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YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity

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Abstract Yeast cells lacking the metacaspase-encoding gene YCA1 (Δ*yca1*) were compared with wild-type (WT) cells with respect to the occurrence, nature and time course of acetic-acid triggered death. We show that Δ*yca1* cells undergo programmed cell death (PCD) with a rate lower than that of the WT and that PCD in WT cells is caused at least in part by the caspase activity of Yca1p. Since in Δ*yca1* cells this effect is lost, but z-VAD-fmk does not prevent both WT and Δ*yca1* cell death, PCD in WT cells occurs via a Yca1p caspase and a non-caspase route with similar characteristics.

Keywords: PCD; Acetic acid; YCA1; Caspase; z-VAD-fmk; *Saccharomyces cerevisiae*

1. Introduction

In the last decade it has been shown that *Saccharomyces cerevisiae* may undergo a form of cell death similar to apoptosis with its own physiological significance [1–3]. In fact, in response to different stimuli *S. cerevisiae* commits to cell death showing typical hallmarks of metazoan apoptosis [4]. Although several yeast orthologues of key apoptotic regulators have already been identified [5–10], how they work in yeast programmed cell death (PCD) remains to be fully established.

In mammalian apoptosis, caspases play a major role [11–13], however the occurrence of a caspase-independent PCD has also been proposed [14,15]. In yeast, YCA1 codes for a protein belonging to the superfamily of caspase-related proteases, termed metacaspases [16]. YCA1 is involved in *S. cerevisiae* PCD triggered by different stimuli [2,6,17–20]. In addition, disruption of YCA1 attenuated the stimulation of apoptosis due to Aifp overexpression [10] or hydrogen peroxide [21]; however, yeast metacaspase-independent PCD has also been reported [22,23].

We have shown that *S. cerevisiae* commits to PCD upon treatment with acetic acid [24,25]. The role of YCA1 in acetic acid-induced PCD (AA-PCD) is still controversial although Yca1p has been indicated as an executor of AA-PCD in *S. cerevisiae* [6]. More recently, a minor role for YCA1 in AA-PCD was proposed [26]. Thus, comparison is made here between WT and YCA1-lacking (Δ*yca1*) yeast cells with respect to the occurrence, nature and time course of the death process. We show that YCA1 contributes to AA-PCD but not as a conventional caspase.

2. Materials and methods

2.1. Strains, media, growth conditions and YCA1 gene deletion

The *Saccharomyces cerevisiae* strain used in this study was W303-1B (*MAT*α *ade2 leu2 his3 trp1 ura3*) and cells were grown at 30 °C in rich medium (1% yeast extract and 2% Bacto-peptone) containing 2% dextrose (YPD). To delete the YCA1 gene, genomic DNA was isolated from BY4743 (*MAT*a/*MAT*α *leu2/leu2 his3/his3 ura3/ura3 lys2/LYS2 MET15/met15 yca1*::*kan*MX4/*YCA1*) cells, kindly provided by Prof. R. A. Butow, and amplified using the oligonucleotide couple 5′-TTA TTG GCC GAG TTG CGC T-3′ and 5′-GGA AGA ACA GGA AGA GTC TG-3′. The Δ*yca1*::KanMX4 cassette-containing PCR product was purified from agarose gel and used to replace the *YCA1* gene in W303-1B cells. Recombinant clones were selected for resistance to the antibiotic G418 disulfate (Sigma–Aldrich). Gene disruption was verified by PCR analysis. Δ*yca1* cells were grown in YPD medium supplemented with 200 mg/l G418.

2.2. Acetic acid treatment

Exponential phase (OD_{600} = 0.5–0.8) wild-type (WT) and Δ*yca1* cells were grown in YPD medium at 26 °C, at 150 rpm and were suspended (10^7 cells/ml) in fresh YPD medium. For acetic acid treatment this medium, containing 80 mM acetic acid, was adjusted to pH 3.0 (set with HCl). Cells were incubated for different times at 26 °C as described previously [24]. To induce hyperosmotic stress, wild-type cells were transferred to medium containing 60% (w/w) of glucose as described [18] and incubated for 300 min at 26 °C.

