensitivity. In MBA sensitivity assays under high toxin concentration, the resulting growth inhibition zones induced by either K1 or K28 did not differ significantly from the basal sensitivity of the isogenic Yca1$^+$ wild type (unpublished data). However, in liquid medium, the Δ*yca1* deletion mutant displayed a slightly better survival compared with wild type (Fig. 3 A). The time course of cell survival was reproduced in three independent experiments. Furthermore, the occurrence of apoptotic markers was strongly reduced compared with wild type (Fig. 3 B, exemplarily shown for K1), indicating that Yca1p is required for the efficient occurrence of apoptotic markers. Residual cell killing seen in the Δ*yca1* mutant after K1 toxin treatment at the 6-pmol level is caused by the toxin's primary lethal effect, which can be partially separated from the apoptosis effects in a dose-dependent manner (as shown in section Killer toxins can induce both apoptotic and necrotic cell death in yeast; Fig. 1 F). Next, we simulated the natural environment of yeast, where toxin concentration is usually low, by application of K28 in a significantly lower concentration of 1 pmol, corresponding to only 32 ng of the purified protein toxin. Under these conditions, deletion of *YCA1* strongly reduced toxin sensitivity; although the deletion mutant continued to proliferate even during toxin treatment for 20 h, cell growth in the Yca1$^+$ wild type ceased and viable cell numbers remained constant (Fig. 3 C).

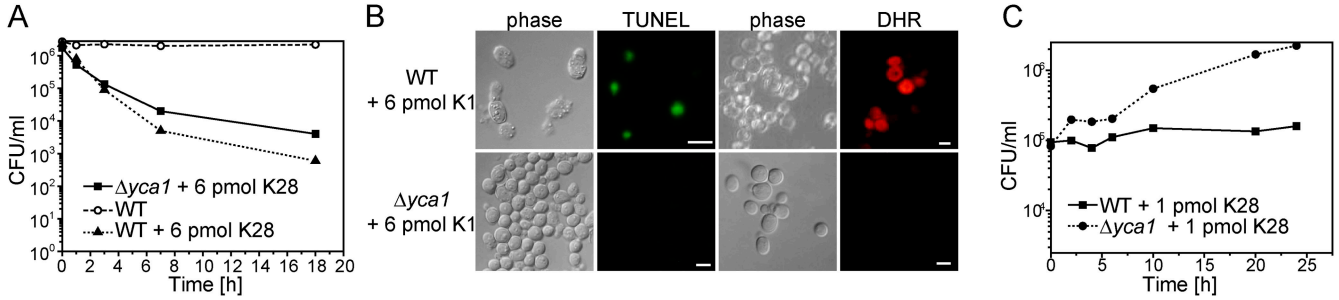

Figure 3. **Yeast caspase 1 is required for an efficient apoptotic cell response against the K28 virus toxin.** (A) Kinetics of cell survival of a yeast Δ*yca1* null mutant and its isogenic Yca1$^+$ wild type (strain 192.2d) in the presence of moderate K28 toxin concentrations (6 pmol). (B) DHR and TUNEL staining of Δ*yca1* mutant cells and its isogenic wild type (strain 192.2d) after treatment with moderate concentrations of K1 toxin (6 pmol). Bars, 5 μm. (C) Kinetics of cell survival of a yeast Δ*yca1* mutant and its isogenic wild type (strain 192.2d) under low K28 toxin concentrations (1 pmol).

Table II. ***S. cerevisiae* strains used in this study**

Strain	Genotype	Reference
192.2d	*MATα ura3 leu2*	Schmitt et al., 1996
192.2d Δ*yca1*	*MATα ura3 leu2 YOR197w::kanMX4*	This study
YPH98	*MATa ura3-52 lys2-801 ade2-101 leu2-3, 112 trp1-Δ1*	Sikorski and Hieter, 1989
YPH98 Δ*gsh1*	*MATa gsh1::URA3 ura3-52 lys2-801 ade2-101 leu2-3, 112 trp1-Δ1*	Brendel et al., 1998

Based on the data presented here, we could show that in low concentrations all three virally encoded yeast toxins induce apoptotic cell death that is accompanied by DNA fragmentation, chromatin condensation, and (as shown for K28) PS externalization. This process is mediated through yeast caspase Yca1p and the generation of ROS. In contrast, high concentrations of killer toxins induce nonapoptotic necrotic cell death, which is independent of Yca1p and ROS. Therefore, killer toxin action can trigger two modes of cell death. Under high toxin concentrations induction of apoptosis plays a minor role, whereas under moderate or low toxin doses, resembling the in vivo situation in the natural habitat of killer yeasts (Starmer et al., 1987), it might be of general importance for a toxin-secreting yeast to induce apoptosis in competing yeast cells, in particular at toxin concentrations that are per se too low to kill via the toxin's primary mode of action.

