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 H2O2, 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 Δyca1 and Δgsh1 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. 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 (Martina 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; Eiffeld 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).

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 H2O2, 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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**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. In contrast, high concentrations of all three toxins led to nonapoptotic cell death independent of yeast caspase 1 and ROS.
efficient necrotic cell killing occurs, whereas apoptotic cell concentration close to 6 pmol resembles the breakpoint at which up to 12% (Fig. 1 F). Thus, in the case of the K1 virus toxin, a con-crotic cell killing dramatically increased, whereas the percent-age up to 6 pmol. At higher concentrations (8 and 12 pmol K1), ne-matically increasing until K1 reached molar concentrations of minimally increasing as well as a TUNEL-positive yeast cell response, both con-struted in Fig. 1 F, the K1 virus toxin caused a rapid cell kill-ing. At these concentrations, DNA fragmentation could be detected, whereas negative control samples 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 chro-matin 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 de-termine if an in vivo breakpoint exists at which efficient cell killing is induced in high concentrations of K1. In contrast to K1, K28, or zygocin, a strong green fluorescence indicative of 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).

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 dihydrophodamine (DHR) 123, which is a cell permeable leukocyte dye 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 ∆gsh1 mutant cells that are genetically blocked in glutathione biosynthesis. Glutathione plays a central role in inducing apoptotic markers and mediates 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 dihydrophodamine (DHR) 123, which is a cell permeable leukocyte dye 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).

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

| Toxin  | Size  | Structure               | Receptor on target cell | Lethal effect    |
|--------|-------|-------------------------|-------------------------|------------------|
| 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).
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 wild type (unpublished data). However, in liquid medium, the 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 Δyca1 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 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. Bars, 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. Bars, 5 μm. (C) DHR staining of _S. cerevisiae_ 192.2d (wild type) after treatment with high concentrations of K1 toxin (12 pmol). Bars, 5 μm. (D) Toxicity assay on MBA illustrating K1 hypersensitivity of yeast Δgsh1 mutant cells compared with its isogenic Gsh1 wild type. (E) Kinetics of cell survival of a yeast Δgsh1 mutant and its isogenic Gsh1 wild type under moderate K1 toxin concentrations (6 pmol).

**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 Δyca1 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 Δyca1 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 Δyca1 mutant and its isogenic wild type (strain 192.2d) under low K28 toxin concentrations (1 pmol).
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 Δyca1 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$_2$HPO$_4$ 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 192.2d (Schmitt et al., 1996) and incubated in osmotically stabilized cells. 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.

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

| Strain   | Genotype     | Reference                  |
|----------|--------------|----------------------------|
| 192.2d   | MATA ura3 leu2 | Schmitt et al., 1996      |
| 192.2d Δyca1 | MATA ura3 leu2 YOR197w:kanMX4 | This study                |
| YPH98    | MATA ura3-52 lys2-801 ade2-101 leu2-3, 112 trp1 Δ   | Sikorski and Hieter, 1989 |
| YPH98 Δgsh1 | MATA gsh1:URA3 ura3-52 lys2-801 ade2-101 leu2-3, 112 trp1 Δ | Brendel et al., 1998 |

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