Cycloheximide (Sigma–Aldrich) dissolved in water or carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone (z-VAD-fmk) (Calbiochem) dissolved in dimethylsulfoxide, were added to yeast cell cultures grown to OD_{600} = 0.5–0.6 at a final concentration of 100 μg/ml or 20 μM, respectively, and the cultures incubated for 30 min before acetic acid treatment. Cell viability was determined by measuring colony-forming units (cfu) after 2 days of growth on YPD plates at 30 °C.

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Abbreviations: PCD, programmed cell death; AA-PCD, acetic acid-induced PCD; PI, propidium iodide; WT, wild-type; Δ*yca1*, YCA1-lacking; DAPI, 4,6-diamidino-2-phenylindole dihydrocloride; z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone; FITC-VAD-fmk, fluorescein isothiocyanate conjugate of z-VAD-fmk; D_2R, $(Asp)_2$-Rhodamine 110

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2.3. Fluorescence microscopy analysis

Exponential cells were treated with acetic acid in YPD pH 3.0 medium as described above. At different times, 10^8 cells were collected and resuspended in water in the presence of 0.25 μg/ml propidium iodide (PI) and 50 μg/ml 4,6-diamidino-2-phenylindole dihydrocloride (DAPI) at 37 °C for 15 min. Then, live cells were observed using an Axioplan 2 microscope (Zeiss) equipped with a UV HBO 50/AC lamp and a 100× objective. Digital images were acquired with an Axiocam CCD camera using Axio Vision software.

2.4. Detection of caspase activation

Detection of caspase activation was performed using the "CaspACE, FITC-VAD-fmk In Situ Marker" (Promega). Briefly, 1×10^6 cells were washed in PBS, suspended in 100 μl staining solution containing 50 μM of the fluorescein isothiocyanate conjugate of z-VAD-fmk (FITC-VAD-fmk) and incubated for 20 min at 30 °C in the dark. Then, cells were washed once and suspended in PBS. For double staining with PI, cells were subsequently incubated with 2 μg/ml of PI for 10 min at room temperature. For the assays with the broad caspase inhibitor z-VAD-fmk, cells were prepared as described above, washed with PBS and incubated with 20 μM z-VAD-fmk for 30 min at 30 °C before incubation with FITC-VAD-fmk.

Flow cytometric analysis was performed using an Epics® XL-MCL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15 mW, and with biparametric detection of FITC fluorescence (488/525 nm; FL1; log units) and PI fluorescence (488/620 nm; FL3; log units). Signal compensation that eliminates optical overlap between the dyes was set at 30%. Twenty thousand events were acquired for each analysis. Data were analyzed using WinMDI 2.8 software.

3. Results

3.1. Deletion of the YCA1 gene decreases the rate of yeast programmed cell death triggered by acetic acid

To ascertain the role of the YCA1 gene in AA-PCD, comparison was made between WT and Δ*yca1* W303-1B yeast cells in exponential phase with respect to their viability up to 200 min after PCD induction (Fig. 1A). In both cases the yeast cells died but the cell death patterns were different. After 30 min of acetic acid treatment, wild-type and Δ*yca1* cells showed about 74% and 83% cell viability, respectively. This progressively decreased to 0% for WT as in [25] and 7% for Δ*yca1* cells at 200 min. Δ*yca1* cell viability was significantly higher ($P < 0.001$) than that of WT cells from 60 to 120 min after acetic acid challenge with death rates of $\mu_d = 0.015 \pm 0.0021\ min^{-1}$ and $0.0074 \pm 0.0002\ min^{-1}$ for WT and Δ*yca1* cells, respectively.