Materials and methods

Strains

S. cerevisiae strains used throughout this work are listed in Table II. Experiments with Δ*yca1* mutant cells and their isogenic wild-type strains were performed in two different strain backgrounds with similar results. Data shown in this paper were performed with the toxin-sensitive tester strain *S. cerevisiae* 192.2d (Schmitt et al., 1996) and its isogenic knockout mutants. Yeast cultures were grown at 20°C in complex YPC medium, which corresponds to YEPD medium supplemented with 1.92% citric acid; pH was adjusted to 4.7 by the addition of K_2HPO_4 as previously described (Riffer et al., 2002).

Toxin production and killer assay

Killer toxins K1, K28, and zygocin were isolated and partially purified from cell-free culture supernatants of the killer yeasts *S. cerevisiae* strain K7 (K1 toxin), strain MS300c (K28 toxin), and *Zygosaccharomyces bailii* strain 412 (zygocin toxin) as previously described (Schmitt and Tipper, 1990; Schmitt and Neuhausen, 1994; Weiler and Schmitt, 2003). To determine toxin-specific cell killing, agar diffusion assays on MBA, pH 4.7, were performed against a lawn of 10^5 cells of the toxin-sensitive yeast strains 192.2d, YPH98, or YPH98Δ*gsh1* (Schmitt and Tipper, 1995). To quantify activity and concentration in each of the three virus toxin preparations, 0.1-ml toxin samples were pipetted into wells (10 mm in diameter) cut into the agar, and the plates were incubated for 4 d at 20°C as previously described (Schmitt and Tipper, 1990). The diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity, which is expressed in arbitrary units; 1,000 arbitrary units each of K1, K28, and zygocin correspond to 0.3, 1.0, and 0.4 μg of the purified killer toxin, respectively. In the present study, low, moderate, and high toxin concentrations are defined as 1, 6, and 12 pmol each of the three virus toxins, corresponding to protein concentrations of 30 ng (K1), 32 ng (K28), and 16 ng (zygocin) that were used in the experiments in which the in vivo killing kinetics of each toxin had been determined.

Killing kinetics

To examine killer toxin activity in liquid media, yeast strains were incubated under shaking at 30°C in YPC medium, pH 4.7, until cells had reached early exponential growth phase (2×10^7 cells/ml). Thereafter, cells were spheroplasted by treatment with Zymolyase 20T as described previously (Eisfeld et al., 2000) and incubated in osmotically stabilized medium containing 0.6 M KCl. The appropriate killer toxin was added to a final concentration of 1–12 pmol, and cell survival was determined by plating aliquots of appropriately diluted cell suspensions onto YEPD agar plates containing 0.6 M KCl. After incubating the plates for 3 d at 30°C, colony-forming units were determined on plates with at least 400 cells.

Detection of apoptotic phenotypes

To detect an apoptotic phenotype, cells were analyzed by TUNEL test (In Situ Cell Death Detection Kit, POD; Roche), Annexin V staining (ApoAlert AnnexinV Apotosis kit; CLONTECH Laboratories, Inc.), DAPI staining (incubation with 1 mg/ml DAPI), and DHR staining as described previously (Madeo et al., 1997, 1999). Fluorescent light microscopy involved a microscope (model BX5; Olympus) with GFP, DAPI, and Rhodamine filters under standard settings.

Disruption of *YCA1*

A disruption cassette consisting of a geneticin resistance gene (Kan^R) with flanking 45 bases of 5′ and 3′ sequences of the *YCA1* ORF was PCR generated with primer YcaDelUp (5′-CGGGTAATAACAACTATTGAAAAAGCATGGCTTCGCATTAATAGGTTCGTACGCTGCAGGTCGAC-3′) and YCADelDown (5′-CGTTAAAAAAACACATGGTCTTATTTTCCAAAATGCCTATTCCCCCACTAGTGGATCTGATATC-3′) and transformed into the toxin-sensitive *S. cerevisiae* strain 192.2d. Geneticin-resistant colonies were confirmed for correct disruption by PCR with three primers (YCA1DelTestf, 5′-CAGTTCTCCTTAAAATCCACATAA-3′; YCADelTestr, 5′-GTCGAAACAAGAAGAGCAAAC-3′; KanRTestr, 5′-AAACAGGAATCGAATGCAACC-3′).

Reproducibility of the results

All experiments were repeated at least three times. Quantitative data from TUNEL tests, ROS staining, and Annexin V staining are from one representative experiment, whereby at least 400 cells were counted.

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