To determine whether death of Δ*yca1* cells occurs via PCD, given that AA-PCD is dependent on *de novo* protein synthesis [24], the effect of cycloheximide on survival of Δ*yca1* and WT cells was investigated. In both cases cycloheximide prevented cell death in a similar way (Fig. 1B). Chromatin condensation, another PCD hallmark, was also analyzed during AA-PCD of both WT and Δ*yca1* cells, through analysis of nuclear morphology and plasma membrane integrity by co-staining cells with DAPI and PI (Fig. 2). With both cell types chromatin condensation along the nuclear envelope was detectable after 60, 120 and 200 min of acetic acid treatment in cells with an integral plasma membrane as shown by lack of staining with PI.

Thus, yeast cells lacking the metacaspase YCA1 gene undergo the process of AA-PCD in a manner similar to that of the WT cells but at a lower rate.

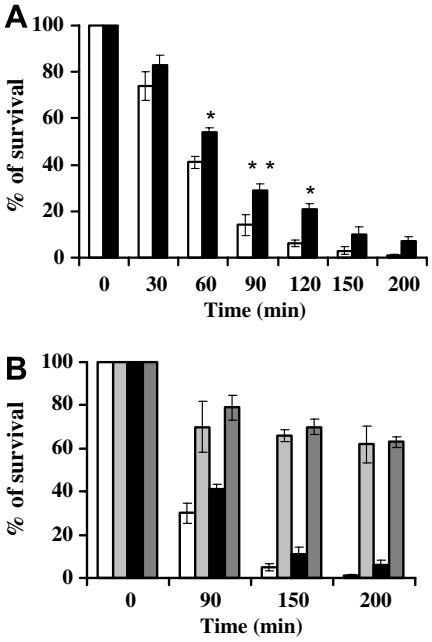

Fig. 1. Effect of YCA1 deletion and/or cycloheximide on viability of *Saccharomyces cerevisiae* W303-1B exponential cells exposed to acetic acid. WT (white bars) and Δ*yca1* (black bars) cell death was induced with 80 mM acetic acid in the absence (A and B, white and black bars, respectively) or in the presence of cycloheximide (B, light and dark grey bars, respectively) and cell viability analyzed at indicated times. Cell survival (100%) corresponds to cfu at time zero. Reported values are the mean of three experiments with standard deviations. ANOVA and Bonferroni test: statistically significantly different with (*) $P < 0.001$, (**) $P < 0.0001$ when comparing WT and Δ*yca1* cells. Death rate μ_d (see text) was calculated by GraFit 3.0 software as the slope of the linear part of the semilogarithmic plot of the number of cfu as a function of incubation time.

3.2. z-VAD-fmk does not prevent yeast AA-PCD even though it partially inhibits caspase-like activity

In the light of the above results, to ascertain whether and how caspase activity is involved in yeast AA-PCD, we monitored caspase-like activity by simultaneous staining of cells with FITC-VAD-fmk [6,18] and with PI to differentiate between FITC-VAD-fmk specific and unspecific staining [27] (Fig. 3A). Cells treated with 60% (w/w) of glucose, which die via PCD [18], were used as a control for positive staining with FITC-VAD-fmk. About 36.9% of WT cells displayed FITC-VAD-fmk positive and PI negative staining (specific staining); after acetic acid treatment the corresponding percentage was 11.8%. Pre-incubation with the pan-caspase inhibitor z-VAD-fmk before staining with FITC-VAD-fmk, caused a reduction in the percentage of FITC-VAD-fmk positive cells both in acetic acid and 60% glucose treated cells.

Staining with FITC-VAD-fmk was very low in WT cells held at pH 3 but without acetic acid (Fig. 3A) and did not increase with time (Fig. 3B). In WT cells treated with acetic acid the staining with FITC-VAD-fmk increased progressively up to about 20% at 200 min after induction of PCD. By contrast, with Δ*yca1* cells the percentage of FITC-VAD-fmk staining remained virtually constant at about 5% between 60 and 200 min of PCD. These results are consistent with YCA1 acting as a z-VAD-fmk sensitive caspase-like protease and/or as a protease activator. In parallel, cell survival was monitored either in the